Isolation and identification of polyhydroxyalkanoates producing bacteria from biopolymers waste in soil

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Abstract: The production of polyhydroxyalkanoates PHAs from biopolymer degrading bacteria was examined in situ by screening isolates using Sudan B Black staining process as potential PHAs detecting, and Nile Blue staining as a proof method detection. Five bacterial strains isolated from biopolymer waste buried in a garden soil were able to produce high rate of PHA. AK1P and AK2P strains demonstrated high productivity of biopolymer by converting 5% (w/v) lactose as the only carbon source to PHA during fermentation. AY2P strain converted 5% (w/v) of glucose with less PHA accumulation. The favorite temperature for those strains to produce a high rate of PHA was at 30º C.

Keywords: Polyhydroxyalkanoates (PHA); Nile Blue A; Sudan Black B; biopolymer; lactose; glucose.

1. Introduction
Petroleum-derived plastics which already in widely use are being regarded as major threat of pollution. they are harming wildlife, deterioration of natural beauty, blockage of sewage systems [1] (Okunola A et al.2019). Biodegradable materials are including polyglycolic acids, polyhydroxyalkanoates (PHAs), polylactides, polysaccharides, aliphatic polyesters, PHAs considered as particular interest due to their thermoplastic characteristics and are similar to many synthetic polymers to a large extent. Polyhydroxyalkanoates (PHAs) is a term given to a family of biogenic polyesters that can be produced by in microorganisms [2] (Bastioli 2020). It is known that, Poly(3-hydroxybutyrate) (PHB) is a homo polymer of 3-hydroxybutyrate, the best characterized and most widespread member of the PHA family.

In the meantime, polyhydroxyalkanoates (PHA) attracting increasing global attention as renewable, bio-based polymers and biodegradable alternatives to petrochemical origin plastics [3] (Kourmentza et
al. 2017). The molecular weights (MW) of PHAs can range from 10 to several thousand, depending on bacterial strain, growth conditions, and carbon source. PHB as a group labeled to PHAs that has similar physical properties to polypropylene, including an aroma barrier and moisture resistance [4] (Mathuriya and Yakhmi 2017). It has been demonstrated by several that PHAs products are not toxic to the soft issues and cells [5] (Chen and Zhang 2018). PHAs have diverse properties and structures to contribute in medical applications. The objective of this study is to isolate bacteria that able to degrade biopolymer wastes in soil and examine its’ potential to produce PHA by fermenting lactose or glucose as a carbon source with other nutrients. Our results demonstrate that these strains are a PHA synthesizers and we consider them as a good candidate for further research on optimization of growth, cost effective methods for PHA extraction and determining the PHAs types.

2. Materials and Methods

2.1. Sampling

2.1.1. Polyhydroxyalkanoates Degrading Bacteria. A Bioplastic sheets, made of specific PHA granules manufactured by German company, were buried in a garden at the depth of 5 cm in a garden soil under three zones. These sheets allowed to degrade naturally for 7 days, sampled using sterile forceps and transferred to the laboratory by aseptic plastic bags for the isolation of PHAs producing bacteria. Serial dilution technique was performed using sterile water. Aseptically, 1 ml of the solution poured in a petri dishes of nutrient agar medium (LabM, England) containing 5% of one of the two sugars Glucose and Lactose, equally. Samples were incubated at 37º C for 3 days.

2.1.2. Isolation of Polyhydroxyalkanoates Producing Bacteria. At the end of incubation period mentioned in previous step, each colony was picked out and streaked individually in polyhydroxyalkanoates medium (PM) agar and incubated for 3 days at 37º C. The medium containing (g l⁻¹): 0.6 K₂HPO₄, 3H₂O; 0.2 KH₂PO₄; 0.2 MgSO₄.7H₂O; 0.2 (NH₄)₂SO₄; 50.0 Glucose (as the only carbon source); 20 agar; supplemented with 2.0 ml l⁻¹ of trace element solution containing (g l⁻¹): 0.10 ZnSO₄.7 H₂O; 0.03 MnCl₂.4 H₂O; 0.3 H₃BO₃; 0.2 CoCl₂.6 H₂O; 0.01 CuCl₂ · 2 H₂O; 0.02 NiCl₂.6 H₂O; 0.03 Na₂MoO₄. 2 H₂O. pH adjusted to 7.5, autoclaved at 121º C for 15 minutes. This medium was used in the next experiments in this study.

3. Screening of PHA producing by isolated bacteria

3.1. Sudan Black B (SBB) staining

All isolates of bacteria were examined for PHA accumulation using Sudan Black B staining (Merck, Germany). Stain procedure was done as described by Balakrishna Pillai et al. (2018) [6]. The smear was examined under (100×) oil immersion objective of light microscope (Olympus CH20i, Japan).

3.2. Nile Blue A staining

Nile blue stain is a more effective and specific stain method for the confirmation of PHAs accumulation. The procedure was performed according to Ostle and Holt (1982) [7] and Legat et al. 2010 [8]. The smears were prepared on a glass slide and fixed by heating, stained with 1% of aqueous solution of Nile Blue A (Sigma-Aldrich, Germany) at 55 °C for about 10 minutes, slides washed with a sterile water and blot dried. The slides were examined under fluorescence microscope (ZEISS, GERMANY) at 460 nm.

4. Identification of Isolates
The successful cultures in the selective Glucose medium were subjected to Gram staining and identified using biochemical test procedure and VITEK 2 Compact system (Biomerieux, France). Five isolates were examined their ability to produce PHA.

5. The role of Sugar Source and temperature in PHA production
Many factors are significantly affect the PHA production by various type of bacteria. Among these factors, temperature and carbon source (sugar) were investigated which are mainly affect the growth of bacteria and PHAs accumulation. Monitoring of Colony growth and PHAs production were examined using fluorescence microscope. Isolates were cultured separately on (PM) agar (as mentioned above) at 30 °C and 37 °C respectively, for 3 days.

6. Results and Discussion
6.1. Isolation and screening of PHAs production
In this study, twenty-one of bacterial isolates were tested for the PHAs production. fifteen isolates were observed in lactose containing plates, while only six isolates overserved in Glucose-nutrient agar plates. Each colony was grown clearly on the PM agar that contained the same lactose or glucose as the only carbon source.

The ability of isolates to produce PHA was screened by Sudan black staining, cultures showed granules filled up with dark staining, as PHA granules can be observed as dark spot under microscope light. Sixteenth isolates showed positive for Sudan black staining that considered to produce PHA granules (figure 1). Only five isolates L1, L2, L5 (lactose depending), G1 and G5 (glucose depending) were selected for the second stain procedure due to the high color intensity. These isolates observed good growth when re-cultured on the same agar and showed positive to Nile blue staining, indicating a presence of PHAs in the cells.

Zain, et al. (2016) [9] confirmed the production of PHAs granules using Nile blue staining as a specific stain and noted that producing isolates are a strong PHAs producers. Tan, et al. 2014 [10] mentioned that Nile blue staining provides evidence for PHAs production which enabled microbe producers to be isolated and identified. Tufail, et al. (2017) [11] screened the PHAs producers as a direct method by observing blue fluorescence under ultraviolet (UV).

Figure 1. Sudan Black B staining observed dark PHAs granules in selected isolates under microscope (100x).
Gram stain procedure was done to differentiate between Gram positive and negative bacteria, results showed 13 isolates Gram negative (grown on PM-Lactose agar) and 2 Gram positive, 1 Gram negative (grown on PM-glucose agar). Table 1, showed screening of PHAs production by isolates according to sugar source and Gram stain.

**Table 1.** Screening of PHAs production by isolates.

| Isolates | Sugar source | Gram stain | Sudan Black B stain* | Nile Blue stain* |
|----------|--------------|------------|----------------------|------------------|
| L1       | Lactose      | -ve        | ++                   | +++              |
| L2       | Lactose      | -ve        | ++                   | +++              |
| L3       | Lactose      | -ve        | +                    | -                |
| L4       | Lactose      | -ve        | +                    | -                |
| L5       | Lactose      | -ve        | ++                   | +++              |
| L6       | Lactose      | -ve        | +                    | -                |
| L7       | Lactose      | -ve        | +                    | -                |
| L8       | Lactose      | -ve        | +                    | -                |
| L9       | Lactose      | -ve        | +                    | -                |
| L10      | Lactose      | -ve        | +                    | -                |
| L11      | Lactose      | -ve        | +                    | -                |
| L12      | Lactose      | -ve        | +                    | -                |
| L13      | Lactose      | -ve        | +                    | -                |
| G1       | Glucose      | +ve        | ++                   | +                |
| G2       | Glucose      | +ve        | +                    | -                |
| G3       | Glucose      | -ve        | ++                   | ++               |

*Note: Granules intensity/fluorescence: + = slightly observed. ++ = high. +++ = fully. - = none.

6.2. Identification of isolates and carbon source dependent

Bacterial isolates with high PHAs production have specific enzymes differs in their functions according to the surrounding conditions such as nutrients (availability or depletion), stress and other limiting growth factors. In this study, some isolated bacteria which were already degrade or consume biopolymer waste exhibited their ability to utilize lactose and glucose as the only carbon source and producing intracellular PHAs granules this gives the idea that PHA degrading bacteria have enzymatic system with multi-function whether to degrade PHA by extracellular depolymerase, or to produce it by PHA synthase in certain circumstances.

![Figure 2. Isolated bacteria on: PM-Lactose agar: (a) L1. (b) L2. (c) L5. PM-Glucose agar: (d) G3. (e) G1.](image-url)
Table 2. Isolated bacterial identification and its’ PHA production.
*Details is hidden.

| Isolates | Strain* | Carbon source | PHAs  |
|----------|---------|---------------|-------|
| L1       | AK1P    | Lactose       | Fully |
| L2       | AK2P    | Lactose       | Fully |
| L5       | AK2P    | Lactose       | Fully |
| G3       | AY2P    | Glucose       | High  |

Four isolates were identified and exhibited high fluorescence intensity by Nile Blue staining (figure 2) and (table 2).

Figure 3. Fluorescence microscopy of strains with Nile Blue stain. (incubation at 37 °C for 3 days). (a): L1; (b): L2; (c) & (d): L5; (e): none fluorescence (Control).
The strains AK1P and AK2P re-cultured on PM agar at 30 °C for 3 days. Results show good growth and high fluorescence intensity due to the high rate of intracellular PHA produced. This mean the ability of those isolates to produce biopolymers by metabolizing lactose and glucose at temperatures range between 30 °C to 37 °C. According to this study, 30 °C gave higher productivity of PHAs (figure 4). The same results illustrated by several researchers, Singh, et al. 2019; Ocampo-López, et al. 2015; Karbasi, et al. 2011 [12,13,14], found that 30º C was the optimum temperature for PHB production by different bacterial species and cyanobacteria.

![Figure 4](image)

**Figure 4.** Fluorescence microscopy of strains with Nile Blue stain. (Incubating at 30 °C for 3 days). a = AK1P sp. b = AK2P sp.

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