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Research Article

The potential of exopolysaccharide-producing bacteria from rhizosphere of rubber plants for improving soil aggregate

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Abstract: This study aimed to examine the effect of bacteria found in the rhizosphere of rubber plants in producing exopolysaccharides to improve aggregate stability of sandy soils. Samples of soil have been taken in rhizosphere of rubber plants in West Kalimantan. Serial soil samples were diluted and cultured on ATCC no.14 medium to select potential bacteria to produce exopolysaccharides. Forty-five isolates of exopolysaccharide-producing bacteria isolated from the rhizosphere of rubber plants was inoculated on ATCC no.14 medium. Based on the observations of morphological colony of these isolates, most of them had similarities in colour and shape so that only ten different isolates were obtained based on the morphological colony. Ten isolates were re-grown on MacConcey medium. Three isolates formed thick or slimy mucus when cultured on MacConcey medium. Three isolates grown on the medium of ATCC 14 resulted in dry weight of exopolysaccharide (mg/mL) varying from 0.28 to 7.59 mg/mL with sucrose and glucose as carbon sources. The results of the molecular identification of the three isolates of *Klebsiella sp.* LW-13, *Klebsiella pneumoniae* strain DSM 30104 and *Burkholderia anthina* strain MYSP113 showed that *Klebsiella sp.* LW-13 and *Burkholderia anthina* strain MYSP113 with 2% organic matter increased soil aggregate stability from highly unstable (30.67%) to unstable (45.01-48.20%). This aligned with the results by scanning electron microscopy (SEM) on treated soil and without bacteria treatments.

Keywords*: exopolysaccharides, sandy soil, rubber, bacteria, soil aggregate*

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Introduction

Sandy soil has very low aggregate stability, factors affecting aggregate stability among other soil tillage, soil microorganism activity and cover crop on the soil surface that can avoid splash erosion due to high rainfall. Soil aggregate are formed due to flocculation and fragmentation processes. Flocculation occurs when soil particles that are initially in a dispersed state, then combine to form aggregates. Fragmentation occurs when the soil is in a massive state, is then fragmented into smaller aggregates (Santi et al., 2010). According to Kusuma et al. (2016), soil aggregation is an important factor for the development of agricultural land and plantation functions. Stability of soil aggregates can be defined as the ability of the soil to withstand the forces that will destroy it. Strong soil aggregates will retain good soil properties for plant growth, such as porosity and water availability for longer periods than with unstable soil aggregates (Rachman and Abdurachman, 2006). A stable aggregate can create a good physical environment for root plant development. The aggregate soil is less stable when exposed to disturbance then the aggregate of the soil will easily be destroyed. Fine grains of crushed results will inhibit the soil pore so that the weight of the soil content increases, the aeration is poor and the permeability becomes slow (Santi et al., 2008). One effort to improve aggregate stability is with indigenous bacterial

producing exopolysaccharide. Exopolysaccharide-producing bacteria are currently receiving considerable attention in improving aggregate stability.

Exopolysaccharide (EPS) produced by Gram-negative and Gram-positive bacteria. According to Alami et al. (2000), the increase of soil aggregate stability in the area around rooting with the addition of inoculant exopolysaccharide-producing bacteria. Therefore, the importance of this research is to explore the role of bacteria in improving aggregate stability of sandy soils having very unstable aggregate stability.

The purpose of this study was to examine the effect of bacteria found in the rhizosphere of rubber plants in producing exopolysaccharides to increase the aggregate stability of sandy soil.

Materials and Methods

The initial stage of exploration was the process of a screening and isolation of target bacteria from rhizosphere of rubber plants in West Kalimantan. Examination of exopolysaccharide-producing bacteria interaction with organic matter related to bacterial ability to form soil aggregate and bacterial ability to increase aggregate stability of sandy soil proved from result of analysis of Scanning Electron Microscopy (SEM).

Isolation and identification of exopolysaccharide-producing bacteria

One gram of soil material was aseptically suspended in physiological saline solution (0.85%) and serial dilutions were made to 10^{-6} with Duplo and incubated in medium ATCC no. 14 (per liter of medium): 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 extract; 20 g sucrose; and 15 g agar bacto with pH 7.2 and Nutrient broth medium for seven days at a temperature of 28^{o}C (Remel, 2005; Santi et al., 2008). Selected bacteria which produce EPS that are characterized by colonies of bacteria that form thick slime (mucoid) (Tallgren et al., 1999) were purified by streaking the four quadrants to obtain single colonies.

The potential exopolysaccharide-producing bacteria form thick slime in a MacConkey medium. The selected EPS isolates were identified molecularly with 16S rRNA sequence analysis of pure DNA.The nucleotide sequence data of selected isolates was searched for by its nearest homology with another strain in the 16S rRNA gene database using BLAST (http://www.nebi.nlm.nih.gov).

Total population of exopolysaccharide-producing bacteria

Isolates of exopolysaccharide-producing bacteria were grown on Nutrient Broth medium with pH 4, 5, 7 and 8, incubated for two days at room temperature. After that, bacterial population was calculated by the Total Plate Count (TPC) according to the method proposed by Enriquez et al. (1995).

Sandy soil aggregation

Types of soil materials that have high sand fractions (FPT) 80-90%, were taken at a depth of 0-30 cm. The analyzes included texture, N (Kjeldahl method), P_2O_5 and K_2O (25% HCl extract), Mg (AAS), CEC (Soil Survey Staff, 1993), soil pH and C-organic of Walkley-Black method (Eneje et al., 2007).

The soil material was weighed 500 g and put into a heat-resistant plastic bag. Sterilization of soil used autoclave with a temperature of 121^{0}C, 1 atm for 20 minutes. Meanwhile, two loops of selected bacterial inoculants were each grown in 500 mL Erlenmeyer containing 300 mL of medium ATCC no. 14. Cultures were grown at room temperature for ± 72 hours above the shaker with a speed of 200 rpm. After 72 hours, the population of bacterial cells were counted until it reached a population of 10^9 cells/mL. The incubation was carried out at room temperature for 30 days under static conditions for comparative use of soil materials with sterilized inoculants.

The experiment was arranged in a completely factorial randomized design with two factors.The first factor was organic matter dosage 1% (v / b), 1.5% (v / b), 2% (v / b) based on fertilization recommendation of PT. Hutan Ketapang Industri was 10 kg/plant. The second factor was isolates of *Klebsiella sp.* LW-13, *Klebsiella pneumoniae* strain DSM 30104 and *Burkholderia anthina* strain MYSP113. Each treatment was repeated three times.

The determination of aggregate stability was made using multiple sieving methods (dry sieving and wet sieving) that was developed by De Leenheer and De Boodt (1959). Furthermore, the aggregate stability index was calculated by the formula: Stability Index = (1 / index of non-steadiness x 100). The aggregate stability index values are as follows:> 80 (very stable), 80-65 (stable), 65-50 (somewhat stable), 50-40 (unstable), and <40 (very unstable). The physical exopolysaccharide and its interaction on sandy texture soil material were observed by scanning electron microscopy (SEM). The treatments tested were:

1. Control
2. *Klebsiella sp.* LW-13
3. *Klebsiella pneumoniae* strain DSM 30104
4. *Burkholderia anthina* strain MYSP113
5. 1.0% OM + *Klebsiella sp.* LW-13
6. 1.0% OM + *Klebsiella pneumoniae* strain DSM 30104
7. 1.0% OM + *Burkholderia anthina* strain MYSP113
8. 1.5% OM + *Klebsiella sp*. LW-13
9. 1.5% OM + *Klebsiella pneumoniae* strain DSM 30104
10. 1.5% OM + *Burkholderia anthina* strain MYSP113
11. 2.0% OM + *Klebsiella sp*. LW-13
12. 2.0% OM + *Klebsiella pneumoniae* strain DSM 30104
13. 2.0% OM + *Burkholderia anthina* strain MYSP113

Data analysis

The data of this study were analyzed by analysis of variance, and results of the study were analyzed by F test, while the difference between treatments was tested by Duncan's Multiple Range Test at 5 % level using SAS software version 9.4 (Steel and Torrie, 1980).

Results and Discussion

Isolation and identification of bacteria

Forty-five isolates of exopolysaccharide-producing bacteria isolated from the rhizosphere of rubber plants were inoculated on ATCC no.14 medium. Based on the morphological colony observations of these isolates, most of them had similarities of colour and shape so that only ten different isolates were obtained based on morphological colony after it was purified by streak method to obtain single isolate.

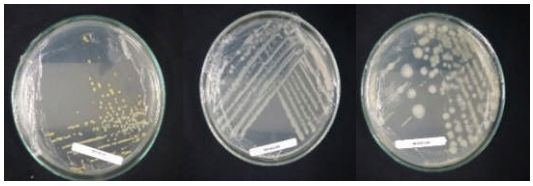

Figure 1. Purification of isolates by streak method to obtain fixed colonies on ATCC no.14 medium

Ten isolates were re-grown on MacConcey medium. Bacterial isolates that are able to grow well on a MacConcey medium are characterized by a thick slime. Isolates capable of growing on a MacConkey medium are grouped in Gram-negative bacteria (Mu'minah et al., 2015). Three isolates formed thick or slimy mucus when cultured on a MacConcey medium. Test results of three isolates grown on the medium of ATCC 14 resulted in the dry weight of exopolysaccharide (mg/mL) varying from 0.28 to7.59 mg/mL with sucrose and glucose as carbon sources. Data presented in Table 1 show that at 2% sucrose concentration, three isolates (RB51, RB292, and RB241) yielded exopolysaccharide dry weight of 7.53, 5.55, and 7.59 mg/mL, respectively. However, if glucose of the same concentration of 2% was used, the three isolates produced only exopolysaccharide dry weight of 4.27, 0.28, and 1.41 mg/mL, respectively. The results of this study corresponded to a study done by Emtiazi et al. (2004) that sucrose is the best source of carbon for the production of exopolysaccharides from *Azotobacter* strains AC2 and *Pseudomonas diminuta*.

Table 1. Average dry weight of exopolysaccharide in ATCC no.14 medium with two carbon sources incubated for 72 hours

Isolate	Source of carbon	Dosage (% b/v)	Dry weight EPS (mg/mL)
RB51	Glucose	1	5.22
		1.5	0.34
		2	4.27
	Sucrose	2	**7.53**
		2.5	6.07
		3	2.71
RB241	Glucose	1	0.29
		1.5	1.23
		2	0.28
	Sucrose	2	**7.59**
		2.5	7.29
RB292	Glucose	1	0.73
		1.5	0.75
		2	1.41
	Sucrose	2	**7.55**
		2.5	4.79
		3	1.97

Based on the analysis of exopolysaccharide formation, three potential exopolysaccharide-producing bacteria were obtained. The result of sequence analysis of 16S-rRNA showed that three isolates capable of producing high exopolysaccharide were *Klebsiella sp.* LW-13 that had a homologous index of 97% (RB51), *Klebsiella pneumoniae* strain DSM 30104 that had a homologous index of 98.65% (RB241), and *Burkholderia anthina* strain MYSP113 that had a homologous index of 98.83% (RB292). Extensively studied bacterial fucose-containing extracellular polysaccharides including colanic acid, fucogel and clavan were found in several

genera of *Enterobacteriaceae* including *Enterobacter*, *Salmonella*, *Escherichia* and *Klebsiella* (Alves et al., 2010, Freitas et al., 2011, Ratto et al., 2006). Fucogel is a polysaccharide produced by *Klebsiella pneumonia* I-1507. It is composed of galactose, 4-O-acetyl-galacturonic acid and fucose. It has also been reported that *Klebsiella oxytoca* can produce EPS by using glucose (Feng et al., 2009) and lactose (Dlamini et al., 2009) as a carbon source.

Population of exopolysaccharide-producing bacteria at various pH

To establish the ability to grow bacteria in soil environments with acidic pH, the isolates of potential exopolysaccharide-producing bacteria were grown in 50 mL of Nutrient Broth medium (NB) with pH 4, 5, 7 and 8, respectively. Growing bacteria in the Nutrient Broth medium with a given pH treatment is one of the criteria for determination of potential exopolysaccharide-producing bacteria. The bacteria were used for further testing. The results of testing the viability of selected bacteria on the pH treatment are presented in Table 1.

Table 2. Total population of exopolysaccharide-producing bacteria in Nutrient Broth medium (NB) at various pH.

Isolates	**pH**	**Total Population (CFU/mL)**
Klebsiella sp. LW-13	4	1.4×10^6
	5	7.5×10^6
	7	9.1×10^7
	8	2.2×10^8
Klebsiella pneumoniae strain DSM 30104	4	4.1×10^6
	5	4.9×10^6
	7	8.3×10^6
	8	2.0×10^7
Burkholderia anthina strain MYSP113	4	1.9×10^6
	5	1.6×10^7
	7	3.2×10^7
	8	1.4×10^8

Three potential exopolysaccharide-producing bacteria could grow well in the pH ranging from 4 to 8. The resistance of bacteria were with the *Klebsiella sp*, *Klebsiella pneumoniae* and *Burkholderia anthina* isolates on NB medium with pH 4 (10^6 CFU/mL). Based on testing of bacterial resistance to acid pH showed that all isolates of exopolysaccharide-producing bacteria were able to grow and adapted well in pH 4-8. According to a research done by Torres et al. (2012), the maximum production of exopolysaccharide (>7 g/L) at a temperature of 25-35^{0}C and pH 6.0-8.0. Other studies suggested that optimum pH and temperature are 7.0 and 30^{0}C, for growth synthesis and EPS by *E. cloacae* WD7 (Prasertsan et al., 2008) and *E. agglomerans* WD50 (Prasertsan et al., 2006). According to Imran et al. (2016), at neutral pH, EPS production increased in both *L. plantarum* NTMI05 (0.35± 0.03 g/L) and NTMI20 (0.32 ± 0.02 g/L).

Testing of exopolysaccharide-producing bacteria in increasing aggregate stability

In the early stages of testing in the laboratory used organic materials with dosages of 5, 7.5 and 10 g/pot and three selected isolates, while the type of soil material used had 91.70% sand fraction content. This stage was aimed to obtain an initial description of the potential of exopolysaccharide-producing bacteria in an aggregation of sandy soil. The sandy soil used in this study had very low cation exchange capacity, N, P, K, and C-organic contents. Soil material with sand fraction reached 91.70% has no structure so that it can be said that soil aggregate stability is very unstable (30.61%). Sandy soils have porosity of less than 50%, with the number of macro-pores larger than the micro-pores making it difficult in storing water and low nutrient content. Exopolysaccharide-producing bacteria testing with the addition of organic matter interacted significantly with the soil aggregate stability index. Table 3 shows that increasing the dosage of organic matter (1, 1.5 and 2%) increased the aggregate soil aggregation index value. The highest aggregate soil index value was significantly different with the other treatments obtained with 2% organic matter, the increase of soil aggregate stability index value reached 30-50% from the initial condition. Research conducted by Yatno (2011) indicated that giving of organic material in the form of manure, compost from the rest of bagasse, and mulch of plant residues of 5 to 15 t/ha had the significant effect to increase aggregate stability.

Table 3. Effect of organic matter on aggregate stability index (ASI) of soil with 30 days incubation time

Organic matter	**Dosage % (b/v)**	**ASI (%)****
Manure	1	39.52 b (STS)
	1.5	40.66 b (TS)
	2	44.89 a (TS)
Coefficient of variation (%)		9.04

Remark : Mean values within a column followed by the same letters are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test. **note: STS (very unstable), TS (not stable)

Testing of combination of bacterial inoculum and organic material presented in Table 4 found that increasing dosage of organic material in the form of animal waste from 1 to 1.5 and 2% could increase the aggregate stability index, even in isolate RB51 and RB292 with 2% organic matter increased soil aggregate stability from highly unstable (30.67%) to unstable (45.01-48.20%) or the increase of 35-60% compared to without organic matter. According to Santi et al. (2008), the inoculation treatment of *Flavobacterium sp.* PG7II.2, raised the milk from 31.95 (very unstable) to 41.34 (unstable).

Table 4. Influence of bacterial inoculums and organic material to aggregate stability index (ASI) of soil with 30 days incubation time

Isolate	**Dosage of Organic Matter % (b/v)**	**ASI**	**(%) ****
Klebsiella sp	1	42.42 abc	(TS)
	1.5	43.75 abc	(TS)
	2	47.87 a	(TS)
Klebsiella	1	37.94 c	(STS)
pneumoniae	1.5	40.51 bc	(TS)
	2	41.01 bc	(TS)
Burkholderia	1	38.21 c	(STS)
anthina	1.5	38.67 c	(STS)
	2	45.79 ab	(TS)
Klebsiella sp	0	31.11 d	(STS)
Klebsiella pneumoniae	0	30.97 d	(STS)
Burkholderia anthina	0	30.48 d	(STS)
Control	2	30.61 d	(STS)
Coefficient of Variation (%)		8.32	

Remark : Mean values within a column followed by the same letters are not significantly different at p<0.05 according to Duncan's Multiple Range Test. *note: STS (very unstable), TS (not stable)

The ability of bacteria in improving sandy soil aggregate stability was evidenced from the results of analysis of Scanning Electron Microscopy (SEM) which showed the existence of binding between aggregate granules of sandy soil material by exopolysaccharides bacteria. Data presented in Figures 2 and 3 show that *Klebsiella sp.* LW-13 and *Burkholderia anthina* strain MYSP113 underwent ammonification reactions and carbonate precipitation to form exopolysaccharides that acted as clogged pores of the soil. Exopolysaccharides that are attached to the wall of soil particles will further fill the pores of the soil through a process called bio-cloging. While in soil material without bacterial inoculation showed no aggregate grain binding and there were still open pores (Figure 4).

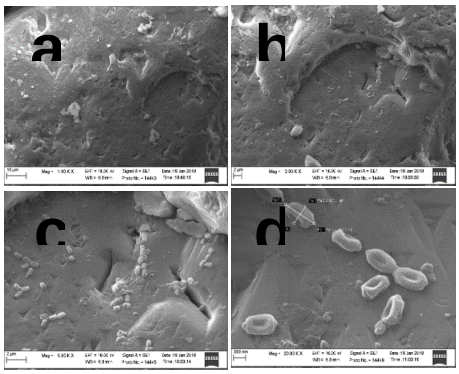

Figure 2. SEM test results of soil samples with *Burkholderia anthina* inoculation. LW-13 (left) a. magnification 1.00 K, b. magnification 2.00 K, c. magnification 5.00 K, d. magnification 20.00 K

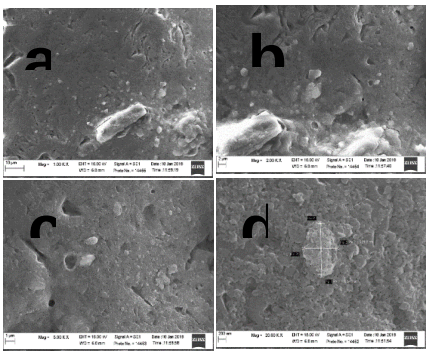

Figure 3. SEM test results of soil samples with *Klebsiella sp.* inoculation. LW-13 (left) a. magnification 1.00 K, b. magnification 2.00 K, c. magnification 5.00 K, d. magnification 20.00 K

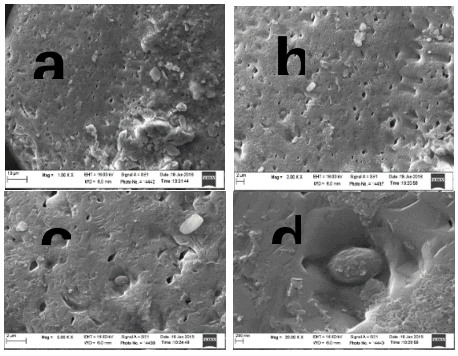

Figure 4. SEM test results of soil samples without inoculation (left) a. magnification 1.00 K, b. magnification 2.00 K, c. magnification 5.00 K, d. magnification 20.00K

Conclusion

The result of isolation of exopolysaccharide-producing bacteria showed that three isolates were capable of producing high exopolysaccharide, namely *Klebsiella sp.* LW-13, *Klebsiella pneumoniae* strain DSM 30104 and *Burkholderia anthina* strain MYSP113.Those which were able to increase sandy soil aggregate were *Klebsiella sp* LW-13 and *Burkholderia anthina* strain MYSP113 with 2% organic matter. The two bacteria increased soil aggregate stability from highly unstable (30.67%) to unstable (45.01-48.20%) aligned with the results of scanning electron microscopy (SEM) on treated soil and without bacteria treatments.

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