Potential and phylogenetic of superior bacterial isolates in biogas sludge from anaerobic digestion of palm oil mill effluent

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Abstract. The study obtains potential and identifies Superior Bacterial Isolates (SBI), i.e., nitrogen-fixing and phosphate solubilizing from biogas sludge. The potential test was conducted using a Completely Randomized Design (CRD) within three replications, and the means were determined by ANOVA and DMRT at P< 0.05. The molecular identification of SBI is used by the PCR-16S rRNA sequencing method. This study was conducted from January to August 2020. The result found in the N3 and P7 from biogas sludge was more potential in the availability of total-N and available-P compared to other isolates. It also was identified as similar to Bacillus paramycoides and Bacillus cereus, respectively. This information can be used as a reference that biogas sludge can be used to support soil fertility.

1. Introduction

The palm oil industry produces several types of waste in the process, solid and liquid waste. The most important liquid waste from the palm oil industry was Palm Oil Mill Effluent (POME). The oil palm fresh fruit bunches per tonne can produce around 0.7 to 0.8 m³ of POME waste [1]. POME processing can be done using the anaerobic fermentation method, and this method is considered more effective in terms of cost and conversion into valuable products such as biogas [2]. Adela et al. [3] said that the lowest part of the first tank that received the incoming effluent showed the highest microbial population was 1.16×10⁶ CFU/ml, thus facilitating the anaerobic digestion of POME for biogas generation. Mustamu and Triyanto [4] reported that the biogas sludge has macro and micronutrients along with the nitrogen-fixing and phosphate solubilizing that have the potential for availability of nitrogen and phosphate. The characteristics of biogas sludge from palm oil waste also reported by Tepsour et al. [5]; Choorit and Wisarnwan [6]; Alvionita et al. [7] such as C/N was 8; nitrogen of 0.14%, carbon by 1.12%, NH₃-N ranged of 91 to 112 mg.l⁻¹, pH ranged by 6.8 to 8.3; and the highest bacterial populations was 7.21×10⁷ cells/ml, and the lowest was 3.15×10⁷ cells/ml.

The diversity of bacterial populations such as nitrogen-fixing and phosphate solubilizing in biogas sludge has the opportunity to increase soil fertility. Sharma et al. [8] described the Bacillus genera such as B. circulans, B. cereus, B. fuziformis, B. pumilus, B. megaterium, B. mycoides, B. coagulans, B. chitinolyticus, B. subtilis had been reported as phosphate solubilizing. Ambrosini et al. [9] said that Bacillus cereus showed the highest nitrogenase activity among 42 different strains of Bacillus spp.
et al. [10] also reported the dominant bacteria found in the biogas sludge from anaerobic processing using the pyrosequencing and clone library methods, i.e., *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Thermotogae*. Thus, it is necessary to test the potential in increasing the availability of nitrogen and phosphate along with identifying microbes in biogas sludge from anaerobic digestion of POME, especially one of the palm oil mills in Labuhanbatu District, Indonesia, which has never been recognized molecularly. The study aimed to obtain the potential and identify superior bacterial isolates (nitrogen-fixing and phosphate solubilizing) from the biogas sludge.

2. **Materials and methods**

2.1. **Study area**

The isolate potential was tested at the Laboratory of Soil Biology, Faculty of Agriculture, Universitas Sumatera Utara, Medan. The analysis of total-N and available-P was conducted at the Analytical Laboratory of PT. Socfin Indonesia, Medan. Molecular identification of bacterial isolates was conducted at the Genetics Laboratory of PT. Genetics Science Indonesia, Banten. Soil sampling for the potential test was conducted in Simalingkar, Medan, Indonesia. The study was conducted from January to August 2020.

2.2. **Selection of superior bacterial isolates**

The biogas sludge derived the isolates used from the digester tank at the palm oil mill of PT. Nubika Jaya, Pinang City, Labuhanbatu District. A total of 1 ml of the bacterial isolate suspension obtained from the characteristic stage was put into a test tube containing 9 ml of distilled water and homogenized. It put a total of 1 ml of the suspension from the dilution into 9 ml of distilled water. The dilution was made to $10^{-5}$. A total of 0.1 ml of the suspension from the last dilution was spread over the James Nitrogen Free Malat Bromothymol Blue (JNFB) medium for the Nitrogen-Fixing Bacteria (NFB) isolates test. While the suspension Phosphate Solubilizing Bacteria (PSB) isolates, the test was spread over Pikovskaya (PVK) medium. The culture medium was incubated for 2 to 3 days at room temperature.

Bacteria isolates were purified and cultured in Nutrient Broth (NB) medium for two days, then inoculated one ml of isolate into an Erlenmeyer flask containing 100 g of ultisols sterilized and incubated for 14 days. The cultures were harvested by centrifugation at 100 rpm. The NFB isolate test was characterized by the presence of colonies growing on the JNFB medium. The growth of PSB isolates is indicated by a halo zone around the microbial colonies on the PVK medium. At the end of incubation, the total-N was determined by the Kjeldahl method and the available-P using the Bray-II method.

The result of the potential test was found in seven nitrogen-fixing and seven phosphate solubilizing isolates to produce total-N and available-P. The potential test was conducted in a Completely Randomized Design (CRD) within three replications and analyzed using ANOVA, followed by DMRT at $P<0.05$.

2.3. **Molecular identification of bacterial isolates**

The bacteria with superior ability to produce the highest total-N and available-P were selected for each bacterial isolate, N3 and P7 isolated and identified by PCR-16S rRNA sequencing method. Identification using DNA markers in the 16S rRNA gene. 16S rRNA was amplified by the Polymerase Chain Reaction (PCR) technique using universal primers in the 27R and 1492R primers. Amplicons were visualized by electrophoresis technique on 0.8% agarose gel. The visualized DNA bands measure around 1300-1400 base pairs (Figure 1). The PCR product is continued to the sequence stage to obtain the nucleotide sequence of the target bacteria.

Sequences of N3 and P7 bacterial isolates were aligned with the bacterial sequence data through the Basic Local Alignment Tool (BLAST). The BLAST results showed that the genus of the species homologous to the two isolates was *Bacillus* and then formed phylogenetically by involving 31 nucleotide sequences from the National Center for Biotechnology Information (NCBI) (Table 1) using the MEGA 5 software [11].
3. Results and discussion

3.1. Potential of superior bacterial isolates

The types of superior bacterial isolates (nitrogen-fixing and phosphate solubilizing) from biogas sludge significantly increased total-N and available-P (Figure 2). It was found that the N3 and P7 bacterial isolates from biogas sludge had the highest total-N and available-P abilities by 62.56% and 36.21%, respectively, compared to the untreated.

Table 1. The accession number of nucleotides of the genus Bacillus

| No | Accession number | Bacteria strains |
|----|------------------|------------------|
| 1  | AB049195.1       | Bacillus sp. NAF001 |
| 2  | AB271757.1       | Geobacillus stearothermophilus |
| 3  | ACMX01000133.1   | Bacillus pseudomyxoides DSM 12442 |
| 4  | ACNF01000156.1   | Bacillus thuringiensis serovar ATCC 10792 |
| 5  | AF290553.1       | Bacillus anthracis strain Vollum |
| 6  | AJ276351.1       | Bacillus subtilis strain DSM10 |
| 7  | AJ320493.1       | Paenibacillus polymyxa strain DSM 36T |
| 8  | AJ419629.1       | Bacillus luciferensis strain LMG 18422 |
| 9  | AJ781029.2       | Bacillus herbersteinensis strain D-15a |
| 10 | DQ374637.1       | Bacillus acidiceler strain CBD 119 |
| 11 | FJ416489.1       | Bacillus gaemokensis strain BL3-6 |
| 12 | FJ416490.1       | Bacillus manliponensis strain BL4-6 |
| 13 | FN995265.1       | Bacillus kochii strain WCC 4582T |
| 14 | HM460884.1       | Bacillus zhanjiangensis strain JSM 099021 |
| 15 | HQ433453.3       | Alteribacillus bidgolensis strain P4B |
| 16 | JN885201.1       | Bacillus bingmayongensis strain FJAT-13831 |
| 17 | KJ733017.1       | Bacillus solisilvae strain NEAU-cbsb5 |
| 18 | MK183820.1       | Bacillus paramycoides strain MCCC 1A04098 |
| 19 | MK184150.1       | Bacillus albus strain MCCC 1A02146 |
| 20 | NR028865.1       | Solibacillus silvestris strain HR3-23 |
| 21 | NR043403.1       | Bacillus thuringiensis strain IAM 12077 |
22 NR074540.1 Bacillus cereus ATCC 14579 (rrnA)
23 NR074914.1 Bacillus cytotoxicus strain NVH 391-98
24 NR114582.1 Bacillus cereus ATCC 14579
25 NR152692.1 Bacillus wiedmannii strain FSL W8-0169
26 NR157729.1 Bacillus albus strain MCCC 1A02146
27 NR157735.1 Bacillus proteolyticus strain MCCC 1A00365
28 NR170494.1 Bacillus fungorum strain 17-SMS-01
29 X76437.1 Bacillus cohnii DSM 6307 T
30 X76443.2 Bacillus horikoshii DSM 8719
31 X76447.1 Bacillus halmapalus DSM 8723

Figure 2. The potential test of Nitrogen-Fixing Bacteria (NFB) and Phosphate Solubilizing Bacteria (PSB) isolates from biogas sludge to increase the total-N and available-P of ultisols. Values followed by a different letter explain the significant difference in the DMRT at P<0.05. The vertical line indicates the standard error (SE). Data PSB has been published by Mustamu et al. [12].

The results showed that bacterial isolates from biogas sludge could increase the total-N and available-P of ultisols with the highest increase in N3 and P7 isolates compared to other isolates. The nitrogen-fixing bacteria isolate (N3) contribution was higher than the phosphate solubilizing isolate (P7). This result was similar to Rodriguez-Gonzalez et al. [13]; nitrifying and denitrifying bacteria use nitrogenase enzymes to metabolize nitrogen to be available, and 54.42% are reported nitrogen-fixing bacteria. It was due to the P7 isolate as phosphate solubilizing bacteria produce organic acids characterized by a decrease in pH that the availability of phosphorus increases. Richardson and Simpson [14]; Khan et al. [15] said that phosphate solubilizing bacteria produce organic acids that solubilizing unavailable-P (PO$_4^{3-}$) into available-P (HPO$_4^{2-}$, H$_2$PO$_4^-$) and Perez et al. [16] reported a decrease in pH from 7 to 2 along with the acidification process. Mustamu et al. [12] also said that isolates of phosphate solubilizing bacteria (P7) from biogas sludge had the highest organic acids sequentially, namely lactic, oxalic, acetic, and citric acids.

3.2. Phylogenetic of superior bacterial isolates

The phylogenetic results of nitrogen-fixing (N3) and phosphate solubilizing bacteria isolates (P7) can be seen in Figure 3. It was identified the nitrogen-fixing bacteria isolate (N3) was nearby, similar to Bacillus paramycoides strain MCCC 1A04098 (MK183820 accession) and the phosphate solubilizing isolate (P7) to Bacillus cereus ATCC 14579 (NR114582 accession).
Figure 3. Phylogenetic tree of N3 and P7 bacteria isolates from biogas sludge with 31 nucleotides of the genus *Bacillus* using the neighbor-joining method with a bootstrap repeat value of 1000x with MEGA 5 software.

Both bacteria superior (N3 and P7) were identified as having the nearby similarity to the *Bacillus* genus, namely nitrogen-fixing bacteria (N3) with *Bacillus paramycoides* and phosphate solubilizing bacteria (P7) with *Bacillus cereus*. It was also reported by Ndubuisi-Nnaji et al. [17] that the anaerobic processing bioreactor contained a group of nitrogen-fixing bacteria (*Clostridium* sp, *Bacillus* sp, *Enterobacter* sp, *Clostridium* sp, *Salmonella* sp), phosphate solubilizing bacteria (*Clostridium* sp, *Bacillus* sp, *Staphylococcus* sp, *Lactobacillus* sp, *Salmonella* sp, *Enterobacter* sp, *Citrobacter* sp, *Clostridium* sp, *Pseudomonas* sp, *Micrococcus* sp). There are groups of bacteria, including nitrogen-fixing and phosphate solubilizing (*Clostridium sp, Bacillus sp, Enterobacter sp*). Safitri et al. [18] reported that the thermophilic bacteria strain D41A had 99.79% similarity to *Bacillus paramycoides*. Suksong et al. [19] said that bacteria of palm oil solid waste from anaerobic digester include: *Ruminococcus* sp., *Thiomargarita* sp., *Clostridium* sp, *Anaerobacter* sp, *Bacillus* sp, *Sporobacterium* sp., *Saccharofermentans* sp, *Oscillibacter* sp., *Sporobacter* sp., and *Enterobacter* sp. Liaquat et al. [20] also reported an abundance of *Bacillus, Clostridium*, and *Enterobacter* spp in an anaerobic digester of wastewater in producing biogas.

**Conclusion**

The N3 and P7 isolates from the biogas sludge had more potentials in the availability of total-N and available-P by 62.56% and 36.21%, respectively, compared to un-isolated. The nitrogen-fixing bacteria (N3) and phosphate solubilizing bacteria (P7) isolates from the biogas sludge were identified similar to *Bacillus paramycoides* and *Bacillus cereus*, respectively.
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