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Research articles

Exploration of indigenous soil bacteria producing-exopolysaccharides for stabilizing of aggregates land potential as biofertilizer

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Abstract : Steady soil aggregationis important for agricultural land which is formed by the micro-aggregate to become a macro-aggregate. This formation is mediated by organic material and various kinds of macro-organisms such as fungi, worms, ants and insects. An organic agencyinvolved in soil aggregation stabilityis exopolysaccharide (EPS) derived from bacterial, fungal mycelium, and products synthesized by plants. However, the use of EPS producing microorganisms as a biofertilizer has not been reported. This study was aimedto explore indigenous EPS-producing bacteria to solidify soil aggregationpotential for biofertilizer. Bacterial strains were isolated from soilsofthree regions at Malang East-Java; two areas of green bean plantation in Kendal Payak and Jambe Gede, as well as forest land. Soil samplewas derived from forest had hasa total bacteria population of 9.3 x 10^{11} CFU/mL.While soil samples from area Kendal Payak and Jambe Gede had total bacteria population of 1.5 x 10^{9} CFU/mL and 2.4 x 10^{9} CFU/mL, respectively. We selected three bacteria that could potentially produce abundant slime, namely as SPE-2, SPE-10 and SPE-20. The three selected bacteria are potential for biofertilizer because oftheir abundant slime, no antagonism and no symtoms as pathogen.

Keywords*: bacteria, biofertilizer, exopolysaccharide,indigenous*

Introduction

Soil fertility is the most important thing in agriculture because as a growing medium that is commonly used. Factors affecting soil fertility are texture, climate, organisms, altitude and water availability of the soil. Soil fertility is determined by physical, chemical and biological properties of the soil. The physical properties of the soil include effective depth, structure, soil texture, aggregation, field capacity of water, drainage, topography, humidity, climate and soil aeration.

Meanwhile, steady soilaggregateis very important for agriculture. The structure of soil with a steady aggregate will provide a positive influence on the growth of plant roots, water availability, and the movement of air and also able to enhance the resistence to disintegration when disruptive forces associated with tillage and water or wind erosion (Bronick and Lal, 2005). Soil aggregate is a group of sand, silt, clay and organic particles such as microbial cells that clot due to gum (adhesive), polysaccharides or other metabolites secreted by microbes (Hattori, 1998; Bertin et al., 2003). The formation of micro-aggregate becomesmacro-aggregates is mediated by organic materials and various kinds of macro-organisms such as bacteria, fungi, worms, ants and insects. Organic agency that can increase soil aggregate stability is exopolysaccharide (EPS) derived from bacteria, fungi mycelium and plant products synthesis.The formation of soil aggregates is generally influenced by EPS which is the result of the activity of microorganisms (Lynch and Bragg, 1985; Goenadi, 1995).

Some exopolysaccharide-producing bacteria that have been reported include *Pseudomonas aeruginosa*, *Erwinia*, *Ralstonia* and *Azotobacter vinelandii*. Exopolysaccharide protects the bacteria from a variety of environmental stresses (Iqbal et al., 2002), protects cells from antimicrobial compounds, antibodies, and bacteriophages, or for sticking to other bacteria,

animal and plant tissues (Wingender et al., 1999). Indigenus bacteria are bacteria that naturally live freely in nature and known to have high capability to survive in their habitat. Some researches utilizing indigenus bacteria have been reported, for example as a bioremediation agent(Arfarita etal., 2016; Nuraini et al., 2015).However, the use ofindigenous microorganisms producing-EPS as a biofertilizer, especially in Indonesia has not been reported. The study was aimed to obtain indigenous bacteria in areaof Malang-East Jawa, that are potential in producing exopolysaccharide (EPS).

Materials and Methods

The initial stage of exploration was to process a screening and isolation of the target bacteria around the rhizosphere of green bean plants in the three regions. Three bacteria had been selected and then observed its potential as a bacterial consortium that will be used as biofertilizer agent. Its potential was also supported by antagonism and pathogenicity test on germination of green beans.

Soil samples

Soil samples were taken in April 2016 from the rhizosphere of mung bean plants (*Vigna radiata* L.) in the area of Kendalpayak (SK) and Jambegede (SJ), as well as forest area in Junrejo (FS) Malang, East Java. Soil samples were taken by pullingout the plant along with its roots carefully.Plant shoot was cut and then the roots along the soil was added to plastic bags. They were kept in a cooling box and then taken to the laboratory or stored at 4-8°Cto isolate the bacteria immediatelly.

Total population of soil bacterial

Ten grams of soil samples were suspended in physiological saline solution (0.85%) and then made serial dilution to 10^{-8}. Calculation of the total population of soil bacteriawas made bythe Standard Plate Count (SPC) method. Certain dilution series of soil samples were made by taking10 µL suspension and spreading to PCA 9Palte Count Agar)medium on a petridish. Petridishes were then incubated for 2 days at room temperature.

Isolation and selection bacteria producing-EPS

The initial selection of bacteria potential of producing exopolysaccharide was performed by a screening process.Soil samples at specific dilutions of suspensions in a solid ATCC no. 14 medium were processed using spread plate method. Media ATCC No.14 per liter medium was prepared as follows: 0.2 g KH_2PO_4; 0.8 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 0.1 g $CaSO_4.2H_2O$; 2.0 mg $FeCl_3$; Na_2MoO_4. $2H_2O$ (trace); 0.5 g Yeast Ekstrac; 20 g sucrose) and 15 g bakto agarwith pH 7.2. Bacterial colonies appeared on selective medium were isolated based on differences of colony morphology for further study (Emtiazi et al., 2004). In the early stages of this exploration, three bacterial strains that produced abundant slime were selected based on morphological differences.

Morphological characteristicswere observed microscopically and macroscopically. At the macroscopic observation, the colony was observed the colours, shape of surface, edge, elevation, and the structure of colony. Microscopic was observed in gram staining properties and cell morphology.

Antagonism test

Antagonism test was performed by streaking selected bacteria on NA (Nutrient Agar) medium in the same petri dishes and incubated for 24 hours at room temperature. The test results of association among consortium isolates wereto show negative results if it didnot form a inhibation zone when cultured simultaneously. Thus isolates used in this consortium werenot antagonistic that couldbe cultured and used in a formulation of biofertilizer.

Phatogenicity test

The pathogenicity test was performed usinggreen bean sprouts as the test plants. Yosida medium was usedfor sprouts growth. Media stock was prepared by mixing 1000 mL solution consisting ofNH_4NO_3 80 g, NaH_2PO_4 40,3 g, K_2SO_4 71,4 g, $CaCl_2$ 88,6 g, $MgSO_4$ 32,4 g, $MnCl_2$ 1,5 g, $(NH_4)6Mo_7O_{24}$ 0,074 g, H_3BO_3 0,93 g, $ZnSO_4$ 0,035 g, $CuSO_4$ 0,031 g, $FeCl_3$ 7,7 g.The solution for planting was prepared by mixing 30 mL of stock solution with 4 liters of distilled water and heated the solution to a mixed material, divided into reaction tubes and sterilized.

Green bean sprouts (1-2 pieces) were inserted aseptically into tubes containing cotton and sterile Yosida medium and then inoculated 1 mL of pure cultures of bacteria into the tube (repeated 6-7 tubes). All of tube was incubated at the place with indirect sunlight. Observations were made every day for 7-10 days. Phatogenicity testwas to observe the presence of necrosis, lesions or abnormalities of sprouts growth comparing with control.The data of sprouts growth were analyzed using analysis of variance

(F test)with significant value ($p<0,05$) andif there were significant effectsthey were then followed by LSD test($p<0,05$).

Results and Discussion

Total population of soil bacteria

Total population of bacteria on PCA medium (Table 1) shows the highest number of population is SF soil sample of 1.5 x 10^{11} CFU/mL. This is due to the high content of organic matter in SF soil samples (Chemical soil analysis are presented in Table 2). These results are consistent withSuntoro (2003) that the organic matter content of soil affects to biological properties that can increase the activity and microbiological populations in the soil, especially related to the activity of the decomposition of organic matter. As for SK and SJ soil samples have a total population of bacteria2.4 x 10^9 CFU / mL and 9.3 x 10^9 CFU / mLlower then FS due to the organic matter content is lower than SF samples. SF soil samples are derived from forest area, where it has been known that forest soils are rich in organic material and have not been disturbed by chemical and human activities.

Selected bacteria producing-EPS

In this screening process had been obtained twenty-producing-exopolysaccharide isolates randomly. The presence of bacteria producing exopolysaccharide (EPS) on a selective medium were characterized by the ability of the growth and slimy colonies.

Exopolysaccharide-producing bacteria will grow well in a medium with a carbon source that is easily oxidized. ATCC medium which was used in screening process contained carbon sources used for optimization EPS bacterial growth medium consisting of sucrose, glucose, lactose, and 4-hydroxy-phenil acetic acid (4-HAA) and trace elements that were specific to the growth of EPS-producingbacteria.Name of exopolysaccharide (EPS) is the common name for all forms of bacterial polysaccharide that is found extrasellular and one of the bioactive products produced by microorganisms(Flemming and Wingender, 2001). Exopolysaccharide is a high molecular weight polymer composed of long-chain sugar residues and secreted by the microorganisms into the surrounding environment. Bacterial exopolysaccharide is a complex mixture of electrolytes macro-polymolekul included polisakrida, proteins and nucleic acids, each of which is composed of variable molecular mass and structural properties(Kumon et al., 1994).

Of the 20 isolates of slimy bacteria were then selected three isolates based onthe abundance of slime and morphological differences that significant for both macroscopic and microscopic morphology (Table 3).

Table 1. Total population of bacteria in Nutrient Agar (NA) media.

No	Soil Samples	Dilution	The average of EPS bacteria population and other bacteria
1	FS (Junrejo Forest)	10^{-7}	1.5 x 10^{-11} CFU/mL
2	SK (Kendal Payak)		2.4 x 10^{-9} CFU/mL
3	SJ (Jambegede)		9.3 x 10^{-9} CFU/mL

Table 2. Chemical analysis of soil samples

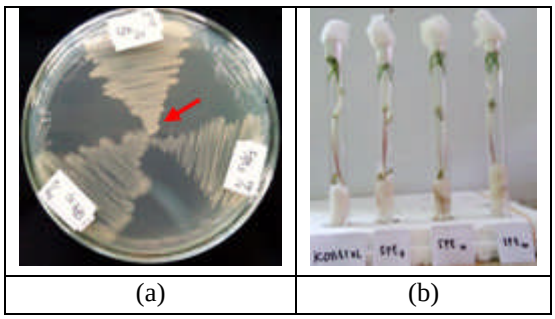
Code	C- Organic (%)	N-Total (%)	C/N	Organic Material (%)	P. Bray I (mg/kg)
Sample FS		0.19	7	2.31	4.67
Sample SJ	1.12	0.11	10	1.94	10.56
Sample SK	1.31	0.15	9	2.26	27.44

Table 3. Microscopic observation of 20 isolates of EPSBacteria Present on the ATCC Media, isolated Randomly Based on Differences of Morphological Colonies.

No	Isolate Code	Gram	Stainning	Morphology	Slime Formation
1	SPE $_1$		-	Staphylococcus	Slimy
2	SPE $_2$		-	Monobasil	Very Slimy
3	SPE $_3$		-	Coccus-Basil	Very Slimy
4	SPE $_4$		-	Monobasil	Very Slimy
5	SPE $_5$		-	Staphylococcus	Slimy
6	SPE $_6$		-	Staphylococcus	Slimy
7	SPE $_7$		-	Palisade	Rather Slimy
8	SPE $_8$	+		Palisade	Not Slimy
9	SPE $_9$		-	Streptobasil	Very Slimy
10	SPE $_{10}$		-	Staphylococcus	Very Slimy
11	SPE $_{11}$		-	Palisade	Rather Slimy
12	SPE $_{12}$		-	Streptobasil	Rather Slimy
13	SPE $_{13}$		-	Coccus -Basil	Rather Slimy
14	SPE $_{14}$	+		Coccus -Basil	Very Slimy
15	SPE $_{15}$		-	Coccus -Basil	Very Slimy
16	SPE $_{16}$		-	Staphylococcus	Very Slimy
17	SPE $_{17}$		-	Coccus	Rather Slimy
18	SPE $_{18}$		-	Streptokokus	Not Slimy
19	SPE $_{19}$		-	Palisade	Not Slimy
20	SPE $_{20}$		-	Coccus	Very Slimy

Antagonism activites

Figure 1(a) shows theresult of antagonism test of three isolates (SPE2, SPE10 and SPE20), which were then cultured simultaneously on NA media. It shows negative antagonism because it didnot form a zone of inhibition. Test antagonism in all selected three isolates were not antagonistic so that it can be cultured in fertilizer formulations as a potential bacterial consortium.



(a) (b)

Figure 1. (a) Antagonism test of three selected EPS- bacteria shows no inhibition after 5 days of observation, in which the ends of the streak can be fused (arrow direction). (b) Pathogenicity tests after 7 days of observation since inoculation of each EPS-bacteria show no pathogenicity.

Antagonistic bacteria commonly found around the root system of the plant roots or known as bacterial rhizosfir. Microbial antagonists are microorganisms that have an adverse effect on other microbes. Each microbe has its own mechanism and have more than one mechanism of inhibition. The mechanism of inhibition of biological control agents is the workings of biological control agents in the control of plant pathogen. The biological control agent typically works by using the results of secondary metabolism, either in the form of antibiotics, toxins, enzymes, hormones, and parasitism which do not involve the metabolism of secondary.

Phatogenicity activites

Based on the results of pathogenicity tests showed that three isolates of bacteria producing EPS that had been selected, namely SPE2, SPE10, SPE20 were not pathogenic. As shown in Figure 2 (b),there is nosymptoms ofnecrosis, lesions or abnormalities in growth compared to controls.

Observations on the growth of green bean sprouts were also performed by analysis of variance with a randomized block design (CRD) with 7 replicates. The results indicated that the length of the plant, total root length, and fresh weight showed a significant effect on the growth of green bean sprouts (Table 4). Average in the growth of green bean sprouts showsthat there is no inhibition on growth. With a growth rate greater than control, the possibility of selected

three bacteria were to produce growth hormone. In this study, exploration of bacterial EPS production potential as a biological fertilizer limited only tocharacterization ofthe antagonism test and pathogenicity test.

Biochemical analysis and identification of bacteria to the molecular level will be observed in the next study. These observations are to determine the bacterial species are really not a pathogen and to determine other potential of bacteria that had been selected in this study. Analysis of growth hormone is also necessary, because the growth in the average observation of green bean sprouts showed a higher growth than the control.As reported by Mu'minah et al. (2015), of 34 EPS bacterial isolates, 4 isolates produced high value of IAA.

Table 4. Average of the growth of green bean sprouts

Treatments	Average		
	Long Sprouts (cm)	Total length Roots (cm)	Fresh weights (g)
Control	14.33 ab	37.42 c	0.96 c
SPE2	12.92 a	31.67 b	0.84 a
SPE10	17.5 b	23.29 b	0.85 b
SPE20	19.00 a	35.00 a	0.84 a
F. Table 5%	Significant	Significant	Significant
LSD	4.06	9.65	0.08

Conclusion

The total population of bacteria producing exopoly-saccharide of each soil sample was different. Soil sample from Forest had has a total bacteria population of 9,3 x 10^{11} CFU/mL.While soil samples from area Kendal Payak and Jambe Gede were much lower than that of forest land,with total population of 1,5 x 10^{9} CFU/mL and 2,4 x 10^{9} CFU/mL, respectively. Three potential bacteria that produced EPS had been selected, namely SPE2, SPE10, and SPE20. Antagonism tests on three selected isolates showed no inhibitory zone occurs simultaneously when cultured on NA. Pathogenicity test did not indicate the nature of the pathogen against green bean sprouts. All three isolates are suspected growth hormone-producing, after germination was observed in green bean sprouts.Biochemical analysis and identification of bacteria to the molecular level need to be performed to determine the bacterial species are really not a pathogen and to determine the other potential of bacteria that had been selected in this study.Test aggregation on the ground also needs to be done in the laboratory and field conditions.

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