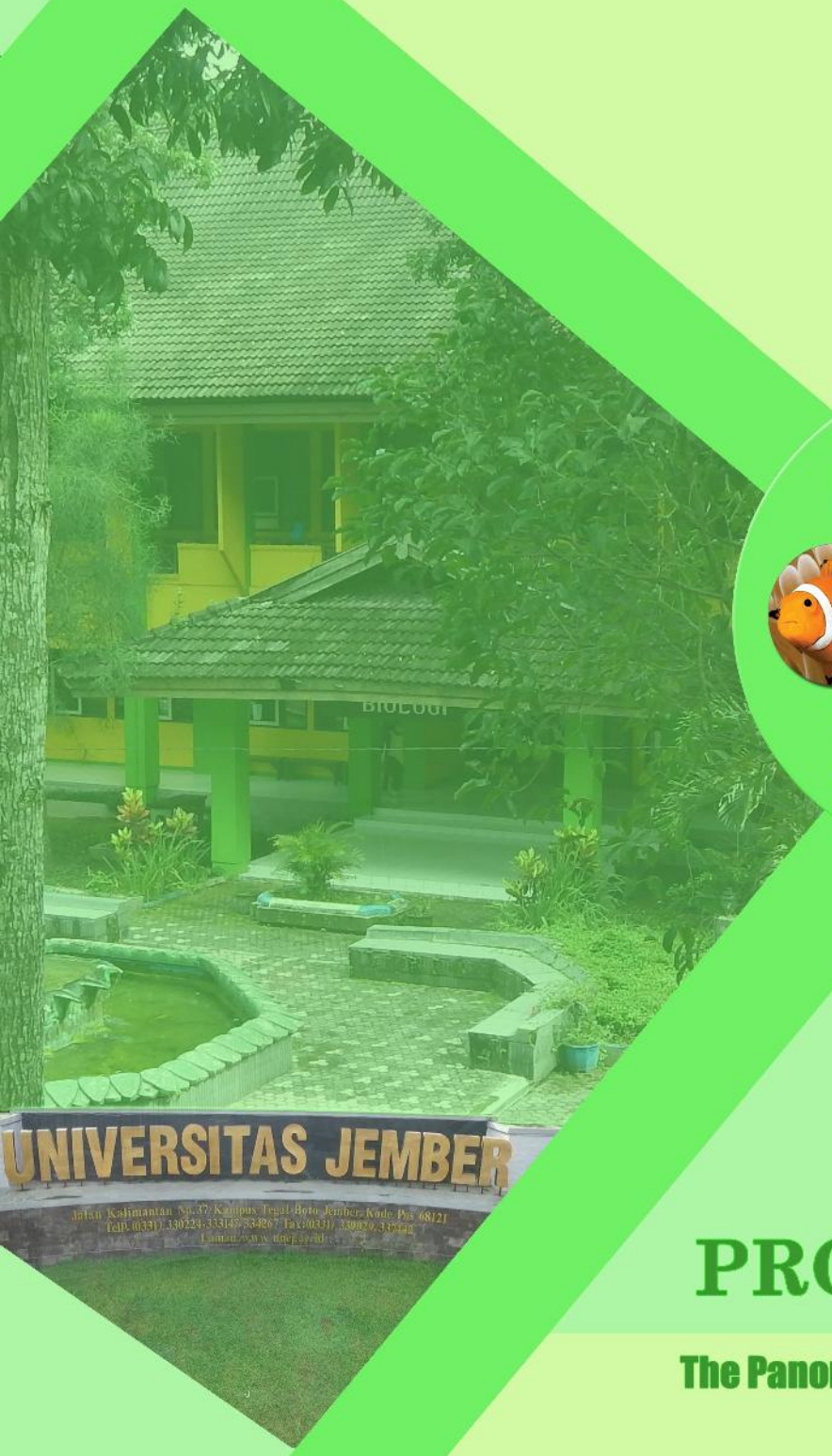
The 2nd ICOLIB

International Conference on Life Sciences and Biotechnology

Biology Department, Faculty of Mathematics and Natural Sciences, University of Jember

(ICOLIB BIO-UNEJ 2017)

Integrated Biological Sciences for Human Welfare



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The Panorama Hotel and Resort Jember
East Java, Indonesia
August 7 - 8, 2017

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PROCEEDINGS
THE 2nd INTERNATIONAL CONFERENCE ON LIFE SCIENCES AND BIOTECHNOLOGY (ICOLIB)

INTEGRATED BIOLOGICAL SCIENCES FOR HUMAN WELFARE

The Panorama Hotel and Resort Jember
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THE 2nd INTERNATIONAL CONFERENCE ON LIFE SCIENCES AND BIOTECHNOLOGY (ICOLIB): INTEGRATED BIOLOGICAL SCIENCES FOR HUMAN WELFARE

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WELCOMING ADDRESS (CONFERENCES)

The International Conference of Life Science and Biotechnology (ICOLIB) was organise by Biology department Faculty Mathematic and Basic Sciences, The University of Jember, Indonesia. This conference has been held biannually at different venues. The last one, (First ICOLIB) held in Aston Hotel Jember 2015, Indonesia. Now, we are held the 2nd ICOLIB at Panorama Hotel and Resort Jember, Indonesia. The ICOLIB is a forum for students, researchers, educators, observers and practitioners from university, research institutions, industry and general public, policy maker to exchange ideas and latest information in the field of life science and its application. The theme of the 2nd ICOLIB 2017 '**Integrated Biological Sciences for Human Welfare**' will underpin the need for collaboration and cooperation of individuals from a wide range of professional backgrounds. The scope of the 2nd ICOLIB covers several fields of studies, namely life sciences, environmental sciences, medical and pharmaceutical sciences, science of renewable energy, agricultural science and food security. This conference will also offer opportunities for discussion and sharing as well as encouraging for international research collaboration. Furthermore, the scientific articles will be peer-reviewed and published in Serial book volume publish with Cambridge Scholar Publishing UK. The selected scientific articles in the 2nd ICOLIB will be further reviewed and will also be published in Scopus-indexed Journal.

The 2nd ICOLIB have been fortunate to have Prof. Harald zur Hausen, 2008 Nobel Laureate in Physiology or Medicine for his discovery of human papilloma viruses causing cervical cancer. Prof. zurHausen and his team has made a breakthrough in 1982 and 1983 when they were able to isolate HPV 16 and HPV 18 as the virus types responsible for cervical cancer. Based on these findings, vaccines have been developed against cervical cancer, one of the most common forms of cancer among women. This work led to improved methods for predicting which women are in the risk zone. We are very honoured to present Prof. Harald zur Hausen, as a keynote speaker, and 6 distinguished scientists as invited speakers.

I sincerely hope that the results of this conference will enable all participating scientists from all over the world to have the opportunity to exchange knowledge through lectures and posters.

Purwatiningsih

Chairwoman of The 2nd ICOLIB 2017

PREFACE FROM EDITOR

Welcome to the The 2nd ICOLIB Proceedings, **Integrated Biological Sciences for Human Welfare,** The theme of this conference reflects our attention Biological science to support the human welfare across of different of fields and contexts. Indeed, the 17 contributions in these proceedings—including keynotes, invited and contributed papers—with authors from at least 6 different countries showed the great results of Biology.

All the manuscript have been peer reviewed with at least 2 people who competence with their subjects. It has been a great previlege for being Editor of the 2nd ICOLIB. Hope these output had a great support for human welfare needs in many levels. Thus enable all participating scientists from all over the world to have the opportunity to exchange knowledge through the conferencere.

Sincerely,

Editorial team of The 2nd ICOLIB

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General Information for the Participant

Registration Information
Conference Venue

- The venue for the conference is the Panorama Hotel, Jember, East Java, Indonesia

Registration

- Registration includes:
  - 2nd ICOLIB Abstract Book
  - ID Card
  - Document Bag
  - Refreshment (coffee & tea) during the conference day
  - Buffet Lunch

ID Card

- Participants are requested to display their ID Card during the conference for entry to scientific sessions, melas and the wellcome reseption. Please also show the ID Card to committee before transportation to the conference venue.

Instruction for the Moderator

- Please ensure that the sessions and speaker presentations are kept stricly on time

Instruction for Speakers (Keynote Speaker and Oral Presenter)

- Speaker are requestes to submit their presentation to staff in the audio-visual room at the least 1 hours before each presentation, then upload and ensure that the proper presentation is in the computer provided
- 45 minutes have been allocated for each keynote speakers (please allow time within this period for answering the questions)
- Free oral presenter will last 10 minutes only (please allow time within this period for answering questions)
- Please be aware that the above times must be strictly adhered to

Instructions for Poster Presenter

- Poster presentations will be located in the front of the conference space along the second floor.
- Poster will be display throughout the conference, and presenters are responsible for putting them and removing them.

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List of Submitted Paper

Keynote Speakers

INFECTIOUS AGENTS IN BOVINE RED MEAT AND MILK AND THEIR POTENTIAL ROLEIN CANCER AND OTHER CHRONIC DISEASES

Harald zur Hausen[1], Timo Bund[2] and Ethel-Michele de Villiers[3]

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Abstract

A large number of global epidemiological studies linked specific cancers and neurodegenerative diseases to the consumption of red meat and dairy products. The geographic pattern suggested a species-specific role for the consumption of such products from Eurasian cattle. We initiated a search for potential infectious agents from serum and dairy products of these cattle and isolated and sequenced more than 30 single-stranded circular DNAs, consistinag of ~1.000 to ~3.000 nucleotides in length. Four different familieswere identified and named as bovine meatand milk factors (BMMF). Except for one group, the other isolates revealed remarkable nucleic acid homologies to plasmids of *Acinetobacter* and *Psychrobacter* bacteria. Upon transfection of human cells, all those tested were transcriptionally and translationally active. In some human cells replication and synthesis of infectious progeny was noted. The infectivity depended on specific sialinic acid components, apparently required as components of cellular receptors.Four isolates were obtained from sera or an autopsy brain sample of multiple sclerosis (MS) patients. Serological analyses with consensus protein epitopes of BMMF-1 group revealed antibodies in healthy controls, but significantly elevated titers in MS patients. A model for the pathogenesis of MS has been published. Presently we analyze the seroreactivity of patients with malignant tumors and neurodegenerative diseases against antigens of all four isolated BMMF groups.Our data represent a first example of bacterial plasmid-derived sequences adapted to gene expression, autonomous replication and synthesis of infectious progeny in human cells. This opensnew approaches to study their involvement in diet-linked cancers, neurodegenerative disorders, and autoimmune diseases.

STUDY ON MOLECULAR MECHANISMS ON PLANT ARCHITECTURE IN RICE

Chang-deok Han

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Abstract

Crop grain yield is simply determined by grain number per unit area and grain filling rate. In rice, around 40% of biomass is carbon and over 1% is nitrogen. 80-90% of the whole carbon in biomass are made from light photosynthesis. In order to increase yield, total photosynthesis (net carbon gain) per unit area and grain filling capacity should be enhanced. Plant architecture determines the efficiency of canopy photosynthesis and plant number per unit area.In rice, lamina and tiller angles are ones of the key agronomical characters determining plant architecture. *MPT1* (Modifier of Plant Type 1) belongs to a transcription factor family carrying a zinc finger "ID“ (indeterminate) domain in rice. The study shows that *MPT1* determines plant architecture. Studies demonstrated that *MPT1* is an ortholog of Arabidopsis *SGR5* (Shoot Gravity Response 5) that has been shown to be involved in shoot gravity response. *MPT1* is specifically expressed in metaxylem and pulvinal tissues (gravity sensing organ in grasses). Mutant shoots exhibit severely reduced gravitropism while overexpressors showed hyper-response to gravity.Meanwhile. a main action of *MPT1* on lamina inclination has been demonstrated to suppress the interaction between IAA (auxin) and BR (brassinosteroids) compounds. In order to further understand molecular mechanisms of *MPT1*-mediated molecular mechanisms, Y2H (Yeast Two Hybrid) and RNA transcriptome analysis have been performed to identify*MPT1*-interacting and target genes, and subsequent studies have been conducted to elucidate their functions. Current progresses will be presented in the seminar. Since overexpression of *MPT1* leads to erect phenotype, current efforts will be discussed to create ideal plant types by manipulating *MPT1*expression and evaluatingagricultural utilities of *MPT1*.

QUALITY MANAGEMENT IN THAI MANGO SUPPLY CHAINS TO MEET THE NEEDS OF CONSUMERS: A CASE STUDY OF MANGO EXPORTING TO JAPAN MARKET

S. Kanlayanarat[1,2], C. Watanawan[1], B. Mathulaprungsan[1] and C. Wongs-Aree[1,2\]

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Abstract

Thai mango cv. 'Nam Dok Mai' is an important commercial fruit of Thailand for exporting to Japan market where sets up a very high quality standard of the fruit. A Thailand exporter established the capital infrastructure for preparing high quality mango for Japan market under the Japan-Thailand Economic Partnership Agreement (JTEPA). Since the high stringency of investigation of pesticide residue remaining as well as fruit fly and disease contamination at the Japan plant quarantine, the mango quality management has to be started from the upstream supply chain at farm level and followed by the supply chain operation to the market. The mango growers must affirm their orchards under the Japan Good Agricultural Practice certification when the best practice manual was prepared by the Department of Agricultural, Ministry of Agriculture and Cooperatives, Thailand. GAP-certified orchards were selected to be a partner of mango exporting company. Mango fruit were bagged 40 days before harvesting in order to minimizing anthracnose disease caused by *Colletotrichum gloeosporioides* which is a very serious postharvest problem at the market places. A week before harvesting, the fruit were randomly sampled to check pesticide residues under the plant quarantine regulation whereas after harvested, the fruit were thoroughly checked for a suitable maturity, primarily sorted and uniformly weighed at local farm packing houses. At the packing house of exporting company, the fruit have to be washed and cleaned by chlorinated water, then dipped in 50°C hot water for 5 minutes. All fruit were cooled down in normal temperature water for 1-2 minutes, quickly dipped in 400 ppm ethephon, and then air dried. After graded and sized according to the quality standard, the fruit were passed through vapor heat treatment at 47°C constant of pulp temperature for 20 minutes for killing habitat insects. Fruit were then protected by individual foam net and packed in a carton box containing 3/5 kg/box. Boxes were transported by refrigerator trucks at 13°C to the airport and shipped by airfreight.

Keywords: Supply chain, quality management, mango

THE IMPORTANCE OF N-TERMINAL DOMAIN ON THE POST-TRANSLATION REGULATION OF SUCROSE-PHOSPHATE SYNTHASE FROM SUGARCANE (*Saccharum officinarum*)

Bambang Sugiharto[1,2] and Widhi Dyah Sawitri[1,3]

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Abstract

Sucrose-phosphate synthase (SPS; EC 2.4.1.14) is believed to be the key enzyme controlling photosynthetic carbon flux into sucrose in plants. Studies on carbon assimilating enzymes revealed that among photosynthetic enzymes, the SPS activity determined sucrose synthesis and accumulation in the *Saccharum* species. Futher study at molecular levels found the presence of SPS gene family in sugarcane; they were photosynthetic *SoSPS1* and non-photosynthetic *SoSPS2* genes. To identify their function, the *SoSPS1*-cDNA was overexpressed in plants and resulted in elevation of SPS activity and sucrose accumulation in leaves of transgenic tomato and sugarcane. However, when the *SoSPS1*-cDNA was overexpressed in *Escherichia coli*, two forms of SPS1-A and SPS1-B proteins were detected by immunoblotting, one with a full length size equivalent with the authentic enzyme from sugarcane leaves and the other with a truncated form shorter by ca 20 kDa, respectively. Molecular and biochemical characterization of the truncated SPS1-B showed that the protein was lacking N-terminal domain, but has higher specific activity and no regulation by an allosteric effector of glucose 6-phosphate (G6P).These results indicated that the N-terminal region of sugarcane SPS is play acrucial role for the allosteric regulation and may the function like a suppressor domain for the enzyme activity.In addition, it is well documented that SPS activity is regulated by light/dark transition facilitated by phosphorilation-dephosphorilation processes, active during light and less-active in dark time, and the regulation is envolved metabolite of G6P. Thus, in planta studies on the importance of N-terminal domain on theregulation of the SPS are needed to determine the regulation. The N-terminal digested of *SoSPS1*-cDNA was constructed in an expression binary vector and overexpressed in transgenic tomato. This presentation will also discuss about a possibility regulation of SPS by the allosteric effector in respond to dark/light transition. The structural analysis on sugarcane photosynthetic SPS1 is also necessary to have a better understanding on the allosteric property.

Keywords: sucrose-phosphate synthase, sucrose accumulation, post-translational regulation, sugarcane.

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LONG-TERM CHANGES IN WATER QUALITY IN LAKE BIWA WITH SPECIAL REFERENCE TO ORGANIC MATTER DYNAMICS, MICROBIAL ECOLOGY AND DIVERSITY

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Abstract

During the last three decades, the water quality of Lake Biwa, the largest freshwater lake in Japan, has been improved through collaboration among multiple stakeholders. Mysteriously, a portion of organic matter expressed by chemical oxygen demand (COD_{Mn}) in the lake has been gradually increasing every year. Some researchers have reported that the increase in COD_{Mn} might be due to the accumulation of refractory and/or semi-labile DOM, and those DOM might be autochthonously produced. We have partly clarified the microbial processes with special reference to production of those DOM in Lake Biwa, especially in the lake's hypolimnion. In the epilimnion of the lake, phytoplankton biomass is producedthrough primary production, followed by sinking into the hypolimnion. In the hypolimnion, a part of the phytoplankton biomass is converted into and produced as humic-like DOM through decomposition by planktonic bacteria. Fluorescence *in situ* hybridization (FISH) showed that bacterial clade, CL500-11 (phylum *Chloroflexi*), predominates in the hypolimnion. We made further analyses on prokaryotic community composition by high throughput 16S rRNA gene amplicon sequencing which showed the dominance by members of *Planctomycetes* exclusively occurred in the hypolimnion. In addition, FISH on eukaryotes showed that bacterivorous kinetoplastid flagellates are the dominant eukaryotes in the hypolimnion. So, the results indicate the presence of unique microbial food webs in the hypolimnion of Lake Biwa, where humic-like DOM is produced by the hypolimnion bacterial assemblages, and those bacteria are grazed by the dominant kinetoplastids and other hypolimnion dwelling bacterivorous protists.

Keywords: Eutrophication, Chemical Oxygen Demand, Dissolved Organic Matter, Phytoplankton, Bacteria, Decomposition, Microbial loop, Protists

1. Introduction

Lake Biwa is the largest and the most socially important lake in Japan. About 14 million people who live in Kinki Area use the water of the lake. During 1960' and 1970's due to the large loading of phosphorus and nitrogen, eutrophication in Lake Biwa was serious, leading to phytoplankton blooms (since the late 1960's), freshwater red tide (since the late 1970's) and cyanobacterial bloom (since the early 1980's). In 1970's, Japanese Government and Shiga Prefectural Government had started some measures to reduce high phosphorus loading in Lake Biwa. Due to the efforts, the water quality of the lake has been improved during the last 40 years (for example, N, P and chlorophyll *a* concentrations have been decreased).

Mysteriously, a portion of organic matter

expressed by chemical oxygen demand (COD_{Mn}) in the lake has been gradually increasing every year. COD_{Mn} is the indicator of organic matter loading, and a large portion of organic matter in Lake Biwa water is dominated by dissolved organic matter (DOM). The most important source and origin of DOM in Lake Biwa is primary production by phytoplankton [1], [2], [3]. However, chlorophyll *a* concentrations whose high values indicate high production of dissolved organic matter (DOM) have been decreased. Some researchers have reported that the increase in COD_{Mn} might be due to the accumulation ofrefractory and/or semi-labile DOM, and those DOM might be autochthonously produced [4].

When algal derived DOM is decomposed under chlorination conditions used for drinking water purification and disinfection, some toxic substances such as trihalomethane may be produced [5]. So, for management and conservation of better water quality in lakes for human well-beings, it is important for us to investigate the origin of unknown DOM and find appropriate measures for the reduction of DOM. So, we must identify origin and composition of DOM, together with elucidation of relationships between DOM, N and P levels and chlorophyll *a*, and this thus helps to understand the biogeochemical cycling of DOM and the reason for DOM changes in lakes.

1. The microbial loop

The planktonic food linkage from dissolved organic matter to bacteria to protists, the so-called microbial loop [6], has been intensively studied in marine and freshwater ecosystems. It was regarded that the herbivorous food web where phytoplankton are preyed on zooplankton functionsmajor matter cycling in pelagic ecosystems. On the other hand, according to numerous previous studies, microbial loop also functions as an important process for the matter cycling in pelagic food webs. Phytoplankton release DOM as intermediate products of photosynthesis and/or as autolytic products [7]. DOM thus released is utilized for the growth of heterotrophic bacteria, followed by protistan grazing on bacteria. The roles of planktonic protists, such as heterotrophic nanoflagellates and ciliates, in microbial loop are to consume bacteria that are too small to serve directly as major prey items for most zooplankters, and to be themselves

2. Microbial loop in Lake Biwa

As mentioned previously, in pelagic systems, matter cycling or DOM dynamics is mainly driven by microbial loop.Microbial loop in Lake Biwa has and this became a good reason for numerous sometimes predominates the total heterotrophic biomass in lakes [8], researchers to conduct their utilized by the zooplankton [6]. Biomass of heterotrophic bacteria research on organic matter transfer from DOM to protistan bacterivores via heterotrophic bacteria.also been intensively studied during the last three decades with special reference to DOM production through phytoplankton primary production [9].

We have partly clarified the microbial processes with special reference to production of those DOM in Lake Biwa, especially in the lake's hypolimnion (Figure 1). In the epilimnion of the lake, phytoplankton biomass is producedthrough primary production, followed by sinking into the hypolimnion. In the hypolimnion, a part of the phytoplankton biomass is converted into and produced as humic-like DOM through decomposition by planktonic bacteria (in [4], "humic-like DOM" is expressed as "humic-like fluorescent dissolved organic matter, $FDOM_M$). The genus *Synechococcus,*which is the free-living cyanobacterial genus with small cell size (usually <2 □m), sinks so slowly (no faster than 0.01–0.02 μm s^{-1}) that the motion of the water is believed to keep them in suspension. Thus, it has been considered that

they are too small to sink to the lake hypolimnion.

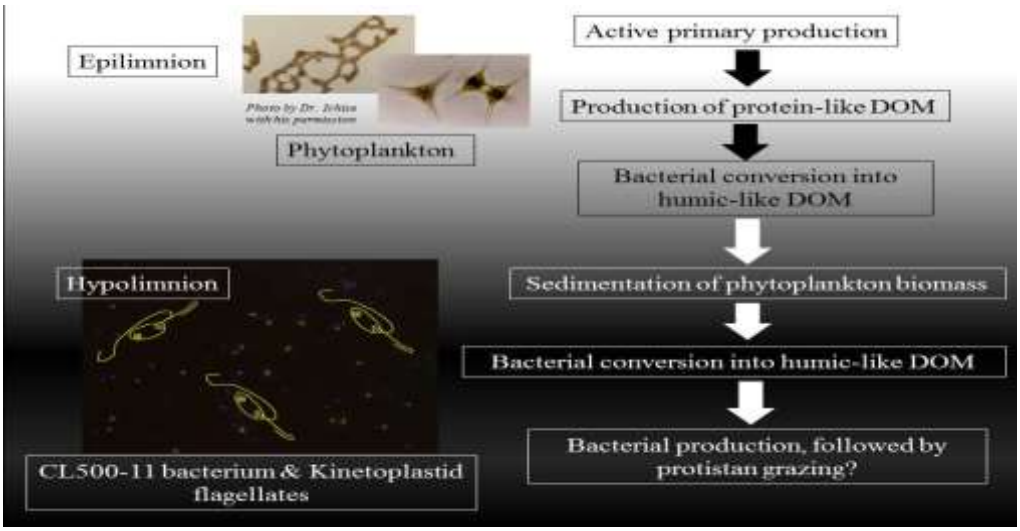

Figure 1. Fate of dissolved organic matter (DOM) produced by phytoplankton primary production. DOM freshly produced in the epilimnion is converted into humic-like DOM through bacterial decomposition in the hypolimnion. The dominant bacterium, CL500-11, may use the DOM for its biomass production and be consumed by Kinetoplastid flagellates.

However, recently, we have found that *Synechococcus* also sank to the hypolimnion of Lake Biwa, and that the contribution of *Synechococcus* chlorophyll *a* amount accounted for about 30% to total chlorophyll *a* concentration [10]. So, it is likely that *Synechococcus* biomass is also converted into humic-like DOM. Usually, humic substances including humic-like DOM are biologically refractory (not easily degradable) and or semi-labile, and, due to this, the humic-like DOM does not have much further bacterial decomposition. So, the reason for the increase in COD_{Mn}in Lake Biwa might be due to the accumulation of refractory and/or semi-labile DOM.

In Lake Biwa, using fluorescence *in situ* hybridization (FISH), we have found that bacterial clade, CL500-11 (phylum *Chloroflexi*), predominates in the hypolimnion [11]. Further analyses on prokaryotic community composition by high throughput 16S rRNA gene amplicon sequencing demonstrated that ubiquitous tribes in the epilimnion can also dominate in the hypolimnion (e.g. bacI-A1 & acI-B1), and that members of *Planctomycetes* (e.g. CL500-15, CL500-37 & CL500-3), together with CL500-11 exclusively occurred in the hypolimnion [11]. In addition, FISH on eukaryotes showed that bacterivorous kinetoplastid flagellates are the dominant eukaryotes in the hypolimnion [12]. Furthermore, high throughput 18S rRNA gene amplicon sequencing showed the presence of possibly novel hypolimnion dwelling bacterivorous flagellates (e.g. cercozoans, choanoflagellates and telonemids).

The seasonal changes in vertical abundance of kinetoplastids were similar to that of CL500-11 bacterium which suggested that similar environmental conditions may favor the growths of those two microorganisms in Lake Biwa. Large size and curved shape of CL500-11 bacteria might protect them from grazing and thus allow this group of bacteria to dominate in

the hypolimnion of Lake Biwa. However, some flagellates, especially bodonids which belong to kinetoplastids, are capable of feeding on large bacteria. Therefore kinetoplastids might have an advantage over other flagellates in the hypolimnion due to their ability to feed on large bacteria dominant in the deeper layers of Lake Biwa.

So, the results indicate the presence of unique microbial loop in the hypolimnion of Lake Biwa, where humic-like DOM is produced by the hypolimnion bacterial assemblages, and those bacteria are grazed by the dominant kinetoplastids and other hypolimnion dwelling bacterivorous protists. Thus, the deep waters of Lake Biwa harbor active microbial loop consisting of novel hypolimnion-specific groups, which might play an important role in the production of refractory DOM.

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ECOLOGY, BIODIVERSITY AND HUMAN WELFARE

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Abstract

The paper discusses the interaction between ecology, biodiversity and human welfare. Mountain forest zonation and environmental science research studies of the author from the Cordillera mountain ranges, southern Luzon mountains, Cebu remnant forests in comparison with each other. One common characteristic in these mountains is the general ascent of lower altitude dominants once higher altitudes underwent disturbances like deforestation. The pines of the Cordillera encroached and dominated higher altitudes after oaks were cut. Definitely, there is reduction of ecosystem services with the destruction of oaks. Sound ecology means meaningful human-nature interaction, resulting to rich biodiversity and sustainable ecosystem services enhancing human wellbeing amidst changing climatic regime. Hence, there is a need to have an in-depth study of the general trend and zonation pattern in Philippine mountains to come up with appropriate ecosystem landscape management strategies to have sound ecology and healthy environment. A number of strategies are discussed to enhance sustainability and resiliency. These include land use planning, establishing landscape corridors, and an effective community biodiversity education among others.

Keywords: ecology, ecosystem services, landscape, resiliency, land use, climate.

1. Introduction

Ecology is becoming a more important and popular science as never before. This is because, a number of people have now recognized its significant contribution to biodiversity science and biodiversity conservation and hence, ecosystem services for humanity and society.

It was in 1869 that Haeckel first coined the term ecology from *oikos* and *logos* which means the *study of the home*. Since then, ecology has always been associated with the study of relationships and interactions among organisms and between the organisms and their environment to ensure a beneficial or mutual relationship and interaction that would lead to stable environment and abundant ecosystem or environmental services. This is especially a concern with the yet unresolved issue on climate changes causing damage and potential damage to many organisms and habitats of organisms.

The main purpose of this paper is to clarify the relationship of a sound ecology to biodiversity and human welfare through some cases.

2. Methods and Framework

Data from the past studies of Buot and colleagues [2] [3] [7] [6] [4] [8] [9] [13] [15] were examined. A framework (Fig. 1) can be used to illustrate the relationship and interaction of the various environmental components that are into play to have environmental health and abundant ecosystem services for the welfare of humans. The interlocking components imply that each component should move in concert or in similar direction with the rest, otherwise, there would be confusion and disharmony causing ecosystem or environmental health problems and remarkably reducing ecosystem services for the human communities.

Figure 1. Framework illustrating the relationship of the various environmental components of ecosystem or environmental health.

Table 1. Woody vegetation zones of Mount Pulag, Benguet, Philippines (Buot 2014).

Zones	Elevation (meters above sea level, masl)	Dominant woody vegetation
ZONE I	2000-2400	*Pinus-Deutzia-Schefflera*
Sub-zone IA	2000-2300	*Pinus*
Sub-zone IB	2300-2400	*Pinus-Deutzia-Schefflera*
ZONE II	2400-2600	*Syzygium-Leptospermum-Eurya-Dacrycarpus-Lithocarpus*
ZONE III	2600-2700	*Rhododendron-Clethra-Eurya*

3. Results and Discussion

Results of field zonation studies showed anomalous vegetation zonation on Mount Pulag (Table 1), Luzon's highest mountain peak (2924 meters above sea level), Mount Akiki (2760 meters above sea level) (Table 2), Mount Mayon (2400 meters above sea level) (Table 3) and Mount Makiling (1100 meters above sea level) (Fig. 2).

It is very clear in the tables and figure that lower elevation dominant species are encroaching into the higher altitudes replacing the original dominants. The lower dominants extend distribution range and adapt to the agroclimatic conditions of the upper slopes. The agroclimatic conditions of the upper elevations have greatly changed following disturbances such as logging, shifting cultivation, natural calamities triggering landslides and gully formation. Fig. 3 illustrates the summary of events after the destruction of the upper slope dominants.

It is very clear that when the oak forests of Mt Pulag and Mt Akiki had been destroyed to give way to vegetable farms, the microclimate had changed. And when the farmer left the farm after the soil had been depleted of the nutrients, oaks will not regenerate anymore obviously due to the infertile soil

Table 2. Woody vegetation zones of Mount Akiki, Benguet, Philippines (Buot 2014).

Zones	Elevation (meters above sea level, masl)	Dominant woody vegetation
ZONE I	1685-2640	*Pinus-Lithocarpus-Deutzia-Leptospermum*
Sub-zone IA	1685-2200	*Pinus*
Sub-zone IB	2350-2640	*Pinus-Lithocarpus-Deutzia Leptospermum*
ZONE II	2300- 2500	*Eurya-Lithocarpus*
ZONE III	2700-2750	*Drimys-Eurya-Rhododendron-Leptospermum*

condition and a warmer climate. Oaks would need a fertile soil and a favorably lower temperature togrow and survive. The steep slopes of the Cordillera would allow only the pine to colonize and dominate the oak zone (Zone II for both Mt Pulag and Mt Akiki). Once the pines established in the

area, there is no more chance for the oaks to recolonize.

Table 3. Woody vegetation zones of Mount Mayon, Albay, Philippines (Buot 2014).

Zones	Elevation (meters above sea level, masl)	Dominant woody vegetation
ZONE I	500 - 800	*Erythrina-Ficus-Astronia-Glochidion*
ZONE II	900-1500	*Astronia-Cyathea-Weinmannia*
ZONE III	1600-2200	*Eurya-Clethra-Neonauclea-Fagraea-*
		Vaccinium

This had happened to Mt Mayon and Mt Makiling as well. Frequent eruptions of Mt Mayon (every 6-10 years) already is a stress for the vegetation [3]. Additionally, the local community does the usual swidden farming in the lower slopes and collect plant biota throughout the altitudinal range of Mt Mayon. These are sold to tourists visiting Mt Mayon everyday. Indeed, Mt Mayon is experiencing both natural and anthropogenic disturbances to a large scale.

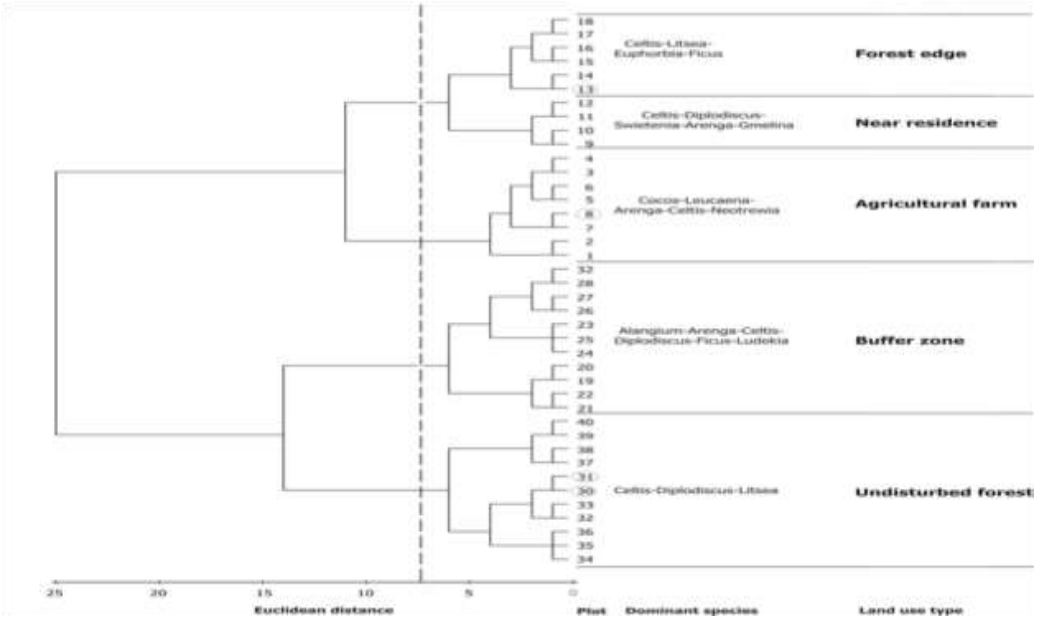

Figure 2. Vegetation zones on Mount Makiling showing encroachment of the dominating *Diplodiscus* [8]

Mt Makiling, though managed by the University of the Philippines Los Banos, yet, there were lots of disturbances too. The logging during the war in the 1940s [14] still has a profound impact on the original vegetation described by Brown [1]. [8] [12] found out that the native dipterocarps are still struggling to recuperate in the wilderness. The native species were cut during the war and natural regeneration is very slow. They are occupying the low DBH classes until now. The University of the Philippines is trying its best to manage the ecology well to facilitate regeneration of the

native species with the goal of restoring the abundant ecological services for the surrounding human communitiesthat had been diminished considerably. Same is true to Mt Tabunan and Aborlan Guba System in Palawan.

4. Research Direction

1. *Succession studies.* There is a need to have an in-depth study of the process of succession in heavily disturbed habitats. This could pave better understanding of the anomalous ascent of lower dominants upon destruction of upper elevation species (Fig. 3).

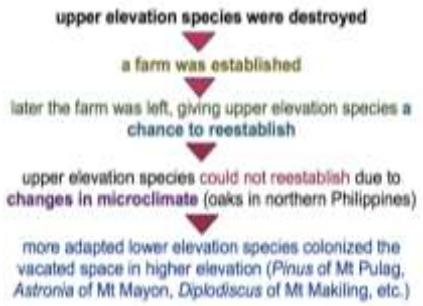

Figure 3. Flow diagram indicating the summary of events after the destruction of uppler elevation dominants in Philippine mountains.

2. *Establishment of a biodiversity corridor*. Past landscapes were fragmented due to natural or anthropogenic disturbances especially in the uplands (Fig. 3). This fragmentationhas created a barrier on the movement of species from one fragment to the other. Hence, there is a necessity to connect these isolated fragments through the establishment of biodiversity corridor network. In the biodiversity corridor, we encourage the planting of native species of both fragments being linked or connected. In doing so, the species in the fragments now, will not be alienated as they start moving from one fragment to another fragment through the newly created biodiversity corridor.

3. *Community land use planning. After a massive* biodiversity education, then a community land use planning and zoning by the local communities (Fig.4) should be done next. The community should be guided and empowered to do the land use planning and zoning by themselves. In this way alone, we can hope for a sustainable land use plan implementation by the locals.

Figure 4. Community land use planning and zoning.

Figure 5. Biodiversity education among adults, youths and kids.

4. *Biodiversity education.*Massive biodiversity education at the community level is important (Fig. 4). This biodiversity education should not be among adults only. It has to be involving the youth and even the kids as well. We found out that there is a need for kids to be involved in the process. Once everyone know the real importance, then they should be willing to help.

5. *Strict enforcement of existing environmentallaws and ordinances*. There is a need for a strict

implementation of existing environmental laws and ordinances. A very strong political will even at the local level is extremely necessary before we can even hope for a success.

6. *Network and collaboration*. Local, regional and international collaboration are a must ingredients. Through these networks, we can have interdisciplinary and transdisciplinary discussions that can help everyone gain insights in solving local challenges. Finally, there is an urgent need to have intergenerational cooperation. This will encourage the young to participate and ensure sustainability of our plans and programs towards sound ecology and ecosystem services for the benefit of human society.

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ISOLATION AND BIOASSAY OF PHOSPHATE BIOFERTILIZER FOR MAIZE

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Abstract

Phosphate biofertilizer is a low cost and environmentally friendly of tropical bioresources for increasing the P availability, soil health, the fertilizer efficiency and the productivity of maize on acid soils. The phosphate Solubilizing Rhizobacteria (PSR) were screened and isolated from agricultural soils (maize rhizosphere) and natural forest ecosystem. Laboratory work and green experiment has been conducted to selected the best of PSR isolate and to investigate the response of maize to PSR as inoculant for P biofertilizer. Based on the phosphate solubility and phosphate activity were isolated five of PSR isolates. The bioassay test of the five isolates using the maize seedling until 21 days were obtained three isolates of PSR that most potential for P biofertilizers which have capability in improving plant root length and ratio of plant-root dry weight of maize seedling.

Keywords: bioassay, biofertilizer, phosphate solubilizing rhizobacteria (PSR), bioresources, maize.

1. Introduction

Maize is one of the important food crops that have strategic role and high economic value in Indonesia, because maize is a source of carbohydrate and protein after rice. The demand for maize is increasing continually due to the increasingly diverse use of maize for food. In addition maize is also used for animal feed and others industrial sector and its demand has reached 50% of national needs (Directorate General of Food of Indonesia, 2015). On the other hand, The average national productivity of maize in 2014 is about 4.8 ton ha^{-1} with harvested area 3,84 million ha. The productivity by 2015 was increased to 5.1 ton ha^{-1} but the crop harvested area was reduced to 3.79 million ha. The data of the last 5 year indicated that the maize harvested is decreasing about 1.31% yearly (Bureau of Indonesia Statistic, 2016).

Since the adoption of green revolution, the effort to boost the maize productivity are highly depend on the intensive uses of inorganic fertilizers and other agrochemical products. Consequently, not only increased the productivity significantly, but has accelerated the land degradation and caused environmental problems. Mostly of agricultural has been deprecated and exhausted. About 90% of dry land has belong to marginal soils and categorized as sick soils which is low organic carbon and high acidity [1,2]. The dryland ecosystems are dominated by the Ultisols, Oxisols and Inceptisols and deals with the main constraints such as acid soil reaction, poor nutrient, retention of P, low organic matter content, high iron and aluminum content [3,4]. Consequently, the biofertilizers and ameliorant are needs to improve the nutrients status and soil productivity of the marginal soils [2,5].

Phosphorus (P) as an essentially major elements plays an important role for plants growth. The need of P fertilizers is increasing along other major elements, such as N and K. The dominant of P fertilizers produced and used in Indonesia are (triple superphosphate, 46 percent P_2O_5). More recently Indonesia replaced

TSP with SP-36 (superphosphate, 36 percent P_2O_5) and produced the compound fertilizer Ponska (15 percent N, 15 percent P_2O_5 and 15 percent K_2O [6]. In addition, more attention has been given to direct use of rock phosphat (RP) as low cost fertilizers contain about 28-32% of P_2O_5 but has a low solubility [7,8]. In addition, RP also the improve of chemical and physical properties and contain relative high of Ca that could contribute to plant nutrition [9].

Since the the last 3 decades, the use of beneficial microbes, known as phosphate solubilizing rhizobacteria (PSR) has gained more attention to improve the availability of fixed P, solubility of P and to promote the environmentally friendly agriculture [10-12]. Moreover, The PSR also produced phytohormone, such as Indole acetic acid, and gibberellin acid [13]. This research is focused on the selection and characterization of superior PSR isolates that potentially formulated as phosphate biofertilizer inoculant for maize. The use of PSR is expected to be able to increase the P fertilizer efficiency and maize productivity.

2. Materials and Methods

The experiment aimed to study the characteristic and ability of selected superior phosphate solubilizing rhizobacteria (PSR) that could be used as phosphate biofertilizer that has been conducted in laboratory and green house. Thirty of composite soils samples for the isolation of beneficial phosphate rhizobacteria were taken from five location of maize rhizosphere which are Majalengka, Tasikmalaya, Bandung, Garut, and Sumedang and natural virgin forest ecosystems in Garut District of West Java Province. The rhizobacteria were isolate using dilution methods and grown in plated agar contain Pikovskaya media. The bacteria that produced the holozone were isolated and subject to test the phosphate solubility, phosphatase activity, and bioassay. Five of superior PSR isolates that has largest of holozone diameter were selected and subjected to the characterization of PSR isolates, phosphatase activity, organic dissolved P, and the bio-assay using the maize seedling. The isolate of PSR were grown on agar plate contain the Pikovskaya media. Sebsequently, its colony form, total population, colour, diameter were recorded.

Activity of Phosphatase enzyme

The enzyme of phosphatase activity was determined according to Eivazi and Tabatabai method, by giving p-nitrophenyl substrate so that p-nitrophenol compound formed by enzyme activity was then stained by sodium hydroxide solution which could be detected by 400 nm spectrophotometer.

Bioassay of PSR Isolates

Bioassay PSR was done according to Murphy method. The experiment was arrange as randomiezed block design consisted six treatments (control and five of PSB isolate) and provided with 5 replication. The 100 mL reaction tube is filled with a 95 mL liquid Murphy medium. Corn seedlings sterilized with 0.2% $HgCl_2$ and 70% ethanol were added to petri dishes sterilized in sterile paper paper that had been moistened with sterile aquades. The Seeds of maize were added and germinated at 30^0C for 3 days. Sprouts are grown in the medium with the help of sterile gauze and buffer tubes and grown in screen house for 14 days. The growth of maize seedling, the content of phosphatase were measured and subject to the statistical analyses.

3. Results And Discussion

Characteristic of PSB Isolates

Based on the characteristics and the diameter of the holozone, has been selected 5 best phosphat solubilizing rhizobacteria (PSR) isolates for further testing of Activity of phosphatase, phosphate solubility and bioassay. The isolated bacteria has shown a different characteristic and ability to solubilize the insoluble P as indicated by the holozone (Table 1).

The five isolates of Phosphate Solubilizing Rhizobacteria (PSR) are J_3B, J_2G, J_3T, J_5H and J_1M have the largest diameter of holozone with the same characteristic of bacterial colony morphology as white opaque, convex, rough, circular, dull, rough.

Table 1 The capability to solubilize the insoluble P and characteristic of bacterial isolated from different rhizosphere

NO	Isolates Code	Ecossytem	Dilution	Holozone	Characteristic of Bacterial Colony Morphology
1.	J1B	Maize	10^8	+++	White opaque, convex, rough, circular, dull, rough
2.	J2B	Maize	10^8	+++	White opaque, convex, rough, circular, dull, rough
3.	J3B	Maize	10^7	++++	White opaque, convex, rough, circular, dull, rough
4.	J4B	Maize	10^7	+++	White opaque, convex, rough, circular, dull, rough
5.	J5B	Maize	10^8	++	White opaque, convex, rough, irregular, dull, rough.
6.	J1S	Maize	10^8	++	White opaque, convex, rough, irregular, dull, rough.
7.	J2S	Maize	10^8	++	White opaque, convex, rough, irregular, dull, rough.
8.	J3S	Maize	10^8	+++	White opaque, convex, rough, irregular, dull, rough.
9.	J4S	Maize	10^7	++	White opaque, convex, rough, irregular, dull, rough.
10.	J5S	Maize	10^7	+++	White opaque, convex, rough, circular, dull, rough
11.	J1G	Maize	10^7	+++	White opaque, convex, rough, irregular, dull, rough.
12.	J2G	Maize	10^8	++	White opaque, convex, rough, circular, dull, rough
13.	J3G	Maize	10^8	++++	White opaque, convex, rough, circular, dull, rough
14.	J4G	Maize	10^7	++	White opaque, convex, rough, circular, dull, rough
15.	J5G	Maize	10^7	++	White opaque, convex, rough, irregular, dull, rough.
16.	J1T	Maize	10^8	++	White opaque, convex, rough, circular, dull, rough
17.	J2T	Maize	10^8	++	White opaque, convex, rough, circular, dull, rough
18.	J3T	Maize	10^7	++	White opaque, convex, rough, circular, dull, rough
19.	J4T	Maize	10^8	+++	White opaque, convex, rough, irregular, dull, rough.
20.	J5T	Maize	10^7	++	White opaque, convex, rough, circular, dull, rough
21.	J1M	Maize	10^7	++	White opaque, convex, rough, irregular, dull, rough.
22.	J2M	Maize	10^8	+++	White opaque, convex, rough, irregular, dull, rough.
23.	J3M	Maize	10^7	+++	White opaque, convex, rough, irregular, dull, rough.
24.	J4M	Maize	10^8	+++	White opaque, convex, rough, circular, dull, rough
25.	J5M	Maize	10^7	++	White opaque, convex, rough, irregular, dull, rough.
26.	J1H	Natural forest	10^7	++	White opaque, convex, rough, circular, dull, rough
27.	J2H	Natural forest	10^7	++	White opaque, convex, rough, circular, dull, rough
28.	J3H	Natural forest	10^8	+++++	White opaque, convex, rough, circular, dull, rough
29.	J4H	Natural forest	10^7	+++	White opaque, convex, rough, irregular, dull, rough.
30.	J5H	Natural forest	10^8	++	White opaque, convex, rough, circular, dull, rough.

Biological Test (Bioassay), Activity of phosphatase and Dissolved P

Shoot and root dry weight were not different signficantly by PSR Isolate inoculation. In contrast, the shoot-root ration was influenced the PSR isolate (Table 2). Compared to control, the inculated tube with PSR isolate tend to decrease the shoot-roots ratio. The lower shoot –roots is indication that the growth of roots are increase.

The lowest shoot – root ratio was`obtained by J_3T and J_2G isolate. The dry weight ratio of the shoot-Root describes the development of the plant toward the canopy or root [3]. Seeds of maize inoculated with J_3T isolates tended to increase their root weight compared to canopy. In addition, the plant height was not affected significantly, but the roots lengts was influenced by PSR isolate (Table 3). The roost length was inrease about 68-242% compared to control. The longest roots was obtained by J5H isolate. Phytohormone and other growth subtances produced by PSR isolate promote the roots growth [10,11,13].

Furterer test revealed that PSR isolates has increased the content of phosphatase, and soluble P or dissolved P in the growth medium of maize (Table 3).

The highest phosphatase content was produced by J_3T, J_1M and J_5H. The ability to produce phosphatase enzymes depends on the type of PSR, biomass and energy source provided. According to Dinesh et al. (2000) microbial biomass and phosphatase enzyme activity depend on the energy sources present in the media. The production of phosphatase enzymes may increase after the addition of an energy source derived from organic matter.

Meanwhile, according Sarapatka (2002) phosphatase activity is strongly influenced by the content of nitrogen media. It is further explained that an increase in the nitrogen content of the medium may increase phosphatase activity in the medium. While the results of research Fitriatin et al. (2008) show that the pH of the medium affects phosphatase activity.

Microbial phosphate solubilizers secrete a number of organic acids, among others, formic acids, acetates, propionate, lactonate, glycolate, fumarate, lactate and succinate which can form chelates with cations such as Al and Fe so as to effect effective phosphate dissolution so that P becomes available And can be absorbed by plants [11,12]. The soluble P generated by the five isolates of BPF is significantly higher than that treated without PSB. Isolate J5H produces higher dissolved P than other isolates although the magnitude of the organic acid phosphatase enzyme it produces is similar to that of other isolates. The difference is suspected because the different organic acids produced so that the ability of these organic acids in mengkhelat P binders will be different too. Lactic acid is one type of organic acid produced by BPF, but still many other organic acids are suspected not the same quantity. According Whitelaw (2000), PSB can secrete organic acids that can form complex compounds that are difficult to dissolve. The formation of this complex compound will cause P fixation to decrease thus increasing P-available.

Table 2 Effect of PSR isolate on dry weight of shoots, dry weight of roots and shoot-roots ratio of maize seedling

PSB Isolates	Dry Weight of shoot (g)	Dry Weight of Roots (g)	shoott-Root Weight Ratio
Control (untreated)	0.12 a	0.06 a	2.00 c
J_3T	0.11 a	0.07 a	1.57 a
J_1M	0.12 a	0.07 a	1.71 b
J_5H	0.12 a	0.07 a	1.71 b
J_3B	0.13 a	0.07 a	1.85 bc
J_2G	0.10 a	0.06 a	1.66 ab

Average value followed by the same letter within column is not different significantly according to DMRT (P = 0.05)

Table 3 Effect of PSB isolate on plant height and root length in maize for 14 days

Treatments	Plant Height (cm)	Root Length (cm)
Control	18,50 a	3,50 a
J_3T	19,90 a	6,50 b
J_1M	18,90 a	6,50 b
J_5H	19,50 a	8,50 c
J_3B	19,00 a	5,90 ab
J_2G	19,50 a	6,50 b

Average value followed by the same letter within column are not different significantly according to DMRT (P = 0.05).

Table 4 Effect of PSB inoculation on content of phosphatase and dissolved P in Pikovskaya media

Treatments	Phosphatase (mg/mL)	Dissolved P (ppm)
Control	0,01 a	20,24 a
J_3T	0,62 c	44,50 b
J_1M	0,51 c	30,09 b
J_5H	0,63 c	35,53 b
J_3B	0,19 b	31,54 b
J_2G	0,11 b	29,19 ab

Average value followed by the same letter within column are not different significantly according to DMRT (P = 0.05).

4. Conclusion

The five selected of PSR isolates had shown a different characteristic and ability to improve the solubility of P. Based on the phosphatase activity were obatined three isolate of PSR the most potential isolates that could be used for the formulation of phosphate biofertilizers. The isolate of J_3T, J_1M and J_5H were the superior isolate in a liquid culture with maize. These Isolates were superior in resulting the phosphatase content and dissolved P.

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IDENTIFICATION OF OSMOPHILIC YEASTS ISOLATED FROM MOLASSES SUGARCANE BIOETHANOL AS STARTER

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Abstract

Osmophilic yeasts was second generation to increase of the bioethanol production. The yeasts were isolated from the high sugar ontain material like honey, juice or by-product materials such as molasses. Bioethanol was usually produced from sugarcane molasses at brix degree of 14%. The aim of the research Reviews These were isolation and identification of phenotypic identification of indigenous-molasses yeast. A phenotypic characteristics were based on typical colony, morphological and fermentation profile. The yeasts were isolated from original molasses (> 80% brix) on malt extract agar (MEA). There were two isolates of osmophilic yeasts. Isolate A regular was clear white colony, sharpe cell with multilateral budding, while isolate B was murky white colony regular, cylindrical cell with multilateral budding. Isolate A and B optimum grown at 30°C and produce high ethanol at 34° Brix but the pH of the growth of yeast were difference. Isolate A growth optimum at pH 5, while isolate B optimum growth at pH 4. The fermentation profile using the API 20C Aux kit Showed that the yeasts can Ferment glucose, glycerol, calcium-2-keto-gluconate, arabinose, adonitol, galactose, sorbitol, methyl-αD-glucopiranoside, N-acetyl-glucosamine, Cellobiose, maltose, sacharose, trehalose, and raffinose. Isolate A Ferment can inositol and melezitose, while isolate B can Ferment xylose and xylitol. Isolate A was identified as *Candida famata* (63.2%) and B isolates was identified as *Candida guilliermondii* (84.3%).

Keywords: osmophilic yeast, blackstrap sugarcane, bioethanol fermentation

1. Introduction

Molasses used as a medium for the production of commercial alcohol on alcoholic fermentation industry, because it is easily obtained widely, cheaply and as the quality of raw materials [1]. Industry uses a substrate with a sugar concentration is 16-18%, if the sugar concentration is higher than 18% will cause osmotic pressure which reduces the efficiency of the fermentation process [2].

In the bioethanol industry, the sugar concentration on the substrate used is 16-18%, and if it is higher than 18% will cause osmotic pressure which reduces the efficiency of the fermentation process, so as to isolate the species or strain of yeast osmofilik origin of molasses that are resistant to high osmotic pressure by characterizing the morphology, physiology and molecular identification. Several studies have isolation of yeast osmofilik of Indonesian food such as honey, milk and fruit jams, obtained yeast isspecies *Candida metapsilosis*, C. *etchelsii*, C. *parapsilosis*, C. *orthopsilosis* and *Sterigmatomyces halophilus* [3].

This study aimed to isolate the origin osmofilik molasses yeast, characterize morphology and physiology as well as identify the molecular origin of yeast osmofilik molasses. Yeast osmofilik molasses origin was expected to be applied to the production of bioethanol with high brix sugar cane molasses, or more than 14 °. It is hoped that the yeast will have a good

adaptability to the production of molasses alcohol with the substrate.

2. Materials and Methods

Molasses was take from PG. Jatiroto sugarcane milling at season 2015. The molasses was preparated by taking a sufficiently concentrated molasses and molasses brix measured 14 °, 24 ° and 34 ° using a refractometer. Molasses brix 34° was made of 200 ml of concentrated molasses and diluted with 350 mL of distilled water. Molasses brix 24° was made of 200 ml of concentrated and diluted with 555 mL of distilled water. Molasses brix 14° was made of 200 ml of concentrated and diluted with 935 mL of distilled water.

Isolation Yeast

Isolation of molasses indigenous yeasts was carried out by taking 1 ml of concentrated molasses and spread on a solid MEA media with dispersive method at 2 petridish. Every isolates were grown from each petridish, purified on MEA solid media with a 3x quadrant scratch method. Isolates were purified, scratched back on a slanted MEA media as saving culture and work culture.

Morphological Characterization

Morphological characterization was done by macroscopic and microscopic observation. Macroscopic observation was conducted by observing the yeast colonies that grow directly on the surface of the MEA media, including colony shape and color of the colony. Microscopic observations done by creating a wet mount. Wet mount was made to fix the yeast cells on a *glass* object,given thedye, *crystal violet*and do repainting with a mordant. The final stage, was observed under a microscope magnification of 1000X with the addition of immersion oil and covered with a *cover*glass. Microscopic observations made include cell shape, presence *cell budding* and type of *budding* cell.

Physiology Characterization

Physiological characterization of yeast was conducted on the test temperature and pH growth, endurance test molasses brix grow at 14°, 24° and 34°, and analyse of alcohol production. Profile of fermentation was analyzed by using API 20C-Aux Kit.

a) Test of Yeast Growth Temperatures

This test was performed by taking 0.03 ml of 3 ml of yeast culture MEB age of 48 hours, then inoculated in 1 ml of sterile MEB media. Each yeast culture was incubated at different temperatures: 10 ° C, 20 ° C, 30 ° C, 40 ° C and 50 ° C for 48 hours. Observations yeast growth at any temperature, was done by measuring the absorbance values of each yeast culture using a spectrophotometer at OD 600 nm on incubation time of 12 hours, 24 hours, 36 hours and 48 hours.

b) Test of Yeast Growth pH

This test was performed by taking 0.03 ml of 3 ml of yeast culture MEB age of 48 hours, then inoculated in 1 ml of sterile MEB medium with a pH of 3, 4, 5, 6. Each yeast culture was incubated at 30 ° C for 48 hours. Observation of yeast growth on any media pH MEB, carried out by measuring the absorbance values of each yeast culture using a spectrophotometer at OD 600 nm on incubation time of 12 hours, 24 hours, 36 hours and 48 hours.

c) Test of Yeast Growth on Molasses at Brix 14 °, 24 ° and 34 °

Test was done by taking a 0.03 mL of work culture of yeast in 3 ml MEB age of 48 hours, then inoculated in 3 ml of sterile molasses brix 14 °, 24 ° and 34, and incubated at 30 ° C for 24 hours and 48 hours. Growing endurance test was conducted using the scatter. On the control, yeast culture in 3 ml MEB life of 48 hours, inoculated in 3 ml physiological saline. Furthermore, the yeast culture was taken as 1 ml and diluted in 9 ml physiological saline solution at a dilution series of 10^{-1} to 10^{-7}. Yeast culture (age of 24 hours and 48 hours) was taken as 1 ml and inoculated at 9 ml physiological saline solution and diluted with a series of 10^{-1} to 10^{-7}. Dilution series of 10^{-4},10^{-5},10^{-6} and 10^{-7} in the control and treatment groups was taken each of 1 ml, plated on solid MEA medium and incubated at 30 ° C for 48 hours. Yeast colonies growing on each plate, count the number of

colonies compared to the control (Log CFU / ml).

d) Fermentation Test Pattern

This test was performed using the API 20C-Aux kit, consisting of 20 wells were wells first as a negative control (O), pitting both as a positive control containing glucose (GLU), and 18 sinks more were glycerol (Gly), calcium 2-keto-gluconate (2KG), arabinose (ARA), xylose (XYL), adonitol (ADO), xylitol (XLT), galactose (GAL), inositol (INO), sorbitol (SOR), methyl-αD-glucopyranoside (MDG), N- acetyl-Glucosamine (NAG), Cellobiose (CEL), lactose (LAC), maltose (MAL), saccharose (SAC), trehalose (TRE), melezitose (MLZ) and raffinose (RAF). Fermentation profile was performed by standard procedures. Observations pattern of fermentation after 48 hours and 72 hours was based on whether or not the substrate turbid sugar on pitting. If the media becomes cloudy, it mean a positive reaction and if the media was clear (not cloudy), it mean negative reaction. The results were written on a *sheet* result, accumulated and acquired on 7 digit biocode. The code was compared with the identification book or dikonfimasi on apiweb™ (https: //apiweb.biomerieux.com//) to identify the type of yeast.

3. Results and Discussion

Morphological Characteristics

Isolation of osmophilic-molasses indigenous yeasts, obtained two isolates that isolates 1 on the first petridish was coded A and isolates 2 on the second petridish was coded B. Purification of isolates was conducted by using quadrant scratch with three times. Single colony was purified again using the scratch method as savings culture and work culture.

Macroscopic characteristics of yeast was based on the appearance of colonies grown on a solid medium, including textures, shapes, colors, margins, elevation and the surface of colony. Microscopic charactertics included cell shape, cell size, formation of *budding,* sprouting type *(budding cell),*and the presence or absence pseudohyphae/hyphae [4,5]. Microscopic observations used observation under the microscope Leica DM 2500, 1000x magnification by addition of emersi oil. Macroscopic and microscopic characters of yeast isolates A and B were shown in Table 1.

Microscopic characteristics of isolate A were round cell shape, cell budding with multilateral type. While the microscopic characteristics of isolate B were cylindrical cells, cell budding with multilateral type. Microscopic characteristics of isolate A and B were shown in Figure 1.

Physiology Characterization

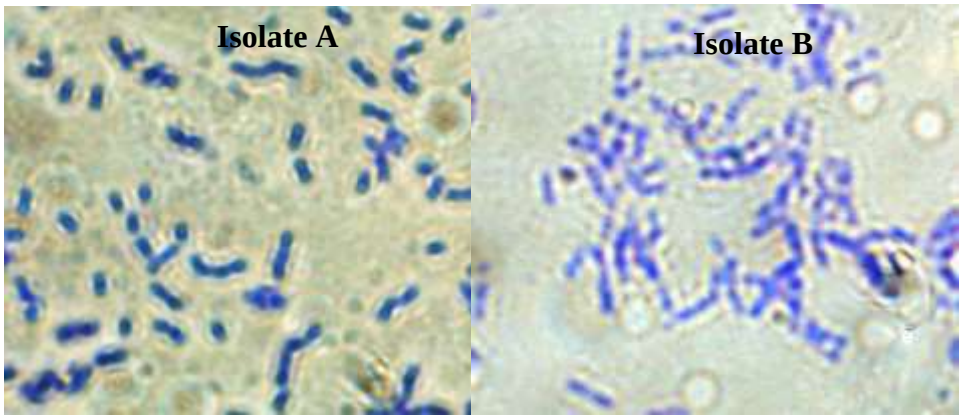
The isolate A can good growth at 20 - 30°C, while the isolate B can good growth at 30-40°C, but the both of isolate can optimum growth at 30°C. Fardiaz reported that the optimum growth at 25-30°C [6]. Figure 2 and Figure 3 showed the growth of yeasts isolate A and B at some growth temperature (20°C, 30°C, 40°C, 50°C).

The yeasts isolates A and B able to growth at pH 3, 4, 5, 6 (Figure 4 and Figure 5). Both isolates had the optimum growth pH i.e pH 5. Prescott and Dunn reported that the yeast growth pH was between 3.0 to 6.0 [7].

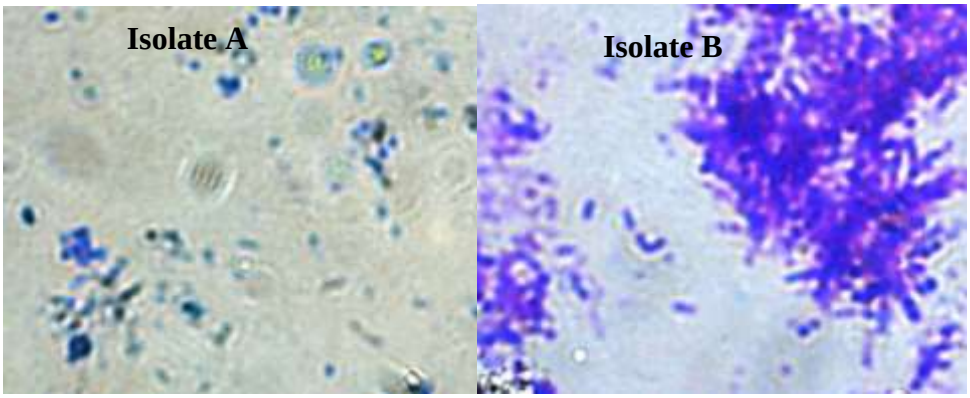
The isolate A and isolate B were able to grow on molasses brix 14 °, 24 ° and 34 °. The highest yeast population were at 34 ° brix molasses i.e 6.91 LogsCFU/ml for isolate A at 48 h incubation and 6.27 LogsCFU/ml for isolate B at 24 h incubation. The yeast population at 24 ° molasses brix were 6.24 log CFU/ml for isolate A at 48 h incubation and 6.23 log CFU/ml for isolate B at 24 h incubation.

Table 1 Morphological characteristics of A and B isolates

Isolates Yeast	Macroscopic characteristics		Microscopic characteristics		
	Shape of colonies	Colonies color	Cells form	Budding	budding type
A	Bulat	White	Round	There	Multilateral
B	Round	White	Cylinders	There	Multilateral



Isolate after 20 h incubation



Isolate after 40 h incubation

Figure 1. Microscopic characteristics of isolate A and isolate B after 20 h and 40 h incubation

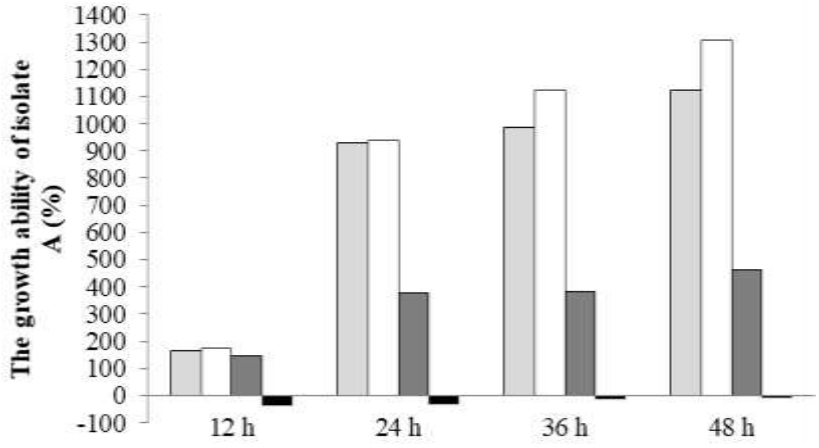


Figure 2. The growth ability of isolate A on MEB medium at at temperature: 20°C (□), 30°C(□),40°C(■) and 50 ° C(■).

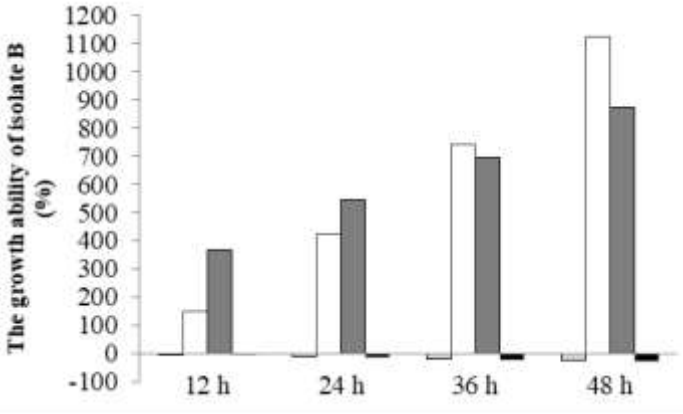


Figure 3. The growth ability of of yeast isolate B on MEB medium at temperature: 20°C (▭), 30°C (▭),40°C (▭) and 50 ° C (▭).

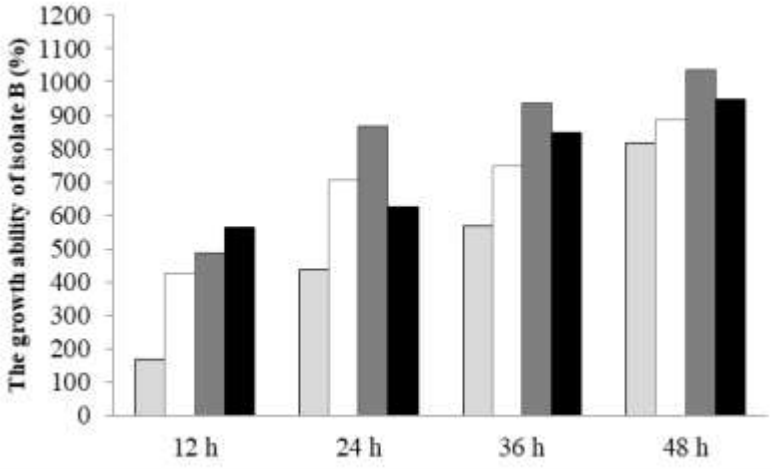


Figure 4. The growth ability of of yeast isolate A on MEB medium at pH: 3(▭),4 (▭), 5 (▭), and 6 (▭).

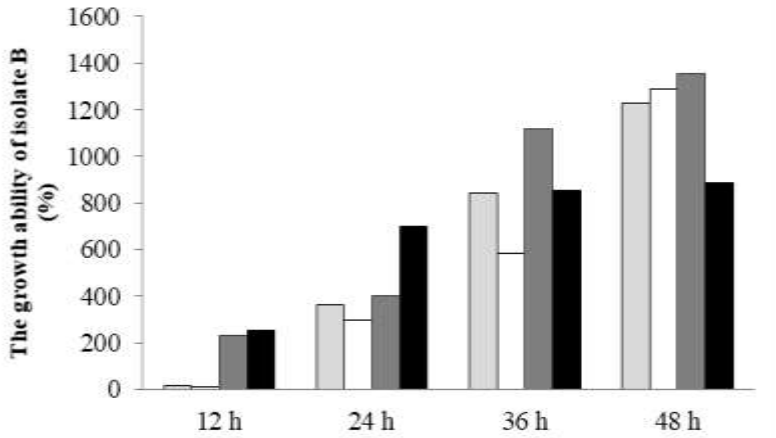


Figure 5. The growth ability of of yeast isolate B on MEB medium at pH: 3°C (▭),4°C (▭), 40°C (▭) and 50 ° C(▭).

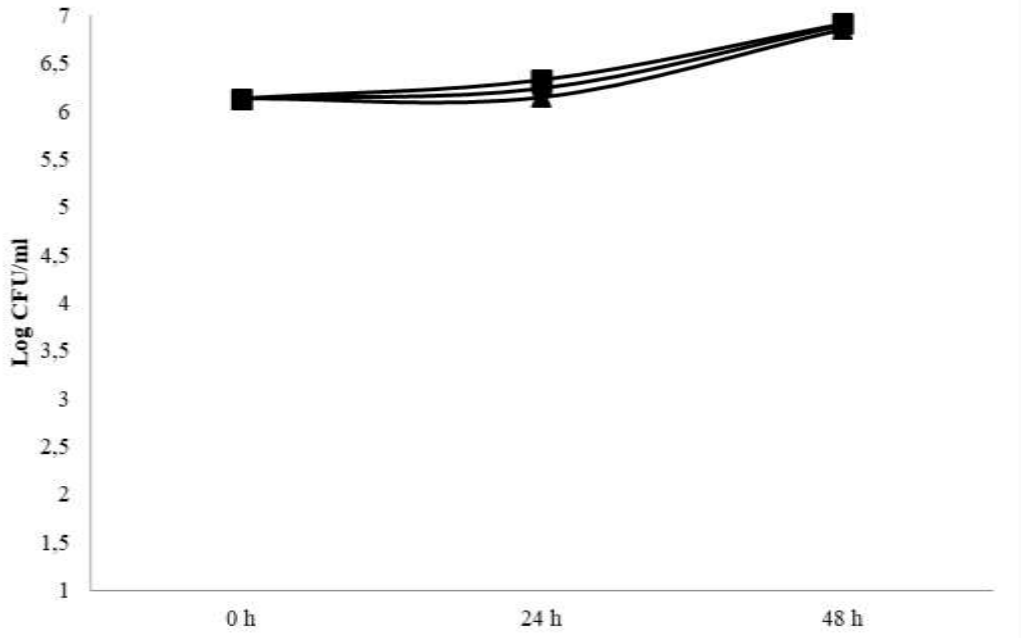


Figure 6. The growth ability of isolate A media molasses brix: 14 ° (▲), 24 ° (●), 34 ° (■) for 24 h and 48 h incubation

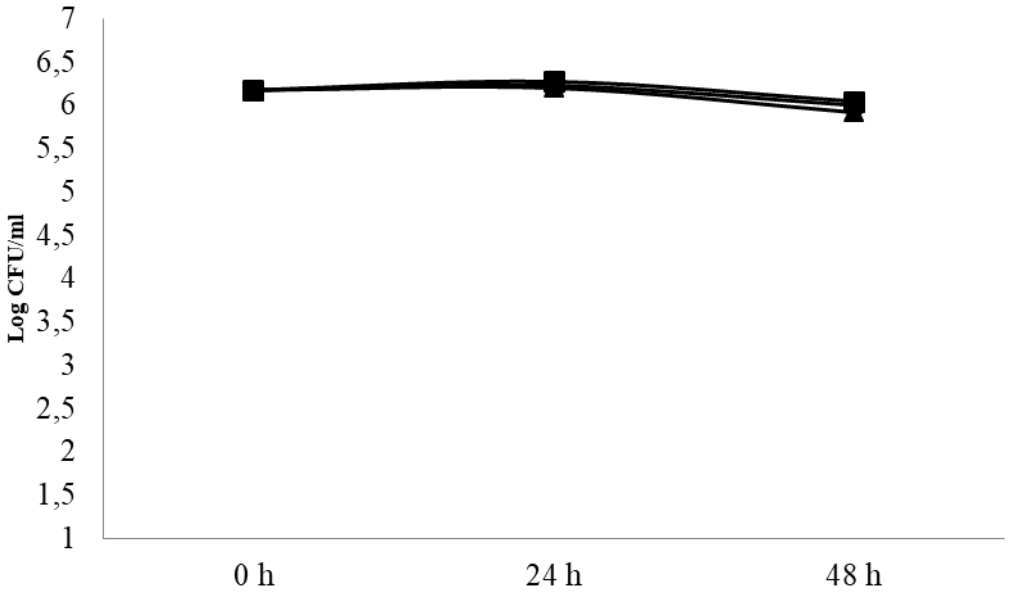


Figure 7. The growth ability of isolate B media molasses brix: 14 ° (▲), 24 ° (●), 34 ° (■) for 24 h and 48 h incubation

The yeast population at 14 ° molasses brix were 6.86 log CFU/ml for isolate A at 48 h incubation and 6.20 log CFU/ml for isolate B at 24 h incubation. Isolates A and B the highest growth at 34° brix molasses, because it has the highest sugar concentration than molasses brix 14° and 24°. It indicated that the isolate A and B were osmophilic yeast.

Isolates A increased the highest growth at 48-hour incubation. That was in accordance with the fermentation time needed for fermentation is 2-3 days. Isolates B increased the highest growth at 24-hour incubation. After 20 hours, it was optimum use of sugar by yeast to produce the primary metabolite (ethanol). The ability to grow isolates A and B on molasses brix 14°, 24° and 34° can be reached 6,17 LogCFU/ml (0 hours), resulting in an increase to the highest growth (3.15%) for isolate A and (1.60%) for isolate B (Figure 6 and Figure 7).

Evaluation of yeast profile fermentation were determined by incubate the isolates at 20 C Aux API kit for 72 hours. A and B isolates were grown on 18 kinds of media on the strip kit API 20C Aux. Results obtained in the form of negative or positive reactions, indicated by cloudy or absence of media after 72 hours of incubation. A positive reaction, if the isolates were inoculated make media becomes cloudy, it means that isolates A or B can ferment the carbon source in the kit substrate and the negative reactions if the kit substrate was clear which means the yeast isolate was not able to ferment the kit substrate. The result showed that isolates A can ferment some sugars such as GLU, Gly, 2KG, ARA, ADO, GAL, INO, SOR, MDG, NAG, CEL, MAL, SAC, TRE, MLZ, RAF on Aux kit API 20C, so that the reaction is positive (+). Isolates A can not ferment kind gulaseperti XYL, XLT and LAC on the kit API 20C Aux, and have no *hyphae* or *pseudohyphae*,so that the reaction is negative (-) (Table 2).

Table 2. The fermentation profile of isolates A in Auxkit API 20C

CODE	Sugar type kit API 20C Aux																				
	O	GLU	Gly	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	H/PH
A	-	+	+	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	-

Results of a positive or negative reaction of the isolates A, was determined to be 7 biocode i.e 6 3 5 7 3 7 3 after incubation for 72 hours. The code was a code to identify isolate A. Code 6 3 5 7 3 7 3 confirmed on programe webto determine the species of osmophilic yeast. The identification result sreported that isolate A was *Candida famata* (with identify confidence 63.2%). According to [8], carbon assimilation test for C. *famata* that was positive assimilate glucose, galactose, maltose, sucrose, trehalose, D-xylose, melezitose, glycerol, raffinose, Cellobiose, L-arabinose; possibility assimilate lactose, starch dissolved, L-rhamnose, D-arabinose; can not assimilate potassium nitrate and inositol. According to [9], *C. famata* isolated from palm wine beverage, characterization morphological and biochemical tests. The results obtained by the *Candida famata* can assimilate D-glucose, D-galactose, D-xylose, sucrose, maltose, α, αTrehalose, Cellobiose, lactose, raffinose, and Glycerol; while unable to assimilate Myo-inositol, 2-Keto-D-glucose, starch and L-ramnose. Isolates A has some similarities with the characteristic C. *famata* above, which was able to assimilate D-glucose, D-galactose, maltose, sucrose, trehalose, melezitose, glycerol, raffinose, Cellobiose, L-arabinose, and did not have the *pseudohyphae* or *pseudomycellium.* The isolate A have the characteristics of some differences with C. *famata* above, isolates still incapable of assimilating inostitol and unable to assimilate D-xylose and lactose; while C. *famata*a can assimilate D-xylose and lactose; and can not assimilate inositol.

The isolate A was *doubtful profile* so isolate A still needed to be further identified by molecular identification. Conventional identification based on morphological, physiological and biochemical, can lead to misidentification especially in closely related species. The isolate A were identified only 63.2%, which still requires the identification of more accurate, and require the identification of yeast easily, quickly and accurately the method of molecular identification [10].

Table 3 showed that isolate B can ferment several types of sugars, such as GLU, Gly, 2KG, ARA, XYL, ADO, XLT, GAL, SOR, MDG, NAG, CEL, MAL, SAC, TRE, RAF the kit API 20C Aux, so that the reaction is positive (+). Isolates B can not ferment sugars, such as INO and LAC on the kit API 20C Aux and have no *hyphae* or *pseudohyphae*so that the reaction was negative(-).

Table 3. The fermentation profile of isolates B in Auxkit API 20C

CODE	kit API 20C sugar type Aux																				
	O	GLU	Gly	2KG	ARA	XYL	ADO	XLT	GAL	INO	SOR	MDG	NAG	CEL	LAC	MAL	SAC	TRE	MLZ	RAF	H/PH
B	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-

Results of positive or negative reaction from isolates B, was determined to be 7 biocode ie 6 7 7 6 3 7 3 after incubation for 72 hours. The results showed that isolate B was *Candida guilliermondii* (84.3%) with identifying information *excelent identificaton to the* genus. According to [11], species characterization *Candida* was performed using kit identification *Candida microexpres* a test for the assimilation and fermentation of sugar. One of the species *Candida* that characterized that C. *guilliermondii* can ferment melibose, sucrose, galactose, cellibiose, xylose, raffinosa and dulcitol, but can not utilize sugars such as lactose, maltose and inositol. Isolate B had some similarities with the characteristics of C. *guilliermondii* above, which was able to assimilate sucrose, galactose, cellibiose, xylose, raffinosa and can not assimilate lactose, inositol. Isolate B had differences with *C.guilliermondii* that assimilate maltose whereas the isolate B can not assimilate maltose. Conventional identification based on morphological, physiological and biochemical, can lead to misidentification especially in closely related species.

4. Conclusion

There were obtained two osmophilic yeast isolated from sugarcane molasses. Morphological characteristics of isolate A and B were round colonies, colony color white and rounded cell shape and type of multilateral budding; while isolate B had cylindrical cell shape and type of multilateral budding. Optimal physiological characteristics isolate A grew at 30 °C and pH 5; had the ability to grow on a substrate molasses brix 14 °, 24 ° and 34 °; and able to ferment glucose, glycerol, calcium-2-keto-gluconate, arabinose, adonitol, galactose, inositol, sorbitol, methyl-αD-glukopiranosida, N-acetyl-glucosamine, cellobiosa, maltose, sucrose, trehalose, melezitose, raffinosa. Isolate B grew optimally at 30 ° C and pH 5; had the ability to grow on a substrate molasses brix 14 °, 24 ° and 34 °; and able to ferment glucose, glycerol,calcium-2-keto-gluconate,arabinose, xylose, adanitol, xylitol, galactose, sorbitol, methyl-αD-glukopiranosida, N-acetil-glucosamine, cellobiosa, maltose, sucrose, trehalose, raffinosa. Isolate A was identified as *Candida famata* (63.2%) and isolate B was identified as *Candida guilliermondii* (84.3%).

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EFFECTS OF POTASSIUM FERTILIZER AND GIBERELLIN ON YIELD OF CHILI PEPPER *(Capsicum frutescens* L.*)*

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Abstract

Chili pepper (*Capsicum frutescens* L.) is one of horticulture commodities which is categorized in annual crop and has high economic value and widely developed in Indonesia and has an important role in the fulfillment of food. Production of this plant each year decreased one of them due to loss of flowers and fruit. One effort that can be done to increase the productivity of cayenne pepper is the addition of potassium fertilizer and gibberellin hormone is known to suppress the occurrence of flower and fruit loss. The purpose of this study is to obtain the dosage of potassium fertilizer and the appropriate concentration of gibberellin hormone to increase the productivity of pepper cayenne plants. This research was conducted in plastic house from June to December 2016, located in Tugusari Village, Bangsalsari district, Jember Regency, with Complete Random Design (RAL) pattern and repeated twice. The first factor is the fertilization of Potassium K0 (0 g KCl / plant), K1 (5 g KCl / plant), K2 (10 g KCl / plant), K3 (15 g KCl / plant). The second factor is G0 (0 ppm), G1 (50 ppm), G2 (100 ppm) and G3 (150 ppm), with doses of 20 ml / plant at the beginning of the flower, ie 40 HST (day after planting) and 40 ml / The plant at the beginning of the fruit pentil that appears 62 HST (day after planting). The results showed: (1) Combination treatment of potassium fertilizer 10 g/plant with Giberelin 100 ppm significantly affect the weight of harvest fruits; (2) Potassium Fertilizer 10 g/plant produces The highest crops, the greatest number of total flowers and the lowest percentage of the lowest flower; (3) The concentration of Giberellin 150 ppm The highest total interest and the lowest percentage of the fall.

Keywords: Chili pepper (Capsicum frutescens L.), potassium fertilizer, gibberellin , productivity.

1. Introduction

Chili pepper (*Capsicum frutescens* L.) is one of the most developed horticultural commodities in Indonesia, has an important role in the fulfillment of food, and has fluctuating economic value as well as the price of this commodity often increases due to the stock limitations caused by harvest failure [1].

The falling flower and fruit is a major problem in chili cultivation. The high rate of falling flowers and fruit makes production of chili decrease. Chili in a certain area of land will produce many flowers, but not all flowers can be formed into fruit; so that, the number of chilies will be reduced when they are harvested [2]. The efforts that can be made to increase the production of chili that is technological improvement including the fertilization and giving growth regulators (ZPT) [3]. Potassium is one of the nutrients needed in some plants in high enough quantities and can be used to reduce the occurrence of falling flowers and fruits. This element is easy to washing which causes the availability for low plants and has a weak bond, so it is easily replaced by other elements [4].

Gibberellin is one of ZPTs that can be used to prevent the occurrence falling flower and fruit in plants besides auxin [5]. Gibberellin (GA3) is a plant-growing hormone affecting to genetic properties, flowering, photosynthesis, and stems lengthening [6].

2. Materials and Methods

The research was conducted in plastic house from June to December 2016 which was located in Jember. The used materials included seeds of chili pepper (Bara variety), top soil, polybag, urea and TSP fertilizers, insecticide, KCl and GA3. This experiment was carried out factorially with the basic

design of Completely Randomized Design (CRD) consisting of two factors: potassium fertilizer (0g / plant (K0), 5g / plant (K1), 10g / plant (K2), 15g / plant (K3), and gibberellin (0 ppm (G0), 50 ppm (G1), 100 ppm (G2), 150 ppm (G3), so it was obtained 16 treatment combinations and repeated 2 times. The implemnetation of experiments included several stages, they were:

Sowing: it was done by using the small plastic bag as medium container. two seeds puted in medium.

***Making Media*;** Preparation of media was done at the beginning of the research by preparing the soil media having low potassium content and air dried. The media were inserted into 40 cm diametered polybag as many as approximately 15 kg.

***Transplanting*;** were done by making planting holes in polybag. 35 days aged seedling, had 4-6 leaves, where in one polybag.

***Plant Maintenance*;** it included urea fertilization as many as 3 times of application, SP-36 as many as once of application, and KCl as many as 3 times of application. The next maintenance was watering that was done in the morning or in the afternoon. When there was pest attact, pest control was performed by using chemical pesticides and weeding routinely. The application of gibberellins was done at the beginning of the flower and fruit valve appearing. Moreover, harvesting was done at 80 days after planting was done as many as 10 times of harvest with time interval of 5 days.

***The variables*;** they included plant height, number of leaves, flowering age, total flower, percentage of falling flowers, number of harvest fruits, and weight of harvest fruits.

3. Results

The experimental results on all observation variables are presented in Table 1 as following.

Table 1.Summary of F-Counts of all Observation Variables

No.	The Observation Variable	Nilai F-hitung		
		Kalium (K)	Giberelin (G)	(KxG)
1	The Plant Height	39,26 **	0,38 ns	0,14 ns
2	The Total Number of Flowers	36,14 **	3,49 *	0,61 ns
3	The Percentage of Falling Flowers	63,01 **	5,29 **	0,37 ns
4	The Number of Harvest Fruits	80,40**	5,18*	2,92*
5	The Weight of Harvest Fruits	55,88**	6,94**	2,62*

Note: ns = Not significant different; * = Significant different; ** = Highly significant different

Based on the summary of variance analysis on (Table 1), the interaction of potassium fertilizer (K) and gibberellin (G) treatments on the observation variables of the number of harvest fruits and the fruits weight at harvest showeda significantly different. Whereas the plant height, total flower and falling flower showed anot significantly different. Potassium fertilizer (K), it gave highly significantly different effect on the plant height, total flower, number of falling flower, the number of harvest fruits, and the weight of the harvest fruits. Gibberellin (G) application showedhighly significantly different effect on number of falling flowers, and it gave significantly different on the total number of flowers as well as it gave a not significantly different on plant height.

Table 2. Interaction Effect of Potassium and Gibberellin on The Weight of Fruits (g)

Gibberellin	Potassium			
	K0 (0 g)	K1 (5 g)	K2 (10 g)	K3 (15 g)
G0 (0 ppm)	171,00 c	219,00 b	246,50 a	239,00 ab
	C	C	C	A
G1 (50 ppm)	192,00 d	221,00 c	258,00 a	239,00 b
	B	BC	B	A
G2 (100 ppm)	204,50 d	229,00 c	270,00 a	239,00 b
	AB	B	AB	A
G3 (150 ppm)	213,00 d	243,50 b	262,50 a	234,00 c
	A	A	A	A

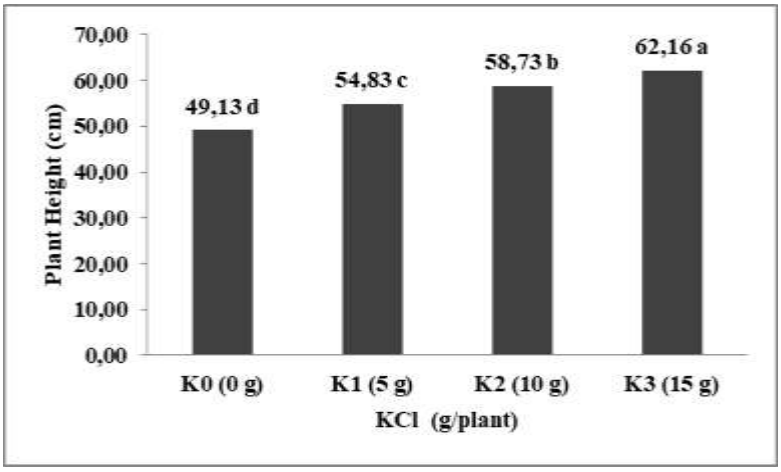
Based on Figure 1, it showed significantly different on the weight of harvested fruits which was the simple effect of factor K at the same G level, and G factor at the same K level showed that both treatments (Potassium 10 g/plant + Gibberellin 100 ppm) could increase the weight of the harvest fruits.

Tabel 3.Interaction Effect of Potassium and Gibberellin on The Number of Harvested Fruits

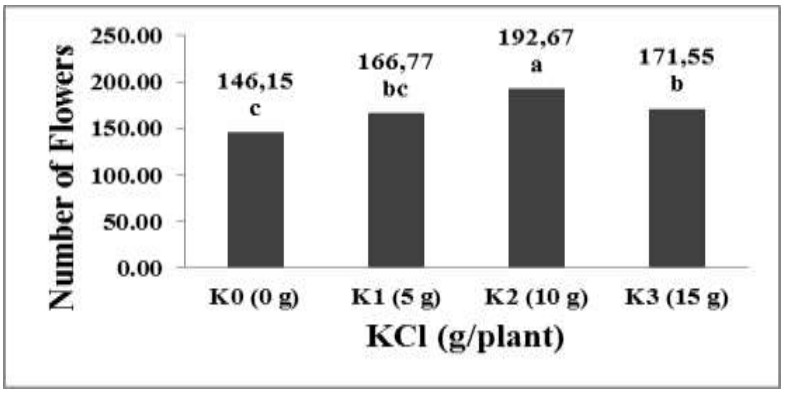
Gibberellin	Potassium							
	K0 (0 g)		K1 (5 g)		K2 (10 g)		K3 (15 g)	
G0 (0 ppm)	114,00	d	123,00	c	170,50	a	158,00	b
	B		B		A		A	
G1 (50 ppm)	123,00	d	141,00	c	174,50	a	159,50	b
	A		B		A		A	
G2 (100 ppm)	124,00	c	158,00	b	176,50	a	157,50	bc
	A		A		A		A	
G3 (150 ppm)	124,00	d	165,50	b	175,00	a	154,50	c
	A		A		A		A	

The simple effect of factor K at the same G level and factor G at the same K level (Table 3) indicated that the interaction of potassium and gibberellin (K x G) treatments gave significantly different of the number of harvest fruits. Duncan test results at5%; the simple effect of factor K at the same G level and factor G at the same K level indicated that potassium 10 g / plant + 50 ppm gibberellin could increase the numberof harvested fruits.

Application of potassium on plant height showed that it was significanlty different. Duncan test results level 5%; the main influence of potassium factor on plant height showed that the best plant height was found in the treatment of potassium 15 g / plant with the average of plant height of 62.16 cm, while the lowest plant height was found in treatment 0 g / plant that was 49.12 cm.



Picture 1. The Main Influence of Potassium Fertilizer Factor (K) on The Plant Height



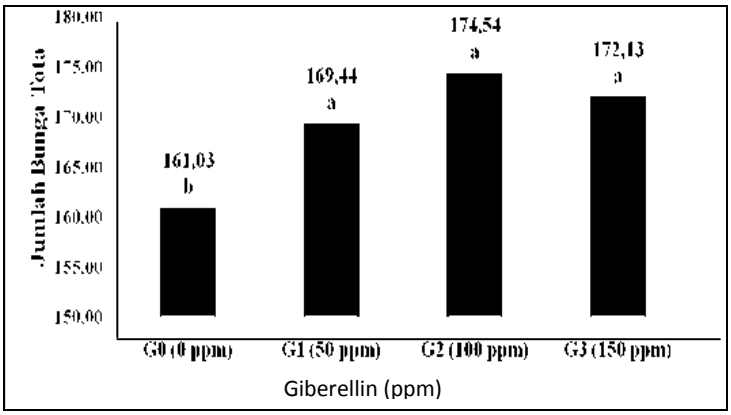
Picture 2. The Main Influence of Potassium on The Total

Number of Flowers

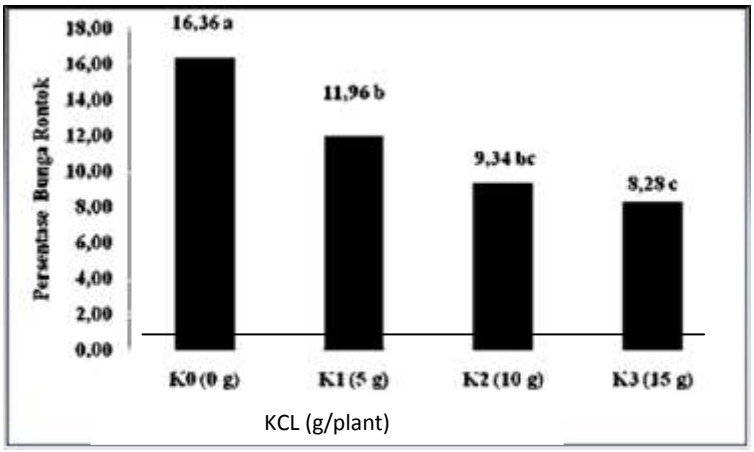
Duncan test results level 5%; the main influence of potassium factor on total number of flowers showed that the highest number of flowers was in the treatment of 10 g / plant potassium with the average number of flowers was 192.67 while the lowest number of flowers was in the treatment of 0 g / plant potassium that was 146.15. The treatment of K2 was significantly different from the treatments of K3, K1 and K0, but K3 was significantly different from K1 treatment.

Duncan test results level 5%; the main influence of gibberellin factor on total number of flowers showed that the largest total number of flowers was found in the treatment of giberelin G2 (150 ppm) with the average number of flowers that was 174.50 while the lowest number of flowers was in the treatment of G0 (0 ppm) that was 161.03. Treatment of G2 was not significantly different from treatments of G3 and G1, but it was significantly different from treatment G0.

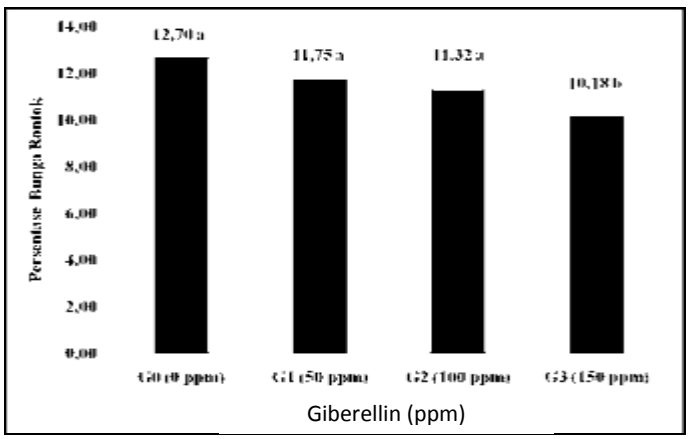
Duncan test results level 5%; the main influence of potassium factor on the percentage of falling flower indicated that the largest number of falling flowers was found in the treatment of potassium fertilizer K0 (0 g / plant) with the average of falling flowers that was 16.36 while the lowest number of falling flowers was in the treatment of K3 (15 g / plant) that was 8.28.



Picture 3. The Main Influence of Gibberellin (G) on The Total Number of Flowers



Picture 4. The Main Influence of Factor Potassium Fertilizer



Picture 5. The Main Influence of the Factor Giberelin Hormone (G) on The Percentage of Falling Flowers.

The treatment of K3 was not significantly different from K2 treatment, but it was significantly different from the treatments of K0 and K1.

Duncan test results level 5%; the main influence of gibberellin factor on the number of falling flowers showed that the largest number of falling flowers was found in the treatment of giberelin G0 (0 ppm) with the average number of flowers that was 12.69 while the lowest number of falling flowers was in treatment of G3 (150 ppm) that was 10.18. Treatment of G3 was significantly different from treatments of G0, G1 and G2.

4. Discussion

In the observation variable of the weight of harvest fruit and number of fruit, there was treatment interaction between potassium fertilizer and gibberellin hormone. According to Mapegau (2006) in [7], In general, the potassium element in the soil is able to provide the balance to other elements. Potassium can provide a significant effect for the plant. [8]stated that potassium affects plant production. By increasing the number of fertilizer K on the plant, the growth and the weight of the crops will increase. [9], based on research conducted on tomato plants with potassium fertilization, it showed that the highest fruit weight (kg) was obtained in potassium treatment of 3.75 g. The size and weight of the fruit can be increased by adding potassium fertilizer.

Giberalin (GA3) is a plant-growing hormone that is very influential on genetic properties, flowering, photosynthesis, and stems lengthening [6]. [10]stated that the GA3 application in plants increases the number of flowers as well as the number of fruits. The number of fruits increasing due to adding GA3 produced smaller size of fruits when the fruit was ripe. The decrease of fruit size along with the increase of GA3 concentration was given by the role of GA3. It also played a role in stimulating the growth of shoots and suppressed the development of the fruit as the result of the asymilat production competition; so that it decreased weight, size, and number of fruits.

[2]stated that giving gibberellin hormone 0, 50, 100 and 150 ppm gave a significat effect on the production of chili. His research result showed that the application of gibberellin hormone 150 ppm was able to show the highest chili production (39.58 g / plant) and the number of fruit harvest (32.96 fruit / plant). The effect of potassium fertilizer on plant height parameter showed that the best chili pepper was found with potassium fertilizer treatment. Potassium is one of the elements having an important role in the processes of physiological, carbohydrate metabolism, the formation, breaking and translocation of starch. The availability of sufficient potassium in plants causes the formation and enlargement of cell size in plant parts normally. The availability of the K element in the plant in sufficient quantities will grow faster because K can keep the cell turgor pressure constantly. The constant cell turgor pressure causes the enlargement of the cells that set up the maristem tissue; so that, they can produce not easily falling plants [11]. The addition of plant height on each potassium fertilizer treatment showed that the height of chili pepper plant is affected by the application of potassium fertilizer [12].

Flowering plants is one of the detreminant indicators of plant production. The number of flowers will determine the number of production obtained. The number of flowers appearing is also influenced by the speed of flowers appearring. According to [13], stated that the availability of nutrients for plants obtained through fertilization can affect the growth of vegetative plants. Based on his research, it also showed that the average number of flowers of chili pepper appears at age 33 days after planting [14].

Potassium application 10 g / plant besides gave an effect on the appearance of flowers; it also affected the number of plant flowers. The more increase of the number of potassium is given, the more increase of the crop production through increasing plant activities that are photosynthesis, protein formation, and carbohydrate that will affect the growth and production of plants [15].

Gibberellin is one of the growth regulators playing a role in flowering, preventing miscarriage of buds, increasing the large size of fruits, and regulating

carbohydrate mobilization. The number of flowers on the plant is one of determinants of the number of fruits that will be produced. The more the number of flowers was formed, the higher risk of falling flowers and fruit will be [10].

A decrease of the percentage of falling flower by giving gibberellin showed a difference in the level of falling compared to the control. Gibberellin (GA3) is a plant-growing hormone that is very influential on genetic properties, flowering, photosynthesis, and stems lenghthening [6]. Giving GA3 is able to accelerate cell enlargement and meristematic activity on the flower; so that, the opening of the flower crown will be faster, so it can shorten the fruit harvest age. Haryantini and Santoso (2001) in [10] showed that giving GA3 with a concentration of 100 ppm to chili given at 30 and 50 days after planting can decrease the falling flowers up to 16%.

Based on the objectives and experimental results that have been obtained, it can be concluded as follows:

1. Combination treatment of potassium fertilizer 10 g/plant with Giberelin 100 ppm significantly affect the weight of harvest fruits.
2. Potassium Fertilizer 10 g/plant produces The highest crops, the greatest number of total flowers and the lowest percentage of the lowest flower.
3. The concentration of Giberelin 150 ppm The highest total interest and the lowest percentage of the fall.

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THE EFFECTIVENESS OF *Syzygium samarangense* LEAVES ON HEALING PROCESS OF BURNS BASED ON COLLAGEN

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Abstract

Background: The World Health Organization (WHO) estimates the number of death in 2014 caused by burns is 265,000 . Burns can caused skin damage as well as other complication problems such as dehydration, infection, and other multiple organ failures. *Syzygium samarangense* leaves contain flavonoids and saponins that can increase the activation of macrophages and TGF-B which is important to accelerate the process of collagen formation and wound healing process. Objective: Knowing the effect of *Syzygium samarangense* leaf extract on the healing process of burn based on collagen. Method: This in vivo study use true experimental design . We made burns by placing a coin that already heated in oven at 70ºC for 10 seconds. Rattus Wistar as experimental animals divided into 6 groups (n = 4) with details of Group A (normal), B (positive), C (negative). Groups D, E, and F were the groups that given ointment extract topically in doses of 15%, 30%, and 45% each day's. Termination is done on day 14. Test statistics by using Kruskal Wallis. Results and discussion: From this research, *Syzygium samarangense* leaf extract can reduce the wound area ($p < 0,05$) and increase the amount of collagen ($p < 0,05$). Conclusion: *Syzygium samarangense* leaf extract can accelerate the healing process of burns.

Keywords : Burns, *Syzygium samarangense* Leaves, Saponin, Flavonoid, Collagen

1. Introduction

World Health Organization (WHO) estimated 265,000 death caused by burns in 2014. Prevalence of burn injuries in Indonesia in 2013 is 0.7% and has decreased 1.5% compared to 2008 (2.2%). Burns are cause by contact with heat source including water, fire, chemical substances, electricity and radiation which lead to not only skin damage but also in patients with extensive (major) burn injuries, the body could not compensate therefore inducing various complications which need special treatment [1,2].

The epithelium is usually burned out in burn injuries, therefore the main principle of mild burns treatment is to cool the injuries with water, prevent infection and give the remaining epithelium to proliferate and close the wound surface. Wound healing process usually needs approximately 25 days to complete. The process can be accelerated by administering medication. Medications commonly used by the people in treating burns are drugs containing 10% placenta extract, 0.5% neomycin sulfate and gel base, but in several cases, irritation may occur marked with red spots on the skin with topical uses.

Semarang rose-apple (*Syzygium samarangense*) leaves contain flavonoids and saponins which can induce macrophage and TGF-B activation which can accelerate collagen formation responsible in wound healing process. Moreover, those substances act as antiseptic and antibacterial. From above statements, it is most likely that Semarang rose-apple has the potential as a material for ointment medication suitable for second degree burn injury [3,4].

2. Methods

Semarang rose-apple (*Syzygium samarangense*) leaves were obtained from

Ponorogo region, Indonesia, in March 2017. This plant is determined by specialists from Pest and Plant Health Laboratory, Agrotechnology Department, Darussalam University Gontor, Ponorogo, Indonesia. The leaves were then extracted using maceration method. Oven dried Semarang rose-apple (*Syzygium samarangense*) leaves were softened using a blender. A total of 800 grams of Semarang rose-apple leaves powder was extracted using 96% ethanol with the ratio of 1:5. Macerates were strained using filter paper to produce evaporated filtrate until 27 grams of concentrated extracts are obtained. Those concentrated extracts were mixed with Vaseline according to ointment concentration dose, then given to test animals for experiments.

This study uses true experimental laboratories method with post test only randomize control group design. Animals used are wistar rats obtained from a supplier in Malang. A total of 96 male wistar rats weighing 100-150 grams were divided into 6 groups (n=4), which are A (normal), B (positive), C (negative), D (15% ointment), E (30% ointment), and F (45% ointment).

The rats were adapted for 7 days then given burns by pressing hot coin (put into oven for 5 minutes) for 10 seconds. The wound were treated everyday for 14 days with the following, group A (no treatment), B (second degree burn treated with bioplacenton), C (second degree burn treated with aquades), D (second degree burn treated with 15% ointment), E (second degree burn treated with 30% ointment), and F (second degree burn treated with 45% ointment). On the fourteenth day continued by termination to collect skin tissues for histopathology examination with HE staining.

Collagen examination were performed by determining collagen density level using microscope with 400x magnification. The scale used in collagen examination was according to Novriansyah 2008:

0: No collagen fiber found in wound area

1: Low collagen fiber density

2: Moderate collagen fiber density

3: High collagen fiber density in wound area

4: Very high collagen fiber density in wound area *Wallis test.*

3. Results

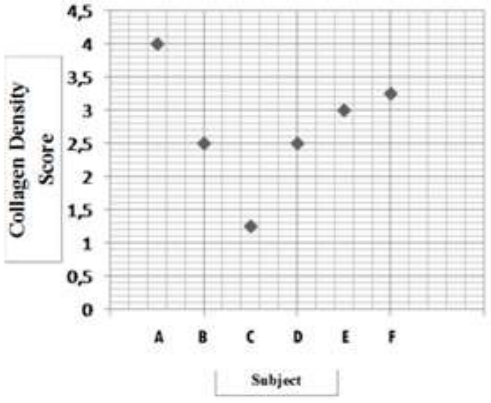

Figure 1. Collagen density scroring result on fourteenth day.

Results of collagen examination by determining collagen density using Novriansyah method showed a tendency of increasing collagen formation in groups treated with ointment as shown on the scoring graph. The highest collagen density level is in group F (45% ointment). ANOVA test results showed $p < 0.05$ ($p = 0.025$) in group F compared to group C (negative) which showed that there is a significant difference of collagen increase when given 45% Semarang rose-apple leaves extract ointment compared to negative group.

4. Discussion

Results showed accelerated wound healing in groups treated with ointment. Negative control group has lower healing level compared to groups treated with ointment. This is caused by the lack of substances which accelerate the process or debridement of wound from foreign objects sticking to the skin.

The highest healing percentage were shown in group F (45% ointment), followed by group E (30% ointment) and group D (15% ointment). This results were reinforced by statistical analysis which showed $p < 0.05$ ($p = 0.025$), which implies that there is a significant difference between 45% ointment group and negative group. This happened because the substances contained in Semarang rose-apple leaves with moisturized preparations, which is ointment. There are flavonoids, tannins, and saponins contained in Semarang rose-apple leaves which play important roles in wound healing process. Flavonoid is an anti-inflammatory substance, meanwhile tannin and saponin are antibacterial. Both of these substances play a role in improving wound healing of burns by increasing macrophage activation which produces growth factors and cytokines such as EGF, TGF-B, IL-1, IL-4, and IL-8 which induces fibroblasts proliferation and migration. These fibroblasts will produce collagen, therefore increasing wound closure.

5. Conclusion and Suggestion

In conclusion, Semarang rose-apple leaves extract given topically can accelerate wound healing of burns in rats based on results and data analysis of this study. Further studies involving Semarang rose-apple leaves experiments are needed at the fractional level.

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MICROBIAL MODIFICATON OF GADUNG (*Dioscorea Hispida* Dennst) TUBER FLOUR THROUGH FACULTATIVE SUB-MERGED FERMENTATION USING *Lactobacillus Plantarum*

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Abstract

Gadung (*Dioscorea hispida* Dennst) has been recognized as one of the most underutilized tubers in the world due to its antinutrients content, such as dioscorin and cyanogens. Its significant resistant starch content, but without gluten has suggested the utilization of gadung tuber in reducing the risk of obesity, diabetes and the incidence of celiac diseases. With these benefits in mind, an effort on gadung tuber processing into functional food materials was undertaken. The objectives of this research were to study the effect of flour consistency (5-25% w/v), microbes loading (2.5-15% v/v) and fermentation time (0-144 hours) on the swelling power, solubility, carbonyl and carboxyl group's content and amylose content during microbial modification of gadung tuber flour. The flour modification was conducted via facultative sub-merged fermentation using Lactobacillus plantarum in a series of Erlenmeyer flasks fitted on a thermo-controllable shaker. Samples were withdrawn from the fermentation flasks at 24 hours interval for swelling power, solubility, carbonyl and carboxyl group's content and amylose content analysis. In general, the swelling power and solubility of modified flour was lower than the native flour. Carboxyl group content increased with fermentation time, while no clear trend was found for carbonyl group. The amylose content of the modified flour was higher than that of the native, which most probably due to the depolymerization of amylopectin branches to form new amylose molecules with various molecular weights. The best fermentation conditions were flour consistency of 10% (w/v), microbes loading of 5% (v/v) and fermentation for 48 hours.

Keyword: gadung flour, fermentation, consistency, microbes loading, time, swelling power, solubility, amylose content.

1. Introduction

Dioscorea hispida Dennst or locally known as gadung is a woodbine belongs to the genus of Dioscorea within the Dioscoreaceae family. This plant has a thorny stem twinning to the left, which may reach up to 20 meters in height with a hairy trifoliate leaves and generates small pale yellow flowers [1]. This plant is reported easily found in the shade or near stream of secondary forest of South East Asia and its surrounding archipelagos [2]. Being planted in the beginning of rainy season (October-November), a mature bulky tuber with white to yellow flesh is usually harvested in summer (April-September). Unfortunately, this tuber contains antinutrients, such as cyanogens, alkaloid (dioscorin), tannins and saponins, which may attribute to the bitterness and toxicity of the tuber [3]. With respect to its high carbohydrate content, this tuber has long been served as staple food for people in the rural areas, specifically during World War II. In Indonesia, Thailand, Vietnam, Malaysia and the Philippines, gadung tubers are mostly consumed boiled, steamed, or fried after being detoxified through a complicated cyanogens removal

process. With no gluten content, but rich in resistant starch content, gadung tuber can be a potential food source for people in reducing the risk of obesity, diabetes, wheat allergy and the incidence of celiac diseases. At present, numerous person in many Western communities apply a gluten-free diet, keeping away of wheat, rye and barley [4]. The markets for gluten-free products have been increasing rapidly [5], widening the opportunities for the development of new technologies to create new products using gluten-free ingredients as alternative for traditional manufacturing bakery products [6]. With these encouraging conditions and benefits in mind, an attempt on the processing of gadung tuber into functional food materials was carried out.

Gadung tuber starch gelatinizes at a high temperature range, which is almost equal to the pasting temperature of cereal starches. This property indicates the potential application of gadung tuber starch as a thickening agent in retort foods or foods that need heat stable viscosity. Starches with restricted swelling and stabilized granular structure are highly desired in the food and pharmaceutical industry [7]. With swelling power of 15.6 g/g dry starch at 90^{o}C [8], gadung tuber starch falls in the restricted swelling category [9]. Based on this characteristic, gadung tuber starch is desirable for the production of value-added products such as noodles. However, due to its low setback viscosity, gadung tuber starch has low retrogradation tendency and, consequently, fails to form strong gels [8]. A higher retrogradation tendency of a starch suggests that the starch is suitable for the use in manufacturing of jelly foods and noodle [10]. Therefore, gadung tuber starch will not be suitable for application in foods that need firm texture.

Commonly, breeders and the food industry use amylose content as the most important determinant in food texture development. In general, stronger starch gels can be well correlated with higher amylose content [11]. Amylose-based networks are believed to give elasticity and strength against deformation on starch gels. In contrast, soft gels containing aggregates in the absence of networks display easier penetrability and greater stickiness and adhesiveness [12]. Starches with low amylose content show a higher degree of crystallinity [13]. Amylose retrogradation determines the initial hardness of a starch gel and the stickiness and digestibility of processed foods [14]. Based on this point of view, one of the suitable efforts to increase the gel strength of gadung tuber flour is by increasing the amylose content, which finally increases the retrogradation tendency. Fermentation has shown its capability to increases the breakdown viscosity value, which is a measure of the retrogradation tendency of the starch [15]. A higher value of breakdown viscosity of the starch is an indication of lower ability of the starch to restrain heating and shear stress during cooking [16].

Fermentation is one of the oldest biotechnological methods of food processing and preservation that widely applied in the world. Basically, fermentation is a metabolic process involving bacteria, yeast, moulds or their combination in which carbohydrates and related compounds are oxidized with the release of energy in the absence of any external electron acceptor under anaerobic or facultative condition. Over millenniums, the demands of fermented foods have greatly increased due to the increased demand for nutritious, safe, natural, additives-free, and well-preserved foods [17]. Therefore, these foods become an important part of human diet worldwide [18]. During the fermentation processes, microorganisms produce specific metabolites such as enzymes, acids, alcohols, antibiotics, carbohydrates, and inhibitory compounds which contribute to the safety and nutritional quality of fermented foods. The lactic acid bacteria (LAB) that is generally regarded as safe (GRAS), plays an essential role in the majority of food fermentations and preservation. These properties are important for the use of LAB as starters or adjuncts to maintain and improve the nutritional, sensory, and safety qualities of final products [19]. In this case, various strains of LAB have been routinely applied as starter cultures in the production of dairy, meat, vegetable, and bakery products [20].

The objectives of this present study are to study the effect of flour consistency (5 to 25% w/v), microbes loading (2.5 to 15% v/v) and fermentation time (0 to 144 hours) on the swelling power, solubility, carbonyl and carboxyl group's content and amylose content during microbial modification of

gadung tuber flour using *Lactobacillus plantarum*.

2. Materials and Methods

a. Microorganism and Materials

Lactobacillus plantarum sp CCRC 12251 was procured from Food and Nutrition Inter-University Center, Gadjah Mada University Yogyakarta-Indonesia and maintained in Mann Rogassa Sharpe (MRS) agar slant at 4^{o}C. Gadung flour was obtained by milling of dried detoxified gadung tuber chips of matured gadung tuber harvested from Gunung Pati-Semarang, Indonesia. All of the chemicals and reagents used in this study were of analytical grade with purity of ≥ 99.5 % (w/w) and manufactured by Sigma-Aldrich. They were purchased from authorized chemicals distributor in Semarang-Indonesia and directly used without prior treatments.

b. Inoculum Preparation

Inoculum was prepared in a 250 mL Erlenmeyer flasks containing 100 mL of modified MRS liquid medium (peptone, 10; beef-extract 10; yeast extract 5; glucose 20; Na_2HPO_4; sodium acetate 5; triammonium citrate 2; $MgSO_4$ 0.2; $MnSO_4$ 0.2; and $CaCO_3$ 4 g/L, Tween 80 0.1 mL and pH 6.8) by transferring a loop full of microorganisms (*Lactobacillus plantarum*) from a stock culture and incubated at 35^{o}C and 120 rpm for 48 hours in an orbital incubator-cum-shaker. The number of viable bacteria was counted by the total plate count (TPC) method as also used by previous researchers [21]. The inoculums were found to contain 3 × 10^7 CFU/mL.

c. Fermentation

Slurries of detoxified gadung flour with various flour consistencies (5 to 25 % w/v) were prepared in 250 mL Erlenmeyer flasks containing 100 mL distilled water. To prevent starch gelatinization, no thermal sterilization was done. Then, the slurries were inoculated with various size (2.5 to 15% v/v) freshly prepared inoculums and covered with aluminum foil. The fermentation was kept at room temperature and let to proceed till 144 hours with continuous shaking at approximately 120 rpm on a horizontal incubator shaker. Samples were withdrawn from the fermentation system at 6 hours, 12 hours and followed by every 24 hours. The samples were filtered under vacuum to obtained fermented flour. The fermented flour was dried in an electric tray drier at 60^{o}C for 24 hours to dryness and stored in an air tight container or directly subjected to swelling power, solubility, amylose, carbonyl group and carboxyl group determinations. All fermentations were carried out in duplicate, and hence the reported values are the average of two experiments.

d. Analytical Methods

The proximate composition of all flour samples was determined following the official method of analysis [22]. The amylose content of the flour samples was determined following the method of Juliano [23]. The swelling power and solubility of the starches were determined as in [24]. The carboxyl content of the flour was determined as previously described [25]. The carbonyl content was determined according to the titrimetric hydroxylamine method [26].

3. Results and Discussion

Based on the swelling power value, gadung tuber flour has already met the requirement for its applications in the food and pharmaceutical industries [7]. The facultative-submerged fermentation of gadung flour using *Lactobacillus plantarum* is expected to change the molecular structure of its starch granule into a more stable form which leads to improve the retrogradation tendency of the starch granules. An indirect method to ensure the retrogradation tendency is by observation of the change in amylose content [13]. Therefore, one of the criteria for the selection of the best operating condition is the amylose content of fermented gadung flour.

a. Proximate Composition of Native and Fermented Gadung Flour

Results of the proximate analysis of gadung flour samples in Table 1 shows that the moisture content of the samples were 12.70% and 12.85%, respectively for native and fermented gadung flour. The moisture content of food samples is an indicator of shelflife and quality of solid foods. The moisture contents of gadung flour obtained in this study were within the acceptable values for dried foods [27].

As shown in Table I, carbohydrate and fiber contents of gadung flour were lowered by fermentation. The loss of nutrients has been attributed to leaching out or due to utilization of the nutrients by the microorganisms. During fermentation, lactic acid bacteria play significant role in the starch breakdown and acidification [28]. The microorganisms will produce amylase and glucoamylase to degrade starches and other type of polysaccharides [29].

Table 1. Proximate Composition Per 100 Gram Flour

Component	Native Flour (NF)	Fermented Flour (FF)
Carbohydrate (g)	81.94	81.28
Protein (g)	1.81	1.58
Fat (g)	1.60	1.36
Ash (g)	0.70	0.43
Fiber (g)	2.40	2.27
Moisture (g)	11.55	12.88

In addition to the loss of carbohydrate, there were also reduction of lipid and protein contents in the fermented gadung flour due to leaching and activity of microorganism. Most of lactic acid bacteria also have the ability to digest proteins and lipids into simpler molecules through the production of enzymes such as protease and lipase [29]. Lipid was depolymerized to free fatty acid during fermentation with the help of lipase as biocatalyst, while protein was decomposed to amino acids by proteases [30]. The ash content, which is a measure of mineral presents in the fermented gadung flour, was lower compared to that of the native one. The reduction in ash content may be caused by microorganisms require nutrients and minerals for growth and development. In addition, the heat used during drying of the fermented flour could also reduce some minerals such as calcium, phosphorus and iron, which are strongly affected by heat. This finding is in a good agreement with ash content of fufu flour inoculated with starter culture strains and those traditionally fermented, which were lower than the unfermented fufu flour [28].

b. Effect of Flour Slurry Consistency

The level of degrading enzymes production used for flour granules modification is maximal during the exponential phase of lactic acid bacteria growth. Since the exponential phase of *Lactobacillus plantarum* exists between 32 to 72 hours, therefore the study of the effect of flour consistency was conducted with 48 hours fermentation time [31]. The flour consistencies were varied from 5 % w/v to 25 % w/v using microbes loading of 5% v/v. The results of this investigation are presented in Table II.

Table 2. Effect of Flour Slurry Consistency

Flour Consistency (% w/v)	Swelling Power (g/g)	Solubility (g/100g)	Carbonyl Group (%)	Carboxyl Group (%)
5	4.90	3.50	0.22	0.66
10	5.10	5.50	0.17	1.07
15	4.80	5.00	0.20	0.38
20	4.35	4.50	0.33	0.30
25	4.16	5.00	0.29	0.26
NF	5.90	7.00	0.54	0.24

Table II reveals that the swelling power and solubility of fermented gadung flours obtained in this study were lower than those of native gadung flour. Reference [32] described a similar phenomenon on the reduction of swelling power of cassava starch to the extent of 12.1% in natural fermentation and 15.5% in inoculum

provided fermentations. The lower value of swelling power can be explained on the basis of weakening of associative forces in the granules, specifically in the amorphous regions. Basically, the side branches function to prevent intermolecular association of carbohydrate polymers [33]. However, when some branches are hydrolyzed, as happens during fermentation, there will be possible intermolecular hydrogen bonding of the fragments. As a result, the number of free hydroxyl group where the water molecules would usually hydrogen-bond decreased significantly. This situation would lead to less water uptake and less swelling during heat treatment [32]. The lower value of swelling power of gadung tuber flour may also be associated with its high protein content. Starch and protein in the flour granules could interact due to the attraction of their opposite charges and form inclusion complexes which finally restrict swelling [9].

The lower solubility of fermented flour than native flour has been reported earlier for sweet potato flour [34]. Reference [32] also mentioned that the solubility of cassava starch was reduced by 26.5 and 37.8%, respectively, during natural fermentation and mixed culture fermentations. The distributions of chain length in the starches molecules also cause differences in solubility [35]. In this case, the reduction in solubility has been explained to be due to alteration in the internal granule structure of the starch following enzyme/acid action [32]. The starch in the flour particles have been degraded into shorter polymer chains as action of enzyme produced by lactic acid bacteria. These shorter polymers, which mainly simple sugars were more soluble and probably were dissolved in the fermentation medium resulted in longer chain polymer retain in the flour. In addition, granular size also affects solubility of the starches where the smaller the granule size, the higher the starch solubility. In this study it was revealed that based on SEM analysis (not shown), the granular size of the fermented flour was bigger than that of native flour [36].

The carbonyl group content of all the fermented flours was lower than the native flour. However, no clear trend was observed on the reduction of carbonyl group content as a function of gadung flour slurry consistency. As expected, the carboxyl group content of the fermented gadung flours was higher than the native one. The increase in carboxyl group contents of fermented flour compared to native flour was probably due to the effect of fermentation and acidification. Similar result was reported on the fermentation of cassava starch [37]. The highest carboxyl group content was achieved when gadung flour slurry with 10% w/v consistency was used. However, the carboxyl group content decreased when the flour consistency was increased further; that was due to inhibitions caused by the high substrate concentration. The other reason decreased the utilization of starch beyond 10% (w/v) substrate, which might be due to increase in osmotic effects or due to hydrolysis of starch to reducing sugars or the microorganisms were incapable of hydrolyzing the starch present in flour at 10% (w/v) or above because they generally grow and being productive at higher water activity [38-39]. In addition, a higher flour consistency will lead to increase the viscosity of the culture medium, which lead to decrease water activity as the process might have shifted from submerged dispersed solid to semi-solid state fermentation [38].

According to the European Union Scientific Committee for Food, the safe level of carboxyl group in food material is at maximum of 1.1% [40]. Therefore, gadung flour slurry with 10% w/v consistency is selected as the best flour consistency for fermentation using *Lactobacillus plantarum*.

c. Effect of Microbes Loading

In industrial applications, the microbes loading range for lactic acid fermentation is usually between 3 to 10% (v/v) of the fermentation broth volume [41]. Suitable microbes loading would eliminate the probable occurrence of lag phase or shorten the lag phase period. The effect of microbes loading on the swelling power, solubility, carbonyl group and carboxyl group contents during fermentation of gadung flour using 10% w/v flour consistency for 48 hours is presented in Table III.

Table III shows that the swelling power, solubility and carboxyl group content of fermented gadung flours obtained in this study were lower than those of native gadung flour. On the contrary of that, the

Table 3. Effect of Microbes Loading

Microbes Loading (% v/v)	Swelling Power (g/g)	Solubility (g/100g)	Carbonyl Group (%)	Carboxyl Group (%)
2.5	5.10	5.00	0.15	0.48
5.0	5.10	5.50	0.17	1.07
10	5.70	5.00	0.16	0.95
15	6.40	5.50	0.12	0.81
NF	5.90	7.00	0.54	0.24

carboxyl group content greatly increased as the microbes loading was increased from 2.5 to 5.0 % v/v. However, the use of higher microbes loading caused gradual reductions of carboxyl group content of the fermented gadung flour. Therefore, 5.0 % v/v was chosen as the optimum *Lactobacillus plantarum* inoculums loading for fermentation of gadung flour. Reference [42] also reported similar result on liquid fermentation of starch for lactic acid production using free and immobilized cells of *Lactobacillus amylovorus* NRRL B-4542. Lactic acid production from paneer whey by *Lactobacillus delbruckii* under submerged fermentation process was reported to increase substantially from 2.8 to 5.6 g/L, when inoculum loading increased from 3 to 8% v/v [43]. However, no significant effect on lactic acid production was observed when inoculum loading was further increased beyond 8% v/v. A long lag phase is undesirable in fermentation process because it is time - consuming and the medium is used to maintain a viable culture prior to the growth. Therefore, 5% v/v inoculum loading was performed better than 10% v/v because the lag phase of 5 % v/v inoculum loading was a little shorter than 10% v/v during fermentation of whey using *Lactobacillus bulgaricus* for lactic acid production [44].

In submerged liquid fermentation, an appropriate loading of inoculum is utmost important parameter for obtaining high product yield and productivities. At low value of inoculum loading the substrate is slowly utilized by microorganisms and prolongs incubation time. On the other hand, high value of inoculum loading will lead to competition of growth of microorganism over the limited substrate supply [42].

d. Effect of time

During the exponential phase of growth, lactic acid bacteria produce the highest amount of degrading enzymes that play important roles in flour modification [45]. Therefore, an optimum fermentation time should exist for microbiological modification of gadung flour through fermentation using *Lactobacillus plantarum*. Table IV presents the effect of fermentation time on swelling power, solubility, carbonyl group, carboxyl group and amylose contents.

T 4. EFFECT OF FERMENTATION TIME

Time (hour)	Swelling Power (g/g)	Solubility (g/100g)	Carbonyl Group (%)	Carboxyl Group (%)	Amylose %
4	4.75	3.00	0.25	0.47	38.41
6	4.99	2.50	0.52	0.52	40.93
12	4.30	3.50	0.48	0.53	41.59
24	4.56	3.00	0.16	0.65	41.92
48	5.10	5.50	0.17	1.07	43.05
72	4.31	2.50	0.21	0.76	43.89
96	4.45	2.00	0.44	0.86	41.63
120	4.60	1.50	0.41	0.92	41.35
144	4.94	3.50	0.30	1.05	39.52
NF	5.90	7.00	0.54	0.24	34.72

As tabulated in Table IV, the swelling power of fermented gadung flour decreased sharply in the beginning of fermentation. Then, the swelling power values started to increase when the fermentation reach 6 hours and achieved a maximum value of

5.10 (g/g) at 24 hours fermentation. This pattern is repeated as the fermentation was further prolonged till 144 hours. No clear trend was observed for solubility, carbonyl group and carboxyl group contents of fermented gadung flour as a function of fermentation time. The amylose content of fermented gadung flour increased with fermentation time to a maximum value of. 43.89% at 73 hours and then leveled off. As expected, the amylose contents of the fermented gadung flours in this study were higher than that of native flour. High amylose starches are useful in the confectionery industry where candy pieces need a stabilizer to supply individual piece shape and integrity [46]. Based on carboxyl group and amylose contents of the fermented flour, 48 hours is selected as the optimum fermentation of gadung flour using *Lactobacillus* plantarum. The incubation period of 48 h has been generally used for lactic acid production using different lactobacilli [47]. The shorter fermentation time is additionally advantageous to increase the economics of the process.

Formation of amylose-like material resulting from enzyme/acid hydrolysis of amylopectin in the amorphous regions of starch granules during fermentation may be the cause of the apparent increase in amylose content of starch and flour after fermentations [32]. In this case, during fermentation the glucoamylase degrades amylopectin into amylose [48]. On the other hand, the organic acids produced during fermentation may form complex molecules with the soluble amylose fraction thereby leading to an apparent reduction in soluble amylose content. This complexation leads to enhancement in gelatinization temperature and also enhancement in fermented flour qualities, as the stickiness due to soluble amylose has been lowered. In addition, amylose may be depolymerized by α-amylase into short chain and lost into water [48].

4. Conclusion

A study on microbiological modification of gadung tuber flour through facultative-submerged fermentation using *Lactobacillus plantarum* has been successfully conducted. The flour slurry consistency, microbes loading and fermentation time significantly affected the physicochemical properties of the flour. The best fermentation conditions were flour consistency of 10 % (w/v), microbes loading of 5% (v/v) and fermentation for 48 hours. Based on its physicochemical properties, the modified gadung flour obtained in this study is suitable for the manufacture of value-added products such as jellies, noodles and bakeries.

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INCREASING RESISTANT STARCH TYPE 3 OF MODIFIED CASSAVA FLOUR (MOCAF) USING ONE CYCLE OF AUTOCLAVING COOLING TREATMENT FOLLOWED WITH DEBRANCHING ENZYMES PULLULANASE

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Abstract

Modified Cassava Flour (Mocaf) is widely used as food ingredients either because they have good functional values such as rich in fiber and contain no gluten that believed related to the occurrence of certain diseases such asautism. By increasing the content of resistant starch (RS) in Mocaf we can increase its role as aprebiotic. One type of RS that widely used is RS type3 (RS3) which formed through retrogradation process using heat and cooling (autoclaving- cooling) treatment. Previous study by Asbar *et al*, demonstrated that 3 cycle of autoclaving-cooling treatment on Mocaf can increase the levels of RS3 as much as 8.73 percent. In the other study conducted by Zahruniya et al, demonstrated that by adding debranching enzyme pullulanase after one cycle of autoclaving-cooling treatemment can increase levels of RS3 incassava starch by 87.64%. The purpose of this study is to increase the levels of RS3 in Mocaf by using one cycles autoclaving-cooling treatment followed with debranching enzyme pullulanase. The results demonstrate that this method can increase the levels of RS3 in Mocaf from 9.66% to 60.87 %.

Keywords: Modified Cassava Flour (Mocaf), Resistant Starch type 3, autoclaving cooling, Pullulanase.

1. Introduction

Currently Modified Cassava Flour (Mocaf) is widely used as food ingredients such as a flour substitute either because they have good functional values such as rich in fiber and contain no gluten that believed related to the occurrence of certain diseases such as autism. By increasing the content of resistant starch (RS) in Mocaf we can increase its role as a prebiotic. RS is part of starch that resistant to hydrolysis process from digestive enzyme such as amylases of that it become poorly digested and cannot be absorbed in the small intestine. In the colon, RS is then fermented by intestinal microflora to produce short chain fatty acids (SCFA) [4] Many research suggests that by consuming food that contain RS can improve glucose metabolism and improve insulin sensitivity. Short chain fatty acids (SCFA) from fermented RS inside the colon will increasethe expression of glucagon like peptide- 1 (GLP-1) gene of Lcell in the intestine wall so that it can increase the production and secretion of GLP-1 in the blood plasma. Glp-1 is a proinsulin peptide. Increasing concentration of GLP-1 in the plasma will also increase the production of insulin in the pancreatic cell. Thus foods containing RS has a great potential in the prevention and treatment of type 2 diabetes mellitus [2, 6, 7] One type of RS that widely

used is RS type (RS3) wich formed through retrogradation process using heating treatment with an autoclave (121°C) followed by cooling at low or at room temperature (autoclaving-cooling). Retrogradation occurs through a process of reassociation (realignment) hydrogen bonding between short chain amylose formed after the heating process [5].

Increasing the content of the RS3 is not enough if only processed using the method of autoclaving-cooling alone. In a study by Asbar et al, demonstrated that 3 cycle of autoclaving- cooling treatment of Mocaf can only increase the levels of RS3 as much as 8.73 percent. By adding starch hidrolizing enzymes such as Pullulanase that hidrolize the α-1,6 branch of amylopectin (debranching) will increase the content of the RS3 even with one cycle of autoclaving-cooling treatment. The longer of the debranching process more short chain amylose will be produced which can multiply the opportunities of RS3 formation. In a study conducted by Zahruniya et al, demonstrated that one cycle of autoclaving-cooling treatemment followed with debranching process for 24 hours using pullulanase 1.04 U/g can increase RS3 in cassava starch up to 88.64% [1,9].

Research on modification of Mocaf to increasethe levels of RS3 with autoclaving-cooling followed with debranching process using pullulanase yet ever done. The purpose of this study is to increase the levels of RS3 in Mocaf by using one cycles of autoclaving-cooling treatment followed with debranching enzyme pullulanase.

This study conducted in April 2017. The modification and analysing process of Mocaf were conducted in several laboratories; The Laboratory of Biochemistry Faculty of Medicine University of Jember and Integrated Analysis Laboratory Faculty of Engineering of Agricultural Products University of Jember. There are three stages in this study: (1) characterization and analysis of native Mocaf (2) Increasing of RS3 in Mocaf using one cycle of autoclaving-cooling treatment followed with debranching process (3) characterization and analysis of RS3 in Mocaf after treatment.

Increasing of RS3 in Mocaf using one cycle of autoclaving-cooling treatment followed with debranching process

In the early stages, Mocaf gelatinized at high temperature using an autoclav. A total of 200 g of Mocaf were suspended with 300 mL of aquadest in Erlenmeyer flasks (20%w/v). Samples were then heated in an autoclave at 121° C for 1 hour. This process will make the starch granules swell and make amylose out from starch granules. Furthermore, the starch is incubated at-20°C for 6 hours. Then proceed with the debranching process. As much as 25 grams sample in 100 ml of acetate buffer pH5.2 heated at 95 °C for 10 minutes, then resuspended in 125 mL of acetate buffer pH 5.2 and heated in an autoclav at121 °C for 30 minutes, after the temperature down to 50 °C. Sample were hydrolyzed by adding pullulanase 1.04U/g. Samples were then incubated at 50°C for 24 hours while shaken at a speed of 160 rpm. Termination of the enzymatic reaction carried out by heating the sample in an autoclav for 1 hour and then dried in an oven at 50°C and then analyzed (Zahruniya 2014)

2. RESULTS

Characterization And Analysis Of Native Mocaf

Analysis showed the water content of native Mocaf was 14.87 %, the starch content was 53.23%. Native Mocaf contain amylose as much as 27.29%. Digestibility analysis showed that native Mocaf digestibility was 15.32%. Furthermore, the analysis showed RS content in native Mocaf was 9.66%, Rapid digestable starch (RDS) content was 43.19% and Slow digestable starch (SDS) was 47.16%.

Increasing of RS3 in Mocaf using one cycle of autoclaving-cooling treatment followed with debranching process

After heated with an autoclave at 121°C for one hour, sample then cooled at low temperature of 20 °C for 6 hours followed with debranching process using pullulanase for 24 hours then driyed at 50 °C in an oven. The starch inside the sample were retrograded. Retrogradation occurs through a process of

reassociation (rearrangement) of hydrogen bonds between short chain amylose that is formed after the heating process [5]. The morphology of RS3 can be seen using electron microscope as seen in Figure 1.

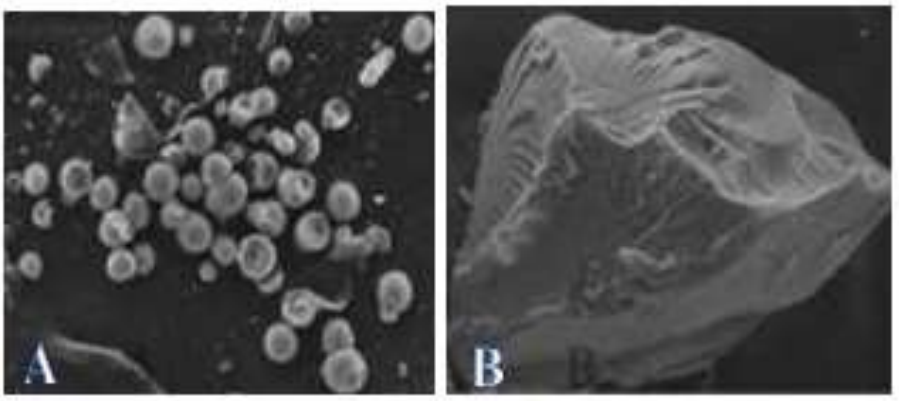

Figure 1. Morphology of starchgranule usingelectron microscope600x:A.NativeMocaf..B.RS3 Mocaf.

Using an electron microscope on the natural starch we would seem starch granules are spherical. While RS3 has been turned into an amorphous shape. Between another starch, RS3 is preferrable because it is more stable against heating so it is not damaged when processed into food products [8]

Characterization and analysis of RS3 in Mocaf after treatment

After the modification process of native Mocaf with one cycle ofautoclaving-cooling followed with debranching process using pullulanase the analysis showed that the water content was 71.09%, the starch content was 3.72%. Amylose content was 7.05%. The result of digestibility analysis was 11.66%. RS level analysis shows that with one cycles autoclaving cooling treatment followed with debranching process using pullulanase produce RS3 as much as 60,88%, and as much as 18.18 % of RDS and 20.94 % of SDS.

3. Discussion

The use of native starch is currently very limited because of the physical and chemical properties that are less appropriate in wide spread use. It is therefore necessary modifications to the starch have added value. Modifications can be done physically, chemically, or a combination of both. One type of modified starch is Resistant Starch (RS) which is resistant to the hydrolyzing process of digestive enzymes [3, 9]

Starch can be classified based on its digestibility by amylase and of the method in producing them. Based on the digestibility by amylase, starch can be classified into 3 namely starches that can be digested quickly or rapidly digestible starch (RDS), slowly digestible starch (SDS), and cannot digested or resistant starch (RS). Based on the way of producing, RS grouped into 5 groups one of which is the RS3. RS3 formed through a process of retrogradation by heating with an autoclave at 121°C, followed by cooling at low temperature orat room temperature. Retrogradation occurs through a process of reassociation (rearrangement) of hydrogen bonds between short chain amylose that is formed after the heating process and form double helix structure that will inhibit water absorption and enzymatic process [5]

The morphology of RS3 can be seen using electron microscopy. Using an electron microscope on the natural starch we would seem starch granules are spherical. While RS3 has been turned into an amorphous shape. Between another starch, RS3 is preferrable because it is more stable against heating so it is not damaged when processed

into food products (Kouamé et al, 2015; Vatanasuchart et al, 2010)

Several in vivo studies conducted on animals and humans indicate that RS has the potential to support human health one of them is as a prebiotic ingredient. Research on RS3 indicate that the granules of RS3 starch form a pattern of attachment specifically in the upper intestines that it can become an attachment for probiotic bacteria.(Brighenti et al,2006)

The fermentation of RS3 by probiotic bacteria result in the form of short chain fatty acids (SCFA) such as acetate, propionate and butyrate. Acetate and propionate has a role in increasing the expression of precursor gene of GLP-1. By getting a lot of content RS3 in food consumed the higher levels of GLP-1 in the intestinal L cell will produced and the higher levels of GLP-1 secreted in blood plasma. Increased levels of GLP-1 will induce pancreatic β cell proliferation, increases the production and secretion of insulin and glucagon to control glucose concentration in the blood and in muscle cells. That is way Food containing RS3 has a potential role as an important nutrient in the prevention and treatment of type 2 diabetes mellitus. (Brighenti et al.2006)

Increasing the content of the RS3 is not enough if only processed using the method of autoclaving-cooling by autoclav alone. The addition of starch hydrolyzing enzymes will increase the content of the RS3. The enzyme frequently used to hydrolyze the starch is pullulanase. Pullulanase has a spesific acts that is hydrolized the α-1,6 branch of amylopectin (debranching).(Zahruniya 2014)

This study has prove that by using one cycles of autoclaving-cooling treatment followed with debranching the α-1,6 bond of amylopectin on Mocaf using pullulanase 1.U/g can increase the levels of RS3 in Mocaf as much as 60.88 % or 6 times higher than the levels of RS in native Mocaf. The comparation between native Mocaf before and after treatment can be seen in table1. Further research related to the type of SCFA formed, the type of probiotic bacteria grown in the colon due to consumption of foods containing RS3 Mocaf as well as its role in the prevention and treatment of type2 diabetes mellitus or another diseases were still need to be done.

Table1. Comparation between native Mocaf and RS3 Mocaf

Parameters	Native Mocaf	RS 3 Mocaf
Starch Content	53.23%	37.2%
Amylose	27.29%	7.05%
RS	9.66%	60,88%
SDS	47.16%	20.94%
RDS	43.19%	18.18%
Digestibility	15.32%	11.66%

4. Conclusion

From these results it can be concluded modification of Mocaf with one cycle of autoclaving-cooling treatment followed with debranching process using pullulanase successfully increase the levels of RS3 in Mocaf as much as 60,88 % or 6 times higher than the RS levels in native Mocaf.

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EFFECT OF PROCESSING METHOD AND FERMENTATION TO ENHANCE QUALITY OF ARABICA COFFEE

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Abstract

Research on the influence of processing methods and fermentation on arabica coffee has been done. The study aims to determine the effect different processing methods on pH and temperature change, and cupping score. This research was conducted through two stages of treatment. First stage is the variation of fermentation time from 4, 6, 8, 10 and 12 hours and with the addition of yeast at 12 hours. Then treatment II with variation of processing process that is stored in plastic bags and laid out without roof over from 24, 48 and 72 hours and continued with fermentation process about 12 hours. The results of the first stage showed that the best fermentation time was at 12 hours with the decrease of pH reach 3.93 and the cupping score of 85.50. The addition of yeast did not give effect to the flavor with the total cupping score about 84.0. The results in treatment II gave the cupping score 84.50 for green bean which obtained from coffee cherries stored in plastic bags much higher than coffee cherries which laid out in open area with cupping score 84.00. The study showed that the coffee cherries can be stored into plastics bags for several days at rainy seasons before the coffee can be sold.

Keyword: Cupping score, fermentation, coffee processing, pH.

1. Introduction

In normal practise, coffee preparation by the dry and wet method in producing countries depends on the species grown, and on the conditions and resources in each production region [1]. Semi-dry processing is a variant that combines a wet mechanical process to remove the pulp and a dry process in which the depulped beans are spread in a thin layer on cement patios to allow further aerobic degradation of the mucilage [2].

The fermentation of coffee fruit is the processing step in which the mucilage is degraded while the fruits are simultaneously dried to 11–12 % moisture. The length of time required for fermentation differs among the three processing methods. During fermentation, physicochemical changes occur in grains, such as a reduction in water content and simple sugars and the formation of aroma and flavor precursors [3]. The fermentation of coffee fruits occurs naturally regardless of the processing method. In all processing methods (dry, wet and semi-dry), the objective of fermentation is to remove the mucilaginous layer, which is rich in polysaccharides (pectin), and to reduce the water content of the fruits. Coffee fruits contain indigenous plant enzymes that degrade the mucilage layer, but such activity is not sufficient for a complete and adequate process [4].

In fact, the two main types of post-harvest process, dry and wet, different treatments exist for these two processes when there were in a rainy seasons. At a rainy seasons, it was difficult to prepare green coffee with the dry method, even it is technologically much simpler than the wet method.

Since the coffee harvest coincides with the rainy seasons. Drying the coffee sufficiently is difficult. The coffee are often placed on plastic bags or laid out without a roof over it invariably gets at least a little wet between the start of a rain storm and when it is gathered up and stored,

prolonging the time needed for drying. It may also pick up moisture from the ground. The coffee is not dried to the levels recommended for commercial purposes, increasing the likelihood of fungus which create undesirable tastes in the coffee. Yet little is known about the impact of these kind processing on the quality of the coffee, or on its aroma potential.

Several studies have been done to improve the quality and taste and aroma of coffee. Similar research has been done by [5], with the addition of yeast in the process of making instant coffee microencapsulation. The results showed that yeast concentration and fermentation time had significant effect on cafeine content, moisture content, ash content and organoleptic value (aroma and flavor). The best results were obtained at 1% yeast concentration and 5 hours fermentation time. However, the research is applied to the wet-way treatment and is devoted to improving the taste of instant coffee.

The study of [6] found that interaction of container type and fermentation time did not significantly influence the weight of coffee beans, coffee period, and hygroscopic but had a very significant effect on the color of coffee beans. However, the study lack of information about organoleptic modification to the coffee especially about cupping score. Nowadays, coffee buyers and consumers as the end stage of specialty coffee much concern to quality of the coffee.

In this research, there are three stages: phase I by measuring pH and temperature changes during fermentation process, stage II is fermentation with addition of yeast and stage III that is done semi wet processing process, because this process is very easy to do in coffee farmer's house with varying the method of storing a closed container using a plastic bags and, laid out coffee cherries without roof over.

The purpose of this work was to study how the treatments of the coffee cherries that placed on plastic bags and the other way coffee cherries laid out without a roof over affected the aroma quality of green coffee. This study had the merits of identifying the pH and temperature changed during fermentation and cupping test for each stage of treatments by two professional coffee cuppers from Gayo Cuppers Team. In addition, fermentation process with adding yeast was included in the comparison.

2. Material and Methods

a. Biological Material

The Coffea arabica samples used in this study came from CV. Nutrisi Aceh (Central Aceh). The coffee cherries were utilized for different treatments and divided into 20 kg batches of cherries are reported in Table 1.

Table 1. Description of the coffee cherries post-harvest processing treatment used

Treatment	Stored time (hours)	pulping	Fermentation (hours)
Stored in plastic bags	24	Disc pulper	12
	48	Disc pulper	12
	72	Disc pulper	12
Laid out without roof over	24	Disc pulper	12
	48	Disc pulper	12
	72	Disc pulper	12
Natural fermentation		Disc pulper	4
		Disc pulper	6
		Disc pulper	8
		Disc pulper	10
		Disc pulper	12
Natural fermentation + yeast		Disc pulper	12

b. Dry post-harvest processing treatments

In all treatment, pulping was carried out without water with a disc pulper. The samples were washed as soon as the fermentation time had been judged sufficient. Once the mucilage had been removed, the beans were dried in the sun on metal trays, in layers approximately 2 cm thick until moisture content of about 12% attained. The samples were frozen at -80 °C in plastic flasks pending their use.

Preparation of coffee samples to study organoleptic score carried out according to SCAA methods [7]. In treatment 3, temperature and pH change were observed periodically in one hours. For the treatment 4, this was done by following the procedure described in previous work [5].

3. Results and Discussion

a. Identification of temperature and pH changed during fermentation

Research on the effect of yeast addition and fermentation time on organoleptic score have been done. Table 2 displayed a result of pH and temperature changes during the fermentation process that is measured periodically every one hour.

One of the stages of processing *semi wet way of arabica coffee* that determines the quality of the final steeping is fermentation. Fermentation aims to remove the remaining sticky mucilage layer from the beans after the ferment process. In addition, arabica coffee fermentation aims to reduce the bitterness and encourage the formation of mild impression on the brew.

This is due to the activity of microorganisms during fermentation to break down glucose and pectin into acids. In line with [6], the longer the fermentation time will cause pectin content in coffee bean mucilage tends to decline. The decrease in pectin levels is due to the degradation of pectin compounds into pectat acid which makes the fermentation environment to acidic.

Table 2. Temperature and pH change identified in green coffee from natural fermentation.

Natural Fermentation				
Time (hour)	**F6**		**F12**	
	pH	T (^{o}C)	pH	T (^{o}C)
0	4,69	21,7	4,69	21,7
1	4,30	22,0	4,34	23,6
2	4,51	22,6	4,31	23,0
3	4,42	24,2	4,27	23,8
4	4,18	22,5	4,29	23,2
5	4,31	24,9	4,15	23,7
6	4,26	23,6	4,12	24,1
7			4,11	24,2
8			4,08	24,3
9			4,06	24,4
10			4,03	24,4
11			4,00	24,5
12			3,93	24,7

F6 : natural fermentation until 6 hour
F12 : natural fermentation until 12 hou

The most important part of this slimy layer is the protopectin component that is an "insoluble complex" where the meta cellular lactice occurs. The enzyme contained in the coffee includes a kind of catalase that will break down the protopectin in the coffee fruit.

In this study, the process of decreasing pH as a result of the decomposition of the compounds into acid takes place both on the fermentation of 12 hours. Naturally coffee beans contain compound precursor forming flavor so that the variation of fermentation from 4 hours to 10 hours also cause the pH decreased as a result of changes in the compound to acid. However, the process to break down the sticky mucilage layer until ten hour is believed to be incomplete. Based on physical observation of fermented samples at 4 hours to 10 hours it is known that in the coffee beans there is still a layer of mucilage (mucilage) so it is difficult to wash.

According to [8], there is an indication that acidity should increase and pH should decrease as cup quality diminishes. This

could be associated to the effect of sour beans on cup quality. The same thing is reported by [9], that the fermentation of arabica coffee should be done 12 hours i n order to reduce and facilitate washing of mucilage and improve flavor taste. The research of [10], shows that the optimum process conditions to produce the best flavor are at 250C fermentation temperature and 12 hours fermentation time.

b. Comparison of the different treatments on organoleptic score

The combination of treatments obtained as in the following Table 3.

Table 3. The cupping score of natural fermentation at different fermentation time

Natural fermentation	Cupping score	Explanation
6 hours	84.75	Low viscosity, slightly flavor like tea, short fermentation time
12 hours	85.50	Herby, fruity, nutty, sweet
12 hours + yeast	84.00	Normal taste, short taste

Tabel 3 shows the quantities of cupping score in green coffee derived from the different fermentation time. Coffee from process fermentation six hour and twelve hour have highest cupping score compared to coffee from fermentation with adding yeast. The green coffee in last process, which was adding yeast did involve a fermentation stage, it displayed cupping score about 84.00 much lowest than the other process. This result might have been due to the yeast cannot improve the quality of coffee, even the rule of the yeast due to complete mucilage removal.

Furthermore, the research done with the addition of yeast as much as 20 grams in 1.5 kg sample of coffee beans. After the pulping process, microorganisms of the Saccharomyces Cerevisiae and Acetobachter Aceti species are naturally present in small amounts in the coffee sample. But due to the limited amount, the fermentation process is slow. Therefore in this study added a number of yeasts for helping the process of fermentation.

Research conducted from [5], showed that the concentration of yeast has an effect on the aroma and taste. From the analysis results obtained the best results at 1% yeast concentration and 5 hours fermentation time.

The results obtained in this study indicate a contrast result that yeast concentration does not affect coffee flavor. The cupping score is lower than the coffee that is not added yeast. In line with [8], not all species of bacteria have high PL activity, and thus not all species would enhance pectin hydrolysis at the beginning of the fermentation.

c. The cupping score for different treatment

Normaly, the coffee cherries should be pulped and fermented until process completed. However, many farmers in the study area stored and ferment the bean for longer periods of time, up to several days. This gives the coffee an identifiable “overfermented’ taste that is considered undesirable from a quality viewpoint. In fact, as a result in Table 5 it can be seen that the results of the cupping score 84.50 are similar for green bean from stored in plastic

bags.

Meanwhile, the green coffee from laid out without roof over have decreased cupping score from 84.00 until 83,25 as long as stored time. This is indicate that coffee cherries cannot stored at the open air because it would decrease quality of the coffee.

Table 4. The cupping score for different treatment and characteristics

Treatment	Stored time (hour)	Cupping score	Characteristics
Stored in plastic bags	24	84.50	herbaceous, sweet melon, short taste
Stored in plastic bags	48	84.50	Nutty, chocolate, tobaco
Stored in plastic bags	72	84.50	Sweety, nutty, sweet lemon
Laid out without roof over	24	84.00	nutty, sweet lemon
Laid out without roof over	48	83.50	fruity, sweet lemon, balanced
Laid out without roof over	72	83.25	Over ripe fruit

Based on visual observations, there are different colors of the two storage methods. Supported result by [6], who tested the color of *arabica* coffee beans stored in a container bucket and inside the sack. The results show that coffee seeds stored in bucket containers have a higher brightness than in sacks.

Table 5. The cupping score categories comparing between different treatments.

Category	A1	A2	A3	B1	B2	B3
Aroma	8.0	7.8	7.8	8.0	8.0	8.0
Flavor	7.8	8.0	8.0	7.8	7.8	7.5
After taste	7.8	7.8	7.8	7.5	7.5	7.3
Acidity	7.5	7.5	7.5	7.5	7.5	7.8
Size	8.0	8.0	8.0	8.0	7.8	7.8
Uniform	7.8	7.8	7.8	7.8	7.5	7.5
Balance	10	10	10	10	10	10
Cup clean	10	10	10	10	10	10
Sweetness	10	10	10	10	10	10
Overall	7.8	7.8	7.8	7.5	7.5	7.5
Total Score	84.50	84.50	84.50	84.00	83.50	83.25

Furthermore, [11], reported that on the surface of the skin of coffee bean shell attached mucilage layer as much as 13.7% dry weight with 0.5 - 2 mm thick, 30% dry weight of reducing sugar and 8.9% dry weight Protein. Under these conditions, high temperatures and long processing times will have an impact on increasing the rate and intensity of the browning process. Besides marked by the occurrence of browning on the surface of the seed, the phenomena is also indicated by a decrease in the mucilage attached to the surface of the shell. In general, heat treatment in the fermentation

process will affect the more layer of mucilage to decompose. Relatively warm conditions will accelerate to the decomposition of sugar during the fermentation process. However, a less controlled process will result in a browning reaction.

4. Conclusions

Given the result obtained from an organoleptics score, temperature and pH changes during fermentation provided enough information to distinguish between green coffees obtained by different treatment. The results of the first stage showed that the best fermentation time was at 12 hours with the decrease of pH reach 3.93 and the cupping score of 85.50. The addition of yeast did not give effect to the flavor with the total cupping score about 84.0. The results in treatment II gave the cupping score 84.50 for green bean which obtained from coffee cherries stored in plastic bags much higher than coffee cherries which laid out in open area with cupping score 84.00. The study showed that the coffee cherries can be stored into plastics bags for several days at rainy seasons before the coffee can be sold.

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THE CORRELATION BETWEEN RESISTANCES OF BACTERIA AGAINST HEAVY METALS AND ITS TOLERANCES TOWARD ANTIBIOTICS

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Abstract

A variety of contaminants has been exposed into the river worldwide. Heavy metal is one of contaminant groups in the environment, especially in the river system, which has to be taken into account intensively. Numerous environmental studies have concern about the impact of heavy metals polluted environment to the growth of microorganisms, such as bacteria. Bacteria which were exposed to the highly polluted environment somehow will have the ability to develop their own defense mechanism to survive in such environmental conditions. Moreover, it has been reported in some publications which estimate the correlation between the resistances of bacteria against heavy metals and its susceptibility decreasing toward antibiotics. In this literature study, several scientific reports have been reviewed regarding those estimations. It is proposed that the heavy metals contaminated stream ecosystem will lead to a co-selection towards antibiotic resistant bacteria. Consequently, the contaminated environment not only will give a bad impact to the ecosystem in direct ways but also endanger life indirectly. People will face a big health problem if many strains of bacteria which exposed to the heavy metals polluted environment have high resistances against antibiotics.

Keywords: heavy metal pollution, bacteria resistance, antibiotics resistance, co-selection

1. Introduction

The human activities around river catchment area, somehow, will cause the pollution effect to the river systems. The introduction of pollutant to the river will seriously degrade the water quality and the river ecosystem as well. River sediment is one of the river systems that may be contaminated by the pollutants. Many pollutants which are released into surface waters will be attached to the particulate matter and incorporated in sediments [1].Various anthropogenic activities and the variety of land use along river catchment can be the primary sources of the river sediment pollutants.

There are several kinds of river sediment pollutants which are commonly known as the causes of the degradation of river ecosystems. Heavy metal is one of contaminant groups in the environment, especially in river sediments, which has to be taken into account intensively since metals are not removed by natural degradation processes [2]. Due to this reason, metals can be accumulated in river sediments for long periods and can be the cause of river contaminations. The introduction of metals in to aquatic systems can be both by natural processes and anthropogenic activities as well [3].

The exposure of heavy metals into river ecosystems may cause toxic effects to the aquatic biota and human life. Nowadays, numerous environmental studies have concern about the impact of heavy metals polluted environment to the growth of microorganisms, bacteria for instance. Bacteria which were exposed to the highly polluted environment somehow will have the ability to develop their own defense

mechanism to survive in such environmental conditions. The modification in structure and function of microbial communities can be occurred when it introduce to various form of heavy metal polluted environments [4]. Thus, some bacteria become resistant to some kind of heavy metals.

It has been reported in some publications which estimate the correlation between the resistances of bacteria against heavy metals and its susceptibility decreasing toward antibiotics [5, 6 ,7]. As mentioned by reference [7], the metal contamination functions are considered as a selective agent in the proliferation of antibiotic resistance. It is proposed that the heavy metals contaminated stream ecosystem will lead to a co-selection towards antibiotic resistant [8].

Consequently, the contaminated environment not only will give a bad impact to the ecosystem in direct ways but also endanger life indirectly. People will face a big health problem if many strains of bacteria which exposed to the heavy metals polluted environment have high resistances against antibiotics. Therefore, the resistances of bacteria against antibiotics will become an emerging public health problem [9] and has received a remarkable attention from scientists' community.

This literature study was focused on collecting and reviewing information from previous researches regarding the co-selection of antibiotic resistance of isolated bacteria which driven by heavy metals contaminated river systems.

2. Discussion

a. Heavy metal contamination within river system

A variety of contaminants has been exposed into the river worldwide. Many contaminants are still present in bottom sediments even though the regulation and contaminants control have been arranged to reduce their release. Sediments are persistent which can cause potential risk to the environment and toxic impacts on aquatic life, organisms and on human health as well [10].

At the present time, the gradually increasing of heavy metals contamination in environment can be observed, especially within aquatic ecosystem. Dissolved heavy metals or heavy metals which attached to solid particles can be transported within the river [11]. Heavy metals are defined in various ways by the scientists. The definition can be based on its density, atomic number or atomic weight, chemical properties or based on its toxicity [11]. Reference [12] defines heavy metal as elements that have metallic properties which include the transition metals, some metalloids, lanthanides, and some actinides. About 59 elements from more than 90 elements in the periodic table, which have density > 5 g/cm^3, are characterized as heavy metals, while 20 are define as metals [13].

Heavy metals cannot be broken down [14] and tend to be accumulated or transferred within organisms in the food web which lead to the ecological impacts and health problems. Even in low concentration, metals may cause the toxicity effect [15].

The concentration of a number of heavy metals like copper, cadmium, chromium, lead, nickel, cobalt, zinc and their impact to bacteria have been investigated in this study. Copper, chromium and cadmium were trace elements in the environment which categorized as harmful to the aquatic organism due to their impact on tissue metabolism [16]. Kidney dysfunction, skeletal injure and reproduction low rate are the example damages that caused by the accumulation of cadmium in the human body [16]. Likewise, lead (Pb) known as a primary contaminant which normally found in water, soil and air. Reference [17] defines lead as hazardous waste and has very high toxicity to human, animals, plant and microbes.

Not only detected in the water column, heavy metals are also encountered in high

accumulation within river sediment. Therefore, the contamination of heavy metals in aquatic sediment becomes the major environmental problem, especially in densely populated region and industrial area. The anthropogenic sources such as mining, chemical and manufacturing industries as well as municipal wastewater flows are considered as the causes of heavy metals content increasing in the aquatic ecosystems [18].

In particular environmental condition, sediment may act as a source of contamination. Due to its character as transporter for particle-associated pollutants, nowadays, aquatic sediments are often used to identify, control and monitor the sources and the waste of metals, and then evaluate its impact to the environment. The contamination of heavy metal in river sediments could be resulting from diffuses and point sources [19]. Such examples of diffuse sources are atmospheric wet and dry depositions as well as soil wash out in agricultural areas. Likewise, the emissions from industrial plants, municipal sewage plants, waste disposal sites and disposal site of dredged sediments and mining wastes are categorized as point source contamination [20].

Concerning the negative consequences that may caused by contaminated sediments, numerous studies have been conducted to observe the concentration of contaminants within freshwater sediments, including heavy metals contamination.

b. Bacterial resistance to heavy metals

Bacteria has been known as one of the smallest entities on earth and part of prokaryote microorganisms. They are important to the environment due to their ability to convert various inorganic and organic pollutants into harmless minerals and recycled back into the environment [21]

Basicly, metal ions are necessary for a range of metabolic activities and structural arrangements of bacteria, magnesium for instance. This compound is essential component for the forming of bacteria structure and also used to build up the ribosomes [22]. However, the author also noted that some metals are only occasionallly used and often very toxic to the bacteria.

Beside act as essential nutrient for bacteria, some metals may also be toxic agents which affecting their cell formation and inhibit the growth processes of this microorganisms [22]. As already noted, for survive and face the toxicity pressures that are encountered in the environment, bacteria has developed such of tolerance mechanisms which then result some resistant bacteria strains toward heavy metals. The susceptibility state of bacteria to heavy metals was detectable by defining its minimium inhibitor concentration (MIC) value.

The fact that the toxicity of heavy metals will influence bacteria as first microorganism in trophic web should be taken into consideration. This means that the toxic heavy metal can be accumulated in bacteria and be passed to another organism in the food chain [23] which possibly lead to widely health problem.

There are several mechanisms how heavy metals harm the bacteria. As mentioned by reference [22], the influences of heavy metals to microorganisms are by affecting their growth, morphology and biochemical activities. Reference [23] has reviewed some examples of how specifically heavy metals impacted bacteria in term of growth inhibition from different sources (Table 1).

Table 1. Examples of heavy metals impacts on bacteria

Examples	Sources
1. The lag phase of *Agrobacterium tumefaciens* was extended from 3 to 6 h by 5- 10 ppm of Cd	Reference [24] Reference [25]
2. The lag phase of *Arthrobacter marinus sp. nov.* was lengthened by 4 x 10^{-4} M Ni from 3 to 72 h	Reference [26]
3. The lag phase of *Vibrio sp.* was extended by 5 ppm of Hg from 3 to 72 h; extended that of *Bacillus sp.* from 3 to 41 h by 15 ppm of Hg; and extended that of *Citrobacter sp.* from 3 to 70 h by 20 ppm of Hg	Reference [27] Reference [28]
4. The growth of *Escherichia coli* was declined by Cd, Zn, Cu, Hg and Ni	Reference [29]
5. The growth of *Pseudomonas tabaci* was completely inhibited by 10^{-2} M Ni and partially blocked by 10^{-3} M Ni	Reference [30]
6. The *Streptococcus faecalis* growth was hampered by 100, 500, 1000 µM of Zn and Cd	
7. 50% of 58 species of bacteria were intolerant of 5 ppm Cd but tolerant of concentration <5 ppm	

However, the coexistence of bacteria cell and toxic heavy metals is not a new phenomenon. Since the early form of life, when the volcanic activity as one of toxic heavy metals source was very intensively happened and causes the abundant of toxic heavy metals on the earth environment, the bacteria have develop such of mechanisms to survice in those kind of environment.

It is a fact that all living cells accumulate particular type of metallic ions, including bacteria. However, at the same time, bacteria are resistant to several heavy metals as well. One of the reasons for this heavy metals resistance could be due to the expression of factors encoded by special plasmids [22].

Furthermore, reference [31] has been mentioned the basic mechanisms concerning bacterial resistances to toxic heavy metals in the following way:

- The presence of enzymes, oxidases and reductases, which alter metal ions from high toxicity into lower toxicity.
- The possibility of the binding of toxic heavy metals either in the cell wall or intracellular which will stop the heavy metals from achieving the intracellular cytoplasm.
- The chance of blocking cellular uptake by varying the available uptake mechanisms in sensitive cells.
- The existence of a highly specific efflux system that able to pump out again quickly if toxic metal ion has closed to the intracellular cytoplasm.

Reviewing this phenomenon, we can simply come out with the overview of how bacteria can be able to build an incredible resistance mechanism under deleterious environmental condition. Reference [32] has been investigated the resistances of bacteria which isolated from heavy metals polluted river water and sediment. The strains of bacteria that have been isolated in that study were *Aeromonas* spp and *Pseudomonas* spp. *Aeromonas* spp are gram negative bacteria which are originally part of the family *fibrinocea*. They can be isolated from foods as green vegetables, raw milk, ice cream, meat and seafood. *Aeromonas* spp is categorized as normal water inhabitants. Numerous epidemiological studies have shown *Aeromonas* spp in feces was more often linked with diarrhea which correspondent to the untreated water as well ([33]. *Aeromonas* spp is widely distributed in both fresh and saltwater and among fish

and invertebrate species of animals.

Meanwhile, the genus *Pseudomonas* is part of a gram negative bacteria group which belongs to the Pseudomonadaceae family [34]. Pseudomonads are abundant and widespread in nature. They can be found in soils, fresh water, marine environment and many other habitats. *Pseudomonas* species is one of environmental bacterium that known as the top three causes of opportunistic human infections [35].

According to reference [32], there was a positive correlation between the increasing of heavy metals concentration in river sediment of investigated regions with the rising of bacteria tolerance toward metals that has been observed along Western Bug River, Ukraine.

Other important invention which has been reported by a number of studies was the estimation of relationship between metals resistant bacteria and its low susceptibility toward antimicrobial.

c. *Antimicrobial susceptibility of bacteria*

According to reference [36], antimicrobial which is also named antibiotics, are molecules that inhibit the growth of microbes, both bacteria and fungi, or kill them directly. Antibiotics that inhibit the bacteria growth, for instance the drug chloramphenicol , are called bacteriostatic, while the antibiotics which cause the cell death of bacteria are named bactericidal [36].

There were nine antibiotics from six antibiotic classes that have been used in this study which were amikacin, gentamicin, tobramicin, ciprofloxacin, aztreonam, piperacilin, piperacilin-tazobactam, ceftazidime and imipenem.

The presence of these antibiotics are very useful in medical field worldwide. However, the fact that bacteria have developed the ability to avoid and become pathogens and resistants to one or more antibiotics has been apparently detected nowadays. Obviously, antibiotic resistant bacteria will causes worldwide public health problem and hampering the infectious desease treatment process [37].

Bacteria can avoid the effect of antimicrobial through a number of mechanisms, which comprise enzymatic inhibition, altered poring channels, alterations of outer or inner membrane permeability, modification of target proteins, antibiotic efflux and altered metabolic pathways [38]. Moreover, reference [39] stated that the rapid growing of antibiotic resistance in bacteria is greatly caused by the tandem-assembling of the resistance genes within single mobile genetic elements which then create the multi drug resistance clusters.

Further reason for the resistances of gram positive and gram negative bacteria to antibiotics is the existence of specific enzyme which modify the antibiotics and incapable the antibiotics interaction with their target in the bacteria cell [40].

Additionally, based on his research, reference [41] has summarized that the variation of existing enzyme genes in various organisms enable them to develop intermediary metabolism and produce huge number of antibiotic chemicals. As the result, selection for resistance mechanisms occurred in the target organisms when these natural antibiotics are introduced to them. Moreover, reference [41]mentioned the classification of possible mechanisms that develops in the target organisms, which include:

a. The reduction of permeability
b. Antibiotics inactivation
c. Modification of the target within the host
d. The expansion of a substitute (but resistant) biochemical pathway
e. The improvement of efflux pathway

Corresponding to those facts, numerous researches have been estimated that one of possibilities that affect the increasing of bacteria resistancy toward antibiotics occur when the bacteria was exposed to the high

metals contaminated environment [8, 5].

d. Co-selection of antibiotic and metal resistance

The bacteria strain which show resistancy to heavy metals will indicate the resistancy to antibiotics as well [7, 42]. As mentioned by reference [5], the resistance of bacteria to different metals and antibiotics can be genetically linked. When the bacteria has exposed to toxic metals, it may cause the selection for strains resistant to antibiotics and vice versa. This phenomenon is known as co-selection process [7]. Antibiotic resistant bacteria could be arising in the polluted environment through co- or cross resistance to metals or co-regulation to metals pathways [45]. Co-resistant is identified when different resistance determinants within bacteria is introduced by the same genetic substance [7]. It can be also explained as a mechanism within bacteria when the encoding genes for metal and antibiotic are obtain on the same plasmid and/or transposon of bacteria [43]. The author also noted that cross-resistance as the mechanisms where one genetic element in bacteria have resistance encoding to metal as well as to antibiotic.

Numerous studies has been conducted based on the estimation of metals and antibiotic co-selection occurrence of bacteria [45, 7, and 44]. From the result of their research, reference [45] have noted that in an environment where bacteria is selected directly to heavy metals, the rising of antibiotic resistant bacteria also occcured and this antibiotics resistance genes is distributed uniquely along the river systems.

Moreover, the research of reference [44] at Iskenderun Bay, Turkey, revealed that the isolates from seawater and sediment which mostly were *Escherichia coli*, *Aeromonas hydrophilia* and *Stenotrophomonas maltophilia* have showed resistance to metals and also high resistance to streptomycin, ampicilin and trimethoprim-sulphamethoxazole.

Furthermore, reference [46] has investigated *Pseudomonas aeruginosa* which isolate from water and sediment of Lake Ontario and sewage treatment plants regarding its susceptibility toward mercury, cadmium, arsenic, lead, gentamicin, carbenicilin and tetracycline. The author noted that the same plasmid was responsible for the occurrence of metals resistance and drug resistance as well.

Studies of reference [47] and [32] also performed the antibiotic susceptibiliy test of the isolates from Western Bug River, Ukraine. Reference [47] reported that isolates from Western Bug River which showed high tolerance to aminoglycosid were also had resistances to betalactam antibiotics. While resistance to more than one antibiotics was detected for 6 % of isolated *Aeromonas* spp from Western Bug River [32].

Reference [8] have reviewed that the selection of metals and antibiotics resistance strains was not encountered within unexposed communities. Furthermore, the authors also informed the significant positive correlation between antibiotics resistance and the rising of metals concentration of their research.

According to reference [7], the development of bacteria tolerance to particular antibiotic when they also expand their capacity to cope with certain heavy metal stress environment is known as co-selection process. Commonly, the co-selection occur through co-resistant and cross-resistant mechanisms.

In summary, river systems which highly contaminated by heavy metals will cause remarkable damage to ecological factors. The indirect impacts of heavy metals to human life concerning the increasing probability of antibiotic resistance which enhance the extra health problem in the society may be of similar importance.

3. Conclusion

Heavy metals contaminated river

systems was found influencing the susceptibility of bacteria within the river sites. Some bacteria strains which were resistant to heavy metals were also found having tolerance to some antibiotics.

Even though the misuse and the overuse of antibiotics for the health purposes have been known as direct causes of the antibiotic resistant bacteria increasing, heavy metals contamination in an environment is already discovered as indirect reason for this occurrence. This undeniably becomes the human health problem worldwide such as insufficient health treatment, ineffective health cost due to longer stays in hospital, even rising the death risk because of the infection.

Finally, in brief, metals contamination environment will not only provide negative impact concerning ecological state itself, but also terribly affected clinical sector by potential contribution in rapid spreading of antibiotic resistant bacteria.

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THE EFFECT OF ENCAPSULATION MATERIAL ON THE QUALITY OF PROBIOTIC CONTAINING *Lactobacillus fermentum*

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Abstract

This research was conducted to ascertain the effect of encapsulate material on the quality of probiotic containing *Lactobacillus fermentum*. The probiotic was isolated from the intestine of Japanese quail (*Coturnix japonica*). A fully randomized factorial design was used to assign four kind of encapsulate material, that was maltodextrin-starch corn (MC), maltodextrin–skim milk (MS), gum arab-starch corn (GC), gum arab-skim milk (GS). The first factor was non-encapsulated probiotic and the second factor was encapsulated. The inclusion levels of encapsulate material was (25, 30 and 35%). Data were analyzed by analyzes of variance by two-way Nested of Completely Randomized Design. The results showed that the best of encapsulate material is maltodextrin-corn Starch 35% (MC 35). *L. fermentum* that encapsulated with MC 35 was produce highest of lactic acid (1.13 %), lowest of pH (4.067), lowest total sugar (0.518 %) and highest amount of probiotic cell (10.66 log cfu/ml).

Keywords: *encapsulation, L. fermentum, probiotic.*

1. Introduction

Lately safety of food products of animal needs special attention, this is due to information about antibiotic residues in poultry products and other animal's products that cause adverse effects not only for animals but also for consumers. The use of antibiotics is carried out by farmers had caused antibiotic residues in animals products up to 63-65%. Most of the liver and poultry meat contaminated with residues of antibiotics, especially penicillin and tetracycline (Setiawan, 2009). The European Union has set regulations as the basis for the prohibition 1831/2003 number of various kinds of antibiotics (FDA, 2012), which is much earlier some countries have restricted the use of these additives in animal feed such as in Sweden in 1986, Denmark in 1995, Germany in 1996 and Switzerland in 1999 (Riley, 2012).

The use of probiotic in the poultry diets has been increasingly important to substitute the role of antibiotic. In the previous experiment Kalsum et al (2010) have isolated several Lactobacillus spp from the small intestine of Japanese quail. One isolates identified as Lactobacillus fermentum has been proved to survive at acidic pH and bile salt tolerant and able to inhibit the growth of microbial pathogens especially *Salmonella typhimurium* and *Escherichia coli* (Afdora et al 2010). Utilization of these probiotics for poultry feed is necessary to protect the area of the mouth to the gizzard by using encapsulation technology. Encapsulation is commonly used spray drying, but this process has a weakness that using high temperatures (140-180 °C), whereas endogenous probiotics are not resistant to high temperatures, because it necessary an alternative to using a low temperature (50 ° C) by using a modified oven. Those efforts are expected to produce a stable and safe probiotics so ready to be used by stakeholders to produce commercially.

2. Materials and Methods

A. Location of the study area

The experiment was carried out in Microbiology laboratory Faculty of Science and the experimental farm of Faculty of

Animal Husbandry, Islamic University of Malang, East Java, Indonesia.

B. Preparation of probiotics

Lactobacillus fermentum was isolated from the intestine of Japanese quail and cultured in agar medium as described by Kalsum et al (2012). The aim of this research was to determine the type and dose of encapsulation material whichis able to produce the best encapsulation material measured by the content of organic acids and dissolution ofthe encapsulated material.

C. Experimental design and treatments

The experimental design of this research was use the method completely randomized design with three treatments combination (maltodextrin-corn starch, maltodextrin-skim milk, and gum arabic-corn starch) with five replications. Before trial types of encapsulation material were implemented, carried out optimization of dose of encapsulation material (25%, 30%, and 35% of the substrate) at a temperature of 50° C and for 60 minutes by the formation of physical encapsulation (density, pH, and total acid).

D. Statistical analysis

Statistical evaluation of the result was performed by the analysis of variance (ANOVA) program in the Minitab (2003) software (version 14). Significant differences between treatments determined by Duncan's multiple range test according to Steel and Torrie (1992).

3. Results and Discussion

This research used the feed additive containing endogenous probiotic i.e. *Lactobacillus fermentum* isolated from the small intestine of quail. Endogenous probiotics in the gut ecosystem will naturally occupy compete in the host location (Servin and Coconnier 2003). But the Lactobacillus genus most susceptible to stress than other bacteria in the digestive tract (Bhunia 2008), and thus the necessary protection to the cells from endogenous lactic acid bacteria in order to maintain the quality of the probiotics.

3.1. Maltodextrin-corn starch

The data of lactic acid levels in the combination treatment of maltodextrin-cornstarch is shownin Table1.

Table 1. Lactic acid levels of maltodextrin-com starch (MC)

Time (hours)	The average levels of lactic acid (%)		
	MC 25%	MC 30%	MC 35%
0	0.25	0.31	0.53
6	0.28	0.36	0.46
12	0.34	0.57	0.74
18	0.43	0.80	0.84
24	0.51	0.81	0.91
30	0.54	0.84	1.13
36	0.49	0.70	0.93

The results of analysis of variance showed that the amount of maltodextrin-corn starch very significant effect on the levels of lactic acid (P <0.01). The highest of lactic acid levels obtained in the treatment of maltodextrin-cornstarch 35% at the 30 th hour. The Ph value in the treatment of maltodextrin-cornstarchis shownin Table2.

Table 2. The Ph value in the treatment of MC

Time (hours)	The average levels of lactic acid (%)		
	MC 25%	MC 30%	MC 35%
0	6.1	6.1	6.1
6	5.4	5.3	5.3
12	5.06	4.7	4.9
18	4.57	4.3	4.4
24	4.4	4.23	4.33
30	4.3	4.167	4.067
36	4.2	4.2	4.06

Analysis ofvarianceofthe Ph value indicatesthat theamountof maltodextrin-cornstarch very significant effect onthe pHvalue(P <0.01). Lowestthe pHvalues obtained inthe treatmentof maltodextrin-cornstarch35% at36thhour. The data of probioticcell countsin the treatment maltodextrin-cornstarchis shownin Table3.

Table 3. The count of probiotic cell inthe treatment of MC

Time (hours)	The average count of probiotic cell (log cfu/ml)		
	MC 25%	MC 30%	MC 35%
0	6,5	6,58	6,58
6	7,6	8,03	8,67
12	9,65	10,58	9,57
18	10,3	10,56	10,66
24	9,68	9,85	9,95
30	8,61	9,246	8,8
36	8,61	8,55	8,83

The Results showedthat theamountof maltodextrin-cornstarchvery significant effect onprobioticcell count(P < 0.01). The highest numberof cellsofprobiotics have been producedin thetreatmentof maltodextrin-cornstarchencapsulation35% at18hours.

Table 4. The lactic acid levels in maltodextrin milk treatment (MS)

Time (hours)	The average levels of lactic acid (%)		
	MC 25%	MC 30%	MC 35%
0	0.312	0.243	0.151
6	0.38	0.314	0.198
12	0.402	0.428	0.329
18	0.469	0.506	0.401
24	0.548	0.542	0.442
30	0.548	0.585	0.582

5.2. Maltodextrin-Skim milk

Analysis of variance showed that the amount of maltodextrin-skim milk significantly effect of lactic acid levels (P <0.05). The highest levels of lactic acid was obtained in 30% of MS encapsulation treatment at the 30 th hour. The pH value in the MS encapsulation treatment is displayed in Table 5.

Table 5. The pH Value in Maltodextrin-skim milk (MS)

	The average change in pH		
Time (hours)	**MS 25%**	**MS 30%**	**MS 35%**
0	5	6	7
6	4.9	5.6	5.9
12	4.7	5.1	5.3
18	4.6	4.8	4.6
24	4.3	4.6	4.6
30	4.2	4.3	4.5
36	4.2	4.3	4.4

Results of analysis of variance showed that the amount of maltodextrin-skim milk significantly affect the pH value ($P < 0.05$). The lowest pH values obtained in the treatment of maltodextrin-skim milk 35% at the 30 th hour. The data of probiotic cell counts in maltodextrin-skim milk treatment is displayed in Table 6.

Table 6. The probiotic cell count in Maltodextrin-skim milk (MS)

	Average of probioticcell counts (log cfu/ml)		
Time (hours)	MS 25%	MS 30%	MS 35%
0	7.422	7.428	7.435
6	8.386	8.409	8.352
12	9.188	9.446	9.387
18	10.307	10.455	10.281
24	10.459	10.447	10.248
30	9.356	10.462	10.472
36	9.330	10.413	10.344

The results of analysis of variance showed that the amount of maltodextrin-skim milk very significant effect on of probiotic cell count ($P <0.01$). The number of cells obtained at the highest of probiotic-treated maltodextrin-skim milk 35% at 30 hours.

5.1. Gum arabic-corn starch

The data of lactic acid levels in the combination treatment of Gum arabic-corn starch is shown in Table 7.

Table 7. The lactic acid levels in the treatment of Gum arabic-corn starch (GC)

	Average of probioticcell counts (log cfu/ml)		
Time (hours)	MS 25%	MS 30%	MS 35%
0	0.040	0.262	0.492
6	0.039	0.214	0.461
12	0.037	0.248	0.452
18	0.041	0.245	0.518
24	0.038	0.227	0.476
30	0.039	0.223	0.496
36	0.037	0.213	0.457

The results of analysis of variance showed that the amount of Gum arabic-corn starch very significant effect on the levels of lactic acid($P < 0.01$). The highest of lactic

acid levels obtained in the treatment of Gum Arabic-corn starch 35% at the 18 th hour. The pH data on gum arabic-corn starch encapsulation is displayed in Table8.

Table 8. The level of pH in Gum arabic-corn starch (GC)

Time (hours)	The average change in pH		
	GC 25%	GC 30%	GC 35%
0	5.400	5.400	5.367
6	5.233	5.267	5.200
12	5.133	5.133	5.033
18	5.033	5.000	4.967
24	5.067	4.900	4.933
30	4.867	4.833	4.867
36	4.833	4.800	4.800

The results of analysis of variance showed that the amount of gum arabic-corn starch very significant effect on the pH value($P < 0.01$). Lowest pH values obtained in the treatment of gum Arabic-corn starch 35% at 36 th hour The data of probiotic cell counts in treatment-gum arabic-corn starch is displayed in Table 9.

Table 9. The count of probiotic cells in Gum arab-corn starch (GC)

Time (hours)	Average of probioticcell counts (log cfu/ml)		
	GC 25%	GC 30%	GC 35%
0	5,65	5,86	5,77
6	6,58	6,64	6,59
12	6,70	6,76	6,59
18	6,45	6,34	6,45
24	6,58	6,83	6,85
30	6,62	6,52	6,29
36	5,82	5,78	5,59

The results of analysis of variance showed that the amount of corn starch-Gum arabic very significant effect on of probiotic cell count ($P < 0.01$). The number of cells obtained at the highest of probiotic treatment Gum arabic-corn starch 35% at 24 hours. The best results of various types of material encapsulation with the highest levels of lactic acid is displayed in Figure1.

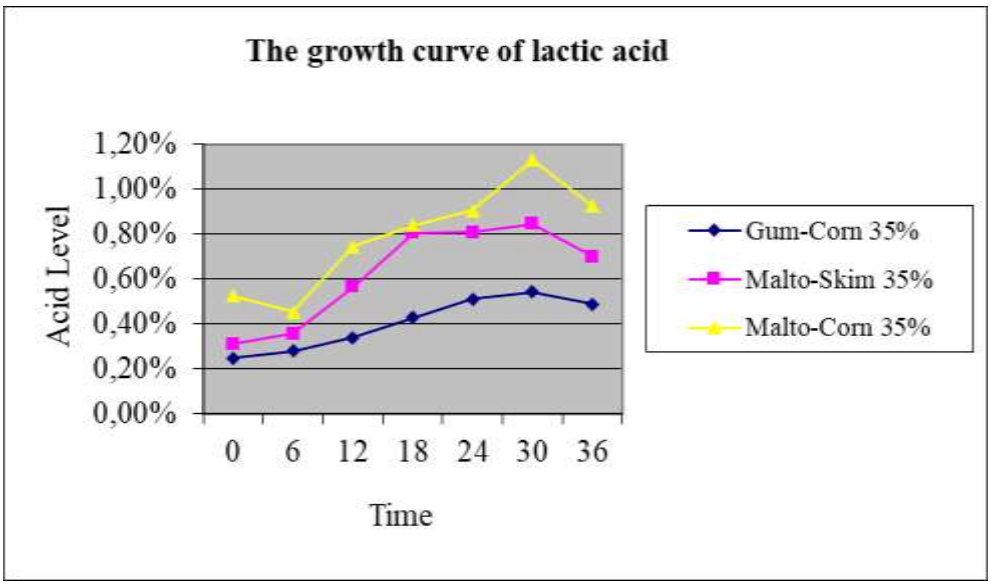

Figure 1. The curvea change of lactic acid levels in various types of encapsulation

The figure1 showed that the encapsulation in maltodextrin-corn 35% produce the highest levels of lactic acid. The best results with the lowest pH levels is displayed in Figure2, where it appears that encapsulation in maltodextrin-corn 35% yield low pH. The best results with the highest number of probiotic cells in Figure3, where it appears that the highest yield of probiotic cell counts is encapsulation in maltodextrin35%.

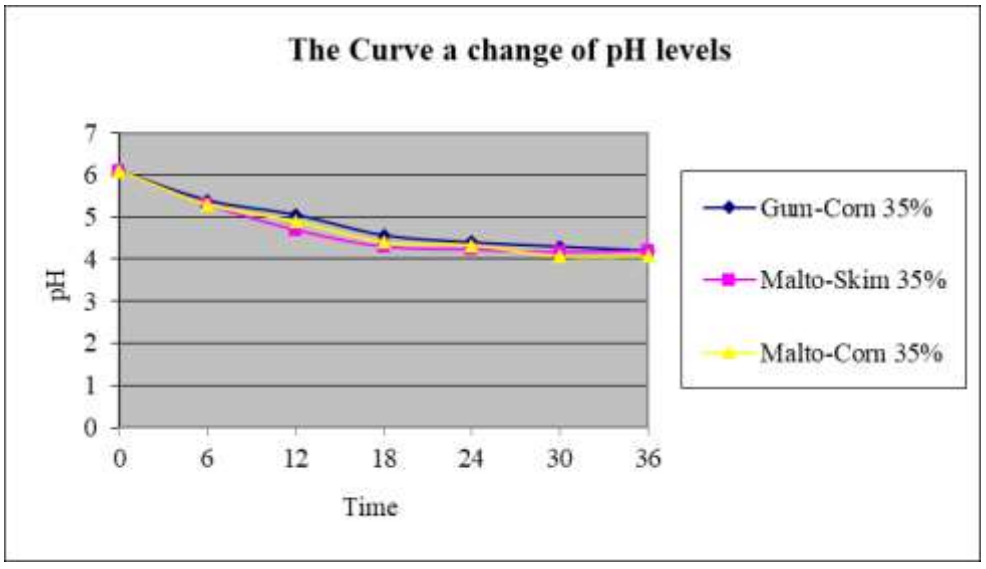

Figure 2. The curvea change of pH levels in various types of encapsulation

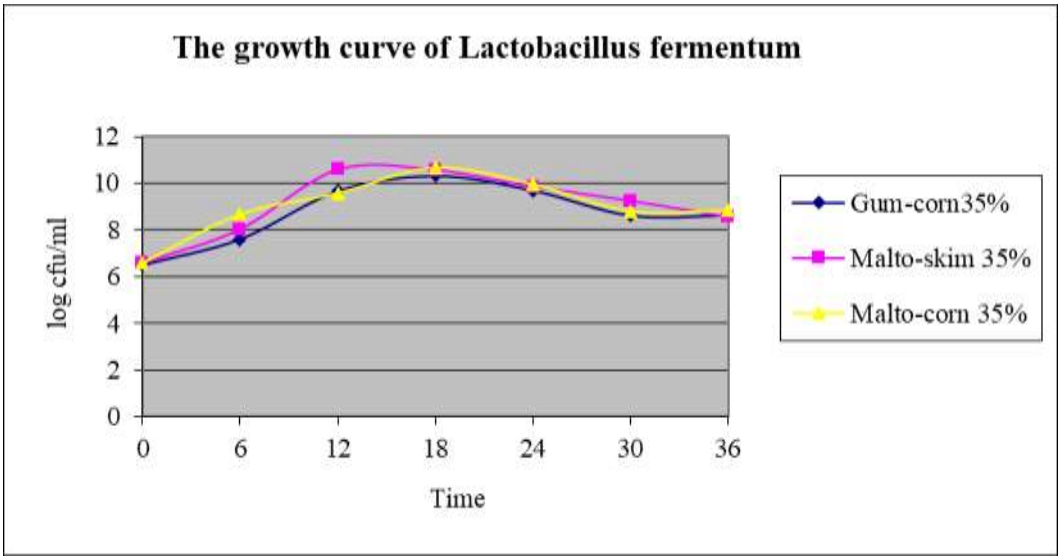

Figure 3. The growth curve of probiotic invarious types of encapsulation

4. Conclusion

The best of encapsulate material is maltodextrin-corn Starch 35% (MC 35). L. fermentum that encapsulated with MC 35 was produce highest of lactic acid (1,13 %), lowest of pH (4,067), and the highest amount of probiotic cell (10,66 log cfu/ml).

Acknowledgment

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EFFECT OF BIO-ACTIVATOR AND *Thitonia Diversifolia* ADDITION ON ORGANIC FERTILIZER PRODUCTION FROM AGAR WASTE

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Abstrak

The purpose of this research was to utilize the waste agar processing into organic fertilizer and determine what types bioactivator and add rate of *Thitonia diversifolia* most appropriate in the manufacture of organic fertilizers. Research design used is Randomized Block Design consisting of two treatments, namely the addition of bio-activator, which consists of B_0 (without the addition of bio-activator), B_1 (the addition of EM-4 bioactivator) and B_2 (addition Petrofast bioactivator) and the ratio of the addition Thitonia diversifolia consisting of T_0 (without the addition of Thitonia) , T_1 (Extra Thitonia 1 : 1) and T_2 (Extra Thitonia 1 : 3). The parameters tested in this study are temperature composting, water content, acidity (pH), C-organic, and C/N-ratio. Data were analyzed using ANOVA analysis of variance followed by LSD test with a significance level of 0.05. The results showed that in general the treatment Thitonia adding more influential than bioactivator treatment, it is caused by high of water content of the materials. Petrofast significant effect on decreasing the pH in the 3 rd week, the increase in organic C as well as an increase in total during the composting process. While the addition ratio greater Thitonia significant effect on the increase of moisture content during the composting process, very significant effect on pH decrease at week 3, the increase in organic C as well as the very significant effect on the value of C/N ratio. Treatment B_2T_2 obtain N-organic value amounted to 5.63%, organic-C by 24.8%, and the C/N-ratio of 20.74. The results showed that the physical properties, content of organic C and C/N ratio, organic fertilizer from the waste in order to qualify as organic fertilizer.

Keywords : Bioctivator, agar waste, *Thitonia diversifolia*

1. Introduction

Gelatin processing industry has produced wastes, either solids or liquids and residues as well. Kim *et al* (2007) asserted that gelatin extraction from seaweed can deliver 65-75% wastes of total output. But, the processing and utilization of gelatin waste are not well designed yet. If it is not strictly handled, it may cause an environmental problem. The waste of gelatin processing was potential to be processed as fertilizer because it had quite higher level of organic matter. Seaweed as raw material of gelatin contained with calcium, phosphor, zinc and natrium [1]. Solid waste is processed to produce artificial fertilizer (Yustin *et al*, 2008). Cellulose content of solid waste from gelatin processing was surely very high rated at 27.38-39.45% (Fithriani *et al*, 2007). The solid waste of gelatin processing outputs is used as the alternative of organic fertilizer.

Factors can influence the length of composting. These factors include early C/N ratio of raw material and bioactivator addition. The greater early C/N ratio is, the longer is the decomposition process, and so is the reverse. *Tithonia diversifolia* has quite high N content, that is 3.5%, and it can decompose quickly. It was a weed from shrub species that can grow every year [2]. The general objective of research was to utilize the wastes from gelatin processing as the organic fertilizers to take benefit from its additional value, and also to resolve the

environmental problem due to waste pollution.

2. Material And Methods

Material

The material of research was gelatinous soil waste derived from UD Srigunting Randuagung Malang, *Tithonia diversifolia*, EM4 and Petrofast as bioactivator, and also molasses and bran.

Equipment

Equipments used in this research were measuring glass, material batch, weight, pH-meter, thermometer, oven, distiller, and *buret*.

Methods

Research method was Group Random Planning (RAK) with factors. It involved 2 treatments and 3 replications. The variables are the type of bioactivator (B) and the ratio of gelatin waste and *Tithonia diversifolia* (T). Data analysis uses ANOVA and it is continued with a more advancing test using BNT 5%. The design of research was shown in Table 1.

Table 1. Research design

Bioactivator (B)	*Thitonia* : Gelatin Waste (T)		
	Without Addition (T_0)	1:1 (T_1)	1:3 (T_2)
Without of Bioactivator (B_0)	B_0T_0	B_0T_1	B_0T_2
EM4 (B_1)	B_1T_0	B_1T_1	B_1T_2
Petrofast (B_2)	B_2T_0	B_2T_1	B_2T_2

3. Result And Discussion

pH (Degree of Acidity)

Composting pH showed a distinctive trend. The most fluctuated reduction of pH was observed at day-8 or about entering the second week. Result of analysis of variance against pH at the second week of composting indicated that the effect of bioactivator addition was obvious (5%<Fhit<1%) on composting pH. The addition of *Tithonia* has a very obvious effect (> 1%) on composting pH. Interaction between both treatments does not have obvious effect (5%>Fhit<1%) on pH of composting process. More advancing test must be arranged separately. Result of this test was displayed on Table 1 and Figure 1.. The change of pH during the composing was also used as the indicator of microorganism activity in decomposing organic matter.

Table 1.The effect of bioactivator type and ratio of gelatin wastes to *Tithonia diversifolia* were added to pH

Treatment	pH
Bioactivator of Type	
B_0	4,33 ab
B_1	4,44 b
B_2	4,06 a
BNT 5%	0,305
Ratio of Gelatin Waste : *Thitonia diversifolia*	
T_0	5,13 b
T_1	3,72 a
T_2	3,98 a
BNT 5%	0,305

Note: The number followed by similar letter in each column is obviously different at level of 5% in BNT test.

At day-8, bioactivator addition and the application of bioactivator and *Tithonia*, had reduced pH into acid rated at 3.87-6.07. The control never experienced pH change. Regular pH remains in acid condition, similarly to its condition at the first day. The decline of pH was caused by the activity of bacteria that decomposed organic matters that would change pH. At day-15, pH decreased into more acid than day-8, except

for the control that did not show the reduction of pH compared to its previous week.

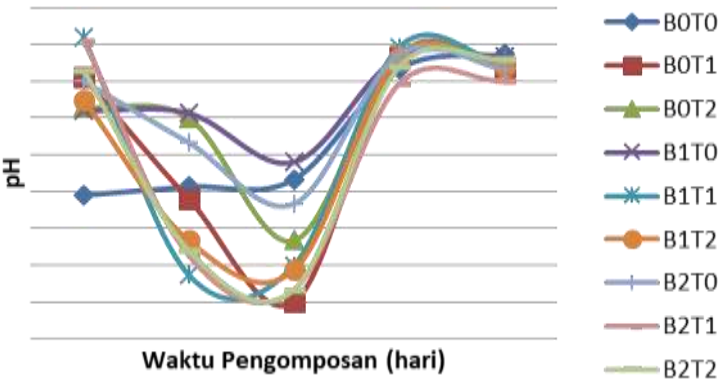

Figure 1. pH value during the composting

According to [3], pH reduction indicated the role of acid-producing bacteria and fungi that produced heat during the decomposition of complex organic materials into simple organic acid. When *Tithonia* was added in ascending quantity, the organic matters in material mix were also developing. Therefore, bacterial activity to produce acid was also maximizing. It differed from the control, and it produced pH change. It may be because organic matters inside gelatin solid wastes were hardly decomposed. Gelatin wastes had a texture of dense and compact, possibly because it had higher lignin content. Equipment Engineering Research Team (2003) found that cellulose total content of chemical component in seaweed was 45.9%, whereas lignin content was 4.02%.

Therefore, without the addition of organic matters or bioactivators, thus the organic decomposition was not optimum. Indeed, pH at the control (B_0T_0) does not decrease. [4] declared that the restructuring of organic matters occurred at aerobic and anaerobic conditions. The reaction of aerobic system restructuring was described as following:

$$\text{Organic Matters} \xrightarrow[\text{Acid-Producing Bacteria}]{} CO_2+H_2O+\text{nutrient+humus+E (484-67 kcal/mol glucose)}$$

There is no channeling for alkali water. Lower layer possibly did not receive very good oxygen aeration, thus triggering anaerobic decomposition process. This anaerobic process was then causing the composting into acid. This reaction was explained by [4] as following:

$$\text{Organic Matters} \xrightarrow{\text{Acid-Producing Bacteria}} x\,CO_3\,COOH \xrightarrow{\text{Methanomonas}} CH_4 + CO_2$$

At day-22, pH of all treatments, including the control and the treatment group with bioactivator and *Tithonia* addition, was increasing and then changing

into neutral at day-29. As said by [3], composting pH started with relatively acid because it derived from the compilation of simple organic acids. Moreover, pH increased at further incubation because protein was decomposed comprehensively and releasing ammonia. In compost maturity phase, the decomposition of organic matters was completed, and microorganism activity was reduced, thus stabilizing pH. This literature insisted that at day-22 or entering the third week, the decomposition of organic matters decreased and the resultant pH of decomposition from microorganism activity started to be stable.

Bioactivator addition in Table 3 indicates the following result. The addition of Petrofast bioactivator (B_2) produced more acid pH, in average, if compared to that without bioactivator (B_0) or that with EM-4 bioactivator. Both later treatments had similar effect as shown by similar notation across treatments.

It may indicate that the addition of Petrofast may degrade organic matters optimally if compared to without bioactivator or also compared to EM-4. It is possible because Petrofast has been more enriched with microbes than EM-4, and therefore, it decomposes organic matters maximally. Petrofast contained with decomposer bacteria such as *Actinomycetes bacterium* (Lignolitic), *Streptomyces sp.* Strain LD021 (Lignolitic), *Streptomyces tumescens* (Cellulotic), and *Trichoderma sp* (Cellulotic). Meanwhile, EM-4 only contained with the fermentative microorganism, particularly photosynthetic one (*Rhodopseudomonas sp*) and *Actinomycetes*. More advance test with *Tithonia* addition as shown in Table 1 showed that *Tithonia* addition can influence pH during composting process.

Different notations were provided. *Tithonia* addition at ratio 1:1 was denoted with T_1, *Tithonia* addition at ratio 1:3 was denoted by T_2, and without *Tithonia* addition was indicated by T_0. It means that *Tithonia* addition increases the quantity of organic matters in the composted material. It was then increasing the number of organic acids during composting process. Final pH of organic fertilizer was exhibited at Figure 1. Analysis of Variance against final pH of organic fertilizer has shown that the effect of bioactivator and *Tithonia* addition on the making of organic fertilizer from gelatin wastes, and the interaction of both treatments were not very obvious (F-5%>F-hit<F-1%) on the final pH of the resultant organic fertilizer. Table 1 also showed that final pH of organic fertilizer remained in neutral rate, precisely from 6.5 to 6.9. If compared to the Minimal Technical Standard of Organic Fertilizer set by The Decree of Agriculture Minister Number 70/Regulation Minister of Agriculture/SR.140/10/2011, the final pH of fertilizer output from all treatments had met the standard. The optimum pH, based on the Decree, must be 4-9. It can be said that the addition of bioactivator and *Tithonia* only influences the degree of acidity during composting process, but not influence final pH of the resultant organic fertilizer.

Furthermore, pH during composting process experienced reduction into more acid category at day-15 or while entering the third week, as rated at 3.3-5.0. Based on the Analysis of Variance, the composting process showed that bioactivator addition was obviously influential to pH result. *Tithonia* treatment was influential very obviously.

The addition of Petrofast bioactivator has the most acid pH at the third week if compared to other treatments. It was said so because Petrofast contained with decomposer bacteria such as *Actinomycetes bacterium* (Lignolitic), *Streptomyces sp.* Strain LD021 (Lignolitic), *Streptomyces tumescens* (Cellulotic), and *Trichoderma sp* (Cellulotic). Meanwhile, EM-4 only comprised of fermentative microorganism, such as lactate acid bacteria (*Lactobacilus sp*), fermentation fungi (*Saccharomyces sp*), photosynthetic bacteria (*Rhodopseudomonas sp*), and *Actinomycetes*. The decomposition of organic matters with Petrofast addition was more optimizing.

The effect of *Tithonia* addition was found at the third week where *Tithonia* addition can increase the quantity of organic matters in the composted material, and therefore, it also increases the number of organic acids during composting process.

Final pH of composting temperature was ranged from 6.5 to 6.9. It means that the addition of bioactivator and *Tithonia* can

influence composting pH but not influence final pH of the resultant organic fertilizer

C-organic

Result of Analysis of Variance against C-organic during composting process has shown that *Tithonia* addition gives very obvious effect (> 1%) on C-organic content during composting process. Bioactivator treatment did not have obvious effect (5%>F-hit<1%) on C-organic content during composting process. Interaction of both treatments also did not have obvious effect. Result of more advancing test was shown in Table 2. Figure 1 exposed the result of observation on C-organic during composting process

Table 2. The effect of bioactivator type and ratio of gelatin wastes to *Tithonia diversifolia* on C-organic.

Treatment	C-organic
Bioactivator of Type	
B_0	23,92 b
B_1	21,67 a
B_2	25,48 c
BNT 5%	2,029
Ratio of Gelatin Waste : *Thitonia diversifolia*	
T_0	21,18 a
T_1	25,72 b
T_2	23,94 ab
BNT 5%	2,029

Note: The number followed by similar letter in each column is obviously different at level of 5% in BNT test.

Table 2 displayed that *Tithonia* addition can improve C-organic during composting process. It is possible because *Tithonia* addition may increase the quantity of organic matters to decompose, thus increasing C-organic content. Bioactivator treatment was not influential because water content exceeded above 70%, and therefore, microorganisms within Petrofast and EM-4 were hardly living optimally. According to [5], composting run well when water content reached 40-60%.

C-organic in early composting (at day-1) for the control (B_0T_0), treatment B_1T_0 (EM-4 without *Tithonia*) and treatment B_2T_0 (Petrofast without *Tithonia*) did have C-organic of 16.27%. This rate must be lower if compared to treatment with *Tithonia* addition at ratio 1:1 or 1:3. C-organic in treatment of *Tithonia* addition at ratio 1:1, either without bioactivator (B_0T_0) or with bioactivators of EM-4 (B_1T_1) or Petrofast (B_2T_2), had C-organic of 21.83%. It explained that organic fertilization with *Tithonia* addition can increase C-organic content in early mix of composted material. More *Tithonia* addition will increase C-organic content. Treatment with bioactivator, either EM-4 or Petrofast, into early composting process was not influential. It seems that after microorganism was added into compost mix, it did not decompose organic matters directly. At day-1, C-organic content in treatment of without bioactivator was similar between EM-4 and Petrofast.

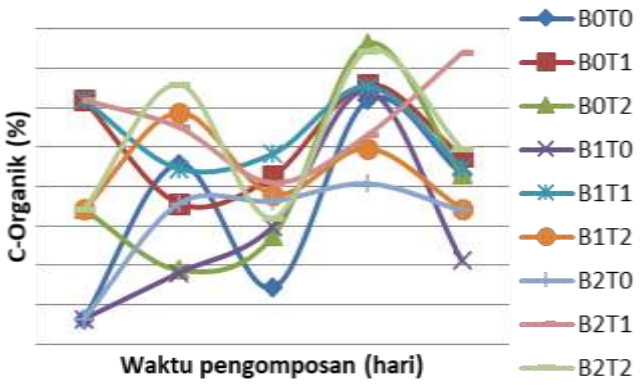

Figure 3. The content of organic C during composting process

At day-8, C-organic of the compost for all treatments was reducing. It indicated that microorganisms started to decompose organic matters such that C-organic content in the composting system decreases. At day-15, C-organic rate increases possibly due to the high level of humidity in the composted materials. A very humid condition triggers fungi to grow on composting system. The mixing may blend fungi with composting materials which then increases C-organic rate.

Bacteria may die. According to Winda (2008), the dead bacteria did not degrade organic compounds, but remained as the organic. Thus, the content of organic compounds of the compost was still high.

At day-29, C-organic reduces due to the decomposition by microorganism. This decomposition occurred because of the introduction of bacteria in the bioactivator or of natural process with the entering of airborne bacteria, recalling that the composting used aerobic system.

Figure 1 showed that the changing C-organic rate during composting process from day-1 to day-29 in all treatments was fluctuated. The fastest bioactivator in decomposing materials was not known. As said by Epstein (1997) in Amanah (2012), carbon in the composting was used as energy source. As temperature declines, the activity of microorganisms slows down and so does carbon decomposition. It is not surprising when the remaining carbon is few. The longer C-organic rate content remained, the slower the composting process was. But the author of this current research denies this finding. When brand is added in day-7, fungi grow on the compost and dead bacteria are observed possibly due to humid compost and higher water content.

Table 2 indicated that *Thitonia* addition was increasing C-organic rate during composting process. Indeed, the addition of *Thitonia* means increasing the quantity of organic matters to decompose, and it also builds up C-organic content. The effect of bioactivator addition was not found because water content was high exceeding above 70%, meaning that microorganisms, either in EM-4 and Petrofast treatments, did not develop optimally. As said by [5], compost making may run well at water content of 40-60%.

Fgure 1, the final rates of C-organic in organic fertilizer made from gelatin solid wastes were not far different to each other in treatment of without bioactivator (B_0) either without *Thitonia* addition (B_0T_0), without *Thitonia* addition at ratio 1:1 (B_0T_1) or without *Thitonia* addition at ratio 1:3 (B_0T_2), in range from 23.62% to 24.53%. For treatment with EM-4 and Petrofast, C-organic content of organic fertilizer without or with *Thitonia* addition was varying. The addition of *Thitonia* can increase C-organic rate. It means that bioactivator addition has influenced the decomposition of organic matters.

The advancing test on the effect of bioactivator addition on C-organic contents in Table 2 has showed that the addition of Petrofast (B_2) bioactivator gives the biggest increase of C-organic if compared with other treatment. The addition of EM-4 (B_1) has

produced the lowest C-organic content of other treatments, and it only counts to 21.54%.

Petrofast consisted of decomposer bacteria such as *Actinomycetes bacterium* (Lignolitic), *Streptomyces sp.* Strain LD021 (Lignolitic), *Streptomyces tumescens* (Cellulotic), and *Trichoderma sp* (Cellulotic). It was enriched by microorganisms and therefore, the decomposition of organic matters became faster. Petrofast was also advantageous because it facilitated the composting of cellulotic organic matters or hard/lignin organic matters. The content of cellulotic bacteria was quite high in Petrofast, and this bioactivator must be suitable for composting gelatin wastes, recalling that cellulose rate of gelatin wastes was very high. According to Setyorini (2011), cellulose was a compound with decomposition difficulty. It was also a complex compound needing relatively longer decomposition but easily broken down by cellulase enzyme produced by bacteria. The product of this breakage included such compounds as monosaccharide, alcohol, CO_2 and other organic acids.

C-organic content in organic fertilizer added with EM-4 bioactivator was only 21.68 %. The reason was because EM-4 bioactivator contained with fermentative microorganisms such as lactate acid bacteria (*Lactobacilus sp*), fermentation fungi (*Saccharomyces sp*), photosynthetic bacteria (*Rhodopseudomonas sp*), and *Actinomycetes*. High humidity in the composting system was caused by higher level of water content. There is a possibility that EM-4 bacteria do not grow well and remain incapable to degrade organic matters maximally. As a result, C-organic content is always low.

Each treatment of *Thitonia* addition gives different effect on the resultant C-organic. Different notation was given to three treatments applied, and it was shown in Table 4. The higher rate of *Thitonia* addition was the higher also C-organic content in organic fertilizer.

As reported by Supriyadi (2004), *Thitoniadiversifolia* was used as alternative organic matter. *Thitonia* contained with organic acids such as citrate acid, oxinate acid, oxalate acid, acetate acid, maclate acid, butyrate acid, propionate acid, phthalate acid, and benzoate acid. Pursuant to [7], C-organic content in *Thitonia* was 38.5%. More *Thitonia* addition will increase the quantity of organic matters in the composting mix, and C-organic content also increased.

Higher water content in the composting mix was influential. Bioactivator addition into the composting process did not give very obvious effect but it influenced the final rate of C-organic content in the fertilizer. *Thitonia* addition has a very obvious effect on C-organic content during the composting process or also on the final rate of C-organic content. More *Thitonia* addition can increase C-organic content. If compared to the Minimal Technical Standard of Organic Fertilizer set by The Decree of Agriculture Minister Number 70/Regulation Minister of Agriculture/SR.140/10/2011, the resultant C-organic content for all treatments has met the standard where the optimum C-organic content required by the Decree was minimally 15%.

C-organic content during composting process was fluctuated where at day-8, the content increased after the addition of brand at day-4. At day-15, C-organic content decreased but it increased again at day-22. The reason may be due to the growth of fungi on the composting process possibly because of humid condition of the compost. Mixing the fungi on the compost may increase C-organic content. Dead microbes were also counted as organic matters. Result of analysis of variance in the composting process showed that *Thitonia* addition was very obviously influential to composting rate. Based on [7], C-organic content in *Thitonia* was 38.5 %. Therefore, more addition of *Thitonia* will increase the quantity of organic matters in the composting materials, and so will C-organic content.

C/N ratio

The effect of *Thitonia* addition can be seen in the early process of composting. More *Thitonia* addition was related with the lower level of early C/N ratio if compared to a treatment of without *Thitonia* addition. At treatment of without *Thitonia* addition (T_0), C/N Ratio of the material at week-0 was 75.

By adding *Thitonia* at ratio 1:1 (T1), early C/N ratio was 47. In the addition of *Thitonia* at ratio 1:3 (T2), early C/N Ratio was 61. As shown in Graphic 4.15, the decrement of C/N Ratio of organic fertilizer occurred quickly at day-8. Next, at day-15, until week-29, C/N Ratio still reduces but not significant. The decrease of C/N Ratio in the compost was evident because materials started to decompose.

Analysis of Variance on C/N Ratio during composting process showed that only *Thitonia* addition was with very obvious effect (> 1%) on C/N Ratio during composting process. Bioactivator treatment and interaction of bioactivator and *Thitonia* additions do not have obvious effect (5%>Fhit<1%) on C/N Ratio during composting proces

Table 3. The effect of bioactivator type and ratio of gelatin wastes to *Tithonia diversifolia* on C-organic.

Treatment	Average	Notation
B_0T_1	14,82	a
B_1T_1	15,81	a
B_1T_2	16,23	a
B_0T_2	17,96	b
B_2T_1	18,59	c
B_2T_2	20,74	c
B_1T_0	24,09	d
B_2T_0	28,05	e
B_0T_0	29,91	e
BNT 5%	3,260	

Note: The number followed by similar letter in each column is obviously different at level of 5% in BNT test

As reported by [8], C-organic content in the material was becoming food source of microorganisms. But, the content reduced because it decomposed into airborne CO_2. Besides, N-total in the material increased because the decomposition of compost material by microbes produced ammonia and nitrogen. The reduction of C-organic content and the increase of N-total have forced C/N Ratio to stand down. Apriwulandari (2008) also reported that microorganisms solved C compounds into energy source. It leads to a competition across microbes and finally accelerates the death of microorganisms. The decomposition of organic matters risks from stoppage but C/N ratio becomes stable. The lowest was observed from treatment of without bioactivator addition and of with *Thitonia* addition at ratio 1:1, and the rate was only 14.82%.

Analysis of Variance indicated that the effect of bioactivator addition, *Thitonia* addition, and interaction of both additions, did have a very obvious effect (> 1%) on final C/N Ratio of organic fertilizer made from gelatin wastes. Result of advance test with BNT at 5% level is displayed in Table 3. Also, Table 3 showed that the lowest C/N Ratio was found at treatment of without bioactivator and with *Thitonia* addition at ratio 1:1 (B_0T_1), whereas the highest C/N ratio was observed in the control, precisely without *Thitonia* and without bioactivator additions (B_0T_0). It means that the addition of organic matters, including *Thitonia*, can accelerate decomposition in the making of organic fertilizer from gelatin wastes. *Thitonia* addition may reduce C/N ratio of the compost materials because of higher level of N as the base material of the compost. Also as indicated by Srihartini (2008), the more nitrogen rates, the faster was the dissolving of organic matters. It was said so because microorganisms that decomposed composting materials always needed nitrogen.

4. Conclusion

1. The addition ratio greater Thitonia significant effect on the increase in the moisture content of compost during the composting process, very significant

effect on pH decrease at week 3, the increase in organic C during the composting process and also the end of compost, an increase of N-total for the composting process and also N-total end, as well as the very significant effect on the value of C/N ratio during the composting process and the final compostingThe most effective bioactivator was Petrofast because it was able to influence pH, and also could increase C-organic content and N-total.

2. The higher ratio of *Thitonia* addition would increase C-organic content, N-total and be influential to C/N Ratio.
3. Treatment of B_2T_2 has produced N-organic rate of 5.63%, C-organic content of 24.8%, and C/N Ratio of 20.74.

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THE INTENSITY ATTACK OF COFFEE BERRY BORER (*Hypothenemus hampei* Ferr.) AFTER APPLICATION OF *Acorus calamus* L. AND ORGANIC INSECTICIDE IN COFFEE PLANTATION

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Abstract

Hypothenemus hampei Ferr. is a damaging insect on coffee berry. The borer activity *H. hampei* is one of the causes of reducing coffee production in Indonesia. So far, to control this insect, chemical insecticide were applied. These methods gave a bad impact on the coffee been quality. Therefore, the use of insecticide with safe friendly- environment is needed. One of the plants with potential insecticide compound is *Acorus calamus* L. Currently, several commercial organic insecticide are readily found in the market and used by several farmers. The aim of this study is to evaluated the intensity attack of coffee berry borer (*H. hampei*) in coffee plantation after application of hexane fraction of *A. calamus* and organic insecticide. The organic insecticide at 0.74 % and hexane fraction at concentration 1.2 % were evaluated in the field by spraying methods. The study concluded that the group had the lowest intensity of *H. hampei* attack is organic Insecticide group, but overall percentage of *H. hampei* attacks in each group is low with the value of percentage less than 10%.

Keywords—*Acorus calamus* L., coffee berry, insect, organic insecticide, regular spraying

1. Introduction

Coffee (*Coffea* sp.) is one of Indonesia's export commodities. In 2013, Indonesia exported coffee to many coutries as 534.023 tones that have export value at US$ 1.174.029 [1]. Coffee production in Indonesia is not correspond with total area of coffee. Data coffee production in 2010 was 689 921 tones and in 2011 showed a decline to 638 646 tones. While the coffee land area increased in 2010 covering an area of 1,210,365 hectares in 2011 to 1,233,698 [1]. This is because the activity of *H. hampei* coffee berry borer (CBB) [2]. The level of attacks by CBB in some provinces, among others, reaching 64.0% in Lampung and 61.5% in East Java [3].

H. hampei is a type of beetle that became coffee berry borer pest. Adult females beetles of *H. hampei* make holes on fruit coffee and lay egss in the coffee fruit. After the eggs hatch, the larvae of *H. hampei* eat the endosperm of coffee beans. Damage of coffee beans endosperm can reduce the quality of the seed [4].

So far, chemical insecticide is used to control *H. hampei.* The use of synthetic insecticides can cause some problems, among others, resistance, resurgance, secondary pest explosion, water and soil pollution, as well as threats to human health [5]. Therefore, the use of insecticide with safe friendly- environment is needed. This insecticide is botanical insecticide. Botanical insectide is insecticide made from plants and easily degradable in environment (biodegradable) [6].

One of the plant that have potential as insecticide is *Acorus calamus* L. *A. calamus* is a herbaceous plant that contains the active compound in the form of (*E*)-*Methylisoeugenol*, β- *Asarone*, *α-Asarone*, and *Methyleugenol* [7]. Oil and steam from *A. calamus* rhizone have toxic effect and steril effect against insect pest, such as flea rice (*Sitophilus oryzae*) [8]. The value of LC_{50} at 24 hours from hexane fraction of *A. calamus* rhizome extract against *H. +hampei* was 2.96%. Furthermore, semi-scale research field indicate the value of LC_{90} at 1 week of hexane fraction of *A. calamus* rhizome extract on *H. hampei*

was 1.14% [9]. In addition, there are several commercial organic insecticides that have been circulating in the market.

The composition of the organic insecticide are a waste of tobacco (*Nicotiana tabacum* L.), yam tuber (*Dioscorea hispida* Dennst.), and lemongrass (*Cymbopogon nardus* L.). The other compositions are cow urine and local microorganisms in cow.

The aim of this study is to evaluated the intensity attack of coffee berry borer (*H. hampei*) in coffee plantation after application of *A. calamus* and organic insecticide. Concentration used was 0,74% for organic insecticide and 1,2% for hexane fraction of *A. calamus*. The place used in this study is coffee plantation in Sidomulyo Village, Sub-distric of Silo, Distric of Jember. Sidomulyo village is one of the main coffee production center in Jember [10].

2. Methods

A. Time and Place

The study was conducted for 10 months started in September 2015 to June 2016 at Laboratory of Zoology and Botani Department of Biology, Laboratory of Basic Chemistry Department of Chemistry, Faculty of Mathematic and Natural Science, University of Jember and Coffee Plantations Sidomulyo Village, Sub-district of Silo, District of Jember.

B. Equipments

The tools used in this study include scale, blade, cutter, rotary evaporator BUCHI R-114, beakers PYREX® 10 mL, measuring cups PYREX® 50 mL, beaker glass PYREX® 1000 mL, Erlenmeyer tube PYREX® 500 mL, Erlenmeyer tube Schott Duran® 500 mL, stock bottles Schott Duran® 1000 mL,stock bottles Schott Duran® 500 mL, separating funnel, spatula, grinding machine, and sprayer SABARA® size 5L, Luxmeter HIOKI, THM (Temperature Humidity Meter) VA8010, GPS, handcounter, camera, and bucket.The material used in this study is filter paper, ethanol 96%, hexane, $MgSO_4$, rhizome of *A. calamus*, tween 80, aluminium foil, organic insecticide, gloves, masks, and water.

Preparation of A. calamus rhizome extract with Hexane Fraction

The rhizome for this study was obtained from Gebang area, Sub-district Patrang, Jember, taken in September 2015. The rhizome was washed, thinly sliced, and dried in the open area without being exposed to direct sunlight for 3-5 days [11]. Rhizome slices that have been dried, are milled by grinding machine into a powder.

A. calamus powder which has been finely is weighed as much as 100 grams, then soaked with 400 mL of 96% Ethanol which has been added by $MgSO_4$. *A. calamus* soaking in ethanol for 24 hours and the process is repeated 2 times. After 24 hours, the marinade is filtered with filter paper. The filtrate of *A. calamus* were taken and concentrated using rotary evaporator at a temperature 0f 50-55^{o}C with the pressure of 250-300mmHg until the filtrate become brown [12]

Filtrate of *A. calamus* that has been concentrated is stored in bottle stock. 500 mL filtrate from bottle stock is added by 1500 mL hexane. The addition of 1500 mL hexane in the filtrate is done gradually and stirring to form a 2-phase solution (Fig. 1 A). The solutions are inserted into the separator funnel to take hexane that binds the active compounds from *A. calamus.* The upper part of the solution is hexane that binds the active compounds from *A. calamus* and lower part is sediment (Fig. 1 B). Part of hexane solutions that binds active compound taken and stored in storage bottle.

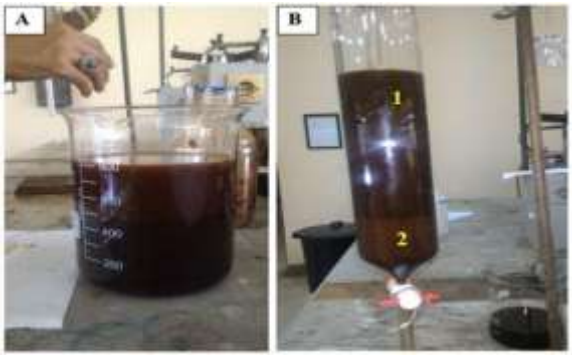

Fig 1 (A) 2 phases of solutions is formed from hexane and *A. calamus* filtrate (B) 2 phases of solutions inserted to separator tunnel (1. Hexane Fraction that binds active compounds of *A. calamus* filtrate 2. Residue or sediment)

As much as 2 Liters of Hexane solutions, after partition process, are concentrated by using rotary evaporator. *A. calamus* solutions which have been separated from hexane known as Hexane fraction of *A. calamus* extract. Extraction and partition produces 315 mL of hexane fraction of *A. calamus*. The solutions are stored in the stock bottles.

Hexane fraction of *A. calamus* coupled with tween 80 before being mixed with water. The fraction of hexane has a low degree of solubility in water, because it has non-polar molecules. Addition of Tween 80 is in order to facilitate Hexane Fraction soluble in water.. Volume of Tween 80 and Hexane Fraction of *A. calamus* extract use 1:1 comparison.

Fig 2 Four Selected Branchs in wind Directions

After labeling, the number of cherries attacked by *H. Hampei* (Fig 2). The number of total coffee berry on each branch are counted as data for H-1 days before spraying. The spraying were carried out by knapsack sprayer 5L which were applied on each group. Concentration of Hexane fraction of *A. calamus* is 1,2% and concentration of organic insecticide is 0,74%. Spraying is done only once at the coffee fruit on branches that have been labeled. Once it is observed at intervals of 7 days, 14 days, 21 days, and 28 days after spraying.

In addition to observation of the coffee fruit, abiotic factors are measured include temperature, humidity, light intensity, and rainfall in each group. Abiotic factor measurements performed daily for ± 1 month on each group. The time span abiotic factor measurements performed during the study were between 09.00 am - 13.00 pm.

Observations were made on the amount of coffee fruit affected to determine the percentage of the coffee fruit that was attacked. The formula for

calculating the percentage of *H. hampei attack* in coffee trees by [15] are as follows:

60 mL of tween 80 in 4880 mL water. Tween 80 is used for simplify of Hexane fraction to soluble in water [14]. Then, the soluble of Hexane fraction is inserted to knapsack sprayer.

C. Procedures

Testing of Hexane fraction of *A. calamus* and organic insecticide effect against *H. hampei* were done in the land of 1 hectare with 800 coffee trees in it. 90 coffee trees was used in this study. Coffee trees are derived into three groups (Figure 3). 30 the first group was sprayed with water (group –A‖), 30 the second group was sprayed with hexane fraction of *A. calamus* (Group –H‖), and 30 the third group was sprayed with organic insecticide (group –I‖).

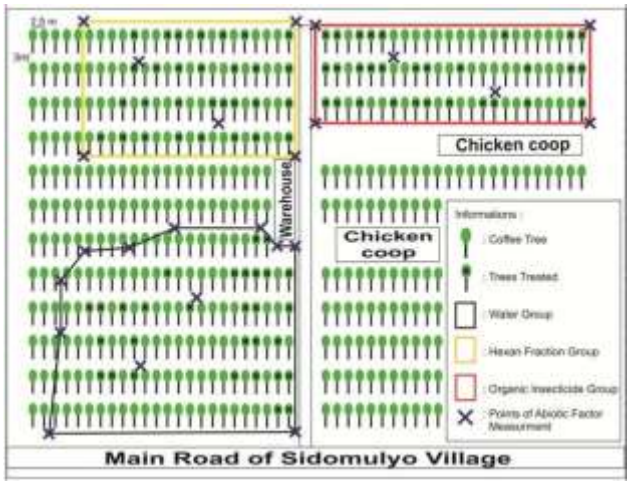

Fig 3 Study Design of The intensity attack of *H. hampei* after application of *A. calamus* and organic insecticide in coffee plantation. In each test group is determined 30 trees with the criteria of the trees have a minimum of four branches that lead to the four winds, namely North, East, South and West. Four branches have been labeled in accordance with the treatment given, –A‖ for group water, –H‖ for group of hexane fraction of *A. calamus*, and –I‖ for group of organic insecticide. I.

D.Data Analysis

Analysis of the data used to determine the effect of the use of hexane fraction of *A. calamus* and organic insecticide against *H. hampei* of the coffee plant is the Kruskal-Wallis test ($\alpha = 5\%$) [16]. Statistical analysis use SPSS Windows Version 15.0

3. Results

The results of this study about the average percentage of *H. hampei* attacks in each treatment group can be seen in Fig. 4.

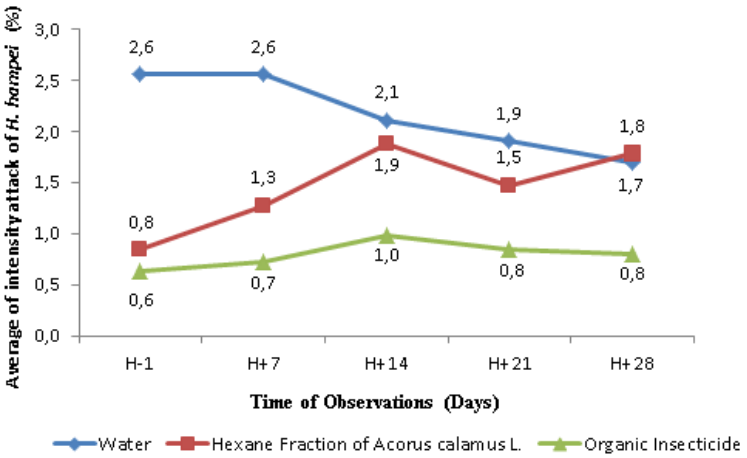

Fig. 4 The average percentage of *H. hampei* attacks in each treatment group

It can be seen from the graph, the average percentage of attackes by *H. hampei* in each treatment group, the smallest is the organic insecticide group. But the overall percentage of attacks *H. hampei* in each group is low with the value of percentage that attacks less than 10%. According [17], the intensity of attack is said light ihe the percentage <25%, moderate (25-5- %), heavy (50-90%) and puso (>90%).

The average percentage of the coffee fruit that was attacked by *H. hampei* in the water group decrease at 14 days after spraying with the decline reached 0,5%. This is because the amount of infected coffee fruit decreased, while the number of whole coffee fruit increased. So that, the percentage of infected fruit will decrease. The availability of coffee fruit effect on population if *H. hampei* because coffee fruits become eating and laying eggs [18]. Reduced the amount of coffee fruit can reduce the number of population of *H. hampei* in the field [19]. At 1 day before spraying, there was difference of average value of infected coffee berry between water group and treatment group (*A. calamus* and organic insecticide group) by ±2%. This is because there are differences in berry that appears in each group. Coffee berry that appears in water group at 1 day before spraying not only green coffee berry but also yellow and red coffee berry, while the coffee berry in the treatment groups did not have yellow or red coffee berry. According to [20], *H. hampei* prefers red coffee berry (ripe berry) compared to green coffe berry (young berry), although in younger fruit *H. hampei* infestation is also found. This is because there are volatile compounds released by ripe berry causing *H. hampei* to draw closer.

Based on the analysis of Kruskal-Wallis test (α = 5%), the intensity of the attack *H. hampei* significant in all treatment groups at 1 day before spraying ($p=0.00 < 0.05$) and 7 days after spraying ($p=0.041 < 0.05$). This shows that the spraying of hexane fraction of *A. calamus* and organic insecticide can suppress *H. hampei* attacks against coffee fruit for 7 days after spraying.

Hexane fraction of *A. calamus* rhizome extract is known contain components of the active compound α-asarone and β- asarone which act as a stomach poison on *H. hampei* [9]. Oil from *A. calamus* rhizome extract also has active compounds in the form of *Methyleugenol* and (*E*)-*Methylisoeugenol* [7]. *Methyleugenol* compounds is known act as a stomach poison and insect repellent [21]. Giving hexane fraction of *A. calamus* rhizome extract allegedly resulted *H. hampei* attack on hexane fraction group doesn't exceed 2% at 7 days after spraying.

The active compounds in organic insecticide that have insecticidal character are include nicotine derivide from tobacco, citronella of lemongrass, and dioscorin of yam tubers. Nicotine compoud is a nerve poison that can react quickly. Alkaloid nicotine, sulfate nicotine, and the others of nicotine may be used as a contact poison, fumigants, and stomach poison. In general, the symptoms of poisioning nicotin

strat from the stimulation, seizures, disability, and death [22]. Tobacco extracts with 5 times dilution may cause mortality of *H. hampei* at 3.75% on 10 days after application in the field [23].

Other active compound are citronella. Citronella compounds have toxic nature dehydration (*desiccant*). The poison is a contact poison that can cause death of insects due to fluid loss continously [24]. Essential oil of citronella can cause mortality of *H. hampei* by 80% at 24 hours after treatment in laboratory scale [25]. The other active compounds are dioscorin. Dioscorin cause seizures and nerve paralysis in insect [26]. Yam tuber extract at concentration of 25-100% can inhibit feeding activit of the larvae of *Plutella xylostella* [27]. Yam tuber extract at concentration of 150 grams can cause mortality of larvae of *Spodoptera litura* by 45% at 72 hours after application [26].

Based on the results of *post hoc test* using *Mann-Whitney* test, for data to 7 days after spraying, there are significant differences between the hexane fraction of *A. calamus* and with water ($p=0.039 < 0.05$) and organic insecticide with water ($p=0.027 < 0.05$). But among hexane fraction of *A. calamus* and organic insecticide is not there significant difference ($p=0.689 > 0.005$). This shows that hexane fraction of *A. calamus* and organic insecticide can inhibit *H. hampei* attacks on the coffee fruit as compared using water. But among hexane fraction of *A. calamus* with organinc insecticide is not known which better to inhibit *H. hampei* attack.

Based on the analysis of Kruskal-Wallis test (5%), the intensity of the attack in all groups at 14 to 18 days showed not significant results ($p < 5\%$). This shows that hexane fraction of *A. calamus* and organic insecticide can't inhibit *H. hampei* attack. This is because one day after spraying occurred heavy rainfall with an average value of precipitation was 155.3 mL. Relatively high rainfall can result in botanical pesticides leaching process [28]. The compounds in pesticides can bemoved along the water or dust particles, water in high rainfall washes potentially active compounds in pesticides in plants is faster [29]. The loss of most of the active compound which is repellant in hexane fraction of *A. calamus* because of washed by rains and an increase in total coffee fruit is thought to be the cause of the attack *H. hampei* increased.

4. Conclusion

The hexane fraction of *A. calamus* showed prospective results to reduce the infestation of coffee berry borer (*H. hampei*). It needs further evaluation the effect of hexane fraction of *A. calamus* on differents fields and seasons.

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ENFLEURATION AND CHARACTERIZATION OF ESSENTIAL OIL FROM CANANGA ODORATA

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Abstract

Enfleuration is a method for extracting essential oils using cold fat as an adsorbent. This research has explored the use of enfleuration for extracting essential oils from *Canangaodorata* using snow white (butter). The enfleuration were carried out in the duration of 1, 3, and 5 day enflueration, with a 24-hour interval of flower change; and without any flower changes during enfleuration. The results showed that the longer duration of enfleuration increased the yield of Cananga oil extract in both methods, even by changing or without changing the flower samples. However, changing the flower samples in every 24 hour during five day enfleuration resulted in the highest yield of Cananga oil extract, 0.777 %, while the lowest yield of cananga oil was obtained from one day enflueration, only 0.090 %. The physical characteristics of Cananga oils obtained in this research were corresponded to SNI 06-3949-1995. Five major chemicals on this Cananga oil extracts were quite similar, i.e.caryophyllene, -humulene, germacrene, .-cadinene, and bergamotene.

Keywords: cananga, enfleuration, essential oils.

1. Introduction

Essential oil is one of the export commodities from Indonesia that has been running for long time. Some kinds of essential oil from Indonesia are citronella oil or citron palm oil, Patchouli oil, nutmeg oil, vetiver oil, cananga oil, clove oil and sandalwood oil [1]. The data shows that Indonesia now can produce 40 kinds of essential oils of 80 types that have been traded in the world and only a small part in which can enter the world market [2]

Essential oil is a liquid extract obtained from the plant part either root, stem, leaf, bark, seeds and even flowers with various methods of extraction. Essential oil, which is a volatile substance in plants, can be used as a characteristic of a kind plants because every plant produces different aroma of oil [3].

Cananga flower or *Cananga odorata* is one of flower that has a very distinctive aroma. Cananga flower comes from several countries in Southeast Asia such as Thailand, Philippines and Indonesia. Original cananga flower Indonesia can be found mainly in Java Island, ie species of *Cananga odorata Baill. F. Macrophylla* and *Cananga odorata Baill f. genuine* which is yellow greenery [4].

Cananga flower contains essential oil that has been used as perfume and mixed base material of foodstuffs, without any indication of cause health problems [5]. But, there is only a few people which is still surviving as a cananga oil supplier because of various production problems. However, some region with a relatively large number of cananga flowers only utilize this flower as a sowing flower during the pilgrimage, so it needs more exploration to extract the essential oil contained so can support the needs of cananga oil-based industries [6].

Cananga oil extraction has been done by several parties along with the increasing demand for essential cananga oils. Extraction methods have been done before such us water distillation, steam distillation, expression, extraction with volatile solvent, cold fat extraction (enfleuration) and supercritical fluid extraction [7]. The essential oil extracted from the steam distillation method has a yield of 1.5-2% with length time of 8 hours [8]. The yield of canangaoil produced by the steam distillation, hydrodistillation and supercritical fluid extractionare 0.936%, 0.41% and 1.8% respectively [9].

Extraction of essential oils of plant can also be done by cold fat extraction method or called enfleuration. This method is best

suited to the type of essential oil from flowers. Some previous research on extraction of essential oils on various flowers such as roses, jasmine and tuberose flower using the enfleuration method gives the best result, has stronger scents and clear colors [10]. [11], also explain the enfleuration method on jasmine flowers provide a higher yield compared with solvent extraction with a rendement of 0.416%: 0.320%.

The enfleuration process is simple enough to do, with animal or vegetable fats are used as adsorbent of essential oil from flower samples. Animal fats that can be used include of beef fat, pig fat, goat fat and chicken fat, meanwhile vegetable fat that has been used is shortening. These fats may be used singly or combined with another. One of them is a mixture of 1 part of beef fat and 2 parts of lard. It can be a perfect combination of fat to extract higher amount of essential oil on enfleuration extraction methods [12]. The length of the enfleuration process is also one of determinant factor to extract the essential oils. Some previous research has used different enfleuration length i.e. using the same enfleuration time with different replacement interval or otherwise, using different enfleuration time with same replacement interval. The research of 13], mentions that yield of rose oilusing a mixture of animal fat as adsorbent (cow:goat:chicken = 2:1:1) for 7 days with 12 hour replacement interval is 0.076% while the 24 hour replacement interval produces 0.174% of volatile oil. Different research about the enfleuration of jasmine flowers with vegetable fat as adsorbent with variation of long enfleurasi 3 days, 6 days, and 9 days with 24 hour intervals also produce a yield of 0.285%, 0.178% and 0.108%,respectively [14].

So far, there is no information for a research on the extraction of the cananga oil by enfleuration method. Therefore, in this study extraction Cananga oil is done through enfleuration method with variation time of enfleuration. The cananga essential oil results then were characterizedfor their physical properties and composition of chemical compounds.

2. Materials and Methods

This research was conducted in Organic Chemistry Laboratory Department of Chemistry, Faculty of Mathematics and Science, University of Jember.

Equipment that were used in this research are glassware, chassis with thickness glass 5 mm, vacuum evaporator, a set of distillation apparatus, refractometer, and GC-MS.

The materials used in this research are including cananga flower (*Cananga odorata*), snow white (butter, shortening), ethanol 96%, indicator phenolphtalein, KOH solution, $H_2C_2O_4$ solution, CH_3COOH solution, $CHCl_3$ solution, IBr reagent, KI solution, KIO_3 solution, $Na_2S_2O_3$ solution,starch solution, HCl solution, and distilled water.

Sample Preparation

The samples of cananga flowers are obtained from Karangpring Village, Sukorambi District, Jember city. This flower has identified by Purwodadi Botanic Garden as *Cananga odorata*

Determination of moisture content

Moisture content was measured as water content. Cananga flower is weighed as much as 5 grams in a weighing glass and recorded as initial weight. Weighing glass and samples were put into oven at 105°C for 3 hours, then were kept in desiccator and weighed as the first weighing. Such treatment was repeated until a constant weight is obtained as the final weight.

Fat Analysis

Tests of Acid Numbers

5 grams of fat was put into erlenmeyer 100 ml and was added with 10 ml of 96% alcohol and heated until it was boiling. Then added 3 drops of indicator PP 1% to solution and titrated with 0.1 N KOH. Titration was stopped when this solution turn to pink color.

Tests of Iod Numbers

0.1 grams of fat was put into erlenmeyer 250 ml and dissolved with 10 ml of chloroform and 5 ml of iodine-bromide reagents, then closed the erlenmeyer. It was stored for 30 minutes in a dark place. The solution was added with 2 ml of KI 15% and

100 ml of distilled water. The erlenmeyer was then closed and shakedfor a while. The solution was titrated with 0.1 N $Na_2S_2O_3$ solution and added with 2 ml of 1% starch solution as indicator. Continue titration until blue color disappears. Blanko was prepared using the same method exclude the fat as sample.

Tests of Peroxide Number

5 grams of fat was put into erlenmeyer 250 ml and added with 30 mL of solvent (60% CH_3COOH and 40% CH_3Cl) and shaken up to the sample dissolved. 0.5 ml of saturated KI was added to this solution and then stored the solution for 2 minute in the dark room. Solution was added with 30 mL of distilled water, then titrated with 0.01 N of $Na_2S_2O_3$until the yellow color almost gone. The solution was added with 0.5 mL of starch 1% starch and was titrated back until the blue color disappears.

Enfleuration

450 grams of fat was applied evenly over the enfleurationchassis. The fat surface was etched with a fork to expand the surface of the fat so that the aroma of cananga flowers could be absorbed maximally. 900 gram of cananga flower was placed on the surface of the fat and adjusted until the entire surface of the fat was covered by flowers, then tightly closed and allowed to be left for 1, 3, and 5 days at room temperature. At the final day, the cananga flowers are removed from the chassis. Fat that has been saturated with oil was scraped from the chassis. The fats were inserted in erlenmeyer and dissolved in 96% of ethanol with a ratio of 1 (fat): 2 (solvent), then cooled in refrigerator and left for 24 hours. The filtrate was separated from the fat by filtration. The extraite was then evaporated under pressure at 40°C. The resulting liquid was called as absolute enfleuration. Meanwhile, this method was done by two different way on changing the flower: without replacement and with replacement every 24 hours.

3. Results and Discussion

The cananga flower was obtained from Karangpring Village, Sukorambi District, Jember. Identity test at Balai Konservasi Tumbuhan LIPI, Purwodadi Botanical Garden showed that this species of cananga flower is *Cananga odorata (Lmk) Hook.f. & Thoms*.

Characteristics of The Shortening

The fat used for this enfleuration method is snow white (butter) which is made from vegetable fat. This fat was tested before and after the usedon enfleuration as an adsorbent. This was aimed to determine the quality of the fat (Table 1).

Table 1. The Characteristic of the Fat (snow white, butter)

Characteristics	Before	After Enfleuration		
		1 day	3 days	5 days
Acid Number (mg/g)	0,145	0,145	0,145	0,145
FFA (%)	0,073	0,073	0,073	0,073
Iod Number (mg/g)	26,24	34,94	36,24	34,94
Peroxide Number (meq/kg)	5,25	4,65	5,25	4,65

Based on the characteristics of the fat,it show that shortening has a low free fatty acid content, low peroxide number, high iodine number, odorless and colorless. The characteristic of white butter after the use as an adsorbent did not show a significant changing in free fatty acid content, peroxide number and iodine number. Therefore, it can be concluded that snow white butter remained in good condition though after enfleuration.

Extraction of Essential Oils Kenanga

Enfleuration is the process of extracting essential oils using cold fat as an absorbent. This enfleuration uses fresh cananga flowers which were still have physiological activities. Yellowish green cananga flower was used since it produces a very strong cananga aroma. Flowers were sorted before use to separate with other undesirable materials such as stalks and leaves. Flowers used should not be wet because it will cause oxidation of fat and causes rancidity. The

flower was spread over the surface of the white butter that has been applied to the chassis, so that the scent of flowers can be directly adsorbedby fat. Then the chassis was covered with plastic warp and left for 1, 3, and 5 days without any replacement and we compare to other enfleuration with replacement every 24 hours.

The flowers were further separated from the fat and produce fat containing essential oils which was commonly called as pomade. The pomade was extracted with ethanol solvent and was cooled in the freezer for 24 hours to separate the essential oil from the pomade. Ethanol will extracts the essential oils which were entrapped in fat. After extraction process, filtration was carried out to separate the fat and extrait. The extrait was purified by evaporating the ethanol using a vacuum rotary evaporator to obtain a light yellow oil, clear and distinctive smell of cananga. The yield of essential oil by enfleuration method can be seen in table 2.

Table 2.Characteristic of Cananga Oil from Enfleuration Method

	Enfleuration					SNI
	1 day	3 days (a)	3 days (b)	5 days (a)	5 days (b)	
Color	LY	LY	LY	LY	LY	LY-DY
Refractive index	1,499	1,499	1,499	1,497	1,497	1,493-1,503
Density	0,618	0,669	0,669	0,680	0,680	0,902-0,940
Odor	Cananga	Cananga	Cananga	Cananga	Cananga	Cananga

Notes: (a) without replacement (b) with replacement; LY: light yellow; DY: dark yellow

Chemical Compound of Cananga Essential Oil

The canangaoilswere analyzed using GC-MS. The results showed that 1, 3 and 5 days non-replacement enfleuration successively yielded 50 compounds, 52 compounds and 51 compounds, resepectively.While 3 and 5 days enfleuration with replacement flower yielded 50 compounds and 60 compounds. Major compounds obtained in the enfleuration are almost identical to those seen in Table 3.

Table 3. Major Compounds of Cananga Essential Oils from Enfleuration Method

No	Compounds	Quantity (%)				
		E 1	E 3 (a)	E 3 (b)	E 5 (a)	E 5 (a)
1.	ß-kariofilen	31,06	31,13	32,15	30,73	30,55
2.	a-humulen	9,88	9,86	10,36	9,75	10,38
3.	Germakren-D	8,04	8,12	8,10	7,97	8,01
4.	d-cadinen	5,66	5,69	5,71	5,72	5,65
5.	a-bergamoten	5,56	5,57	5,57	5,53	5,63
6.	Benzil benzoat	4,18	5,04	4,85	4,84	4,70
7.	a-farnesen	5,41	4,14	3,98	4,08	4,19
8.	T-muurolol	3,57	3,51	3,06	3,53	2,91
9.	Kariofilen oksida	2,4	2,58	2,45	2,58	2,36
10.	Geranil oksida	2,16	2,16	-	2,09	-
11.	Linalool	1,82	1,72	-	1,76	-
12.	Geranil asetat	2,16	2,16	2,09	2,09	2,28
13.	ß-cubeben	-	-	-	-	-
14.	Copaen	1,73	1,77	-	1,72	-

Notes: (a) without replacement;(b) with replacement

4.Conclusion

Based on the results, it can be concluded that the longer of enfleuration time with replacement of flower every 24 hour produces more yield of essential oil. The resulting cananga essential oil has the characteristic of yellow color, typical cananga odor, refractive index of 1,499 for enfleuration 1 day; 1,499 for 3 day enfleuration and 1,497 for enfleuration 5 days. The major components of canangaessential oil are ß-cariofilen, a-humulen, germakren-D, d-cadiene, abergamotene, benzyl benzoate, a-farnesen,

T-muurolol, kariofilen oxide, and geranil acetate.

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COMPOSITE OF ZEOLITE AND ARROWROOT STARCH BASED HYDROGEL AS MATRIX FOR CONTROLLED RELEASE AMMONIUM SULFATE FERTILIZER

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Abstract

Agricultural productivity can be improved intensively through fertilization by common practice of spreading the fertilizer directly on to the soil. The fertilizer which is normally in the form of simple chemical compound, will be dissolved by water in the soil before it can be utilized by plants. However this common practice of fertilization can pollute the environment since only small portion of the dissolved fertilizer can be absorbed by plans. One method that being developed to solve this problem is by using matrix for controlled release fertilizers (CRF) such as a hydrogel composite. Application of hydrogel composites based CRF has several advantages such as being able to keep soil moisture while controlled the amount of fertilizers released to the plants. In this paper, the hydrogel composite was synthesized from arrowroot starch, acrylamide, partially hydrolyzed acrylic acid, natural zeolites, and ammonium sulfate fertilizer (ZA) through radical polymerization process in one pot solution polymerization technique. The ZA CRF based on composites of zeolite-hydrogel was then characterized its structure by IR spectroscopy. The water absorption capacity and ammonium release rate ware measured at various pH . The results shown that the water absorption capacity and ammonium release rate of the ammonium CRF was the highest at pH 9 and the lowest at pH 5.

Keywords: composites, arrowroot starch, crf, ammonium

1. Introduction

The agricultural productivity needs to be improved considering the diminishing availability of land. One easy way to improve productivity is through fertilization [1]. One type of nutrients supply fertilizer is ammonium sulfate with nitrogen content of 21% and 24% of sulfur [2]. The fertilizers applied to plants are not fully absorbed by the plant due to largely leached together with rain water and irrigation water. One of the ways that being developed to overcome the inefficiency of fertilization is by using controlled release fertilizer (CRF) based hydrogel [3].

The use of CRF may improve the availability nutrients in soil at longer time and may also reduce the risk of environmental pollution [4].Fertilizer in the form of CRF is relatively easy to prepare from a variety choice of matrixes [5]. The CRF can be prepared simply by loading the fertilizer into the matrixes such as hydrogel composite. The enrichment can be done by fertilizer-starch impregnation, soaking the hydrogel in fertilizer solution, and loading the fertilizer into a hydrogel composite. Coating of fertilizers with inorganic compounds such as sulfur also can improve the ability to control the release of fertilizers so that it is more efficient [6]. Hydrogel as a controller for release of fertilizers that previously investigated were CRF of NPK fertilizer by coating technique [7], CRF of urea [5], urea CRF from cellulose mulberry [8], urea CRF based on encapsulation [9], CRF of phosphate [10], CRF of potassium nitrate [11], and CRF of potassium phosphate-ammonium sulfate based methylcellulose-polyacrylamide hydrogel [12].

Hydrogels are three-dimensional polymer which have crosslinking structure between the main polymer chains and have the ability to absorb water [13]. Hydrogels from natural materials have been widely developed. Natural materials are more

favorable in terms of safety for the environment because they are non-toxic, biodegradable and biocompatible [14]. One type of natural material that can be developed as materials for making hydrogel is starch. Starch is composed of two types of glucose polymer namely amylose and amylopectin [15]. The hydroxyl group of starch has the potential to form a complex copolymer networks and cause the tissue is able to absorb large amount of water [3]. The synthesis of hydrogels from starch which have been studied previously were hydrogels from yam starch [16], corn starch [17], wheat starch [18], banana weevil starch [19], potato starch [11], and cassava starch [1]. The hydrogel grafting results can be modified by the addition of inorganic minerals such as zeolite to form hydrogel composite. This modification aims to make the formed hydrogel composite has better mechanical properties, and have stronger interaction with the soil compared to hydrogel [20]. The presence of inorganic mineral in the composites of zeolite hydrogel makes stronger interaction with soil may cause the hydrogel composite not too easily carried away by the water flow.

This research was carried out by synthesis of hydrogel and hydrogel composite from arrowroot starch by grafting it with acrylamide monomer for CRF of ammonium sulfate fertilizer by one pot solution polymerization techniques. Arrowroot starch as composite hydrogel based material has advantage prepared by solution polymerization technique because of arrowroot starch solution at high concentration, i.e. up to 12% can still form a flow-able liquid which is needed to facilitate the polymerization process. Acrylamide monomer partially neutralized (10%) by KOH to increase the swelling capacity of hydrogel composite. The synthesis of CRF based hydrogel composite was performed by adding the fertilizer during polymerization process so that all fertilizer can be loaded into the composite. The structure of hydrogel composite produced was characterized from their IR spectrum. The ammonium release rate was measured in buffer solution of pH 5, 7 and 9 as the simulations of various agricultural soil pH.

2. Materials And Methods

Equipments and Materials

The experiment was conducted using common laboratory glassware, electric bath, magneticstirrer, pestle, mortar, porcelain cup, thermometer,oven, Buchner funnel, spray bottle, 120 mesh sieve, and analytic balance. The commercial arrowroot starch from local market and natural zeolite were washed thoroughly before used. The chemicals such acrylamide, *N*, *N*'-methylenebisacrylamide (MBA), potassium persulfate ($K_2S_2O_8$), KOH, NaOH, NH_4Cl, sodium salicylate ,sodium nitroprusside, trisodium citrate, sodium hypochlorite, sodium dihydrogen phosphate, disodium hydrogen phosphate ,and trisodium phosphate are all from Merck and were used as it is.

Procedure

The clean and dry arrowroot starch and zeolite were sieved using a 120 mesh sieve. The ZA CRF based on hydrogel composites was prepared by mixing all ingredients with determined weight as in Table 1 into 100 mL solution of arrowroot starch 8.0% in glass containers, the glass containers cupped and then put in the oven set at 60^{o}C for 24 hours.

Table 1. The Composition of CRF ZA Composite zeolite/hydrogel Based on Arrowroot Starch

Materials (g)	Hydrogel and Hydrogel Composites						
	H	*HZ*	*P2*	*P4*	*P6*	*P8*	*P10*
Arrowroot starch	8	8	8	8	8	8	8
KPS	0.5	0.5	0.5	0.5	0.5	0.5	0.5
KOH	1.89	1.89	1.89	1.89	1.89	1.89	1.89
Acrylamide	24	24	24	24	24	24	24
MBA	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Zeolite 100%	0	32	32	32	32	32	32
ZA fertilizer	0	0	6.4	12.8	19.2	25.6	32.0

The arrowroot starch solution 8% (w/v) was prepared by dissolving arrowroot in water at about 75^{o}C. The ZA CRF based on hydrogel composites were cut into thin chips and dried in a oven at 60°C for 6 days and then grinded on 30 mesh in size. The dried ZA CRF composites were then characterized

their chemicals structure by FTIR and also their water absorption capacity and ammonium release were measured at pH 5, 7 and 9 according to salicylic method [22,23].

3. Results and Discussion

Synthesis of Hydrogels and Hydrogel Composites

The CRF composites of zeolite/hydrogel show different physical appearances when they were synthesized from different composition. The composite without minerals or fertilizers (H), whis is actually a hydrogel based on arrowroot starch, has a lighter colour and looks transparent. The composite zeolite/hydrogel without fertilizers (HZ) has stronger texture and greenish. Zeolite fills the polymer network and interacts with the polymer components so that the polymer network becomes increasingly rigid and tight in accondance with literature cited [27]. The CRF composites hydrogel which was contain fertilizer with various concentrations all have stronger texture than composite without fertiliser HZ and looked greenish color. The higher the ZA fertilizer added, the CRF composites were harder textures. This is because the ionic fertilizer of ZA interact with the composites of zeolite/hydrogel and they were becomes more tightly interact each other.

Chemical Structures of CRF Composites zeolite/ydrogel.

The IR spectra of arrowroot starch before polymerization (Fig. 1. (a)) shows the absorptions at 3394, 2933, 1155, and 1020 cm^{-1}. These wave numbers are absorption the O-H group of alcohol, C-H sp^3,C-O of alcohol, and C-O of ether respectively. The arrowroot starch which has been made into the hydrogel H (Fig. 1. (b)) shows new absorption peaks at wave numbers 3433, 1664, and 1402 cm^{-1} that are absorption of the N-H group of acrylamide, stretching C=O (acrylamide, acrylate, and MBA), and bending N-H vibrations. These peaks indicate that acrylamide-co-acrylic has been successfully grafted and the MBA has successfully crosslink to the polymer chain. The IR peak of C-O alcohol at 1155 cm^{-1} regiondid not appear on the IR spectrum of hydrogel H which indicated the hydroxyl groups of starch are successfully converted to C-O ether. The successfulness of grafting can also be seen in the absorption of vinyl group CH_2=CH_2 of acrylamide monomer (acrylamide-co-acrylic acid) which did not appear at 900-990 cm^{-1} wavenumber.

The IR spectrum of the composites of zeolite/hydrogels (HZ) (Fig. 1. (c)) shows an increase in the intensity of the absorption peak and new absorption peaks at 1109 and 617 cm^{-1} wavenumbers. These peaks indicate a vibration of Si-O (Si) or Si-O (Al) and internal (Si, Al)-O of zeolite [13]. Absorption peaks at 1109 and 617 cm^{-1} wave numbers do not appear on the IR spectrum of hydrogel H, so those peaks show the specific features of composite containing zeolite. The composite of zeolite/hydrogel with contain ZA fertilizer (P6) showed a wider absorption peak with higher intensity at 1109 cm^{-1} (Fig. 1. (d)). This peak indicates the presence of SO_4^{2-} groups derived from ZA fertilizer [24]. The differences in absorption of this P6 composite show the specific features of composites containing ZA fertilizers.

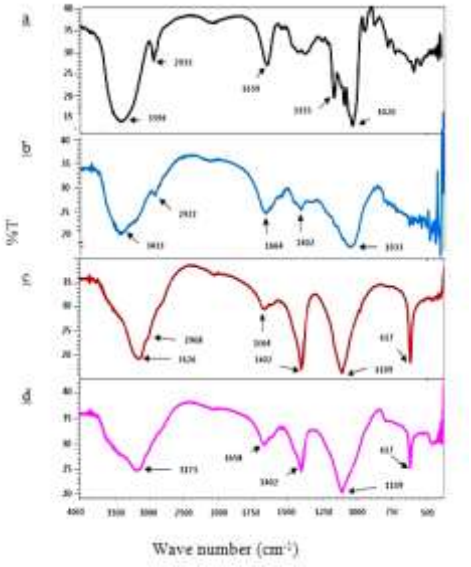

Fig. 1. The IR spectrum (a) of arrowroot starch; (b) hydrogel (H); (c) composite hydrogel-zeolites HZ; (d) composite HZ-fertilizer (P6)

Swelling Capacity of Hydrogels and the Composites Hydrogel

The swelling capacity test of the hydrogel and composite hydrogel in solution of pH 5, 7, and 9 indicated that swelling

capacity of the CRF composites increased with increasing the pH of the solution and the swelling capacity decreased with the increasing concentration of zeolite and ammonium sulfate (ZA) fertilizer in the CRF composites. The swelling capacity of the hydrogel and hydrogel composite are shown in Fig. 2 below.

In more acidic solutions (pH 5) causes the $–NH_2$ groups of acrylamide and $-COO^-$ groups of acrylic to be protonated to produce $-NH_3^+$ and -COOH. The cationic $-NH_3^+$ interacts strongly, due to ion-dipol interaction, with negative charge on the zeolite so that the network gets closer and tighter. Swelling capacity increases at pH 7 because the number of protonated $–NH_2$ and $-COO^-$ groups decreases, thereby reducing ionic interactions. The $–NH_2$ group can also form hydrogen bonds with water molecules while the $-COO^-$ groups will undergo electrostatic repulsion with each other as well as with negative charge of zeolite. This causes the hydrogel network increasingly tenuous so that the swelling capacity increases. Swelling capacity is higher at pH 9 as more hydrogen bonds occur between $-NH_2$ with water and the acrylic ions that undergo the electrostatic repulsion becomes more frequent. Electrostatic repulsion causes the hydrogel network to expands and a large amount of hydrogel volume is filled by solution [16].

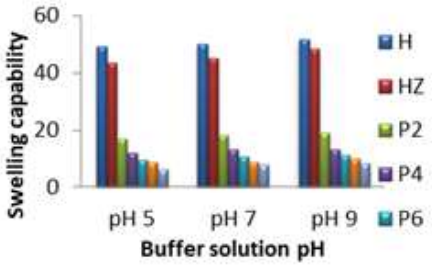

Fig. 2. The swelling capacity of the composite of zeolite/hydrogel

The swelling capacity of the composite of zeolite/hydrogel is also influenced by the composition of the CRF composites. The swelling capacity of the hydrogel H is higher than the composite HZ and the ZA CRF composite is lower than the HZ composite. The swelling capacity ofcomposite HZ is lower than the hydrogel H because presence of zeolite in the polymer affects the swelling capacity.The amount of zeolite loaded in this study is 48% from the composite total mass. The addition of excess zeolite (above 10% of the composite total mass) can decrease the elasticity of the bonds in the polymer as more crosslink points are formed [25]. Zeolite can also be trapped in the network cavities thereby which results in decreasing the capacity of swelling [13]. The advantage from loading large amounts of zeolite is the hydrogel becomes heavier when applied to agricultural soils so that it is not easily carried away by water. The composite P has the lowest swelling capacity because the fertilizer loaded into the composites will interact with the hydrogel.

The ammonium release analysis was performed by salicylic and spectrophotometric methods at maximum wavelength of 640 nm. The ammonium release analysis in buffer solution pH 5, 7 and 9 shows that the amount of ammonium released increases with increasing amount of fertilizer being loaded. The fertilizer release ability is based on the difference of concentration gradient inside and outside the hydrogel matrix. The highest ammonium release was obtained in the composite P10 and the lowest in composite P2. The higher the concentration of ZA fertilizer in the ZA CRF composite, the higher the number of soluble fertilizer. The rate of ammonium release from the composites P2, P4, and P6 tend to be gradually increased. This is because the amount of fertilizer loaded is not too much so that the buffer solution is only able to dissolve a little amount of fertilizer for each interval time. The effect of variation of ZA fertilizer addition to the ammonium release ability on buffer solution pH 5, 7, and 9 can be seen in Figure 3.

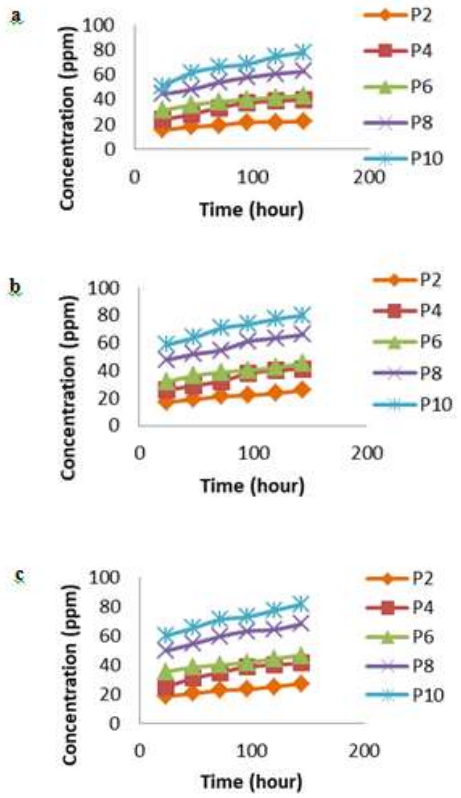

Figure. 3. The ammonium release of composites P2, P4, P6, P8, and P10 in solution at (a) pH 5; (b) pH 7; (c) pH 9

Ammonium release rate in Figure 4 shows thatafter 120 hours (6 days) the amount of ammonium released from each composite of zeolite/hydrogel is getting steady, that mnean there is no more ammonium is being released. The amount of ammonium released after 120 hours for the all composites are relatively low and there was a small amount of ammonium was released compared to the total ammonium contained in the ZA CRF composite. The small number of ammonium being released by the ZA CRF composite of zeolite/hydrogel to the solution up to 140 hours could be one consideration for the application of this ZA CRF system in real situation.

Ammonium released measurement indicated that the number of ammonium released in solution pH 5, 7 and 9 are in accordance to the swelling capacity at these pHs. The ammonium released is higher when the swelling capacity of the ZA CRF composites at this pH is higher. The release.

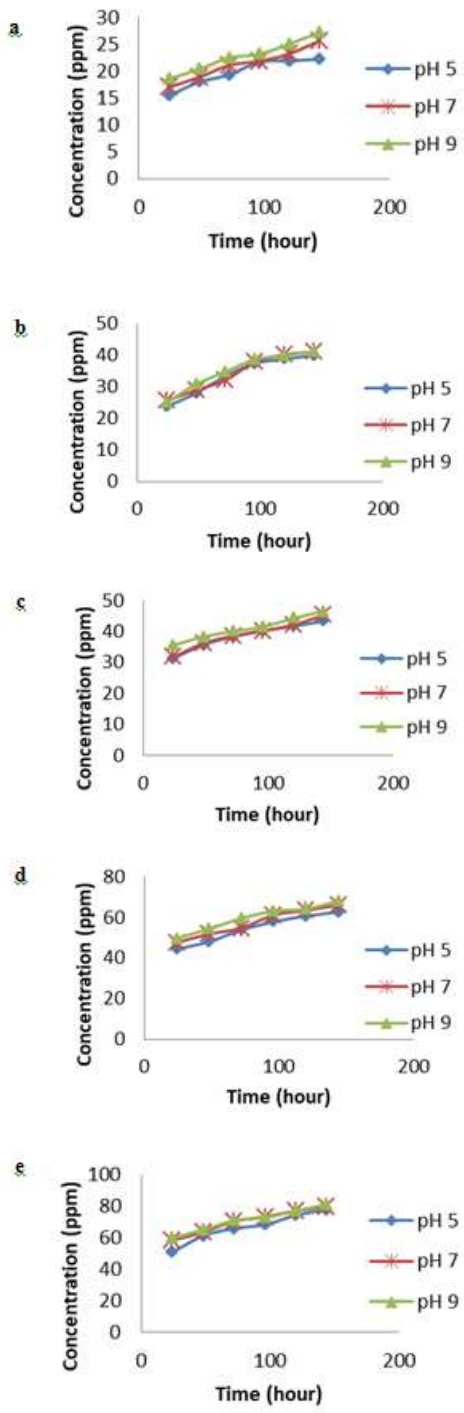

Figure. 4. The ammonium release rate in solution pH 5, 7 and 9 of (a) composite P2; (b) composite P4; (c) composite P6; (d) composite P8; (e) composite P10.

of ammonium is lower in acidic solution, say at pH 5 than in pH 7 or pH 9. At higher pH the ZA CRF composites absorbs more water, which make them more swelling and give

better diffusion of ammonium ion in the composite to enter to the solution. This causes the composite network to be tenuous so that more ammonium ion can be dissolved by the solution. Ammonium release graph is presented in Figure 4 b

4. Conclusion

The swelling capacity of the ZA CRF composite of zeolite/hydrogel decreases with the increasing the amount of ZA fertilizer concentration in the composites, and increases as the pH of solution increases. The presence of ZA fertilizer in the composites was characterized by it IR spectrum showed an absorption peak at 1109 cm^{-1} due to vibration of the SO_4^{2-}. The ammonium released from the composite of zeolite/hydrogel increases when the amount of ZA fertilizer loaded in the CRF composites were increased at any solution.

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THE EFFICACY OF BIOLOGICAL CONTROL AGENTS OF *Heterorhabditis* sp. AND *Serratia* sp. TO THE POPULATIONS OF *Nilaparvata lugens* AND *Scirpophaga incertulas* PESTS ON RICE PLANT IN PANCAKARYA VILLAGE, JEMBER

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Abstract

One of the problems in rice farming techniques is the presence of insect disturbances. Some insects that interfere with rice plants are the brown planthopper (*Nilaparvata lugens*) and the yellow stems borer (*Scirpophaga incertulas*). Conventional control measures are still using chemical insecticides with high spraying frequency poses negative effects on environment, health, resurgence and insect resistance. Therefore, it is necessary to look for an environmentally friendly alternative with the use of biological agents such as bacteria, entomopathogenic nematodes, and vegetable pesticides. The purpose of this study not only to determine that the efficacy of *Heterorhabditis* sp. and*Serratia* sp. can reduce the population of brown planthopper pests and yellow stem borer pests, but also to determine the effect on growth and production of rice crops. The results showed that the application of several biological agents to *N. lugens* population before and after treatment was different but not significant (p-value = 0,053). Similarly, the application of some biological agents on the before and after treatment of population *S. incertulas* showed different results, but not significant (p-value = 0.637).

Keywords: *Heterorhabditis* sp., *Serratia* sp., *Nilaparvata lugens*, *Scirpophaga incertulas*

1. Introduction

Rice is one of the staple foods in Indonesia. Rice is a crop of berberun grass. One of the obstacles in rice farming techniques is the presence of insect disturbances. Some insects that interfere with rice plants are brown planthopper and stem borer. According [1], conventional control efforts are still being done that is using chemical insecticides with high spraying frequency. The excessive use of insecticides has a negative effect on the environment, health, resurgence and insect resistance [2]. Therefore, it is necessary to find an environmentally friendly alternative. One of the controls of insects with the use of biological agents such as bacteria, entomopathogenic nematodes, and vegetable pesticides.

2. Materials And Methods

Place and time

This research was carried out on the land owned by residents in Pancakarya Village, Kab.Jember. The research time is September-December 2016.

Tools and Materials

The tools used in this research are: pot with diameter 28 cm, hoe, bucket, funnel, sprayer, sickle, stationery, nameplate, measuring tube, scisors, manila paper, plastic, yarn, digital balance, THM and camera. The materials used in this research are: pandan scented rice plant, entomopathogenic nematodes *Heterorhabditis*sp. (Production of CV Nemadic ®), *Serratia* sp., vegetablepestcides (Organeem), Decis ® (chemical pestcides) and NPK Mutiara® organic fertilizer.

The Research Design

This research was conducted using Group Random Design. Consisting of 6 treatments including control, each treatment consisted of 12 replications, so the total pots were 72 pots with the following treatment details:
A0 = control
A1 = NEP *Heterorhabditis*sp. doses of 12,000 juvenile infectives (IJ) / 2 ml in 500 ml of water (nemadic® cultures)
A2 = *Serratia* sp. dose of 2 ml / 500 mlwater
A3 =*Heterorhabditis* sp. dose 12.000 IJ / 2 ml + *Serratia* sp. dose of 2 ml / 500 ml of water
A4 = Organic Pesticide (Organeem) dose 2 ml / 500 ml water
A5 = Chemical Pesticide (Decis) dose 2 ml / 500 ml water

The Research Implementation

This research used pandan wangi variety of rice plant that has been sowing and aged ± 20 days, then transferred into pot with diameter 28 cm. After ± 27 days old rice plants fertilization with NPK Mutiara ® fertilizer as a base fertilizer with a dose of 10 grams per pot. The pots are placed with a distance of 50 cm between blocks. Each pot contains 3 rice plants.

The application of treatment on rice plants is done after 7 days of fertilization, ie when the rice plants aged ± 34 days up to the age of 92 days after planting, with treatment time range every 7 days and all treatment applications done in the afternoon.

Method of Collecting Data

The population of insects to be observed was calculated by the direct method of the entire treatment plant. Observation of insect population was done on one day before treatment and 3 days after treatment.

The growth of rice crops observed is the height of rice crops. The measurement of plant height is done by measuring the highest leaf length in rice plants using a ruler.

Measurement of rice production is done by weighing the weight of the grain of rice seed during harvest including wet weight and dry weight. Wet weight can be measured immediately after the harvesting process, while the dry weight measurement of rice plants must wait several days after the drying process takes place. Wet weight and dry weight are measured using a digital balance sheet gauge.

Data Analysis

The data obtained were analyzed by SPSS using Paired Sample T-test and One Way Anova for biological agent test on *Nilaparvatalugens*, *Scirpophagaincertulas* and wet weight and dry weight of rice. If the results are significantly different, then continued with LSD test with 5% confidence level. In the test of biological agents on the average height of rice plants conducted One Way Anova analysis.

3. Results and Discussion

Based on the result of paired sample t-test, there were different but not significant results between *N. lugens* population before treatment and after treatment (p-value = 0,053) with mean population *N. lugens* before treatment of 8.39 tail and the average population of *N.lugens* after treatment of 3.06 tails.

The result of One Way Anova analysis between treatment to the average population of *N. lugens* at before treatment (F = 1,840; p-value = 0,135) and after treatment (F = 2,271; p-value = 0,073) showed no significant difference. The result of the difference in the average population of *N. lugens* is shown in Table 1.

Table 1. Average of populations of *N. lugens* before and after treatment

Treatment	Average of populations of *N. lugens* before treatment	Average of populations of *N. lugens* after treatment	Difference average of populations of *N. lugens*
Control	0,83	0,33	0,5
Heterorhabditis sp.	0,67	0,33	0,34
Serratia sp.	0,83	0,17	0,66
Heterorhabditis sp. + *Serratia* sp.	1,67	0,67	1
Organeem	12,5	0,67	11,83
Decis	33,83	16,17	17,66

The population of *N. lugens* before and after the application of different biological agents showed no significant difference (F = 1,840; p-value = 0.135) and (F = 2,271; p-

value = 0.073). The amount of *N. lugens* in after treatment showed a decrease in all treatments. The largest population decline was found in the Decis treatment (17,66), whereas the lowest decrease in *N. lugens* population was found in *Heterorhabditis* sp. application (0.34) lower when compared with controls having the average population of *N. lugens*(0.5) (Table 1).

The result of paired sample t-test between population of *S. incertulas* before treatment and after treatment showed significant different but not significant (p-value = 0,637) at 5% The population of *S. incertulas* in amounted to 10.25 head, whereas in the average number of *S. incertulas* population was 9.67 tails. The result of one way anova test between the treatment of the average population of *S. incertulas* before treatment (F = 0,381; p-value = 0,858) and after treatment (F = 0,775; p-value = 0,575) real. The result of the average population difference of *S. incertulas* is shown in table 2.

Table2.Average of populations of *S. incertulas*before and after treatment

Treatment	Average of populations of *S. incertulas* before treatment	Average of populations of *S. incertulas* after treatment	Difference average of populations of *S. incertulas*
Control	8,67	7,83	0,84
Heterorhabditis sp.	11,5	12,83	-1,33
Serratia sp.	16,17	15,5	0,67
Heterorhabditis sp. + *Serratia* sp.	11,5	12,67	-1,17
Organeem	11,67	7,67	4
Decis	2	1,5	0,5

Note: sign "-" showedthe increases average of populations of *S. incertulas*.

Based on the data in table 2 it can be seen that the lowest average population of *S. incertulas* is in the treatment of Decis with 0.5 tail, then the *Serratia* sp. with the average population *S. incertulas* 0.67 tail, then control with the average population *S Incertulas* 0.84 tail, the next organeem with an average population of 4 tails, whereas for *Heterorhabditis* sp. and *Serratia* sp. combined the average increase in population of *S. incertulas* is 1.17, and the last one in the *Heterorhabditis* sp. application occurs on average increase Population of *S. incertulas* amounted to 1, 33 head.

The result of one way anova test showed that the treatment between several biological agents on the growth of rice plant height showed no significant difference (F = 0.092; p-value 0.993) at 5% confidence level, while for the time between application per week to plant height showed the result Significantly different (F = 157,682; p-value = 0,000).

The result of one way anova test between several biological agents on the production on wet weight of rice plants was found to be significantly different (F = 203,442; p-value = 0,000), as well as on the results of one way anova test between the biological agents on dry weight of rice plants Obtained a significantly different result (F = 224,669; p-value = 0,000). Result of LSD test of wet weight of paddy and dry weight at 5% confidence level can be seen in table 3.

Table3.Result of one way anova between treatment in crop productions

Treatment		Average of wet rice productions (g)	Average of dry rice productions (g)
Control	A 0	135,56^{e}	112,92^{f}
Heterorhabditis sp.	A 1	112,72^{d}	87,04^{d}
Serratia sp.	A 2	70,34^{a}	55,87^{a}
Heterorhabditis sp. + *Serratia* sp.	A 3	75,06^{a}	60,88^{b}
Organeem	A 4	105,98^{c}	76,48^{c}
Decis	A 5	95,74^{b}	94,05^{e}

Note: Numbers that followed by the same*superscript* in the same coloumn showed not significant by LSD test (α=5%)

When the process of cooking and filling of seeds there are factors that affect the process. These factors are internal factors and external factors. Internal factors include: plant species and the diversity of genes between varieties within species, whereas on external factors include: climatic conditions,

land conditions and cultivation techniques [3].

Internal factors include leguminous plant species (dikotil) and cereal plants (monocots). The fundamental difference is seen in the process of sucrose translocation between the two. If in cereal plants the translocation process has reached maximum when the anthesis occurs. However, in leguminous plants the translocation process begins when the formation of fruit (pollination has occurred). Furthermore, in one species even in one plant variety, there is considerable genetic diversity. Genetic diversity is also very influential on the process of filling and cooking seeds. For example, in PETA with INTANI rice plants have different physiological cooking time (Ma'rufah, 2008).

External factors such as climate conditions such as moisture will also affect the filling and cooking seeds. Seeds that are in high humidity conditions will inhibit the achievement of physiological cooking so that the process takes a long time to achieve. This is because the reduction of water content in the seeds inhibited / requires a lot of energy. The condition of the land affected by the nutrients in the soil is very diverse also play a role in filling and cooking seeds, there are soils that are rich in nutrients and there is also a soil that is less nutrient. When the nutrients in a low condition then the growth of plants will be hampered so that the process of growth and filling of seeds is also hampered and vice versa. Cultivation techniques also affect the filling and cooking of these seeds, such as when planting, water supply, fertilization, plant spacing, and when harvesting. When the right planting is related to the season that best suits the growth of the plant. If the plant is planted in the right season (the right time) then photosythesis will be generated and affect the development of seeds. Similarly, the provision of water, fertilization, planting distance treatment and when the right harvest will have a positive effect on cooking and seed development [3]

4. Conclusions

Based on the research that has been done can be concluded that:

a. Treatment of biological agents *Heterorhabditis* sp. and*Serratia* sp. can decrease the population of *N. lugens* by 2% in the pre- and post-treatments, while the *Heterorhabditis* sp. and*Serratia* sp.can not decrease *S. incertulas* population.
b. Treatment of biological agents *Heterorhabditis* sp. and*Serratia* sp. do not affect the average height of rice crops and rice grain production.

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ANTIBACTERIAL ACTIVITY OF *Averrhoa carambola* L. AGAINTS METHICILLIN RESISTANT *Staphylococcus aureus* (MRSA)

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Abstract

Star fruit (*Averrhoa carambola* L.) has been used as treatment for curing illness traditionally. Purpose of this study is to investigate the effect of methanol extract of A. *carambola* leaves and its barks on the growth of Methicillin Resistant *Staphylococcus aureus* (MRSA). The extraction was done by maceration method while antibacterial test by Kirby-Bauer method. Antibacterial activity test used three concentrations namely 25, 50, 75%. For negative and positive control, methanol and linezolid was used respectively. The result showed the extraction of barks at concentration of 75% gave the widest inhibition zone to MRSA.

Keywords: *Averrhoa carambola L.*, MRSA bacteria, maceration, Kirby-Bauer method.

1. Introduction

Indonesia as a tropical country has a very high number of plant biodiversity consisting of various tribes and species. Plants are used for various purposes, one of them is as material in the treatment of many diseases. According to [1], the use of medicinal plants in Indonesia has been empirically done by our ancestors. It is proved by the discovery of sculptures on Borobudur temple regarding health and beauty care activities. Plants have medicinal properties because they contain secondary metabolites such as alkaloids, flavonoids, terpenoids, saponins, sterols and coumarin [2].

One of the medicinal plants is starfruit. (*Averrhoa carambola*). Amalia and Zumaidar (2007), found that the people in Pidie area, Aceh Province, utilize some part of starfruit as inflammatory bowel, antimalarial and antirheumatic [3]. Research on the ability of secondary metabolite compounds of the genus *Averrhoa* has also been conducted by others, Zakaria *et al*. (2007) [4] and Safitri (2011) [5]. Aqueous and chloroform extracts of *Averrhoa bilimbi* leaves can inhibit *Staphylococcus aureus* growth with inhibit zone ranging from 7 - 11 mm [4]. Methanol extract of *A.carambola* leaves and barks in concentration of 30% against *S. aureus* ATCC 29213 resulted in inhibit zone of 8 and 16 mm respectively.

Based on the results of those studies it is interesting to perform further testing of leaves and barks methanol extracts of starfruit against bacteria Methicillin Resistant Staphylococcus aureus (MRSA). There have been several types of Staphylococcus aureus resistant to the penicillin-type β-lactam antibiotic. The existence of these bacteria is increasingly widespread, so appropriate handling is needed. This study aims to examine the ability of starfruit plants in inhibiting the growth of MRSA bacteria.

2. Materials and Methods

Plant materials collection

The leaves and barks of A. carambola were collected from its natural habitat in Kabupaten Aceh Besar, Aceh Province.

Bacteria culture

Bacteria tested in this study were MRSA obtained from Microbioloy Laboratory of Zainal Abidin Hospital (Rumah Sakit Umum Zainal Abidin).

Preparation of A. carambola methanol extracts

Fresh leaves and barks of *A. carambola* were air dried for 7 days at room temperature. A total of 500 grams of dried sample was then macerated with n-hexane solvent for 2x24 hours. The residue was then filtered and re-macerated using n-hexane. This step was repeated until a clear color of filtrate was obtained. The residual result of n-hexane was further macerated using

methanol solvent for 1x24 hours. The residue was further filtered and macerated with methanol until the filtrate is clear. The methanol filtrate is then concentrated using a rotary evaporator. The methanol extract was then made in concentrations of 25, 50, and 75% [6].

Antibacterial study

Antibacterial testing was performed using Kirby-Bauer method (modified) [7]. Mueller Hinton Agar (MHA) medium which had been sterilized poured into each petri dish as much as 15 – 20 ml and left for a while until solid. In solid media, a 0.1ml suspension of 24-hour MRSA bacteria synchronized with a standard of 0.5 Mc Farland was spread using a sterile dispersion stem until the bacterial suspension was evenly distributed across the surface of the media. The first MHA medium was divided into 3 sections (A, B, and C) where a disc containing methanol extract of 25%, 50%, and 75% at concentration of 20 µl were placed. Discs containing positive control (linezolid as an antibiotic) and negative control (0%) were placed on the second medium in different regions (D and E). This test was performed as triplicate. Furthermore, the media were incubated at 37ºC for 24 – 48 hours and the formation of inhibition zone were observed. The inhibit zone formed was then measured in diameter using a ruler in millimeters.

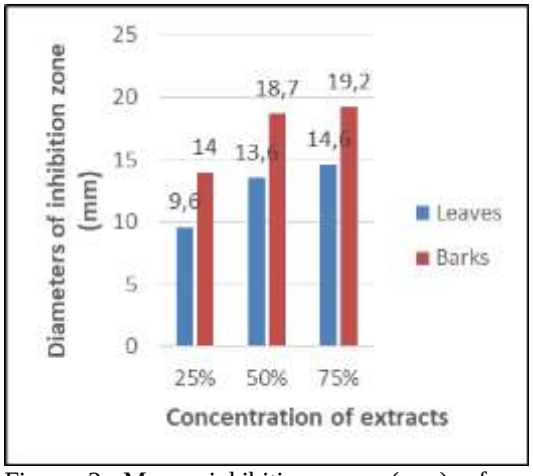

Figure 2. Means inhibition zone (mm) of methanol extracts of *A. carambola* leaves and barks diameter at 25, 50 And 75% against MRSA

On the graph shows the diameter of inhibitory zone produced by methanol extract of barks is wider than leaves. The methanol extract of leaves at 75% concentration yield

3. Results and Discussion

The result of antibacterial activity test showed that the methanol extract of leave and bark have ability to inhibit the growth of MRSA bacteria. This can be seen from the formation of inhibition zones showed by clear zones around paper disc containing test extracts (figure 1.)

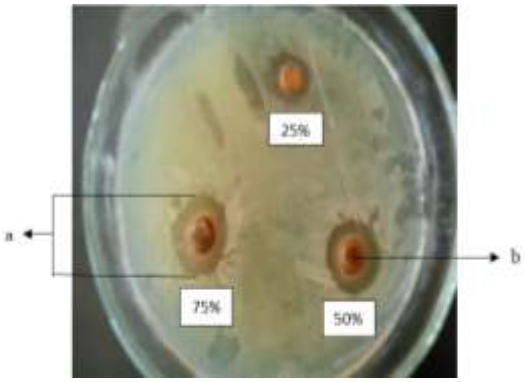

Figure 1. Inhibition zone of methanol extract of *A. carambola* bark against MRSA; (a) diameter of inhibition zone; (b) disc

diameter of inhibitory zone 14.6 mm while the extract of bark at the same concentration produce inhibitory zone diameter up to 19.2 mm.Diameter of inhibitory zone produced by the two extracts increased as it concentration increased. The resistance response generated by the two extracts include in strong category because it is in the range of 11 – 20 mm [8]. The positive control of linezolid has a zone of inhibition of 33.7 mm which belongs to a very strong category.

Antibacterial ability showed by methanol extracts of *A. carambola* leave and bark against MRSA growth is very potential, considering the tested bacteria used is a resistant bacteria type. MRSA resist to methicillin and all beta-lactam antibiotic group because it had mutation on *Protein Binding Penicillinase* 2 (PBP2) converted to PBP2a [9]. This condition cause beta-lactam antibiotics affinity become low. PBP2 is a protein in the cell membrane of bacteria

which plays role in the process of peptidoglycan synthesis.

Antibacterial potency of the *A. carambola* leaves and bark extracts is viable due to the secondary metabolite compound it possesses. The result of phytochemical test on both extracts (leave and bark) showed the presence of alkaloid compounds, phenols, steroids, coumarins and saponins [5]. The presence of these secondary metabolites makes a workable synergistic relationship. This is confirmed by Saifudin (2014), that the presence of a metabolite compound will strengthen the effects of other compounds [10]. Each secondary metabolite compound has different antibacterial mechanisms of action.

Alkaloid compound interferes peptidoglycan synthesis, so that the bacterial cell wall layers are not formed completely. This condition leads bacterial cells become lysis. Steroid compound that belong to triterpenoid classes can disrupt the cell of bacteria, especially in the lipophilic parts. The coumarin which is also a phenol compound has an antibacterial action mechanism in binding to cell membrane protein cause the protein denaturized [11]. Furthermore, saponin which has toxic ability can disrupt the stability of bacterial cell walls, especially groups of Gram-positive bacteria. Some herbs are reported as inhibit bacterial growth of MRSA agent, including methanol extract of *Anacardium ocidentale* bark [12], the alcohol extract of *Erythropheleum suaveolens* [13], and methanol extract of *Hibiscus rosa-sinensis* leaves [14].

4. Conclusion

1. Methanol extract of *A. carambola* leaves and barks can inhibit growth of MRSA bacteria.
2. Diameter of inhibitory zone produced by methanol extract of *A. carambola* leaves and barks at concentration of 75% were 14,6 mm and 19,2 mm, respectively.

ACKNOWLEDGMENT

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CHARACTERIZATION OF TERRESTRIAL SPORES FERN PLANTS FROM WILDLIFE HIGHLAND "YANG" THE ARGOPURO MOUNTAIN

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Abstract

This study was conducted to determine the spore characteristics of terrestrial fern plants. It covered in highland “Yang" wildlife moss forest, Argopuro Mountains. The specimens of terrestrial fern collections were carried out by a structured random method along 2 km mossforest climbing route that was divided into 20 points. In each pointwas 100 meters away then made a rectangular plot with a length 15 meters and 6 meters wide. In each plot measurements of abiotic data such as temperature, humidity, light intensity, geospatial location and site altitude. The spores obtained were prepared by using acetolysis, then it observed under a microscope with magnification 400 times. Spore parameters were type, shape, size, and exinornamentation. Twelve kinds of terrestrial fern plants with 2 types of spores were trilete and monolete. The trilete spore was found in a single fern plant of *Pteristripita*. While monolete type were found in *Aspleniums colopendrium, Asplenium excisum, Asplenium normale, Blechnum nudum, Lastreopsis rufescens, Lastreopsis munita, Lastreopsiss mithiana, Lastreop sisgrayi, Sticherus lobatus, Diplazium pallidum and Athyrium mearnsianum*. The spores form observed were suboblasts (1 species), prolate (3 types), peroblics (2 types), oblate (1 type) and subspheroidal (5 types) with 5 exine ornamentation forms were psilate, verrucate, scabrate, echinate and regulate. This study that characteristics spore of fern plants in moss forest, Argopuro Mountains for better understanding the key characters from morphological features of spore fern plant.

Keywords: characterization of spore, terrestrial fern plants, Argopuro Mountains.

1. Introduction

Pteridophyteis one of flora diversity in Indonesia. It has a vessel system (kormophyhte). The spore as generative propagation tools instead of seed. Fern plants hadmetagenesis phase between gametophyte phase and sporophyte phase. Spore is an early development the gametophyte phases of fern plants [1]

Fern plants produce 2 kinds of spores, homospores or heterospores. In heterosporaproduce two types of spores, namely macrospores and microspore, whereas in the homospores produce only one type of spore. The spores of fern plants are composed of a thick outer part called an exine, and a thin inner part called an intin. According to Kapp [2] spore fern plants is divided into 2 types namely monolete and trilete. The difference between each type of spores is based on the presence or absence of a thin structure that resembles the aperture of tetrad spore scars.

Fern plants are pioneer plants in every type of forest area and it plays an important role for compiling forest ecosystems. Fern plants mostly live in a high humidity levels such in highland forests. This relates to the adaptation of epiphytic and terrestrial fern plants requiring the presence of water to maintain its viability and as a medium for sperm transfer during fertilization (Loveless, 1999).

One of highland in East Java is the Argopuro Mountains. It has a peak with an altitude of 3088 meters above sea level. One of the conservation areas is a moss forest it located in the altitude range of 1000-2000 meters above sea level which it located in the "Highlands Yang" Wildlife. Argopuro Mountains is located in 4 districts in East Java: Probolinggo, Jember, Bondowoso, and

Situbondo (Bksdajatim, 2012). Based on preliminary survey on 12thFebruary 2015 in themoss forest area are found many moss plants and fern plants, because both of plants require habitat has a high humidity, enough sunlight, and water availability.

Fern plant exploration and research, especially research on spores in the Argopuro Mountains region has never been done. In addition, the spores of each fern plant division have variations of shape and size [3] and the characteristics of each spore can be basis for distinguishing each species of fern plant. Therefore this study conducted to determine the characteristics of spores terrestrial fern plants in the Highland "Yang" Lichen Forest Argopuro Mountains are very important.

2. Materials and Methods

The tools used in this research are herbarium press tool, GPS (Global Positioning System), knife, microcentrifuge tube, centrifugator, falcon tube, vial tube, beaker glass (50 mL, 100 mL, and 500 mL), OptiLab, plastic cup, measuring cup (10 mL, 100 mL, and 500 mL), object glass, cover glass, waterbath, binocular microscope, stereo microscope, pipette, luxmeter, hygrometer, thermometer, and altimeter.

The materials used in this study were mature spores from terrestrial fern plants obtained in the Argopuro Mountains, aquadest, 70% alcohol, fern polish, 45% glacial acetic acid, glycerin, 45% glacial acetic acid mixture and concentrated sulfuric acid 9: 1 ratio, and safranin.

Research procedure

The Sampling was conducted by a randomly structured method around2km along moss forest tracking. The location were 20 points with each point was 100 meters. From each point, 15 meter straight line is drawn in the forest and then 6 meter transverse line is formed therefore a rectangular plot of 15 meters long and 6 meters wide is formed (Fig 1).

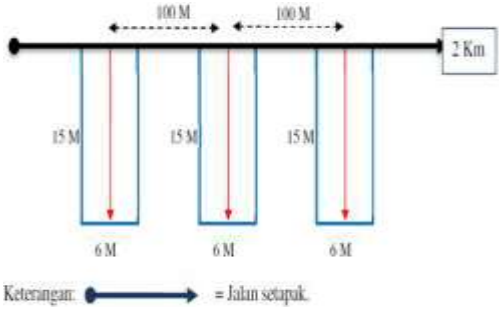

Figure1. Plot design

Sample collections of terrestrial fern plants obtained in each plot were put into large plastic bags for making herbariums. In each plot were mounted abiotic data such as temperature, humidity, light intensity, geospatial location, and height of research place. Each sample of the fern plants was labeled, morphologically characteristic, including the type, shape, color, and position of sorus to the leaf. It purposedfor identifying the species.

The Samples that mature spores (sori) were collected directly from terrestrial fern plants by scraping the sorus with a toothpick and then collected in a small plastic. The mature Soruswere characterized by a blackish brown sorus color and broke. The Identification of fern plants based on the morphological characteristics include shape and color of stem, branching stems, shape and color of leaves, shape of leaf bone, leaf margin, size and sorus location, the shape of the indigenous, the shape of the scales, and the paraphysis [4]. The morphological characteristics result compared with literatures [5], [6], (Winter and Amoroso, 2003a), and collected it to be Jemberiense Herbarium collections.

Preparation of Spore preservation

Preparation of spore preservation based on [7]. Spore preserved without incision (whole mount). The steps of spore preservation were acetolysis, cleaning, staining and mounting (attachment).

a. Acetolysis

The spores put into microcentrifuge tube were lyzed using 1 mL 24 hour glacial acetic acid then centrifuged at 2000 rpm for 30 minutes. The pellet added with a mixture of glacial acetic acid and 1 Ml sulfuric acid with a ratio of 9: 1 while warming it in the

waterbath for 10 minutes. Sample centrifuged at 2000 rpm for 30 minutes then removed the supernatan.

b. Cleaning

The pellet was added by aquadestthen centrifuged at 3000 rpm for 10 minutes three times. It purposed to clean pollen free with chemical substances such as the fixative substances in the spores to be prepared [7].

c. Staining

This step aims to clarify the spore wall ornament shape and facilitate the measurement of spores. This step was used safranin which has strong chloride and alkaline.Safranindissolved with aquadest for about 90 minutes. Spore stained with safranin for 1- 2 minutes. It washed with aquadest twice to remove the remaining dye. This repetition is necessary until the spore is good quality [7].

d. Mounting

Mounting was done by addition of glycerin which has been heated and stirred evenly. Glycerin dripped on the top of glass and trimmed it then spores dripped on top of the object glass and then closed with a cover glass and the edges smeared with fern polish. During the mounting process is kept in order not toformed air bubbles. The last preparations were dried and labeled.

Observation and Determination of Spores

Spore preservation observed under a microscope used 400 times magnification. Spore parameters were spores, size, exine ornamentation, and the presence or absence of thin structures resembling the aperture of tetrad scar on the spores.

This observation determined by comparing the observed features with several literatures such as [2] and Jones (1979).

3. Results and Discussion

1. *A. scolopendrium* Lour.

The spore was monolete, suboblaced, had a polar side length of 36.8 µm, Equatorial side 60.6 µm, and a ratio of P / E 0.83 µm, Exine ornamentation psilate shaped was 4.9 µm. The ornamentation element had a smooth surface that bulging on apical (Figure 2).

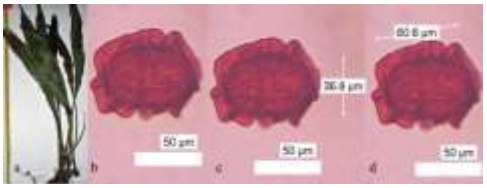

Figure 2. A. *Scolopendrium* Lour. A. Habitus; B. Spore forming; C. Polar side; D. Equatorial side

2. *A. excisum* C.Presl

Monolete spore type, peroblated, had a polar side length of 22.5 µm, Equatorial side 47.1 µm, and had ratio of P / E 0.47 µm, exine ornamentationechinate-shaped was 2.5 µm (Figure 3).

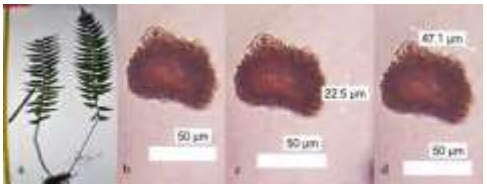

Figure 3. A. *Excisum* C. Presl a. Habitus; B. Spore form; C. Polar side; D. Equatorial side

3. *A. normale* D.Don

Monolete spore type, oblate-shaped, had a polar side length 25.7 µm, Equatorial side 44.80 µm, and had ratio of P / E 0.57 µm, exine ornamentation verrucate-shaped was 4.5 µm. The ornamentation element was Isodiametric shaped (Figure 4).

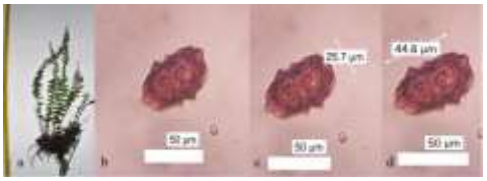

Figure 4 A. *Normale* D.Don a. Habitus; B. Spore form; C. Polar side; D. Equatorial side

4. *B. nudum* (Labill.) Wakef.

Monolete spore, subspheroidal, had a polar side length of 45.8 µm, Equatorial side 35.3 µm, and had ratio of P / E 1.29 µm, the exine ornamentation rugulate-shaped had 7.9 µm and Isodiametric (Fig. 5).

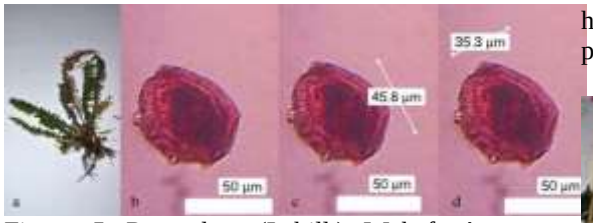

Figure 5 *B. nudum* (Labill.) Wakef. A. Habitus; B. Spore form; C. Polar side; D. Equatorial side.

5. *L. rufescens*(Blume) Ching

Monolete spores, prolate-shaped, had 60.5 µm polar side lengths, Equatorial side 33.6 µm, and had ratio of P / E 1.8 µm, Oxine ornamentation verrucate form measuring 2.9 µm. Ornamentation element is isodiametric shape (Figure 6).

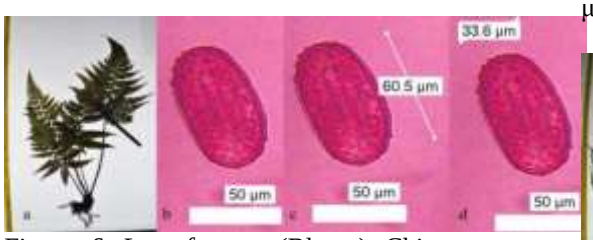

Figure 6 *L. rufescens* (Blume) Ching a. Habitus; B. Spore form; C. Side Polar; D. Equatorial side

6. *L. munita*(Mett.) Tindale

Monolete spore type, prolate-shaped, had 55.7 µm polar side, Equatorial side size 42.0 µm, and had ratio of P / E 1.32 µm, the exine ornamentation psilate shaped 5.1 µm. Ornamentation element is isodiametric shape (Figure 7).

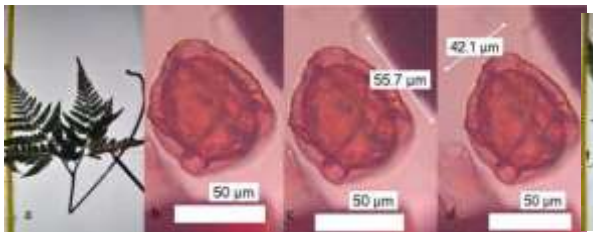

Figure 7 *L. munita* (Mett.) Tindale a. Habitus; B. Spore form; C. Polar side; D. Equatorial side

7. *L. smithiana* Tindale

Monolete spore type, subspheroidal shaped, had a polar side length of 55.6 µm, 38.6 µm equatorial side, and had ratio of P / E 1.44 µm, Oxine ornamentation psilate shaped 8.9 µm. The ornamentation element had smooth surfaces and inflated on apical parts (Figure 8).

Figure 8 *L. Smithiana* Tindale a. Habitus; B. Spore form; C. Polar side; D. Equatorial side

8. *L. grayi* D.L.Jones

Monolete spore type, subspheroidal, had a polarized side length of 28.6 µm, Equatorial side 52.6 µm, and had ratio of P / E 0.53 µm, Oxine ornamentation 2.7 µmrugulate shaped (Figure. 9).

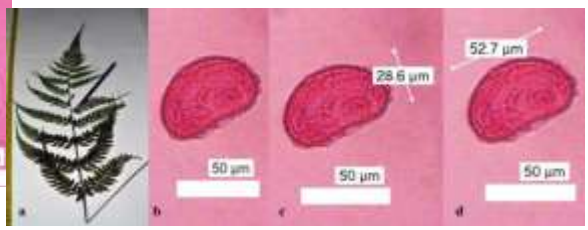

Figure 9 *L. Grayi* D.L.Jones a. Habitus; B. Spore form; C. Polar side; D. Equatorial side

9. *S. lobatus* N.A.Wakef.

Monolete spore type, peroblated, had a polar side length of 17.5 µm, equatorial side 39.3 µm, and had ratio of P / E 0.44 µm, the exine ornamentation psilate-shaped length 0.9 µm. Ornamentation element is isodiametric shape (Figure 10).

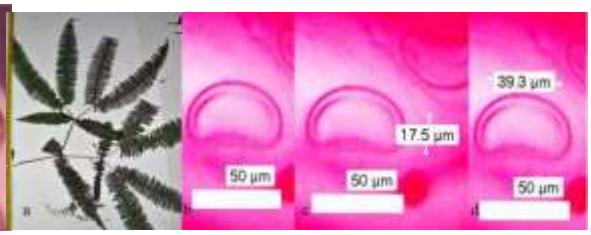

Figure 10 S. *lobatus*N.A.Wakef. A. Habitus; B. Spore form; C. Polar side; D. Equatorial side

10. *D. pallidum* (Blume) T.Moore

Monolete spore type, prolate form, had a polar side length 60.9 µm, Equatorial side 36.9 µm, and hadratio of P / E 0.61 µm, exine ornamentation Psilate-shaped 5.4 µm. The ornamentation element had a surface as

smooth as the wings and inflatable on the apical part (Figure 11).

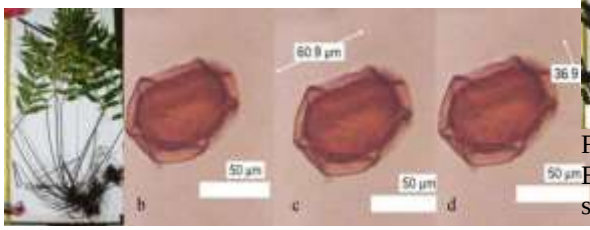

Figure 11 *D. Pallidum* (Blume) T.Moore A. Habitus; B. Spore form; C. Polar side; D. Equatorial side

11. *A. mearnsianum*(Copel.) Aldrew.

Monolete spore type, subspheroidal, had a polar side length 52.9 µm, 42.1 µm equatorial side, and had ratio of P / E ratio of 1.25 µm, the exine ornamentation is a verrucate 5.4 µm. The Ornamentation element isodiametric shape (Figure. 12).

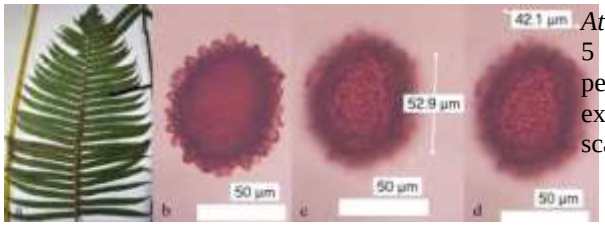

Figure 12 A. mearnsianum (Copel.) Aldrew. A. Habitus; B. Spore form; C. Polar side ; D. Equatorial side

12. *P. tripartita* Swartz.

Trilete spore type, subspheroidal, had a polar side length is 74.1 µm, the equatorial side is 78.5 µm, and had ratio of P / E 0.94 µm, exine ornamentation psilate-shaped is 12.8 µm. The ornamentation element had smooth surface and bulge on the apical (Figure. 13).

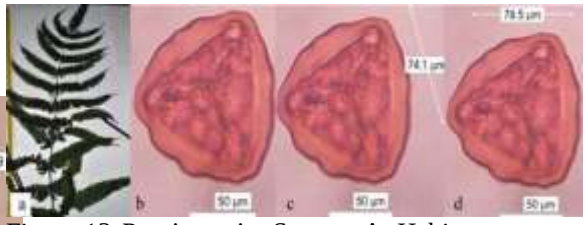

Figure 13 *P. tripaartita* Swartz. A. Habitus; B. Spore form; C. Polar side; D. Equatorial side

4. CONCLUSION

In moss forest Argopuro mountain found 12 types of terrestrial fern plants with 2 types of spores such as trilete and monolete. Trilete spores were found in *Pteristripartita*. Monolete spores were found in 11 species such as *Aspleniumscolopendrium*, *Aspleniumexcisum*, *Aspleniumnormale*, *Blechnumnudum*, *Lastreopsisrufescens*, *Lastreopsismunita*, *Lastreopsissmithiana*, *Lastreopsisgrayi*, *Sticheruslobatus*, *Diplaziumpallidum* and *Athyriummearnsianum*. The spore form were 5 kind namely suboblasts, prolles, peroblasts, oblasts and subspheroidal. The exineornamentation werepsilate, verrucate, scabarate, echinate and regulate.

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DIVERSITY OF JUVENILE AND SMALL FISH IN MANGROVE WITH DIFFERENT ROOT TYPES IN LABUHAN COASTAL SEPULU - BANGKALAN

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Abstract

The complexities of mangrove root types are well known to give effect on communities of fish including larval, juvenile and adult stages. This study aimed to access the difference on species composition and diversity of juvenile and small fish from mangrove area with different root types, especially in mangrove of coastal area of Sepulu, Bangkalan which projected as a mangrove protection area. The samples were collected from November 2016 to June 2017 using modified centipede net and scoop net. Sampling locations including area with the pneumatophores (S), stilt roots (R), combination of both types (C) and area without mangrove (unvegetated area or T). At the end of study, at least 32 species from 16 families of juvenile and small fish were identified. Families of fish with highest number of species are Gobiidae with 13 species, followed by Ambassidae, Mugilidae, Eleotridae and Siganidae with 2 species each. Fish species with highest abundance in all sampling sites and periods are *Oryzias javanicus* (F. Adrianichthyidae, 22.039%), *Ambassis kopsii* (F. Ambassidae, 17.878%), *Liza vaigiensis* (F. Mugilidae, 9.613%), *Am. buruensis* (F. Ambassidae, 9.261%), *Terapon jarbua* (F. Terapontidae, 7.562%), *L. subviridis* (F. Mugilidae, 7.385%) and *Pseudogobius javanicus* (F. Gobiidae, 7.385%), respectively. Result of two-way Anova (p=0.05) suggest that area with stilt roots (R) which dominated by mangrove *Rhizophora* have relatively higher abundance and species richness of juvenile and adult small fish. Meanwhile, average of Shannon-Weaner diversity index (H') value in R area is 2.031 or highest among the other sampling sites.

Keywords: diversity, juvenile fish, small fish, mangrove root, Labuhan coastal area

1. Introduction

Mangrove is a special coastal transition ecosystem occurred only in tropics and some subtropics region [1] and generally restricted to the tidal zone [2]. Mangroves are mostly characterized by several structural aspects such as complexity of roots with special functions [3] and canopy composed by branches and leaves. Because of their nature and position in the boundary of land and sea, mangroves becoming habitat for terrestrial and aquatic organism [1][4]; including shelter for aquatic organism from their predator; as well as providing suitable microhabitat and plentiful supply of food [5]. Mangrove is also important habitat for fish as shelter and many habitat function, including feeding, breeding, spawning and nursery ground [4][6][7].

At least two hypotheses are proposed to explain why mangroves are very attractive for fishes. Predator refuge hypothesis stated that the structural complexity of mangrove roots provides excellent shelter from predators for juvenile and small fishes by migrating into vegetated areas of mangroves, particularly when the trees are inundated by water [5][8][9][10]. Feeding hypothesis explained that there is a greater abundance of food within mangroves due to high productivity and the abundance of associated benthic fauna [9][11].

Most of quantitative studies about juvenile and small fish were focused only to compare the community structure in mangrove and non-mangrove areas, for example are study of [11], [12], and [13]; while information on comparison of juvenile fish and small fish in mangrove with different types of roots is still lacking and difficult to obtain.

This study aimed to fill the gap in the information on diversity of juvenile and small fish in mangrove with different types of roots, especially in coastal area of

Labuhan, Sepulu, Bangkalan – Madura. The mangrove in Labuhan is projected as mangrove conservation and education area. More than 12 species of true mangrove and 25 species of associate mangrove are existed [14] and creating horizontal zonation that could be easily recognized by species difference; each species with different root types [14].

Data obtained from this study could be used as baseline for mangrove management in the area, especially as a consideration in determining which species of mangrove with better habitat function to be planted for rehabilitation efforts.

2. Materials and Methods

Study Sites

Four sampling sites or stations representing vegetated and non-vegetated area were established, as shown in Fig.1. The R station dominated by *Rhizophora* spp with stilt root, S station dominated by *Sonneratia alba* with pneumatophore while C station with mixed species (including *Rhizophora*, *Sonneratia*, *Avicennia* and others) and mixed root type (stilt root and pneumatophore). Non-vegetated area represented by T location.

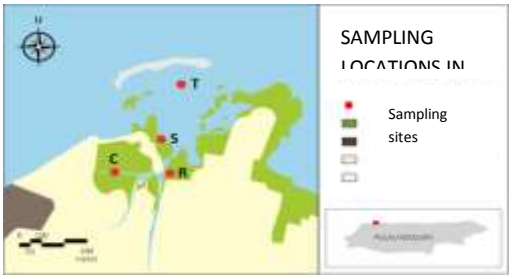

Fig.1 Juvenile and small fish sampling locations in coastal area of Labuhan, Sepulu, Bangkalan – Madura.

Specimen Collection and Processing

Sample collections were conducted monthly from November 2016 to June 2017. The specimens collected by a modified centipede net (200x60x40 cm in dimension, 0.5 cm mesh size) and scoop net (0.5 cm mesh size). The centipede net placed in small creek in the proximity of mangrove and installed at high tide and then checked at low tide (±4 hours after installation) [11]. At each sampling site, three replicate samples were obtained.

In the laboratory, juvenile and small fish samples were sorted and separated and the number of individual of each species were counted. Identification to the level of species refers to [15][16][17].

Ambient parameters measured *in situ* including salinity, water temperature, alkalinity or pH, level of dissolved oxygen (DO), turbidity and abundance of plankton (phytoplankton and zooplankton).

Plankton samples collected by plankton net with mesh size of 0.063 (for phytoplankton) and 0.150 mm (for zooplankton). The net towed subsurface in proximity of mangrove. Towing path is 100-meter-long and duplicate samples were obtained from each location [14]. Collected plankton samples immediately preserved in 10% buffered-formaldehyde for further analysis in the laboratory.

Data Analysis

Two-way analysis of variance (Anova) [11] [18], followed by a Tukey's HSD test (both at p = 0.05) were used to compare the differences in fish abundance and species richness among locations and sampling periods. Species richness represented as number of species in particular location and period; while diversity counted with Shannon-Wiener index counted as follows:

$$H' = -\sum[\frac{n_i}{n}\ln\left(\frac{n_i}{n}\right)]$$

(1)

H' is Shannon-Wiener diversity index; *ni* is individual number of abundance of species-i and *N* is total number of individu in community [19]. Value of H' then used to determine level of diversity of the community; based on standard developed by [20], as shown in Table 1.

Table 1 Criteria Of Diversity

Criteria	Value of H'
High diversity	H' > 3.00
Moderate diversity	2.00 ≤ H' ≤ 3.00
Low diversity	2.00 < H'

Source: [19][20]

3. Results and Discussion

Environmental Variables

Average value of environmental variable and quality standard for marine organism are available in Table 2.

decomposition of mangrove litter [27] or wave action [28].

Table 2 Result Of Environmental Variables Measurement

Parameter	Location				Quality standard	OT
	R	*S*	*T*	*C*		
Salinity (‰)	28 ± 7.62	27.25 ± 7.27	25.75 ± 6.7	30 ± 4.24	to 34	32.62 ± 0.61 [21]
Temperature (^{0}C)	28.5 ± 2.35	28.7 ± 2.11	28.8 ± 1.92	29 ± 2.65	28-32	29.50 ± 1.92 [21]
Turbidity (NTU)	24.75 ± 22.98	42.63 ± 27.91	26.41 ± 25.76	26.78 ± 12.59	<5	20.23 ± 33.5 [14]
Dissolved oxygen	7.88 ± 0.36	7.74 ± 0.36	7.90 ± 0.28	7.44 ± 1.34	>5	4.80 ± 1.36 [21]
pH	7.56 ± 0.92	7.74 ± 0.13	7.78 ± 0.18	7.82 ± 0.31	7-8.5	7.74 ± 0.089 [21]
Phytoplankton (cell/liter)	6015.2 ± 8653.2	4483.4 ± 3672.3	2906.1 ± 2847.5	5084.9 ± 5312	Not blooming	1564.44 ± 1042.88 [22]
Zooplankton (ind./liter)	942.7 ± 872.3	541.7 ± 577.6	343.7 ± 264.8	405.7 ± 339.6	Not blooming	1760 ± 497.8 [22]

Note:
- quality standard based on Appendix III of KepMen LH No 51 2004
- OT, compared to other author(s)

Based on the Table 2, most of environmental variables, except for turbidity, are still in range of quality standard for marine life, based on [23]. In detail, highest salinity recorded only at November and December 2016 in station R and T, with the value is 35‰. However, at January and February 2017, salinity and water temperature were lower, ±23‰ and ±26^{0}C; this is probably due to heavy rainfall prior the measurement of those variables. Most of aquatic organism in estuary composed by eurythermal and euryhaline species, therefore the changing and high range of salinity and temperature could still be tolerated [24]. In some cases, even higher salinity can still be tolerated by several species such as Mugilidae which able to withstand to salinity about 70‰ [25].

Plankton is one of food source for fish; and food availability is very important biotic factor in determining success of fish life cycle. Species composition and abundance of plankton may also affect fish production in their early life cycle [29].

Value of turbidity in all sampling location were higher than quality standard for marine organism [23]. However, several other researches stated that turbidity in estuary usually high and could still be tolerated by fish, either larvae, juvenile or adult fish [14][26]. Higher turbidity in estuary could be possibly caused by

Composition and Abundance of Juvenile and Small Fish

After eight months of sampling from four stations, we identified 32 species of juvenile and small fish from 16 families. In R station, abundance of juvenile and small fish ranging from 37 individuals in November 2016 and 197 individuals in March 2017 whereas in R station ranging from 14 individuals in November 2016 and 71 individuals in June 2017.

The abundance also lowest in November 2016 at C and T stations, however highest abundance in both stations recorded at June and February 2017, as shown in Fig.2.

The sampling area with the largest number of juvenile and small fish was R station with 932 individuals (50.437% from total population), then C station with 506 individuals (27.65%), S station with 286 individuals (15.628%), and area without mangroves (T station) with 115 individuals (6.284%),respectively.

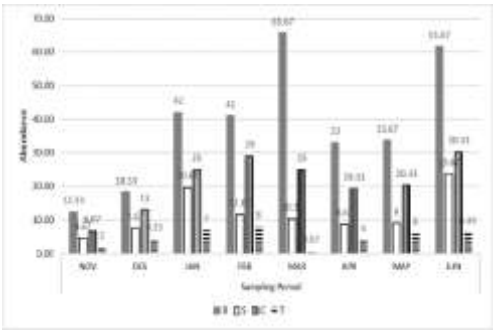

Fig.2 Average of abundance of juvenile and small fish in study area from November 2016 to June 2017

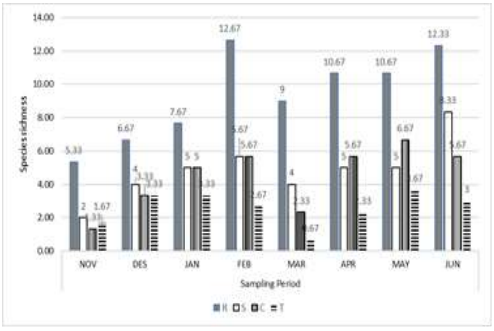

Fig.3 Average of species richness of juvenile and small fish in study area from November 2016 to June 2017

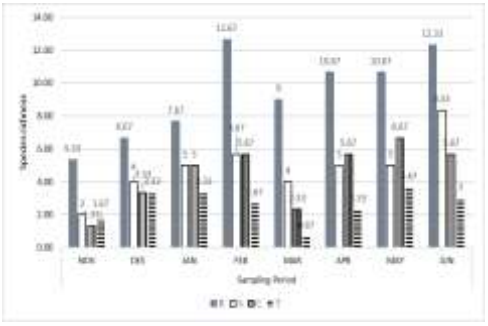

Fig. 4 Result of Two-way ANOVA followed by Tukey HSD test (p = 0.05) comparing abundance of juvenile & small fish among sampling locations; the bar with same alphabet symbol indicate no significant difference

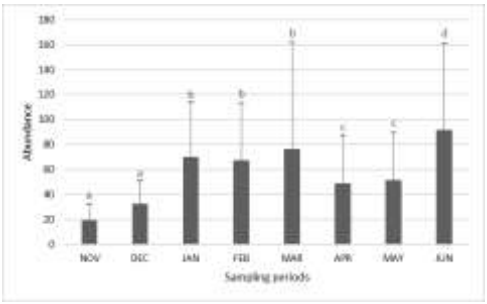

Fig. 5 Result of Two-way ANOVA followed by Tukey HSD test (p = 0.05) comparing abundance of juvenile & small fish among sampling periods; the bar with same alphabet symbol indicate no significant difference

Result of two-way ANOVA and Tukey's HSD test (both with p = 0.05) suggested that abundance of juvenile and small fish is different among location or sampling station (Fig. 4) with R station have the highest abundance. The same analysis also yields conclusion that fish abundances are grouped into four groups, in term of sampling period; namely November-December 2016, January to March 2017, April-May 2017 and June 2017 (Fig. 5).

In general, at each sampling period, the R station was always having highest abundance of juvenile and small fish, followed by C and S station; and the latter was T station. This finding is consistent with several other studies that stated that vegetated coastal habitats support higher abundance of fish compared to non-vegetated habitat [30][31][32].

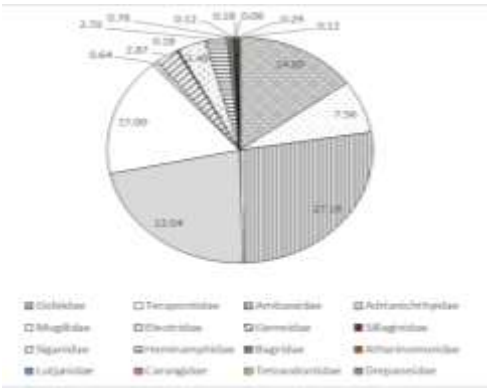

Fig.6 Percent of abundance of juvenile and small fish based on level of families in study area from November 2016 to June 2017

The most abundant species from November 2016 to June 2016 were *Oryzias javanicus* (F. Adrianichthyidae) with 376 individual or 22.039% from total juvenile and small fish population; followed by *Ambassis kopsii* (F. Ambassidae, 17.878%), *Liza vaigiensis* (F. Mugilidae, 9.613%), *Terapon jarbua* (F. Terapontidae, 7.651%) and *Pseudogobius javanicus* (F. Gobiidae) and *L. viridis*; both with 7.385%, respectively. Individuals of *O. javanicus* were mostly caught in R and C stations.

At the level of family, as depicted in Fig.6, highest abundance was belonged to Ambassidae (27.139% from total population of juvenile and small fish), Mugilidae (16.998%), Gobiidae (14.888%) and Siganidae (3.458%).

In study by [33] in mangrove and muddy area of Hongkong, Ambassidae become fish family with highest abundance to found in mangrove area. Similarly, study conducted by [34] on the diversity and abundance of juveniles and small fish in the coast of Thailand also resulted in highest abundance of Ambassidae in all habitats including seagrass beds, mangroves, sandy beaches and muddy beaches. Habitat of Ambassid species are mangroves, estuarine and freshwater in shallow water. They live in groups during the day and forage for food in the night [15] and usually prefer sheltered area [35].

In this study, Gobiidae also found in high abundance and also with highest number of species. These findings were also consistent with results of studies by [11] in Dongzhaigang Bay, Hainan Island and [34] where Gobiidae also recorded as fish family with a high number of species and diverse individual abundance within each species.

Species Richness

Lowest average of species richness in station T occurred at March 2017 and highest at May 2017. For other three stations (R, S and C), lowest species richness detected at November 2016 and highest at February, June and May 2017, respectively (Fig.3).

Fig. 7 and Fig. 8 show the result of ANOVA and Tukey's HSD test (p = 0.05) for species richness among sampling stations and periods. There was significant difference among sampling stations but not for sampling periods, especially for December 2016 to June 2017.

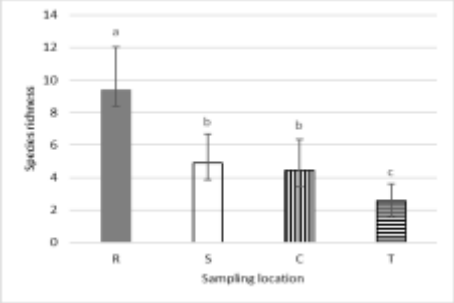

Fig. 7 Result of Two-way ANOVA followed by Tukey HSD test (p = 0.05) comparing species richness of juvenile & small fish among sampling locations; the bar with same alphabet symbol indicate no significant difference

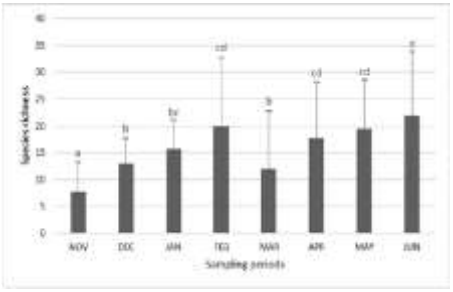

Fig. 8 Results of Two-way ANOVA followed by Tukey HSD test (p = 0.05) comparing species richness of juvenile & small fish among sampling periods; the bar with same alphabet symbol indicate no significant difference

R station also have highest species richness of juveniles and small fish compared to C, S and T station. Highest species richness from all sampling locations recorded at February 2017 and the lowest occurred at November 2016 and March 2016.

Different sampling periods could be correlated to different seasons, where fish abundance is usually higher in the rainy season compared to dry season as reported by [36] and [37]. Rainy season is a period where the abundance of juvenile fish and zooplankton are very high, as occurred in the estuary of mangroves in tropical Australia [36]. Seasonal changes and changes in fish abundance over a given period is also possibly due to the habit of fish reproduction and changes in food availability in the estuary [38].

The function of vegetated habitats in coastal areas is as a nursery ground for juveniles [39], food sources [40][41], and as a shelter [42] for Juveniles from large fish and small adult fish (small fish).

Vegetated areas will be more beneficial to the juvenile life of fish and small fish because they able to avoid predation. The habitat complexity of the roots, branches, and leaves structures provided by mangrove vegetation can serve as a shelter for juvenile fish and small fish from predators [9]. In addition to providing shelter for juvenile and small fish, the complex root structure of mangroves can be symbiotic with epibionts that can be a source of food for small fish and small juveniles [11]. [13] stated that the abundance of epibionts in root surface from mangrove is positively correlated with the abundance of fish in the area.

Shannon-Wiener Diversity Index (H')

The value of H' in R station ranging from 1.758 (Jan.17) to 2.286 (Apr.17), in S station from 1.220 (Nov.16) to 2.074 (Jun.17); ranging from 0.199 (Nov.16) to 1.697 (Apr.17) in C station and from 0.693 (Mar.17) to 1.839 (Dec.16) in T station as shown in Fig.9 below.

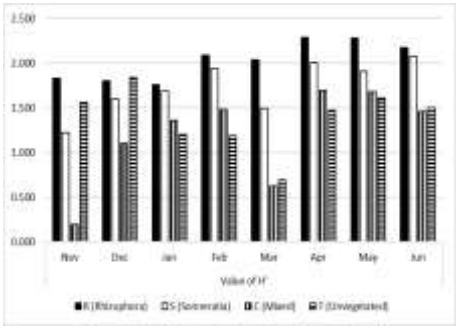

Fig. 9 Value of Shannon-Wiener Diversity Index (H') of juvenile and small fish community in study area from November 2016 to June 2017

Higher value of H' in R station is caused by highest number of species and more varied abundance among species. In addition, R station dominated by *Rhizophora* mangroves with relatively higher root complexity and a denser canopy. [8] stated that fish abundance and diversity will be higher in locations with higher density and more complex root system and denser shading.

Lower value in S and C station is possibly caused by lower number of species and dominance of one or several species present in those location [27]. For example, *O. javanicus* is quite dominant in C station; the species has averaged relative abundance of 38.199% from total population of fish in the area.

Species diversity could be used to measure the stability of community; i.e. the ability of community to be unaffected and withstand from disturbance on their species composition, therefore stable communities will have high value of species diversity [43]. In general, the value of H' of juvenile and small fish community in study area were considered low (H' <2.00) and/or moderate (2≤H'≤3).

4. Conclusion

Based on the results of the study, it can be concluded that among sampling station, the R station always have highest abundance and species richness of juvenile and small fish; which suggested to be caused by more complex root system and denser shading. R station also have greater value of Shannon-Wiener Diversity Index (H').

In general, vegetated (mangrove) sampling areas have relatively higher abundance and species richness compared to non-vegetated area.

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INBREEDING EFFECT ON VIABILITY OF *Drosophilla melanogaster* Meigen. FROM WILD TYPE

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Abstract

One of the system mating or breeding in natural is inbreeding. An inbreeding is the production of offspring from the mating of individuals or organism that are closely related genetically (through common ancestry). Negative effect of inbreeding is inbreeding depression. Inbreeding depression leads to reduce quality, viability, productivity and fitness. The aim of this study to analyze the effect of inbreeding and the type of inbreeding to viability of *Drosophila melanogaster* Meigen from wild type. The research design by using Random Design Completely with 4 treatments i.e outbreeding, and three of type inbreeding (full sib mating, half sib mating and uncle- niece mating), the treatment is 6 replication respectively. The result showed that the inbreeding lead to reduce on viability of *Drosophila melanogaster* Meigen from wild type on all stage development (larva – pupae, pupae – imago and larva – imago). While the differences of type inbreeding didn't effect to viability of *Drosophila melanogaster* Meigen from wild type.

Keyword: Inbreeding, *Drosophila melanogaster* Meigen, viability

1. Introduction

There are two system mating or breeding in natural i.e otbreeding and inbreeding. Outbreeding is mating between unrelated or distantly related individuals of a species. Outbreeding produces genetically diverse offspring that have a higher potential of adapting to new environments. Outbreeding reduces the chance of expression of deleterious recessive mutations in offspring. While inbreeding is a process of the mating of individuals or organism that are closely related genetically (through common ancestry). Inbreeding reduces the amount of variation in a population. (Suryo, 2012; Gardner and Snustad 1984).

Inbreeding effect increases the proportion of homozygotes in population and reduce proportion of heterozygotes. The high probability in homozygosity and lower in heterozygosity in population hence their offspring would be carrying deleterious recessive gene (Crow & Kimura, 1970; Gonzalo, *et al*, 2011). As a result of inbreeding the offspring have the low ability to survive and reproduce, a phenomenon called inbreeding depression. Inbreeding depression is a term used to describe the reduction in performance and viability due to the increase in inbreeding levels (reduced genetic variation). Inbreeding depression leads to reduce survival, viability, fertility, productivity and fitness of the offspring, occurs in wild animal and plant populations as well as in humans. (Charlesworth and Willis, 2009).

Based on the introduction the aim of this study want to analyze the effect of inbreeding and the type of inbreeding to viability of *Drosophila melanogaster* Meigen from wild type.

2. Materials and Methods

The research design by using Random Design Completely with 4 treatments i.e outbreeding, and three of type inbreeding (full sib mating, half sib mating and uncle-niece mating), the treatment is 6 replicate respectively. The type of mating (breeding) are :

P_0 = outbreeding (control)

P_1= uncle-niece mating

P_2= half sib mating

P_3= full sib mating

The data analysis to determine the effect of inbreeding to viability all stage development of *Drosophila melanogaster* by using Anava. Further analysis using by Duncan Multiple Range Test (DMRT) test 5% to analyze effect the mating inbreeding pattern to viability.

3. Results And Discussion

The viability percentage of inbreeding and outbreeding mating from all the development stage (larvae-pupae, pupae-imago, larvae-imago) can be shown in Table 4.1.

Mating treatment	Viability (%)		
	larva-pupae	pupae-imago	larva-imago
P_O	98,74±2,4[a]	97,15±3,0[a]	93,91±2,6[a]
P_1	97,35±1,4[ab]	90,14±1,4[b]	87,77±1,1[b]
P_2	96,30±1,4[b]	91,89±1,4[b]	88,47±1,1[b]
P_3	95,58±2,0[b]	89,23±1,7[b]	86,80±1,3[b]

Based on ANOVA test results showed that the inbreeding mating has significance effect to viability on stage of larva-pupae, pupae-imago and larva-imago of *Drosophila melanogaster* wild type. Thus indicates the inbreeding mating reduce viability of wild type *Drosophila .melanogaster* in all stage development. The viability reduced of the developmental stages of larva-pupae, pupae-imago and larva-imago caused by homozygosity increasing and reducing heterozygosity. increasing of homozygosity leads to a pressure known as inbreeding depression [Charlesworthet *al*.,2009]. According to Chiyokubo,*et al* (1998), inbreeding pressure can be seen from the characters related to the fitness of an individual such as survival, growth or development and the ability of hatching eggs.

Offspring from individuals or organism who are inbreeding mating will have percentage of abnormalities increases and low viability, growth and decreased egg production (Tave,1986). According to Gonzalo *et al* (2011), Inbreeding resulted in most of the adverse genes appearing causing smaller body size changes, decreased fertility, and strength and viability or viability will decrease. Individuals who have abnormal body tend to have low body resistance so it will be susceptible to disease and not resistant to environmental changes.

Futhermore DMRT analysis test results showed that the inbreeding mating pattern (niece- uncle, full sib and half sib mating) have no significance differences (table 4.1). Thus indicated the Inbreeding coefficient (F) didn't effect to viability. The coefficient of inbreeding is the probability that two alleles at a randomly chosen locus are identical by descent and that alleles may be identical, due probability that the alleles have come from a common ancestor.

4. Conclusion

Inbreeding mating lead to reduce on viability of *Drosophila melanogaster* Meigen from wild type on all stage development (larvae – pupae, pupae – imago and larvae – imago). While the differences of type inbreeding didn't effect to viability of *Drosophila melanogaster* Meigen from wild type.

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THE UNSATURATED FATTY ACID CONTENT IN RED KIDNEY BEANS *(Phaseolus vulgaris* L.*)* TEMPE

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Abstract

Tempe fermentation is made from red kidney beans (*Phaseolus vulgaris* L.). The fermentation process causes physical, biochemistrical and microbiological change that benefit the nutritional substance. Dry red kidney beans (*P. vulgaris*) contains important fat nutrition classified as essential linoleat (ω-6) and alfa linoleat (ω-3). This research aims to identify the type and the unsaturated fatty acids substance found in pre or post red kidney bean fermentation process (*P. vulgaris*) using RAPRIMA within 24 and 48 of hours of the fermentation process. The data analysis used in this research is qualitative data based on the result of Gas Chromatography Mass Spectrometry (GCMS) analysis. The result showed that there are two kinds of polyunsaturated fatty acids (PUFAs), namely 9,12- Octadecadienoic acid (ω-6) and 9-Octadecenoic acid (ω-9). The most favorable time of fermentation of the increased unsaturated fatty acid is at the 48th hour of fermentation process in which it increased up to 72, 53% at 9,12 Octadecadienoic acid.

Keywords: *Fermentation, GCMS®,, Phaseolus vulgaris* L., ω-6, ω-9.

1. Introduction

Tempe is one of Indonesian traditional food made by fermented soybean using mold *Rhizopus* sp. [1] According to historical record, tempe came from east Java, Indonesia [2]The fermentation process of tempe causes physical, biochemistrical, microbiological change that benefits to its nutritional content[3].

Tempe in Indonesia is generally found in many fermentation food made by nuts using *Rhizopus* sp and the most popular one is soybean [4]. Moreover, other ingredients that can be processed to produce tempe are; bogor nut, koro, green beans, peanut and red beans (*Phaseolus vulgaris* L.) [5,6]. Recently, red kidney beans tempe is widely produced as one of fungtional alternative food.

The dry red kidney bean seed (*Phaseolus. vulgaris* L.) contains an important lipid nutrition such as linoleat fatty acid (omega 6) and alfa linoleat fat (omega 3) [7]. The nutrition substance in 100 gr red kidney beans tempe is: 336 (kal) energy. Therefore, there must be an alternative to improve fungsional food through fermentation process of red kidney beans tempe (*Phaseolus vulgaris* L.) to analyze the type of unsaturated fatty acid substance classified as alfa linoleat and linoleat acid (ω-6) and the possibility of another unsaturated fatty acid formation from the fermentation process, and the improvement of its usability as the main ingredients of tempe.

This research aims to identify the type and the unsaturated fatty acid substance before and after the fermentation process of red kidney beans (*Phaseolus vulgaris*) using RAPRIMA yeast within 24 and 48-hours of fermentation. The analysis of the type and the unsaturated fatty acid substance was done through *Gas Chromatography Mass Spectrometry* (GCMS) analysis. GCMS is a powerful tool for the quantitative and qualitative analysis of a wide variety of relatively volatile compounds. The parameter of analysis is the type and the unsaturated fatty acid in red kidney beans tempe fermentation (*Phaseolus vulgaris* L.) within 24 and 48 –hours fermentation process.

2. Materials and Methods

The analysis of the type and unsaturated fatty acid contents in red kidney beans tempe (*Phaseolus vulgaris* L.) was done through several sampling processes. The first sample is using fermentation red beans

process of 500 gr red beans with 5gr inoculum RAPRIMA yeast within 24 and 48 hours of fermentation process. The red kidney beans tempe of each fermentation sample is dried at $40^0 - 45^0$C in 2 x 24 hours. Then the dried tempe is blended until become powder.

The second step is exctraction process by combaining 1 gr the powder of dried red kidney beans tempe within Erlenmeyer and then 3 ml methanol-chloroform (1:1) is added to the process to be stirred in 5 minutes. After that, 2 ml buffer Na Phosphate and 1 ml chloroform are adjoined and they are stirred for another 5 minutes. Then the disentrifuge sampling stirred within 2000 rpm speed for another 15 minutes. After disentrifuging, the sample resulted in two phases, get rid of the top part, then add 1 ml chloroform and stir it for another 5 minutes. Then, do the disentrifuge sample for the second time in 2000 rpm speed for about 2 minutes. Move the disentrifuge sample into test tube. Then steam the sample using nitrogen gass (N2), until the fatty acid traced under the test tube.

The third step is transmethylsesterification sample. The transmethylsesterification sample is done by adding 1 ml BF3 Methanol 20% to the extraction of fatty acid product and then do the incubation process on *Hot Plate* at 60ºC for about 30 minutes. Then add 1 ml of hexsana to the product of methyl ester and shake it slowly. Then take the hexana phase and steam it using nitrogen gas (N2), in order to get rid of the rest of water. Then add hexana in each sample with the same volume and analyze it using *Gas Chromatograph and Mass Spectrometer* (GCMS). The sample product of transmethylsesterification sample process tends to be volatile (easy to steam) because the fatty acid is bounded with methyl alchohol, so that it is necessary to immediately analyze it using GCMS.

GCMS is only able to read the volatile sample injected into the column. The injector temperature is already optimized. Then the sample transferred into the column until the sample is separating based on the moving phase of the column within the retention time of each different ion mollecule. The ionization method used is electron effect (EI) that causes ion mollecule to split into smaller fragments. The fragment is sorted based on the mass to form fragmentation pattern that provides important information about the ionized sample molecule structure. Based on the cromatogram peak on each spectrum, they will be analyzed by using database library in *Mass Spectrometer*.

3. Results and Discussion

The red kidney beans tempe of fermentation process in 24 and 48 hours can be seen on Figure 1.

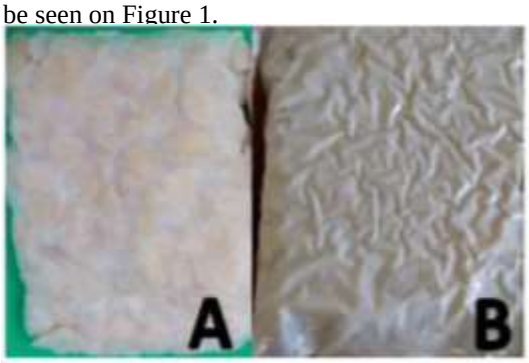

Figure 1. A) The result of red kidney beans fermentation in the 24 hours; B) The result of red kidney beans fermentation in the 48 hours

Table 1. Analysis of GC-MS fermentation of red kidney beans tempe (*Phaseolus vulgaris* L.). It displays percentage area and retention time.

Sample	Peak	Chemical name	Retention time	% Area	Similarity Index
0 hour	2	9,12-Octadecadienoic Acid	40.441	6,76	96
	3	9-Octadecenoic Acid	40.652	60,58	96
24 hours	2	9-Octadecenoic Acid	40.632	64,52	96
48 hours	2	9,12-Octadecadienoyl chloride	40.760	79,29	91

Doubled polyunsaturated fatty acid type

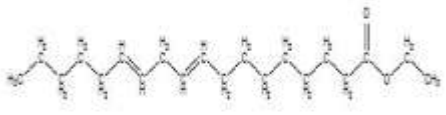

Figure 2. Chemical structure 9,12-Octadecadienoic acid

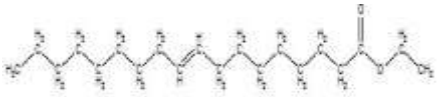

Figure 3. Chemical structure 9-Octadecenoic acid

Table 2. Unsaturated fatty acid in red kidney beans tempe (*Phaseolus vulgaris* L.)

Fatty acid type	Essential/Non Essential	0 hour	24th hour	48th hour
9-Octadecenoic acid	-	+	+	+
9,12-Octadecadienoic acid	+	+	-	+

Chromatogram result of each fermentation treatment red kidney beans (*Phaseolus vulgaris* L.), PUFAs, ω-6, ω-9.

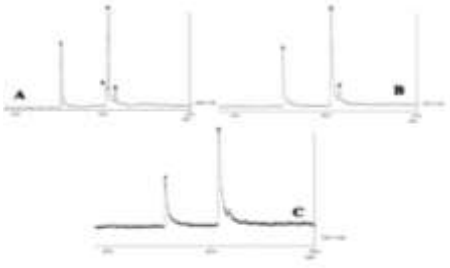

Figure 4. A) Chromatogram result of red kidney beans in 0 hour before fermentation; B) Chromatogram result of red kidney beans in the 24 hours; C). Chromatogram result of red kidney beans in the 48 hours.

4. Discussion

The red kidney beans fermentation in the 24 hours started to form white misselium over the red beans on Figure 1. The misselium formation in red kidney beans tempe on this research started to mold in the 24 hours, on the other hand the misselium of red beans forms in the 36 hours [5]. These variations is possibly caused by the different treatment on fermentation process, the steaming process is done only once before the 24 hours submersion. Mean while in this research, the steaming process is done twice, both before and after 24 hours submersion. That is based on the purpose to accelerate the fermentation process and fat acid renovation [5]. Boiling is done once so it needs more time for longer submersion, then acid smell would likely to arise [6].

The Figure 2 of the red kidney beans tempe shows better result at the 48 hours, which is shown of the emergence of misselium all over the beans. The sign of the emerging misselium identifies the final process of tempe fermentation. [7].

The Table 1 in 0 hour before fermentation shows the result of fatty acid is on the peak 2 with 40.441 minute retention time with 9,12-Octadecadienoic acid type with percentage area of 6,76, the peak 3 with 40.652 minute retention time with 9-Octadecenoic acid type, while in the 24th hour the red beans fermentation obtains unsaturated fatty acid in the 2nd peak with 40.632 minutes retention time with 9-Octadecenoic acid type in percentage area of 64,52 and in the 48th hour fermentation shows 9,12-Octadecadienoic acid type in the peak 2 of 40,760 minute retention time and percentage area of 79,29. Based on the data, each fermentation treatment has the emergence of particular kind of fat acid while another fatty acid is not appeared in which it can be seen on table 4.2.

Area percentage on the table 1 shows that doubled polyunsaturated fatty acid (PUFAs) 9,12 Octadecadienoic acid is increased up to 72,53% after the 48th hour of fermentation, the increasing of fatty acid ingredients 9,12-Octadecadienoic acid in the 48 hour fermentation was possibly caused by the rising of misselium altogether all over the beans which indicates the growth of microba that is getting better and can benefit the substrate as the energy source by changing the complex component to the simple one. Genus Rhizopus is a mold which has a high lipase activity [5]. The increasing linoleat acid of the red kidney beans fermentation process in this research is 72,53% .

The 9-Octadecenoic acid increases insignificantly after the 24 hour fermentation process for about 4%. It is possible that

during the process of the 24th hour, the misselium fermentation of red beans has not appeared yet and reached the optimum condition. The incomplete growth causes enzim production to split oleat acid which has not reached the optimum condition yet and the result of oleat acid is paltry increased.

The different number of typical fatty acid ingredient in each fermentation treatment in table 2 is probably caused by the condition in which the data does not seem to reach the peak within the analysis process so that is possible to notice small peaks (*noise peak*) to accumulate. From the unidentified compound within the accumulation of those small peaks, it is possible that it will appear in the small peaks. The condition can be identified from the chromatogram result on each fermentation treatment, the analysis compound explanation on each peak is different, it is accessible on Figure 2.

The unsaturated fatty acid types on the table 2 can be classified based on its double bond. According to Fennema (1996) the unsaturated fatty acid based on its double bond is classified into two types, they are; *Monounsaturated Fatty Acid* (MUFA) that has 1 (one double bond), and *Polyunsaturated Fatty Acids* (PUFAs) that has two or more double bond. The result of fat acid in this research is calssified as MUFA, that is 9-Octadecenoic acid, while the 9,12-Octadecadienoic acid is classified as PUFAs.

The octadecanoic acid (9,12-Octadecadienoic acid) is a fatty acid with carbon atom 19 that has 2 double bond [9]. 9- octadecenoic acid based on its formula molecule consists of $C_{19}H_{36}O_2$ with the longest double bond from carboxyl cluster in number 9 from methyl cluster so that it is classified as omega 9. Unlike 9,12-Octadecadieboic acid, the longest double bond from carboxyl cluster in number 6 from methyl cluster classified as omega 6 with typical acid of essential fat (figure 4). one of essential fat acid type is linoleat fatty acid (omega 6) [10].

5. Conclusion

From the result of GCMS analysis of unsaturated fatty acid substance of red kidney beans tempe, it was found two types of unsaturated fatty acid, they are: 9,12-Octadecadienoic acid (ω-6) and 9-Octadecenoic acid (ω-9). The optimum fermentation time for the increasing unsaturated fat acid is on the 48 hours which it increased up to 72,53% on 9,12-Octadecadienoic acid. The GCMS standard is necessary to be used in order to know the increasing of unsaturated fat acid on each types of fat acid.

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THE EFFECT OF NATRIUM METABISULPHIT ($Na_2S_2O_5$) EXPOSURED ON BALB'C MICE LIVER HISTOLOGY

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Abstract

Sodium metabisulphite ($Na_2S_2O_5$) is an additive that is often used in food processing. This compound serves as a food preservative because it can prevent browning reaction (browning) and can work as an antioxidant. Excessive and continuous use $Na_2S_2O_5$ of can have adverse health effects. Several studies have shown that sulfites and their derivatives can cause damage to some organs such as the liver, brain, lungs, lymph and stomach in rats. The aim of this research is to know the effect of sodium metabisulfite ($Na_2S_2O_5$) and the effect of dosage of sodium metabisulfite ($Na_2S_2O_5$) on the damage of liver histology structure in Balb-C mice (*Mus musculus* L.). This study used 0.21 mg/gbw, 0.42 mg/gbw and 0.63 mg/gbw of sodium metabisulphite that administered intraperitonially during 30 days. On the 31st day the liver organ were taken and made preparations slides. The conclusion of this research is sodium metabisulfite ($Na_2S_2O_5$) treatment can cause damage of hepatocyte structure in the form of vacuolization of cells, pycnosis, and necrosis in central venous and periportal area.

Keywords: natrium metabisulphit, Balb'C mice, liver.

1. Introduction

Sodium metabisulphite ($Na_2S_2O_5$) is an additive that is often used in food processing. The sodium metabisulphite compound serves as a food preservative primarily for dried food and bleach, as it prevents browning and can act as an antioxidant in foods, beverages and medicines. In the market, sodium metabisulfite is widely used as a preservative for sap, which is a sugar-making ingredient [1] and [2]. In addition sulphite is also known as an antimicrobial agent [2].

Excessive and continuous use of food additives can have adverse health effects. Several studies have shown that sulfites and their derivatives can cause DNA damage and oxidative damage to some organs such as the liver, brain, lungs, lymph and stomach in mice [3]. Metabolism of sodium metabisulfite takes place in the liver by converting sulfite into sulfate with the help of sulfite oxidase enzyme and then excreted to the kidney [4]. The sulfite oxidation process can cause damage to hepatic cells [3]. From the background that has been put forward it is necessary to do research on the effect of giving sodium metabisulfit (Na2S2O5) to the histology structure of liver mouse (Mus musculus L.) strain Balb-C.

2. Methods

The research was conducted from in Zoology Laboratory, Biology Department, Faculty of Mathematics and Natural Science, Jember University. Animals used were 8-9 week old male mice Balb'C strain, ± 25 gram weight. Mice were kept in plastic cages size of 30 x 20 x 15 cm3. Mice were fed by pellet CP511 as much as 1 per 10 gram body weight per day and drinking water ad libitum. In this study were used 24 male mice. The mice were divided into four groups. First group as a control and three other were treated with Na2SO intraperitonially at a dose of 0.21 grams; 0.42 grams and 0.63 grams given daily for 30 days respectively.

In order to do histological assesment, dissection was done in 31st days, one day after the last treatment, to take the hepar. Furthermore, the methods are used for making preparations were paraffin histology method and Haemotoxylin Eosin staining. Paraffin method is done by order of fixation, dehydration, clearing, infiltration, embedding, section, affixing and mounting.

Data were analyzed using one way ANOVA test at a level of 95 % or α = 0.05 followed by Duncan's Multiple Range Test Test (DMRT) to know a significancy difference between the treatment group dose, as well as using T test with a level of 99 % or α = 0.01, to determine the correlation between long day treatment with dose treatment.

3. Results and Discussion

Observations on hepatic histology preparations showed that sodium metabisulphite treatment resulted in damage to vacuolization, pycnotic, and necrotic cells. The average number of hepatocytes undergoing vacuolization in the central and periportal venous regions after the sodium metabisulphite treatment is shown in Table 1

From the results of anava test on the average number of hepatocytes had vacuolization both in the central venous region and periportal obtained p value = 0.00 with p <α (0.01). This shows that the administration of Na2S2O5 has a very significant effect on the average number of hepatocytes had vacuolization in the central venous and periportal region.

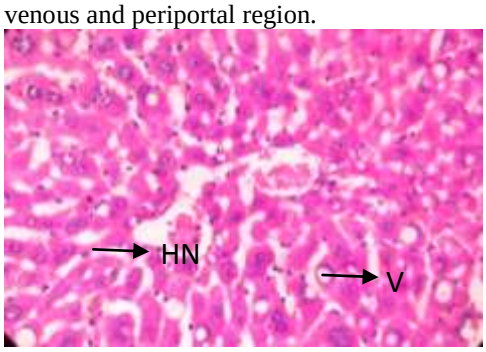

Figure 1. Transverse incission of mice liver (Mus musculus) after treatment of sodium metabisulfite 0.21 mg / g BB, 400x magnification. Notes: HN: normal hepatocyte, V: vacuolization cell.

The values show mean ± SD. Different superscript letters on the same line indicates significant differences at P value <0.01. Hepatocyte vacuolization is one type of cell damage that can be found in the liver. This damage is characterized by enlarged hepatocyte volume, vacuolization of the cytoplasm, and a visible vacuole that appears wide and clear [5] and [6]. In addition, vacuolized hepatocytes are also characterized by the presence of fatty vacuoles present in the cytoplasm, thereby pushed the cell nuclei to the edge [7]. The clear vacuole have seen on the cytoplasm. Hepatocytes undergoing vacuolization after administration of sodium metabisulfite can be seen in Figure 1.

Table 1. The average number of hepatocytes undergoing vacuolization after treatment of sodium metabisulfite.

Treatment (mg/gbw)	Cell Vacuolization (x±sd)	
	Centralis Vein	Periportal
Control	1,12±1,07[a]	0,65±0,86[a]
$Na_2S_2O_5$ 0,21	2,13±1,49[b]	2,68±1,69[b]
$Na_2S_2O_5$ 0,42	5,00±1,88[c]	2,72±2,09[b]
$Na_2S_2O_5$ 0,63	4,85±2,04[c]	4,37±2,19[c]

Vacuolization in hepatocytes occurs, due to fat globuli, especially triglycerides accumulated in the cytoplasm in large numbers. The cell vacuole is begun with lipid degeneration, which is the interference of lipid metabolism in cells [8], because of interference with mitochondrial function [6] and impaired enzymes [8]. This is supported by a statement [9] that sulfur dioxide can cause pathological changes in liver cells in mice including nuclear inflammation and mitochondrial degeneration.

Hepatocyte vacuolization can also occur due to an increase in water influx into cells and then water accumulated into the vacuole [6]. Sodium metabisulphite can increase lipid peroxidation that is known to have the ability to damage the structure and function of cell membrane [10] which results in a disruption of the cell membrane. This results in increased sodium (Na +) and calcium (Ca +) as well as reduced potassium (K +) in the cytoplasm so that water is easy to enter and to rotate the cell [11]. Vacuolization of hepatocytes is due to a disruption in the hepatocyte membrane structure that can increase the entry of water into cells.

In addition to vacuolization cells also showed that hepatocytes undergone

pycnosis. The average number of pycnotic hepatocytes in the central and periportal zone regions after the sodium metabisulphite treatment can be seen in Table 2.

Table 2. The average number of damaged hepatocytes in the form of pycnosis after treatment of sodium metabisulfite.

Treatment (mg/gbw)	Pycnosis (x±sd)	
	Centralis Vein	Periportal
Control	2,82±2,19[a]	3,18±2.11[a]
$Na_2S_2O_5$ 0,21	12,20±3,30[b]	11,43±2,98[b]
$Na_2S_2O_5$ 0,42	16,45±3,73[c]	14,05±3,88[c]
$Na_2S_2O_5$ 0,63	21,80±2,77[d]	14,27±4,55[c]

The values show mean ± SD. Different superscript letters on the same line indicates significant differences at P value <0.01. The result of the anava test on the average number of hepatocytes having pycnosis in both central and periportal venous areas obtained p = 0,00 with p <α (0,01). This shows that the administration of Na2S2O5 has a very significant effect on the average number of hepatocytes that have pycnosis in the central vein and the periportal region.

Pycnosis is an early stage of cell death (necrosis) characterization [12]. This damage is characterized by the shrinking of the cell nucleus, the cell nucleus became smaller, and appears darker [13]. This is in accordance with the results of observations that indicate that the hepatocytes that have pycnosis appear blackened and the nucleus has disfragmented. Hepatocytes undergoing pycnosis after administration of sodium metabisulfite can be seen in Figure 2.

The cause of pycnosis is related to the cause of necrosis, because pycnosis damage is an early stage of necrosis followed by a cariorecsis and cariolysis [14]. Pycnosis can occur because of damage inside the cell that begun by cell membrane damage [15]. Damage to the structure and function of cell membranes by toxic compounds is thought to be the cause of core damage. This is in accordance with research that intragastric administration of sodium metabisulfite may increase lipid peroxidation which is known to have the ability to damage the structure and function of cell membranes [2]. It cause disruption of the incoming regulation of compounds in the cell so that suspected to cause pycnosis.

In addition to vacuolization and pycnosis cells, the common hepatocyte damage is necrosis. The mean number of hepatocytes damaged in the form of necrosis in the central vein and periportal regions after the sodium metabisulphite treatment is shown in Table 3.

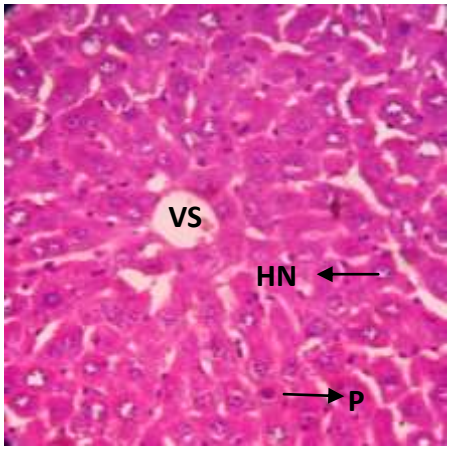

Figure 2. Transverse incission of mice liver (Mus musculus) after treatment of sodium metabisulfite 0.42 mg/gbw, 400x magnification (VS: centralis vein, HN: Normal Hepatocyte, P: Pycnotic cell)

Table 3. The mean number of damaged hepatocytes in the form of necrosis after treatment of sodium metabisulfite.

Treatment (mg/gbw)	Necrosis (x±sd)	
	Centralis Vein	Periportal
Control	2,72±2,17[a]	2,63±1,67[a]
$Na_2S_2O_5$ 0,21	10,77±3,68[b]	9,87±2,98[b]
$Na_2S_2O_5$ 0,42	20,25±3,73[c]	9,25±3,66[b]
$Na_2S_2O_5$ 0,63	20,07±4,37[c]	15,40±4,14[c]

The values show mean ± SD. Different superscript letters on the same line indicates significant differences at P value <0.01. The result of Anava test on the mean of necrotic hepatocyte both at central vein and

periportal area was obtained p = 0,00 with p <α (0,01). This shows that the administration of Na2S2O5 had a very significant effect on the average number of hepatocyte damage in the form of necrosis in the central venous region and periportal.

Necrotic of hepatocytes is the death of cells or tissues in living organisms. This hepatocyte damage is characterized by a blackened and fragmentation nucleus. In addition, hepatocytes appear to be smaller and shrunken and thus have irregular shapes [5]. This damage is also characterized by loss of metabolic function and the integrity of cell membranes [16]. This is in accordance with the results of observations that indicate that the necrotic hepatocytes are smaller and had an irregular shape.

Necrosis is caused by several factors: lack of ATP, decreased oxygen (hypoxia), and oxidative stress. Oxidative stress can increase the amount of oxygen radical that can decrease the activity or amount of antioxidant, so it can directly cause necrosis in hepatocytes [16]. Sodium metabisulfite dissolved in water will form sulfur dioxide, bisulfite ions and sulphite ions. Some of the sulfites that enter the body will be metabolized in the liver by sulfite oxidase into sulfate through the oxidation process [17]. Several studies have shown that sulfur dioxide and its derivatives are systemic toxic agents that can cause DNA damage and oxidative damage to cells, tissues, and other organs including liver [2][9].

The necrosis that resulted from the administration of sodium metabisulphite is caused by increased lipid peroxidation because it is induced by the sulfite oxidation process and the formation of sulfite radicals which can cause damage to the structure and function of cell membranes [10]. The sulphite mechanism in inducing lipid peroxidation increases occurs through the process of sulfite oxidation (SO32-) into sulfite radicals (SO3- ●). The sulphite radicals react with oxygen molecules and form peroxyl sulfite (SO3OO) and sulfate (SO4-β) radicals that can react with phospolipids in cell membranes, which can cause oxidative damage to various organs. The sulfite radicals may also react directly with the lipids, thereby forming an alkyl lipid radical (L)) [10].

Increasing the amount of oxygen radicals can decrease the amount or activity of antioxidants [16]. This is in accordance with previous research which stated that exposure to SO2 causes a decrease in the level of glutathione peroxidase and superoxide dismutase activity [9]. These two enzymes are enzymes that serve as a protective against oxidative damage by inhibiting the production of reactive oxygen metabolites [10]. Thus, hepatocyte damage in the form of necrosis is also thought to be caused by decreased activity of glutathione peroxidase and superoxide dismutase. In addition, a decrease in the amount of glutathione may cause damage to the mitochondria, which may lead to a decrease in ATP production and result in a shortage of ATP in the cell [16]

Research showed that intragastric administration of sodium metabisulfite may cause oxidative stress and increase lipid peroxidation [2]. Lipid peroxidation is known to have the ability to damage the structure and function of cell membranes. The average number of hepatocyte damage in the form of bervakuole, pycnosis, and necrosis cells in the central venous area is higher than in the periportal area. It is thought to be due to the central venous region receiving blood from the periportal region. The blood contains low oxygen and nutrients and toxic substances from the peripheral part hepatocytes. It can cause hepatocytes in the central venous region to have hypoxia and necrotic hepatocytes number higher than the periportal area [18].

4. Conclusion

The conclusion of this study that the damage of hepatocytes in the form of vacuolization, pycnosis, or necrosis is caused by a sulfur dioxide toxic agent contained in sodium metabisulfite. The result of observation of hepatocyte radial structure structure in control showed that the arrangement of hepatocyte was still arranged radial toward the central venous compare to the treatment.

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DECOMPOSITON OF COFFEE PULP UNDER SOLID STATE FERMENTATION BY *Aspergillus* VT12

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Abstract

In coffee bean processing more than 40% coffee pulp hemicellulose wastes were produced with high C/N ratio so that difficult to decompose. An isolate *Aspergillus* VT12 can grew well and produced extracelluler enzyme during solid state fermentation on coffee pulp substrate based. The enzyme was observed actively breakdown or degrade coffee pulp substrate and released reducing sugars. The optimum activity of crude enzyme in pH 5 at 35^{o}C. At 18 hours incubation, the crude enzyme had hydrolysis eficiency 1.49 %.

Keyword: *Aspergillus* VT12, coffe pulp, solid state fermentation

1. Introduction

Coffee pulp is abundant agricultural waste in Indonesia. In every coffee processing will be produced less more 40 % coffee pulp [1]. In 2012, Indonesia produced about 336 tons coffee pulp from total capasity coffee production about 748 tons [2]. Coffee pulp has contain compounds of caffeine, tannins and polyphenol that show toxic effects on the environment [3]. In Addition, Coffee pulp also has a high C/N ratio namely less more 57.2 which cause this agricultural waste takes long time to decompose naturally [4]. So, for those reason, coffee pulp is very potentially to cause pollution. However, coffee pulp has some potensial material as well such as cellulose (18,65 %), hemicellulose 0,98 % nad lignin 12, 25 % [5].

The coffee pulp waste treatment can be helped using microorganism. Microorganism such as bacteria and fungi as a decomposer agent has some benefits, such as need little time to grow and has a cheap production cost relatively. Genus *Aspergillus* is a genus of filamenteous fungi which can decompose organic material from natural resource. *Arpesgillus* VT12 can produce selulase extarselluler enzyme to break polysaccharides chain in the CMC and TKKS substrats [6]. For this reason, need to further study about activity of *Arpesgillus* VT12 extraseluler crude enzyme to decompose polysaccharides contens in the coffee pulp.

2. Materials and Methods

Alkaline extract substrat of coffee pulp

A total of 100 grams of coffee pulp powder with water content of less than 1 % were mixed with 80 g NaOH 2 M and dissolved in 1000 ml distilled water. The resulting mixture is then homogenized using a magnetic stirrer for 24 hours. After 24 hours, the pH of the mixture was adjusted to reach 7 by adding a solution of acetic acid (CH_3COOH) little by little. After the pH to 7, followed by filtering the mixture with a filter paper to be taken filtrate. Then added 97 % ethanol with ethanol filtrate ratio is 6 :4. The resulting mixture is centrifuged and taken it pellet and dried at 50 °C.

Optimization of the production of enzymes

1 ml suspension of *Aspergillus* VT12 containing 4,2 x 10^7 spora/ml into 10 grams of coffee pulp substrate and then incubated at 30°C for 7 days and continued harvesting enzymes begin day 1 to day 7. Enzyme harvesting is done by adding 20 ml of distilled water containing 1 % NaCl and 0.1 % Sodium Azide and shaker 10 rpm for 12 hours then filtered using filter paper. The filtrate centrifuged at a speed of 800 rpm for 10 minutes [14, 15]. Supernatant was taken and tested the it activity using *Somogyi-Nelson* method.

Large -scale production of crude enzyme.

A total of 20 ml suspension of *Aspergillus* VT12 containing 4,2 x 10^7 spora/ml added into 200 grams of coffee pulp substrate, and then incubated at 30 °C corresponding optimum incubation time of enzyme production. Harvesting is done by adding 20 ml of distilled water containing 1 % NaCl and 0.1 % Sodium Azide and dishaker 10 rpm for 12 hours then filtered using filter paper. The filtrate centrifuged at a speed of 800 rpm for 10 minutes. Supernatant was taken and tested using *Somogyi – Nelson* method.

Stability and Optimum pH

PH stability test is divided into 2 test, test X and Y. A total of 500 mL mixed enzyme buffer 50 mM pH varisi (3 ; 3.5 ; 4;4.5 ; 5 ; 5.5 ; 6 ; 6 , 5 ; 7 , 7.5, 8). While the Y test, enzyme treated using sterile distilled water of 500 mL of an enzyme that is mixed using 500 mL sterile distilled water. Then, Each mixture is incubated 4 hours at 37 ° C and reducing sugar testing methods Somogy nelson using coffee pulp substrate 0.5 % and 500 ul enzyme . In the determination of the optimum pH, 500 mL of coffee pulp substrate 0.5% pH 5 60 mM were incubated at 37 ° C for 20 minutes and were divided into 2 groups: test and control. In the test group added 100 mL of crude enzyme in 50 mM buffer pH variations and incubated at 37 ° C for 2 hours. Then added 0.5 ml of reagent Somogyi. While the control group, after incubated 20 minutes then passed back incubation at 37 ° C for 2 hours. After it was added 0.5 ml of Somogyi reagent and 100 mL of crude enzyme in 50 mM buffer pH variations. Each then homogenized with vortex engine. Once homogeneous, the solution is heated in a water bath for 15 minutes. Once cool, Nelson reagent solution was added 0.5 ml and 2.5 ml of distilled water and then, mixed with vortex engine. Each of these is taken into a 1 ml eppendorf and centrifuged at a speed of 800 rpm for 10 minutes. The supernatant is taken and the reduction of sugar measured with a spectrophotometer wavelength of 500 nm as much as 2 repetitions. Reduction sugar test results are compared with standard glucose curve that had been made previously.

Stability and optimum temperature

Temperature stability test is divided into 2 test, X and Y. 500 mL to 500 mL of enzyme in buffer in 50 mM buffer pH optimum at X incubated test using predetermined temperature variation that is a temperature of 30 °C , 35 °C , 40 °C , 45 °C, 50 °C , 55 °C , 60 °C , 65 °C and 70 °C, while the Y test, 500 mL to 500 mL of enzyme in buffer in 50 mM buffer pH optimum directly tested shortly after being taken from the place storage at -20 °C. Test X performed with reducing sugar analysis using Somogyi - Nelson method using soft leather substrate coffee fruit 0.5 % and 500 ul enzyme. After reducing sugar known test through X and Y, then the specified percent of the enzyme activity by comparing the results of reducing sugar X and Y. In the determination of the optimum temperature, 500 mL of coffee pulp substrate 0.5% pH optimum of 60 mM were incubated at 37°C for 20 minutes and were divided into 2 groups: test andcontrol. In the test group added 100 mL of crude enzyme in buffer 50 mM pH optimum and incubated at a temperature variation of 30 °C – 70 °C for 2 hours. Then added 0.5 ml of reagent Somogyi. While the control group, after incubated 20 minutes then passed back to the variation of incubation temperature of 30 °C - 70 °C for 2 hours. After it was added 0.5 ml of Somogyi reagent and 100 mL of crude enzyme in 50 mM pH optimum. Each then homogenized with vortex engine. Once homogeneous, the solution is heated for 15 minutes. Once cool, Nelson reagent solution was added 0.5 ml and 2.5 ml of distilled water and then homogenized with vortex engine. Each of these is then taken into a 1 ml eppendorf and centrifuged at a speed of 800 rpm for 10 minutes. The supernatant is then taken and the reduction of sugar measured with a spectrophotometer wavelength of 500 nm as much as 2 repetitions. Reduction sugar test results are then compared with standard glucose curve that had been made previously.

Coffee pulp hydrolysis optimization by *Aspergillus* sp. VT12 extraseluler enzyme

The aim of this method to determine the highest activity of *Aspergillus* sp. VT12 extracellular enzymes in decomposing coffee pulp waste. A total of 2.5 grams of coffee pulp substrates added into 50 ml crude enzyme and added 500 ul Na Azide 1 %, incubated at 37 °C for 72 hours. Sampling for the reduction of glucose testing is done by taking 1 ml micture between substrate and enzyme at the incubation time 0,6, 12, 18, 24, 30, 36, 48, 60 and 72 hours . Each sample is tested the reducing sugar by Somogyi - Nelson method. Efficiency hydrolysis was calculated using the formula :

$$hydolysis\ efficiency = \frac{\sum reduction\ sugars\ was\ produced}{\sum polysaccharides\ substrats} x\ 100\ \%$$

3. Results

Optimization of *Aspergillus* sp. VT12 extraseluler enzyme production

Enzyme prodution time was known at 4-5 day with reduction sugar less more 1, 122 ug/ml and 1, 127 um/ml (figure 1).

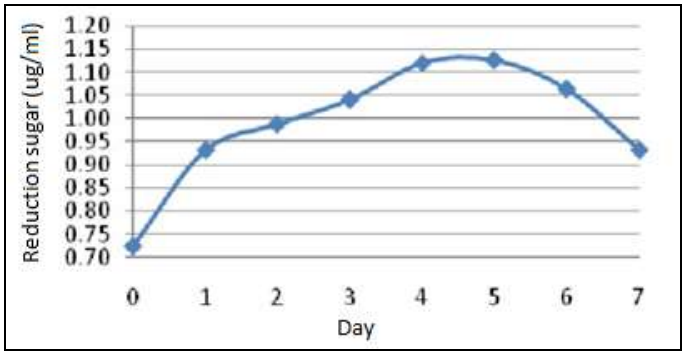

Figure 1. Curve of enzyme production time

[7] said that high relative activity of *Asperigillus sp.* selulase enzyme was obtained after stationary phase at 4 day fermentation . The relative activity decrease at 6nd day to 7nd day, this is happen because *Aspergillus* sp. VT 12 use a simple carbon source from the break of polysaccharides chain in the coffe pulp. [8] in his research on the influence of several carbon sources on the growth of fungi, said fungi will tend to utilize a simpler carbon source first.

Stability and Optimum of pH

Crude extaselluler enzyme of *Aspergillus* sp. VT 12 has a stable activity at range pH 3,5 -7 with % activity less more 85-95 % and the optimum ph was obtained at 5 with 1,2 ug/ml reduction sugars (figure 2).

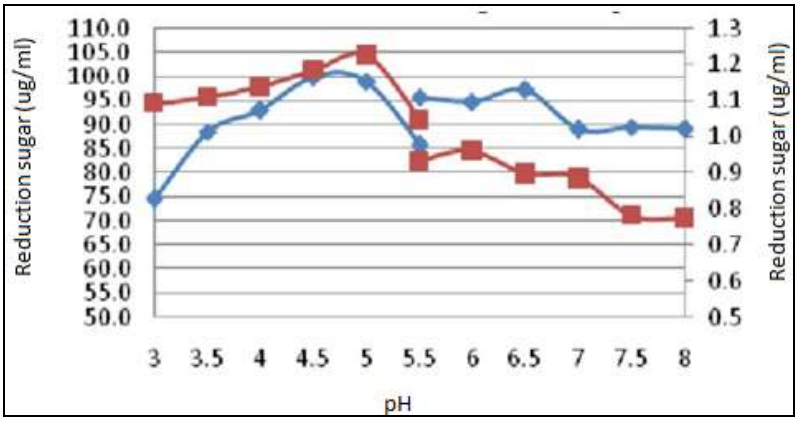

Figure 2. Curve of stability and optimum pH

The enzyme has a structure wich very sensitive to changes in pH. Too acidic pH or too alkaline will cause ionization of the active site of enzymes that will reduce or even inhibit the enzyme activity. Enzyme activity increases concurrently increase in the pH of the environment until it reaches its maximum activity or pH optimum, then fell back when the pH the more alkaline environment [9].

Stability and Optimum of Temperature

Based on the analysis of the *Aspergillus* VT 12 extraselluler enzyme, known that stabilityof temperature at range between 30^{o}C-50 ^{o}C and the optimal of temperature at 35 ^{o}C with 0,9 ug/ml reduction sugars (figure 3).

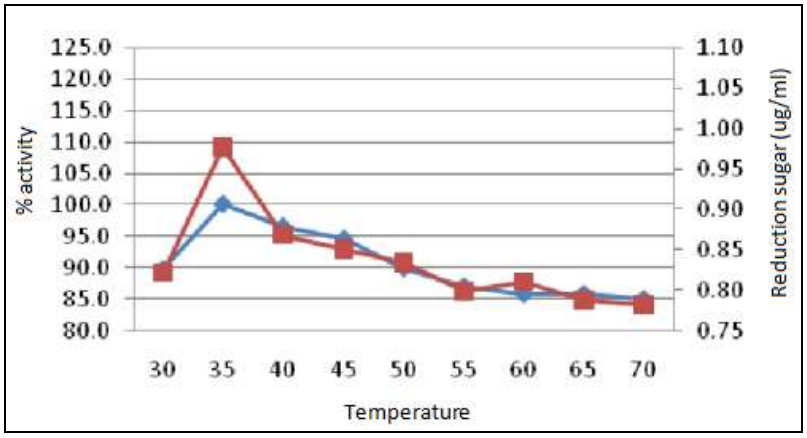

Figure 3. Curve of stability and optimum temperature

The enzyme basically can be a pure protein, or is the result of a combination of proteins with other chemical cluster [10]. Such as proteins, enzymes also have a high sensitivity to temperature. The temperature is too low will reduce or even stop their activities, while the temperature is too high will denature or destroy most enzymes [10]. In the structure of enzymes, there are part thermosensitive easily denaturation when exposed to temperatures above an area of stability, even if any chemical reaction can be increased concurrently increase in temperature to some extent [9]. [11] said that the genus Aspergillus such as *Aspergillus niger* has a temperature range of approximately between 6 °C- 47 °C and automatically influence the temperature range in which the enzymes produced work. Based on the above crude enzyme known that *Aspergillus* sp. VT12 is belong to mesophilic species [12].

Coffee pulp hydrolysis by *Aspergillus* sp. VT12. The highest hydrolysis activity was obtained at 18 hours incubations with hydrolysis efficiency 1,49 % and less more 750 ug/ml of reduction sugars (figure 4).

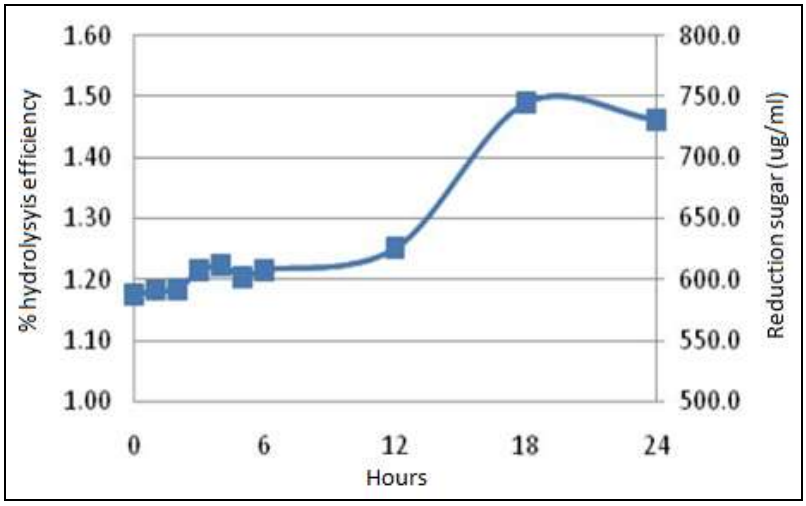

Figure 4. hydrolysis curve of coffe pulp using *Aspergillus* VT12 extracelluler enzyme

For curve above show that the relative activity increase at 3 hours and begin to decrease at 5 hours. This is happen may cause the anzyme break a simple substrat first, and then more complex substrats. This mechanism can produce a inhibitor molecul which stop its own syntesis, in this case cellulase. According to [13] mentioned that the presence of metabolites such as glucose products will inhibit the enzyme activity lignoselulolitic particularly cellulose degrading manner allosterik attached to the side so that the active enzyme can no longer occupied by the substrate.

4. Conclusion

Based on the our research was obtained that the optimum activity was obtained at pH 5 with temperature 35^{o}C and incubation time at-18. The eficiency value of hidrolysis is 1.49 %. The *Aspergillus* sp. VT12 extracelluler enzyme has stable pH between 3.5 -7 and stable temperature at range 30 ^{o}C to 50 ^{o}C.

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NEGLECTED ZOONOTIC-PARASITES ARE CIRCULATED IN RAT POPULATION IN CODE RIVERSIDE, YOGYAKARTA

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Abstract

Rats are well-known as vector more than 60 zoonotic diseases. They acts as very effective transmitter for diseases due its anthropophilic character and very adaptable with human-living. In addition, rats are top rodent in South East Asia causing pre-harvest damage of rice farming reaching 5% to 17%. The management and controls of rats challenge countries worlwide as they have lived close to human being in thousands years. Population density of rats in tropical countries especially oceanic islands is higher than continental land. Increased human demography, urban growth and contruction for living-space enhance contact between rat and human dramatically. Reports and update of rat parasite in Indonesia are little. This investigation documents parasite focused on liver with spot on expanded urban area and settlement construction adjacent to Code River, Yogyakarta, Indonesia. Rats necropsied show 42.31% are infected by *Capilaria hepatica* and 43.59% by *Cysticercus fasciolaris*. This study indicates clearly that species with potentially health risk are circulated in high rates in Code riverside Yogyakarta, Indonesia.

Keywords: rats, parasite, zoonotic

1. Introduction

Rats are well-known as vector more than 60 zoonotic diseases [1]. They acts as very effective transmitter for diseases due its anthropophilic character and very adaptable with human-living (SA 2001). In addition, rats are top rodent in South East Asia causing pre-harvest damage of rice farming reaching 5% to 17% [2]. The management and controls of rats challenge countries worlwide as they have lived close to human being in thousands years. Population density of rats in tropical countries especially oceanic islands is higher than continental land [3]. Increased human demography, urban growth and contruction for living-space enhance contact between rat and human dramatically [4]. Moreover, rat eradication is difficult due to their reproductive behaviour, fluctuation on feed and presence of natural predators if any [3]. The use of toxicants in an eradication program may unsuccesfull due to re-invaded events [5].

Several reports on parasites in rats show implications in several aspects. Previous epidemiology study reports that 17% resident in West Java were positive for *Angiostrongylus cantonensis* by serological tests [6]. Common parasites with public health aspect i.e *Hymenolopis nana*, a neglected zoonotic helminths as the most common tapeworm in children [7] and *H. diminuta* with less cases [8]. Metal accumulation on *Hymenolopis* may lead to be a sensitive indicator for pollution level of an urban area [9]. Therefore, it can be used as sensitive bioindicator in an ecosystem. Nowadays, *C. hepatica* is attracting more investigations since its complete life cycle is not yet clearly known. The progress of liver fibrosis during egg deposition during maturation is of interests and may contribute to liver fibrogenesis study [10,11]. Several hepatic capillariasis in human are reported worldwide and often misdiagnosed due to its unspecific symptoms [12]. In addition, its life cycle generates both worms and eggs are not routinely found in blood or fecal samples of presumed cases. Test by using liver biopsy is irritating and need standard medical procedure. It is assumed that only severe cases are diagnosed by liver biopsy whilst mild or initial infections are not

noticed [13]. Infection of *C. hepatica* are frequently found with *C. fasciolaris*, the larval stage of *Taenia taeniformis* [14,15]. *T. taeniformis* has been found with high numbers in cats by several reports [16-18]. Cat with rat consumption contribute to the circle of cysticercosis in these species. The transmission of diseases to human is in high risk since both of the animals, cat and rat, are closely associated with human environment.

Human cases of rat-transmitted parasites are mainly from developing countries which may correlate with the life-style. Reports and update of rat parasite in Indonesia are little. This investigation documents parasite focused on liverc with spot on expanded urban area and settlement construction adjacent to Code River Yogyakarta, Indonesia. The study give significance to the circulated parasites on rats with closely inhabited in the area under study.

2. Materials and Methods

Ethical clearance was issued from LPPT Universitas Gadjah Mada Yogyakarta.

Rat Traps

Traps were positioned approximately 200-400 m close to Code riverbanks urban area, Yogyakarta, Indonesia. Single-catch traps were used and checked every day. Captured rats were necropsied immediately in Department of Parasitology, Veterinary Medicine, Gadjah Mada University.

Parasites Identification

Cestodes were stained with aceto charmine and identified [19]. Liver sections were kept in 10% formalin for hematoxylin eosin (HE) staining. Histopathologic trimming were performed by observing abnormal lesion on organ, i.e: liver cyst, haemorrhage or presence of white spots.

3. Results

This study was performed in Code Riverbanks Yogyakarta. Parasites examinations of total 78 rats (*Rattus norvegicus*) showed 100% infections rates of various anthropozoonotic species. In this reports, we are focusing on parasite with predilection spot in the liver, i.e: *C. hepatica* and *C. fasciolaris*. Hepatic parasites i.e *C. hepatica* dan *C. fasciolaris* were found in high rates. *C hepatica* infections reachs 42.31% and *C. fasciolaris* 43.59%. Co-infection between *C hepatica* and C. *fasciolaris* occurs on 20.51% (16) rats. *C. hepatica* were found as ova on fresh liver squeeze, adult and histologic section. *C. fasciolaris* was found in dissected cystic lession on hepatic surface, stained worm and histologic section. In our observations, bigger rats showed trends of more parasitic infestation in comparison to smaller rats.

C. hepatica lesion were observed macroscopically by presence of white spots in the surface of liver. Adult nematode was difficult to be found intact by livers squeeze due its fragility. Identification is based on egg morphology that clearly show polar prominences on the fresh and stained liver. Liver of infected rats showed necrotic tissue surrounded by connective formation. Nematode eggs were placed in parenchyma with inflamatory cell infiltrations and liver fibrosis.

Cystic lesions were containing liquid and coiled larva of *T. taeniformis*. In the necropsy, the larva were found alive (moving) when dissected from cystic noduli on liver. Some livers had more than 5 cysts either embeded in parenchyma or distributed on the surface. The cysts were vary on sizes i.e: 0.5 mm-8 mm diameter. Scolex identification showed typical 2 rows of hook.

4. Discussion

Rats are wellknown as reservoir for many diseases worldwide. In the present study we found that hepatic parasitism with health risk to human are highly prevalent in Code River, Yogyakarta, Indonesia. To our best knowledge this is the first report on *C. fasciolaris* and *C. hepatica* of rats in Yogyakarta.

The rate of *C. fasciolaris* infection (43.59%) in Yogyakarta is high. *C. fasciolaris* are distributed in different types area of study, i.e: in Korea 33.8%, Mexico 7.5% [20], Philipines 100% [21], India 39.3% [15], Iran 4.34% [22], Colombia 2.4% [23] and Taiwan 31.37% [24]. It seemed that both in rural and urban areas, *C.*

fasciolaris is prevalent with significant numbers. The definitive host of *C. fasciolaris* is carnivores with higher degree in cat than dog. But it is noticed that human infection of *T. taeniformis* is occured in Srilanka, Argentina and Japan as reviewed elshewhere [2]. High degree of larval form in this investigation suggests the circulatory parasites of carnivores inhabited in Code River. Direct examination of *T. taeniformis* is not commonly performed in pet carnivores since biopsy or necropsy is not generally accepted by owners. This strategy of acquiring information is evidenced previously in *Angiostrongylus terrestris* [25].

This study also shows *C. hepatica* infection is present in high numbers in single and co-infection with *C. fasciolaris*. *C. hepatica* is reported in all over continents, such as in China [12], British [26], Colombia [23] and India [14]. *C. hepatica* colonize liver of 140 mammal species with unknown complete life cycle [27]. *C. hepatica* in human is assumed to be neglected since fecal samples is rarely shedding eggs as diagnostic tool. Although stated as rare infection, human cases are reportedly as many as 163 [27] and may also had occured in the 3rd to 4th centuries ago in France [28].

Our study indicate zoonotic helminths circulate in high frequency in the urban area adjacent to Code River, Yogyakarta. Diseases in rats is easily transmitted to human as it close association with human living especially in the urban areas with dense rats population. Current reports also showed that parasite can drive and manipulate its carrier to be close to its definitive host. This incuding change in behaviour and increase chance of intermediate host predatory as evidenced in *Pomphorhynchus laevis* infection. It is mandatory to perform survey in periodical time to briefly describe dynamic of parasite ditribution on endemic a rea. Since the tropical climate, oceanic and archipelagic condition of Indonesia lead to vary of parasitic diseases occured.

High rates of zoonotic species found in rats suggest the need of controlling parasite vector. Further research about the wider distribution, both area of investigations and species sampled, of these parasites will determine the level of health threat they present for susceptible human and domestic or pet animal populations.

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FAR FIELD METHOD FOR THE EVALUATION OF TROPICAL TUBER PROPERTIES

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Abstract

Detection of the frequency response of tropical tubers has been evaluated using the Far-Field method. The tropical tubers used as targets in this research are Porang (Amorphophallus muelleri Blume) tuber, Iles-iles (Amorphophallus variabilis) tuber, and sweet potatoes- Cileumbu. A horn reflector, appropriate to the minimum range in far field area, is designed as the guide of the electromagnetic wave. The experiments were performed by varying the distance between the horn mouth and the target material (tropical tubers). The optimum distance of 6 up to 10 cm resulted in relatively high reflected power received by the antenna. Different thicknesses of the sliced tubers influenced the reflected power. Finite Difference Time Domain model simulation was conducted for Porang tuber to examine the power and the distance for different tuber thicknesses. The results of the measurement show that Porang, Iles-iles and sweet potatoes-Cileumbu can be detected at range frequency of 1.745 - 1.88 GHz, 2.185 – 2.21 GHz, and 2.055 – 2.17 GHz, respectively. These results will give benefit for modeling the sensor system for detecting the tubers underground and in the line-production.

Keywords: far-field method, Porang tuber, range of frequency

1. Introduction

Tropical tuber, namely Porang (*Amorphophallus muelleri* Blume) is one of high-value agricultural commodities export in Indonesia. Porang tubers contain 80% to 90% of Glucomannan, which can be obtained from the root of *konjac* plants, and it can be used as dietary fiber or as gelling agents in foods, in pharmacy, and in airplane industry [1, 2]. Harvesting of the Porang tubers should be done with caution, since defect tubers (it might be injured by hoe or other equipment) could be rotten rapidly, hence, its economic values also decrease very quickly. A system that can solve the aforementioned problem would be realized to detect existing tubers under-ground. Furthermore, the possibility to detect the size of the tubers under-ground would help farmers to select the tubers during harvesting. Several plantations detection using ground penetrating radar system (GPR) has been reported [3,4]. To build the system, working frequency of the device must be selected carefully and the properties of tubers should be evaluated.

The instruments to detect the quality of agricultural products is growing rapidly such as tools for the detection of rice [5], potatoes and beets [6], watermelon [7,8], the level of ripeness of tomatoes [9], apple [10] and bananas [11,12], and the detection of moisture and dielectric constants of wheat and millet [13].

In this study, we present the measurement of frequency responses of Porang and Iles-iles (*Amorphophallus variabilis*) tubers using far-field method. Iles-iles has the same family with Porang, hence, an evaluation on its response is necessary to be able to distinguish their properties. In order to compare their frequency responses to another tuber, here we also present the measurement of sweet potatoes Cileumbu. The Cileumbu tuber is one of famous tropical tubers

originating from West Java Province, Indonesia, that can produce very sweet taste like honey after being roasted.

This research focused on the investigation of the difference of frequency responses of the tropical tubers, and it is considered as pre-research for the characterization of Porang tuber and further for the under-ground tropical tuber detection system. In addition to the far-field measurement method, a simulation using FDTD (Finite Difference Time Domain) is also presented to model the Porang tuber properties and evaluate their reflected power.

2. Materials and Methods

Far Field Method

Far-Field radiation area can be depicted in Fig. 1, its area is defined as range of R > (2D2/□) + □ from the antenna surface, where D is the antenna aperture and □ is the wavelength obtained from the working frequency. In this area the field distribution is not influenced by distance to antenna, hence, normally the antenna measurement is performed in Far-Field area.

Range of the working frequencies and maximum reflected power are examined in this study. Fig. 2 shows the measurement block diagram. USB-TG44A tracking generator having amplitude of -30 dBm to -10 dBm is used to generate signal with a frequency of 1.4 GHz up to 3.4 GHz transmitting to the antenna transmitter. The generator has three connectors, i.e. 10 MHz Ref Out, USB 2.0 and TG Sync. The 10 MHz Ref Out connector is used for 10 MHz/s signal sampling mode connected to the input of spectrum analyser, while the USB 2.0 is connected to power supply using USB cable, and TG Sync is used to synchronize the frequency between the tracking generator and the spectrum analyser.

Based on theory the radiation area at 1.4 GHz required minimum distance of 23.5 cm, and at 3.4 GHz it required minimum distance of 12.04 cm. This calculation is used to determine the reflector height. The horn reflectors for transmitter and receiver guided the electromagnetic waves. The horn reflector is designed with a height of 23 cm, using aluminium plate with the thickness of 0.5mm. Microstrip antenna used in this measurement has a frequency range of 1650 to 2700 MHz, and its gain reached the highest value of 10 dBi at 1750 MHz.

The antenna transmitter worked to transmit the RF signal generated by tracking generator into the target material (tubers). Meanwhile, the antenna receiver received the reflected RF signal from the tubers and then the results are plotted by the spectrum analyser. The optimum distance between the antennas is 13 cm. USB-SA44B spectrum analyser with a frequency range of 1 Hz to 4.4 GHz worked to measure received signal that is amplified by RF preamplifier, it is supported by USB cable as its supply, hence, the external power is not required. The spectrum analyser showed the signal path loss, i.e. the amplitudes, and RF signal frequencies received by the antenna. Distance between the mouth of horn reflector and the target material were varied, i.e. 4 cm, 8 cm, 12 cm, and 16 cm. The tubers as target measurement are sliced into approximately twice as big as the horn reflector mouth, that is 2x2 cm2.

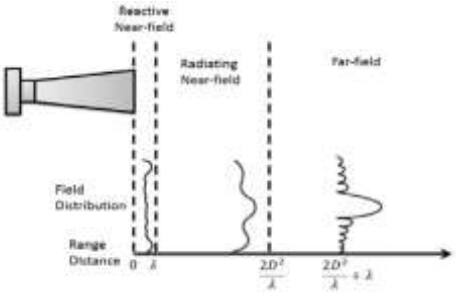

Fig. 1. Far field radiation area (redraw after [14].

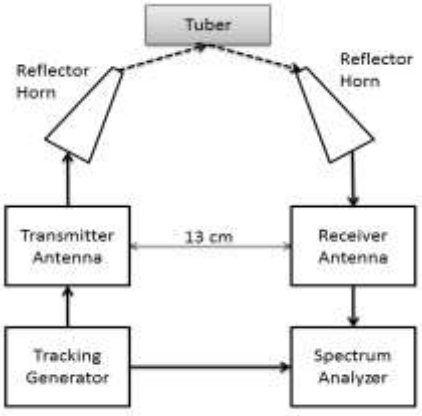

Fig. 2. Set-up measurement to detect the frequency response of tubers.

FDTD Model Simulation

Different thicknesses of Porang tuber, 10 mm to 30 mm, were simulated by FDTD model, and the reflected power is examined at distance of 20 mm to 140 mm between the reflector and the Porang tuber. The relative permittivity of Porang of 5e-12 F/m was selected, the value was obtained from the previous simulation and the measurement [15] for estimating the dielectric properties of tropical tubers.

Model simulation using FDTD used three different thicknesses of Porang tuber, i.e. 10 mm, 20 mm and 30 mm, with the size of 80 mm x 80 mm. The distance between the horn reflector and the target measurement were varied, as depicted in Fig. 3. The horn had a dimension of 40 x 80 x 230 mm^3 with an air hole. The wave source-port and the receiver- port had the same size, i.e. 10 x 10 x 1 mm^3.

3. Results and Discussion

Measurement data were taken based on path loss values measured from the receiver antenna. The first data were obtained from the path loss value during the measurement without the target, and the second data were taken from the value during the measurement with the tuber as the target. The tuber reflected the electromagnetic wave which is transmitted by transmitter, hence, the path-loss value of the transmitter antenna received by the receiver was lower than the path-loss value received without the presence of the tuber. The differences of these path loss values were evaluated as a function of frequency. The experiments were performed ten times for each selected distance, and the results were the average values of the measurement data.

Measurement results of Iles-iles tuber, with the thickness of 30 mm are shown in Fig. 4. At the distance of approximately 8 cm to 12 cm between the horn and the tuber, relatively high reflected power of 0.06 µW is obtained at 2.24 GHz. This tuber can be detected at range frequency of 2.185 GHz up to 2.25 GHz.

The Fig. 5 shows the frequency responses of sweet potatoes Cileumbu, which were sliced into 30 mm in thickness, between 2.055 GHz up to 2.17 GHz. Relatively high reflected power of 0.24 µW was obtained at 2.115 GHz measured at 8 cm distance between the horn and the Cileumbu tuber.

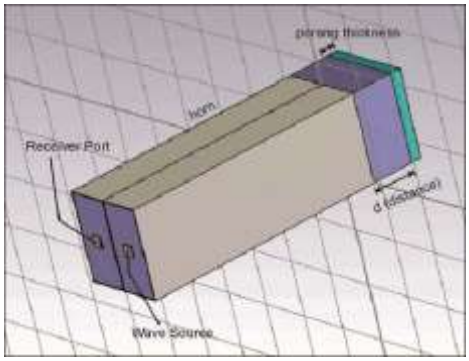

Fig. 3. FDTD model simulation for different thickness of Porang and varied distance (air) between horn and Porang (target).

Porang tubers were sliced into 10 mm and 30 mm for the experiments. Range of frequency responses of 1.745 GHz up to1.88 GHz was detected for the 10 mm tubers´ thickness, revealing a reflected power of 0.15 µW at 1.83 GHz (Fig. 6). Porang tuber with the thickness of 30 mm showed a relatively high reflected power of 0.6 µW at 1.84 GHz, within the same range of frequency.

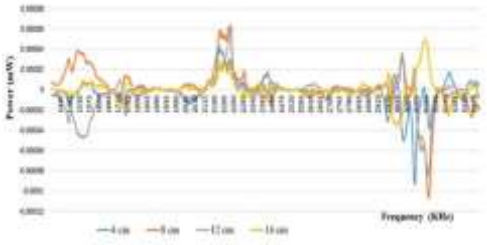

Fig. 4. Graphic of path loss difference as a function of frequency, measurement result of Iles-iles tuber with 30 mm in thickness of a figure caption.

Different thicknesses did not influence the frequency responses, however it affected the power received by the antenna. The thicker tubers resulted in the higher reflected power.

Fig. 5. Graphic of path loss difference as a function of frequency, measurement result of sweet potato Cileumbu with 30 mm in thickness.

Fig. 6. Graphic of path loss difference as a function of frequency, measurement result of Porang tuber with 10 mm in thickness.

The simulation results show at the distance of 6 cm and 8 cm for the 20 mm tuber thickness, the reflected power was $0.073x10^{-9}$ W at frequency of 1.88 GHz. Meanwhile, at the distance of 6 cm, for the thicknesses of 30 mm and of 10 mm (distance of 4 cm – 6 cm), the power of $0.15x10^{-9}$ W and $0.064x10^{-9}$ W were achieved at 1.88 GHz, respectively. Hence, thicker tuber caused higher reflected power. The simulation results also found out that for relatively thicker tubers the optimum distance measurement between the horn and the tuber was located between 6 cm up to 10 cm. The simulation was conducted by modelling a horn reflector made of aluminum, and transmitter and receiver antennas made of copper. Since the dimension of the horn and the antennas did not represent the actual equipments, the simulation did not reveal similar results as the measurement. However, range of frequency responses for the Porang tuber, and also the frequency where the reflected power reached relatively high value was quite similar, i.e. 1.83 GHz from the measurement and 1.88 GHz from the simulation.

4. Conclusion

Detection of the frequency response of tropical tubers, i.e. Porang, Iles-iles, and sweet potatoes-Cileumbu, has been performed using the far-field method. Distance of 6 cm up to 10 cm is optimum between the horn reflector, which is worked as a waveguide, and tubers as the measurement target, in order to obtain relatively high received signal power.

Range of frequency responses of 1.745 GHz up to 1.88 GHz was obtained for the Porang tuber, with the reflected power of 0.15 µW measured at 1.83 GHz. The thickness of thetubers did not affect the frequency responses, however, it influenced the power. Sweet potatoes Cileumbu had range of frequency of 2.055 GHz up to 2.17 GHz, and Iles-iles tuber showed their responses at 2.185 GHz up to 2.25 GHz. The simulation results for the Porang tuber confirmed the optimum distance between the horn and the tuber, and the influence of the Porang tuber thickness on the received power.

Different frequency responses of these tropical tubers allow us to distinguish the tubers among others. This results give benefit for further investigation on the sensor system for Porang tuber under-ground and on the line-production to select the appropriate tubers. Experiments to evaluate the quality of Porang tubers and as a comparison to the far field method is also performed and will be reported elsewhere.

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CLIMATE CHANGE IMPACT TOWARD FISHERIES CATCH IN EAST JAVA: FISH DIVERSITY IN TUBAN

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Abstract

Climatic conditions in recent years have been uncertain, the difference between the dry season and the rainy season can't be determined by the month, even the high rainfall and wind speed conditions make it difficult for fishermen to catch fish in the sea. On the other hand, the increasing demand for food, especially the nutritional requirements of animal protein also increases along with the increasing population density and the food industry. A number of studies have been conducted to determine the impact of climate change on it, but there wasn't integrated data documentation, especially in East Java. The research was conducted by taking the fish catch data by a fisherman in East Java from 2012-2014 in *Diskanla*, then climate change can be taken from *BMKG*. In addition, documentation of fish species was also conducted directly at *TPI* in several districts in Tuban. Interviews with fishermen, fish traders, and *TPI* officers were also conducted to determine the perception and knowledge of the community about climate change and its effect on the fish catch. The results showed that climate change didn't significantly influence the diversity of fish catch. Since other factors that allegedly more influential fishing methods that can damage the marine ecosystem. Although significantly unaffected, the data show fish catches in 2013-2015 tend to be stable and decrease in 2016 which in line with the results of interviews stating that the catch of fish has decreased in 2015 because of the rain throughout the year (El Nino).

Keywords: climate change, diversity, east java, fisheries

1. Introduction

Production of capture fisheries business in East Java has decreased in recent years, reaching 407,575.9 tons in 2009 [1], then decreased by 15.29% to 338,915.2 tons in 2010 [2]. This resulted in a decrease in income for fishermen [3]. On the other hand, the need for fishery resources is increasing, along with the increase of population, especially in East Java.

The decline of fish catch is one of the causes allegedly due to climate change. Climate change occurs due to changes in temperature over long periods of time. The change in temperature is caused by greenhouse gases (CO2) which make the atmosphere to hold more heat from the sun, so there is an increase in temperature in the earth. Human activities such as manufacturing, transportation and logging lead to the release of CO2 emissions into the atmosphere [4]. About 8--20% of CO2 emissions occur due to deforestation and land use change [5]; [6]. A number of studies related to the carbon cycle on Earth to reduce uncertainty in estimating the potential impact of anthropogenic CO2 emissions on Earth's climate have also been done [7]; [8].

Climate change results in rising sea surface temperatures melting of polar ice, sea level rise, rising acidity of seawater, rising tropical storms and extreme weather, seasonal shifts and increasing rainfall and probability of flooding, Water fertility [9]. The extreme weather caused the fishermen difficult to catch fish. Past research proves that climate change (rainfall, wind speed, and waves) causes changes in the volume of catches every month and changes in the number of months of fishing at fishing communities on the north coast of Semarang City [10].

Climatic conditions in recent years have been uncertain, the difference between the dry season and the rainy season can't be determined by the month, even the high

rainfall and wind speed conditions make it difficult for fishermen to catch fish in the sea. On the other hand, the increasing demand for food, especially the nutritional requirements of animal protein also increases along with the increasing population density and the food industry. A number of studies have been conducted to determine the impact of climate change on it, but there wasn't integrated data documentation, especially in East Java.

2. Methods

Study Site

The study was held in Palang Fish Auction Place in Tuban (Fig. 1), the district in northern coastal in East Java. Palang village was located between two village, Gresik Harjo and Glodok. The community activities were dominated by fisherman.

Fig.1. Study site

Data Collecting

The research was conducted by taking the fish catch data by fisherman in East Java from 2012-2014 in Marine and Fisheries Agency of East Java Province, then the temperature were collected from *BMKG*. In addition, documentation of fish species was also conducted directly at Palang Fish Auction Place (*Tempat Pelelangan Ikan, TPI)* on June-July 2017. Interviews with fishermen, fish traders, and *TPI* officers were also conducted to determine the perception and knowledge of the community about climate change and its effect on the fish catch.

3. Results

The documentation of fish species which conducted directly at Palang Fish Auction Place (*Tempat Pelelangan Ikan, TPI)* obtained 55 species on June and 25 species on July.

The secondary data which were collected from Marine and Fisheries Agency of East Java Province indicate enhancement of fishery production, number of fish species, and also average of temperature. The data summary was presented in graphic below.

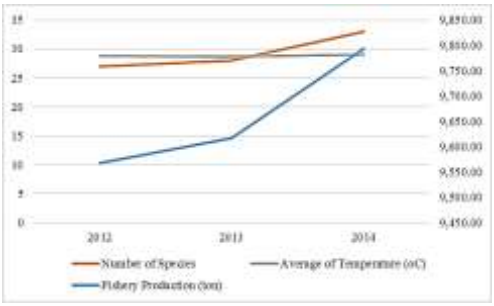

Fig. 2. Correlation of fishery production, number of fish species and temperature average in Tuban

The result of correlation analyzing data from Table II were analyzed revealed the correlation of fishery production and average of temperature was 0.82. This value explain that the temperature has moderate correlation with fishery production. I hasn't different with the correlation of number of fishes species and temperature. The value is 0.85 which temperature has moderate correlation with number of species.

4. Discussion

The results of the analyzing from secondary data reveal different result with the other research. Temperature has effect toward fishery production and the fish diversity. Enhancement of temperature was followed by fishery production and the fish diversity. It could be argued that climate change give positive impact on fisheries in Tuban.

Meanwhile, the direct survey at Palang Fish Auction Place presented higher diversity in June 2017. However the number of species when recorded in July decrease significantly. This result come about the weather condition in the study area. Depend on the direct survey in the location, showed that the wind velocity in July was stronger than in June.

Table 1. Diversity of fishes in Tuban

No	Species	Common name	Family
1	Abalistes stellaris *(Bloch & Schneider, 1801)*	Starry triggerfish	Balistidae
2	Alectis ciliaris *(Bloch, 1787)*	African pompano	Carangidae
3	*Batrachomoeus occidentalis Hutchins, 1976*	Western frogfish	Batrachoididae
4	*Carangoides malabaricus (Bloch & Schneider, 1801)*	Malabar trevally	Carangidae
5	*Carcharhinus dussumieri*	Stingray	Carcharhinidae
6	*Carcharhinus limbatus*	Stingray	Carcharhinidae
7	*Carcharhinus sealei* (Pietschmann, 1913)	Blackspot shark	Carcharhinidae
8	Chiloscyllium plagiosum (Bennet, 1830)	Whitespotted Bambooshark	
9	*Chiloscyllium griseum Müller & Henle, 1838*	Grey bambooshark	Hemiscylliidae
10	*Chirocentrus dorab*	Dorab Wolf-herring	Chirocentridae
11	*Dactyloptena peterseni*	*Starry Flying Gurnard*	Ikan walang
12	*Dactyloptena peterseni*	Starry Flying Gurnard	Dactylopteridae
13	*Diagramma pictum*	Painted Sweetlips	Haemulidae
14	*Diploprion bifasciatum Cuvier, 1828*	Barred soapfish	Serranidae
15	*Drepane punctata* (Linnaeus, 1758)	Spotted sicklefish	Drepaneidae
16	Dussumieria acuta	Rainbow sardine	
17	*Epinephelus areolatus* (Forsskål, 1775)	Areolate grouper	Serranidae
18	*Fistularia petimba* Lacepède, 1803	Red cornetfish	Fistulariidae
19	Gazza minuta *(Bloch, 1795)*	Toothpony	Leiognathidae
20	*Johnius vogleri (*Bleeker, 1853)	Little Jewflsh	Scianidae
21	*Lagocephalus inermis* (Temminck & Schlegel, 1850)	Smooth blaasop	Tetraodontidae
22	*Lagocephalus lunaris (Bloch & Schneider, 1801)*	Lunartail puffer	Tetraodontidae
23	*Leiognathus equulus* (Forsskål, 1775)	Common ponyfish	
24	*Lepidotrigla cf. japonica*	Indonesian Gurnard	Triglidae
25	*Lethrinus ornatus*	Ornate Emperor	Lethrinidae
26	*Lutjanus quinquelineatus*	Fivelined Snapper	
27	*Lutjanus sebae*	Red Emperor	Lutjanidae

28	*Lutjanus lutjanus* Bloch, 1790	Bigeye snapper	Lutjanidae
29	*Lutjanus malabaricus* (Bloch & Schneider, 1801)	Malabar blood snapper	Lutjanidae
30	Megalaspis cordyla *(Linnaeus, 1758)*	Torpedo scad	Carangidae
31	*Nematalosa come* (Richardson, 1846)	Western Pacific gizzard shad	Clupeidae
32	*Nemipterus nematophorus (Bleeker, 1854)*	Threadfin bream	Nemipteridae
33	*Netuma thalassina (*Rüppell, 1837)	Giant Sea Catfish	Ariidae
34	*Parachaetodon ocellatus (Cuvier, 1831)*	*Sixspine butterflyfish*	Chaetodontidae
35	Parastromateus niger	*Black pomfret*	
36	*Platax batavianus Cuvier, 1831*	Humpback batfish	Ephippidae
37	*Platax teira (Forsskål, 1775)*	Longfin batfish	Ephippidae
38	Polydactylus sextarius *(Bloch & Schneider, 1801)*	Blackspot threadfin	Polynemidae
39	*Pomadasys kaakan (Cuvier, 1830)*	*Javelin grunter*	Haemulidae
40	Priacanthus tayenus	*Richardson, 1846*	Priacanthidae
41	*Psettodes erumei (Bloch & Schneider, 1801)*	Indian halibut	Psettodidae
42	*Pterois russelii Bennett, 1831*	Plaintail turkeyfish	Scorpaenidae
43	Rachycentron canadum *(Linnaeus, 1766)*	*Cobia*	Rachycentridae
44	*Rastrelliger kanagurta* (Cuvier, 1816)	Indian mackerel	Scombridae
45	*Sardinella fimbriata*	*Fringescale sardinella*	
46	Selar crumenophthalmus *(Bloch, 1793)*	Bigeye scad	Carangidae
47	*Siganus javus*	Javan Rabbitfish	Siganidae
48	*Stolephorus waitei*	Spotty-face Anchovy	Engraulidae
49	Synodus sp.	*Lizard fishes*	Synodontidae
50	Terapon jarbua (Forsskål, 1775)	Jarbuaterapon	Terapontidae
51	*Upeneus japonicus (Houttuyn, 1782)*	Japanese goatfish	Mullidae
52	*Upeneus moluccensis* (Bleeker, 1855)	Goldband goatfish	Mullidae
53	Uranoscopus cognatus (Cantor, 1849)	*Two-spined yellow-tail stargazer*	Uranoscopidae
54	*Xiphocheilus typus* (Bleeker, 1856)	Blue-banded wrasse	Labridae
55	*Zebrias cancellatus* (McCulloch, 1916)	Harrowed sole	Soleidae

Belong to the results from interviewing fishermen, fish traders, and *TPI* officers, the fishery production very depend on the weather condition. It could be cause the least of number of species which caught in July.

The results showed that climate change didn't significantly influence the diversity of fish catch. Since other factors that allegedly more influential fishing methods that can damage the marine ecosystem. Although significantly unaffected, the data show fish catches in 2013-2014 tend to be stable and decrease in 2016 which in line with the results of interviews stating that the catch of fish has decreased in 2015 because of the rain throughout the year (El Nino).

Faced with decreasing populations of edible fish species and an increasing population of consumers, not only climate change which give the impacts of on marine ecosystems, but also the socio-economic impacts; the competition for "ocean space and resources; and the need for international and national cooperation, managing the world's fisheries, both wild and famed, is challenging.

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MUNG BEAN SPROUTS FLOUR (*Vigna radiate* L.) AND CORN SPROUTS FLOUR (*Zea mayz*) AS MODIFICATION OF FORMULA WHO 75 FOR MALNUTRITION CHILD WITH LACTOSE INTOLERANCE

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Abstract

Modification of Formula WHO 75 with mung bean sprouts flour and corn sprouts flour is one of alternative for children with lactose intolerance. This study aimed to analyze the effects of mung bean sprouts flour and corn sprouts flour as modification of Formula WHO 75 for malnutrition child with lactose intolerance. This study used randomized block design. The treatment was applied with the ratio of mung bean sprouts flour and corn sprouts flour were F1 (55%: 45%); F2 (50%: 50%); F3 (45%: 55%); F4 (40%: 60%); F5 (35%: 65%); and F6 (30%: 70%). The best treatment of this research was F6 (mung bean sprouts flour : corn sprouts flour is 30%: 70%). The results showed significant differences ($p < 0.05$) on osmolarity, lactose content, viscosity, bioavailability protein, colour and the appearance between Formula WHO 75 standart and formula with modified. No significant differences ($p > 0.05$) on energy content, protein, taste and flavour between Formula WHO 75 standard and formula with modified. The mung bean sprouts flour and corn sprouts flour as modification of the Formula WHO 75 can be used for the treatment of malnutrition child at stabilization phase with lactose intolerance.

Keywords: WHO 75, corn sprouts flour, mung bean sprouts flour, malnutrition

1. Introduction

Malnutrition is one of the most serious nutritional problems in Indonesia, especially in children under five years. Nationally malnutrition prevalence decreased from 5.4% in 2007 to 4.9% in 2010 but in 2013, the prevalence of malnutrition increased again to 5.7%. The achievement of Millennium Development Goal's (MDG's) in 2015 is the nationally malnutrition prevalence should be decreased by 4.1% in the period 2013 to 2015 [1].

The high prevalence of malnutrition cases can occur due to various factors such as social, economic, biological, and environmental [2]. Handling of malnutrition infant problem can be done by giving food formula which have been recommended by World Health Organization (WHO). Giving food formula according to phase of malnutrition patient that is stabilization phase given WHO 75 formula and phase transition and rehabilitation given WHO 100 formula [3]. Giving of Formula WHO 75 and 100 are influential in increasing weight in under-fives malnutrition outpatient of stabilization, transition and rehabilitation phase [4].

In the stabilization phase, food or WHO 75 Formula given should be hypoosmolar, low-lactose and low-fiber [3]. The WHO 75 formula made from skim milk is a high quality protein source. Skim milk contains lactose which can cause lactose intolerance [5]. Modified formulas with green beans can be used as an alternative modification WHO 75 Formula which is one source of vegetable protein. Protein content of green beans is 22.2% [6], and the high thiamine content in green beans can increase appetite and child weight [7]. But green beans also contain toxic substances, such as antitrypsin that inhibits the action of trypsin enzyme, hemolysin and hemagglutinin. However, these toxic agents can be removed by heating and germination [6]. Other than green beans, corn is one source of carbohydrates from cereals that have a protein content of 9.2% [6]. Vitamin A or carotenoids and vitamin E are found in corn,

especially yellow corn. Beside its function as a micro nutrient, vitamin A in the body can help the growth process, especially in children [8]. While vitamin E plays a natural antioxidant that can enhance body immunity and stimulate immune reactions. It can be useful in patients with malnutrition [7].

Both of these foods complement each other from the side of the amino acid content. Furthemore, a mixture of cereal and bean sprouts will also affect the organoleptic properties of the product, since the components contained in the different ingredients. Cereal is a source of carbohydrates while nuts are a source of protein. Amylose and amylopectin forming viscosity texture of the product [9]. So the aims of this study was to analyze the effect of the use of green bean sprouts and corn sprouts as a modified material of WHO 75 Formula for malnutritioin children with lactose intolerance.

2. Materials and Methods

Materials

Green bean seeds and corn seeds are obtained from the farmers in Kaliacar village -Gading district, Probolinggo. Others materials are skim milk, sugar, vegetable oil, mineral mix, and warm water (± 60 ° C). All analytical grade of chemical materials for analysis were procured from the Laboratory of Biosciences State Polytechnic of Jember.

Making Green Beans Sprout Flour

Soak bean sprouts for 8 hours then drain. Next put the green bean immersion on the cloth-coated tray. Then the green beans were added in a tray covered with banana leaves for 48 hours. During germination, do watering every 12 hours. The germination was placed on an aluminum tray covered with parchment paper and dried with oven at 50 ° C for 6 hours. It is then milled with a milli disc pin and sieved at 80 mesh until homogeneous flour. Further analysis of water content, ash content, protein content, fat, carbohydrates, and energy content.

Making Corn Sprout Flour

Selected corn, washed thoroughly and soaked for 3 hours for sorting and preparing water reserves. Then the tray is dialed with cotton cloth. Drain the maize yield for 3 hours then transfer it to the tray. Then the corn is left in a tray that is covered with banana leaves to get moisture. Then watered every twelve hours. The germination process was stopped after 36 hours. Corn sprouts are placed on an aluminum tray covered with parchment paper. Then dried with oven at 60 ° C for 3 hours. After that it is ground with a milli disc pin and sieved at 80 mesh until homogeneous flour. Further analysis of water content, ash content, protein content, fat, carbohydrates, and energy content.

Making Modified Formula 75

Sugar, vegetable oil and electrolyte solution (mineral mix) are mixed first until blended. Then add a little bit of green bean sprout flour and cornstarch flour according to the formulation into it (ratio of mung bean sprouts flour and corn sprouts flour were F1 (55%: 45%); F2 (50%: 50%); F3 (45%: 55%); F4 (40%: 60%); F5 (35%: 65%); and F6 (30%: 70%)), stir until gel-shaped. Add water gradually, stirring until homogeneous, reaching 1000 ml. Then boil while stirring for 2 - 7 minutes until dissolved. Serve in container (Ministry of Indonesian Health, 2011).

Analysis

Analyzes conducted on raw materials were moisture oven method (AOAC, 1995), ash content (AOAC, 1995), protein content of semi-micro kjeldhal method (AOAC, 1995), fat content of soxhlet method (AOAC, 1995), and carbohydrate (By different) (Winarno, 2008). The observational parameters for the WHO Formula 75 modification consist of protein content, osmolarity, and organoleptic properties. While the observation parameters for the best formulation consist of energy content, protein content, osmolarity, lactose content, viscosity, protein bioavailability value, and organoleptic properties.

Statistical Analysis

All the experiments were conducted in four replicateds and the mean and standard deviation were statistically analyzed using SPSS.

3. Results and Discussion

Nutritional Composition of Green Bean Sprout Flour and Corn Sprout Flour

Green bean sprout flour produced in light brown with a spesific aroma of green beans. The brown color of the flour is produced from the germination process which hydrolyses proteins and carbohydrates into simpler form which leads to an increase in the amount of protein and reducing sugars [10]. This will lead to more Maillard browning reaction. The process of drying by using the oven also causes a browning reaction [11]. The results of the analysis of nutritional composition of green bean sprout meal can be seen in Table 1.

Corn starch flour has a light yellow color and a typical aroma of corn. The yellow color is produced from the color pigment of the corn itself. The color pigment of corn (yellow corn) is a carotenoid that gives a yellow to orange red [8]. In addition, in corn there are also zeaxanthin pigments which give yellow color to flour [12]. The yield produced from the manufacture of corn starch flour is 64.60%. The results of the analysis of nutrient composition of corn starch flour can be seen in Table 1.

Table 1 shows that protein content and water content of green bean sprouts and maize sprout flour are higher than literature [13]. Fat content of green bean sprouts is lower, but fat content of corn starch sprouts is higher than other research results. This is due to the difference in germination time and steaming. The germination process will increase the levels of protein and water content. The longer the germination process the levels of protein and water content increases. In contrast to the fat content will decrease with increasing length of germination. Because in the process of germination fat is used as an energy source so that the content decreases.

Modified WHO Formula 75

- Protein

Protein is one of the most important nutrients for life processes. The results showed that the protein range in the modified WHO 75 Formula was 0.83-1.10 g / ml (Table 2). Therefore, in this study, the formula with the addition of green bean sprouts flour has higher protein content. Protein content of green bean sprouts is 30.43 gr / 100 gr, while flour protein of maize flour is 10,22 gr / 100 gr. The recommended limit for protein intake is 2 fold from the Nutritional Numbers (AKG) for protein [7]. Protein content of F1 formulation (55% green bean sprout flour and 45% corn sprout flour) and F2 (50% green bean sprouts and 50% corn sprout flour)> 10% of standard formula can be supplied with amounts appropriate to the child's needs And does not exceed 2 fold of Nutritional Needs (AKG).

- Osmolarity

Osmolarity is the concentration of particles per total volume of solvent. Osmolarity is measured by units of miliosmol (mOsm) per liter of solvent. Food osmolarity / Liquid formula about 80% of its osmolality [14]. The results showed that the osmolarity of Modified Formula WHO 75 was about 92.26-96.98 mOsm / l (Table 2).

The osmolarity will be higher with the addition of green bean sprout flour. The highest osmolarity was F1 with the ratio of 55% green bean sprout flour and 45% corn sprout flour ie 96.98 mOsm / l. The lowest osmolarity was F6 with 30% green bean sprout flour and 70% corn sprout flour which was 92.26 mOsm / l. All formulations qualify WHO 75 Formula that must be hypoosmolar evidenced by the value of osmlarity is <413 mOsm / l.

- Hedonic quality test

The hedonic quality test is an organoleptic test that expresses an impression on a product [15]. The hedonic quality is presented in Table 3. The color hedonic quality of all formulations was not significantly differrent. The average hedonic quality score scale of modified WHO Formula 75 of green bean sprout and corn sprout flour is in the range of 4.32 to 4.68 indicating a brownish to brownish yellow color. Brownish yellow to brown is the color of the main ingredient of green bean sprouts flour are light brown and corn sprout flour is yellow.

Table 1. Nutritional composition of green beans flour and corn sprout flour (in 100 grams of material)

Nutrition Component	Green Bean Sprouts Flour		Corn Sprouts Flour	
	Research	Literature *)	Research	Literature *)
Protein (%)	30,43	20,79	10,22	5,99
Fat (%)	2,51	4,98	6,86	3,65
Carbohydrat (%)	54,27	-	71,95	-
Moisture content (%)	8,67	8,16	9,42	7,45
Ash (%)	4,11	-	1,55	-

*) : Aminah dan Hersoelistyorini (2012)

Table 2 Protein content and osmolarity of modified WHO 75 formula

Formulas	Protein content (mg/ml)	Osmolarity (mOsm/l)
F1 (55% green bean sprout flour : 45% corn sprout flour)	$1,10 \pm 0,054^{c}$	$96,98 \pm 1,648^{d}$
F2 (50% green bean sprout flour : 50% corn sprout flour)	$1,08 \pm 0,004^{c}$	$96,04 \pm 1,648^{a}$
F3 (45% green bean sprout flour: 55% corn sprout flour)	$1,00 \pm 0,040^{b}$	$95,09 \pm 1,648^{d}$
F4 (40% green bean sprout flour: 60% corn sprout flour)	$0,97 \pm 0,076^{b}$	$94,15 \pm 1,648^{c}$
F5 (35% green bean sprout flour: 65% corn sprout flour)	$0,87 \pm 0,020^{a}$	$93,20 \pm 1,648^{b}$
F6 (30% green bean sprout flour: 70% corn sprout flour)	$0,83 \pm 0,029^{a}$	$92,26 \pm 1,648^{a}$

Values with the same column are not significantly different by *Duncan* test 5% (protein content) & *Mann-Whitney* 5% test (osmolarity)
Values are the means of four determinations

Table 3 Hedonic quality test of modified WHO 75 formula

Formulas	Hedonic quality test			
	Color	Taste	Aroma	Appereance
F1 (55% green bean sprout flour : 45% corn sprout flour)	4,68	4,52	3,36	$2,52^{b}$
F2 (50% green bean sprout flour : 50% corn sprout flour)	4,44	4,40	3,36	$2,24^{a}$
F3 (45% green bean sprout flour: 55% corn sprout flour)	4,44	4,40	3,32	$2,24^{a}$
F4 (40% green bean sprout flour: 60% corn sprout flour)	4,44	4,40	2,96	$2,20^{b}$
F5 (35% green bean sprout flour: 65% corn sprout flour)	4,36	4,40	2,92	$2,16^{a}$
F6 (30% green bean sprout flour: 70% corn sprout flour)	4,32	4,40	2,60	$1,48^{a}$

Values with the same column are not significantly different by *Mann-Whitney* test 5%
Color : 1 : white; 2 : yellowish white; 3 : yellow; 4 : brownish yellow; 5 : brown
Taste : 1 : very bland; 2 : rather bland; 3 : bland; 4 : sweetish; 5 : sweet
Aroma : 1 : corn aroma is strong; 2 : corn aroma is rather strong; 3 : moderate; 4 : green bean aroma is rather strong; 5 : green bean aroma is strong
Appereance : 1 : many sediment; 2 : rather a lot of sediment; 3 : moderate; 4 : slightly sediment; 5 : no sediment

Table 4 Hedonic test for modified WHO 75 formula

Formula	Hedonic			
	Color	Taste	Aroma	Appereance
F1 (55% green bean sprout flour : 45% corn sprout flour)	3,00	3,16	2,92	2,88
F2 (50% green bean sprout flour : 50% corn sprout flour)	3,00	3,20	2,92	2,88
F3 (45% green bean sprout flour: 55% corn sprout flour)	3,04	3,24	3,00	2,96
F4 (40% green bean sprout flour: 60% corn sprout flour)	3,04	3,36	3,00	3,08
F5 (35% green bean sprout flour: 65% corn sprout flour)	3,04	3,36	3,00	3,08
F6 (30% green bean sprout flour: 70% corn sprout flour)	3,08	3,44	3,12	3,20

Values with the same column are not significantly different by *Mann-Whitney* test 5%
Scale 1 : very dislike; 2 : dislike; 3 : rather like; 4 : like; skala 5 : very like

Table 5 Charateristic of modified WHO 75 formula and standart WHO 75 formula

Charateristic	Formula F6	Standart WHO 75 formula
Energy (kkal)	89,27	83,01
Protein (%)	0,83	0,87
Osmolarity (mOsm/L)	92,26*	160,10
Lactose (g/75ml filtrat)	0,09*	0,53
Viscosity (cp)	4,71*	1,39
Protein bioavaibility		
a. SAA	63,26*	78,59
b. Theoritical MC	86,49*	100,00
c. NPU (%)	54,71*	78,59
d. PST (%)	0,45*	0,70
e. PER (%)	2,37*	3,74
Organoleptic		
a. Color	4,32*	1,28
b. Taste	4,40	4,44
c. Aroma	2,60	2,88
d. Appereance	1,48*	3,72

Values with the same column are not significantly different by *T-test* 5% (energy, protein, osmolarity, lactose, viscosity, and protein bioavaibility) and *Mann-Whitney* for organoleptic tes

The hedonic quality of the taste of all formulations was not significantly different due to the the same addition of sugar in each formulation. The average hedonic quality value of taste ranges from 4.40 to 4.52, the modified WHO 75 Formula has a sweet to sweetish flavor. The sweet taste is caused by the addition of sugar in the formula making.

The hedonic quality of the aroma all the formulations formulations was not significantly different. The average hedonic quality value of aroma ranges from 2.60 to 3.36 which shows a slightly corny smell of corn typical to a slightly greenish-smelling smell of green beans. Green bean sprout flour has a more stinging smell than corn starch. This leads to formulations with a greater ratio of cornstarch (as well as F3, F4, F5, and F6), the aroma of green bean flour still feels and balances the aroma of corn.

On the hedonic quality of the appearance, the average value of scales ranging from 1.48 to 2.52 indicating that the appearance of many sediments to a lot of sediment. The presence of such precipitates indicates that the green bean sprouts andcorn sprout flour do not dissolve completely in the water. The amount of sediment in the modified WHO Formula 75 will increase with the addition of corn sprouts flour. The precipitate is related to the absorption and solubility of each flour.

The absorption capacity shows the percentage of the amount of water that can be absorbed. The absorption capacity of flour can be affected by different the time of soaking of peanut seeds. The longer the soaking time of the peanut seed before it is added, the higher its absorbency [16]. Absorption may also affect the solubility, the higher the absorption, the higher the solubility of the starch [17]. Higher absorption and solubility cause less sedimentation in the formula.

- Hedonic test

The hedonic test used to determine degree of acceptability of the products. This scale is a category-type scale with ranging from “dislike extremely” to “like extremely.” A neutral midpoint (neither like nor dislike) is included. Panelist give rate the product on the scale based on their response [15]. The result of hedonic test in each formulation can be seen in Table 4.

The average color henonic test results ranged from 3.00 to 3.08 which belonged to the rather like to likes category. This may occur because in every formulation of modified WHO Formula 75 of green bean sprouts and corn sprout flour has a brownish yellow to brown color. Color differences that are not too far away can affect the panelist's preference level. The brown color of the formula gives an unattractive impression.

Average hedonic taste test results ranged from 3.16 to 3.44, categorized as rather like to likes. This may be possible because in each formulation the modified WHO 75 Formula has the same taste that is rather sweet. Each panelist has a different preference for sweetness, there are likes sweet there are also who do not like. This affects the panelist's preference to the modified WHO 75 Formula.

The average results of the test of hedonic aroma ranged from 2.92 to 3.12 which belonged to category dislike to likes. This may occur because in every formulation of modified WHO 75 Formula has slightly scented aroma of corn to medium (the aroma of green beans and corn alike). Each panelist

is more like the smell of a typical green bean or a typical aroma of corn. It affects the panelist's favored level on the scent of the WHO Formula 75 modified green bean sprouts flour and corn sprouts flour.

The average results of henonic test appearances ranged from 2.88 to 3.20 which belonged to category dislike to likes. Each formulation has large amount of precipitate. The presence of such precipitates indicates that the green bean sprouts and corn sprout flour do not dissolve completely in the water solvent. This affects the panelist's preference level against the appearance of modified WHO 75 Formula.

Best Formula

Determination of the best treatment of modified WHO 75 Formula was done by using effectiveness index method. The best treatment result showed that the best formulatin of modified WHO 75 Formula was F6 treatment, with the percentage of green bean sprout and corn sprout flour was 30%: 70%. Characteristics of Formula F6 were then compared with the standard WHO 75 formulas presented in Table 5.

Based on the results of One Sample T-test showed that the best treatment of Formula F6 is different from standard WHO 75 formula to osmolarity, lactose, viscosity, protein bioavailability value, except energy and protein content. The content of the protein and protein of Formula F6 was not significantly different. Mann-Whitney test results showed that the oragnoleptic properties of F6 was not significantly differrent in taste and flavor but there was a significantly different on color and appearance with the standard WHO 75 formula.

The energy content of Formula F6 showd no significantly different with the standart WHO 75 Formula. Giving a formula is given in accordance with the needs and conditions of children with malnutrition. The nutritional needs of malnutrition children in the stabilization phase are 80-100 kcal / kg BW / day [3]. Malnutrition children with weight 5 - 10 kg, requires energy intake of about 400 - 1000 kcal. Formula F6 (30% green bean sprouts flour and 70% corn sprouts flour) and standard WHO 75 formulas can be given 6 to 12 times per 100 ml as needed. It is expected to fullfill energy needs and improve nutritional status of malnutrition children.

The protein content of Formula F6 showed no significantly different with standard WHO 75 Formula. Therefore Formula F6 expected to pursue the needs of malnutrition children to protein content and can replace skim milk that contains high lactose. Malnutrition children in the stabilization phase require a high protein of 1-1.5 grams / kg / day [3]. Protein requirement can be fulfilled with protein from standart WHO 75 Formula or modified WHO 75 Formula. Protein requirement of malnutrition children with weight 5 - 10 kg need protein intake of 5 - 15 gr in a day. This can be fulfilled by giving standard F6 and WHO 75 formulas as much as 6-12 times per 100 ml in each day.

Osmolarity in Formula F6 is lower than the standard WHO 75 Formula. The standard WHO 75 formula has a 413 mOsm / l osmolarity [3], but in the calculation results is 160,10 mOsm / L. The Osmolarity of Formula F6 can not be compared with osmolarity in the standard WHO 75 Formula, since the osmolarity value of F6 is only based on the calculation, not using the osmometer tool.

The lactose content in Formula F6 (0.09 mg / 75 ml filtrate) is lower than the standard WHO 75 formulas (0.53 mg / 75 ml filtrate) so it can be given to malnutrition children with lactose intolerance. Malnutrition children with lactose intolerance should be given low-lactose-containing milk (LLM / Low Lactosa Milk). The content of lactose in LLM ranges from 0 to 1 g / 100 ml [18].

The result of the viscosity analysis showed that the viscosity of Formula F6 was higher than the standard WHO 75 formula. Formula F6 needs to improve the texture of green bean sprouts and corn germ flour to make it smoother and softer, so that the resulting formula can be uniformly mixed and homogeneous. Viscosity for liquid food Commercial DM formula at RSCM is 7cp - 13,5cp. The commercial DM formula may pass through a sonde pipe [19]. The viscosity value of Formula F6 4.71cp indicates that Formula F6 can pass through the sonde pipe and can be given to malnourished children through enteral (NGT interval). The results of this viscosity test can not determine valid because Formula F6 has considerable

sediment. Therefore it is necessary to test the flow power on the NGT tube.

The result of the viscosity analysis showed that the viscosity of Formula F6 was higher than the standard WHO 75 Formula viscosity. Formula F6 needs to improve the texture of green bean sprouts flour and corn sprouts flour to make it smoother and softer, so that the formula can be homogeneous mixed. Viscosity for liquid food of commercial diabetes mellitus formula in Cipto Mangunkusumo Hospital is 7cp - 13,5cp. The commercial diabetes mellitus formula may pass through a sonde pipe [19]. The viscosity of Formula F6 4.71cp indicates that Formula F6 can pass through the sonde pipe and can be given to malnutrition children through enteral (NGT interval). The results of this viscosity test can not determine valid because Formula F6 has considerable sediment. Therefore it is necessary to test the flow power on the NGT tube.

The calculation of protein bioavailability of Formula F6 is lower than that of WHO 75 standard formula. Differences in protein bioavailability are also caused by green bean sprout flour and corn sprouts flour are a source of vegetable protein while skim milk (base material of WHO 75 standard formula) is a source of animal protein. Green bean sprout flour and corn sprouts flour will complement each other for the essential amino acids although they will not be comparable to the amino acids in skim milk. Green beans will complement the tryptophan amino acids that are not owned by corn and corn will complement the amino acid methionine in green beans. It is expected to pursue protein digestibility from skim milk.

The organoleptic properties for taste and aroma of Formula F6 showed no significantly different with the standard WHO 75 Formula, but there are significantly different in color and appearance. Formula F6 and Formula WHO 75 standards have significantly different in color, and appearance. Differences in color and appearance due to differences in the main ingredients used.

For the appearance, green bean sprouts flour and corn sprouts flour have a rougher texture than powdered skimmed milk powder so that resulting Formula F6 has many sediment. Therefore, it is necessary to improve the texture of green bean sprouts and corn sprouts flour become smoother so that the resulting precipitate is less.

4. Conclusion

The modified WHO 75 formula of green bean sprout and maize sprout flour contained a standard protein content of 0.83 gr - 1.10 gr per 100 ml, osmolarity of 92.26 mOsm/l to 96.98 mOsm/l, and rather favorable organoleptic properties. The best formulations of the modified WHO 75 Formula green bean sprouts and maize sprouts were F6 (30% green bean sprouts and 70% maize flour). There were no significant differences in energy and protein content, as well as organoleptic taste and aroma, but there were significant differences in lactose content, osmolarity, viscosity, protein bioavailability, and organoleptic properties of color and appearance between standard WHO 75 Formula with Formula F6. Formula F6 as a WHO Formula 75 modification from green bean sprouts flour and corn sprout flour can be used as a formula for the handling of a malnutrition child in stabilization phase with lactose intolerance.

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PARASITOLOGICAL AND MOLECULAR CHARACTERIZATIONS OF CHRONIC LYMPHATIC FILARIASIS PATIENTS IN PROVINCE OF ACEH, INDONESIA

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Abstract

Lymphatic filariasis (LF) is a chronic neglected tropical disease and continues to present challenge to current elimination programs. It is estimated that 1.3 billion people live in LF endemic areas with 120 million people infected worldwide. Indonesia shares the second highest number LF patients worldwide after India. Indonesia is also the only country that has all three species cause the disease; *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. There is no report regarding parasitological and molecular characterizations of chronic LF (elephantiasis) patients in Province of Aceh although the province has the second highest number of LF patients in Indonesia. In the present study, 22 patients in Province of Aceh who had developed chronic lymphedema for more than 10 years were investigated. The blood samples of these patients were also characterized based on their parasitological and molecular profiles using microscopy, immunochromatograpic card test (ICT) and polymerase chain reactions (PCR) methods. None of the patients showed positive result for microfilaraemia nor ICT. PCR also could not detect filarial DNA in the blood. These results suggest that there was no larval and adult stage worm in the patients anymore after long term infection. Nonetheless, the study provides important information regarding microfilaraemic status of lymphatic filariasis patients since the national elimination program is still undergoing in the province.

Keywords: lymphatic filariasis, parasitological and molecular characterization, diagnostic method

1. Introduction

This Lymphatic filariasis (LF) is a chronic neglected tropical disease and continues to present challenge to current elimination programs[1]. It is estimated that 1.3 billion people live in endemic areas for LF with 120 million people infected worldwide [2]. The disease is commonly found in tropical regions especially in Asia and Africa. Indonesia shares the second highest patients worldwide after India, whereas India, Indonesia and Nigeria share more 70% of total cases worldwide[1]. Province of Aceh which has only 2% of total Indonesian population shares the second highest LF patients in the country at 20% of total national cases [3].

There are three species which are known to cause LF in humans: *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*[1]. Indonesia is the only country that has all three species [4]. Although lymphatic filariasis does not lead to direct mortality of the patient, it has huge impact on economy, social and psychology [1]. Additionally, LF also causes permanent disability and reported as the second leading cause of permanent and long-term disability worldwide after trauma [5].

Diagnosis of LF is made by detecting Mf which circulate in the peripheral blood [6] although detection of circulating worm antigen by either enzyme-linked immunosorbent assay (ELISA) or a rapid-format immunochromatographic card test can be use for diagnosing bancroftian and brugian filariasis [7], [8]. Assays for detecting DNA of *W. bancrofti* and *B. timori*

by polymerase chain reaction (PCR) have been developed recently with high sensitivity and specifity [9], [10].

In 2000, WHO launched mass drug administration program (MDA) to interrupt LF transmission by delivering combination of diethylcarbamazine citrate (DEC) and Albendazole annually to entire populations in endemic area [11]. Indonesia started Mass Drug Administration (MDA) in 2001 although not all endemic district was covered while some districts in Province of Aceh started the program in 2010 including Pidie District[4]. After finishing five-round of MDA, LF endemic district should be assessed for its transmission rate by a survey named Transmission Assessment Survey (TAS). TAS examines Circulating Filarial Antigen (CFA) in first and second level of elementary school students in order to ensure no new transmission after 5 years of anti-filarial drug administration.

The study aimed to investigate parasitological and molecular characterization in human infected with *W. bancrofti* and *B. malayi* after five rounds of MDA in Province of Aceh. The information will be very helpful for TAS as TAS assess new transmission while chronic LF patients are potentially source of transmission.

2. Material and Methods

Patients Selection and Study Population

The "The study is conducted in the Province of Aceh, given the second highest prevalence of lymphatic filariasis patients in Indonesia. Specifically, the study is carried out in Pidie regency that has 91 recorded elephantiasis patients of its 443,718 total inhabitants. Pidie regency is located at approximately 110 kilometers southeast of Banda Aceh, the capital city of Province of Aceh. This cross sectional study was comprising 22 chronic lymphedema (elephantiasis) patients both male and female aged between 18-50 years who have been documented by primary health care. All study participants have signed informed consents and being interviewed for their personal biodata and medical history. Physical examination was performed on every participant by experienced medical practitioner.

Blood microfilaria, Circulating Filarial Antigen and IgG4 specific antibody examinations

Blood microfilariae load was determined by microscopy examination. The survey was conducted at night between 9-12 pm. Peripheral blood was also tested for circulating adult *W. bancrofti* antigen (CFA) using BinaxNOW® rapid test from Alere (Waltham, USA). Additionally, IgG4 antibody against *B. malayi* in the blood samples was tested using BrugiaRapid® from Reszon Diagnostics (Subang Jaya, Malaysia).

Sample Collection

As much as 3 ml of venous blood was obtained from all study participants using BD Vacutainer® Push Botton Blood Collection Needle (New Jersey, USA) and transferred into BD Heparinized Collection Tubes (New Jersey, USA). Blood sample was then stored at -20^{0}C at Faculty of Medicine Syiah Kuala University until transferring to University Malaya for PCR examination.

Extraction of total genomic DNA and Polymerase Chain Reaction (PCR)

Total genomic DNA was extracted from heparinized blood using the QIAamp DNA mini kit by Qiagen (Hilden, Germany), according to the manufacturer's instructions. The DNA was used in PCR using a previously described procedure [12], and in the PCR amplification of the ITS1 region. Primers were used are forward ITS1F 5′GGTGAACCTGCGGAA-GGATC-3′ and reverse ITS2F 5′-AGCGGGTAATCACGACTG-3′). PCR was carried out in a 25 ml reaction mixture containing 10 mM Tris HCl (pH 8·3), 2 mM MgCl2, 50 mM KCl, 0·01% gelatin, 200 mM of each deoxynucleoside triphosphate, 20 pmol of each primer, 1 U of Taq polymerase by Fermentas Life Sciences (Canada). The PCR mixture was pre-heated at 95 °C for 10 min for initial denaturation before 30 cycles of amplification, which consisted of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and elongation

at 72 °C for 2 min. Final extension of the reaction was carried out at 72 °C for 10 min.

Data Analysis

Statistical analyses were performed with GraphPad® Prism software using the Mann Whitney t-test (u test) for non-parametric data.

3. Results

Characteristics of the subject

As many as 22 elephantiasis patients were included to the study. Characteristics of the subject of this study are shown on Figure 1. According to their age, majority of the samples are geriatrics where female shares approximately a third of total subject of this study (36.3%). Moreover, some of patients have developed secondary infection on their skin as shown in Figure 1A and 1B. Interestingly, a 15-years old girl with lymphedema was also found in this study (Figure 1C).

All samples showed negative result for microfilaraemia, CFA and parasite specific IgG4.

As "Blood microfilaria in all samples were then analyzed using microscopic examination with thick blood smear. Blood collection was conducted at night (10 – 12 pm) to coincide with the appearance of the microfilariae. Thick smears were then stained with 3% of Giemsa solution in the next day and directly examined with binocular microscope. Unfortunately, no subject showed positive for microfilaremia (data not shown). To test for cilculating adult *W. bancrofti* antigen, periphery blood was then examined using rapid test directly after obtaining blood from the subjects according to manufacturer's instruction. Additionally, IgG4 antibody against *B. malayi* was also analyzed using rapid test card. However, all blood samples showed negative for both rapid test methods (Figure 2B).

Table 1. Characteristic subject of the study

No	Initial	Age (Years)	Sex
1	T	82	Female
2	M	67	Male
3	M	62	Male
4	I	80	Female
5	A	80	Male
6	H	68	Female
7	P	70	Female
8	U	65	Female
9	P	60	Female
10	A	80	Male
11	A	53	Male
12	I	72	Male
13	H	18	Female
14	H	70	Male
15	I	55	Male
16	I	70	Male
17	P	76	Male
18	U	70	Male
19	D	32	Female
20	R	61	Male
21	M	80	Male
22	S	65	Male

All samples showed negative result for microfilaraemia, CFA and parasite specific IgG4

Since adult filarial antigen examined by CFA rapid test showed negative for all sample, we then investigated worm DNA in heparized whole blood by PCR. Primers that were used are ITS1F (5'–GGTGAACCTGCGGAAGGATC-3') and ITS2F (5'–AGCGGGTAATCACGA-CTG–3') as previously described. Unfortunately, no specific band was found in gel for all

samples (Figure 3). However, we were able to see specific band in positive control.

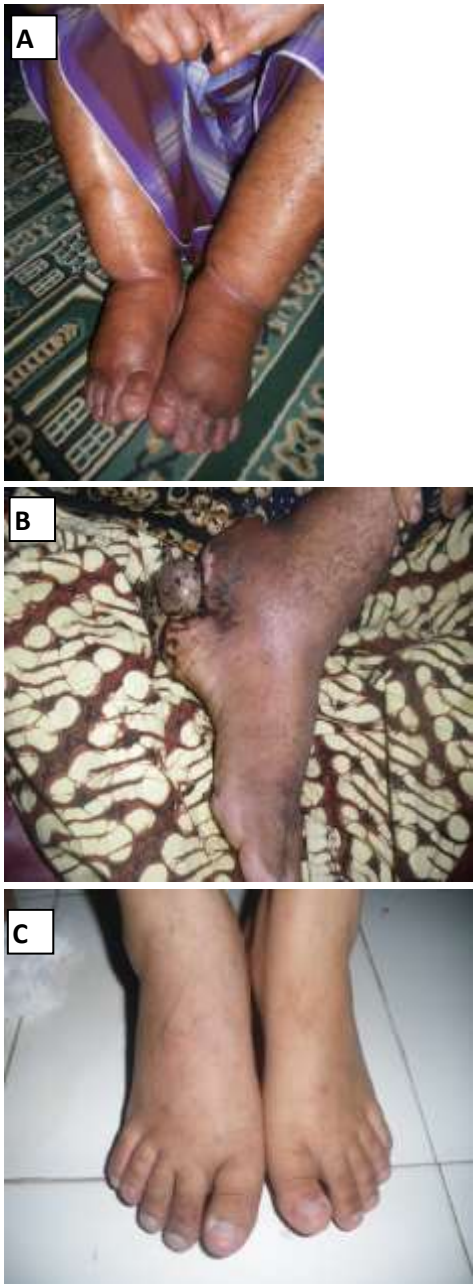

Fig. 1. Bacterial secondary infections were found in majority of patients (A and B). 15-years girl with lymphedema (C).

Fig. 2. Venous blood sample was obtained from lymphatic filariasis patients in their houses at 10 pm. until 2 am. (A) and directly analyzed for circulating antigen and antibody. Results for both rapid tests (B).

4. Discussion

We investigated 22 subjects in Delima and Padang Tiji sub districts which have highest incidence of chronic lymphatic filariasis/elephantiasis in Pidie district. The study showed that majority of elephantiasis patients are at old ages (above 50 years old) and had already developed pathology when they are at young ages. However, we also found a single young patient (below 20 years old) had developed bilateral lymphedema in her lower extremities. This case is rare and should get attention from the stakeholders in the future.

Pidie district is included one of endemic areas that are already finished 5 rounds of MDA program by WHO in 2015 where antifilarial drugs diethylcarbamazine (DEC) and albendazole were delivered to the

community in the district annually usually on November. "This study was conducted middle of 2015 after half year of last round MDA program. The district was also passed first Transmission Assessment Survey (TAS) that was conducted December 2016. TAS surveys 3,000 elementary school students in first and second grade with CFA as the main indicator. An endemic district passes the TAS if CFA positive below than 1% of total samples. Although not as variable, Mf rate is important to predict transmission rate of the disease in the district and used for mini TAS, a survey conducted before TAS. Mini TAS is a requirement for TAS.

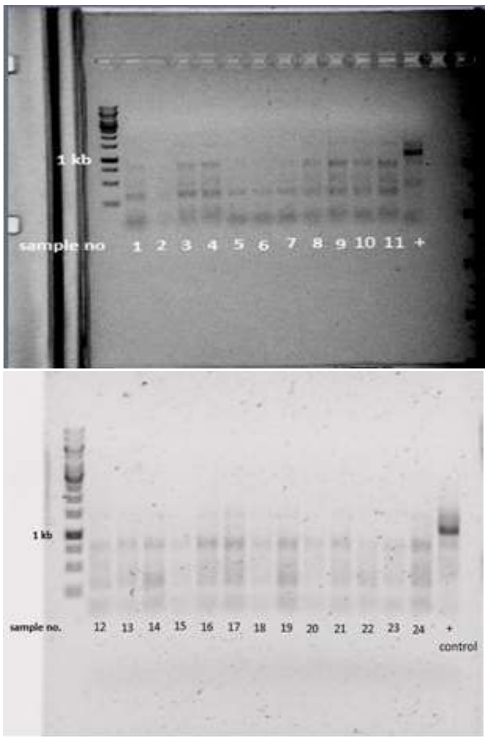

Fig. 3. Result of PCR from heparinized whole blood of 24 patients (No 23 and 24 are endemic normals) showed negative for filarial worm DNA.

However, we could not find any microfilaremia positive in all samples. The possible explanation why microfilaremia negative in all sample is probably due to last MDA drug that had been delivered few months before blood was obtained, although not all subjects took the drug as previously described. Another explanation may be due to rate of blood microfilaria were very low for some subjects that cannot be detected by microscopic examination. Accordingly, we could not detect any adult worm antigen in the blood by rapid tests. This is probably because there may be no adult filarial worm can be found in the lymphatics system of patients anymore since the infection are already last for more than 20 years. Furthermore, filarial worm eradication in the lymphatic system lead to microfilaremia negative as has been discussed previously. Therefore, PCR examination was conducted to confirm the presence of adult worm.

However, PCR showed negative result for all 22 patients as previously described. There were no specific bands shown in the sample, although 1 kb band could be seen in positive controls. These results parallel with microscopic and rapid test results that we conducted in the field previously. Maybe this because no microfilariae or adult worm antigen in the blood could be detected anymore.

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CHARACTERISTICS OF SALTED CATFISH (*Pangasius hypophthalmus*) WITH SALT CONCENTRATION VARIATIONS AND TIME OF FERMENTATION

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Abstract

Fish protein has a privilege that besides more digestible also contains amino acids had a pattern similar to the pattern of amino acids in the human body. One of the many types of fish consumed by people who are catfish (*Pangasius hypophthalmus*). Catfish easily damaged so should the effort to preserve the fish, using NaCl. The process of decay in fish can be caused mainly by the activity of enzymes found in the body of the fish itself, the activity of microorganisms, or the oxidation process in the fat body by oxygen from the air, in addition to the fungus also causes damage to the fish. The study evaluated the effect of salt concentration and time of fermentation on the characteristics of salted fish. The research method with a completely randomized design with 2 factors., the concentration of salt (20%, 30% and 40%) and time of fermentation (24, 36 and 48 hours). Analysis is conducted moisture, ash , protein content, total plate count, total mold and sensory evaluation test. Research shows that the best treatment in this study is in treatment salt content of 30% and fermentation time 24 hours. Characteristics of these treatments is 14.16% moisture content, ash content of 2.87%, 5.73% protein content and organoleptic test results saltiness 3,0, 1,60 crispness, color, 2.50 and 2.90 the overall favorite. Total plate count 1,1 x 10^5 CFU/g and total mold 7,2 x 10^2 CFU/g.

Keywords: antenna, propagation, measurement

1. Introduction

Fish has been commonly used as a source of animal protein. Fish protein has a privilege that is easy to digest and contains amino acids with a pattern similar to the pattern of amino acids in the human body. Advantages of utilizing fish meat as a protein source is high protein (20%), containing slightly woven binder so easily digested, although has high of fatty acid (0.1 - 2.2%) but 25% of this amount is an acid-unsaturated fatty acids that humans need, cholesterol levels are very low, contains a number of minerals (K, Cl, P, S, Mg, Ca, Fe, Zn, F, Ar, Cu and Y), vitamins A and D in an amount sufficient for the human body. The fish meat can be accepted by all levels of society.

Weakness of a fish's body has a high water content (80%) and pH close to neutral so it is a medium growing mold, spoilage bacteria and other microorganisms. Meat fish susceptible to autolysis so that the meat becomes soft and become a medium growth of microorganisms and mold.

The means used to preserve fish, which is often used temperature modification (canning, refrigeration and freezing), chemical means (use of salt and vinegar) and drying (natural and artificial). Salting techniques there are two types, namely dry and wet salting [1].

One of the many types of fish consumed by people who are catfish (*Pangasius hypophthalmus*). Catfish is quite easy to find on the market, so it can become an alternative source of animal protein. As with other fish products, catfish easily damaged so should the effort to preserve the fish.

In fermentation using salt, which plays a role in the decomposition of the compound is the enzyme of the fish itself, especially from the contents of the stomach and microorganisms derived from fish and salt are used. Bacteria that develop during fermentation with salt mainly from the type of *Micrococcus*, *Bacillus*, and *Sarcina* which are halotoleran nature. High salt levels in the product, capable of inhibiting pathogen bacteria Staphylococcus aureus. Salt causes the withdrawal of water from the food (fish) so Aw fish will decline and the growth of some microorganisms will be inhibited [2].

Combination of pH and salt in many fermentation products tends to inhibit the growth of microorganisms. During fermentation, pH is reduced below 5 and safe processing depends on proper salt content and temperature control and pH [3].

This study will assess the effect of salt concentration and fermentation time on the quality of salted fish (jambal) catfish, to avoid microorganism and mold growth. The concentration of salt used is 20%, 30% and 40%. Factors used fermentation time is 24, 36 and 48 hours. Salted fish products will be analyzed levels of protein, moisture content, ash content and microbiology analysis (Total plate count and total mold).

2. Methods

The design used was completely randomized design (CRD) with 2 factors. Factor 1: the concentration of salt (G1 : 20% , G2 : 30 %, G3 : 40 %). Factor 2 : time of fermentation (F1 : 24 hour, F2 : 36 hour, F3 : 48 hour) The data obtained by analysis of variance (ANOVA) with a significant level of 5%. Then, if the calculation result is no real difference followed by Tukey's test. Materials Research is catfish, salt crystals, chemicals for analysis is distilled water and chemicals for ash (AOAC, 1992), water content (AOAC, 1992) and protein analysis (Mikro Kejhldal method), microbiology analysis (Total Plate Count using Plate Count Agar + 5% NaCl and Total mold using Potatoes Dextrose Agar + 5% NaCl) and sensory evaluation (with hedonic test).

Preparation of salted fish : fish Cleaned and cut of visceral fish, Cut with a thickness of 3 cm and added crystal salt. Then Fermented for 24, 36, 48 hour and washed with fresh water. Fish dried 3-4 days, with the cabinet dryer. For sensory evaluation, fermented fish fried with hot oil for 3 minute.

3. Results and Discussion

Chemical Analysis

The water content of salted fish catfish showed a tendency to increase along with the high salt concentration and fermentation time. According [3] that the salting process will stop after a proper balance between the solution in the fish meat with a salt solution on the outside during certain salting. [4] stated that during the process of salting will happen salt penetration into the body of the fish depends on the purity of the salt used. Some important factors that influence the effectiveness of salting is salt concentration, temperature salting, the thickness of the fish meat and the level of freshness of the fish. The length of time determined by the speed of salting salt dissolves to form 'brine', the speed of the salt into the flesh of fish and attract water, the amount of salt or brine density, temperature salting, size of the fish.

Table 1. Chemical analysis

Salt concentration	Time of fermentation	Water content (%)	ash (%)	Protein (%)
20%	24	7,346 ^{a}	3,135 ^{a}	5,888 ^{cd}
	36	8,664 ^{ab}	2,733 ^{ab}	5,828 ^{cd}
	48	12,539 ^{abc}	2,361 ^{ab}	5,509 ^{d}
30%	24	14,157 ^{abc}	2,869 ^{ab}	5,729 ^{cd}
	36	10,502 ^{abc}	2,815 ^{ab}	5,338 ^{d}
	48	15,634 ^{bc}	2,206 ^{a}	5,411 ^{cd}
40%	24	17,239 ^{c}	2,349 ^{ab}	5,746 ^{cd}
	36	13,994 ^{abc}	2,619 ^{ab}	4,892 ^{b}
	48	13,738 ^{abc}	2,357 ^{ab}	4,487 ^{a}

Note: The numbers followed by the same letters show no different with DMRT test on 5% significance.

The high ash content is influenced by the raw materials used. Other materials used are crystal salt, which is quite accounted ash content in the salted fish. The content in between sea salt sodium chloride (> 80%) as well as other compounds such as MgCl, $MgSO_4$, CaCl, and others, which is a mineral [5]. The minerals in the salt contributes to high levels of ash in the salted fish pangasius.

According Rahmani [6] administration of salt causes increase of mineral salts

(especially sodium) in the flesh of the fish so that the ash content is also increasing. Average ash content tends increase with increasing soaking time. This is due to osmosis and if prolonged lead minerals (especially sodium) each person into the flesh of the fish.

The protein content of salted fish catfish tend to decrease with the increase of salinity and fermentation time. The highest protein content in this study is in treatment salt content of 20% and fermentation time 24 hours is 5.888%. According Putri [5] NaCl can interact with proteins. At low concentrations causes the protein to undergo salting in and on the concentration of high protein salting out. In the salting process, will be more soluble protein, in contrast to the events of salting out proteins will settle and not easily soluble. Average protein content of salted fish catfish tends to decrease with increasing soaking time and salinity. This is due to salting in the process so that the protein will be more soluble.

Kurniawan [7] research on the effect of the concentration of the salt solution and the time of fermentation on the quality of fish sauce catfish, showed that the concentration of salt solution 3% protein content which is greater than the concentration of salt solution of 5% and 9%. This may occur due to inhibition of enzyme activity of protease (bromelin enzyme) at concentrations higher salt solution so that the amount of protein resolved into amino acids decreased.

Total Plate Count

The total bacteria in the fermented catfish decrease as the higher concentrations of salt were added. Decrease in the viable count of total bacteria from 10^5 to 10^2 CFU/g. This result is in agreement with Bakhiet et al. [8] who reported that total bacterial count decrease, may be due to the presence of high salt concentration in fessiekh so the pathogenic microorganism growth is controlled.

Pathogenic bacteria most often associated with the living environment of fish, are *Vibrio parahaemolyticus* and *Clostridium botulinum.* Combination of pH and salt in many fermentation products tends to inhibit the growth of microorganisms. During fermentation, pH is reduced below 5 and safe processing depends on proper salt content and temperature control and pH [3].

Tabel 2. Total Plate Count (CFU/g)

Salt concentration (%)	Time of fermentation (hour) 24	36	48
20	1.4×10^3	1.0×10^3	4.2×10^3
30	1.1×10^5	3.2×10^4	2.0×10^3
40	9.4×10^4	8.3×10^3	7.8×10^2

Total Mold Count

The mold in the fermented catfish increase as the higher concentrations of salt were added. Increase in the viable count of total mold from 10^2 to 10^5 CFU/g.

Tabel 3. Total mold Count (CFU/g)

Salt concentration (%)	Time of fermentation (hour) 24	36	48
20	4.2×10^2	8.3×10^3	9.4×10^4
30	7.8×10^2	3.2×10^4	1.4×10^5
40	2.0×10^3	1.0×10^5	1.1×10^5

Microbial action has been known to play a large in the spoilage of fish. Bacterial spoilage is characterized by softening of the muscle tissue and the production of slime and offensive odours. Fungi generally prefer substrate with low water activity and usually vary in dry samples. The water content in dried product is low and in favour spore-former fungi as a result spreading of spores by air since fish is exposed to ambient atmosphere [9].

Sensory Evaluation

Salty Taste Test Appearance Results analysis, saltiness in salted catfish showed no significant difference. All panelists assess salted fish products catfish tasted salty. The salty taste of the highest in the judgment of the panel is in

treatment salt content of 20% and fermentation time 24 hours, which is 3,80.

Results of analysis variance of crispyness showed that no significant between treatments. The panelists rate the less crunchy salted fish catfish. Crispness is also demonstrated by the high water content of salted fish in this study were higher than the ISO standard. The highest value of crispness on salted fish catfish is on the treatment of 20% salt content and fermentation time 24 hours is 2.20. This is consistent with the results of the analysis of water content in this study, in which the same treatment, the water content showed the lowest score.

Tabel 4. Sensory Evaluation

Salt concentration	Time of fermentation (hour)	Taste	crispnes	color	Overall Appearance
20%	24	3,80[a]	2,20[a]	3,10[b]	2,30[b]
	36	3,20[a]	2,00[a]	1,50[a]	2,80[a]
	48	3,60[a]	2,10[a]	2,50[b]	2,90[a]
30%	24	3,00[a]	1,60[a]	2,50[b]	2,90[a]
	36	3,50[a]	2,10[a]	1,40[a]	2,50[a]
	48	3,50[a]	1,60[a]	2,80[b]	2,80[a]
40%	24	2,90[a]	2,10[a]	2,00[a]	2,70[a]
	36	3,30[a]	2,10[a]	1,80[a]	2,10[a]
	48	3,30[a]	1,80[a]	2,10[a]	2,20[a]

Note: The numbers followed by the same letters show no different with DMRT test on 5% significance.

Results of analysis variance of color showed that no significant between treatments. The unstable panelist ratings for each treatment. The higher the value indicates the color of dried fish catfish increasingly dark brown. According Rahmani et al [6] a salt containing Fe and Cu compounds can lead anchovies dirty brown or yellow.The salted fish catfish, before being tested by the panelists, salted fish frying, the frying process is not uniform can cause inconsistent ratings panelists. Color of salted catfish in the judgment of the panel is in treatment salt content of 20% and a 24-hour fermentation period is equal to 3.10 (brown).

Results of analysis variance of Overall Appearance salted fish catfish showed not significantly different combinations. Table 4 shows that the overall favorite salted fish catfish according to panelist is in treatment salt content of 30% and fermentation time 24 hours and 20% salt content and fermentation time of 48 hours, with a value of 2.90, although not statistically significantly different. Average panelists assess not particularly fond of salted fish pangasius products. This is possible because of the tested products containing high levels of salt, so it is very pronounced saltiness. At the time of testing the panelists, the fish is served with the frying process and tested directly into the panel in the form of fried fish with no other additives, so that the salty taste is very pronounced and less panelists liked. Salted fish is usually consumed by the public in the form of a side dish of rice friend and not as a snack (ready to eat).

4. Conclusion

Salt concentration and fermentation time is optimal in salted catfish, the salt content of 30% and fermentation time 24 hours. Total Plate Count in salted Pangasius was decrease the higher concentrations of salt were added, in the viable count of total bacteria from 10^5 to 10^2 CFU/g. Total mold in the fermented catfish increase as the higher concentrations of salt were added. Increase in the viable count of total mold from 10^2 to 10^5 CFU/g. Characteristics of salted fish catfish is 14.157% water content, ash content of 2.869%, 5.729% protein content and sensory test results saltiness 3,00, 1,60 crispness, color, 2.50 and overall 2.90.

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IMPROVEMENT ON IN-VITRO EFFECTIVITY OF IBUPROFEN SOLID DISPERSION ON TRANSDERMAL PATCH FORMULATION

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Abstract

Ibuprofen, a Non-Steroidal Anti Inflammatory Drug (NSAID), frequently used as an analgesic, antiinflamatory agent, Rheumatoid Arthritis and Osteoarthritis therapy. Commonly reported side effects of ibuprofen taken orally were abdominal pain, acid or sour stomach, peptic ulcer and upper gastrointestinal hemorrhage. Those side effect can be overcome by formulating ibuprofen into transdermal patch. The objectives of this research were to developed a new product of transdermal patch containing ibuprofen and to determine in-vitro effectivity of ibuprofen transdermal patch. This research carried out into following step: (1) Ibuprofen:PEG 6000 solid dispersion formulation; (2) Transdermal patch formulation; (3) Transdermal patch dissolution/release and penetration profile. The result showed that ibuprofen were able to be formulated into solid dispersion and transdermal patch which has physical characteristic as expected. Propylene glycol as penetration enhancer agent was proven to be effective in increasing ibuprofen release from transdermal patch and increasing penetration of ibuprofen through the skin. Adding propylene glycol into transdermal patch formula, will increase ibuprofen flux release and penetration value.

Keywords: ibuprofen, solid dispersion, patch transdermal, penetration

1. Introduction

Ibuprofen is one of the drugs known as *Non-Steroidal Anti-Inflammatory Drug* (NSAID) derivatives of propionic acid, commonly used as an analgesic, anti-inflammatory, antipyretic, and reduce *Rheumatoid Arthritis* and *Osteoarthritis* symptoms (Garzon *et al.*, 2004). Gastric ulcer and upper gastrointestinal hemorrahage are common side effect of ibuprofen taken orally (Rainsford, 2003). Those side effects can be prevented by formulating ibuprofen into a transdermal dosage form. Transdermal dosage form will increase the bioavailability of the drug because it does not undergo *first pass metabolism* in the liver and provide a consistent absorption in the long term (Prausnitz, 2008). Most of inflammatory diseases occur locally and at the surface of the body, so that a transdermal preparation can provide direct pharmacological effects and has a local effect with rapid therapeutic action (Arellano *et al.*,1999). Effective transdermal dosage forms used for NSAID delivery is patch form.

Based on the *Biopharmaceutical Classification System* (BCS), ibuprofen classified as class II drug, which has a high membrane permeability, but has a low solubility (Potthast, 2005). Ibuprofen has a solubility of 21 mg / L at 25 ° C and 46.9 mg / L at 37 ° C in water (Yalkowsky and Dannenfelser, 1992; Xu, 2007). Ibuprofen low solubility can cause bioavailability of the drug in the body to be low (Chowdary and Srinivas, 2000). Solid dispersion technique can be used to increase the solubility of ibuprofen (Craig, 2002). Erizal and Salman (2007) proved that solid dispersion composition of ibuprofen – PEG 6000 (1: 1.5) with the fusion method at 80°C has a most excellent release profile. Those ratio have been obtained in previous study are then used as a reference for determining the composition and method of solid dispersion manufacturing in this study.

Factors that may affect the penetration profile of the drug through the skin are solubility, diffusion of drugs pass through the stratum corneum, and the

partition coefficient between the drug and the stratum corneum. Diffusion of drugs pass through the stratum corneum can be enhanced through the addition of penetration enhancers which can disrupt blockage properties of stratum corneum (Williams and Barry, 2004). Propylene glykol is an excipient widely used in topical preparations as penetration enhancer when compared with other glycol groups, because of its non-toxic effects and minimal irritation (Rowe, 2006).

This research wil be developed a new transdermal patch dosage form of ibuprofen with ethylcellulose (EC) - hidroksipropilmetilselulosa (HPMC) as polymer. Quality control carried out in this research were ibuprofen in-vitro release rate from transdermal patch, and rate of penetration through the skin.

2. Methods

Research Materials

Materials used in this study are: Ibuprofen obtained from PT. Indofarma, Indonesia; 1 N NaOH; ethanol 96%; methanol; distilled water; PEG 6000; Phosphate Buffer Saline pH 7.4; HPMC; ethyl cellulose; PEG 600; backing patch; propylene glycol and skin rat (Wistar strain). All chemicals used have the technical standards.

The tools used are: 0.45 µm membrane filter, spektrofotometer ultraviolet-visible (Genesys), FTIR, DSC, SEM, dissolution tester, moisture content analyzer, Franz diffusion cell, quartz cuvette and oven (Memmert).

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Research Procedure

Preparation of ibuprofen solid dispersion

Solid dispersion composition was ibuprofen-PEG 6000 (1: 1.5) and the methods used in dispersion preparation was fusion method. This method was prepared by weighing the amount of ibuprofen and PEG 6000 according to the composition. Carrier (PEG 6000) was mixed with ibuprofen and melted at a temperature of 80° C on a hot plate magnetic stirrer. This mixture was immediately cooled in ice bath and then stored in a desiccator for 24 hours before pulverized using a mortar and stamper. Solid dispers ion sieved with a # 40 sieve.

Formulation of ibuprofen solid dispersions transdermal patch

Ibuprofen solid dispersion transdermal patch formulation can be seen in Table 1.

Table 1. Ibuprofen solid dispersion transdermal patch formulation

Composition	Function	Formula (mg)			
		F (1)	F (2)	F (3)	F (4)
Ibuprofen solid dispersion	Active ingredient	250	250	250	250
HPMC	Polymer	50	50	50	50
EC	Polymer	150	150	150	150
Propylene glycol	*Enhancer*	0	50	100	150
PEG 400	*Plasticizer*	150	150	150	150

3. Results and Discussion

FTIR Analysis of Ibuprofen Solid Dispersion

"FTIR Spectra of ibuprofen (Figure 1) shows the presence of OH groups which can be seen from widened and sharp peak at the position of 3100-2800 cm^{-1}. It also showed that benzene group on streching position at 1500-1400 cm^{-1} and the group C = O showed a sharp peak at the position of wave number 1725-1700 cm^{-1}.

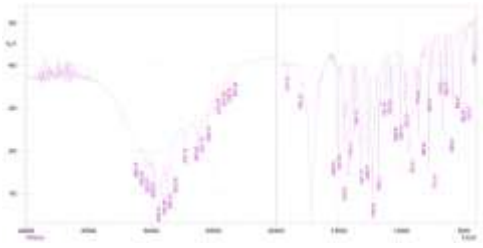

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Figure 1. IR Spectra Profile of Ibuprofen Solid Dispersion

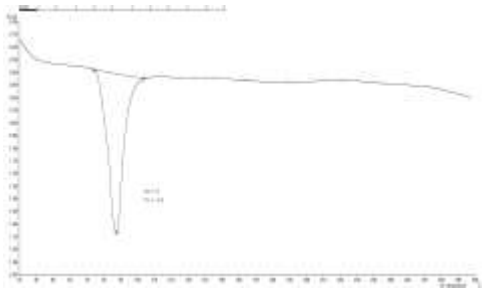
DSC Analysis of Ibuprofen Solid Dispersion

The melting point of ibuprofen before formulated in the form of a solid dispersion was 82.1°C, whereas after formulated in the form of a solid dispersion has a melting point of 54.7°C (Figure 2). This decline in melting point due to the interaction of ibuprofen with PEG 6000 as a carrier. It can also be caused by changes in the characteristics of crystalline ibuprofen. This

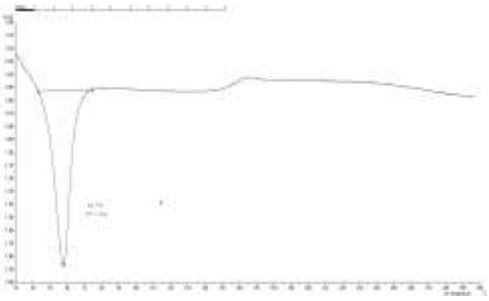
changes was caused by sudden cooling stage in solid dispersion preparation. This can lead to the amorphous powder which has a lower melting point than the crystalline form.

FTIR Analysis of Ibuprofen Transdermal Patch

FTIR spectra profile of ibuprofen transdermal patch showed similar results in spectra of ibuprofen. The OH group can be seen from the widening dan sharp peak at 3.100-2800 cm^{-1}. It also showed benzene group streching at 1500-1400 cm^{-1} and the group C = O showed a sharp peak at the position of 1725-1700 $cm^{-}1$. This result shows that ibuprofen in the transdermal patch formulation did not experience interaction with excipient component. So it can be assured that the ibuprofen in the preparation of transdermal patch will be able to provide pharmacological activity in accordance with the indications.



(a)



(b)

Figure 2. Thermal Profile (a) Ibuprofen, (b) Ibuprofen in Solid Dispersion

Release Profile of Ibuprofen Transdermal Patch

According to the calculation, ibuprofen release flux values obtained from formula 1 to formula 4 were 208.98 $\mu g/cm^2$.menit, 230.07 $\mu g/cm^2$.menit, 298.15 $\mu g/cm^2$.menit, and 421.88 $\mu g/cm^2$.menit respectively. Based on these data, it is known that formula 1 has the smallest value of flux among other formula, because of the absent of propylene glycol. The addition of propylene glycol in transdermal patch formula, will enhance the flux value of ibuprofen. Propylene glycol will increase the solubility of active ingredients in the formula patch, so that higher concentration of prolylen glycol will provide more soluble ibuprofen to be released from transdermal patch.

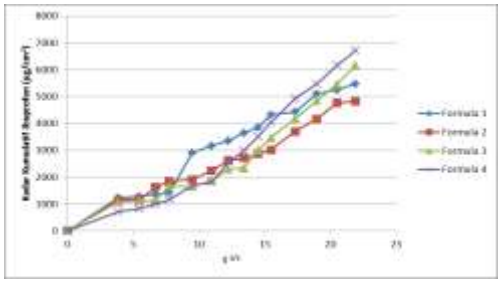


Figure 3. Release Profile of Ibuprofen Transdermal Patch during the 8 hour

Release profile of ibuprofen transdermal patch can be seen in Figure 3. Formula 4 shows the highest cumulative amount of ibuprofen that can be released from transdermal patch for 8 hours. The profile shows that ibuprofen will be provide therapeutic effects for 8 hour application.

Penetration Profile of Ibuprofen Transdermal Patch

In-vitro penetration of transdermal patch was conduct to determine the amount of ibuprofen that can be transported through the skin per unit of skin area and per unit of time. The results of a penetration test expressed in penetration flux. Ibuprofen penetration flux from formula 1 to formula 4 were 5.13 $\mu g/cm^2$.menit, 6.49 $\mu g/cm^2$.menit, 7.01 $\mu g/cm^2$.menit, and 9.82 $\mu g/cm^2$.menit respectively. Penetration profile shows that the addition of propylene glycol will be able to increase the penetration flux of ibuprofen transdermal patch (Figure 4).

Propylene glycol works by affecting the solubility of active ingredients in the skin

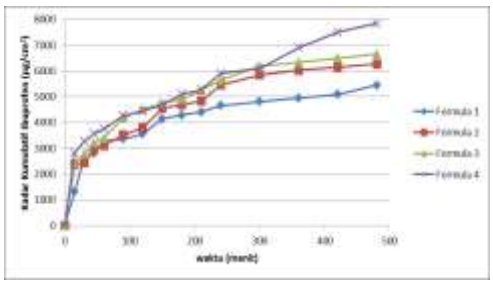


Figure 4. Penetration profile of ibuprofen transdermal patch for 8 hour

and interact with the outer layer of skin, the stratum corneum. Thus, it will increase the diffusion rate of ibuprofen and cause *sweeling* the stratum corneum thereby increasing the hydration of the skin and allows the ingredients to penetrate the skin. Ibuprofen transdermal patch penetration result shows linearity with release flux, the higher concentration of propylene glycol as penetration enhancer will increase both release and penetration flux.

4. Conclusion

Ibuprofen solid dispersions using PEG 6000 has successfully formulated into transdermal patch dosage form. Ibuprofen solid dispersion IR spectra showed that farmakofor group still detected and it does not undergo interaction with PEG 6000 as carrier of solid dispersion. Ibuprofen transdermal patch provide good organoleptis properties such as flat and dry surface, does not crack, and has a homogenous mixture in the surface of the patch. In addition, it shows no significant differences in colour and odour characteristic compared with pure ibuprofen. Formulations of ibuprofen transdermal patch have good physico-chemical characteristics and meets to the requirements.

FTIR analysis has shown that ibuprofen did not undergo an interaction with excipients in transdermal patch formulation. Release dan penetration flux of ibuprofen result shows highest value in formula 4, with highest consentration of propylene glycol as penetrating enhancer. This value indicate that propylene glycol has the ability to enhance release characteristic of ibuprofen from transdermal patch base, and to penetrates the skin so it will be able to provide the expected pharmacological effects.

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BIODIVERSITY LOSS IN LAKE TOBA ECOSYSTEM

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Abstract

Lake Toba, like other lakes in the world, provided services to people live in surrounding area. It provided plants from which people utilised food, herbs, wood, as well as types of fish from the lake. In addition to this provision service, the lake also provided regulating service and cultural service. Some studies revealed that the ecosystem has been changed especially in past decades due to population growth and development purposes. Human activities caused change of ecosystem. Widespread deforestation, shifting of land use pattern, and intensive agricultural practices in catchment area of Lake Toba contributed both directly and indirectly to the plant diversity. Not many available references documented the variety of indigenous flora and fauna in the area, however through interview with local inhabitant in the area, it was found that many of known local species were become difficult to find. This paper spelt out some of rare if not endangered plant species such as pine, *mobe, antarasa, andalehat, and sijungkot*. As to fish, ihan Batak is already listed as endangered species. Some of them have direct or indirect supports to the food security and sustainable environment.

Keyword: Lake Toba, ecosystem, biodiversity

1. Introduction

Lake Toba is Indonesia's largest lake located 176 km from the capital city of North Sumatera Province, Medan. Situated at 904 meter above sea level with depth about 505 meter it is known as the largest caldera lake in theworld. Holding about 280 km^3 fresh water, Lake Toba is recognised as the largest permanent lake body in Southeast Asia.

The ecosystem of Lake Toba and its surrounding serves the people living in the area. The basin provides abundant fresh water, and catchment area supplies food to the community through agricultural and fisheries activities, whilst forest provides wood and various herbs. Lake Toba catchment area covers 3,658 km^2 embracing parts of area of seven districs.

Ecosystem of Lake Toba Catchment Area (LTCA) has been changed dramatically especially in last decades. The Lake Toba catchment area faces an environmental crisis characterized by widespread deforestation, drought, decline of the water level, water quality degradation, invasive species and loss of biological diversity [1]. Changes of ecosystem of Toba region may be a result of numerous variables. Agricultural practice has been changed a lot. Traditional agriculture was no longer sufficient to maintain productivity for food production. Economic forces led to increasing of specialization of productive plant species and utilised more land than before.

Habitat destruction was also occurred not only due to land use change for planting industrial commodities, in the same time housing and industrial development purpose should be one of the factor. Loss of plant biodiversity was also occurred, and it is believed hundreds of species of flora and fauna in LTCA region are currently extinct or in a state of endangered. Some of them have direct or indirect supports to the food security and sustainably of environment.

2. Agrobiodiversity Loss

Endangered Plant Species

Toba land, which is inhabited by the Batak ethnics group, is blessed with a variety of plants that are being used for multiple purposes. Plants provide fuel, food, medicine and wood for shelter, as parts of provision service of the Lake Toba ecosystem. Some of the domesticated plants species are listed on Table 1, however this data gained from statistic bureau is not enough to show complete plant species

grown in the area, as wild plant species for example are absent in the list.

Table 1. Flora grown at Lake Toba catchment Area (LTCA)

Agricultural	Plantation and Forestry
Arachis hypogaea L.,	*Artocarpus heterophyllus* Lam.,
Allium cepa L. forma	*Alaurites moluccana* (L.) Wild.,
ascalonicum,	*Arenga pinnata* (Wurmb.) Merr.,
Allium sativum L.,	*Anacardium occidentale* L.
Avenus communis (L.) Merr.	*Areca catechu* L., *A*
A. maricata,	*Pinciflorum*,
Brassica rugosa Prain.,	*Bambusoideae* spp.
Capsicum annuum L.,	*Coffea arabica* L.,
Cucumis sativus L.,	*Cocos nucifera* L.,
Carica papaya L., and	*Citrus aurantium* L.
Curcuma longa L.	*Cinnamomum burmanni* (C.G. &
C. annuum	Th.Nees) Bl.,
Daucus carota L., *Glycine max*	*C. burmanni*.,
(L.) Merr., *Ipomoea batata* (L.)	*Cassia siamana* Lamk
L., *Ipomoea aquatica* Forsk.,	*Durio zibethinus* Murr.,
Lycopersicon esculentum Mill.,	*Eugenia aromatica* O.K.,
Manihot esculenta Crantz.,	*E. subumbrans*
Musa paradisiaca L.,	*E. dagliqata*,
Oryza sativa L.,	*G. tapium*,
P. grandis,	*Gnetina arborea* Roxb.,
Psidium guajava L.,	*Mangifera indica* L.,
Pisonia grandis R.Br.,	*Melia azadarach* L.
Phaseolus vulgaris L.,	*Myristica fragrans* Houtt.,
Solanum aculeatissimum Jacq.,	*Macadamia hildebrandii* Steenis.,
Solanum melongena L.,	*Persea americana* Mill.,
S. aculeatissimum,	*Parkia speciosa* Hask.
Solanum torvum Sw.,	*Pinus merkusii* Jungh. & De Vr.,
Vigna unguiculata (L.) Walp.,	*Sandoricum koetjape* (Burm.f.)
Zea mays L.,	Merr.,
Zingiber officinale Rosc.,	*Syzygium aqueum* (Burm.f.)
	Alston,
	Theobroma cacao L.,
	T. sinense

Source: Central Bureau Statistic (CBS) of Simalungun District (2015); CBS Samosir District (2015); CBS Karo District (2015)

Lake Toba catchment area currently faced not only the losses of the existing ancient Toba forest but also lose its biodiversity, as well as hectars of critical lands due a worst of enviromental destruction. Some plants species are found only in a very small number, and left without any action for conservation.

Not many available references documented the variety of indigenous flora and fauna in Toba region. Through a direct observation and interview with local people, following are some example of rare if not endangered plant species in Lake Toba catchment area. Some of endangered plant species have a high economomic value, socio-cultural meaning, as well as functional/health benefit and medicinal properties.

Artocarpus hypargyreus

Artocarpus hypargyreus or kwai muk is a tropical fruit tree belongs to family Sapotaceae. It is native to southern China, widespread and common in tropical and southern subtropical evergreen forest in South China [2,3]. This species is globally vulnerable. It is not very widely known around the world, but very popular in Toba area which is called *mobe* by its vernacular name. However, the tree is becoming rarely seen in the area of Toba.

The tree has a dense and rounded canopy, grows up to 20-25 m tall. The fruit is ovoid to globous with yellow skin/surface. It has a soft orange-red pulp with a melting texture and a pleasent and excellent acid flavor. In Toba region, this fruit is very popular and has been used traditionally but together with lemon pepper are limited as ingridients for local and traditional cuisine "*arsik*". However, the ripe fruit could be eaten ripe or preserve with salt or sugar syrup or dried. The leaves could also mix with mango leaves which are treated as tea and used as mouth washing. According to [3], not many information has been published on its nutritive and medicinal values.

Styrax benzoin

Styrax benzoin is a tree species belongs to family Styraceae, and native to the nothern hemisphere, including eastern and southeastern Asia. The most populer ones come from Sumatera and Laos. Styrax tree in Indonesia, known as kemenyan by its vernacular name, is commonly referred as to Sumatera Benzoin [4].

Its produce benzoin resin, which is tapped and dried by farmer and used for incense, perfume and pharmaceutical industries [5] as dried resin produced fragrant aromas when burn which made it become valuable source of incense.

In North Sumatera, Batak farmers manage benzoin trees in agroforest system. However, community forest where styrax benzoin grown has partially shifted to industrial plantation forest.

Chrysophyllum roxburghii

Known as apple star, and locally popular as *andalehat* (Batak), *Chrysophyllum roxburghii* is a member of Sapotaceae family. This tree species reaches a heigh of 30 metres, with a trunk diameter of up to 40 cm. The fruits are

eaten fresh, and contain considerably higher minerals B, Ca, Fe, Mn and P compared to other fruits. It is also high in aspartic acid and essential non essential amino acids [6]. It is currently found very rare in the area of Toba.

Lactuca canadaensis

Lactuca canadaensis is a plant species in the Asteraceae family. It is a type of wild lettuce and leafy vegetables, and locally known as *sijungkot* (Batak) by vernacular name. Wild lettuce commonly refers to the more bitter cousins of common. The three main species of this group are *Lactuca virosa*, *Lactuca canadensis*, and *Lactuca serriola*. Constitute a mild sedative and cough suppressant, calmative, anticancer activity of *Lactuca steriolla*. The leaves of this vegetable plant are eaten fresh, posses a very faint bitterness, and has a good crunch.

***Litsea cubeba* (Lour.) Pers**

Litsea cubeba is locally known as *antarasa* (Batak) belongs to Lauraceae family, and is an evergreen aromatic tree with dioecious flowers and small pepper-like fruit. The species could grows up to 30 metres, in the wild of secondary tropical forest in southeast Asian countries including Indonesia, India, China, Korea and Taiwan [7].

L. cubeba has been collected from forest, and the fruits of this vegetable plants are normally eaten fresh during meal for local Toba people. It is a very aromatic, and has a good crunch as eaten and promote digestion. However it is not much known in Toba region that essential oil could be extracted from *Litsea cubeba*. Generally known as May Chang in China, aromatic essential oil of *L. cubeba* is produced in China with an annual production of about 2000 tonnes and more than 50% of their production is exported to worldwide.

Mangifera foetida

Mangifera foetida belongs to family Anacardiaceae. Known as horse mango or bacang (Indonesia), the plant is endemic plant to Sumatera, Borneo, Peninsular Malaysia and Peninsular Thailand. Mostly are found wild in lowland natural wet evergreen forest [8]. *M. foetida* is grouped as least concern in IUCN Red List of Threatened Species.

M. foetida is perennial tree up to 30 – 40 m high. The fruit is vibrous, has a strong turpentin aroma and is normally eaten fresh, but in Malaysia is used to make chutney and pickle. It has a yellow pulp which contained flavonoid, carotenoid, and ascorbic acid. In the case of carotenoid, it was found higher in its fiber than in fresh and powder form. Overall, it was also found that high content of these component in *M. foetida* has a positive coorelation with its antioxidant activity [9].

***Pinus merkusii* Junghuhn & de Vriese**

Pinus merkusii known as pine is a large tree up to 50 – 70 metres tall, with a straight and cylindrical bole with average diameter of 55 cm. Needles come in pairs, slender but rigid, cylindrical 16-25 cm long. Its distribution is in Asia, and mainly in Philippines and Sumatera. The resin of this pine is used in turpentine, medicine, paint, printing and in the parfume industry.

This species is native to Sumatera – Indonesia and Philippine, fragmentation and exploitation are puting the species as a vulnarable as listed in The IUCN Red List of Threatened Species. Around Lake Toba, logging and over exploitation has progressed the population to near extict if the region. Industrial need for rayon is the factor responsible for most of the population of Sumatera pine that have been destroyed. Since 1988 a pulp factory was established in Porsea, near the lake, and consumed existing indigenous *Pinus merkuusi* from Toba surrounding area as raw material.

Several actions should be accomplished to conserve Sumatera pine. Natural forest management permit given to industrial company should be restricted to forest where no pines are grown. It should then follow by conserving and managing unprotected populations in cooperation with landowners; restoring populations impacted by off road vehicle use and other impacts, as well as studying Toba pine seed set, and threats to the species such as climate change.

Sandoricum koetjape

Sandoricum koetjape or santol (English) is belonging to genus of Meliaceae. In Indonesia is locally known as kecapi, with other vernacular names sentul and ketuat. In Toba region it is known as *sotul*, which fruit were normally collected by local people for domestic purpose. Santol is deciduous, small to large tree and native to Indochina, and from there has been introduce to South East Asia, and was spread to Australia. It s found in primary or sometimes secondary tropical forest below 1000 m. However, it has become increasingly rare in Toba land.

The fruit is about a clenched fist size, with 1-5 locular drupe. The seed is large, without aril and surrounded by a translucent or pale, acid, edible pulp of good flavor. Food nutrient composition of fruit of santol per 100 g edible portion is reported as: energy 57 kcal, moisture 84.5%, protein 0.4 g, fat 0.7 g, carbohydrates 13.9 g, dietary fibre 1.0 g, ash 0.5 g, Ca 9 mg, P 17 mg, Fe 1.2 mg, Na 3 mg, K 328 mg, B-carotene equivalent 5 mg, thiamin 0.05 mg, riboflavin 0.03 mg, niacin 0.09 mg, ascorbic acid 14 mg [3]. The fruits are eaten raw plain or with spice added. It could also be added as ingridient for local traditional recepy. In Philippine, however, santol marmalade is popular and had become export commodity. They also produced alcohol drink by processing the pulp of very ripe santol fruit with rice. In Malaysia, young fruits of santol are processed to produced candies [3].

Extract of the stem bark has anticancer activity and become good candidate for cancer treatment (Aisa *et al*., 2009), and *S. koetjape is* a terpenoids-rich traditional medicinal plant belonging antiangiogenic [10].

Zanthoxylum acanthopodium DC.

Zanthoxylum acanthopodium DC. or lemon pepper tree (English), is a unique aromatic cushion-forming perennial plant of Rutaceae family. It is known as *andaliman* by its vernacular name by Batak people lived in Toba catchment area. Lemon pepper is local endemic plant to Toba land in North Sumatera province. In the wild forest of Toba, it grows as a shrub and small tree up to 2.5 m tall.

Concerning culinary used, the fruit of lemon pepper has been traditionally used freshly as spicy on several Bataknese food, such as *arsik*, *naniura*, *natinombur* or sauces. Yanti et al. [11] revealed that the fruits are used as folk medicine for remediation of diarrhea and stomach ache. Their work also revealed that the extract of lemon pepper could be potentially used as herbal medicine to heal inflammatory particularly gastrointestinal inflamantation.

Conservation

Healthy ecosystems with plants diversity is vital for livelihoods and well being of human kinds. Until now, continuous degradation of natural habit and fragmentation is still occurred in Toba region. It is a big concern regarding ecosystem. Deforestation and land degradation caused a continuous decline of biodiversity, which could reduce life sustaining ecosystem services. Often it will lead to loss of species diversity or even potentially to a loss of genetic diversity.

In the face of current situation for sustaining future human well-being, there is a need for conservation of the plants for sustainability of the nature and protection of ecological regions. Conservation could be define as the management of human use of the biodiversity so that it may yield the greatest sustainable benefit to present generation while maintaining its potential to meet the needs and aspirations of future generations. By protecting the indigenous variety of species and preserving genetic diversity we maintain the essential ecological processes and life support systems on which human survival and economic activities are depend. Conservation action could be start by a proper of documentation, monitoring, and proper training of the locals and reducing anthropogenic pressure.

3. Closing Remarks

Although North Sumatera government authorities have made some efforts to nature conservation, much work will remain to be done to strengthening biodiversity conservation in Lake Toba catchment area. Absence and lack of environment and biodiversity commitment both at goverment and grass root level, has lead to extiction

and irrepairable damaged to the biodiversity in Toba Land.

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ANTIFUNGAL POTENCY FROM WALUR (*Neonauclea gigantea* (veleton) Merr.)

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Abstract

This study aims to determine the anti-fungal activity of Candida albicans from leaves, bark and wood of Walur (*Neonauclea gigantean* (veleton) Merr.) plant. Extraction uses three kinds of solvents (n-Hexane, ethyl acetate, and methanol). Phytochemical testing and anti-fungal activity were performed on nine extract samples obtained. The results of phytochemical tests on leaves, wood, and bark showed that all samples contain carbohydrates. Only methanol extracts of bark containing alkaloids. Flavonoids are present in 7 extracts except in bark and wood extracted n-hexane solvent. Saponins are present in methanolic extracts from leaves and wood. The results of terpenoid testing showed only the methanolic extract from the bark gave a positive result, while the steroid was found only in the ethylacetate extract of the leaves. Anti-fungal testing of n-hexane extract on wood obtained inhibition results of C. *albicans* fungi at a concentration of 500 µg of 12 mm, 250 µg of 9.33 mm, and 125 µg of 8.66 mm. While the n-hexane leaves extract obtained by 500 µg inhibition of 13.33 mm, 250 µg of 14.33 mm, and 125 µg of 10.33 mm. From these results it can be seen that the samples of n- Heksan extracted leaves have higher inhibition rates.

Keywords: antifungal, *Candida albicans*, Walur plant

1. Introduction

The use of plants as a drug tends to increase with the issue of back to nature and a prolonged crisis resulted in the decline in people's purchasing power of modern medicine which relatively more expensive. Medicinal plants are also considered to have almost no harmful side effects. This is supported by the rise of herbalist medicinal products that are traded today. Utilization of a nutritious forest plant as a medicine in Kalimantan, is different from one tribe to another. This is due to the ecological differences and diverse cultures in Kalimantan.

The development of herbs that are used as medicine in Kalimantan is necessary because data on plant species that are used as medicines from all regions of Kalimantan have not been fully collected. Indonesia is a well known country as a producer of medicinal plants.Itt is about 30,000 species of flora are exist in tropical forests of Indonesia, about 9,600 species of plants have been known to have efficacy as a medicine. About 283 species of plants are recorded as important medicinal plants for traditional medicine industry. East Kalimantan is rich in medicinal plants that have considerable potential when compared with quite expensive modern medicines.

In addition to its medicinal plants, plants that exist in East Kalimantan is quite potential as a natural cosmetic ingredients as well as skin care and based on traditional information utilization by the community in the area of West Kutai. One of them especially the Dayak tribe inland with the name Walur plant or with scientific designation *Neonauclea gigantean* (Veleton) Merr is used as a cold powder and also believed (myth) can make a child can quickly talk hen eat leaves buds. Because of reference to the above it is deemed necessary to conduct this research to identify and know the potential of Walur (*N. gigantean* (Merr) as a natural antifungal agent in this case *Candida albicans* fungus capable of inhibiting and stopping the growth or activity of fungus on the human skin.

The purpose of this study was to verify the results of phytochemical tests and to know the activity of anti-fungal skin of *C.albicans* on Walur plant extract. Scientific

study conducted to see the utilization of walur plant extract as a material to inhibit and stop the activity of skin fungus through its active compounds, and further can be followed up its utilization in the field of pharmacology or as herbal product.

2. Experimental

Materials

Raw materials used in this research are leaves, stem and skin of walur plants (*Neonauclea gigantea* (veleton) Merr.) from Family *Rubiaceae* taken from Samarinda East Kalimantan. Antifungal testing conducted with a culture of fungus *C. albicans* derived from the collection of Forest Products Chemistry laboratory Faculty of Forestry Mulawarman University. Other substances used in this study were ethanol, acetone, methanol, n-hexane, ethyl acetate, aquades, chloramphenicol, ascorbic acid, gallic acid, Potato, white gelatin, dextrose, glucose, alcohol, spirtus.

Equipment

Equipment used in this research include: blender, glass beaker, measuring cup, shaker, scissors, Whatman 42 filter paper, funnel, Erlenmeyer, aluminum foil, round flask, vaselin, rotary evaporator, spatula, vial, Analytical, oven, cup, dropper, micro-pipette, stative and clamps, shaker, petry dish tube, autoclave, desiccator, incubus burner, clamp, UV lamps, laminar flow, etc.

Methods

Leaves, wood and bark samples of (*N. gigantean* (veleton) merr.) were dried in an oven at 35 ° C, and then powdered using a blender. All powdered samples were extracted by a multilevel maseration method using three solvents (n-Hexan, Ethyl Acetate and Methanol, then the samples were soaked and shaken using Shaker for 2 x 24 hours for each solvent and thus the next stage was filtered to separate the extract from plant material. Extracts filtrat were concentrated / evaporated using a rotary vacuum evaporator at 40^{o}C to obtain a crude extract. The obtained crude extracts were tested for phytochemical analysis, the test was only performed on the ethanolic extract of leaves,wood and bark. The phytochemical analysis was performed by color change test to examine the presence of active compounds including carbohydrates, alkaloids, flavonoids, saponins, tanins, triterpenoids and steroids. Furthermore, anti fungal test against *C. albicans* fungus used acetone as negative control, however positive control used chloramphenicol. The extract concentrations for anti fungal testing were used a concentration of 500 µg, 250 µg, and 125 µg.

3. Results and Discussion

Extraction

Total extracts from leaaves macerated by *n*-Hexan yielded of 0.8516g, ethyl acetate of 1.8656g, methanol of 6.69g. The bark extracts from maceration by *n*-Hexan yielded of 0.3263g, ethyl acetate of 0.4006g, methanol of 4.6129g. The wood sample successively extracted by *n*-Hexan, ethyl acetate, and methanol yielded of 0.1723g, 0.3147g, and 0.3147g respectively.

Phytochemical Analysis

The analytical of phytochemistry referred to Harborne (1987), Kokate (2001) . The results of analysis of phytochemical testing showed that all samples possessed carbohydrate content. The methanolic bark extract was positive containing alkaloids dan triterpenoid, while other extracts showed negative results. The flavonoid active compound contained in almost all extracts except in n-Hexane soluble wood- and bark extracts. The only one extract contained saponin is methanolic leaves extract. The tannin active compound is not contained in nearly all extracts except methanolic bark and wood extract. The steroid active compound was contained in leaves extracts soluble in *n*-Hexan and ethyl ecetate. The results of the phytochemical test are presented in Table 1.

Antifungal Activity

Testing of the antifungal activity referred to Kusuma et al. (2005) . The results of antifungal testing of 9 samples against *C. albicans* indicated soluble n-Hexane leaves- and wood extracts showing inhibition of the tested fungus. Anti-fungal testing of n-hexan soluble wood extract against *C. Albicans* fungus showed inhibition at a concentration of 500 µg of 12 mm, 250 µg of 9.33 mm,

Table 1. The results of the phytochemical test from leaves, bark, and wood of Walur (*N. gigantea*) plant

Nr.	Sample	Solvent	Car	Alk	Fla	Sap	Tan	Triter	Ster
1	Leaves	n-Hexane	+	-	+	-	-	-	+
2	Leaves	Ethyl acetate	+	-	+	-	-	-	+
3	Leaves	Methanol	+	-	+	+	+	-	-
4	Bark	n-Hexane	+	-	-	-	-	-	-
5	Bark	Ethyl acetate	+	-	+	-	-	-	-
6	Bark	Methanol	+	+	+	-	+	+	-
7	Wood	n-Hexane	+	-	-	-	-	-	-
9	Wood	Ethyl acetate	+	-	+	-	-	-	-
10	Wood	Methanol	+	-	+	-	-	-	-

Table 2. Antifungal Activity from leaves, bark, and wood of Walur (*N. gigantea*) plant extracts against *C. albicans*

Nr.	Sample	Solvent	Inhibition Zone of C. albicans (mm)			
			Control (+)	500µg	250µg	125µg
1	Leaves	n-Hexane	28.66	13.33	14.33	10.33
2	Leaves	Ethyl acetate	29.22	0	0	0
3	Leaves	Methanol	27.88	0	0	0
4	Bark	n-Hexane	30.88	0	0	0
5	Bark	Ethyl acetate	30.66	0	0	0
6	Bark	Methanol	29.33	0	0	0
7	Wood	n-Hexane	31.33	12	9.33	8.66
9	Wood	Ethyl acetate	28.66	0	0	0
10	Wood	Methanol	28.66	0	0	0

and 125 µg of 8.66 mm. While the soluble *n*-Hexane leaves extract gave results at concentration of 500 µg inhibition of 13.33 mm, 250 µg of 14.33 mm, and 125 µg of 10.33 mm. These results indicated that the *n*-hexane soluble leaves extract has higher inhibition rate than the *n*-hexane soluble wood extract against *C. Albicans* (as presented on Table 2).

4. Conclusion

The methanolic extracts showed the highest extract yield, while the n-hexane soluble extracts showed the lowest extract yield. Despite low yield, the n-hexane soluble extract provides a high inhibition against the C. *albicans* fungus. The *n*-hexane soluble leaves extract indicated strong inhibition, however the *n*-hexane soluble wood extract indicated medium inhibition against *C. Albican.*

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MODELING GAMLSS WITH PENALIZED SPLINE SMOOTHING USING INTERACTIVE WEB

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Abstract

Generalized Linear Models for Location, Scale and Shape (GAMLSS) is a model that can be applied to modeling data in a semi parametric with four parameters: location (μ), scale (σ), and the shape composed of skewness (υ), and kurtosis (τ). Besides being able to model the four parameters, the distribution of which is included in GAMLSS an exponential family and supplemental distribution to another. Package in R is used to analyze GAMLSS is a package gamlss. On the other side, the r-shiny also serve to create a web tutorial because the ability of r-shiny is able to integrate HTML and R program which allows use of web based data analysis. One of smoothing method can be used to analyze a non parametric data on GAMLSS is penalized spline. Penalized spline smoothing has two advantages, namely of parametric estimation on spline regression and flexible adjustment of the level of subtlety of the curve resulting from roughness penalty on spline smoothing ($\lambda \geq 0$). The purpose of this research create interactive web using r-shiny so that a statistician or user can easily analyze data or modeling data using GAMLSS. The results of this study in the form of an interactive web can be accessed at the address http://statslab-rshiny.fmipa.unej.ac.id/JORS/GAMLSS/ and this study also successfully modeled deaths due to dengue fever in all provinces in Indonesia by using GAMLSS.

Keywords: GAMLSS, penalized spline, r-shiny

1. Introduction

Regression analysis is a technique for determining the statistical relationship between two or more variables to be functional modeling. Regression analysis is divided into three namely parametric, non parametric, and semi parametric. Parametric regression is basically a strong and rigid assumption that regression curve forms are known, for example, linear, quadratic, and cubic. Unlike the parametric regression, nonparametric regression does not require certain assumptions. With non parametric regression view, the data itself will look for the estimation form of the regression curve [1]. Besides the parametric and non parametric regression approach there is also a regression approach called semi parametric, which consists of two components, namely parametric component (known regression form) and non parametric component (unknown regression form). Generalized Additive Model (GAM) is a generalization of the additive model and can be used to solve data problems that can not be resolved parametrically. For that matter, GAM uses non parametric by smoother. The distribution of GAM is an Exponential Family. In GAM also contained link functions such as log, logit, identity, etc. (Generalized Additive Model Generalized Additive Model for Location, Scale, and Shape (GAMLSS) is a model that can be applied for modeling data in a semi parametric with four parameters *:* location (μ), scale (σ), and the shape composed of skewness (υ), and kurtosis (τ). GAMLSS is an extension of the Generalized Additive Model (GAM), therefore in this model also provides smoothing for predictor variables that are modeled with non parametric. The advantages of GAMLSS than GAM response variables not only from exponential family distribution but also addition of other distributions, including for discrete and continuous distributions with highly skewed and kurtosis. Other researchers have applied GAMLSS to death data due to pneumonia with local regression (loess) smoothing [2].

In the research will be different from the previous one using the script to analyze the regression, this research will create an interactive web based tutorial so that readers can read the theory of GAMLSS and directly practice using GAMLSS. Research on interactive web making with r-shiny between [3] and [4] respectively for the implementation and development of Generalized Estimating Equations (GEE). In this study the type of data used is discrete data or counters, namely death due to dengue throughout provinces in Indonesia 2013. The data used are secondary and obtained from the Health Profile of Indonesia Year 2013, Maluku in Figures 2015, Statistical Yearbook of Indonesia 2015. The purpose of this study create an interactive web using r-shiny so that a statistician can easily analyze data or model data using GAMLSS.

Generalized Additive Model for Location Scale, and Shape (GAMLSS)

Let $\boldsymbol{y_i} = y_1, y_2, y_3, \dots, y_n$ be the vector of the response variable observations, $k = 1,2,3,4$ and $g_k(.)$ be a known monotonic link function relate distribution parameter to explanatory variables by

$$\boldsymbol{g_k(\theta_k) = \eta_k = X_k\beta_k + \sum_{j=1}^{J_k} h_{jk}(x_{jk})}$$

if $\mathbf{Z}_{jk} = \mathbf{I}_n$, where $\mathbf{I}_n$ is an $n \times n$ identity matrix and $\gamma_{jk} = \boldsymbol{h_{jk}} = h_{jk}(\boldsymbol{x_{jk}})$ for specific combinations of j and k then the resulting GAMLSS model, this gives

$$\boldsymbol{g_k(\theta_k) = \eta_k = X_k\beta_k + \sum_{j=1}^{J_k} h_{jk}(x_{jk})}$$

$$\boldsymbol{g_1(\mu) = \eta_1 = X_1\beta_1 + \sum_{j=1}^{J_1} h_{j1}(x_{j1})}$$

$$\boldsymbol{g_2(\sigma) = \eta_2 = X_2\beta_2 + \sum_{j=1}^{J_2} h_{j2}(x_{j2})}$$

$$\boldsymbol{g_3(v) = \eta_3 = X_3\beta_3 + \sum_{j=1}^{J_3} h_{j3}(x_{j3})}$$

$$\boldsymbol{g_4(\tau) = \eta_4 = X_4\beta_4 + \sum_{j=1}^{J_4} h_{j4}(x_{j4})}$$

Where $\boldsymbol{\mu, \sigma, v, \tau,}$ and $\boldsymbol{\eta_k}$ as vector of length n, $\boldsymbol{\beta_k^T}$ is a parameter vector of length $\boldsymbol{J_k'}$, $\boldsymbol{X_k}$ is a known design matrix of order $n \times \boldsymbol{J_k'}$ and h_{jk} as smoothing function non parametric from predictors variable $\boldsymbol{x_{jk}}$. $\boldsymbol{x_{jk}}$ with $j = 1,2, \dots, j_k$ and $k = 1,2, \dots 4$ as vector from predictors variable $\boldsymbol{X_k}$ and $\boldsymbol{h_{jk}} = h_{jk}(\boldsymbol{x_{jk}})$ is a vector that evaluate function h_{jk} to (x_{jk}).[5]

Estimator Penalized Spline

Let n is $\{(x_1, y_1), (x_2, y_2), \dots, (x_n, y_n)\}$ that follow regression model

$$y_i = f(x_i) + \varepsilon_i, i = 1,2, \dots, n \quad (1)$$

with,

f : regression function unrecognized
y_i : response variable
ε_i :error random with mean 0 and varian $\sigma^2 \boldsymbol{I}$

Orde and knots regression function non parametric can be declared with this equation $\kappa_1, \kappa_2, \dots, \kappa_K$ and can be declared with this equation $f(x) = \beta_0 + \beta_1 x + \cdots + \beta_p x^p + \sum_{k=1}^{K} \beta_{pk}(x - \kappa_k)^p_+$ (2)

Where $p = 1,2 \dots, n$. from equation (2) can be made into matrix

$$\boldsymbol{y = C\beta + \varepsilon} \quad (3)$$

Or can be written in the form,

$$\begin{bmatrix} y_1 \\ y_2 \\ \vdots \\ y_n \end{bmatrix} = \begin{bmatrix} 1 & x_1^1 & x_1^2 & \dots & x_1^p & (x_1-\kappa_1)^p_+ & \dots & (x_1-\kappa_K)^p_+ \\ 1 & x_2^1 & x_2^2 & \dots & x_2^p & (x_2-\kappa_1)^p_+ & \dots & (x_2-\kappa_K)^p_+ \\ & & & & \vdots & & & \\ 1 & x_n^1 & x_n^2 & \dots & x_n^p & (x_n-\kappa_1)^p_+ & \dots & (x_n-\kappa_K)^p_+ \end{bmatrix} \begin{bmatrix} \beta_0 \\ \beta_1 \\ \vdots \\ \beta_{pK} \end{bmatrix} + \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \vdots \\ \varepsilon_{pK} \end{bmatrix}$$

and penalized spline estimator can be written as

$$\hat{\boldsymbol{y}} = \boldsymbol{C}\,\hat{\boldsymbol{\beta}} \quad (4)$$

Penalized spline estimator is obtained by minimizing the Penalized Least Square function (PLS)[6]. PLS is a standard measure of conformity to data (goodness of fit) consisting of least square $\sum_{i=1}^{n}(y_i - f(x_i))^2$ and measure of natural smoothing $\sum_{k=1}^{K} \beta_{pk}^2$, can be written as

$$\sum_{i=1}^{n}(y_i - f(x_i))^2 + \lambda \sum_{k=1}^{K} \beta_{pk}^2 \ , \ \lambda \geq 0$$
(5)

with,

λ : smoothing parameter

k : number of knots
p : polynomial degree.
The smaller the value of λ then the estimated function obtained will be more coarse and in another hand, the bigger the value of λ then the function estimate will be more smooth [7].
Next on the steps to minimize PLS function are

1. Change $\sum_{i=1}^{n}((y_i - f(x_i))^2$ into matrix $\sum_{i=1}^{n}(y_i - f(x_i))^2 = \boldsymbol{y^T y - 2\beta^T C^T C\beta + \beta^T C^T \beta}$ (6)
2. Change $\sum_{k=1}^{K} \beta_{pk}^2 = \beta_{p1}^2 + \beta_{p2}^2 + \cdots + \beta_{pK}^2$
3. There is a matrix D which is a diagonal matrix, defined as

$$D = \begin{bmatrix} a_{11} & 0 & \cdots & 0 \\ 0 & a_{22} & & 0 \\ \vdots & & \ddots & \vdots \\ 0 & 0 & \cdots & a_{(pK+1)(pK+1)} \end{bmatrix}$$

with $a_{11} = a_{22} = \cdots = a_{pp} = 0$

and $a_{(p+1)(p+1)} = \cdots = a_{(pK+1)(pK+1)} = 1$.

If function $\sum_{k=1}^{K} \beta_{pk}^2$ is written into matrix form, then

$$\sum_{k=1}^{K} \beta_{pk}^2 = [\beta_0 \ \ \beta_1 \ \ \cdots \ \ \beta_{pK}] \begin{bmatrix} a_{11} & 0 & \cdots & 0 \\ 0 & a_{22} & & 0 \\ \vdots & & \ddots & \vdots \\ 0 & 0 & \cdots & a_{(pK+1)(pK+1)} \end{bmatrix} \begin{bmatrix} \beta_0 \\ \beta_1 \\ \vdots \\ \beta_{pK} \end{bmatrix}$$
(7)

Subtitution equation (6) and (7) to equation (5) so that PLS function can be written with this equation

$$\boldsymbol{L = y^T y - 2\beta^T C^T y + \beta^T C^T \beta + \lambda \beta^T D\beta}$$
(8)

Value of $\boldsymbol{\beta}$ can be obtained by minimizing the equation $\boldsymbol{L}$ so that

$$\hat{\boldsymbol{\beta}} = (\boldsymbol{C^T C} + \lambda \mathbf{D})^{-1} \boldsymbol{C^T y}$$ (9)

Subtitution equation (9) to (4) Produce a penalized spline estimator form of $f(x)$ can be written to be

$$\hat{\boldsymbol{y}} = \boldsymbol{C(C^T C} + \boldsymbol{\lambda D)^{-1} C^T y}$$ (10)

R-Shiny

Although R programs already have many packages to identify the problem but most of the packages / modules can only be accessed via script commands (CLI = Command Line Interface), only a small part of the analysis package that can be accessed through the menu. This makes R less attractive to users statistics that is somewhat layman in terms of computer programming capabilities. R shiny allows to overcome the R deficiency.

The r-shiny module is a module created by the rstudio group that can be utilized to create a web-GUI (Graphical User Interface) menu that uses an interactive graphical web interface that interacts with R [8]. Components on R-Shiny are

a. User Interface.

User interface can be used as:

1. Control panel is used for controlling input data, variable, model, etc
2. Entering input data (data with different types of variables required, model selection, type, and statistical test criteria).
3. Output presentation, output can be displayed in the form of graphics, numbers, and mathematical notation with latex format. Shiny provide various format like tableOutput, plotOutput, textOutput, etc. User interface presentation can be presented into ui.r file and file HTML.

b. Server

The server is the center of the program in which it performs the simulation. Various results from the input data processed then directly send the results to the output. This section has been supported by various procedures and data analysis that are generally available on the R package.

2. Research Methods

In This research will be discussed about GAMLSS modeling by using interactive web. Steps in this research there are two, namely data processing and data analysis. In the data processing will be discussed about the research undertaken is the creation of interactive web-based tutorial program for the application of gamlss package using mathjax and r-shiny for data analysis online. On the other hand data analysis is discussed about what to do to model data using GAMLSS. The data analyzed in this research is secondary data. Secondary data obtained are data of death from dengue fever in 33 provinces in Indonesia obtained from [9], [10], and [11]. These data include the count of cases of death from dengue fever (y), count of malnutrition cases (x_1), number of districts / cities infected by dengue fever (x_2) , number of healthy and clean households (x_3), number of community health center in 2013 (x_4), and total of rain fall 2013 (x_5) .

The steps of data processing are:

a. Specifies the input and output of the program. These inputs and outputs are based on what is provided in GAMLSS.
b. Transfer the theory documents into the latex format so they can be displayed online.
c. Creating r-shiny programs in the form of user interface and server. The user interface is an HTML file used to input values and output presentations. The server is the brain of the program so that in this section we will apply GAMLSS semiparametric program to R into R-Shiny program.

The steps of data analysis are:

a. Entering Data
b. Determining matching distribution.
c. Determining parametric and non parametric models on a variables
d. A combination of parametric and non parametric regression called semiparametric.
e. Testing Significance. In this case to be tested is a test of the significance of parameters to the regression model by:

H_0 : There is no significant regression parameter to the semiparametric regression model and can be denoted ($\forall \beta_j = 0$ for $j = 0,1,2,3, \dots n$)

H_1: There is influence (at least one) from where $j = 0,1,2,3, \dots n$ can be ($\exists \beta_j \neq 0, j = 0,1,2,3, \dots$).

f. Provide visualization term plot and worm plot.

The scheme of data processing and data analysis can be seen in the figure 1 and 2,

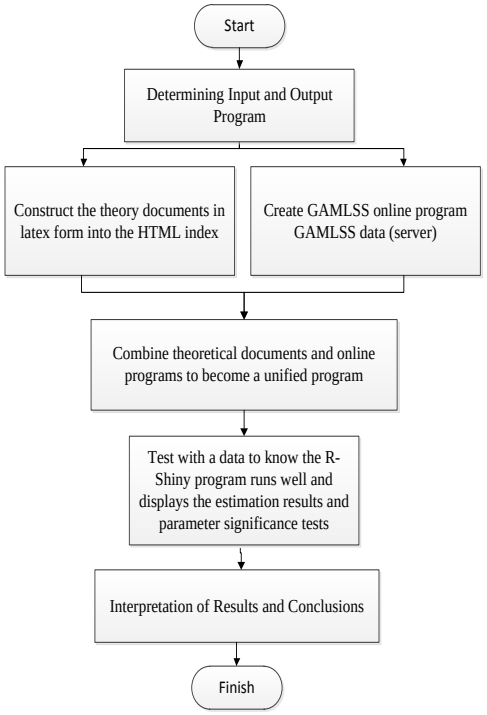


Figure 1. Interactive Web Procedure

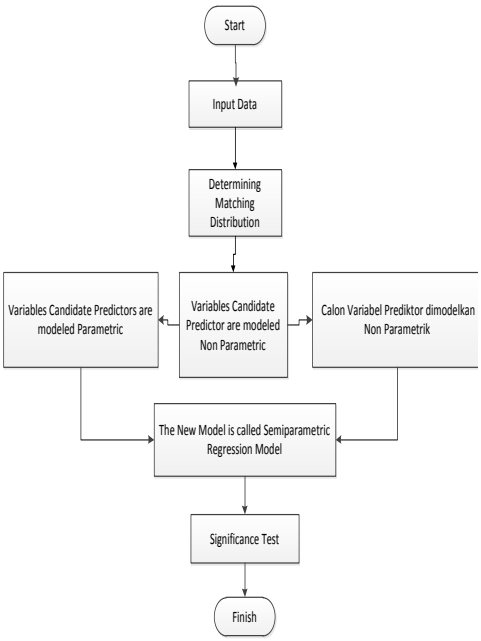


Figure 2. Data Analysis Web

3. Result and Discussion

Results and Explanation Web Features

In this research we will discuss about making interactive web for gamlss package with penalized spline smoothing and determining the best model for data analyzed by GAMLSS penalized spline smoothing. There are matters that will be made on the interactive web in the form table of contents, theoretical summary, the steps of data analysis, data analysis, conclusions, and references. The theoretical summary is intended to give an explanation of GAMLSS and penalized spline smoohhthing so that readers can understand what to do in analyzing a data. In the next matter is in the form of data analysis, on this matter is intended for the reader to process data according to the steps given. This section will also discuss the results of interactive web programs and data analysis to model GAMLSS especially penalized spline smoothing. Based on the steps of creating an interactive web-based GAMLSS program using r-shiny following the steps of compilation.

a. Compile Document Theory from Latex

Theories on GAMLSS modeling with penalized spline smoothing are written in index.html using latex. The included theoretical documents are explanations of parametric regression, non parametric regression, semi parametric regression, skewness, curtosis, GAMLSS and penalized spline smoothing.

b. Identification of Input and Output

The program inputs for modeling web based GAMLSS consist of input data and input models in the form of response variable, AIC or SIC options, GAIC optional penalty options, predictor input variables, variable inputs for formulas, non parametric parameter input variables, inputs for variable scale, input for skewness formula, input for kurtosis formula, distribution option.

1. *Input* data

Data Input is a script used to make a selection (text option) entered in the database. The option can be taken data from program R or user. The options of the R program can be selected as rock data, aids data ,cars data and trees data. Each of which can be selected as rock, aids, cars and trees. If the user wants to analyze data that does not come from program R then can choose menu Impor(CSV) after that select “choose file” and the selected file must be csv.

2. *Input* Model

Input Model is a script to make a selection of components in the GAMLSS model and its smoothing, in which case the important components included are response variables, AIC or SIC Options, optional GAIC penalties, predictor variables, formulas for μ, option non parametrik variables, scale formula, skewness formula, formula kurtosis, distrbution option, df option, method option. Examples of scripting for both response and predictor variables can be seen in [3] and [4]. In addition to the example of making the option,

<select name="md.ter">

<option value="AIC">AIC</option>

<option value="SIC">SIC/BIC/SBC</option>

</select>

The script above is a script to choose between AIC and SIC

3. Output Data Processing

Outputs in this web based GAMLSS program include visualization of distribution histogram, AIC value, SIC value, GAIC value, estimated result. Examples of output scripts can also be viewed at [3] and [4].

c. Program Server Creating

The server program basically response to the existing input in index.html. All requests sent by index.html file is processed by r-shiny server via server.r. After that the output is issued at the address given in the index.html file. The following will provide some examples of server creation.

1. Defines the data on the server.r file to be called for analysis. Creation of data input script can be seen on [3] and [4].

2. Next are steps to read and call the variable names that exist in the data and labeling to be able to communicate with index.html and other server components. Examples of scripts for reading and calling variables in the index.html file can also be viewed on [3] and [4]. One way of calling a function on r-shiny is to use do.call. Application of do.call in general can be written,

   do.call(what, args, quote = FALSE, envir = parent.frame())

   with,

   what : The name of the function will be called

   args : List of arguments for the call function

   quote : A logical value that indicates whether to quote an argument

   envir : Used if the string or argument is a symbol An example using do.call can be viewed on [3] and [4].

Program Display Results

The web view for GAMLSS modeling that has been created can be accessed on http://statslab-rshiny.fmipa.unej.ac.id/JORS/GAMLSS/, with

1. A place to enter new data or select the data already available in the program.

Figure 3. Display of Data Options

2. Determine response variables, distribution selection, and AIC or SIC.

Figure 4. Variable Respon , Distribution, AIC or SIC Selection

3. Determine the predictor variable as a formula of μ,σ, ν, and τ .

Figure 5. GAMLSS Model Analysis

4. Estimation of the distribution parameters from GAMLSS model

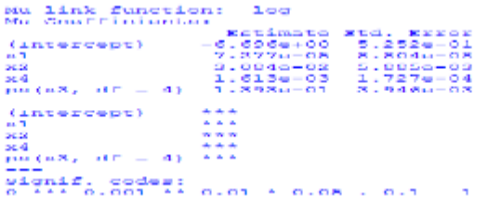


Figure 6. Analysis GAMLSS Result

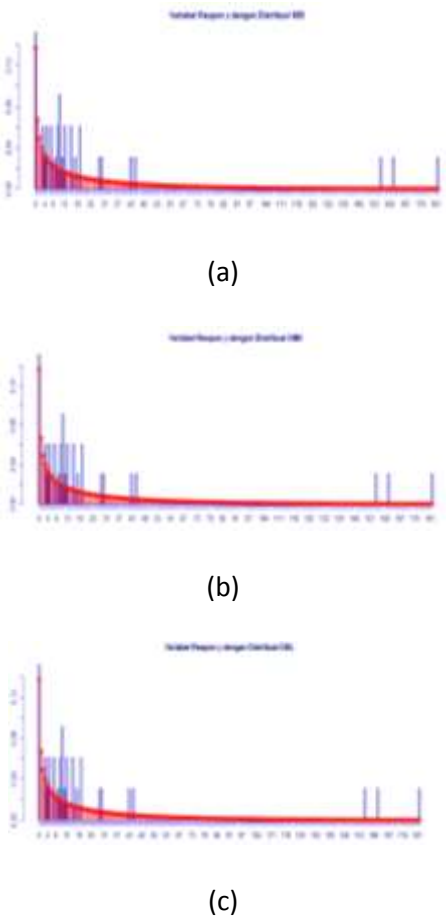
The overall appearance of web programs to analyze data can be summarized as :

Tabel 1. Overall Web Features

Features	Displayed and Used Distributions	Yes /No	Information
Exploration	Distributions	Yes	
	Scatterplotmatrix	Yes	
	μ	Yes	Only linear variable is alvailabe
	ν, σ, and τ.	Yes	Modeled only one varable
	Response Variable	Yes	
	Predictor Variable	Yes	
	Smoothing:		
	Penalized Spline	Yes	
	Pb Spline	Yes	
	Loess	Yes	
	Without Smoothing	Yes	
Diagnostic	AIC atau SIC	Yes	
	Term plot	Yes	
	Worm plot	Yes	

Analysis Data

These data include the count of cases of death from dengue fever as response variable (y), count of malnutrition cases as predictor variable (x_1), number of districts / cities infected by dengue fever as predictor variable (x_2) , number of healthy and clean households as predictor variable (x_3), count of community health center in 2013 as predictor variable (x_4), and total of rain fall 2013 as predictor variable (x_5). The first step after getting the data is to determine the best distribution of data. The tool for finding suitable distributions of data is histDist(). Using histDist() intuitively the appropriate distribution applied to death data due to dengue fever is Negative Binomial type 1 (NBI), Negative Binomial type 1I (NBII), and Delaporte which can be seen in Figure 7.



(a)

(b)

(c)

Figure 7. Distribution Histogram of (a) NBI, (b) NBII, and (c) Delaporte

The advantages of using histDist(), besides being able to view intuitively this command also provides AIC and SIC calculations. If determining the best distribution using AIC and SIC then all you have to do is look at the smallest AIC and SIC values. The AIC and SIC values are summarized in the table 2.

Table 2. AIC and SIC Value Model Distribution

VariabelRespon	Distribusi	AIC	SIC
y	**NBI**	**277.3678**	**280.3608**
y	**NBII**	**277.3678**	**280.3608**
y	Delaporte	149.4690	283.8573

Based on Table 2 it can be seen that the model with the distribution of NBI and NBII has a smaller AIC value than Delaporte so it

is known that NBI and NBII is the most suitable distribution of death data due to dengue fever.

Next is determining parametric and non-parametric Components. In this research, the model used is semi parametric model. Specifically on non parametric models will be estimated by using penalized spline smoothing. The choice of variables that will be used to select the parametric and non parametric sections is to use the distribution of data. Distribution of data can be seen by using function in R that exist in car package that is scatter plot Matrix (). The results from scatter plot Matrix () will provide an overview data distribution of the response variable with all predictor variables present in the data. Another advantage of using scatter plot Matrix () is that it can display linear regression and display the predictor variable's against response variables using loess smoothing. The selection of predictor variables that will be modeled with non parametric uses scatter plot Matrix () through the loess smoothing of the predictor variable to the unknown and low-fit response variables on linear regression. The red curve is the curve generated by the loess smoothing for predictor variable to the response variable and the linear regression is colored by green.

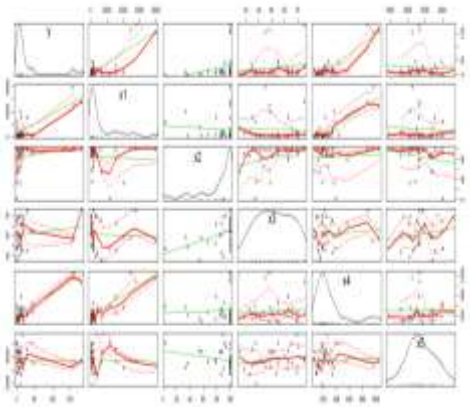


Figure 8. Data Exploration

According to Figure 8, it is seen that the relation of y with x_5 a is the lowest fit to the linear regression (green). The curve of loess (red) y with x_5 too many curves can be said as curve shape (red) is unknown, so x_5 is more suitable to be modeled non parametric. Next is to determine the combination of df, y, σ, and NBI and NBII best on the fitting model. This research will compare with values $df = 1,2,3,4,$ and 5. This research will also compare with some models σ one of x_1 , x_2, x_3, x_4, or x_5. The model analysis of the four best results can be seen in Table 3.

Table 3. Best Results from Model Analysis

Model	df	Dist	Scale	AIC	SIC
1	3	NBII	x_5	240.0651	256.5267
2	1	NBII	x_3	240.3189	253.813
3	**1**	**NBII**	**Cons**	**241.4108**	**253.4157**

Based on model 1 in table 3 has the smallest AIC and model 3 in table 3 has the smallest SIC. One could use the BIC-preferred model as a minimum size and the AIC-preferred model as a maximum, and make further choices based on other kinds of fit criteria, on theory, or on subjective inspection of the results [12]. Because of those things, the best model is model 3. Signification Model Test The Significance test is useful for knowing the significant parameters with,

H_0: $\beta_i = 0$ (Have no significant effect).

H_1: At least one $\beta_i \neq 0$ (Have a significant effect) The parameters are said to be significant when the value of p-value < α. α value indicates the effect of data should be so that it can show any difference with other data. In this research take α with value of $0,05$.

Table 4. Test of Model Estimation Significance

Part of : log function link					
Coefficient	Estimation	P-Value	Info	AIC	SIC
Interc	-8.79e-01	0.3497	No Significant	241.41	253.41
x_1	8.756e-05	<2e-16	Significant		
x_2	3.193e-02	0.0046	Significant		
x_3	4.979e-03	0.6010	No Significant		
x_4	2.361-03	3.17e-11	Significant		
$ps(x_5, df=1)$	-1300e-04	<2e-16	Significant		
Part of (scale): log function link Significant					
Interc	1.9252	<2e-16	Significant		

Based on the significance test in Table 4, coefficient from x_3 is not significant. When x_3 is thrown out and do re-modeling, new model is obtained and illustrated in Table 5.

New model of re-modeling is the best model because it has AIC and SIC smaller than before and SIC count is smallest than others. In mathematic new model can be written as

$$\log(\mu) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_4 x_4 + ps(x_5, df = 1)$$

$$= -2821e + 00 + 8.334e - 05\, x_1 + 3.351e - 02 x_2 +$$

$$2.346e - 03 x_4 + ps(x_5, df = 1)$$

and model of σ is

$$\log(\sigma) = 1.95713$$

Table 5. Re-Test of Model Estimation Significance

Part of : log function link					
Coefficient	Estimation	P-value	Information	AIC	SIC
Interc	-2821e+0	0.00525	Significant	239.78	250.29
x_1	8.334e-05	<2e-16	Significant		
x_2	3.351e-02	0.00172	Significant		
x_4	2.346e-03	1.96e-11	Significant		
$ps(x_5, df=1)$	6.042e-04	<2e-16	Significant		
Part of (scale): log function link Significant					
Interc	1.95713	<2e-16	Significant		

The non parametric modeling sections that have been fitted are difficult to describe in mathematics but nonparametric sections can be displayed using term.plot () [13]. term.plot () can be used to describe smoothing within each distribution parameter. The using of term.plot can be seen in figure 9.

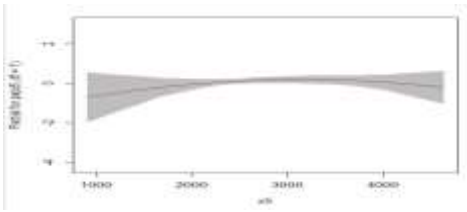


Figure 9. Term Plot of Model Smoothing $ps(x_5, df = 1)$

Results of Figure 9 is rain fall still stable. Good model can be checked the residual of the model. The gamlss package provides a tool for knowing whether this model is a good model or not with wp(). The application of wp() in this model can be seen in Figure 10.

In Figure 10 This model overall looks good because the residual points (gold) worm is perfectly between the elliptic curves.

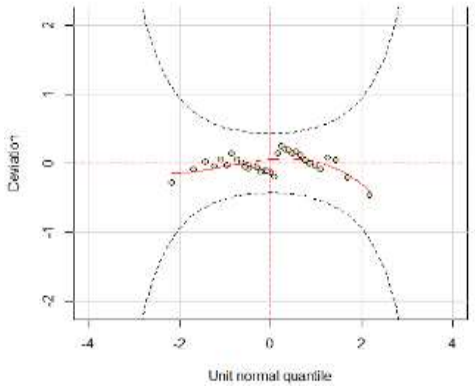


Figure 10. Residual from Model

4. Conclusions

Based on the results and discussion, the implementation of GAMLSS smoothing penalized pline made with interactive web are obtained some conclusions:

a. The interactive web-based GAMLSS program has web features such as, scatterplot, data menu options, smoothing options: (pb(),lo(),cs(), and ps()), location , scale and shape formula option, option , span option for loess, degree option for loess, term.plot(), worm plot wp(()), and distribution histogram. The advantages of interactive web that is tutorial other than the user to read the theory can directly apply the data prepared by the web or data prepared by the user, more practical when changing the formula than using R.

   Disadvantages of this interactive web is no menu knot position, polynomial degree and λ setting, Smoothing used only spline and loess families only, non parametric section is modeled only one predictor variable. σ, ν, and τ are only linearly modeled (no smoothing and no combination of predictor variables), μ is not yet modeled in polynomial and smoothing only modified one predictor variable.

b. the best model of GAMLSS fitting model with penalized spline on death data due to dengue after analyzing is a model with a combination of NBII distribution $df = 1$ and σ formula is modeled as constant . μ formula are modeled by x_1, x_2, x_4, and x_5 without x_3. The best model death from dengue fever is

$$\log(\mu) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_4 x_4 + ps(x_5, df = 1)$$

$$= -2821e + 00 + 8.334e - 05\, x_1 + 3.351e - 02 x_2 + 2.346e - 03 x_4 + ps(x_5, df = 1)$$

and model of σ is

$$\log(\sigma) = 1.95713$$

where by x_1, x_2, x_3, x_4 and x_5 are the count of malnutrition cases, number of dengue-affected districts / cities, and number of healthy and clean households, and count of puskesmas and total of rain fall on 2013.

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1D MAGNETOTELLURIC MODELING AT TIRIS GEOTHERMAL AREA USING RECURSIVE FORWARD MODELING

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Abstract

The 1D Magneto-telluric forward modelling has been carried out at the Tiris Geothermal area to obtain apparent resistivity and impedance phase responses as function of frequency due to the effect of resistivity and different layer depth. The resistivity and layer depth model are obtained from tentative model that result from previous research, using forward modelling with recursive technique. Base on apparent resistivity and impedance phase curve, the value of apparent resistivity varies by frequency and resistivity of each layer. In general, the apparent resistivity value approaches resistivity of the bottom layer determined by skin depth. The impedance phase varies around 45 degrees, affected by the frequency and resistivity of each layer. Finally, the magneto-telluric method can be used for more detailed exploration at Tiris geothermal area.

Keywords: 1D Magneto-telluric, recursive method, geothermal area, Tiris.

1. Introduction

The Tiris geothermal area is located to the East of Mount Lamongan, and has hot springs as a manifestation which is located in the Segaran village. This hot spring has a temperature of around 77.6^{0}C [1] and the reservoir temperature of around 280^{0}C [2] Due to the geology of this area is very complex, the control of heat source that exists in this area is still not certain, whether controlled by Mount Lamongan, Mount Argopuro or by maar around manifestation [3][4]. In order to know the geothermal system in this area, several studies using geophysical methods have been done using magnetic and gravity method, but very small in scope [5][6].

Application of magnetotelluric method will obtain the sub-surface information more detailed, but very expensive. In this study, we conduct of magnetotelluric modeling to see frequency response and phase that's correlated with sub-surface layer as a preliminary study. Exploration Geophysics is an activity using Geophysical technology by applying the concepts of geophysics, one of them using the Magnetotelluric method that use electromagnetic waves. Magnetotelluric method is a passive method of utilizing natural resources in the form of electromagnetic waves to know sub-surface structure corresponding to its electrical conductivity [7]. The basic concept of the magnetotelluric is an electric field and a magnetic field perpendicular to each other [8]. The purpose of this research is to know the influence of Earth resistivity and depth with response modeling of 1D Magnetotelluric.

The propagation of electromagnetic waves is present on the Earth the conductive medium, where meeting the current $\vec{j}$ is no longer equal to zero but proportional to the electric field $\vec{E}$, So Maxwell's equations become:

$$\nabla x \vec{E} = -\frac{\partial \vec{B}}{\partial t} \quad (1)$$

$$\nabla x \vec{H} = \vec{j} + \frac{\partial \vec{D}}{\partial t} \quad (2)$$

Where, $\vec{E}$ is the electric field (V/m), $\vec{B}$ is magnetic field (A/m), $\vec{j}$ is current density (Cm^3), and $\vec{D}$ is the electric displacement (C/m^2).

In this 1 D modeling, magnetic fields are considered propagate vertically into the Earth and the electric field perpendicular to it, so that each component of horizontal electric and the magnetic field varies to the depth of layers only. The impedance is defined as the comparison between electric field and a magnetic field perpendicular to each other can write as,

$$Z_{yx} \equiv \frac{E_y}{H_x} = \sqrt{i\omega\mu_0\rho} \quad (3)$$

Where, z is the impedance, ω is the angular frequency, μ_0 is the permeability in vacuum, and ρ is the resistivity of the layer.

$$\hat{Z}_n = Z_{n-1}\frac{Z_{n+1} + Z_n \tanh(ik_n h_n)}{Z_n + Z_{n+1}\tanh(ik_n h_n)}$$

where, z is the impedance, k is the complex wave number and h is the layer thickness.

Based on the above equation, homogeneous Earth impedance is a complex scalar number which is a function of the resistivity of the medium and the frequency of the electromagnetic waves. Resistivity of medium on the 1D model varies to the depth and interpreted as a horizontal layers, each with a homogenous resistivity and certain thickness [9].

According to Grandis [10] solved in the complete calculation of the impedance in the Earth's surface layers has done and produce a recursive equation that can be implemented numerically to 1D Magnetotelluric forward modeling. Pseudo resistivity and phase can be calculated using the following equation,

$$\rho_a = \frac{1}{\omega\mu_0}|Z_1|^2 \; ; \; \Phi = \tan^{-1}\left(\frac{Im Z_1}{Re Z_1}\right) \quad (4)$$

where, ρ_a is apparent resistivity of the layers, and Φ is phase.

2. Methods

The first step is to make a tentative model of litologi layer in the Tiris Goethermal area in Probolinggo based on tentative model made by Fernania using magnetic method. A tentative model of sub-surface lithology from magnetic data is shown by Figure 1 below.

The next stage is to determine the point of observation and determine input form the layer thickness and the resistivity values of each layer.

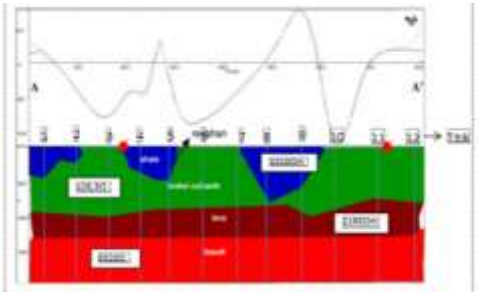

Fig. 1. Tentative model of litology at TGA from magnetic data

After this type of rock is known, the value of specified resistivity and thickness used to compute a 1D MT response using frequency range 10^{-1} Hz to 10^3 Hz. The Response of 1D MT modeling are two curves, are pseudo-resistivity and phase curves impedance for various frequencies and resistivity of each depth layer using equation (5) below,

$$\hat{Z}_n = Z_{n-1}\frac{Z_{n+1} + Z_n \tanh(ik_n h_n)}{Z_n + Z_{n+1}\tanh(ik_n h_n)} \quad (5)$$

3. Result and Discussion

Pseudo resistivity and phase of the impedance is the output of the 1D MT modeling. The station that is used in this research are 12 point, with the distance between the MT station is 83 m.

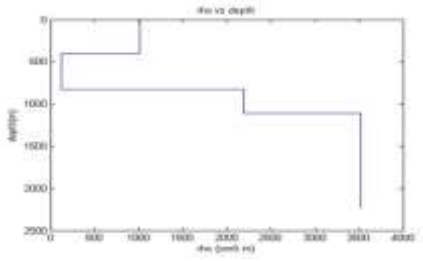

Fig. 2. 1 D resistivity model of rocks at station 1

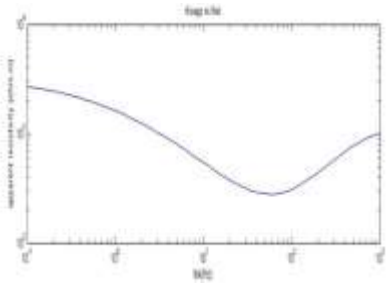

Fig. 3. Pseudo resistivity vs frequency curve at first station.

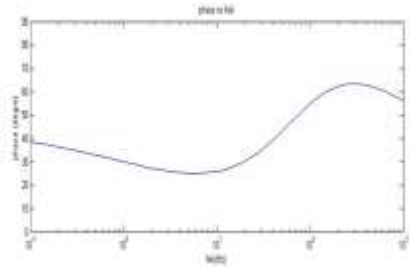

Fig. 4. The phase impedance vs frequency at first station.

The waves penetration on the 0.1 Hz frequency indicates the value of 2900 Ωm pseudo-resistivity, which can penetrate all layers with a depth of 1500m. On the frequency of 0, 6Hz up to the 2Hz pseudo-resistivity value decrease from 2000 to 1000 Ωm, so the waves passed through the layer 1, 2 and 3 from 0 – 1100 m depth. 3rd layer resistivity values (Ωm 2195) is the value of the most dominant influence on the value of the pseudo resistivity. At 2-60 Hz frequency, resistivity values of pseudo decrease again until it reaches 290 Ωm. At a frequency of 60 Hz-1000 Hz pseudo-resistivity value increase due to the influence of rock shale formation (1010 Ωm). In this frequency range, the value of apparent-resistivity is 1000 Ωm.

In the curve of Figure 4 impedance phase values can be grouped into 2 parts, namely the value of the impedance phase more than 45^0 and less than 45^0. In the frequency range between 0.1 Hz to 50 Hz impedance phase has a value of less than 45°. On the frequency range, the phase value of the impedance decreases, because the value of resistivity on layer 3 of 2195 Ωm-resistivity values smaller than on layer 4 (3520 Ωm), which is assumed to have an effect on the attenuation of electromagnetic waves. At the 50 Hz – 300 Hz frequency range, waves phase rising extremelly from 25° to 63°, it is affected by the change in resistivity of 2195 Ωm at 3rd layer be 126.5 Ωm at 2nd layer. At a frequency of 1000 Hz having a bit of a downturn into 58°, this is a concern because it is affected by the value of resistivity on Shale rocks (1010 Ωm).

On the next simulation, namely at the 3rd station results shown by Figure 5, 6 and 7 below,

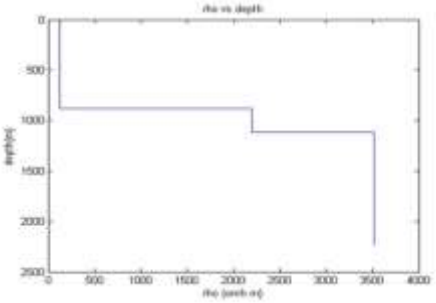

Fig. 5. 1 D resistivity model of rocks at 3rd station.

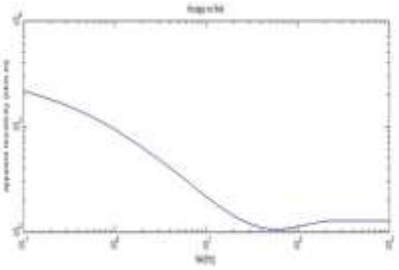

Fig. 6. Pseudo resistivity vs frequency curve at 3rd station

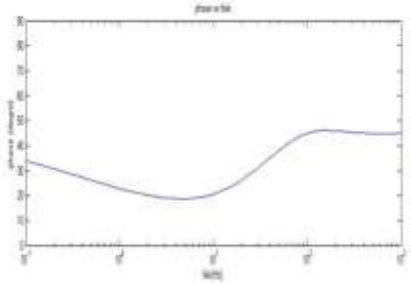

Fig. 7. The phase impedance vs frequency at 3rd station.

Figure 6 shows the resistivity curves all with a frequency on the 3rd station. At 0.1 Hz frequency have a value resistivity of 2100 Ωm. The value of the pseudo resistivity affected layers that have a resistivity value of 126.5- 2195 Ωm, but most predominantly influenced by the layers of lava that has a resistivity of 2195 Ωm with 875 m-1100 m depth. Then occurs at a frequency of 1 Hz to 10 Hz pseudo resistivity value has decreased from 900 to 200 Ωm. On the frequency 10Hz to 1000Hz experienced a slight decrease in the value of the constant challenges tends to resistivity of 120 Ωm. Same as Figure 4, in Figure 7 the value of impedance phase can be divided into 2 parts applies a value less than 45 phase and greater than 45, Impedance phase curve changes influenced by resistivity layers.

As for the value of apparent-resistivity in 3rd station is almost the same as with station 6, 7, 10, 11 and 12, since at that point passes through the 3 layers. The resulting phase value at that point also depends on the value of resistivity layers.

4. Conclusion

With 0.1 Hz frequency range 1000 Hz, litologi funds in TGA can be mapped properly. Base on apparent resistivity and impedance phase curve, the value of apparent resistivity varies by frequency and resistivity of each layer. In general, the apparent resistivity value approaches resistivity of the bottom layer determined by skin depth. The impedance phase varies around 45 degrees, affected by the frequency and resistivity of each layer.

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OPTIMATION OF PALM EMPTY FRUIT BUNCH AND PALM KERNEL SHELL BIO-BRIQUETTES CHARACTERISTICS USING RESPONSE SURFACE METHODOLOGY

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Abstract

Palm empty fruit bunches (EFB) and palm kernel shells (KS) are the abundant waste of palm oil plantation and the palm oil industry. This study used the raw material of EFB and KS to produce bio-briquettes. The research objectives were to determine the effect of EFB to KS ratio and starch adhesive concentration on the quality of the bio-briquettes, the optimal ratio and adhesive concentarion were studied using Response Surface Methodology. The raw materials were carbonized in less oxygen conditions and sieved to 40/60 mesh. The ratios of EFB to KS were 0: 1; 1: 3; 1: 1; 3: 1; and 1: 1 (w/w) were mixed, and 4%, 6%, 8%, 10%, and 12% (w/w) starch adhesive were added. Each biobriquet was shaped with a cylindrical mold and pressed by hydraulic pressure. Furthermore, they were dried under the sunshine. The heating values were carried out using bomb calorimeter K88890. Response Surface Methodology was applied in order to obtain the optimal result among the the response variables. The results showed that bio-briquett made of KS with 8% starch adhesives, gave a heating value up to 5634 cal/g. The other characteristics such as moisture content, relaxation, and density were respectively 7.62%, 3.03% and 0.857 g/cm^3. The biobriquettes meet the national standar and suitable to apply as green energy.

Keywords: bio-briquettes, palm kernel shells, palm empty fruit bunches, starch, heating value, response surface methodology

1. Introduction

Briquettes have been promoted as one of green energy. Many studies have been conducted to find out the efective formula in constructing the best briquettes from biomass. Some advantages of using biomass as the energy source are abundantly available, very cheap, easy to find, even come from the rubbish, and less pollution.

Densification of biomass has been performed in many areas in the world. However, many other areas with abundant biomass waste, densification is not familiar. In consequence, the scarcity of energy for cooking being a problem for years.

Biobriquettes from biomass can prevent the wood exploitation for energy source. The energy sources in Indonesia including the waste of palm oil, the waste of rice mills, sawdust, wood, polywood, sugar mill waste, cocoa [1], the waste of coffee plantation [2], rice straw, bagasse leaves [3, 4] and many others. Palm empty fruit bunches (EFB) and palm kernel shells (KS) are some of the abundantly available biomass wastes of palm oil plantation and industry. Palm oil plantation in Aceh Utara named Nusantara I, produce 45 tons/h of Fresh Fruit Bunches (FFB) , which are 20-23% wastes, and 7-9% of the KS are wastes. The wastes were mainly used directly as the boiler fuels in the industry and as the compos for the plantatation itself, even only burned to prevent the waste build up. On the other hand they have high energy, so it potential enough for the biobriquettes production.

Using EFB and KS directly as the fuel is not recommended due to lower calorific value per volume of the biomass and may polute air [3]. Briquetting serve the biomass to be more dense so it will be easy to transport, competitive bulk and energy per volume, less emission, and less moisture [5]. Densification is very important in the briquetting process, it produces a higher density of the briquette. Commercial densification including pelletizing, cubing rolling-compressing and briquetting [6].

Briquettes from the EFB and KS have higher heating value (HV) compare to the HV of sawdust [6]. Heating value of the raw

material of EFB and KS are 4,463 cal/g and 4,629 cal/g. After carbonization, the heating value become accordingly 5,077 cal/g and 6,448 cal/g [8]. Carbonization has significantly improved the heating value of the biomass for briquetting.

Product quality is determined by some treatments in the production process such as using high pressure to improve the strength of the product. This method has significantly produced high strength of briquettes but required higher cost of production [9, 11]. As a solution, using binder can be considered as a cheaper option. Shuma and Madyra [12] stated that type of binder is one of the factors affecting the quality of biomass densification. Starch adhesive has been studied to have a good binding property for briquettes, and inexpensive.

This research aimed to determine the ratio of using EFB to KS charcoal and starch binder concentration to the biobriquettes quality. Characteristics of the briquettes including density, water content, relaxation and thermal property were determined according to standard. Response Surface Methodology was applied in order to find out the optimal interaction among of the indenpendent variables in which 11 treatments have been randomly implemented following Box-Behnken design. These factors can produce the optimal ratio of the materials and optimal adhesive concentration to support green energy production for society.

2. Methodology

The EFB KS were collected from local palm oil industry, PKS Cot Girek, North Aceh. Starch as binding agent was from local market in North Aceh. The research was conducted in Chemical Engineering Laboratory of Lhokseumawe State Polytechnic. EFB and KS were carbonized using a reactor for 2 and 4 hours respectively. The charcoal was grinded and sieved to 40/60 then mixed together with the ratio of 0: 1; 1: 3; 1: 1; 3: 1; and 1: 1 (w/w). Starch was prepared using water to the ratio 1:2, and boiled for 5 minutes. The adhesive of 4%, 6%, 8%, 10%, and 12% (w/w) from total charcoal added to the charcoal, stirred and placed into cylinder shape mold of 2.5 cm diameter and 3.5 cm height. Furthermore, a hidraulic press of 2000 lb/in^2 was applied to form briquettes and dried. Density of the briquettes was conducted by caliper according to ASAE S269.2 DEC96, in accordance with relaxation test, where it was measured during weeks. The water content was tested using Moisture Analyzer Mx-50. Heating value of the briquetttes were analyzed using Bomb Calorimeter K88890 mode isoperibolic 25^{o}C according to ASTM D 4809.

Design expert 6.0.8 of Response Surface Methodology (RSM) was applied in the optimation of independent variables. Central Composite Design (CCD) of two factorial: X1 (starch adhesive concentration, %) and X2 (EFB to KS ratio), with 25 treatment combinations and four responses: water content, relaxation, density, and heating value were studied. These combinations then reduced randomly in which 11 treatment left.

3. Results and Discussion

Briquette has been found to be a sustainable solid fuel for any purposes. Biomass briquetting produces a competitive fuel due to extremely better quality compared to the raw materials. Briquetting creates a dense, high heating value, compact, less volume, and easy handling solid fuel. Briquetting of biomass directly is not recommended due to high volume on smoke produced, may contain organic materials, and some raw material such as KS is very hard to break before carbonized [3, 8]. Carbonization under certain temperature removes volatile matters and enhances the heating value.

Optimation of the Response Variable

In order to obtain competitive EFB and KS solid fuels with a low operating cost, response surface methodology has been applied. High quality of densified rice straw has been found with a less cost of production [11]. Response surface methodology (RSM) has been used widely in chemical engineering and related process to analyze the interaction among variables effectively [4] by reducing some interactions arbitrarily. According to the results from RSM in this research, eleven treatment combinations have been randomly selected to optimize the

research. The result of the experiment is shown in Table 1.

According to experimental results by randomly run variables, water content was 7.62 – 13.47%, density was 0,763 – 0.891 g/cm^3, relaxation was 1.51-3.9%, and heating value was 4,834.1 – 5,633.8 cal/g (Table 1). The prediction result from the plots of the independent variable (factor 1 and factor 2) to the response variables would be disscussed in the next section in order to acquire optimum result.

Water Content

Water content in the briquettes comes from the raw materials of the briquettes and the adhesive used. Carbonizing of the raw materials have a significant effect to alleviate moisture of the briquettes. Drying after the briquetting is very important to reduce water content. However, some of the water stays persistent in the briquettes.

Tabel 1. Briquettes characteristics according to experimental results

Run	Factor 1 X1 : adhesive (%)	Factor 2 X2 : ratio of EFB to KS	Respon 1 water content (%)	Respon 2 Density (g/cm^3)	Respon 3 relaxation (%)	Respon 4 heating value (cal/g)
1	8	1 : 1	11.13	0.771	3.03	5,266.6
2	10	3 : 1	12.28	0.776	3.90	4,834.1
3	8	0 : 1	7.62	0.857	3.03	5,633.8
4	6	3 : 1	11.05	0.871	3.33	5,058.0
5	8	1 : 1	10.97	0.825	2.10	5166.48
6	4	1 : 1	8.80	0.891	3.12	5385.6
7	6	1 : 3	7.90	0.785	2.94	5553.6
8	8	1 : 1	11.28	0.814	2.33	5203.7
9	8	1 : 0	10.24	0.763	1.56	4903.2
10	12	1 : 1	13.47	0.876	1.61	5106.2
11	10	1 : 3	8.70	0.848	1.51	5416.1

Tabel 2. Standard of briquette in some states

Characteristics	Standard			
	Japan	British	USA	Indonesian (SNI 01-6235-2000)
Water content (%), max	6-8	3-4	6	8
Density (g/cm^3), min	1-2	0.84	1	0,44
Heating value (cal/g), min	6.000-7.000	6.500	7.000	5.000

According to the national standard, water content of briquette not more than 8%, while in Amerikan, UK, and Japan are 6%, 3-4%, and 6-8 % respectively. Less moisture is favorable due to better heating value of the briquettes.

The response surface plots fot the interaction between EFB to KS ratio and starch adhesive concentration to the water content in the briquettesis is ilustrated in Fig.1. The more EFB ratio, the more moisture of the product. EFB is naturally less dense compared to KS, and bind more water. Eventhough the product has been dried for some time, some amount of water in the biobriquettes cannot be avoid. Using 100% KS for biobriquettes production gives the biobriquettes moisture content less than 7%. According some standards, water content of briquette not more than 6-8% (Japan), 3-4% (British), 6% (USA), and 8% (Indonesian). Water content of briquettes in current research follows Japan and Indonesian

standard particularly for the variable of using 100% KS charcoal and less than 8% starch paste.

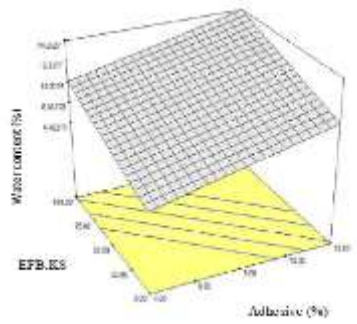

Fig. 1 Response Plots between EFB to KS ratio and adhesive concentration to the water content of the briquettes.

Starch adhesive plays an important role in the moisture of the biobriquettes. Fig. 1 shows that utilize of 4% starch paste generated water content of the briquettes less than 7%. The highest moisture content in the briquettes obtained when using 12% paste. Adhesive, in one hand, make the briquettes more dense, on the other hand increase the briquettes moisture content.

According to RSM plots, to produce water content of the biobriquette which corresponds to the standard, the starch paste should be as less as possible, and using KS 100%. However, using less than 8% starch paste yielded less compact and fracture of the products, consequently, higher pressure than 2000 kg.cm^{-2} should be applied.

Furthermore, in order to generate apropriate and qualified biobriquettes, pressure applied should be optimum. Utilizing beyond the appropriate pressure may lead the rupture of the products and rough combustion [9]. On the other hand, minimum pressure of briquetting yielded the minimum production expense [11]. Present research applied low pressure in the biquetting process, hence more binding agent would be required.

Density

Density of the briquettes is one of the most important characteristics of product quality. One of the inadequacy of biomass solid fuel is the low bulk density, ordinarily ranges from 0.080 to 0.100 g/cm^3 [10]. One of the briquetting purpose is to increase the density of solid fuel.

Many research have been conducted to find out sucessful densification. Less dense briquette means high volume, so it will require more storage area, and more transportation cost. Additionally, less dense briquettes correspond to less energy in the same volume of product. Density of the products is related to the the pressure of briquetting, more pressure produces more dense briquettes. On the other hand, as has been explained before, more pressure means more production cost.

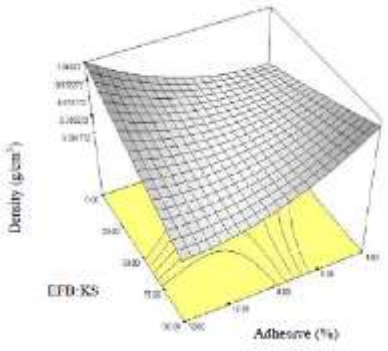

Fig. 2 Response Plots of EFB to KS ratio and adhesive concentration to the briquette density.

In present research, the density around 0,6 – 1 g/cm^3 obtained. According to response plots which is demonstrated in Fig. 2, more density of briquettes achieve when more KS used. Using 100% KS produce more dense briquette. Naturally, palm kernel shell has higher density compare to palm empty fruit bunche. To gain more dense EFB charcoal briquette, applying higher pressure than 2000 kg/cm^2 can be an option, but consequently higher production cost required.

Starch paste concentration, in addition, also affects the briquette density. Fig. 2 shows that, more starch concentration applied in the densification process, produce less density. Lower starch adhesive concentration is desired to overcome the appropropriate density. However, less starch adhesive concentration produce breakage briquettes. In this study, using 4% adhesive to combine charcoal praticles created the density up to 0,89 g/cm^3 but physically, they are very rupture. In addition, using 100% KS and 12% starch binder yielded briquettes density up to 1,06 g/cm^3. More binder

produce briquette more compact and tend to have the highest density.
Utilizing of 8% (w/w) adhesive concentration for the densification of the EFB and KS charcoal is apropriate enough to obtain optimum briquettes density with the sufficient strength, where 0,857 g/cm^3 density is achieved.
Density of briquette corresponds to Japan, British, USA, and Indonesian standard are accordingly 1-2 g/cm^3; 0,84 g/cm^3; 1 g/cm^3; 0,44 g/cm^3. The density of the briquettes produced in this experiment meet the standards.

Relaxation

Relaxation is necessary to predict the extra volume demand of the briquettes in the storage for some time. The relaxation of briquettes is significantly influent by the concentration of binding agent. Using less starch adhesive concentration (4%) result in the highest realxation, up to 5,25% according to response plots. Larger amount of starch adhesive makes the particles bond far better, hence the relaxation was lower. In contrast, Fona et al [8] reported that the relaxation of of EFB-KS briquette with sago adhesive up to 2.8%, sago binder concentration did not affect significantly to the relaxation.

It was also predicted the effect of raw materials in briqueting. Fig. 3 shows that using more KS tends to develop more relaxation. This is in accordance to Fona et al [8], where more KS applied tends to produce higher ralxation of the product.

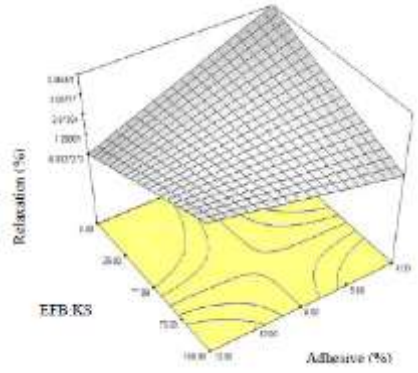

Fig. 3 Response Plots of EFB to KS ratio and adhesive concentration to the briquette relaxation.

Heating Value of the Briquettes

Heating value of the briquettes characterizes the energy content of the briquettes. It is the main point controls the briquettes quality. Higher calorific value means higher briquette quality. According to Jittabut [3] heating value is very important in selecting a fuel. High amount of fuel will be required when the calorific value is very low and it is inefficient.
In corresponds to world standard, calorific value of briquettes for Japan, USA, British and Indonesian are accordingly 6,000 – 7,000 cal/g; 7,000 cal/g; 6,500 cal/g; and 5,000 cal/g.

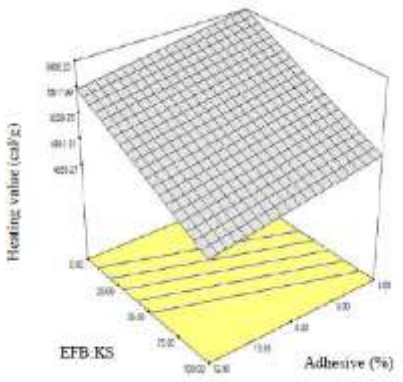

Fig. 4 Respon plots between EFB to KS ratio and adhesive concentration to briquettes heating value.

Fig. 4 demonstrates the effect of EFB to KS ratio to the heating value of the briquettes. The more KS charcoal applied, the more heating value of the products. According to respons plots (Fig.4), using of 100% KS charcoal materials siginificantly increase the calorific value of the product, up to 5,933 cal/g. Heating value of the EFB and KS charcoal before briquetting are 6.448,32 and 5.077,20 respectively [8]. It informs that KS charcoal naturally has a higher calorific value than EFB's. However, mixing both materials is very important to convert the waste of EFB for solid fuel with low cost process to produce high quality of fuel with the paremeters refer to minimum standard.

In addition, Fig.4 illustrates also the effect of binding agent concentration to the calorific value. As indicated before, using 4% or 6% adhesive make the rupture products, due to less sufficient binding between particles. Binder type and concentration have a significant impact to the characteristics of briquettes [13]. Using of petroleum products and tar as briquettes binder have performed a comparable strength of briquettes, but produced air pollution. Natural binders have been studied to be save from poisonous emision but lower binding power [14]. Starch adhesive is one of competitive natural binder cellulose. To develop higher binding quality, higher concentration would be required. Hence, appliying 8% (w/w) starch adhesive and at least ¼ portion of KS charcoal produces the briquettes heating value fit to Indonesian standard.

4. Conclusion

The more KS charcoal ratio for the raw materials of briquettes produce less water content, higher density, less relaxation, and higher heating value of the briquettes. The starch adhesive concentration of 8% can be applied to produce the briquettes from EFB and KS. Using at least ¼ portion of KS and 8% starch adhesive can generate the briquette quality fit to minimum standard. The optimum combinations is using 100% KS and 8% adhesive, where the heating value of the briquette 5,634 cal/g was obtained. The other characteristics such as moisture content, relaxation, and density were respectively 7.62%, 3.03% and 0.857 g/cm^3. The biobriquettes meet the national standar and suitable to apply as green energy.

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THE RELATIONSHIP OF PIPERACEAE BASED ON MORPHOLOGICAL CHARACTER OF VEGETATIVE ORGAN IN MERU BETIRI NATIONAL PARK JEMBER EAST JAVA

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Abstract

The family of Piperaceae in Meru Betiri National Park Jember East Java is represented by ten species: *Peperomia pellucida*, *P. Sarmentosum*, *P. aduncum*, *P. auriculatum*, *P. retrofractum*, *P. nigrum*, *P. canicum*, *Piper* Sp 1, *P. betle* and *Piper* Sp 2. Taxonomic studies were performed to determined the relationship between each species based on morphological characters. By using hierarchical cluster analysis, the relationships between species were illustrated in a dendrogam. The results show that from 10 species collected can be grouped into 7 clusters as follow: (*P. aduncum* + (*Peperomia pellucida*) + (*P. retrofracum*) + (*P. canicum*) + (*P.betle*) + (*P. sarmentosum*) + (*P. auriculatum* + *Piper Sp. 2* + *P. nigrum* + *Piper Sp.1*)). The closest relationship was obtained between species of SP 4 (*P. auriculatum*) and SP 10 (*Piper* Sp. 2) with 0.7% similarity level and 24.307 of the coefficient value. The farthest relationship was obtained between SP 1 (*Peperomia pellucida*) and SP 3 (*P.Aduncum*) with 25% similarity level and a coefficient value of 387.415.

Keywords: relationship, *Piperaceae,* morphology, taxonomic characters.

1. Introduction

Piperaceae is an aromatic herbs, shrubs or small trees there are often rhizomatous, and can be terrestrial or epiphytic. The stems can be either simple or branched. Leaves are simple with entire margins, and are positioned at the base of the plant or along the stem, and can be alternate, opposite, or whorled in arrangement [1]. Piperaceae contains most of the species in the order, with Peperomia and Piper as the largest genera (approximately 1600 and 2000 species respectively), but also including the smaller genera Verhuellia, Manekia and Zippelia [2,3].

Piper species occur in the understory of tropical forests as herbs, trees, tree lets and climbers. All species of the genus are easy to recognize in the field by their thickened nodes. Due to the rather uniform floral morphology and the number of species in the genus, classification is complicated, as well as distinction of species based on morphological characters [4]. In contrast to Piper, Peperomia shows a larger morphological variation. While Piper occurs mostlyas trees and shrubs, life forms of Peperomia are herbs, geophytes, succulents, epiphytes and more.Not only interspecific variation is remarkably large, it is even observed within species. Processes like hybridization and rapid speciation could lead to this variation, but little is known about this in Peperomia [3].

Piper and Peperomia are the largest genera of Piperaceae and have wide geographical distribution, among them in Meru Betiri National Park (TNMB). The members of piper and Peperomia shows the diversity of species and variations of morphology between the both. Diversity of species with variations of morphology in piper and peperomia can be accurately studied with morphometry, in order to know the relationship between types of the species.

2. Materials and Methods

The materials used in this research were GPS Garmin e-Trex 10, ruler, slide, knife, scissor, glove, labels, raffia rope, Munsell Color Charts for Plant tissues book, used newspaper, stationary and camera. The raw materials were vegetative organ (root, stem and leaf) of family Piperaceae and alcohol 70% for preservation.

The sample had been taken by roaming forest in left side of the main road of Andongrejo-Bandealit resort. The location where the Piperaceae specimens found is marked by the position of the coordinate point using GPS.

The observed parameters were qualitative and quantitative parameters of vegetative organ, qualitative parameter were scored in order to quantified. Data obtained from each observation parameter had been analyzed using descriptive statistical analysis and then followed by group analysis (Cluster Analysis) SPSS 16.0. Then, the result is dendrogram which depicting the relationship of the existing species [5-7].

3. Results and Discussion

Results of the research was represented by ten species of family Piperaceae in Meru Betiri National Park Jember Jawa Timur: *Peperomia pellucida*, *Piper Sarmentosum*, *Piper aduncum*, *Piper auriculatum*, *Piper retrofractum*, *Piper nigrum*, *Piper canicum*, *Piper* Sp 1, *Piper betle* and P*iper.* Sp 2.

Based on qualitative and quantitative measurement and descriptive statistical analysis in SPSS 16.0, the highest result of general measurement was *Piper aduncum* which is the only type of tree habitus Piperaceae found in the research area. Its highest result was on PIT parameter (Length of Center Petiol). Otherwise, the lowest results of measurement were *P. sarmentosum*, *P.retrofractum* and *Piper* sp. 1 with 0,00 on RPDPIT (Length of Leaf and Length of Center Petiol Ratio) which indicated uniformity between the two parameters as shown in Table 1.

Table 1. Measurement Average of Qualitative and Quantitative Parameters

No.	Jenis / Parameter	P.P	P.S	P. A	P. AU	P. R	P. N	P.C	P.1	P.B	P.2
1.	PB (cm)	2.15	6.33	14	3.347	8	4.043	6.357	2.43	6.857	4.23
2.	DB (mm)	0.61	0.4	0.67	0.147	0.245	0.267	0.235	0.269	0.336	0.14
3.	RPDB (cm)	1.43	5.91	10.16	3.17	7.754	3.77	6.12	2.164	6.52	4.09
4.	PHD (cm)	1.56	10.53	16.52	6.72	13.37	8.52	7.79	7.07	9.97	7.22
5.	LHD (cm)	1.64	7.79	7.5	4.53	5.37	5.023	3.93	6.8	5.7	6.37
6.	RPLHD(cm)	-0.09	2.68	9.03	2.19	8	3.5	3.857	0.27	4.31	0.85
7.	PIT (cm)	1.56	10.53	16.52	6.7	13.37	8.49	7.7	7.07	9.9	7.2
8.	PBM (cm)	0.78	3.82	3.97	2.43	2.73	2.39	2.03	3.43	2.9	3.13
9.	DTD (cm)	0.107	0.15	11.81	0.151	0.18	0.202	0.2	0.22	0.165	0.12
10.	PTD (cm)	0.88	3.58	1.3	2.19	1.653	2.243	1.97	3.3	2.57	1.57
11.	RPDPIT(cm)	0.01	0	0.01	0.023	0	0.03	0.123	0	0.077	0.02
12.	RPDPTD(cm)	0.68	6.96	15.27	4.53	11.713	9.09	5.83	3.73	7.143	5.65
13.	KHD (cm)	0.023	0.03	0.15	0.01	0.03	0.029	0.02	0.03	0.021	0.02
14.	RPTDDTD(cm)	0.77	3.454	-10.47	2.053	1.473	2.201	1.77	3.113	2.4	1.45
15.	RLHDPBM(cm)	0.87	3.97	3.53	2.1	2.63	2.63	1.9	3.37	2.77	3.2
16.	RPITPTD (cm)	0.69	6.957	15.19	4.5	11.71	6.147	5.7	3.73	7.33	5.63
17.	RLHDKHD (cm)	1.621	7.759	7.48	4.523	5.337	4.993	3.913	6.77	5.645	6.35
18.	AA	2	1	2	1	1	1	1	1	1	1
19.	BB	1	1	1	1	1	1	1	1	1	1
20.	ATB	1	4	1	3	3	3	3	3	3	3
21.	SPB	1	3	3	4	3	3	1	3	3	4
22.	WB	4	4	1	3	3	4	2	4	4	3
23.	BD	3	3	4	3	4	3	3	3	3	3
24.	PD	4	4	1	4	1	4	4	4	4	4
25.	UD	2	2	1	2	2	2	2	2	1	2
26.	TD	1	1	1	1	1	1	1	1	1	1

27.	BPD	2	3	1	2	1	2	1	2	2	2
28.	SPAD	1	1	3	2	1	1	4	1	1	2
29.	SPBD	1	3	3	2	3	3	3	3	1	2
30.	WPAD	2	4	4	2	4	4	2	4	4	2
31.	WPBD	4	4	4	3	4	3	3	3	4	3
32.	TID	1	1	2	1	1	1	1	1	1	1
33.	AD	1	2	2	2	2	2	2	2	2	2

Note: P.P (*Peperomia pellucida)*, P.S (*Piper sarmentosum)*, P.A (*Piper aduncum)*, P.AU (*Piper auriculatum)*, P.R (*Piper retrofractum)*, P.N (*Piper nigrum*), P.C (*Piper canicum*), P.1 (*Piper* Sp.1), P.B (*Piper betle*), P.2 (*Piper* Sp.2), PB (Length of stem),DB (Stem Diameter),RPDB (Length of stem and Stem Diameter Ratio), PHD (Length of Lamina), LHD (Lamina Wide), RPLHD (Length and wide of Lamina Ratio),PIT (Length of Center nervatio),PBM (Basal-Marginal Length of leaf), DTD (Petiol Diameter),PTD (Lenght of Petiol), RPDPIT(Length of Leaf and Length of center nervatio Ratio), RPDPTD(Length of Leaf and Length of center Petiol Ratio), KHD (Lamina Thickness), RPTDDTD (Length of center Petiol and Petiol Diameter Ratio), RLHDPBM (Lamina Wide and Basal-Marginal Length of leaf Ratio), RPITPTD (Length of Center nervatio and Lenght of Petiol Ratio), RLHDKHD (Lamina Wide and Lamina Thickness Ratio), AA (Adventious Root),BB (Stem Shape),ATB (Growing Direction Stem),SPB (Structure Surface of stem), WB (Color of stem), BD (Leaf Shape), PD (Length of Leaf),UD (end of leaf), TD (Leaf edge), BPD (Nervatio Shape of Leaf), SPAD (Upper surface structure of Leaf), SPBD (Bottom surface structure of Leaf), WPAD(Upper surface Color of Leaf), WPBD(Upper surface Color of Leaf), TID (Leaf Type), AD (Leaf Aromatic).

Table 2. Showed on the highest deviation standard was in the RPDPTD parameter (Length of Leaf and Length of center Petiol Ratio) with the amount 4.42665, it indicated that this parameter has the greatest variation or diversity. The lowest deviation standard was in the parameter BB (Stem Form) and TD (leaf edges) with the amount 0.0000 which means it has a very small variation, it can be seen from the stem form and leaf edges which is almost the same of the obtained ten species of Piperaceae.

Table 2. Deviation Standard of Qualitative and Quantitative Parameters Measurement

No.	Jenis / Parameter	P.P	P.S	P.A	P.AU	P.R	P.N	P.C	P.1	P.B	P.2
1.	PB	.30089	1.84218	3.96863	.91686	3.86264	.53631	1.71949	.60277	2.04246	.20817
2.	DB	.79674	.12166	.24007	.01155	.01528	.06110	.05033	.18502	.06110	.01155
3.	RPDB	.44185	1.81997	5.94172	.88794	3.87145	.59702	1.69193	.78341	1.99369	.21166
4.	PHD	.09815	2.30290	3.92895	1.35596	.37859	1.21173	2.69115	1.40119	1.38298	.31177
5.	LHD	.24826	2.62246	1.00000	1.73183	.89629	.04041	.60277	2.49800	1.01160	.11547
6.	RPLHD	.15588	.48952	3.22542	1.60758	1.15326	1.23406	2.08869	1.49778	.88504	.27301
7.	PIT	.12503	2.30290	3.90636	1.35277	.37859	1.23988	2.51661	1.40119	1.30610	.34641
8.	PBM	.16523	1.39366	.45092	.68069	.57735	.10149	.30551	1.40119	.43389	.11547
9.	DTD	.00577	.02517	.61587	.07810	.01732	.12166	.00000	.01732	.01155	.01155
10.	PTD	.21362	1.01717	.57735	1.98023	.06807	.64694	.30551	1.89297	.65744	.05774
11.	RPDPIT	.01732	.00000	.01732	.04041	.00000	.03512	.21362	.00000	.08083	.03464
12.	RPDPTD	.16623	2.15073	3.65559	2.20303	.43317	3.63831	3.00888	1.53731	1.98888	.25403
13.	KHD	.00577	.00000	.11358	.00000	.00000	.00577	.00000	.00000	.00000	.00000
14.	RPTDDTD	.21656	1.02510	1.19274	1.92516	.05568	.83345	.30551	1.91014	.64532	.04619
15.	RLHDPBM	.09074	1.22882	.55076	1.07000	.32146	.06506	.30000	1.09697	.57735	.17321
16.	RPITPTD	.18520	2.15073	3.76116	2.25167	.43317	1.94104	2.82135	1.53731	1.91709	.28868
17.	RLHDKHD	.25120	2.62041	1.00000	1.72653	.89629	.03464	.60277	2.49800	1.00898	.11547
18.	AA	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
19.	BB	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
20.	ATB	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
21.	SPB	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
22.	WB	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
23.	BD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
24.	PD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
25.	UD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
26.	TD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
27.	BPD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
28.	SPAD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
29.	SPBD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000

30.	WPAD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
31.	WPBD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
32.	TID	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000
33.	AD	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000	.00000

Note: P.P (*Peperomia pellucida)*, P.S (*Piper sarmentosum)*, P.A (*Piper aduncum)*, P.AU (*Piper auriculatum)*, P.R (*Piper retrofractum)*, P.N (*Piper nigrum*), P.C (*Piper canicum*), P.1 (*Piper* Sp.1), P.B (*Piper betle*), P.2 (*Piper* Sp.2), PB (Length of stem),DB (Stem Diameter),RPDB (Length of stem and Stem Diameter Ratio), PHD (Length of Lamina), LHD (Lamina Wide), RPLHD (Length and wide of Lamina Ratio),PIT (Length of Center nervatio),PBM (Basal-Marginal Length of leaf), DTD (Petiol Diameter),PTD (Lenght of Petiol), RPDPIT(Length of Leaf and Length of center nervatio Ratio), RPDPTD(Length of Leaf and Length of center Petiol Ratio), KHD (Lamina Thickness), RPTDDTD (Length of center Petiol and Petiol Diameter Ratio), RLHDPBM (Lamina Wide and Basal-Marginal Length of leaf Ratio), RPITPTD (Length of Center nervatio and Lenght of Petiol Ratio), RLHDKHD (Lamina Wide and Lamina Thickness Ratio), AA (Adventious Root),BB (Stem Shape),ATB (Growing Direction Stem),SPB (Structure Surface of stem), WB (Color of stem), BD (Leaf Shape), PD (Length of Leaf),UD (end of leaf), TD (Leaf edge), BPD (Nervatio Shape of Leaf), SPAD (Upper surface structure of Leaf), SPBD (Bottom surface structure of Leaf), WPAD(Upper surface Color of Leaf), WPBD(Upper surface Color of Leaf), TID (Leaf Type), AD (Leaf Aromatic).

From the ten types of cluster analysis based on morphological characters of vegetative organs can be grouped into 7 clusters as follow: (*P. aduncum* + (*Peperomia pellucida*) + (*P. retrofracum*) + (*P. canicum*) + (*P.betle*) + (*P. sarmentosum*) + (*P. auriculatum + Piper Sp. 2 + P. nigrum + Piper Sp.1*)).

The closest relationship was obtained between species of SP 4 (*P. auriculatum*) and SP 10 (*Piper* Sp. 2) with 0.7% similarity level and 24.307 of the coefficient value. The farthest relationship was obtained between SP 1 (*Peperomia pellucida*) and SP 3 (*P.Aduncum*) with 25% similarity level and a coefficient value of 387.415 as shown in Figure 1.

Average Linkage (Between Groups)

Agglomeration Schedule

Stage	Cluster Combined		Coefficients	Stage Cluster First Appears		Next Stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	4	10	24.307	0	0	3
2	6	8	42.537	0	0	3
3	4	6	57.591	1	2	4
4	2	4	88.832	0	3	5
5	2	9	103.859	4	0	6
6	2	7	125.691	5	0	7
7	2	5	141.476	6	0	8
8	1	2	245.869	0	7	9
9	1	3	387.415	8	0	0

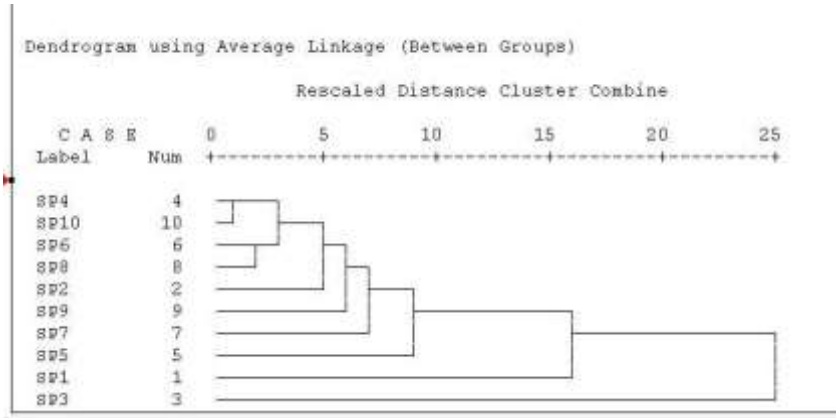

Figure 1. Dendrogram The result of 10 Species Piperaceae Relationship

4. Conclusion

The results show that from 10 species collected can be grouped into 7 clusters, the closest relationship was obtained between species of SP 4 (*P. auriculatum*) and SP 10 (*Piper* Sp. 2) with 0.7% similarity level and 24.307 of the coefficient value. The farthest relationship was obtained between SP 1 (*Peperomia pellucida*) and SP 3 (*P.Aduncum*) with 25% similarity level and a coefficient value of 387.415.

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ANTI-INFLAMMATORY EFFECT OF ARABICA COFFEE EXTRACT (*Coffea Arabica* L.)

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Abstract

Coffee is a beverage that contains some bioactive compounds, such as caffeine, chlorogenic acid, and trigonelline, that provide health benefits. Phenolic and flavonoid compounds in coffee were expected to have anti-inflammatory effects. Nowadays, arabica coffee is a type of coffee that is popular and has increased consumption every year. This study aims to evaluate the anti-inflammatory effects of arabica coffee extract (*Coffea arabica* L.) by in-vitro model. The anti-inflammatory effect on arabica coffee was tested on lipopolysaccharide (LPS)-stimulated murine RAW 264.7 macrophages. To clarify the specific effect of arabica coffee extract, we also evaluated its cytotoxic activity by viability assay using WST-8 assay. Anti-inflammatory activity in arabica coffee extracts was demonstrated by increased IL-6 production as well as decreased protein concentrations in each sample concentration reduction. The results of cytotoxic activity assay showed that arabica coffee extracted in hot distilled water solvent and distilled water had no cytotoxic effect for RAW 264.7 cell.

Keywords: arabica coffee, anti-inflammatory effect, RAW 264.7

1. Introduction

Inflammation is a protective response of the body that plays a role against agents that cause cell damage in injured local areas [1]. Inflammation stimulates cells to release chemical factors (such as histamine, bradykinin, serotonin, leukotrienes, and prostaglandins) as a mediator in the body's system to keep the surrounding tissue from spreading the infection. Inflammation will subside by itself when the spread of infection stops, which then followed by the healing process of the damaged cells. There is some evidence that some foods have anti-inflammatory effects [2,3,4,5]. Because the anti-inflammatory substances of the natural food ingredients are less toxic and have no significant side effects, screening and discovery of foods that have anti-inflammatory effects are essential. According to some previous studies, coffee is one of the agricultural commodities that has potential as an anti-inflammatory agent [6,7,8].

Coffee has been known for a long time as a beverage by many people. Coffee contains several active compounds, such as caffeine, chlorogenic acid, and trigonelline. This active compounds can provide health benefits, such as a source of antioxidants, anti-cancer, anti-bacterial, and anti-inflammatory. There are three types of coffee cultivated for consumption, namely arabica coffee, robusta, and liberica. Arabica coffee is the most widely produced type of coffee [9]. In recent years, the consumption of arabica coffee has also increased significantly [10]. The role of coffee as a functional food also the increased production and consumption of arabica coffee, make research on the health benefits of arabica coffee needs to be done, especially for anti-inflammatory activity test.

According to [11], arabica coffee is shown to have anti-inflammatory activity by testing on animals model. Specific content in coffee, such as kahweol, has also been studied and showed positive results on anti-inflammatory effect [7]. The aim of this study was to evaluate the anti-inflammatory effect of arabica coffee extract on RAW 264.7 murine macrophage cells from blood.

2. Materials and Methods

Materials

The tools used in this study were 15 mL & 50 mL centrifuge tubes, pH-meters, 5 mL syringe, 0.22 µm filters, 1.5 mL eppendorf tubes, 50 mL beaker glass, blade grinder, electronic balance, autoclave, rotary mixer, high speed centrifuge, cell counter, 96-well plate for protein assay, 96-well plate for ELISA, and plate reader.

While the materials used in this study were light-roasted arabica coffee beans, sterilized distilled water, RAW 264.7 cells, PBS, 10% FBS-1 × DMEM medium, sterlilzed 0.55% EDTA-Trypsin, LPS, BSA standard solution (2.0 Mg / mL), Reagent A, Reagent B, 0.05% tween-PBS, capture antibody, coating buffer, detection antibody, carbonate buffer (pH 9.5), 1% BSA-PBS, ELISA/ELISPOT Diluent, IL-6 standard (1000 Pg / mL), Avidin-HRP, ELISA POD Substrate TMB kit (Nacalai Tesque., Inc.), 2 N H_2SO_4, and WST-8 Solution.

Methods

The research was divided into 5 stages, namely sample preparation, cell culture, protein assay, ELISA, and viability assay.

Sample Preparation

The roasted arabica coffee beans were mashed using a plate grinder to become a coffee powder as a research sample.

a. For distilled water solvents

Mix 2 grams of sample with 20 mL of solvent into centrifuge tube. Shake the mixture using shaker. Then, mix the mixture using rotary mixer for 24 hours to gain the coffee extract.

b. For hot distilled water solvent

Mix 2 grams of sample with 20 mL of solvent into the erlenmeyer tube. Cover the bottle using aluminum foil. Heat it at 120^{o}C for 20 minutes.

Separate the supernatant from the residue using a high speed centrifuge with the following settings: temperature (4^{o}C), time (20 minutes), speed (20,000 xg), and adjust the rotor to the number. After the centrifugation process, pour the supernatant into the new centrifuge tube. Check the pH and adjust to close to 7.4. Then, filter the supernatant by using 0.22 µm filter. After that, move the supernatant into eppendorf tubes (@600 µL), and label it. Keep the sample supernatant in the freezer.

Cell Culture

RAW 264.7 cells was tore off by Sterlilzed 0.05% EDTA-Trypsin, stored in 15 mL centrifuge tube and centrifuged at 1,000 rpm for 5 min. This supernatant was removed, precipitate was suspended with 10% FBS-1×DMEM medium and it was centrifuged at 1,000 rpm for 5 min. This supernatant was removed and precipitate (Cell concentration) was prepared to be 3.0×10^5 cells/mL. This suspension was added to each well at 200 µL 96 well plate. This plate was incubate for 16 hours in 5% CO_2 and 37℃. Supernatant of each well of this plate was removed and added solution (20% FBS-2×DMEM medium: sample= 1:1) to each well at 200 µL. This plate was incubated for 6 hours in 5% CO_2 and 37℃. Use collected supernatants for ELISA.

Protein Assay

Prepare BSA standard solution with PBS. Add BSA standard solution and sample to 96-well plate at 5 µL/well. Add Reagen A and Reagen B to the plate at 25 µL/well and 200 µL/well. Stir the plate for 15 minutes at 500 rpm. Measure the absorbance at 665 nm and the reference at 415 nm by plate reader.

ELISA

Dillute IL-6 Capture antibodies with 1 % BSA-PBS or ×1/2 coating buffer to 400 times or 500 times and add to 96-well plates at 100 µL/well. Keep the plate at 4℃ for overnight. Wash the plate by 0.05% tween-PBS 3 times and then add to each well with 200 µL of 1% BSA-PBS or ×1/10 ELISA/ELISPOT Diluent. Keep the plate for 1 hour at room temperature. Prepare IL-6 standard solution. Add collected supernatant and standard solutions to plate at 50 µL and put for 2 hours at room temperature. Dillute IL-6 Detection antibodies with 1% BSA-PBS or ×1/10 ELISA/ELISPOT Diluent to 400 times or 500 times. Wash the plate with T-PBS 3 times, then add this to the plate at 100 µL/well and put for 1 hour at room emperature. Dillute IL-6 Avidin-HRP solution with 1% BSA-PBS or ×1/10 ELISA/ELISPOT Diluent to 2,000 times or 500 times in shielding the light. Wash the plate with T-PBS 3 times, then add it to the

plate at 100 µL/well and put for 1 hour at room temperature in shielding the light. Dillute IL-6 substrate with distilled water to 2 times in shielding the light. Wash the plate with T-PBS 3 times, and then add it to the plate at 100 µL/well. After coloring reaction, add each plate with 1 N H_2SO_4 at 50 µL/well. Measure the absorbance with plate reader at 450 nm and the reference at 595 nm.

Viability Assay

Wash the plate of collected supernatants with 1×PBS to each well at 200 µL. Add solution (10% FBS-1×DMEM medium :WST-8=10:1) to each well at 110 µL in shielding the light. Measure the absorbanace at 450 nm and the reference at 655 nm by plate reader.

3. Result and Discussion

Inflammation is a protective response caused by tissue damage. If the tissue in the body are injured for example by burning, slicing or due to bacterial infection, then the tissue will occur a series of reactions to destroy agents that endanger the tissue or to prevent the agent spread more widely. These reactions then also cause injured tissue to be repaired or replaced w ith new tissue. This series of reactions is called inflammation. The large number of inflammation cases made the researchers interested in finding new sources derived from natural food ingredients that contain anti-inflammatory effects.

Infection causes microbes to have access to body tissues. Pathogenic products from microbes such as lipopolysaccharide (LPS) and other compounds that enter the body tissues will cause inflammation that causes increase production of cytokines, both pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines include tumor necrosis factor (TNF), interleukin-1 (IL-1), and interferon-γ (IFN-γ), which help the cell to destroy infected microorganisms. Anti-inflammatory cytokines include interleukin-1 antagonist receptors (IL-1ra), IL-4, and IL-10 that have task on modulation, coordination or repression of excessive responses. While IL-6 may be both pro- and anti-inflammatory cytokines at the same time. IL-6 is one of the interleukin that plays an important role in inflammatory reactions and induces antibody production [13].

The measurement of protein content in this experiment was done by using standard curve that made from the relationship between concentration of the solution and its absorbance. The standard curve is made from standard solutions of known concentration value. The standard solution used Bovine serum albumin (BSA) solution, which is an albumin protein derived from cows. This solution is required to calculate the concentration value of the protein sample measurement using the line equation of obtained standard solution. Measurement of absorbance value of standard solution and sample solution used spectrophotometer by passing light of a certain wavelength on a glass or quartz object called cuvette. Some of the light will be absorbed and the rest will be missed. The absorbance value of the absorbed light is proportional to the concentration of the solution in the cuvette [14]. The formula generated from the standard curve is further used to calculate the protein concentration in the sample (R^2=0.9909).

Table 1. Protein Concentration (Dillution at 4^2)

Sample	Absorbance at 655 nm			Protein Conc. (µg/mL)			Protein Conc. Average	Final (µg/mL)
	#1	#2	#3	#1	#2	#3		
Coffee-Hot DW	0,100	0,097	0,094	927,6	899,9	872,2	899,9	449,9
Coffee-DW	0,075	0,076	0,066	697,1	706,3	614,1	672,5	336,3

In the inflammatory process, proteins are produced during the occurrence of fever which is essential to help cell survival during stress. Studies show that this protein may have anti-inflammatory effects by decreasing the level of pro-inflammatory cytokines. From Table 1, it can be seen that in treated cells using coffee extract in a hot distilled water solvent (coffee-hot DW), it produced higher final protein concentrations than those produced in treated cells using coffee extract in a distilled water solvent (coffee-DW). Furthermore, it should be proven that the produced protein was an anti-inflammatory protein that will help decreasing the level of pro-inflammatory cytokines. For this purpose, ELISA testing

was performed to determine the production of cytokines (IL-6) in various concentrations of samples (4^0, 4^1, 4^2).

Table 2. IL-6 production

		Absorbance			IL-6 (pg/mL)			Average	Dillution	Final Conc.			Average	SD	Protein Conc.(g/mL)
Blank Hot D.W.		0,004	0,005	0,002	-5,0	-4,6	-5,8	-15,4	1	-5,0	-4,6	-5,8	-15,4	1	
Control Hot D.W.		0,215	0,211	0,201	95,8	93,7	88,4	277,8	15	1437,1	1405,0	1325,3	4167,4	58	
Hot D.W.	4^0	0,009	0,006	0,002	-2,9	-4,1	-5,8	-12,8	15	-43,2	-62,2	-87,3	-192,7	22	7199,24
	4^1	0,021	0,04	0,024	2,2	10,5	3,5	16,2	15	33,3	156,8	52,6	242,7	66	1799,81
	4^2	0,147	0,144	0,169	60,6	59,1	71,7	191,5	15	909,2	886,8	1075,8	2871,8	103	449,95
D.W.	4^0	0,013	0,006	0,003	-1,2	-4,1	-5,4	-10,7	15,0	-17,9	-62,2	-81,0	-161,0	32	5380,16
	4^1	0,05	0,031	0,039	14,9	6,5	10,0	31,4	15,0	223,1	97,9	150,3	471,3	63	1345,04
	4^2	0,154	0,075	0,092	64,1	26,2	34,0	124,3	15,0	961,8	392,4	510,5	1864,7	300	336,26

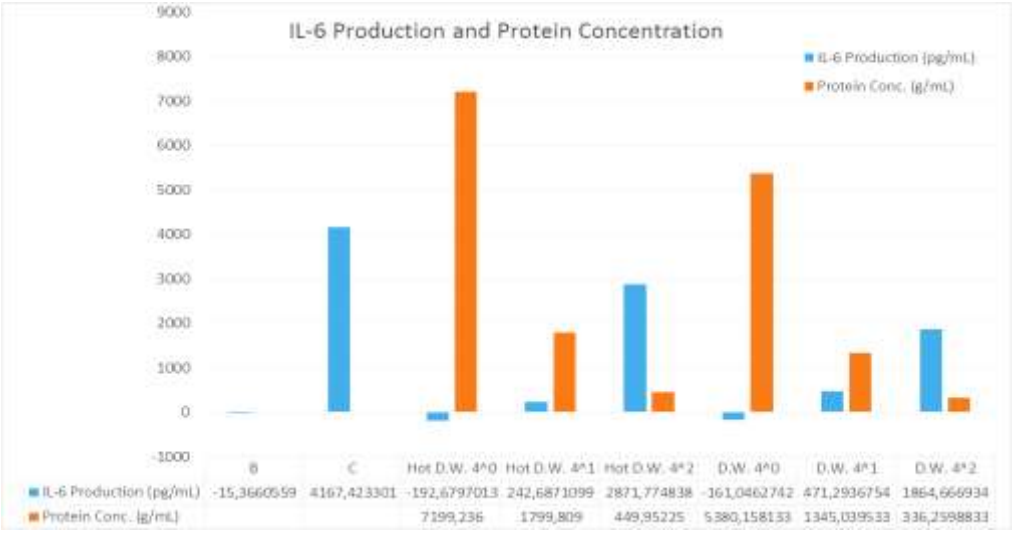

Figure 1. Graphic of IL-6 Production

The formula generated from the standard curve was further used to calculate the production of IL-6 in the sample. From Table 2 and Figure 1, it can be seen that the production of IL-6 decreased along with the increasing of protein concentrations in each sample concentration enhancement. This proved that the produced protein is an anti-inflammatory protein that is capable of inhibiting pro-inflammatory cytokines production, in this case is IL-6. Furthermore, viability assay was done to know whether coffee sample has toxic properties to the cells (Table 3). The WST-1 and WST-8 are the prototype of water soluble tetrazolium salts for viability assay. The research which was conducted by [14] concluded that WST-8 has higher sensitivity than those of conventional tetrazolium salts.

Table 3. Viability Assay (WST-8)

		Absorbance				Viability (%)					
Sample		#1	#2	#3	Average	#1	#2	#3	Average	SD	Protein Conc.
Blank		0.39	0.26	0.22	0.29	87.82	58.62	49.70	65.38	19.94	
Control Hot		0.57	0.42	0.35	0.45	127.49	93.83	78.68	100.00	24.98	
Hot-Coffee	4^0	0.47	0.46	0.39	0.44	104.98	102.97	87.37	98.44	9.64	7199.24
	4^1	0.39	0.47	0.45	0.44	87.15	105.42	100.74	97.77	9.49	1799.81
	4^2	0.55	0.52	0.52	0.53	122.81	115.45	115.45	117.90	4.25	449.95
DW-Coffee	4^0	0.42	0.47	0.46	0.45	92.72	104.09	102.08	99.63	6.07	5380.16
	4^1	0.44	0.52	0.52	0.49	98.51	116.12	115.23	109.96	9.92	1345.04
	4^2	0.42	0.53	0.52	0.49	94.06	117.46	114.78	108.77	12.81	336.26

The results of cytotoxic activity assay of arabica coffee extract with WST-8 showed that arabica coffee extracted in hot distilled water solvent and distilled water had no cytotoxic effect for RAW 264.7 cell.

Figure 2. Graphic of Viability Assay (WST-8)

4. Conclusion

In this study we found that arabica coffee extract has anti-inflammatory effect on RAW 264.7 murine macrophage cells from blood. Anti-inflammatory activity in arabica coffee extracts was demonstrated by increased IL-6 production as well as decreased protein concentrations in each sample concentration reduction. The results of cytotoxic activity test of arabica coffee extract showed that arabica coffee extracted in hot distilled water solvent and distilled water had no cytotoxic effect for RAW 264.7 cell.

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MAMMARY GLAND HISTOLOGY OF SWISS WEBSTER MICE (*Mus musculus* L) OVARIECTOMY AFTER SOYTEMPEH FLOUR EXTRACT EXPOSURE

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Abstract

Estrogen plays important role in the growth of the mammary gland which stimulategrowth of the stroma, the duct system and stimulate the accumulation of fat in the mammary gland that can provide mass on the mammary gland. Decreased estrogen levels caused mammary gland atrophy and duct dilation that lead to inflammation. Tempe is a food containing phytoestrogen compounds that have a structure resembling 17β estradiol. This study was conducted to determine the effect of soybean tempeh flour extract against mammary gland histology in Swiss Webster mice (*Mus musculus*) ovariectomied. Tempe soybean flour extract dosage given to the mice was 0,21g / ml, 0.42 g / ml and 0.63 g / ml by gavage. Mice were dissected on 11, 21 and 31 day. Histological mammary gland preparations using paraffin method and HE staining (Haematoxylin eosin). The results showed that the extract of soybean tempeh flour for 10, 20, and 30 days can decreased the average diameter of the lumen and increased the ductal intralobularisepithelium thickness. The average value of the lowest lumen diameter was found at a dosage of 3 (0.63 g / ml / day) during of 30 days is 23.40 µm and the average value of the highest epithelium found in 3 dosage (0.63 g / ml / day) for the treatment of 30 days is 8.96 µm.

Keywords: Mammary gland, Ovariectomy,Soybean tempeh flour extract

Background

Estrogen plays major role in the growth of the mammary glands, which stimulates the growth of stroma, duct systems and can stimulate fat accumulation in the mammary glands that can give mass to the mammary glands [13]. Decreased estrogen levels as in menopausal conditions in women will have an impact onfemale reproductive system. In addition to naturally occurring, menopause can also occur due to ovarian removal or damaged by the effects of radiation used in treatment of cancer patients. The presence of this radiation effect causes premature menopause if radiation therapy concerns in ovary region [18]. Due to decrease estrogen levels of the mammary glands, the structure changes in the form of involution, the lobular connective tissue changes from a loose structure into a solid structure. Additionally, the basal membrane around the asinus is thickened and the asinus-limiting cells disappear, the alveoli and ductal epithelium are atrophy and only a few ducts are seen in the lobes. In some ducts the connective tissue appears solid and homogeneous [16]. Changes in the structure of the mammary glands in the form of increased connective tissue and widening of the ducts can lead to inflammation [4].

Soybean and its derivative food products namely tempeh is a food containing phytoestrogen compounds [7; 20].

Phytoestrogens are compounds that have structures resembling 17β estradiol [5].

Several studies on the effect of phytoestrogens on mammary glands have been performed. According to [1], it was mentioned that giving Fenugreek seed extract (*Trigonella foenum-graecum* L.) containing diosgenin at a dose of 60mg / 200g BB can increase the development of the mammary lobe in female Wistar rats. In addition 15 stated that rhizome extract of turmeric containing phytosteroid with a dose of 230 mg / kg bb, 310 mg / kg bb, and 390 mg / kg bb may increase the diameter of the mammary gland ducts.

Tempeh was known has higher isoflavones content when compared to 901.24 mg / kgBK [26]. Therefore it is necessary to do research about the effect of soybean flour extract as one of the sources of phytoestrogens to the mammary glands in Swiss Webstermice ovariectomy.

Methods

The research was conducted in Zoology and Botany Laboratory in Department of Biology, Faculty of Mathematics and Sciences University of Jemberand Pathology and Anatomy Laboratory Faculty of Medicine, Gadjah Mada University, Yogyakarta.

The animal probandus was 90 days old Swiss Webster mice weighing 30 g after ovariectomy of 45 mices. Mice were placed in a plastic tub 40x30x15 cm3 with wire to cover the top of cage. Mice were placed in a room with a room temperature of 27 ° C and a relative humidity of 77% RH. The mice were fed standard feed pellets (CP 511) by administering 1/10 of body weight and drinking sterile aquadest on ad libitum.

Determination of dosage was obtained based on [22] i.e giving of soybean and tempehflour to mouse. The provision of tempeh flour was 10 g dw / 100 g bw / day, then converted from mouse to mice to obtain the dose of tempeh flour extract of 0.21 g / ml, 0.42 g / ml and 0.63 g / ml. Provision of soybean flour extract is done orally using stomach feeding tube in accordance with the dose, once daily. Provision of soybean flour extract was given for 10, 20 and 30 days.

Taking the mammary glands on the 11th, 21st and 31st days by placed mice on the surgeon with supine position, then shaved hair around mammaein right and left inguinal part, then mammary gland tissue taken by cutting the skin to subcutaneous then affixed to the white cardboard paper so the tissue does not shrink.

Histological preparations are prepared by means of embedding paraffin with HE staining (Haematoxylin Eosin). The parameters observed were the diameter of lumen and the thickness of epithelial lining of the intralobular ducts. The measurement of the lumen diameter and the thickness of intralobular duct layer were performed on one point of view with 3 measurements horizontally, vertically and diagonally.

The data were analyzed using One Way ANOVA test, then continued by Duncan test at 99% confidence interval (α = 0,01) to know the real difference between treatment group.

Results and Discussion

The average calculation result of luminal ductal intralobularis of mammae glands after treated with soybean flour extract can be seen in Table 1.

Table 1.Average intracellular lumen duct diameter of the mammary glands of Swiss Webster mice ovariectomy after giving

soybean flour extract for 10, 20, and 30 days.

Treatment	Diameter of luminal ductal intralobularis(µm), after giving soybean flour extract (days)		
	10 days	20 days	30 days
Control (-)	21,86 ± 1,25^{a}	21,76 ± 2,02^{a}	22,03 ± 0,77^{a}
Control (+)	48,03 ± 4,36^{d}	55,26 ± 2,75^{c}	58,06 ± 1,82^{b}
0,21 g/ml	37,80 ± 0,30^{c}	35,53 ± 7,41^{b}	32,80 ± 3,31^{a}
0,42 g/ml	33,20± 2,65^{bc}	31,60 ± 0,93^{ab}	25,30 ± 7,25^{a}
0,63 g/ml	30,86 ± 1,33^{b}	24,46, ± 0,83^{a}	23,40 ± 4,95^{a}

Description: numbers in the same column followed by different letters show significantly different p <0.01.

Based on the results of One Way Anovaanalysis obtained the value of day-to-day significance of 10, 20 and 30 p = 0,000 <0.01. This shows that the dose of soybean flour extract treatment has a very significant effect on the decrease of luminal ductal intralobularisdiameter. Based on the Duncan test there was very significant difference between negative control group andpositive control group. The diameter of luminal ductal intralobularisbetween positive control and treatment at all doses showed very significant difference. Treatment between soybean flour extract doses showed no significant difference in decrease of luminal ductal intralobularisdiameter. The duration of giving soybean flour extract showed no significant difference in doses 1, 2, and 3 for 10, 20 and 30 days.

In treatment for 10, 20 and 30 days there was very significant difference between the negative control group and the positive control group. The results were suspected that ovariectomy for 30 days had an effect on the mammary glands, which was an increase in luminal ductal intralobularisdiameter. According to [8], decreased ovarian activity can cause mammary gland duct dilate and form a cyst. The dilated ducts are larger than normal with a flat layer of cuboid epithelial cells [17].

A very significant difference between positive control group and treatment group showed that giving of soybean flour extract had an effect on the decrease of luminal ductal intralobularisdiameter. These results are suspected because soybean flour extract has a structure of isoflavone compounds similar to estrogen that affect to mammary glands. There are similarities between the structured of isoflavones in plants with estrogen (17β estradiol) in humans that can affect women's health [3].

The treatment between soybean flour extract doses showed no significant difference. However, this treatment tended to decrease the diameter of luminal ductal intralobularis. The results are suspected because in the treatment group get addition of exogenous estrogen. Phytoestrogen compounds in the form of isoflavones may replace the role of endogenous estrogens that do not directly affect the decrease luminal ductal intralobularisdiameter due to ovariectomy. The induction of estradiol may increase proliferation and branching of the ducts [2]. Therefore, givingsoybean flour extract can increase the proliferation of epithelial cells then decrease the diameter of luminal ductal intralobularis. Estrogens binding to REα in stroma can activate the paracrine factor of EGF (Epidermal Growth Factor), EGF binds to the protein kinase receptor present in the epithelium. The EGF bond complex with the protein kinase receptor activates the kinase proteins present in the cell cytoplasm. The result of activation of protein kinase is MAPK (Mitogen Activated Protein Kinase). Which is a major signal of transcriptional and translational activation resulting in protein synthesis used in the process of mitosis of

epithelial cells thus causing the epithelium to proliferate [10].

Duration of giving soybean flour extract showed no significant difference in doses 1, 2, and 3 for 10, 20 and 30 days. However, inter dose tends to decrease the diameter of luminal ductal intralobularisof the mammae gland. It is suspected that the longer of soybean flour extract the more exogenous estrogens that accumulate in the body so as to decrease the diameter of luminal ductal intralobularis. Potential phytoestrogens 10-3- 10-5 than natural estrogens, so exogenous estrogen in soybean flour extract can have an estrogenic effect by binding to estrogen receptors but not provide an equally strong oxygenic effect to natural estrogens [14].

The average calculation of the thickness of the epithelial layer of ductal intralobularisof the mammae glands can be seen in Table 2.

Table 2 Average thickness of the epithelial lining of ductal intralobularisof the mammary glands of Swiss Webster mice ovariectomy after giving soybean flour extract for 10, 20 and 30 days.

Treatment	Thickness of the epithelial lining of ductal intralobularis (μm), after giving soybean flour extract (days)		
	10 days	20 days	30 days
Control (-)	$9,23 \pm 0,30^{c}$	$9,26 \pm 0,25^{c}$	$9,30 \pm 0,26^{c}$
Control (+)	$5,43 \pm 0,15^{a}$	$4,40 \pm 0,36^{a}$	$3,23 \pm 0,15^{a}$
0,21 g/ml	$7,70 \pm 0,36^{b}$	$7,90 \pm 0,30^{b}$	$8,03 \pm 0,51^{b}$
0,42 g/ml	$7,96 \pm 0,15^{b}$	$8,06 \pm 0,47^{b}$	$8,40 \pm 0,30^{bc}$
0,63 g/ml	$8,20 \pm 0,60^{b}$	$8,66 \pm 0,50^{bc}$	$8,96 \pm 0,55^{bc}$

Description: numbers in the same column followed by different letters show significantly different p <0.01

Based on the results of One Way Anova analysis, there were significance values of 10, 20 and 30 p = 0,000 <0,01. This showed that the dose of soybean flour extract treatment had a significant effect on the thickness of ductal intralobularisepithelial layer. Based on the Duncan test there was very significant difference between negative control group and positive control group. The thickness of ductal intralobularisepithelial layerbetween positive controlsand all treatment groups of soybean flour extract showed very significant difference. Treatment between treatment doses showed no significant difference in the thickness of the ductal intralobularisepithelial layer. Based on One Way Anova analysis, the duration of soybean flour extract showed no significant difference at doses 1,2 and 3 at 10, 20 and 30 days.

Treatment for 10, 20 and 30 days there was very significant difference between negative control group and the positive control group. The results showed that ovariectomy for 30 days had an effect on the mammary gland, which was decrease in the thickness of ductal intralobularisepithelial layer. Treatment of ovariectomy may cause the mammary gland atrophy and cause the ductal epithelium to have flat atrophy [24]. Expression Transforming Growth Factor Beta 1 (TGF-β-1) may inhibit ductal morphogenesis in prepubertal mammary glands [23] and inhibit normal breast epithelial cell proliferation [19]. Estrogen may inhibit the expression of TGF-β-1 [12], so estrogen deficiency may allow for a decrease in the thickness of ductal intralobularisepithelial layer.

The thickness of ductal intralobularisepithelium between positive control and all treatment groups of soybean flour extract showed very significant difference. These results suggest that exogenous estrogen administration may have an estrogenic effect on the thickening

of ductal intralobularisepithelial of the mammary glands. Long-term estrogen administration for 8 months in menopausal monkeys causes a thick increase in epithelial tissue and an increase in epithelial cell proliferation [11]

Treatment between doses of treatment showed no significant difference. However, between doses of treatment tend to increase thickness of the epithelial layer of ductal intralobularis. These results suggest that isoflavone compounds in soy flour extract can have an estrogenic effect on epithelial cell proliferation. Genestein may have a stimulating effect of epithelial cell proliferation on the mammary glands [21].

The duration of soybean flour extract showed no significant difference in doses 1, 2, and 3. However, inter dose tended to increase thickness of the epithelial layer of ductal intralobularis. It is suspected that the longer the supply of soybean tempeh flour extract is more exogenous estrogen intake that can meet estrogen in the body so it can give estrogenic effect on the mammary gland. The half-life of genestein and deidzein is 6-8 hours after the administration of pure components [9].

Conclusion

Administration of soybean flour extract can decrease the lumen diameter and increase the thickness of the epithelial layer of ductal intralobularisof the mammary gland after ovariectomy. Giving at a dose of 0.63 g / ml / day with 30-day duration was the most influential treatment in decreasing lumen diameter and increasing the thickness of ductal intralobularisepithelial layer.

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THE EFFECTS OF METHOXYCHLOR ON EPIDIDYMIS STRUCTURE AND THE QUALITY ON MICE SPERMATOZOA (*Mus musculus* L)

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Abstract

The aim of this experiment is to analyze the effects of methoxychlor on epididymis structure and spermatozoa quality on mice (*Musmusculus* L). Mice were intraperitonial by methoxychlor dose 0.14 mg/g bw, 0.28mg/g bw and 0.42 mg/g bwonce in two days for 36 days. On the 37th mice were sacrificed right cauda epididymis was removed for histology preparation by paraffin method and Hematoxylin-Eosin stained. Except that left the cauda epididymis was prepared for measurement of spermatozoa quality.The result shows that MXC administered affected to epididymis structure and sperm quality. Thickness of epididymis epithel tended to decreased but decreased spermatozoa quality included the decreasing of spermatozoa motility and increasing of abnormal spermatozoa morphology. The conclusion from this research is MXC at the highest doses (0.42 mg/g bw) showed the worst effect on male reproduction system.

Keywords: methoxychlor, epididymis structure, spermatozoa quality, mice

1. Introduction

The progress of agriculture is accompanied by the production of various kinds of pesticide which most of the compounds can be pollutants to the environment. The use of pesticide can affect human health and population. One of the pesticides is methoxychlor which is mostly used as pest control of vegetables and fruits.

Methoxychlor (MXC) is one of organo-chlorine pesticides which is used as the alternate of dichlorodiphenyltrichloroethane (DDT) (Cupp*et al*., 2003). MXC is acompound that is estrogenic, anti-estrogenic and anti-androgenic that can interfere with hormone work or endocrine disrupter (Gaido*et al*. 1999). This compound binds to steroid hormone receptors that will interfere with endocrine function resulting in impaired reproductive ability, infertility, endometriosis, breast, uterine and prostate cancers (Gaido*et al*. 1999).

Latchoumycandane and Mathur (2002) claim that oral exposure of MXC at a dose of 100 mg/kg bw/day for 45 days decreases the weight of the testes, epididymis, seminal vesicles and prostate gland and it causes oxidative stress in rat testes. According to Latchoumycandane*et al* (2002), the administration of MXC with doses of 50, 100 and 200 mg/kg/day for 7 days resulted in a decrease in the weight of epididymis, seminal vesicles and spermatozoa, while MXC exposure for 4 days and 7 days reduced spermatozoa motility.

Research on the effects of methoxychlor on epididymis has not much been done. Based on this background, it is necessary to further examine the effect of methoxychlor on the epididymis structure and the quality of mice spermatozoa (*Musmusculus* L) Balb'C Strain.

2. Methods

The test material to be studied is Methoxychlor and the solvent is corn oil produced by MP Biomedicals, Inc. of France. Male mice (*Musmusculus* L) Balb'C strain obtained from *Pusvetma* Surabaya age 10 weeks, husk, feed in the form of pellet CP 511 production of PT Charoen Phokphand Surabaya.

The study was conducted using a completely randomized design, with one control and three treatments. Mice were given methoxychlor with dose 0.14 mg/g bw, 0.28 g/bw and 0.42 mg/g bw while control group was given corn oil. The treatment was given intraperitonally once 2 days for 36 days (18x injections). On the 37th mice were sacrificed, rightcauda epididymis was removed for histology preparation by paraffin method and Hematoxylin-Eosin stained. Except that left the cauda epididymis was prepared for measurement of spermatozoa quality.

The parameters observed were the structure of epididymis and the quality of spermatozoa including motility and abnormality of spermatozoa (Soeharno& Winarso, 1987)The data obtained were analyzed by one-way ANOVA, followed by DMRT test, at 5% confidence lever.

3. Results and Discussion

The results of the observation on epididymis after methoxychlor treatment for 36 days which include epithelial thickness and spermatozoa quality can be seen in Table 1.Statistical results with ANAVA showed that epididymal epithelial thickness after MXC administration was not significantly different from the control but there was a decrease in thickness by increasing doses.

Table 1. Epididymis epithelial thickness and quality of spermatozoa after treatment of methoxychlor

Dose (mg/g bw)	Epididymis epithelial thickness (µm) X±SD	Quality of spermatozoa (%) X±SD	
		Motility	Abnormal permatozoa
0 (control)	7,2477± 1,7129 [a]	72,7042 ± 5,7899 [a]	41,8143± 7,6784 [c]
0,14	7,0241± 1,5017 [a]	61,6182 ± 14,1471 [b]	45,9909± 12,0730 [bc]
0,28	6,9813± 0,8594 [a]	55,0167± 12,1688 [b]	54,2143± 10,6342 [ab]
0,42	5,0258 ± 0,7155 [a]	54,2315± 12,0636 [b]	59,5222± 8,6233 [a]

The same letter in the same column shows no significant difference

The percentage of motility of spermatozoa after MXC treatment with ANAVA showed a distinct difference with control. A further 5% DMRT test showed that MXC administration significantly reduced the percentage of spermatozoa motility from a dose of 0.14 mg/g bw. The percentage of abnormal spermatozoa after being analyzed by ANAVA showed that MXC administration had significant effect

on percentage of abnormal spermatozoa morphology. The results of 5% DMRT test showed that MXC administration significantly increased the percentage of abnormal spermatozoa morphology at doses of 0.42 mg/g bw, while MXC dosage 0.14 and 0.28 mg/g bw were not significantly different from the control.

MXC is a pesticide that has estrogenic, anti-estrogenic and anti-estrogenic properties. In the liver MXC undergoes metabolism into HPTE [2.2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane].
HPTE is a MXC metabolite that has a potentially more estrogen-like effect of MXC(Akingbemi*etal*., 2009).The decline in epididymal epithelial thickness and the quality of spermatozoa is suspected that MXC or HPTE metabolites are endocrin disrupter, which inhibits the function of Leydig cells in producing testosterone. According to Gore (2002) MXC provides negative feedback to the hypothalamic-pituitary-testis shaft. MXC metabolites bind to estrogen receptors that cause GnRH synthesis inhibition. Decreased synthesis GnRH causes decreased secretion of FSH and LH (Rochira *et al*., 2006), which causes disruption of Leydig cells to produce testosterone. Testosterone and FSH are required for the process of spermatogenesis. If the scretion of terstosterone and FSH is inhibited then spermatogenesis is also disrupted resulting in an increase in primary abnormalities in spermatozoa. Inhibition of testosterone secretion also causes disrupted maturation of spermatozoa in epididymis (Mahsa & Bernard, 2009).

Latchoumycandane dan Mathur (2002), free radical is a compound containing one or more free electrons that is unstable and reactive. Free radicals that have a high oxidative ability are refereed to Reactive Oxygen Species (ROS). Hydrogen peroxide is one of the ROS compounds that play a role in the process of reproduction. When ROS production exceeds the existing antioxidant capacity, it causes oxidative stress in cells. Furthermore Latchoumycandane and Mathur (2002) claim that administering MXC in male mice orally at a dose of 100 mg/kg/day for 45 days may induce oxidative stress caused by the increased hydrogen peroxide (H2O2) and lipid peroxide in the testes. Lamarande*et al* (1997) states that spermatozoa membrane is the main target of ROS while lipid is a potential target. Lipid plasma membranes of spermatozoa have high levels of phospholipids, causing spermatozoa very susceptible to ROS. High levels of ROS can also oxidize lipids, proteins, and DNA. Lipid oxidation of membrane spermatozoa produces a malondialdehyde (MDA) compound, which is toxic to the cell causing damage to the spermatozoa membrane. Damaged plasma membrane spermatozoa cause cell metabolism disorders thus increasing sperm abnormalities.

In this research the motility of spermatozoa decreased with increasing dose. The motility of spermatozoa is said to be normal when the percentage of motile spermatozoa (2 + 3) ≥ 50% (Ogli*et al.*, 2009). The mean of motility percentage of control group spematozoa, MXC dose 0.14; and 0.28 mg/g bw included normal category because the value is above 50%, while MXC dose 0.42 mg/g bw has a mean percentage of 43.16%, which means below the normal percentage. Decreased spermatozoa motility is expected because free radicals inhibit oxidative phosphorylation processes. Oxidative stress caused by increased production of ROS (Reactive Oxygen Species) causes disruption to the oxidative phosphorylation process in spermatozoa (Latchoumycandane & Mathur, 2002). Oxidative phosphorylation is a process of energy formation that involves complex enzyme which found in the inner membrane of mitochondria. Mitochondria spermatozoa are located in the central part of the spermatozoa, while the neck and tail function in the movement of spermatozoa.

After being synthesized in the mitochondria, ATP is transported to the axonem in the tail of the spermatozoa, then converted by dynein to the axonem that will decompose ATP into energy for spermatozoa movement. The inhibition of ATP release to the axonem resulting unfulfilled or reduced energy demand to move the tail, then the result is, spermatozoa cannot move fast or not move at all (Tremellen, 2008). The effect of MXC indirectly on the quality of spermatozoa can occur hormonally through inhibition the function of Leydig cells. Spermatozoa maturation is one of the endogenous factors that affect the motility of spermatozoa so that the disturbance in the process can decrease spermatozoa motility and increase secondary abnormalities in spermatozoa. The velocity movement and motility of spermatozoa is closely related to the morphological condition of spermatozoa. If the morphology of spermatozoa is abnormal then the movement of spermatozoa becomes disturbed. The percentage of spermatozoa abnormalities increased with the higher doses of MXC administered (Table. 1) and between treatment doses MXC showed significant differences. The morphology of normal mice spermatozoa is a hook-shaped head with normal size, long tail is neither circular nor double. The morphological observation of spermatozoa showed the existence of primary abnormality in morphology of mice spermatozoa like round head and flat head. Secondary abnormalities were found, head cracked, neck curled, broken neck, scab, broken tail, head without tail, and (Fig. 1). This is in accordance with the statement of Latchoumycandane *et al*. (2002) that the administration of methoxychlor (MXC) doses of 50, 100 and 200 mg/g bw for 4 or 7 days may increase "oxidative stress" by decreasing antioxidant enzymes accompanied by decreased epididymis weight and epididymis sperm quality. The decline in epididymis epithelial thickness and sperm quality leads to decreased fertility.The histological structure of the epididymis can be seen in Figure 1.

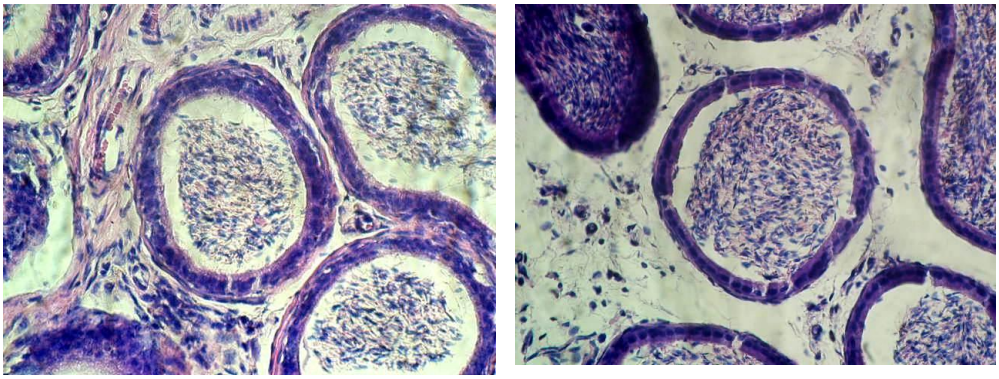

Figure 1. Epididymis histology structure after MXC treatment A.Control; B. MXC dose 0.42 mg / g bw

MXC treatment causes abnormalities in spermatozoa. There are two spermatozoa abnormalities, namely primary and secondary abnormalities. Primary abnormalities found are double-headed spermatozoa and head without acrosome whereas secondary abnormalities include neck forming loops, tail, tail fold, tail angle, neck bending, neck and tail forming loops. Images of morphological abnormalities can be seen in Figure 2.These abnormalities occur because of a decrease in testosterone levels.

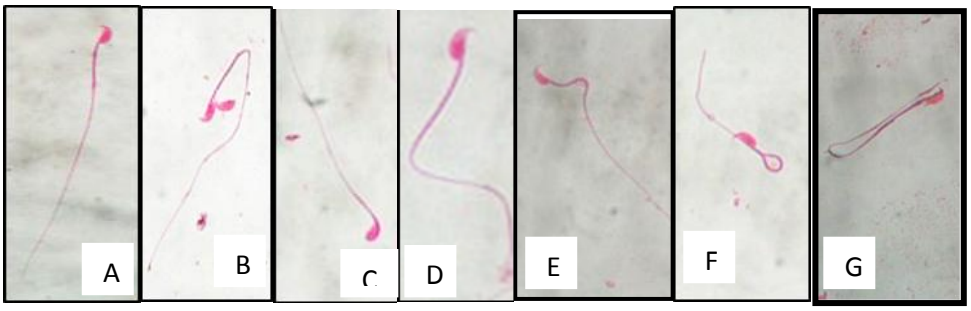

Figure 2. Morphological abnormalities of spermatozoa after methoxychlor treatment

A. Normal spermatozoa; B. Double head; C. head without acrosom; D. The tail forms an angle; E. neck bending; F. Neck forming a loop. G. The tail is over

Primary abnormalities occur during the development of spermatozoa in the testis seminiferous tubule. Primary abnormalities occur suspected due to spermatogenesis disorder caused by decreased testosterone levels. Secondary abnormalities occur during the process of maturation of spermatozoa in the epididymis. The function of epididymis depends on the hormone testosterone. Therefore if the testosterone levels decrease the maturation of spermatozoa becomes disturbed.

4. Conclusion

From this study, it can be concluded that administration of MXC at the highest dose (0.42 mg / gbb) tends to decrease epididymis epithelial thickness and significantly decrease the quality of spermatozoa.

5. Suggestion

From the results of this study can be found suggestion as follows:

For further research it is necessary to measure testosterone and FSH hormone levels

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