Screening of polyhydroxyalkanoates (PHA)-producing bacteria from soil bacteria strains

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Abstract. Plastic wastes and petrochemical-based polymer materials have become a serious problem to the environment due to the characteristics of these materials that are difficult to degrade in nature. Polyhydroxyalkanoates (PHA) is one type of biodegradable plastics that have a great potential to replace the widely-used hydrocarbon plastics since it will decompose completely into carbon dioxide and water after burial for several months in the soil. PHA can be produced by microorganisms such as bacteria and algae through a fermentation process. The objective of this research is to obtain bacteria that can produce PHA. Screening was carried out by two sequential steps, qualitative and followed by quantitative methods. An amount of 29 bacteria strains isolated from Indonesians soil were screened for this purpose. The qualitative screening was conducted by growing the bacteria in a specific medium containing Nile red dye. The results showed that 19 strains were positive, generated pink to orange colonies under UV light at 235 nm. It was also confirmed by fluorescence microscope. The quantitative screening was performed by measuring the intracellular materials (predicted as PHA) of the bacterial cells by gravimetric method. The results indicated that the highest average of PHA content was 52.9%, 35.6% and 35.4 of dried cell weight, respectively for the Burkholderia sp B73, Bacillus sp B58, *Bacillus toyonensis* B50 and *Staphylococcus cohnii* B66.

1. Introduction
The use of plastic as packaging is increasing along with the development of the plastic industry. Plastic packaging for food products dominates the food industry in Indonesia and even the world. The plastic packaging is used to store, package and wrap food and beverages. The increased plastic packaging was driven by the properties of plastic that are lightweight, flexible, stainable, strong, stable, transparent, not easily broken and resistant to chemical materials [1]. Plastics in the market are petroleum-based plastics, so they are not easily degraded biologically by microbes [2]. Consequently, plastic waste will settle for a long time in the soil and need a long time to decompose in nature [3] and as a result, plastic waste will accumulate and become an environmental problem. One of the solutions to replace conventional plastic materials is to substitute them with environmentally friendly plastics that can be degraded by microbes, called biodegradable plastic or bioplastic [3]. Polyhydroxyalkanoates (PHA) is one type of biodegradable plastics that have great potential and is now being developed by researchers to replace the widely used hydrocarbon plastics. This biodegradable plastic group will decompose completely into carbon dioxide and water after burial for several months in the soil [4].
Polyhydroxyalkanoates (PHA) are a group of polyester synthesized by various types of bacteria and accumulated in the cytoplasm as energy and carbon reserves in the form of granules when the condition of excess carbon with nutrients (phosphorus, nitrogen, sulfur, oxygen) are limited [5–8]. PHA is natural, renewable and biocompatible biopolymers which can be made into plastic materials with properties similar to petrochemical plastics [6]. There are various microbes from negative and positive bacteria that can accumulate PHA as an energy reserve material including Alcaligenes, Nocardia, Bacillus, Pseudomonas, Azotobacter, Rhizobium, Cupriavidus necator, Ralstoniaeutropha, Corneybacterium B. circulans, B. thuriengiensis and Micrococcus sp [9–13]. Each microorganism will produce a different composition of PHA [7,9] and the type of carbon source consumed by each microorganism also determines the type of PHA produced [14]. Because of its biodegradability, biocompatibility and ability to be produced with various types of sources, PHA is interesting to be studied and developed [15]. Studies on PHA production have been widely reported, including screening for the PHA producing bacteria. The bacteria can be obtained from landfill, palm oil mill effluents and others [3,16].

Different carbon sources were used for the production of PHA. Ralstonia picketii was reported to produce PHA in a medium containing fructose as a carbon source [10]. Bacillus subtilis and E. coli were grown on cane molasses fortified with ethanol to produce PHA [7], while Ralstonia eutropha was grown on hydrolyzed sago starch medium as the carbon source [2]. Various inexpensive carbon sources (molasses, corn oil, starch, wheat flour, corn flour, rice brand, cassava starch hydrolysate) were also utilized for PHA production [5,16–18]. The high cost of production is related to the use of carbon sources. Therefore, it is necessary to use a cheap carbon source, one of which is molasses. Molasses is a by-product of the cane or beet sugar processing industry which still contains sugar and organic acids in the form of thick liquid like syrup and dark brown or acidic reddish brown [19–22]. Molasses has a high sucrose content ranging from 48-55% while the pH ranges from 5.5-5.6 [18,23]. The organic C content in molasses has the potential as a carbon source for the growth of PHA-producing bacteria. The goal of the study was to obtain potential bacteria strains for PHA accumulation in the first step, that will be used in the next study to produce PHA by utilizing molasses as fermentation substrates.

2. Material and methods

2.1. Materials

Soil bacteria strains were obtained from InaCC–Research Center for Biotechnology, Indonesian Institute of Sciences. The carbon-rich nutrient agar media containing Nile Red was used as a medium for qualitative screening with the following composition: glucose 1%, beef extract 0.3%, peptone 0.5%, sodium chloride 0.8%, agar 1.5% and Nile red 0.5µg/mL in DMSO. Minimal medium was used for PHA accumulation [24]. The composition (per liter) were: glucose 10 g, (NH₄)₂SO₄ 1 g, Na₂HPO₄·7H₂O 6.7 g, KH₂PO₄ 0.1 g, MgSO₄·7H₂O 0.2 g, ferrous ammonium citrate 60 mg, CaCl₂·7H₂O 10 mg and trace element 1 mL. Trace element solution contained the following components per liter: MnCl₂·4H₂O 1.98 g, CoSO₄·7H₂O 2.81 g, CuCl₂·2H₂O 0.17 g, ZnSO₄·7H₂O 0.29 g and FeSO₄·7H₂O 2.78 g.

2.2. Methods

2.2.1. Maintenance of bacterial cultures. Bacteria cultures were grown on nutrient agar, incubated at 30°C for 24 hours and stored at 4°C before further use.

2.2.2. Qualitative screening of PHA-producing bacteria. Qualitative determination for PHA accumulation of the bacterial isolates was checked by Nile red staining according to the method of Bhuwal et al [25]. The bacterial isolates were grown by streaking method onto carbon-rich nutrient agar media containing Nile red, incubated at 30°C for 24 hours. The colonies grown were observed under UV light at a wavelength of 235 nm. The positive colonies would show bright orange or pink fluorescence.

The positive bacteria for PHA accumulation were confirmed by observing the cells under fluorescence microscope following the method of Aarthi and Ramana [26]. The positive colonies were
grown in nutrient broth medium and incubated at 30°C for 24 hours. The nutrient broth culture of 1.0 mL was inoculated into 10 ml of minimal medium broth containing 0.2 % Nile Red in DMSO. The culture was incubated at 30°C for 24 hours and 10 μl of the culture was then dropped on an object glass and observed under a fluorescence microscope with a magnification of 400. The positive cells would give pink color fluorescence.

2.2.3. Quantitative screening for PHA-producing bacteria. The quantitative screening was carried out by measuring PHA accumulation in positive bacterial cells. Each suspected positive bacterial isolate was grown in nutrient broth medium and incubated in a shaker incubator at 150 rpm, 30°C for 24 hours. The nutrient broth culture of 3.75 mL was inoculated into 75 ml of minimal medium and incubated in an incubator at 150 rpm, 30°C for 72 hours. The culture containing PHA was centrifuged at 7000 rpm for 15 minutes. The supernatant was removed and the pellet was suspended in 5 mL of distilled water and centrifuged at 7000 rpm for 15 minutes. The pellet was added with 3 mL phosphate buffer pH 7.0 and 1 mL NaOCl 5%, then incubated at 180 rpm, 25°C for 24 hours for lysis of cells. The obtained cell extract was centrifuged at 7000 rpm for 15 minutes and subsequently washed with 5 mL distilled water, 3 mL acetone, 3 mL diethyl ether and let stand for 5 minutes. The discard of each rinsed solvent was done very slowly and carefully to avoid removing some of the PHA pellets. Afterwards, the pellet was dried at 40°C until dry and then weighed as PHA.

To determine the accumulation of PHA inside the cells, dry cell weight (DCW) was carried out first. A 1.5 mL microtube was dried at 70°C for 24 hours until constant weight, then 1 mL of cell suspension was added into the microtube and dried in an oven at 70°C for 24 hours until constant weight. The percentage of PHA accumulation was estimated as a percentage of PHA present in the dry cell weight:

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\text{PHA accumulation} \, (\%) = \frac{\text{Dry weight of extracted PHA (g/L)}}{\text{Dry Cell Weight (g/L)}} \times 100\%
\]

3. Result and discussion

In this study, we attempted to screen a number of 29 bacterial strains with different genera and different species of Bacillus, Pseudomonas, Staphylococcus from the genera previously reported. The strains were isolated (previously in another work) from various land sources in several parts of Sulawesi Island, Indonesia including Mekongga, North Kolaka, Papalia, South Konawe and Bantimurung [27]. Out of the 29 bacteria screened with Nile red staining, there were 19 isolates suspected of having the ability to accumulate PHA. Then, the confirmation test was carried out using a fluorescence microscope to see the shape of PHA granules. Table 1 presents the result of the screening of PHA-producing soil bacteria strains based on qualitative analysis.

3.1. Qualitative screening for PHA-producing bacteria

Determination of PHA was qualitatively carried out by Nile red staining, a specific dye for the of PHA granules, which gave luminescence results in bacterial colonies that accumulate PHA [16,28,29]. The fluorescence intensity increased with the increase of PHA content from bacterial cells. They are accumulated intracellularly under the condition of nutrient stress and act as a source of carbon and energy [30]. The screening result from 29 soil bacteria isolates which are planted in carbon-rich nutrient agar medium showed qualitatively, by spreading bright orange or pink fluorescence on UV light irradiation at 235 nm, that 19 soil bacteria isolates were able to accumulate PHA granules (Table 1). The Nile red-stained isolate is indicated by the symbol (+) with a similar fluorescence intensity of all positive strains. The results of planting bacteria on the carbon-rich nutrient medium are presented in Figure 1.
Table 1. Screening of PHA-producing soil bacteria strains based on qualitative analysis.

| No. | Sample sourcea | Name of bacteria     | PHA accumulation | Color  |
|-----|----------------|----------------------|------------------|--------|
| 1   | Soil           | *Aeromonas hydrophylia* | +                | orange |
| 2   | Paddy field soil | *Aeromonas jandae* B925 | -                | -      |
| 3   | Stone, Satondan Island | *Alcaligenes faecalis* | -                | -      |
| 4   | Soil, Mekongga Forest, North Kolaka | *Bacillus altitudinis* B959 | -                | -      |
| 5   | Soil, Mekongga Forest, North Kolaka | *Bacillus cereus* B62 | +                | pink   |
| 6   | Soil, Papalia, South Konawe | *Bacillus sp* B49 | +                | pink   |
| 7   | Soil, Mekongga Forest, North Kolaka | *Bacillus sp* B51 | +                | pink   |
| 8   | Soil, Mekongga Forest, North Kolaka | *Bacillus sp* B58 | +                | pink   |
| 9   | Soil, Mekongga Forest, North Kolaka | *Bacillus toyonensis* B50 | +                | pink   |
| 10  | Collection of Lausanne University, Switzerland | *Bacillus thuriengensis* B332 | +                | orange |
| 11  | Soil, Papalia, South Konawe | *Brevibacterium* sp B 45 | +                | pink   |
| 12  | Soil, Papalia, South Konawe | *Brevibacterium* sp B446 | +                | orange |
| 13  | Soil, Mekongga Forest, North Kolaka | *Burkholderia* spB64 | +                | orange |
| 14  | Soil, Mekongga Forest, North Kolaka | *Burkholderia* spB73 | +                | pink   |
| 15  | Soil, Papalia, South Konawe | *Cupriavidus* spB47 | +                | orange |
| 16  | Soil, Mekongga Forest, North Kolaka | *Cupriavidus* sp B71 | +                | orange |
| 17  | Soil, Mekongga Forest, North Kolaka | *Enterobacter asburiae* B65 | -                | -      |
| 18  | Soil, Mekongga Forest, North Kolaka | *Enterobacter radicincitan* B92 | -                | -      |
| 19  | Soil, Mekongga Forest, North Kolaka | *Enterobacter* sp B30 | -                | -      |
| 20  | Soil, Papalia, South Konawe | *Enterobacter* spB48 | +                | pink   |
| 21  | Soil | *Pseudomonas aeruginosa* B52 | +                | pink   |
| 22  | Soil, Mekongga Forest, North Kolaka | *Pseudomonas* sp B68 | -                | -      |
| 23  | Soil, Mekongga Forest, North Kolaka | *Pseudomonas* spB69 | +                | orange |
| 24  | Soil, Mekongga Forest, North Kolaka | *Pseudomonas* sp B70 | -                | -      |
| 25  | Soil, Mekongga Forest, North Kolaka | *Ralstonia* sp B57 | -                | -      |
| 26  | Soil, Mekongga Forest, North Kolaka | *Rhodococcus equitii* B67 | +                | orange |
| 27  | Paddy field soil, Surabaya | *Pseudomonas* sp B959 | -                | -      |
| 28  | Soil, Bantimurung, Sulawesi | *Staphylococcus caprae* B442 | +                | orange |
| 29  | Soil, Mekongga Forest, North Kolaka | *Staphylococcus cohni* B66 | +                | orange |

a Catalogue of Microorganism, Indonesian Culture Collection (InaCC) [27]

Confirmation was carried out by growing the positive colonies in minimal broth media containing Nile red. Minimal media was enriched by glucose as a carbon source for bacterial growth so that bacteria could form PHA granules as carbon stocks and energy in cells, due to an imbalance of growth conditions (excess carbon and limited nutrients i.e. phosphorus, nitrogen, oxygen, potassium and sulfur) [31].

Observation of the cultures under a fluorescence microscope at 580 nm would show fluorescence for all colonies that accumulate PHA in the cell. PHA in the form of granules absorbed Nile red and fluoresce when excited by fluorescent light. In this study, all of 19 positive colonies grown on nutrient-rich agar medium were subjected to confirmation for PHA accumulation. As shown in Figure 2, all of the positive colonies were confirmed to be able to present cells that fluoresce under a fluorescent microscope. From 19 isolates, only four isolates were showed excellent by nutrient-rich carbon in the plate assay method.


Figure 1. Orange/pink fluorescence under UV light by PHA-producer (1-19) and non-fluorescence under UV light by non-PHA-producer (20).

3.2. Quantitative PHA Analysis

Strains that were detected to be able to accumulate PHA were then first grown in nutrient broth as a pre-culture media with the aim of enriching the bacteria. Afterwards, the pre-culture was grown in minimal media containing glucose as a carbon source. After three days of incubation, the PHA in the culture was extracted and the weight was determined. Table 2 shows that all of the 19 bacteria strains accumulate PHA. These strains produced PHA from 0.15 to 0.70 g/L, with a yield ranging from 15.7 to 57.7 % PHA of cell dry weight. The highest yield of PHA was presented by Burkholderia sp B73, 52.9% of cell dry weight, followed by Bacillus sp B58 with 35.6% of cell dry weight, Bacillus toyonensis B50 with 35.3% of cell dry weight and Bacillus cohnii B66 with 35.3% of cell dry weight. The four strains were indicated to be able to use glucose source for their growth and convert glucose to PHA as a food reserve when other nutrients in the media were limited. The interesting finding is that all of those four strains were isolated from the same source namely soil samples taken from the Mekongga Forest in North Kolaka.
Similar results were reported by Reddy et al [31], in which 15 municipal sewage strains classified as the genera of *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Aeromonas*, and *Chromobacterium* accumulated PHA ranged from 17-64 % of cell dry weight after 48 hours’ incubation with glucose as the carbon source. Sangkharak and Prasertsan [32] also reported PHA accumulation with similar results from 50 isolates from soil samples taken under selective conditions such as pH, salt concentration and heavy metal types. After incubation for 96 hours with urban wastewater, palm oil mill effluents, glycerol and
molasses as carbon sources, 10-68.80% (w/w) PHA contents were obtained and the PHA-producing bacteria were identified as Bacillus, Proteus, Pseudomonas, Aeromonas, Alcaligenes and Chromobacterium bacteria. However, low PHA content ranged from 1.6 to 9.4% (w/w) was reported from Pseudomonas aeruginosa and Bacillus subtilis after 72 hours incubation using glucose as the carbon source [3]. The highest PHA producing strain in this work, Burkholderia sp B73, will be used for further study. According to the several research previously reported, the finding strain Burkholderia sp B73 as PHA producer is for the first time reported to date.

Table 2. PHA accumulated by the 19 positives cultures.

| No. | Isolates                  | Run | Cell dry weight (g/L) | PHA amount (g/L) | PHA content (%) | Average PHA content (%) |
|-----|---------------------------|-----|-----------------------|------------------|-----------------|------------------------|
| 1   | Bacillus thuringensis     | 1   | 1.2662±0.0377         | 0.3298±0.0194    | 26.1            | 31.8                   |
|     |                           | 2   | 0.9427±0.0162         | 0.3547±0.0000    | 37.62           |                        |
| 2   | Bacillus sp B58          | 1   | 1.1542±0.0470         | 0.3236±0.0108    | 28.1            | 35.6                   |
|     |                           | 2   | 1.6302±0.0143         | 0.7031±0.0194    | 43.13           |                        |
| 3   | Staphylococcus cohnii B66 | 1   | 0.7840±0.0247         | 0.2831±0.0143    | 36.1            | 35.3                   |
|     |                           | 2   | 0.8027±0.0162         | 0.2769±0.0460    | 34.50           |                        |
| 4   | Bacillus cereus B52      | 1   | 1.1293±0.0230         | 0.3018±0.0108    | 22.3            | 34.1                   |
|     |                           | 2   | 1.6520±0.0162         | 0.3111±0.0269    | 41.49           |                        |
| 5   | Staphylococcus caprae B442 | 1   | 1.4280±0.0830         | 0.3204±0.0593    | 30.7            | 22.7                   |
|     |                           | 2   | 1.3098±0.0269         | 0.3827±0.0000    | 23.16           |                        |
| 6   | Cupriavidus sp B47       | 1   | 1.2476±0.0353         | 0.3827±0.0162    | 30.7            | 31.2                   |
|     |                           | 2   | 0.6409±0.0054         | 0.4169±0.0353    | 31.83           |                        |
| 7   | Burkholderia sp B73      | 1   | 0.6876±0.0514         | 0.4013±0.1213    | 57.7            | 52.9                   |
|     |                           | 2   | 0.9644±0.0285         | 0.3080±0.0407    | 48.06           |                        |
| 8   | Bacillus toyonensis B50  | 1   | 1.0516±0.0944         | 0.3951±0.0389    | 37.9            | 35.4                   |
|     |                           | 2   | 1.1853±0.00583        | 0.3173±0.0900    | 32.90           |                        |
| 9   | Aeromonas hydrophilia     | 1   | 1.1387±0.0371         | 0.2987±0.0247    | 26.2            | 26.9                   |
|     |                           | 2   | 1.1853±0.0583         | 0.3267±0.0373    | 27.56           |                        |
| 10  | Bacillus sp B51          | 1   | 1.3751±0.0635         | 0.4138±0.0285    | 30.1            | 30.9                   |
|     |                           | 2   | 1.0018±0.0235         | 0.3173±0.0373    | 31.68           |                        |
| 11  | Brevibacterium sp B45    | 1   | 1.5058±0.0054         | 0.3889±0.0377    | 28.1            | 29.9                   |
|     |                           | 2   | 1.5524±0.0194         | 0.4916±0.0235    | 31.66           |                        |
| 12  | Bacillus sp B60          | 1   | 0.8587±0.0093         | 0.3733±0.0000    | 43.5            | 34.9                   |
|     |                           | 2   | 1.0094±0.0235         | 0.2644±0.0054    | 26.33           |                        |
| 13  | Brevibacterium sp B46    | 1   | 1.5120±0.0583         | 0.3547±0.0093    | 23.5            | 20.6                   |
|     |                           | 2   | 1.2813±0.0108         | 0.2271±0.0235    | 17.72           |                        |
| 14  | Pseudomonas aeruginosa    | 1   | 0.6689±0.0862         | 0.3204±0.0143    | 48.4            | 32.0                   |
|     |                           | 2   | 1.8231±0.0285         | 0.2862±0.0194    | 15.70           |                        |
| 15  | Cupriavidus sp B71       | 1   | 0.9676±0.0389         | 0.3204±0.0143    | 33.1            | 29.9                   |
|     |                           | 2   | 0.9802±0.0247         | 0.2613±0.0612    | 26.67           |                        |
| 16  | Burkholderia sp B64      | 1   | 1.3969±0.0441         | 0.4044±0.0470    | 28.9            | 26.5                   |
|     |                           | 2   | 1.4031±0.0108         | 0.3391±0.0235    | 24.17           |                        |
| 17  | Rhodococcus equi         | 1   | 1.4249±0.1159         | 0.4013±0.0093    | 28.3            | 34.8                   |
|     |                           | 2   | 0.9240±0.0093         | 0.3827±0.0280    | 41.41           |                        |
| 18  | Enterobacter sp B48      | 1   | 1.4591±0.0328         | 0.3578±0.0216    | 24.5            | 22.0                   |
|     |                           | 2   | 1.0928±0.0143         | 0.2707±0.0428    | 19.46           |                        |
| 19  | Pseudomonas sp B68       | 1   | 1.0769±0.0261         | 0.3889±0.0108    | 36.1            | 25.5                   |
|     |                           | 2   | 1.3907±0.0520         | 0.1524±0.0054    | 17.82           |                        |
4. Conclusions
Out of the 29 bacterial strains tested, 19 strains were able to accumulate PHA by giving a positive reaction to Nile red staining. Quantitative measurement of PHA from 19 strains presented four potential strains for PHA accumulation ranged from 35.3–52.9% of cell dry weight. The highest PHA producer *Burkholderia* sp B37 is for the first time reported.

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