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Research Article

Effects of seaweed waste on the viability of three bacterial isolates in biological fertilizer liquid formulations to enhance soil aggregation and fertility

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Abstract: Biofertilizer production in Indonesia should fulfil the minimum requirement for being produced and released to the market. Problems occurred when those products are being absent on informing those expiration dates and the viability of microbial activity which then closely related to the quality of the product. Seaweed composted material are potential resources for producing Biofertilizer, but lacking on the optimization on their process as this material contain a various important component for soil and environment. The production of Biofertilizer from seaweed waste required an optimum condition, i.e.: pH and typical microbe which could germinate under specific formulation and temperature. This study aimed to determine the optimum pH in liquid fertilizer formulations made from seaweed waste in the form of composted material, to test the viability of three bacterial isolates and those pathogenicity properties, to examine the effect of metabolites release from bacterial isolates to green bean seed germination. The experimental design used was a completely randomized design with four treatments, which were as follow: P0 as a control (Peptone), RP1 (seaweed waste), RP2 (seaweed waste and glycerol), and RP3 (seaweed waste and PEG). The three bacterial isolates used were: (1) *Bacillus licheniformis, (2) Psudomonas plecoglossicida* and (3) *Pantoea ananatis*. This liquid fertilizer biological formulation was stored for 8 weeks at pH 5.5 and temperature 25°C. The results showed that the treatment of RP1 (seaweed waste) had high bacterial viability and could stimulate growth for green bean sprouts. The carrier material for seaweed waste with the addition of glycerol and PEG showed no effect of the disease and symptoms of a pathogenic bacterial consortium on germination of green beans.

Keywords: *biofertilizer, microbe, compost, pathogenity, seaweed waste*

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Introduction

The use of chemical fertilizers in the world continues to increase resulting in concern among environmental experts. This can increase the level of soil pollution which in turn can affect human health and soil microflora balance (Lingga and Marsono, 2000). At present farmers have started to use of various biological fertilizers in their farming system. Biofertilizers are inoculants made from active or latent organisms in a liquid or solid form that have the ability to mobilize, facilitate and increase the availability of non-available nutrients into available form through biological processes.

One of the problems on using biological fertilizers is the absence of expiration date information and those of microbial viability which then determine Biofertilizer quality (Husen, 2007). On the other hand, the application of seaweed

waste for agricultural crops has long been carried out in several countries. The use of seaweed waste for plants such as various types or forms of seaweed fertilizer product such as : (1) liquid seaweed fertilizer (LSF), (2) seaweed liquid fertilizer (SLF), (3) liquid fertilizer (LF), and chopped powdered algae manure that are commonly distributed on market (Sedayu et al., 2013).

Seaweed has long been used directly as a soil and fertilizer conditioner in various coastal regions (Haslam and Hopkins, 1996; Cocozza et al., 2011). Seaweed extract has also been widely marketed as an additive for the plant as a fertilizer in which those benefits and advantages of its use has been recently reported (Fornes et al., 2002; Padhiand Swain, 2006; Sivansankari et al., 2006). Chemically, seaweed consists of water (27.8%), protein (5.4%), carbohydrate (33.3%), fat (8.6%) crude fibre (3%) and ash (22.25%). Besides carbohydrates, proteins, fats and fibre, seaweed also contains enzymes, nucleic acids, amino acids, vitamins (A, B, C, D, E and K) and macro minerals such as nitrogen, oxygen, calcium and selenium as well as microminerals such as iron, magnesium and sodium.

In this study, a liquid formulation of Biological fertilizer made from seaweed waste was added by three indigenous bacterial inoculants obtained from previous studies (Arfarita et al., 2016; 2017; 2019), namely: *Pseudomonas plecoglossicida* which acts as exopolysaccharide-producing bacteria to enhance soil aggregation, *Bacillus licheniformis* which acts as a free N fixing bacteria and *Pantoea ananatis* which acts as a phosphate soluble bacteria. The use of all three bacterial indigenous isolates is important to improve nutrient availability, so that when biofertilizers are being applied in specific conditions of agricultural area, it can be repeated to the other regions, as long as it has similar climatic and soil conditions when biological condition of this fertilizers can be maintained in optimum condition. This study was conducted to determine the viability of liquid Biofertilizers made from seaweed waste in the form of composted material with the addition of 3 bacteria isolated and selected from previous studies.

Materials and Methods

Materials

The liquid Biofertilizer formulation was conducted by incubation technique in a white plastic bottle. Three indigenous bacterial isolates from previous studies (*Pseudomonas plecoglossicida, B. licheniformis, Pantoea ananatis*) were added as consortium inoculants. Media used in this study were NA (Nutrient agar), PCA (Plate Count Agar), NB (Nutrient Broth), Yoshida solution, sterile distilled water, 70% alcohol, green bean seed (Vima-1 varieties), glycerol, peptone, PEG 6000.

Purification of bacterial isolates

Each bacterium was scratched on the NA (Nutrient Agar) media using an ose needle. The purification results in the form of a single colony from each bacterium were taken using an ose needle and scratched again on NA media and then incubated for 24 hours. The purity of the isolate was observed using gram staining methods.

Optimizing pH for bacterial consortium growth

The pH optimization process for bacterial consortium growth was carried out under a completely randomized design (CRD), by testing all each pure isolates (*Pseudomonas plecoglossicida, Bacillus licheniformis, Pantoea ananatis*) as consortium bacteria isolates. This consortium isolate was grown on a 100 ml Erlenmeyer tube containing 50 ml NB (Nutrient Broth) with 5 pH treatments (5.0; 5.5; 6.0; 6.5; 7.0). The treatment was repeated four times (using the formula t (n-1) ≥ 15). Determination of bacterial consortium growth was carried out by measuring OD (Optical Density) from each treatment after a storage period of 3 days at room temperature. OD analysis was performed by taking 3-4 ml liquid bacteria from the consortium; then the result was detected using a spectrophotometer at a wavelength of 600 nm.

Manufacturing of liquid biofertilizer

The liquid Biofertilizer formulation was made using seaweed waste in the form of composted material, which then PEG was added into, followed by the addition of glycerol, and the three consortium bacterial isolates. The ingredients were mixed according to the treatment, namely : P0 (Pepton Water 0.1% + 3 bacterial isolates), RP1 (Seaweed waste + consortium of 3 bacterial isolates), RP2 (Seaweed waste + consortium of 3 bacterial isolates + 1% glycerol), and RP3 (seaweed waste + consortium of 3 bacterial isolates + 1% PEG). The carrier materials were sterilized first, then added additional ingredients and liquid cultures of bacteria, and stirred until homogeneous. Then put in a plastic bottle packaging, stored in a dry place, at room temperature and avoid direct sunlight.

Viability test for bacteria

The viability test was conducted using the spread plate method on PCA media. TPC (Total Plate Count) is carried out by taking 10 µl of liquid

bacterial culture in certain dilutions using peptone water. Petri dishes were incubated for 24 hours at 30ºC (Astuti., 2014).

Pathogenicity test

Green bean sprouts are grown in a test tube containing Yoshida's media. As much as 200 µml of liquid biofertilizer formulation was included in each test tube aseptically. Each treatment was repeated 4 times using a completely randomized design. The parameters observed from the pathogenicity test were the presence or absence of malformations, necrosis and decay. The observation was conducted to examine green bean sprout growth and total root length.

Data analysis

The analysis used was the analysis of variance (ANOVA) with a 5% F test to determine the effect of each treatment, followed by a 5% LSD test to find out the differences between treatments.

Results and Discussion

Microbial purification

Three bacterial isolates were purified with the streak plate method on NA (Nutrient Agar) media before added on to liquid culture as a bacterial consortium. After a single colony was obtained, then staining was done to observe the purity of the isolate (Figure 1).

Observation of optimum pH

Observation of optimum pH for bacterial growth consortium in liquid media was observed using a spectrophotometer. The quantity of bacterial growth was measured with light absorbed by the bacterial suspension. This was expressed in OD (Optical Density) at the OD600 value to read the level of turbidity in the Bacterial medium. Based on Figure 2, it can be seen that the optimum pH required by the three bacteria to grow in a consortium was at pH 5.5 with the results of OD 600 of 1.542.

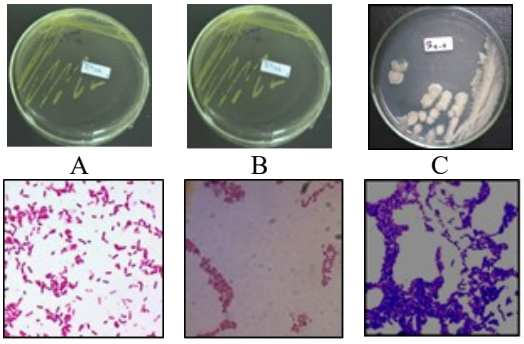

Figure 1. Above: morphology of bacteria colony, Below: morphology of bacterial cell of A) *Pantoea ananatis,* B) *Pseudomonas plecoglossicida,* C) *Bacillus licheniformis.*

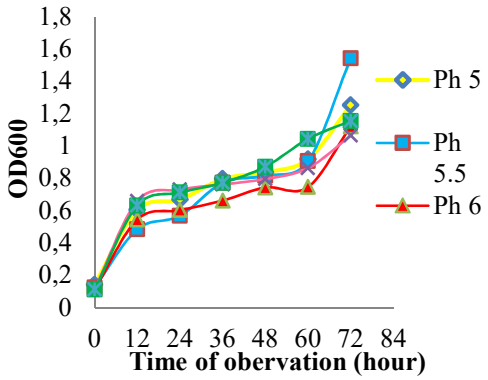

Figure 2. Consortium bacterial growth at liquid media shown by OD_{600} value under different pH status.

Viability test

The viability test was performed using the spread plate method to calculate the TPC (Total Plate Count) colonies. Liquid biofertilizer formulations that have been made were stored at room temperature (20-27° C). The observation was carried out once a week for a storage period of 8 weeks. Analysis of viability and growth was based on TPC log calculations as CFU (Colony Forming Unit), as presented in Table 1.

Table 1. The average number of colony (CFU/ml) across different of the liquid biofertilizer treatments over a period of 8 weeks incubation.

Treatments	Average of number of bacterial colony (CFU/ml), in weeks								
	W0	**W1**	**W2**	**W3**	**W4**	**W5**	**W6**	**W7**	**W8**
P0	6.32ab	10.61a	11.68a	13.52	12.87a	12.87a	12.33	12.32	11.84b
RP1	6.36b	10.98bc	12.36b	13.06	12.94a	12.94a	12.13	12.00	12.75c
RP2	6.66b	10.80ab	11.99a	12.83	14.32b	14.32b	12.47	12.30	12.00b
RP3	6.00a	11.17c	11.73a	13.5	13.50a	12.77a	12.22	12.05	11.07a
LSD 5%	0.34	0.20	0.32	Ns	0.53	1.39	Ns	Ns	0.35

Note: Number with a similar letter within a column showed not significantly different at a significant test of LSD 5%. Ns= Not significantly different.

Graphs of viability and growth of the consortium of three bacterial isolates from all treatments during the 8 weeks of observation showed significantly different ($P<0.05$) are presented in Figure 3. Treatment of P0, RP1 and RP3 reach the peaks of growth at the 3rd week, however, RP2 treatment obtained the best growth for the following week. After the eighth week of cultivation, all treatments meet declining population phase except for the treatment of RP1. By the end week of observation, the highest viability value was obtained at the treatment of RP1 (Table 1).

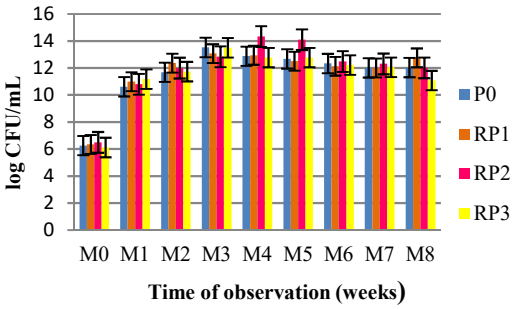

Figure 3. Bacterial viability (CFU/ml) across various liquid biofertilizer treatments. Over 8 weeks of incubation.

Pathogenicity test

Pathogenicity tests were observed until green bean sprouts were 6 days old by observing symptoms of necrosis, decay and sprout malformations compared to controls showed negative results (Figure 4). Whereas sprout growth is presented in Table 2.

Figure 4. The growth of green bean sprout at 1 day after planting (left) and 6 days after planting (right).

From Table 2, it can be seen that in term of the parameters of sprout length, the treatment of control and P0 was not significantly different in sprout length compare to RP1 and RP3 treatments, but not for RP2. In addition, this treatment was not significantly different compared to RP1 and RP3. Furthermore, in term of root length measurement, there was not significantly different across the treatments.

Table 2. Growth of green bean sprout across various liquid biofertilizer treatments.

Treatment	Sprout length (cm)	Root length (cm)
Control	4.74a	2.64
P0	4.71a	2.46
RP1	7.5ab	2.25
RP2	9.3b	2.35
RP3	6.9ab	2.04
LSD 5%	Sig	N-Sig

Note: Numbers with similar letters within a column showed not significantly different at a significant test of LSD 5%.

Discussion

Observation of optimum pH for bacterial consortium growth

The main factors that are affecting the growth of microorganisms include the supply of nutrients, time, temperature, water, pH and the availability of oxygen. Each organism has a range of pH values where growth is still possible, and each usually has an optimum pH. Most microorganisms can grow within the range of pH 6.0-8.0, and the pH value outside the range 2.0 and 10.0 is usually destructive (Winarwi, 2006). From observations of bacterial growth by observing the absorbance value of OD600 (Figure 2), it was found that at the 72nd hour of observation all treatments showed differences and the best results were shown at pH 5.5. Therefore, from this result, we can conclude that soil aggregate-fixing bacteria (*Pseudomonas plecoglossicida*), phosphate solvents (*Pantoea ananatis*) and N fixing bacteria (Bacillus licheniformis) can grow as a consortium with optimum pH 5.5. From the previous studies, it was known that *Pseudomonas plecoglossicida* grew optimally at pH 7.3 and optimum temperature 28ºC (Borica, 2009). Deepa (2009) shows that *Pantoeaananatis* can grow at pH 7.0 (pH minimum 4.0 and maximum 10.0) with optimum temperature 28ºC - 30ºC (minimum temperature 15ºC and maximum 37ºC). *Bacillus licheniformis* forms spores in the soil and produces various extracellular enzymes that are related to the cycle of nutrients in nature. The optimal growth temperature is at 50° C. The optimal temperature for enzyme secretion is at 37 ° C. Because it can grow in alkaline conditions, these bacteria are able to produce proteases that can survive at high pH. Protease *Bacillus licheniformis* has an optimal pH of about 9 and 10 (Pepe et al., 2003).

Viability test of bacteria in liquid biofertilizer

The liquid biofertilizer formulation used in this study is composted material from factory waste for seaweed. The addition of the carrier materials used is to add food content to prolong the life span of the bacteria in liquid biofertilizer formulations.

Hadioetomo (1990) revealed that a medium is a material used to grow microorganisms above or in it. The manufacture of liquid biofertilizer formulation is performed by utilizing seaweed waste added with bacterial isolates. This seaweed waste is wasted material; its existence is abundant and has a low selling price. The use of seaweed waste as fertilizer or fertilizer additives is expected to be an alternative solution to environmental problems because it is safe for soil and plant microbes and also increases the economic value of seaweed in Indonesia.

Chemically seaweed consists of water (27.8%), protein (5.4%), carbohydrate (33.3%), fat (8.6%) crude fibre (3%) and ash (22.25 %). Besides carbohydrates, proteins, fats and fiber, seaweed also contains enzymes, nucleic acids, amino acids, vitamins (A, B, C, D, E and K) and macro minerals such as nitrogen, oxygen, calcium and selenium as well as microminerals such as iron, magnesium and sodium (McHugh, 2003).

The viability test is carried out to determine bacterial growth. The growth of microorganisms is defined as the increase in cell weight. Cell weight is relatively the same in each cell cycle so that growth can be defined as an increase in the number of cells (Purwoko 2009). This phase shows the condition of the cell when it runs out of food, and there is a buildup of toxic metabolic products resulting in a decrease in the number of living cells (Fujikawa et al., 2004).

In general, bacterial media must contain sources of carbon, nitrogen, sulfur, phosphate, vitamins or ingredients that can promote bacterial growth such as meat extract or yeast. Meat extracts contain peptone and amino acid acids. Peptone is used in culture media as a source of nitrogen. Many simple nitrogen compounds are contained in peptones so that the nitrogen element is easily removed. Besides that, there are also bacteria that need fertilizers such as blood, serum and metals from inorganic salts as "trace elements" / microelements such as Ca, Mn, Na, Mg, Zn, Co, Fe, Cu (Collin and Lyne 1987). Peptone is an important component in microbial growth media which acts as a nitrogen source. Peptone by bacteria is broken down into amino acids, then absorbed to be used as an energy source and build cytoplasm (Wiranti, 2014).

Addition of peptone (treatment P0) with all three bacterial isolates able to add nutrients and to supply food to bacteria. The most important feature of peptone is its function as a source of nutrition for microorganisms so that it requires a high amount of nitrogen and amino acids that can support the growth of microorganisms (Astuti, 2014). However, peptone media is very expensive for the sake of commercialization and mass production for field applications. Its use here is as a control or comparison. The concentration used is based on the use of culture in the laboratory, and the appropriateness of costs for field applications. The treatment of RP2 consisting of seaweed waste, a consortium of three bacterial isolates and glycerol is known to be able to extend the viability of bacteria. Because the content of seaweed waste in the form of N, P and K is even rich in protein, flour, sugar, and vitamins A, C and D (Mc Hugh, 2003). The protein contained in seaweed waste is able to become a food source for bacteria to maintain its survival. Glycerol is the simplest compound, with hydroxyl which is hydrophilic and hygroscopic. Glycerol is a component that makes up various types of lipids, including triglycerides. Glycerol also functions as a stabilizer (stabilizer) from the carrier material and can extend the shelf life of the formulation (Advance et al., 2015). Glycerol (I, 2,3-propanetriol) or also called glycerin is a trihydric alcohol compound with the formula of building $CH_2OHCHOHCH_2OH$. Glycerol is a clear, hygroscopic, thick liquid, and tastes sweet. Glycerol is found in vegetable and animal oils and fats but is rarely found in its own form. Glycerol prepares oil and fat after mixing with fatty acids such as stearic acid, oleic acid, palmitic acid, and lauric acid (Kern, 1966).

RP3 is a liquid formulation added by PEG in addition to compost wash seaweed as its basic ingredient. PEG is a polymer consisting of several monomer bonds. Polyethylene glycol (PEG) belongs to the synthesis polymer group. Polyethylene glycol (PEG) is a synthetic compound that is widely used in the food, pharmaceutical, and, cosmetics and agricultural industries. PEG has soluble properties in warm water, non-toxic, non-corrosive, odourless, colourless, has a very high melting point (580 ° F), is spread evenly, hygroscopic (*volatile*) and can also bind pigments, so PEG can be used as an additional ingredient for bacterial viability.

But if seen at week eight, there is a difference in the treatment of RP1. When all other treatments have experienced a decline in the bacterial growth population, the treatment of RP1 is still experiencing a log phase. This shows that there is a need for additional periods to test the viability of RP1 so that it is known when the phase of population decline occurs. Then if viewed from the

base material from RP1, that is only in the form of seaweed waste without any additional ingredients.

Pathogenicity test

The results of the variance analysis of the length of the green bean plant are F count > F table. This showed that the concentration of PEG 6000 has a significant effect on the length of green bean sprouts (*Vigna radiate* L.), then further testing with LSD (Least Significant Difference) was carried out. This was because the carrier content in RP2 in the form of seaweed waste and glycerol was likely to stimulate the growth of green bean plants. Because the content of seaweed waste in the form of N, P and K is even rich in protein, flour, sugar, and vitamins A, C and D (Mc Hugh, 2003) is a nutrient needed during the vegetative period of green bean growth.

The consortium of three bacterial isolates namely *Pseudomonas plecoglossicida, Pantoea ananati*s and *Bacillus licheniformis* did not show pathogenic properties of these plants. At the age of six days, green bean plants continue to grow well as controls. The effect of the consortium's bacterial growth on liquid formulations does not produce substances that can cause necrosis, growth inhibition or growth sprouts.

Judging from the root length observations in all treatments showed a size that was not significantly different. This is presumably because the water requirements and the humidity conditions of the media from all treatments and controls are sufficiently met so that there are no noticeable differences in these parameters. Existing water functions as a nutrient solvent and maintains the temperature in the media. Water is a substance solvent (ingredients) in various things in chemical reactions.

Conclusion

Based on the literature, the optimum pH for growth of each isolate in the bacteria of *Pseudomonas plecoglossicida*, *Pantoea ananatis* and *Bacillus licheniformis* were different. However, when they were cultured under bacterial consortium for formulating liquid biofertilizer, the optimum pH was 5.5. Within the storage period up to 8 weeks, the formulation of bio-treatment RP1 (peptone with the addition of three bacterial isolates and glycerol) was giving the most optimal results for a long-term bacterial stationary period of 3 weeks, and the population of bacteria produced at 8 weeks was 12.75 x 10^{-9} CFU/ml. This was due to the formulation of the addition of glycerol can prevent bacteria cell wall integrity. The consortium of three isolates of bacteria did not produce any exudates in liquid form, which could result in diseases, for the germination of a green bean sprout. The significant difference of green bean sprout length, between the control and treatments, were likely due to the growth of hormones produced by the metabolism of the bacteria, or the effects of the additional material contained in that liquid fertilizer.

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