Isolation and characterization of local *Azotobacter* isolate) producing bio-plastics and consuming waste vegetable oils

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Abstract. Polyhydroxyalkanoates (PHAs) are a family of polyhydroxyesters synthesized by numerous bacteria as an intracellular carbon and energy storage compound under nutrient-limiting conditions with excess carbon. An extensive screening program was previously done to isolate a promising bacterial isolate capable of polyhydroxyalkanoate (PHA) production. Polyhydroxalkanoates are biodegradable thermo polymers like poly-β-hydroxybutyrate (PHB), which can be produced intracellularly as carbon and energy reserves. In this present study, out of 35 isolates, as many as 12 isolates were found to accumulate PHA which isolated from the oil and plastic contaminated soils, collected from different contaminated sites in Baghdad. However only one bacterial colony is selected among others based on maximum PHA yield (4.8g/L) after 48 hrs of incubation at 30°C with 54 % of PHA. Bacteria from this colony was characterized by morphological, biochemical and identified as *Azotobacter*. The maximum PHA yield was recorded under the dry weight basis with 3% of corn oil wastes as the sole carbon source. However, Sudan black stained cells showed the presence of large quantities of granules in the cell cytoplasm when viewed under microscope. The polymer was extracted for the purpose of studying its physical and chemical properties.

1. Introduction
Poly-hydroxy-alkanoates (PHAs) are biodegradable and biocompatible polymers synthesized and accumulated in intracellular compartments in several bacterial species. (PHAs) are synthesized by numerous prokaryotes, such as *Cupriavidus necator* (*Ralstonia eutropha*), *Pseudomonas* spp., *Comamonas* spp., and *Bacillus* spp., in response to stress conditions, under high carbon and low nitrogen [1]. In addition to *Pseudomonas and Bacillus*, [2] reported the isolation of soil PHA producing bacteria belonging to the genera *Citrobacter, Enterobacter, Klebsiella*, and *Escherichia*, all of them Enterobacteria. Among symbiotic bacteria and plant growth-promoting rhizobacteria, PHA production has been reported in members of the genera *Rhizobium, Azospirillum, Herbaspirillum* and *Azotobacter* [3]. These polymers represent a class of compounds with physical-chemical characteristics similar to
petroleum-derived plastics such as polypropylene, polyethylene and polystyrene, but are environmentally compatible and totally biodegradable to carbon dioxide, which makes them important as substitute for replacing plastic and can be used in all fields where synthetic plastics are used and water.[4,,5].

Thus a lot of efforts are still being made for process development for the production of this biopolymer in the large amounts in a cost effective manner utilizing renewable or waste materials. This will facilitate its commercialization and rightly justifies its versatile applications as replacement for plastics. The present investigation was undertaken to harness the potential of local bacterial isolate for production of PHA using oil waste as carbon source.

2. Materials and methods

2.1. Collection of samples

For the isolation of PHA producing bacteria, soil samples were collected from the different contaminated sites (soil contaminated with hydrocarbons Household waste and agricultural soils). The soil and contaminated samples were collected, kept in plastic bags, marked with collection details and then stored at low temperature (4°C) till further use. For further processing, the collected samples were air dried at room temperature, and then gently crushed with the help of pestle and mortar for further analysis.

2.2. Bacterial isolation from collected soil samples

For the isolation of bacterial population, 10 gram of each sample collected from the contaminated sites was suspended in 90 ml of sterile distilled water, shaken vigorously and serially diluted in sterile distilled water. Dilutions ranging from 10^-5 to 10^-8 were then plated on standard nutrient agar medium. After 72 hrs. of incubation at 30°C, well-formed colonies were obtained on the plates. Colonies showing remarkable differences in their morphology were selected and re-streaked on nutrient agar plates to obtain pure culture.

2.3. Screening of isolates for PHA production using Sudan Black dye

All the representative pure isolates were screened for PHA production using the lipophilic stain Sudan Black B [6] on agar plates, and under light microscope.

2.4. Screening for PHA on solid agar

Individual bacterial isolates were streaked on nutrient agar plates (3 isolates on one plate), and the plates were incubated at 30°C for 24 hrs. Ethanolic solution of 0.3% (w/v in 70% ethanol) Sudan Black B was spread over the colonies and the plates were kept undisturbed for 30 minutes. The plates were then destained by washing with ethanol (96%) to remove excess stain from the colonies. The colonies that retained their black color after destaining were attributed as PHA producing isolates [7].

2.5. Screening for PHA production under light microscope

For microscopic studies, smears of respective colonies were prepared on glass slides, heat fixed and stained with a 0.3% (w/v in 70% ethanol) solution of Sudan Black B for 10 min. The colonies were decolorized by immersing the slides in xylene, and were then counterstained with safranin (5% w/v in sterile distilled water) for 10 sec. Bacterial cells appearing black under the microscope were
considered PHB producing strains while others were marked as negative [8]. All the positive isolates were assigned code numbers based on their source of isolation.

2.6. PHA extraction and quantification
Burks Medium is used cultivation of PHA production bacteria such with composition as below: gm/L Magnesium sulphate 0.200, Dipotassium phosphate 0.800 , Monopotassium phosphate 0.200 Calcium sulphate 0.130 Ferric chloride 0.00145 Sodium molybdate 0.000253, Corn oil waste 20.0ml in 1000 ml of D.W. From production media, cells were harvested after 48 hrs. by centrifugation at 10,000 g for 30 minutes. Supernatant was discarded and the cell pellets were dried 24 hours. PHAs were extracted from the dried cells using dispersion of chloroform (CHCl3) and sodium hypochlorite (NaOCl) method. In this method, dried cells were incubated in 80% sodium hypochlorite and chloroform in 1:4.5 ratio for 2 hours at 30°C and 150 rpm. After incubation, the suspension was centrifuged at 10,000 g for 20 minutes. As a result of centrifugation 3 distinct layers formed in the centrifuge tubes. The top layer was an aqueous phase of sodium hypochlorite, the middle layer contained cell debris, and the bottom layer was the chloroform containing dissolved PHAs. The lower most layer of the chloroform containing polymer was then collected and filtered. The extract was concentrated by evaporating chloroform using a rotary vacuum evaporator [9].

2.7. Characterization of PHA Granules
Characterization and determination of native PHA like granules involved precise measurements to analyze their physical properties which were carried out mainly by GCMS chromatography and infrared spectroscopy (IR). Briefly, extracted sample was separately made in to a solid pellet by making an intimate mixture of a powder sample with potassium bromide for IR analysis. The relative intensity of transmitted light was measured against the wavelength of absorption on the region 800 to 4000 cm−1 using IR double beam spectrophotometer. The IR spectra of samples were measured at ambient condition. [10, 11].

2.8. Morphological and biochemical characterization of PHA positive isolates
Bacterial isolate with the greatest efficiency in production of PHA was then studied for its morphological characteristics on the basis of their physical appearance (color, shape and texture), and some biochemical tests, using the criteria of Bergey’s Manual of Systematic Bacteriology [12]. Citrate utilization, H2S production, catalase reaction, utilization of some carbon sources, i.e., glucose, rhamnose, sucrose, arabinose, and methanol, which were assayed according to [13].

2.9. Optimization of Carbon to Nitrogen Ratio (C/N Ratio)
For this, cultures were inoculated in MSM
Mineral Salt Medium (MSM)
Chemicals Composition (g/L) (NH4)2SO4 0.45 Na2HPO4 3.42 KH2PO4 2.38 MgSO4 0.4 [14]. supplemented with different ratios of concentrations of the best C and N source (C/N ratio as 10:1, 15:1, 20:1 25:1 and 30:1). Cultures were incubated at 30°C on a rotary shaker (150 rpm) for 48 h.
After incubation, PHA content was quantified, and based on the content the best C/N ratio was determined.

3. Results and discussion

3.1. Isolation and screening of PHA producing bacteria

Contaminated samples were collected from nine different sites (Table 1). Total bacterial population was enumerated by making serial dilutions of each sample and plating appropriate dilutions on nutrient agar medium. Each of them were picked, purified and maintained as pure cultures for further screening using Sudan Black dye.

The screening was done by staining the isolates with Sudan Black B on petri plates as well as under the microscope.

Table 1 Screening of PHA producing bacterial isolates

| Serial no. | Soil Source of isolation          | Name of Isolate | Biomass (g/L) | PHA conc. (g/L) | PHA Content % (w/w) |
|------------|----------------------------------|-----------------|---------------|-----------------|---------------------|
| 1          | Soil contaminated with hydrocarbon waste | B3              | 2.13          | 0.70            | 33.34              |
| 2          | E4                               | 1.98            | 0.58g         | 29.28           |
| 3          | E2                               | 0.64            | 0.00          | 0.00             |
| 4          | E1                               | 3.6             | 1.92          | 48.20           |
| 5          | Contaminated soil with household waste | B2              | 7.4           | 1.61            | 21.86              |
| 6          | E3                               | 4.8             | 2.35          | 54.16           |
| 7          | B1                               | 4.5             | 1.26          | 39.37           |
| 8          | Agricultural soil                | D3              | 3.34          | 1.40            | 41.80              |
| 9          | D4                               | 7.3             | 1.60          | 21.87           |
| 10         | A2                               | 8.6             | 1.32          | 20.11           |
| 11         | A3                               | 4.4             | 1.38          | 39.32           |
| 12         | A1                               | 3.32            | 1.38          | 41.86           |
Figure 1. Primary screening for PHA producing isolates using Sudan black stain on solid medium (A: ++ev, B+ev, C -ev).

Sudan Black dye has been used as a screening measure for PHA production by several workers [15, 16]. It was observed that out of 35 isolates, as many as 12 isolates were found to accumulate PHA, exhibiting blue/ black color, both under the microscope and on the plates upon staining with Sudan Black (Fig.1, 2). All the positive isolates were assigned code numbers (Table 1).

Figure 2. Sudan black B stain of PHA granules (black section) in *Azotobacter* observed (under 100 X oil immersion objectives, A before staining, B after staining) and C under contrast phase object.

3.2. Characterization of PHA positive isolate
The PHA highest positive isolate was grown on nutrient agar plates and its colony morphology was recorded. The morphological characteristic of the representative bacterial isolate was recorded under four major headings, viz., color, shape, and texture. It was viscous, semi-transparent, Gram-negative with rounded ends, after 48 hrs. growth in nitrogen free liquid culture. Aerobic, catalase positive, producing insoluble pigment creamy changes to brown. The isolates, utilize citrate, and produce H₂S As well as their ability to consume a number of carbon sources as glucose, rhamnose, sucrose, arabinose, and methanol (Table 1).

Table 2. Characterization and diagnosis of high-efficiency isolate in polymer synthesis

| Isolate Test          | E4 isolate |
|-----------------------|------------|
| Gram stain            | –          |
| Oxidase               | +          |
| Catalase              | +          |
| Citrate utilization   | +          |
| Utilization of    carbohydrates |          |
| Glucose               | +          |
| Arabinose             | +          |
| Rhamnose              | +          |
| Methanol              | +          |

Figure 3. A Selected blue black colored bacterial isolate after Sudan Black B staining, B : extracted polymer

As PHA accumulation has been found to be enhanced if the bacterial cells are cultivated in the presence of an excess carbon and limited nitrogen sources [17], therefore, in addition to the determination of the best C and N sources, the effect of different C:N ratios on PHA production was also determined. Results shown that 30 / 1 ratio was the best to produce the polymer (fig4). The PHA granules could exactly indicate the PHA production ability of microorganisms [18].
Figure 4. C/N ratio and its relation to polymer synthesis and biomass for *Azotobacter* isolate

3.3. *FTIR Analysis*

The functional groups of extracted PHA were identified using FTIR Analysis. The functional groups of PHA extracted *Azotobacter* as C=O groups. Analysis showed prominent functional groups CH₃, CH₂, C=O, C-O, CH, and OH, which confirms the extracts as PHA.

Figure 5. FTIR spectra of polymer extracted from *Azotobacter*
Table (3); showed that a common molecular fragment of the PHA ion chromatogram was produced. A predominant peak corresponding to the Tetramer of 3HB (hexadecanoic acid methyl ester) was noted at 19 min. The retention times and ion fragment patterns of the peaks at 20 and 21 and 23 min were identical to those of the dimer methyl esters of Pentadecanoic acid, Undecanoic acid, Octadecenoic acid respectively. The similar results of GC-MS were observed by [19] with 3-hydroxydecanoate (HD or C10) 63% and 3-hydroxyoctanoate (HO or C8) 21% with other medium chain length (mcl) monomers.

Table 3. Monomer composition by Mass spectra (MS) of polymer extracted from Azotobacter isolate

| No. | Monomer composition                      | Mol Weight | Formula     |
|-----|-----------------------------------------|------------|-------------|
| 1   | Hexadecanoic acid, methyl ester         | 284        | C₁₆H₃₆O₂   |
| 2   | Pentadecanoic acid                      | 270        | C₁₅H₃₄O₂   |
| 3   | Undecanoic acid,3                       | 214        | C₁₃H₂₆O₂   |
| 4   | Octadecenoic acid                       | 296        | C₁₈H₃₆O₂   |

Hence, this project focused on the isolation of microorganisms from soil samples of polluted sites and the optimization of conditions for the production of PHB effectively and frugally.

4. Conclusions

The major objective of the present study was to isolate effective polyhydroxyalkanoate producing local Azotobacter isolate are able to use various cheap carbon sources including edible oil for its growth and production of PHA, bio-plastic. It shows methods of isolating, screening and characterizing bacteria producing bioplastics using cheap materials. Polymers resulting from the consumption of cheap materials have been diagnosed with advanced methodology, such as GCMS and IR. In future, application of modern molecular biology techniques for cloning and overexpression of genes will result in achieving higher concentration of PHB. Thus, the role of molecular biology is yet to be exploited predominantly prominently in the vital area of environmental science which is recommended for future investigations.

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