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Production of bioplastic by bacteria isolated from local soil and organic wastes

Wala'a Shawkat Ali[1,*], Neihaya Heikmat Zaki[2] and Somaya Yassen Najim Obiad[2]

[1] *Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.*
[2] *Department of Biology, College of Science, Al-Mustansiriyah University, Baghdad, Iraq*

* Corresponding author: Dr. Wala'a Shawkat Ali; e-mail: microrose.2788@yahoo.com; dr.walaashali@scbaghdad.edu.iq

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ABSTRACT

Polyhydroxybutyrate (PHB), due to similar mechanical properties, has become alternative for petrochemical derived plastic. PHB is biodegradable, ecofriendly, biocompatible and microbial thermoplastic. In order to isolate and screen the ability of bacterial species to produce bioplastic (PHB), samples of soil and organic wastes were collected from different areas near the Al-Mustansiriyha University and Al-Zahraa region in Baghdad. Twenty-eight bacterial isolates were obtained (6 from soil and 22 from organic wastes). Results of screening to produce PHB by Sudan black staining method showed that five isolates (three from organic wastes referred to as W1, W2 and W3, and two from soil samples referred to as S1 and S2) were high efficient to produce PHB granules. PHB was extracted and purified from the five selected isolates by sodium hypochlorite-chloroform method and the results showed that the isolate W3 was the best one for production of it. Additionally, the selected isolates were characterized and the results showed that all the five isolates belong to *Bacillus* spp. depending on cultural, microscopic, and biochemical tests. The amount of polymer that produced by W3 was about 74 mg dry weight. FTIR analysis was performed for characterization PHB and the results showed two intense absorption bands specific for C=O and C–O stretching vibrations at 1730.21 and 1274.99 cm^{-1}, respectively. PHB methanolysed and analyzed by Semi Electron Ionization (SEI) and exhibited that illustrates purified material (target) with a large one dome-shaped. The results of GC-MS analysis appeared that the drawing molecules present in the target similar to the drawing molecules of a graphic library (standard). Also from the data of GC-MS, the molecular weight of PHB that obtained from the isolate W3 was 418 kDa.

Keywords: PHB; bioplastic; screening; polymer analysis.

1. INTRODUCTION

Materials of synthetic plastics are considered as a basic need for human life at the present time [1], but unfortunately these materials are non-biodegradable, so they can cause contamination to our environment [2]. Furthermore, the recycled plastics cause more problems to the environment than the origin plastic due to the mixing of additives, colors and stabilizers [1]. Because of environmental and waste management problems, alternative is the bioplastic [3]. Bioplastic is a family of a new generation of biobased, biodegradable or features both characteristics plastics [4]with different applications [5]. It is eco-friendly alternative to plastics and biodegradable by the microbial enzymes [6] into CO_2, methane, H_2O and biomass [2]. Although bioplastic production will lead to decrease consumption of fossil fuels and CO_2 emissions as well as reduce plastic waste generation [3], the high cost for production of bioplastic from bacteria is still the major limitation in comparison with the production cost of petroleum-derived polymers [4].However, within the next decade, it is expected that bioplastics will capture 30% of the total market of plastics [3].

Polyhydroxylalkanoates (PHAs) are the most studied thermoplastic biopolymers [7], discovered for the first time by Lemogine in 1926 [8] and have numerous applications in different fields of life [4], they are used in clothing, industrial products, fluid containers,

wrapping and building materials, packaging films, toys household, shopping and garbage bags [5]. PHAs can be synthesized by various microorganisms under imbalanced conditions of nutrition [9]. They are water-insoluble compounds and can be divided depending on the number of carbon atoms into long-chain length PHA (lcl-PHA), medium-chain length PHA (mcl-PHA) and short-chain length PHA (scl-PHA)which including; PHB, PHH, PHV, PHD and PHO. PHBs are natural polyesters that are stored as intracellular inclusions by a great variety of bacteria [7] such as *Staphylococcus*, *Alcaligenes, Bacillus, Pseudomonas, Micrococcus* and *Rhodococcus* [3] and they have characteristics similar to those of standard synthetic plastics [1].

This study aimed to screen the ability of bacteria that isolated from soil and organic wastes to produce PHB and characterization it by FTIR and GC-MS, in addition to characterize the producing bacteria.

2. MATERIALS AND METHODS

2.1 Samples collection and isolation of pure cultures

Soil and organic-wastes samples were collected in sterile plastic sacs from different areas at Al-Mustansiriyha University and Al-Zahraa region in Baghdad. One gram of each sample was dispensed in 10 ml of sterile distilled water and mixed vigorously, serial decimal dilutions (10^{-1} – 10^{-8}) were made for each one of them by using physiological normal saline. 0.1ml from each one of the last three dilutions was plated onto a nutrient agar medium by spread plate method, and then plates incubated at 37 °C for 24 hours. The isolated colonies were sub cultured again by streaking method on nutrient agar plates, incubated at 37 °C for 24 hours, the pure cultures of different isolates preserved at 4 °C for future use in screening for production of PHB and maintained by sub-culturing the isolates at an interval of 4-6 weeks [1].

2.2 Screening of isolated bacteria for production of PHB

Bacterial isolates were cultured for 2-3 days at 37 °C in Minimal Davis Medium [5]. From each, a loop full culture was taken on clean, sterile glass slides and stain with Sudan black stain [1].Five bacterial isolates, three from organic wastes and two from soil were selected and used in the subsequent experiments.

2.3 Extraction of PHB

It was performed according to Singh and Parmar [3] by sodium hypochlorite-chloroform method; the five selected isolates were cultured in Minimal Davis Medium at 37 °C for 3 days. After incubation, 10 ml of culture was centrifuged at 6000-rpm for10 minutes and supernatant was discarded. The pellet was suspended in 5 ml of 4 % sodium hypochlorite and 5 ml of hot chloroform and incubated at 37 °C for 1 hour. After incubation, the suspension was centrifuged at 3000 rpm for 10 minutes. Upper and middle phases were discarded, 5 ml of hot chloroform was added to the bottom phase, and then 5 ml of ethanol and acetone mixture (1:1) was added to precipitate the granules. The precipitate was allowed to evaporate for dryness at 30 °C, and then the weight of PHB was measured.

2.4 Polymer analysis

- **Fourier Transform Infra-Red (FTIR) analysis:** In order to know the functional groups present in PHB extract, about 1mg extracted sample of PHB was dissolved in 5 ml of chloroform. Chloroform was allowed to evaporate to get PHB polymer film and then subjected to FTIR analysis by using FTIR spectrophotometer (8400S-SHIMADZU). Spectra were recorded in in the range 4000–600 cm−1 [10].

- **Gas chromatography mass spectrometry (GC-MS) analysis:** For molecular analysis of purified polymer, a coupled Gas chromatography–mass spectrometry (GC-MS) performed using a GC-MS-QP 2010 Plus model, with capillary Column-Rtx-5 MS (30 m × 0.25 mm × 0.25 µm film thickness). The samples were injected (3 µL) in the splitless mode, and the injection temperature was 260°C and column oven temperature was 100°C. The mass spectra obtained were compared with the Nist-08 and Willey-08 mass spectral library [11].

2.5 Identification of the PHB producers

They were identified depending on microscopic examination by using Gram stain , macroscopic examination of colonies on solid media (nutrient agar, blood agar, mannitol salt agar and MacConkey agar) and biochemical tests which included; catalase test, oxidase test, urease test, indole production, methyl red test, Vogues-Proskauer reaction, citrate utilization and starch hydrolysis [12].

3. RESULTS AND DISCUSSION

3.1 Isolation of bacteria and screening for production of PHB

Twenty-eight of bacterial isolates were obtained, six from soil samples referred to as S1-S6 and 22 from organic wastes samples referred to as W1-W22. To screen the ability of bacterial isolates to produce PHB, Sudan black staining method was used. In this staining, microorganism shows positive in blue violet and shows negative in yellow-brown [1]. Twenty of 28 isolates showed positive for Sudan black staining and were able to produce PHB granules but only five isolates, W1, W2, W3, S1 and S2 were selected for further study due to their high color intensity with Sudan black. The accumulation of PHA by bacteria that isolated from different environments such as soil, organic wastes, marine and sewage sludge have been recorded. Bhagowati [5] obtained 32 of bacterial isolates from marine and organic wastes and he noted that most of the isolates from organic wastes showed the production of PHB in cells. Radhakrishnan *et al* [13] recognized 18 isolates from marine sediment and only six of them were found to produce PHA.

3.2 Extraction and quantification of PHB

Extraction of PHB from the five selected isolates was performed by sodium hypochlorite-chloroform method. The results showed that the isolate W3 was the best one for production of PHB; it produced about 74 mg/L of PHB. On the other hand, the amounts of PHB from the rest isolates were very little (table 1 and Figure 1).Solvent extraction is common, simple, rapid and efficient method to obtain very pure PHB from the cell biomass [7,14]. It is involved two main steps; modification of the plasma membrane permeability to release and solubilization of PHA, and then precipitation of PHA [14]. Acetone is the most solvent that used to extract PHB [15] while methanol and ethanol are commonly used to precipitate it [16].

PHAs like PHB are intracellular inclusions and can form approximately in all bacteria. The biosynthesized PHAs can reach up to 90% of the dry cell mass as a response to growth conditions deficiency [17]. Some microorganisms such as *Rolstonia eutropha* , *Alcaligenes latus*, and *Pesudomonas oleovorans* in their wild types forms can produce PHAs between 50% and 80% of the dry cell mass [18].

Table 1: Extracted Polyhydroxybutyrate (PHB) dry weight that produced from the bacterial isolates.

Bacterial isolate	Extracted PHB dry weight (mg/L)
W1	26
W2	2
W3	74
S1	12
S2	1

W= isolate from organic waste samples, S= isolate from soil samples

Figure 1: Extracted Polyhydroxybutyrate (PHB) that produced from the W3 bacterial isolate.

FTIR analysis was performed for characterization PHB that extracted from the selected isolate in order to know the functional groups present in PHB extract, and used for recording IR spectra in the range 4000–600 cm−1. The results of IR spectra showed two intense absorption bands specific for C=O and C–O stretching vibrations at 1730.21 and 1274.99 cm−1, respectively, and C–H stretching vibrations of methyl, methylene groups. These absorption bands confirm the structure of PHB (Figure 2). Similar results to our results were recorded in the other studies [11, 19].

In the presence of sulphuric acid and methanol, the PHB was methanolysed and analyzed by GC-MS. Figure 3 illustrates the purified material (target) with a large one dome-shaped by Semi Electron Ionization (SEI) Mode; it shows the Retention time (RT/min) on the x-axis and the Intensity (I) of the y-axis.

Figure 4(a and b) shows the mass/z (m/z) of x-axis, Intensity (I) of the y-axis, and the top drawing molecules present in the target compared with the drawing below of a graphic library (standard). The best temperature was 140 and similarity Index was 82 % to control. From the data of GC-MS, the molecular weight of PHB that obtained from the isolate W3 was 418 kDa. MS graph showing that the major peak at (m/z 150) is of phthalate, which is a type of plastic, and compound name is (1,2-Benzenedicarboxylic acid, 6-methylheptyl 8-methylnonyl ester, Isooctyl isodecyl phthalate, 1-(6-Methylheptyl) 2-(8-methylnonyl) phthalate); and its formula is $C_{26}H_{42}O_4$.

The structure of components could be clarified by the help of GC-MS analysis and the identification of the compounds is depending on their retention peak. However, the use of GC-MS for characterization the extracted PHA has been reported by others [11, 20, 21].

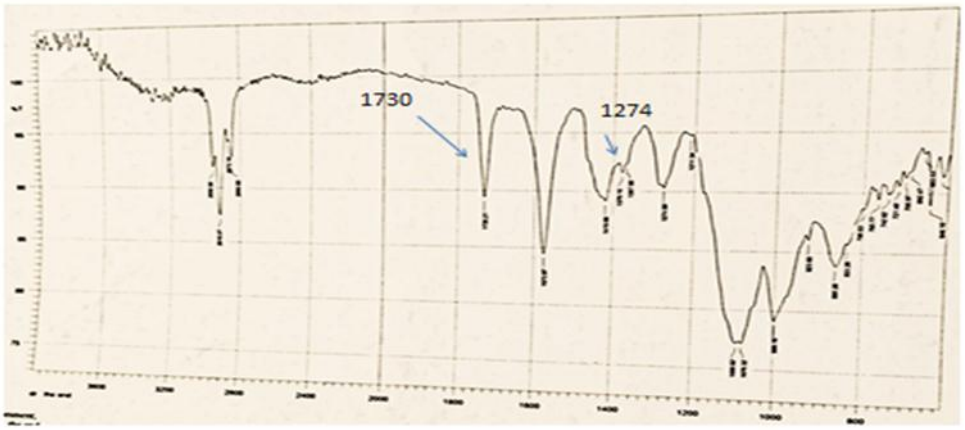

Figure 2: FTIR analysis of extracted PHB

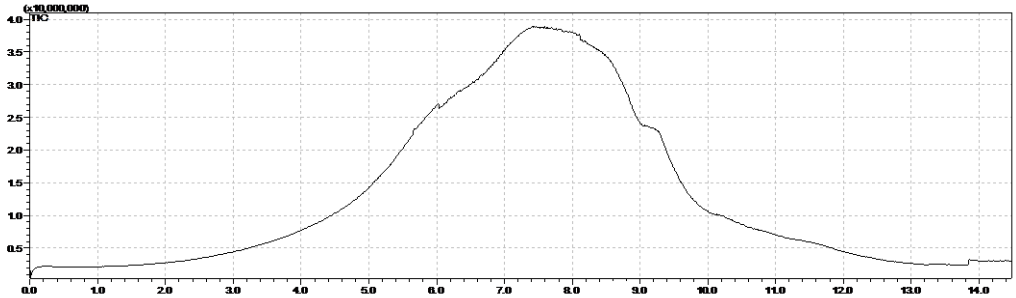

Figure 3: Analysis of PHB by Semi Electron Ionization (SEI) Mode.

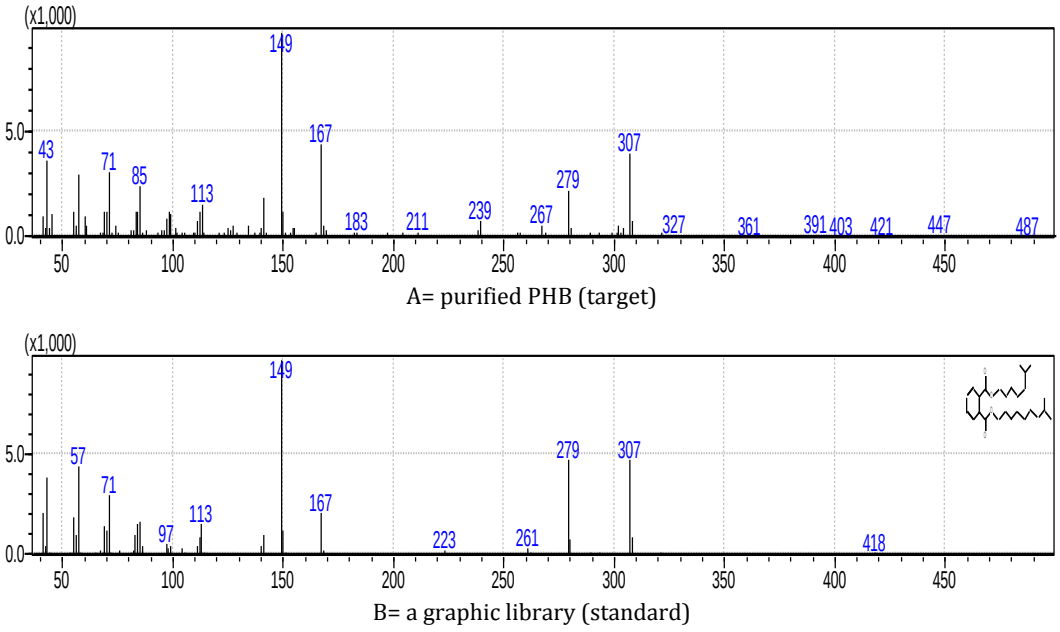

Figure 4: Analysis of purified PHB with GC-MS apparatus.

Characterization of PHB producing isolates

Morphological examination of the five selected isolates grown on nutrient agar medium showed large, cream- to beige-colored, raised, irregular round, with lobate margins colonies, while they were developed large, irregular, rough and waxy colonies by zones of beta hemolysis on blood agar (Figure 5). They were unable to grow on MacConkey agar or mannitol salt agar. MacConkey agar is a selective medium that supports growth of Gram negative bacteria and inhibits Gram positive bacteria. Mannitol salt agar is used for the isolation of Staphylococci [12]. In microscopic examination, the cells of the five selected isolates reacted positively with Gram stain and appeared as large purple rods, often in pairs or chains with rounded or square ends(Figure 6). All the bacterial isolates gave

negative results for catalase and methyl red tests while they showed positive results for indole production, Vogues-Proskauer reaction, citrate utilization, starch hydrolysis, oxidase and urease tests. Depending on the results above, the bacterial isolates could be identified as *Bacillus* spp. [22, 23].Many *Bacillus* species have ability to live in different natural environments [22], so the isolation of this genus from both samples of soil and organic wastes was expected.

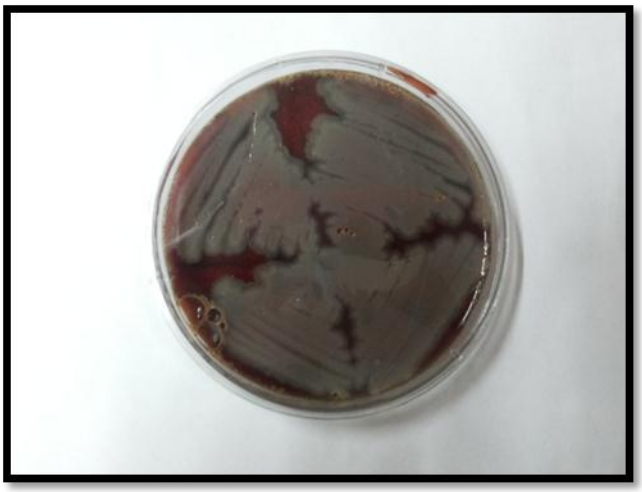

Figure 5: Growth of *Bacillus* spp. on blood agar medium

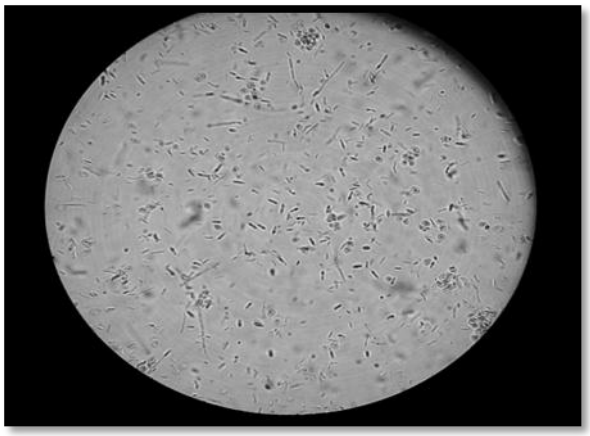

Figure 6: Microscopic picture of *Bacillus* spp.(Imaging was performed under oil- immersion)

4. CONCLUSION

From this study, it concluded that waste isolate W3 (which belongs to *Bacillus spp*.) showed ability to accumulate PHB. It was able to produce 73 mg/L of it. FTIR spectra and GC-MS of the synthesized PHB reveal similar spectra with standard PHB. The bacteria from organic waste were found to be more capable of producing PHB. Hence, the continuous search from the various environmental conditions may provide some more suitable isolates and their genetic modification, for efficient PHB production for commercial use.

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