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MOLECULAR IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM PGPR PRODUCT DERIVED FROM TEMANGGUNG REGENCY

Identifikasi Molekuler Bakteri Pelarut Fosfat Dari Produk PGPR Kabupaten Temanggung

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ABSTRACT

T Phosphate solubilizing bacteria are soil bacteria that can dissolve phosphates from insoluble phosphate bonds so that they can be absorbed by plants. Phosphates in the soil are naturally present in organic and inorganic forms. Both forms are insoluble or slightly soluble forms of phosphate, so their availability for soil biota is very limited. This study aimed to identify the phosphate solubilizing bacteria from locally PGPR product from Temanggung, Indonesia, namely BPF 1. The method in this study was carried out by molecular identification using the 16S rRNA encoding gene which includes bacterial DNA isolation, DNA amplification using PCR, bacterial DNA sequencing and the creation of phylogenetic trees. The results of the study were obtained isolate bacteria solubilizing phosphate BPF 1 from PGPR products of local farmers of Temanggung Regency identified as the genus Bacillus, having the closest kinship with B. subtilis strain SBMP4 (NR_118383.1) with a similarity of 98.42%.

Keywords: *Phosphate Solubilizing Bacteria, Bacillus subtilis strain SBMP4, PCR, 16S rRNA Gene, PGPR*

ABSTRAK

Bakteri pelarut fosfat merupakan bakteri tanah yang dapat melarutkan fosfat dari ikatan fosfat tak larut sehingga dapat diserap oleh tanaman. Fosfat di dalam tanah secara alami terdapat dalam bentuk organik dan anorganik. Kedua macam bentuk tersebut merupakan bentuk fosfat yang tidak larut atau sedikit larut, sehingga ketersediaannya bagi biota tanah sangat terbatas. Tujuan pada penelitian ini mengidentifikasi bakteri pelarut fosfat isolat BPF 1 dari produk PGPR lokal petani Kabupaten Temanggung, Indonesia. Metode dalam penelitian ini dilakukan dengan identifikasi molekuler menggunakan gen penyandi 16S rRNA yang meliputi isolasi DNA bakteri, amplifikasi DNA menggunakan PCR, sekuensing DNA bakteri dan pembuatan pohon filogenetik. Hasil penelitian didapatkan isolat bakteri pelarut fosfat BPF 1 dari produk PGPR petani lokal Kabupaten Temanggung teridentifikasi sebagai genus Bacillus, memiliki kekerabatan terdekat dengan *B. subtilis* strain SBMP4 (NR_118383.1) dengan kesamaan 98,42%.

Kata Kunci: Bakteri Pelarut Fosfat, *Bacillus subtilis* strain SBMP4, PCR, Gen 16S rRNA, PGPR

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INTRODUCTION

Plant Growth Promoting Rhizobacteria (PGPR) is a microorganism that lives in a rhizosphere environment that can spur plant growth and increase crop production. PGPR is capable of producing plant growth hormones such as auxin, gibberellin and cytokinins, as phosphate solvents and nitrogen fixations (Cahyani et al. 2018) . Based on its activity PGPR can induce plant growth both directly and indirectly, and PGPR can be classified as a biofertilizer agent, phytostimulator, rhizoremediator and biopesticide (Geetha et al. 2014)

Microorganisms have a considerable role in the cycle of various elements, one of which is phosphorus. Phosphorus (P) is one of the nutrients that plants absolutely need because it plays a role in the storage and transfer of energy and as part of proteins and nucleic acids (Sharon et al. 2016). Plants absorb P from the soil in the form of phosphate ions, mainly in the form of $H_2PO_4^-$ and $H_2PO_4^{2-}$ present in the soil solution (Musaad 2018).

Phosphate is one of the essential macroelements, not only for plant growth but also for soil biota (Lin et al. 2016). The activity of soil microorganisms has a direct effect on the availability of phosphates in the soil solution. The bacterial mechanism in dissolving phosphates will release organic acids such as citric, glutamate, succinic, lactic, oxalic, malic, fumaric, tartaric and α-ketobutarat (Setiani 2016).

Bacteria that can dissolve phosphates are *Phyllobacterium, Pseudomonas, Bacillus, Rhodococcus, Arthrobacter, Serratia, Chryseobacterium, Delftia, Gordonia*, and *Rhizobium* (Ulfiyati and Zulaika 2015). In addition *Escherichia freundii, Aspergillus niger, B. megaterium, Pseudomonas* sp., *B. subtilis, Arthrobacte*r sp., *B. firmus* B-7650, *P. putida* M5TSA, *Enterobacter sakazakii* M2Pfe, *B. megaterium* M1Pca, *B. amyliliquefaciens* , *B. licheniformis, B. athrophaeus, Penibacillus macerans, Vibrio proteolyticus, Xanthobacter agilis, E. aerogenes, P. aerogenes* and *Chryseomonas luteola* can dissolve phosphates by different mechanisms (Sharma et al. 2013).

The method of identification of bacteria can be carried out by analysis of the phenotype and genotype. In phenotype analysis, there is a disadvantage namely that there are often errors in differences in species and bacterial strains. The error is due to the presence of an unusual character of the bacterial phenotype. Moreover, the character of the bacterial phenotype is not static and can change along with changes in organism conditions and the environment to cause evolution (Rosahdi et al. 2019). The identification of bacteria based on the phenotype also has a low reproducibility because it depends on the culture conditions in different laboratories (Nisa' 2018).

The lack of accurate identification by phenotype encourages the identification of bacteria by other, more accurate methods. A widely practiced bacterial identification method is bacterial genotype analysis through the reading of nitrogenous base sequences in the nucleotides constituting the 16S rRNA gene fragments of bacteria. The 16S rRNA gene is used as a genetic markers because gen 16S rRNA is universal for bacteria. 16S rRNA gene from almost all bacterial species has found nitrogenous base sequences so that it can be used as a guideline if new species are found, and the nitrogenous base sequence of the 16S rRNA gene has a lower intraspecific diversity than other protein coding genes. rRNA encoding genes are used to determine taxonomy, phylogeny, as well as estimate rates of species divergence of bacteria (Johnson et al 2019). In general, the comparison of the sequences of the 16S rRNA gene allows differentiation between organisms at the genus level on the main phylum of bacteria with the aim of classifying strains of different levels and sub-species (Noer 2021).

This study used bacteria isolate BPF 1 (*Bakteri Pelarut Fosfat* 1 / phosphate solubilizing bacteria 1). BPF 1 isolate is a potential phosphate solvent bacterial isolate that was successfully isolated from local PGPR products of Temanggung farmers. Isolate BPF 1 has a high phosphate solubility index (2,625). This index is higher than Ulfiyati and Zulaika (2015) that got 2,52 phosphate solubility index from *Bacillus cereus* ATCC 1178. Based on this background, this study aimed to identify the phosphate solubilizing bacteria derived from locally PGPR product

from Kataan, Ngadirejo, Temanggung, Indonesia, namely BPF 1.

MATERIALS AND METHODS

Location and time

This research was conducted at the Biotechnology Laboratory, Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang. Research activities were carried out from January 2022 to July 2022.

Materials

Bacterial isolates of phosphate solubilizing BPF 1 collection of the Biotechnology Laboratory of the Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Semarang.

DNA isolation

The DNA isolation process is carried out based on the InstaGeneTM Matrix protocol for bacterial DNA isolation (Bio-Rad #732-6030). The colony of bacteria to be isolated is selected and suspended into 1 mL of nutrient broth in a microtube, then centrifugation for 1 minute at 13,000 rpm to separate between the supernatant and the pellets. The supernatant is discharged and pellets are added with an Instagene kit of 200 µL. After which it is incubated at a heat block temperature of 56ºC for 30 minutes, then the vortex at high speed for 10 seconds is then re-incubated at 100ºC for 10 minutes and homogenized using a vortex for 10 seconds. The function of the heat block is for the release of plasmid DNA from cells caused by high heating temperatures, then the solution is centrifuged at a speed of 13,000 rpm for 3 minutes, the supernatant containing the DNA is taken and transferred to a new microtube. Use the resulting 20 µL of supernatant per 50 µL of PCR reaction. Incubated supernatant at −20ºC. Incubated of DNA samples at a temperature of −20ºC aimed at preventing DNA from being denatured by enzymes.

Polymerase Chain Reaction (PCR)

The primer used is a universal primer for the bacterial domain in the form of primer used is forward 27F and reverse 1492R (Sharon et al. 2016). All reaction components were mixed into the microtube with a total mixed volume of 50 µL consisting of a sample of 2 µL, Primary (forward 2µL and reverse 2µL), My Taq Hs Red DNA 25µL and ddH_2O 19 µL and fed into the PCR machine. Amplification was carried out using a gradient PCR thermal cycler for 30 cycles with pre-denaturation steps at 94ºC for 5 minutes; denaturation 94ºC for 1 minute; annealing 54ºC for 45 seconds, extension 72ºC for 2 minutes and final extension 72ºC for 10 minutes (Yu et al. 2022) PCR result was checked by electrophoresis with 1% agarose gel in TAE buffer with 100 V for 30 minutes.

Sequencing analysis

The PCR results of BPF 1 isolates were then sequenced by 1st Base PT. Genetics Science Indonesia for mapping successfully amplified nucleotide base pairs.The data from the sequencing results were then analyzed using the Bioedit program. The results obtained are then compared with the data contained in GenBank using the Basic Local Alignment Search Tool BLAST program (http://www.blast.ncbi.nlm.nih.gov/). Then the sequences are aligned with the ClustalX2 program and create a phylogenetic tree by using the Treeview program to understand a both relationship (Rinanda 2011).

RESULTS AND DISCUSSION

The results of isolation of phosphate solubilizing bacteria obtained in PGPR products are 2 isolates with different characteristics. The best isolate in dissolving phosphates is the BPF 1 isolate. BPF 1 isolate has a higher phosphate solubility index of 2.625 while BPF 2 isolate has a phosphate solubility index value of 2.1. The difference in phosphate solubility index value is closely related to the ability of bacteria to dissolve phosphate. Each strain of bacteria has a different ability to produce organic acids, both in quantity and type during growth, thereby influencing phosphate solubility (Oksana et al. 2020). The ability to dissolve phosphate is indicated by the presence of a clear zone around the bacterial colony. This occurs due to the presence of organic acids excreted by

bacteria and then binds to Ca ions from Ca3(PO4)2 in Pikovskaya media and liberates H2PO4 to form a clear colored area.The higher the phosphate solubility index value produced, the higher the ability of bacteria to dissolve phosphate.

The DNA of the isolate BPF 1 was then isolated using the Instagene kit protocol. The results of DNA purity in phosphate solvent bacterial isolates using nanodrop obtained a DNA concentration value of 476.4 ng/µL with a DNA purity of A260 / A280 which was 1.98. Nanodrop aims to measure the concentration and purity of DNA. In the isolate BPF 1, a concentration of 476.4 ng/µL with a purity value of 1.98 this result can be said to have good quality. DNA purity can be measured by calculating the ratio of an absorbance value of 260 nm to an absorbance value of 280 nm. Fatchiyah et al. (2011) stated that the nanodrop test results are in the form of DNA purity values on A260/A280 and DNA concentration values. Good quality DNA based on the nanodrop test has a purity of 1.8-2.0 and concentrations above 100 ng/µL. Murtiyaningsih (2017) said that the ratio value is less than 1.8 it is likely that the sample is contaminated with protein and organic matter whereas if the ratio value is more than 2.0 then the contamination is caused by phenols and RNA.

The absorption of DNA values is seen in waves of 260 nm while protein values are seen at wavelengths of 280 nm. According to Iqbal et al. (2016) the value of A260 is a value expressing the value of DNA absorbed by a wavelength of 260 nm. The high and low value of A260 will affect the value of concentration and purity. Purity values can be affected by A280 (contaminant) values. The level of purity obtained can affect the results of quantitative analysis by electrophoresis. The results of quantitative DNA analysis are shown in Table 1.

Table 1. Result of BPF 1 DNA quantitative test using nanodrop

Sampel DNA	Nucleic Acid Conc. (ng/µL)	A260	A280	A260/A280
BPF 1	476.4	9.528	4.815	1.98

DNA amplification results

The amplification process is carried out by the Polymerase Chain Reaction (PCR) method. PCR is a technique for synthesis and amplification of DNA in vitro. The PCR technique can be used to amplify DNA segments millions of times in just a few hours. The purpose of PCR is to create a large number of duplications of a gene (Yulistiana et al. 2020). The results of amplification can be done by visualization using GelDoc presented in Figure 1. Based on the visualization of the results of the amplification of the 16S rRNA Gene aligned by the marker, it shows that the DNA of the bacteria isolate BPF 1 was successfully amplified by showing a size of 1500 bp. The results of electrophoresis showed that the DNA band was clearly visible in the absence of mixing bands. The full 16S rRNA gene provides better taxonomic resolution, it is about 1500 bp (Johnson et al. 2019) which consisting of conserved areas (Akihary and Kolondam 2020). The process of amplification requires a primer that can limit the initial region of the gene and at the end of the gene sequence. Although the commonly used sizes for sorting and comparing sequences are 500 and 1500 bp, the order in the database can be more varied.

DNA sequencing is performed to ensure the DNA fragments amplified in the PCR process so that 16S rRNA DNA sequences can be used for identification of bacterial genus and strains. According to Lokapirnasari et al. (2017) DNA sequencing can be used in determining the identity and function of genes or DNA fragments by comparing sequences with other DNA sequences that have been known from GeneBank.

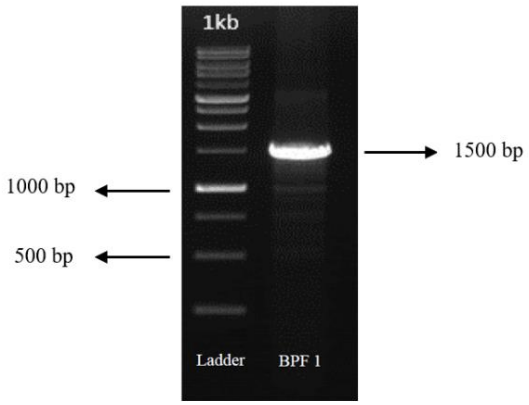

Figure 1. GelDoc DNA Results of gene 16S rRNA Isolate BPF 1

The results of the sequencing analysis obtained two sequence data, namely forward and reverse sequences. DNA sequence data of 16S rRNA BPF 1 were analyzed using the Bioedit application. Sequences are aligned with Muscle (Multiple Sequence Alligment) in a bioedit program for contig analysis. According to Ekblom and Wolf (2014) analysis contig is a set of DNA or RNA segments that represent consensus sequences. Consensus sequences are DNA sequences resulting from the alignment of reverse and forward primers. Contig forms sequences longer than the initial DNA fragments into an intact gene. The contig data will be analyzed using the Basic Local Alignment Search Tool (BLAST) to see the homology with the database on Genebank. BLAST results of BPF 1 isolates using the NCBI programme are presented in Table 2.

Table 2. NCBI BLAST Results of BPF 1 DNA Isolate

Description	Max Score	Total Score	Query Cover	E-Value	Per-ident	Accesion
Bacillus velezensis strain FZB42	1559	1559	98%	0.0	98,42%	NR_075005.2
Bacillus subtilis subsp. Subtilis strain 168	1559	1559	98%	0.0	98,42%	NR_102783.2
Bacillus subtilis strain DSM 10	1559	1559	98%	0.0	98,42%	NR_027552.1
Bacillus inaquosorum *strain* BGSC 3A28	1559	1559	98%	0.0	98,42%	NR_104873.1
Bacillus subtilis strain SBMP4	1559	1559	98%	0.0	98,42%	NR_118383.1
Bacillus vallismortis *strain* NBRC 101236	1559	1559	98%	0.0	98,42%	NR_113994.1
Bacillus subtilis strain JCM 1465	1559	1559	98%	0.0	98,42%	NR_113265.1
Bacillus spizizenii *strain* NBRC 101239	1559	1559	98%	0.0	98,42%	NR_112686.1
Bacillus subtilis strain NBRC 13719	1559	1559	98%	0.0	98,42%	NR_112629.1
Bacillus vallismortis *strain* DSM 11031	1559	1559	98%	0.0	98,42%	NR_024696.1

Phylogenetic Tree Analysis Results

Based on the results of the identification of the BPF 1 isolate strain, it has a similarity of 98% with the 10 bacterial strains contained in NCBI which indicates that the BPF 1 isolate is in the same genus. Sharma et al. (2013) stated that a similarity value of 99% indicates that isolates are considered the same species. If homology ≥ 97% can be considered isolates in the same genus while homology between 83-93% indicates a different family. The construction of the phylogenetic tree of BPF 1 isolates can be seen in Figure 2.

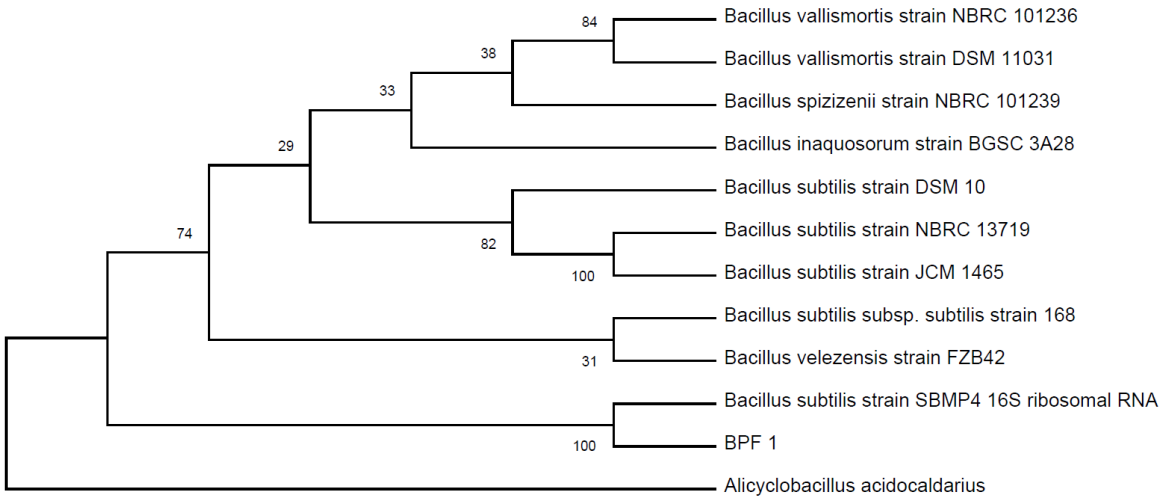

Figure 2. Phylogenetic tree of isolate BPF 1 with Primer 16S rRNA

BPF 1 isolates was close to the diversity *of B. subtilis* strain SBMP4. The microbe *B. subtilis* strain SBMP4 is a biological control agent that has the advantage of being a PGPR, which can function as a biofertiliser, biostimulant, biodecomposer and bioprotectant. *Bacillus subtilis* is a gram-positive bacterium and produces endospores, this is in accordance with the study Naveena and Gowrie (2018) stated that in PGPR it is identified as *B. subtilis* with a accession number KY 614549. Similar studies have been conducted by Anastia and Lubis (2022) stating that in 10 isolates of phosphate solvent bacteria from PGPR mangroves identified as many as 9 isolates having similarities with the genus *Bacillus* sp and 1 isolate with the genus *Pseudomonas*. According to Mandic-Mulec et al. (2015) the Bacillaceae usually are saprophytes, can be found in variety of habitats like soil, and participate in the carbon, nitrogen, sulfur, and phosphorous cycles. The morphology and properties of B. subtilis bacteria are a type of Gram-positive and bacillus-shaped bacteria (rods) that can form oval-shaped endospores in the central part. According to Ulfiyati and Zulaika (2015), *Bacillus* sp. it is known to be able to produce organic acids from carbohydrate metabolism, where the secretion of organic acids is one of the mechanisms for the process of dissolution of phosphates by bacteria. Ali et al. (2014) said that *B. subtilis* bacteria have also been used in the industrial field, namely to produce proteases, amylase, antibiotics, and chemicals.

CONCLUSION

Isolates of phosphate solubilizing bacteria BPF 1 from PGPR products of agriculture of Temanggung Regency were identified as the genus *Bacillus*, having the closest kinship with *B. subtilis* strain SBMP4 (NR_118383.1) with a similarity of 98.42%.

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