MOLECULAR CHARACTERIZATION OF RHIZOBACTERIA (ISOLATE EO-4) AS POTENTIAL SOLVENT OF PHOSPHATE IN-VITRO FROM THE MANGROVE ECOSYSTEM OF TELUK AWUR, JEPARA-INDONESIA

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ABSTRACT

Rejuvenation and conservation of mangrove ecosystems, especially the Teluk Awur mangrove ecosystem, Jepara Regency, can be done by using bacteria that are capable of supporting plant growth or called Plant Growth Promoting Rhizobacteria (PGPR). One of the mechanisms that support plant growth by the PGPR group is phosphate dissolving activity, because the phosphate in the soil is in the form of a compound that is difficult for plants to absorb. This study aims to determine the character of Rhizobacter isolates as a superior phosphate solvent in dissolving phosphates in-vitro from the Teluk Awur mangrove ecosystem, namely isolates coded EO-4. These isolates have similar microbiological and biochemical characters to the genus *Enterobacter*. Molecular characterization of isolates was carried out using the Polymerase Chain Reaction (PCR) method - 16S rRNA sequence analysis (comparing with 16S rRNA sequences in gene banks). The results showed that the phosphate-solubilizing rhizobacteria that were isolated had the same base pair percentage of 48% with *Enterobacter pyrinus* (access number NR_028875).

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A. INTRODUCTION

Phosphorus (P) is the second major macronutrient important for plant growth and development (Wan et al., 2020), which constitutes approximately 0.2% of the dry plant weight (Alori et al., 2017; Chen & Liu, 2019). This element is important for plants because it is involved in various key metabolic processes, such as cellular energy storage, photosynthesis, respiration (Awasthi et al., 2011), and biological oxidation (Wan et al., 2020), as well as protein and nucleic acid synthesis (Chen & Liu, 2019). However, despite the high phosphorus content in the soil (about 0.05% by weight of the soil), The element of

phosphorus that is available to plants (phosphate dissolved) are in concentrations that low (only about 0.1% of total P in soil) (Alori et al., 2017; Lambers & Plaxton, 2015)

The phosphate dissolution reaction is a mechanism that plays a role in increasing the availability of nutrients for plants (Sembiring et al., 2013), in addition to other mechanisms such as free nitrogen fixation, sulfate reduction, production of certain enzymes, and production of growth substances (Sahoo & Dhal, 2009). Microorganisms that play a role in phosphate dissolving can be bacteria, fungi, *actinomycetes* (Premono, 1994; Ginting et al, 2006) and yeasts (Premono, 1994). The use of various types of microorganisms to provide nutrients in the soil, to support plant growth, is known as *biofertilizer* (Suriadikarta & Simanungkalit, 2006).

Phosphate solubilizing rhizobacteria are a group of bacteria that live in the rhizosphere of certain plant roots, which are capable of dissolving phosphate (Sugianto et al., 2019). This phosphate dissolution is important because inorganic phosphate has a low level of availability in the soil, so it cannot be absorbed by plants (Ginting et al., 2006). Purwaningsih (2003) wrote that bacteria from the genus *Pseudomonas*, *Micrococcus*, *Bacillus*, *Microbacterium*, *Flavobacterium*, *Azotobacter* can act as phosphate solvents in the soil.

Research by Oktaviani et al., (2020) has succeeded in characterizing phosphate solubilizing rhizobacteria (isolate EO-4), which was isolated from the rhizosphere soil of the mangrove *Rhizospora* sp., As part of the mangrove community of Teluk Awur, Jepara Regency, Central Java Province. However, characterization was carried out only on microbiological characters (aspects of cell morphology, colony morphology, and biochemical activity), which concluded that the isolate was similar to the genus *Enterobacter*. Molecular characterization has not been carried out.

The most widely used method for molecular-level analysis is based on ribosomal RNA sequencing. Sequences on 16S rRNA which are hypervariable are widely used to detect diversity and place lines in certain species (Suwanto, 1994; Pangastuti, 2006). The 16S type of ribosomal RNA can be used as a molecular marker because it has identical functions in all Prokaryotic organisms and can change according to the evolutionary distance of a particular organism so that it can be used as a good measure of evolution (Pangastuti, 2006). This analysis is said to be practical when compared to the 5S rRNA and 23S rRNA analyzes, because it has a more proportional size (1500 base pairs) when compared to the analysis of the two sequences (Suwanto 1994; Pangastuti, 2006). Molecular data (from ribosomal 16S rRNA sequences) is then used as the basis for the construction of phylogenetic trees (Dharmayanti, 2011), which is a model to describe the relationships between organisms and estimate the differences that exist between certain ancestors and their offspring (Li et al., 2000) According to Waugh (2007) in Irawan et al., (2016), the identification of species based on nucleotide size is considered consistent, accountable, and fast, so it plays an important role in conservation and diversity research. The purpose of this study was to characterize EO-4 isolates based on 16S rRNA sequence data, through phylogenetic tree construction, in order to obtain the relationship between these isolates and other species (in the same genus).

B. METHOD

The molecular characterization stage of EO-4 isolates (the process of genomic DNA isolation to 16S sequence amplification) was carried out at the Genetics Laboratory, Department of Biology, Faculty of Science and Mathematics (FSM), Diponegoro University. Determination of the concentration and purity of DNA was carried out at the Instrumentation Laboratory of the Department of Chemistry, FSM UNDIP. The 16S rDNA sample was then sent to Macrogen Inc. (Korea) for the 16S sequence determination. The tools used in this research are cooling system, test tube, measuring cup, autoclave, analytical balance, spatula, vortex, centrifuge, micropipette (and its equipment), glass bottle, Ultra Violet-Visible (UV-Vis) spectrophotometer device, tube erlenmeyer, micro tube (micro tube), UV transilluminator, electrophoresis device (Mupid Ex) and Mastercycler personal Polymerase Chain Reaction (PCR) machine (Eppendorf).

Some of the materials used include chemicals in the form of distilled water, HCl solution, Tris base powder, Ethylene Diamine Tetra Acetate (EDTA), Sodium Dodecyl Sulfate (SDS) powder, lysozyme enzymes, phenol, chloroform, 96% ethanol solution, agarose powder, acid solution glacial acetate, Ethidium Bromide (EtBr) powder, 1 kb step ladder DNA, bromophenol blue, primer (27F primer (5'-AGA GTT TGA TCA TGG CTC AG-3') and 1500R primer (5'- AAG GAG GTG ATC CAG CCG C-3')), based on research by Xinxian et al. (2011), the AmpliTaq 360 MasterMix PCR reaction kit and aquabides (ddH₂O). Research support materials used include cotton, gauze pads, yarn, distilled water, 70% ethanol and methylated spirits.

The isolation of bacterial genomic DNA was carried out using the midi scale alkaline lysis method (Sambrook J, Fritsch EF, 1989). Determination of concentration and purity of DNA was carried out according to the protocol of (Sambrook J, Fritsch EF, 1989) with modifications. The stages of amplification of 16S rRNA sequences with PCR reaction followed the protocol (Xinxian et al., 2011) with modification. The DNA electrophoresis stage was carried out according to the protocol from Ausubel et al., (1995) with modification. The PCR products obtained were shipped to Macrogen Inc. (Seoul, Korea) for the determination of the 16S rRNA sequence.

Determination of the 16S rRNA sequence was carried out using a unidirectional reaction with 27F forward primary DNA. The sequences obtained were analyzed with Molecular Evolutionary Genetic Analysis-X (MEGA-X) software, which can be downloaded at <http://www.megasoftware.net> (Kumar et al., 2018). The 16S rRNA sequences of the obtained isolates were then aligned (multiple sequence alignment) with 9 (nine) 16S rRNA sequences from various species of the genus *Enterobacter*, the data of which were downloaded from the National Center for Biotechnology Information (NCBI) on the <https://www.ncbi.nlm.nih.gov>. Furthermore, the construction of phylogenetic trees was carried out using the Maximum Likelihood and Tamura-Nei methods (Tamura & Nei, 1993). This analysis involved 10 nucleotide sequences.

C. RESULTS AND DISCUSSION

Based on research by (Oktaviani et al., 2020), isolates with code EO-4 are potential rhizobacterial isolates that have similar microbiological characters to the genus *Enterobacter*. This study conducted further characterization of these isolates, based on the phylogenetic tree construction from 16S rRNA sequence data. The results of the determination of the 16S rRNA sequence obtained 200 bp of the total length of the 16S rRNA sequence obtained in the PCR reaction.

In the phylogenetic tree construction carried out, a total of 1929 positions were obtained in the final dataset. The phylogenetic tree of 9 accessions of 16S rRNA sequence data for various species in the genus *Enterobacter* downloaded from NCBI and 16S rRNA sequences of EO-4 isolates can be seen in **Figure 3.1**. The algorithm used to construct phylogenetic trees is *Maximum Likelihood*. According to Dharmayanti (2011), this method uses a calculation to determine the phylogenetic tree that has the best variation in the sequence set analyzed. Furthermore, Dharmayanti (2011) wrote that this method considers the number of sequence changes or mutations that occur in each tree, which results in sequence variations. The nine accessions used as a comparison can be seen in **Table 3.1**.

Table 3.1. Accessions of comparisons for phylogenetic tree construction downloaded from the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov>)

No. Akses	Spesies	Panjang Basa
EU721605.2	<i>Enterobacter mori</i> strain R18-2	1.285 bp
AJ010486.1	<i>Enterobacter pyrinus</i>	1.929 bp
NR_028993.1	<i>Enterobacter kobei</i> strain CIP 105566	1.451 bp
NR_042154.1	<i>Enterobacter hormaechei</i> strain 0992-77	1.365 bp
NR_042349.1	<i>Enterobacter ludwigii</i> strain EN-119	1.513 bp
NR_044977.1	<i>Enterobacter cancerogenus</i> strain LMG 2693	1.495 bp
NR_145647.1	<i>Enterobacter asburiae</i> strain JM-458	1.422 bp
NR_146667.2	<i>Enterobacter tabaci</i> strain YIM Hb-3	1.522 bp
NR_148649.1	<i>Enterobacter bugandensis</i> strain 247BMC	1.444 bp

Based on the phylogenetic tree (**Figure 3.1**), EO-4 isolates have a closer kinship with *Enterobacter pyrinus*, compared with eight accessions of strains from other *Enterobacter* species. Research by Tahir et al. (2013) also obtained a phosphate solubilizing rhizobacteria of the genus *Enterobacter* (isolated from wheat and cotton growing fields in the areas of Hajira, Sahiwal, and Mailsi, part of Pakistan), which was further identified as a strain of the *Enterobacter amnigenus* species based on phylogenetic tree constructions from data 16S rRNA sequence. Apart from *Enterobacter amnigenus*, other isolates that were also identified were *Azospirillum brasilense* and *Bacillus licheniformis*. Borham et al, (2017) have also carried out the isolation and identification of phosphate solubilizing rhizobacteria isolated from the rhizosphere of healthy rice, maize, cotton, chilies, and cucumber plants in the Kafr Eksheikh Governorate area, Egypt. The results showed that based on morphological, biochemical characters, and 16S rDNA sequence analysis, the isolates that showed the highest phosphate dissolving activity were identified as *Enterobacter cloacae* species (base pair similarity rate was 99% with *E. cloacae* strain ECNIH4). In addition, (Aliyat et al., 2020) also conducted research to carry out the isolation and characterization of phosphate solubilizing bacteria from phosphate

mining mud soil in Morocco. The results of this study indicate that based on the comparison of 16S rRNA gene sequences, 27 (twenty seven) isolates were identified into the genus *Pseudomonas*, *Serratia*, *Pantoea*, and *Enterobacter*. Martínez & Martínez (2007) have also been getting phosphate solvent bacterial isolates were identified as *Xanthomonas maltophilia* and *Enterobacter cloacae*, 100 rhizosphere samples taken from 16 land farming in the area of Tolima, Cundinamarca, and Casanare (Country of Colombia). Kim et al., (2002) have also identified an isolate of *Enterobacter intermedium* from the rhizosphere of grasses, which is reported to have the ability to produce organic acids and dissolve phosphates.

Apart from these studies, isolation and identification of phosphate solubilizing bacteria were also found in other genera, outside *Enterobacter*, from rhizosphere samples of mangrove plants and from other types of samples. Teymouri et al., (2016) reported that based on 16S rDNA sequence data and alignment that had been carried out against existing databases in GenBank NCBI, phosphate solubilizing rhizobacteria isolated from mangrove forests off the coast of Qeshm Island (southern Iran) were identified into the genus *Bacillus*, *Pseudomonas*, and *Acinetobacter*. Bharathkumar et al., (2008) have also carried out the characterization of bacterial populations on rhizosphere soils of several different mangrove plants, namely *Porteresia coarctata*, *Sonneratia apetala*, *Avicennia marina*, *Rhizophora mucronata*, and *Excoecaria agallocha*, which compose the Pichavaram mangrove community (South East Coast of India). The results showed that most of the *Single Strand Conformation Polymorphism* (SSCP) sequence bands were related to the characteristics of the bacteria from the genus found in *rooted* environments and soil-free with root systems, namely *Bacillus*, *Planococcus*, *Planomicrobium*, gram-positive bacteria with a base content of G+C low, glacial ice bacteria, and other bacteria that could not be identified. Based on phylogenetic analysis of 16S rRNA sequences, a close relationship was obtained with Phylum *Firmicutes* and bacteria that were not identified in various clones of bacterial species obtained. Meanwhile, Javadi Nobandegani et al. (2015) reported that analysis of the 16S rRNA gene for phosphate solubilizing bacterial isolates isolated from land planted with oil palm at University Putra Malaysia, Serdang, found that these isolates were included in the genus *Alcalligenes*, *Serratia*, *Bacillus*, and *Vagococcus*.

Figure 3.1. Phylogenetic tree of EO-4 isolates and 9 (nine) accessions of 16S rRNA sequences of various species from the genus *Enterobacter*, generated from MEGA-X software, using the *Maximum-Likelihood* method (numbers on each branch indicate the length of the branches, which describes the number of bases substitution (DNA polymorphism or haplotype)).

D. CONCLUSION

Phylogenetic trees constructed based on 16S rRNA sequences of EO-4 isolates, which in previous studies (Oktaviani et al., 2020) were characterized microbiologically similar to the genus *Enterobacter*, showing a close relationship with *Enterobacter pyrinus* (GenBank accession number AJ010486.1), compared with eight other accessions of species in the genus *Enterobacter*.

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