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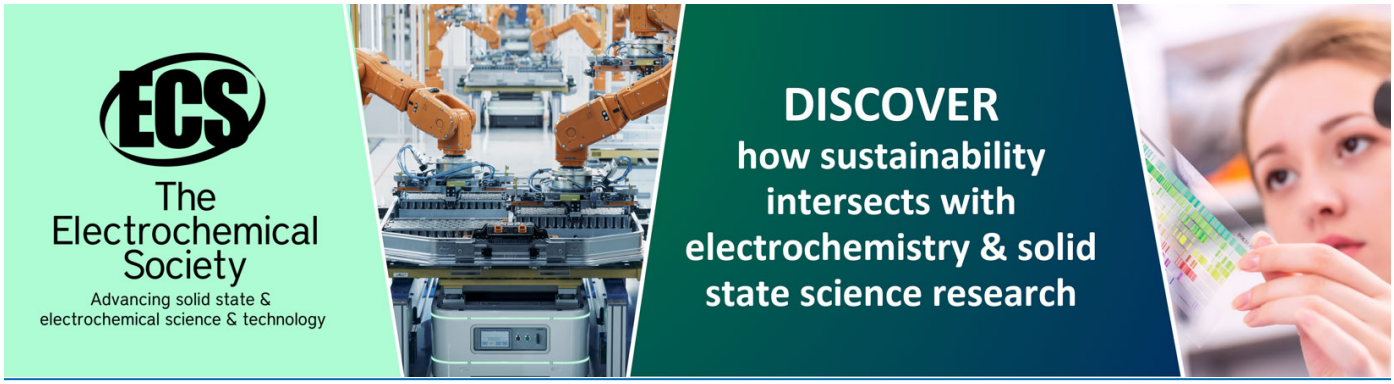

Drought resistant of bacteria producing exopolysaccharide and IAA in rhizosphere of soybean plant (*Glycine max*) in Wonogiri Regency Central Java Indonesia

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Abstract. Drought is one of the main problem which limitating the agriculture productivity in most arid region such as in district Eromoko, Wuryantro and SelogiriWonogiri Central Java Indonesia. Bacteria are able to survive under stress condition by producte exopolysaccharide. This study aims to determine the presence of exopolysaccharide-producing drought-resistant bacteria on rhizosphere of soybean (*Glycine max*) and to determine the species of bacteria based on 16S rRNA gene. Isolation of bacteria carried out by the spread plate method. The decreased of osmotic potential for screening drought tolerant bacteria according to the previous equation [12]. Selection of exopolysaccharide-producing bacteria on solid media ATCC 14 followed by staining the capsule. 16S rRNA gene amplification performed by PCR using primers of 63f and 1387r. The identificationof the bacteria is determined by comparing the results of DNA sequence similarity with bacteria databank in NCBI database. The results showed 11 isolates were exopolysaccharide-producing drought tolerant bacteria. The identity of the bacteria which found are *Bacillus sp*, *Bacillus licheniformis*, *Bacillus megaterium* and *Bacillus pumilus*.

1. Introduction

Drought is one of the main problem whichlimiting agricultural productivity in the arid region [1]. Wonogiri is an area that has low rainfall levels. In 2013, between Augusts to September in three districts in Wonogiri namely, Eromoko, Wuryantro, and Selogiri encounter drought because there is no rainfall. Limited water availability in the soil can inhibit the absorption of nutrients by the roots of plants, especially legumes.

Drought can affect on the growth of microorganisms and plants as the water becomes a limiting factor for plants and microorganisms to survive. Microorganism adaptation to drought is to secrete exopolysaccharide (EPS) in high quantities [2]. Bacteria are able to survive under stressful conditions due to the production of EPS [1]. EPS is a structural component of the extracellular matrix in biofilms that are synthesized by cells of microorganisms in response to physiological stress on the environment [3]. EPS have important role for bacteria to protect against a broad range of environmental stress. Such as the bacterium Pseudomonas sp. which increases the production of EPS during the dry season [4]. Production of EPS of Rhizobium sullae KYGT207 strains isolated from dry land in Southern Algeria is known to have a role in the absorption of water and nutrients. EPS in sandy soils can protect plants from stress, lack of water and contribute to the formation of soil aggregates [2].

Bacteria from a broad range of genus are an important component of the soil. The bacteria involved in the various biotic activity of the soil ecosystem to be dynamic for the exchange of nutrients and support agricultural production [5]. A group of bacteria that can be found in the rhizosphere, which is advantageous in improving the growth of plants, can be classified as plant growth-promoting rhizobacteria (PGPR) [6, 7]. Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, and Serratia have been reported as PGPR able to promote plant growth [7].

Bacteria belonging to the PGPR are one of high producing IAA. One of the PGPR is one of potential IAA-producing bacteria. IAA-producing bacteria touch some physiological processes of plants by inserting the IAA that it generates to the plant. The effect on crops is changing its IAA concentration thus helps in the formation of lateral roots, adventitious roots and primary root elongation [8]. Condensed root can increase the nutrients quickly, thus increasing plant growth [9]. Also, rhizosphere bacteria are able to secrete siderophores, dissolving phosphate, and act as a biocontrol that protect crops from fungi, pathogens, and pests [10].

Soybean (*Glycine max*) is one of the primary sources of protein crops in Indonesia. Soybean becomes one of the primary food commodities other than rice and corn [11]. Soybean is one of the agricultural products commonly grown in upland areas in the district Eromoko, Wuryantro, and Selogiri, Wonogiri. To develop sustainable agriculture, it is needed to know the potentially exopolysaccharide producing bacteria as producers of IAA in the rhizosphere of soybean crops that grow under drought stress conditions.

2. Methods

2.1. Isolation of Rhizosphere Bacteria

Bacteria isolated from the dry soil around the roots of soybean (*Glycine max*) using a dilution series (Platting Method). In the rhizosphere soil of soybean plants as much as 1 gram dissolved in 10 mL of normal saline (0.85% NaCl), vortex, and heated in a water bath at a temperature of 80ºC for 20 minutes, then carried serial dilutions, to obtain 10-1 dilution, Dilution performed until dilution to 10-2. Each series of dilutions taken 100 mL put in a petri dish that already contains media NA and propagated using stem L to rub it on the surface so that droplets of the suspension evenly. The cup and its contents were incubated upside down in an incubator at 38ºC for 24-48 hours. Furthermore, the different morphology of each colony that grows on the medium NA selected and grown on agar slant NA as the pure culture.

2.2. Preparation of Medium and Osmotic Pressure

Nutrient Broth medium are sterilized, added with polyethylen glycol (PEG) 6000 while still hot for lowering osmotic potential to follow the equation according to [12]:

$\Psi s = - (1{:}18 \times 10\text{-}2)\ C - (1{:}18 \times 10\text{-}4)\ C2 + (2.67 \times 10\text{-}4)\ CT + (8{:}39 \times l0\text{-}7)\ C2T$

Description:

Ψs = osmotic potential (Mpa)

C = concentration

T = ambient temperature (oC)

Osmotic potential that is set is -1 MPa, -1.5 MPa, -2 MPa.

2.3. Screening Drought Tolerant Bacteria

1000 mL of a suspension of bacteria grown on NB medium with a population of around 1,5x108 CFU / mL was inoculated into 20 ml NB medium that has been coupled with PEG 6000. These cultures were incubated in an incubator shaker at a speed of 80 rpm at a temperature of 38ºC for 24 hours, Optical Density measurement using a spectrophotometer at a wavelength (λ) 570 nm. Value of drought tolerance to Optical Density (OD) was determined as: Highly Sensitive OD <0.3; Sensitive OD = 0.3-0.4; Tolerant OD = 0.4-0.5; Highly Tolerant OD> 0.5 [13].

2.4. Test of exopolysaccharide production

A selection of potential bacteria producing-exopolysaccharide conducted by growing a loop of bacterial isolates on solid media in a Petri dish ATCC 14 for seven days [4] at a temperature of 38ºC. Colonies of bacteria that form slime or mucus thick exopolysaccharide show that it contains.

2.5. Capsules Staining

Glass objects cleaned using 70% alcohol. Then at the bottom of the glass object is labeled with a marker. As much as one drop of physiological saline dripped on the surface of the glass object. As much as a loop of bacterial culture was swapped on the surface of the glass object and homogenized and then fixed over the Bunsen burner until visible dry evenly. Results of preservation of bacteria dropped with a drop of crystal violet and allowed to stand for 3 minutes. Then rinsed with water and dried. Mixture drops with a Chinese ink at the edges of the glass objects. Mixture smear results are then dried. Furthermore, preparations added immersion oil for observation with a microscope.

2.6. IAA production

IAA produced by the bacteria is measured by a colorimetric method with the addition of Salkowski reagent [14]. Bacterial isolates were inoculated into 10 ml of nutrient broth (NB) medium that has been added 0.2 Mm L-tryptophan and NB media without the addition of L-tryptophan. The cultures were incubated and shaken (80 rpm) at room temperature for 48 hours. 3 ml cultures of each treatment are divided into two microtubes and then centrifuged (10,000 rpm) for 15 minutes. A total of 2 ml of the filtrate obtained is inserted into a sterile test tube and add 2 ml of Salkowski reagent. Then, the suspension was incubated for 60 minutes in the dark at room temperature. After that, IAA levels were measured using a spectrophotometer at a wavelength of 530 nm.

2.7. Amplification of 16S rRNA encoding-gene

16S rRNA gene was amplified using the Polymerase Chain Reaction (Perkin Elmer PCR system geneAmp 2400). PCR mix done by mixing a universal primer for the group of bacteria, which is 63F (5'-CAG GCC CAC ATG TAA CAA GTC-3 ') and 1387r (5'-GGG CGG WGT CAA GGC GTA-3'), 1.25µl, 2X KAPA 2G Fast Ready Mix 12.5 mL, 2 mL total DNA template, as many as 8 mL ddH2O. Cycle used consisted of pre-denaturasi at a temperature of 95ºC for 3 minutes, denaturation at a 95ºC for 15 seconds, annealing at 55ºC for 15 seconds, elongation at a temperature of 72ºC for 5 seconds for 30 cycles and finalizing at a temperature of 72ºC for 1 minute. The process of denaturation, annealing, and elongation each consisting of 35 cycles [15]. The PCR products then sequenced at 1st Base Singapore.

2.8. Data Analysis

Data were analyzed descriptively includes the exopolysaccharide capsule which is produced as the bacteria tolerant to drought, especially PGPR bacteria capabilities as the production of IAA. 16S rRNA gene sequences were analyzed by bioinformatics at GenBank data center by using the program Basic Local Alignment Search Tool (BLAST) on the website National Center for Biotechnology Information (NCBI), (http://www.ncbi.nlm.gov/BLAST/).

3. Results And Discussion

3.1. Drought Tolerant Rhizosphere Bacteria

Drought stress conditions were made by the addition of polyethylene glycol 6000 (PEG-6000) to the culture medium to adjust the osmotic pressure [12]. PEG-6000 can be used to mimic the magnitude of potential ground water or drought stress level [16]. A selection of bacteria that are resistant to drought observed begin at -1 MPa pressure. Bacteria that have a value of Optical Density (OD) greater than 0.5 at the osmotic pressure of certain bacteria are categorized as a genuinely tolerant [13]. The selected bacteria can survive and grow well under conditions of drought stress.

Tolerance to drought stress conditions of 34 isolates was selected 11 best isolates with OD values at pressures above 0.5 MPa -1. At 11 isolates contained 9 isolates were highly tolerant of drought, drought-tolerant 1 isolates, and 1 isolates were highly sensitive to drought (Table 1). Isolates E2, E4, E5, E11, S3, W6, W11, W13, W19 grouped into categories that are very tolerant because it has the OD> 0.5 in the third osmotic pressure, ie -1,0MPa, -1,5MPa, -2, 0MPa. E13 isolates included in the class tolerant because of the decrease in osmotic pressure OD value fell to 0.4879 at the osmotic pressure -2,0MPa. Isolates S6 listed in the class is very sensitive because of the decrease in osmotic pressure OD value decreased to 0.2745 at the osmotic pressure -2,0MPa. The high osmotic pressure outside bacterial cells causes the fluid inside the cell will diffuse out of the cell, so that the destruction of microbial cell walls will cause lysis. Tolerance is a condition where bacteria can survive and persist in the environment is less favorable, namely drought. Meanwhile, the sensitive is a state in which bacteria cannot survive because the environment does not support life.

Table 1. Tolerance levels of rhizosphere bacteria of soybean roots (*G. max*) on NB media added PEG 6000

Bacterial Isolate Code	Optical Density (OD) Value in Osmotic Pressure (MPa)			Category
	-1,0	-1,5	-2,0	
E2	1.7596	0.7493	0.5946	highly tolerant
E4	1.3652	1.1079	0.7866	highly tolerant
E5	0.8516	0.7227	0.6322	highly tolerant
E11	1.6334	1.4092	1.1641	highly tolerant
E13	1.5300	0.8929	0.4879	Tolerant
S3	1.2897	0.7952	0.7357	highly tolerant
S6	0.9746	0.3361	0.2745	very sensitive
W6	1.6741	1.5578	1.3743	highly tolerant
W11	1.7177	1.3042	1.1583	highly tolerant
W13	1.5870	1.4436	1.2403	highly tolerant
W19	1.5428	0.9903	0.5989	highly tolerant

Populations of soil bacteria decreased with stress or stress on the water, but not entirely the same, and certain soil bacteria can withstand extreme drought conditions [13]. Bacteria can survive in drought conditions through several mechanisms such as the production of exopolysaccharide (EPS), biofilm formation, and production osmolit to avoid the shortage of water in the cell [17].

3.2. Producing Bacteria exopolysaccharide

Source sucrose contained in ATCC medium can be used by bacteria to form exopolysaccharide. Colonies of bacteria that form slime or mucus thick exopolysaccharide show that it provides exopolysaccharide. Bacteria then examined the physical structure of the capsule staining. Capsule of the bacteria can not be colored with Indian ink [18]. Exopolysaccharide often found around the outside structure of prokaryotic and eukaryotic microbial cell. The physical structure exopolysaccharide in the form of a capsule to the cell wall of the massive slime formed outside the bacterial cell membrane [19].

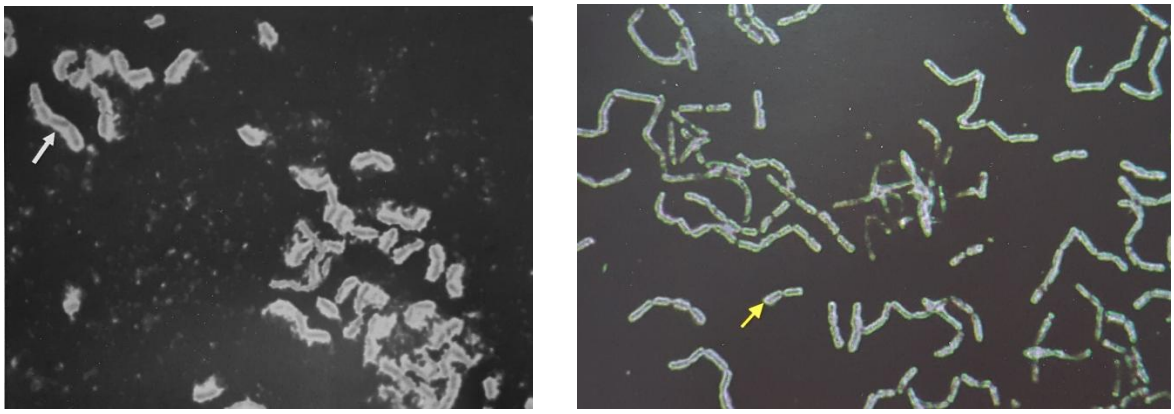

Figure 1. The bacterial capsule (arrow) is seen as a white exopolysaccharide layer not colored by ink dye

3.3. Producing Bacteria Indole Acetic Acid (IAA)

The ability of bacteria to produce IAA was tested by using colorimetric method with the addition of reagent salkowski according to [14]. Salkowski addition of reagents used to detect the presence of the compounds synthesis of IAA. The data (Table 4) show that each bacterial isolate produce IAA less and tend to be low. IAA production has decreased in drought stress conditions [20]. Based on research [17]. All the strains showed the ability to produce IAA with the L-tryptophan as a precursor. Some species of bacteria have been known as a producer of IAA, such as Pseudomonas sp., Bacillus sp., Azotobacter sp. and others [21]. However, some strains show little variation synthesis, and even strains of the same genus (Bacillus) produced a different amount of IAA in liquid media. This can happen because the production of IAA can be affected by conditions of culture, the phase of growth and the availability of substrate.

Table 2. IAA-producing bacteria from soybean plant rhizosphere

Code of bacterial isolate	IAA concentration (ppm)		color reaction
	Without L-triptophan	with the addition of L-triptophan	
E2	-0.5928	1.0652	++
E4	-0.4802	0.5474	+
E5	-0.3221	0.9249	+
E11	-0.6462	0.0573	+
S3	-0.8932	0.5296	+
W6	-0.8557	0.1660	+
W11	-0.5632	0.1996	+
W13	-0.7628	0.1264	+
W19	-0.2707	0.2411	+
E13	-0.7885	0.2984	+
S6	-0.2845	0.4664	+

Annotation: IAA= <1 μg mL^{-1} (+), 1–10 μg mL^{-1} (++), 11–50 μg mL^{-1} (+++), >51 μg mL^{-1} (++++) color: low (+), medium (++), high (+++).

3.4. Bacterial Identity based on 16S rRNA gene sequence

Isolates E5 has a similarity 97% with *Bacillus licheniformis*, isolates E11 has a similarity 97% with *Bacillus licheniformis*, isolate S3 has a similarity of 96% with *Bacillus megaterium*, isolate W6 has a similarity of 98% with *Bacillus pumilus*, isolates W11 has a similarity 97% with Bacillus sp., isolates W13 has a 97% similarity with *Bacillus pumilus* isolate E13 has a 98% similarity with *Bacillus*

licheniformis, isolates S6 has a 98% similarity with *Bacillus pumilus*. BLAST analysis results which have sequences with ≥99% similarity showed the same species. Sequences with ≥97% similarity included in the same genus [22].

Table 3. Species identical to bacterial isolates found in rhizosphere of soybean by the gene encoding of 16S rRNA

Isolate name	The closest relatives	No. Access	% similarity
E5	*Bacillus licheniformis*	JQ965662.1	97%
E11	*Bacillus licheniformis*	JX237861.1	97%
S3	*Bacillus megaterium*	KM279706.1	96%
W6	*Bacillus pumilus*	KC845305.1	98%
W11	*Bacillus sp.*	HQ704718.1	97%
W13	*Bacillus pumilus*	KJ195695.1	97%
E13	*Bacillus licheniformis*	KJ831075.1	98%
S6	*Bacillus pumilus*	KC845305.1	98%

Bacillus are found as PGPR, as in some of the following research. *Bacillus pumilus* and *Bacillus licheniformis* isolated from the rhizosphere alder glutinosa [L.] Gaertn. has an activity as a strong growth driver [23]. *Bacillus megaterium* reportedly included in the solvent phosphate bacteria that help the growth of Tectona grandis and Chukrasia tubularis on plantations [24]. Based on research Kavamura [17] was selected as the strain of bacteria PGPR for *Zea mays* L. under water stress is *Bacillus* sp. and *Pantoea* sp.

4. Conclusion

It is concluded in this study there are 11 isolates were exopolysaccharide-producing drought tolerant bacteria. The bacteria identified as *Bacillus sp*, *Bacillus licheniformis*, *Bacillus megaterium* and *Bacillus pumilus*.

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