Characterization phosphate-solubilizing marine actinobacteria associated with Sargassum Sp from Menjangan kecil island, Indonesia

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Abstract. Phosphorus plays a crucial significant role in all life forms. Microbes have the ability to convert several forms of phosphorus into dissolved forms, so that they can also be used in the phosphorus cycle process. The aim of this research were to explore, screen, phenetic and molecular identify the phosphobacteria naturally colonized and associated with Sargassum sp. from the Menjangan Kecil Island, Karimunjawa. Total of 23 bacterial isolates associated with Sargassum sp. were screened. All isolates obtained were tested for their ability in phosphate dissolution activity in Pikovskaya agar medium. Three different isolates showed the largest dissolution activity around bacterial colonies selected for quantitative estimation of phosphate solubilization. The next screening was to choose one candidate isolate based on its highest ability in dissolving phosphate and then identifying its identity based on phenotypic character and 16S rRNA gene. Quantitative estimation of phosphate solubilization PSB isolates MBS3R was 149.32 μg P ml⁻¹ was analyzed for 120 hours of incubation at room temperature. MBS3R isolate is a strain of Microbacterium sp, which is taxonomically included in the class of Actinobacteria, genus Microbacterium.

1. Introduction
Phosphate can be found in water, soil, and sediment. Sources of phosphate in marine waters in coastal and marine areas can come from rivers or phosphorus cycles. Phosphorus (P) is an essential macronutrient like carbon (C), and nitrogen (N). The phosphate formations most commonly found in the marine are in the form of phosphate salts deposited more in sedimentary rocks. Phosphate salts released from weathering of rocks through soil are usually soluble in water and can be absorbed by plants. Phosphorus in marine water is in the form of organic and inorganic compounds. [1] [2].

It is known that phosphate is also a limiting factor for plant growth in marine ecosystems because phosphate is not so soluble in water [3]. Plants and plant-eating animals are one of the chains in the process of phosphate absorption for fish or aquatic organisms. Phosphorus cycle rotation occurs faster through rocks and sediments. The phosphate cycle will return when animals and plants die [4]. In general, phosphate absorption is carried out by microbes. Phosphate dissolving bacteria are bacteria that can dissolve phosphates that are difficult to dissolve into soluble forms, both from the soil and
from fertilizers, so that plants can absorb them. Organic acids, and enzyme phosphatase is a compound used in the process of phosphate mineral dissolution. Both of these elements are very important in organic phosphate mineralization in the soil [5] [6] [7]. Bacteria and other microbes assimilate inorganic phosphate and mineralization of organic phosphorus compounds. The study was conducted with the aim of exploring and obtaining phosphate-solubilizing bacteria (PSB) associated with *Sargassum* sp. from Menjangan Kecil Island, Karimunjawa.

2. Method

2.1. Algae Sampling

Menjangan Kecil Island in Karimunjawa, Indonesia was chosen as the location for sampling *Sargassum* sp. Sterile sea water is used to wash and rinse samples. Samples that have been cleared of impurities are then stored until used.

2.2. Isolation and purification of marine microbes

The media used for microbial isolation is Zobell Marine Agar 2216 (Hi-Media). Weighed 20 g (wet weight) *Sargassum* sp then added 200 mL of sterile sea water. This solution was then incubated in a rotary shaker at 100 rpm for 15 minutes. [8] with a few modifications. Samples are then serialized and carried out in duplicate. Plates are incubated at room temperature for 48 - 168 hours. Bacterial colonies that were grown were observed then purification of isolates.

2.3. Estimation of phosphate solubilization efficiency

Representation of bacteria from dominant morphology the type on the plate is selected randomly and cultivated on Pikovskaya (PVK) medium. PVK media were supplemented with 1.5% Bacto-agar (Difco) inoculated with test bacterial isolates. The culture is then incubated for 5 days at room temperature for 5 days. After the incubation period is complete, the diameter of the colony and the clear zone formed is then observed and measured. The solubilization index is used to measure the clear zone. The formula stated by Edi-Premono et al [9] is used to measure the solubilization index, which is by comparing the diameter of the clear zone and the diameter of the colony. Medium 0.5% TCP is used as a substrate to qualitatively measure dissolution potential. The cultures were incubated for 5 days at a rotary shaker at a speed of 100 rpm at room temperature, then centrifuged at 10,000 rpm for 10 minutes. Determination of phosphate is dissolved in culture using the Phosphomolybdate [10] method with slight modification.

2.4. Morphological and biochemical characterization of bacteria

Characteristics of potential isolates such as physiology and biochemistry were carried out according to standard methods [11]

2.5. Molecular identify 16S rRNA gene sequencing and analysis

16S rRNA gene potential bacterial isolates amplified by polymerase chain reaction (PCR) method using universal primers for bacteria 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT ) [12] [13]. The PCR products then sequenced. The nucleotide sequences were compared using the BlastN program [14], and the closest match of known phylogenetic affiliation was used to assign the isolated strains to specific taxonomic groups.

3. Result and discussion

Microbes, especially bacteria, play a significant role in biogeochemical cycles and nutrients in the marine environment. Most phosphates and nitrates in the marine environment are associated with aquatic plants, animals, and sediments, depending on the role of the microbes themselves. Microba is also responsible for recycling phosphorus and decomposing organic matter [6]
The primary sources of phosphate and nitrate naturally come from the water itself through the decomposition process, weathering, decomposition of plants, remnants of dead organisms, land waste disposal (domestic, industry, agriculture, livestock, and leftover food) which bacteria will break down into substances nutrients for the marine environment itself. But on the contrary, the excess phosphate content in the waters causes algae growth (eutrophication) blasting events with side effects of decreasing oxygen concentration in water bodies causing the death of aquatic biota.

The researchers used procedures for initial screening to identify PSB isolates usually, on the use of Ca$_3$(PO$_4$)$_2$ on the indicator plate [15]. The phosphate solubilizing bacteria (PSB) are capable of secreting phosphatase enzymes that play a role in the process of dissolving the insoluble inorganic phosphorus into soluble organic phosphorus.

A total of 23 dominant Sargassum sp symbiotic isolates were phenetically selected and tested for halo zone production on Pikovskaya agar plates to detect PSB. Thus, the solubilization index (SI) value is measured for all of them (data not being shown). Based on these results selected three different isolates showed a maximum light circle (solubilization zone) 5 – 9 mm phosphate dissolution zone after five days of incubation at room temperature and found to have the highest SI values and were therefore selected for further experiments (Table 1). One symbiotic isolate was chosen for quantitative estimation of phosphate solubilization (Table 2). Morphological and biochemical characterization performed were presented in Table 3. Molecular analysis based on 16S rRNA gene sequencing for final characterization was shown in Fig 1. Based on these characters, the MBS3R isolates were assigned to class Actinobacteria genera Microbacterium.

### Table 1. Solubilization index (SI) based on colony diameter and halozone for each PSB isolate symbiont

| Isolate Code | Colony diameter (cm) | Halozone diameter (cm) | SI  |
|--------------|----------------------|------------------------|-----|
| MBS3R        | 0.5 ± 0.03           | 0.9 ± 0.02             | 2.8 |
| MBS6R        | 0.4 ± 0.02           | 0.6 ± 0.03             | 2.5 |
| MBS11R       | 0.6 ± 0.03           | 0.5 ± 0.02             | 1.8 |

### Table 2. Estimation of quantitative phosphate solubilization efficiency

| Isolate Code | Incubation Period (h) | P solubilization ($\mu$g mL$^{-1}$) | pH after incubation | pH of the medium |
|--------------|-----------------------|-------------------------------------|---------------------|------------------|
| MBS3R        | 120                   | 149, 32 + 0,24                     | 4,93 + 0,02         | 4,17             |

The efficiency of phosphate solubilization from MBS3R isolates in Pikovskaya Broth indicated the presence of dissolved inorganic phosphate in a medium containing 0.5% tri-calcium phosphate (Table 2). MBS3R selected isolate produces 149,32 mg mL$^{-1}$ dissolved phosphate at PKV broth after 120 hours of incubation period. The publication reported by [16] [17] that the optimal incubation period for phosphate dissolution by various bacterial isolates is to start around three days, up to 15 days. The study has proved that most phosphate solubilizing bacteria were studied have a lowered the pH of Pikovskaya Broth compared to unoinoculated sterile Pikovskaya Broth control incubated for five days under conditions as inoculated (Table 2). The pH value and the amount of phosphorus dissolved in PV Broth inoculated by MBS3R isolates for 120 hours of incubation at room temperature are presented in Table 2. The pH value is a significantly decreased pH value compared to the initial media pH and control of 7.0 ± 0.2. The phosphate solubilization process in this study was obtained at 120 hours.
incubation. This result is relatively the same as the research carried out by [18] that the maximum P solubilization was detected after 120 hours of incubation. Decreasing pH indicates the production of organic acids and phosphatase which are related to phosphate solubilization activity [18][19][20].

This is in accordance with the statement due to the fact that perhaps organic acids are the possibility of organic acids is the main factor responsible for dissolving P, so this is, of course, related to the metabolic process of these microbes. Phosphate solubilizing products by microbes, such as lactic acid, gluconic acid, acid 2-keto-gluconate, isobutyrate acid, acetic acid, oxalic acid, citric acid, etc. [20][6]. Other important things that need attention and observed that some isolate symbiont of PSB lost their ability to produce halo zone in repeated subculturing.

Fig.1. The phylogenetic tree of MBS3R based on 16S rRNA gene sequences isolates were constructed using the neighbor joining method which showed an association between the MBS3R strain and members of the Microbacterium genera. The Gen bank nucleotide accession numbers are listed next to the strain names.

| Table 3. Phenotypic characteristics of MBS3R |
|---------------------------------------------|
| NO  | Characteristic               |                     |
| 1   | Colony morphology            | Smooth, yellow      |
| 2   | Motility                     | -                   |
| 3   | NaCl range (%) for growth    | 0-7                 |
| 4   | Growth at 37 °C              | -                   |
| 5   | Catalase                     | +                   |
| 6   | H2S production               | -                   |
| 7   | Nitrate reduction            | -                   |
| 8   | Hydrolysis of:               |                     |
| 9   | Casein                       | +                   |
| 10  | Starch                       | +                   |
| 11  | D-Arabinose                  | -                   |
| 12  | Ribose                       | -                   |
| 13  | D-Xylose                     | -                   |
| 14  | D-Trehalose                  | -                   |
| 15  | N-AcetylGlucosamine          | -                   |

Microbacterium is a cosmopolitan microbial. The distribution of Microbacterium is very wide both on land and in the waters. [21] published that Microbacterium is a type of actinobacteria found in the
intestines of *Schizothorax zarudnyi* and *Schizocypris altidorsalis* fish and is a microbiota of these fish. The publication of the ability of Microbacterium sp in solubilization of phosphate has been announced by [22] [23], namely *Microbacterium ulmi* sp. nov., PSB was isolated from *Ulmus nigra* sawdust and has the ability as xylanolytic. and a new actinobacterium resin, the JXJ CY 01T strain, isolated from the *Microcystis aeruginosa* FACHB-905 mucilaginous sheath collected from Lake Dianchi, southwest of China can solubilize both inorganic insoluble (calcium phosphate) and organic phosphate (1-α-phosphatidylcholine).

4. Conclusion
The conclusions obtained from the study showed that the presence of PSB isolates was associated with *Sargassum sp*, Quantitative estimation of phosphate solubilization PSB isolates MBS3R was 149,32 μg P ml⁻¹. MBS3R isolate is a strain of *Microbacterium* sp, which was a member of the phylum Actinobacteria. which is expected to represent a valuable source in the phosphorus cycle and plant growth in the aquatic ecosystem.

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