

Original Research Article

Characterization of Polyhydroxyalkanoates Produced by Contaminated Soil Bacteria using Wastewater and Glucose as Carbon Sources

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Received: 10 May 2015

Revised accepted: 9 August 2015

Abstract

Purpose: To isolate polyhydroxyalkanoates (PHA)-producing bacterial strains from contaminated soil using industrial wastewater and glucose as carbon sources.

Methods: The strains were isolated and identified as *Pseudomonas*, *Bacillus*, *Enterobacter*, *Exiguobacterium* and *Stenotrophomonas* using biochemical tests and further confirmed by MacroGen sequencing. Two different sources, namely, glucose and wastewater were used to evaluate and compare the use of wastewater as a carbon source for PHA production. The biomass obtained was analyzed by Fourier transform infra-red (FTIR) to identify the presence of PHA in it. Afterwards, PHA extraction was carried out and then gas chromatography (GC) performed to identify PHA monomers.

Results: Utilization of glucose resulted in the production of PHB, while wastewater yielded copolymers poly-3 hydroxybutyrate-co-3hydroxyvalerate P(3HB-co-3HV) due to its content of volatile fatty acids such as acetic acid, propionic acid and butyric acid, which led to the production of different types of polymers. The maximum PHA production was 41 ± 0.22 % obtained for *Stenotrophomonas* (SM03) using 2 % glucose as carbon source while for wastewater, maximum production was achieved by the *Pseudomonas* strain (SM01).

Conclusion: Wastewater is produced in large quantities daily during various activities and therefore can be used as a cheap carbon source for the production of valuable products such as PHA.

Keywords: Polyhydroxyalkanoates, Wastewater, Glucose, *Pseudomonas* strain, *Stenotrophomonas*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Polyhydroxyalkanoates (PHA), produced by around 300 different bacterial strains, serve as carbon and energy storage material and are similar to synthetic plastics in physical and chemical properties. Therefore, they are considered to be a good alternative to petrochemical plastics [1].

PHA production in industries is carried out using pure microbial cultures, the maintenance of which is the main component of the high cost of

PHA production. This contributes to the high cost of pure-culture PHA relative to conventional plastics [2,3]. Efforts have been made in recent decades to discover ways to achieve low-cost production of PHA. The use of mixed microbial cultures in sludge or wastewater is one of such approaches as it eliminates the need for media and equipment sterilization [4,5].

Industrial wastewater, which is highly loaded with organic materials, has been investigated as a carbon substrate for producing PHA because it showed good results in initial experiments [6]. In

this context, several types of waste effluents were used to determine their ability for PHA production such as sugar industry, oil industry paper or municipal waste. Although the PHA production capacity obtained by these substrates was not as high as obtained with synthetic substrates, a reasonably high capacity of up to 55 % PHA of the cell dry weight could be achieved [7-9]. The highest PHA per cell dry weight was obtained with wastewater by Albuquerque *et al* [10] and it amounted to about 75 %. Nonetheless, the process still needs to be improved and optimized for higher yield of PHA from wastewater effluents. Wastewater is composed of many carbon substrates that are easily biodegraded and converted to PHAs by bacteria instead of utilizing it for the formation of bacterial cell components [11].

Wastewater is composed of a lot of substrates along with acetate and experiments were conducted using different compounds separately or in mixtures that includes propionate, butyrate, glucose, lactate but the metabolism of these organic substrates is not yet well understood [12].

The ability of the bacterial strains to produce PHA using glucose and wastewater as carbon sources was analyzed in this work. The purpose of the study was to evaluate the ability of the bacteria to produce valuable products by using raw substrates such as wastewater in comparison with synthetic feedstock and then checking the produced polymers for their monomer composition.

EXPERIMENTAL

Wastewater preparation and analysis

Industrial wastewater used as a growth media was collected from Hadiara drain, Ferozpur road, Lahore, Pakistan and kept at -18 °C before use until required. Its pH was also measured with a pH meter. To use it as a media heating of wastewater was done to remove the insoluble solid particles then followed by centrifugation at 4000 rpm for 10 min. This clarified wastewater was autoclaved at 121 °C for 15 min [13] and used in different concentrations. Wastewater was analyzed for different components present in it by using APHA standard methods for analysis of water and wastewater [14].

PHA producing strains

Bacterial strains were isolated from contaminated soil near industrial wastewater drain and were purified on N-agar plates. Soil sample was added

into a 125-mL Erlenmeyer flasks containing 20 mL nutrient broth composed of (g/L): beef extract, 3.0 and tryptone, 5.0, and incubation was done on a rotary shaker at 37 °C and 150 rpm for 24 h after incubation, 0.1 mL of the sample was taken out and plated on nutrient agar plates and incubated at 37 °C for 24 h. Morphological and biochemical characterization was undertaken and bacterial genus was identified. Sudan Black B staining was used to confirm PHA producing strains [15]. Heat fixed film of bacterial strains were prepared on a slide and Sudan black B staining was carried out for 15 min. PHA detection agar (PDA) media containing 5 µL/mL Nile blue A was used for the direct screening of PHA producers [16]. Plates were incubated at 37 °C for 24 h. PHA producers show fluorescence when the plates were illuminated with UV light and hence are detected. Five strains were selected for further experimentation named as SM01, SM02, SM03, SM04, and SM05. Genomic DNA was isolated by miniprep method [17] and used as template in PCR reaction using thermocycle Primus96 (PeQLab) for the detection and isolation of 16S rRNA. The isolated DNA fragments were sent to Macrogen, Korea for the identification of the strains. Phylogenetic analysis of the strains was identified using MEGA 6.0 software.

Seed culture media preparation

Seed culture media was prepared containing g/L; Na₂HPO₄ 6.36, KH₂PO₄ 2.7, (NH₄)₂SO₄ 4.70, MgSO₄ 0.39, N-Broth 1, Glucose 9. Trace element solution 1 mL/L was added, (g/L, FeSO₄.7H₂O 10, ZnSO₄.7H₂O 2.25, CuSO₄.5H₂O 1, MnSO₄.5H₂O 0.5, CaCl₂.2H₂O 2.0, Na₂B₄O₇.10H₂O 0.23, (NH₄)₆ Mo₇O₂₄ 0.1, 35 % HCl 10 mL). pH was kept at 7.0 for the growth of isolates. The inoculum was prepared in 50 mL flasks and incubated at 37 °C for 24 h at 150 rpm.

Production of PHA using glucose as substrate

PHA screening media was prepared with the following composition (g/L; Na₂HPO₄ 6.36, KH₂PO₄ 2.7, (NH₄)₂SO₄ 0.50, MgSO₄ 0.39, and 1 mL/L of trace element solution). Glucose was added at 1, 2 and 3 % concentration and the pH of the medium was adjusted at 7.0. Inoculum (2 %) from seed culture media was transferred to screening medium and incubation was carried out in a shaker at 37 °C and 150 rpm. Sample (10 mL) was collected after every 24 h until 96 h to measure cell dry weight (CDW) and PHA content.

Production of PHA using bioindustrial wastewater

PHA screening media was prepared as mentioned above but instead of glucose wastewater was used as a carbon source. Different concentrations of wastewater were used to check the ability for PHA production i.e; 20, 40, 60, 80 and 100 %. pH was adjusted to 7.0. Inoculum (2%) from seed culture was given to the medium and incubated at 37 °C and 150 rpm. Sample (10 mL) was taken after every 24 h until 96 h to measure cell dry weight (CDW) and PHA content.

Determination of cell dry weight

Samples (10 mL) from the culture media were taken at regular time intervals and centrifugation was done at 4,000 rpm for 15 min. supernatant was discarded and pellet collected at the bottom was washed using distilled water and pellet was lyophilized to dry it and then weighed. The pellet samples were stored at room temperature in a dry location until PHA extraction was carried out.

Determination of PHA

PHA in the biomass was determined by examining the dried biomass on the ATR-FTIR. The scanning conditions were spectral range between 4000 cm^{-1} and 400 cm^{-1} , 24–32 scans, at a resolution of 4 cm^{-1} . The specific bands for PHA were observed in the biomass, so it was taken for PHA extraction.

PHA extraction

Biomass (8 grams) was treated with 100 mL sodium hypochlorite and 100 mL chloroform. The stored pellet of the cells was suspended using 35 % sodium hypochlorite to lyse the cells [15] and stop bacterial activity. Following this, chloroform was added and the mixture was incubated for one and half hour at 37 °C with constant shaking

in a shaking incubator. After incubation, the solution was centrifuged at 4000 rpm for 10 min. Three layers were obtained after centrifugation, with the upper layer consisting of sodium hypochlorite, the central layer of cell debris and the bottom layer of a PHA-chloroform solution. This bottom layer was separated and solvent was allowed to evaporate.

Gas chromatography

Extracted PHA was analyzed for its chemical structure. For this purpose, about 8 mg of purified PHA obtained after extraction was methanolized by adding 1 mL acidified methanol and 1.0 mL chloroform and heating was carried out at 100 °C for 140 min. Sample (1.0 μL) was injected using split injection method and nitrogen was used as the carrier gas at a flow rate of 3 mLmin^{-1} . Polyhydroxybutyrate-hydroxyvalerate (PHBV, 30 %) was used as a standard to determine the respective retention times for monomer identification [18].

RESULTS

Identification of bacterial strains

Bacterial strains after isolation and purification on N-agar plates were subjected to Sudan Black B staining. Black granules were observed in the cells under light microscope. With Nile Blue A dye, few of the strains showed fluorescence when exposed to UV light after 24 hrs period. The microorganisms were identified using biochemical tests and then 16S rRNA fragments were submitted to Macrogen for sequencing. The strains belonged to genus *Pseudomonas* SM01 (KF270348), *Bacillus* SM02 (KF270351), *Stenotrophomonas* SM03 (KM234128), *Exiguobacterium* SM04 (KF928335) and *Enterobacter* SM05 (JF901811). The phylogenetic relationship and distances among the strains is shown in Figure 1.

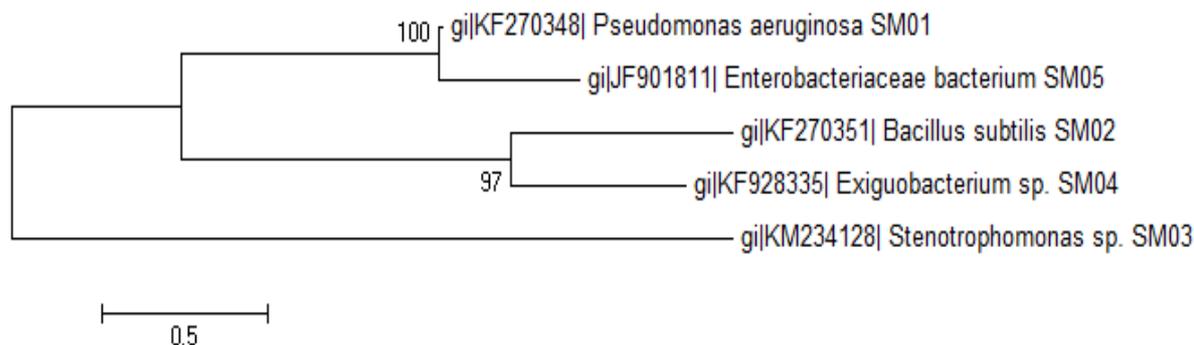


Fig 1: Phylogenetic tree for the sequenced strains

Wastewater analysis

Table 1: Wastewater composition used in this study

Parameter	Concentration (mg/L)
COD	61.0
Magnesium	22.0
Nitrate	1.26
Nitrite	N.D
Copper	0.069
Iron	0.82
Zinc	0.36
Manganese	0.035
Sodium	171.0
Potassium	04
Total sugars	5500
Phosphorus	1.0
Protein	0.87
Total Kjeldhal nitrogen	4

Industrial wastewater was analyzed for its different components using standard methods for analysis of water and wastewater [14]. The composition indicates that the water is composed

of many components such as carbohydrates, proteins, nitrates and trace elements in minor amount as indicated by the data as given in Table 1. Due to this composition, the wastewater proved as a good carbon source for the bacterial strains and helped them to accumulate PHA up to 45 % of cell dry weight.

Assessment of growth in different carbon sources

Three different concentrations 1, 2, and 3 % of glucose were used in this study to evaluate the usage by different bacterial strains. The highest PHA content observed was 41 ± 0.22 % after 48 h at 2 % glucose concentration for strain SM03 as indicated by Figure 2b.

When wastewater was used as a carbon source in different concentrations, the maximum PHA obtained was 45 ± 0.15 % at 100 % concentration of wastewater after 48 h incubation with the strains SM01 as shown in Figure 3b.

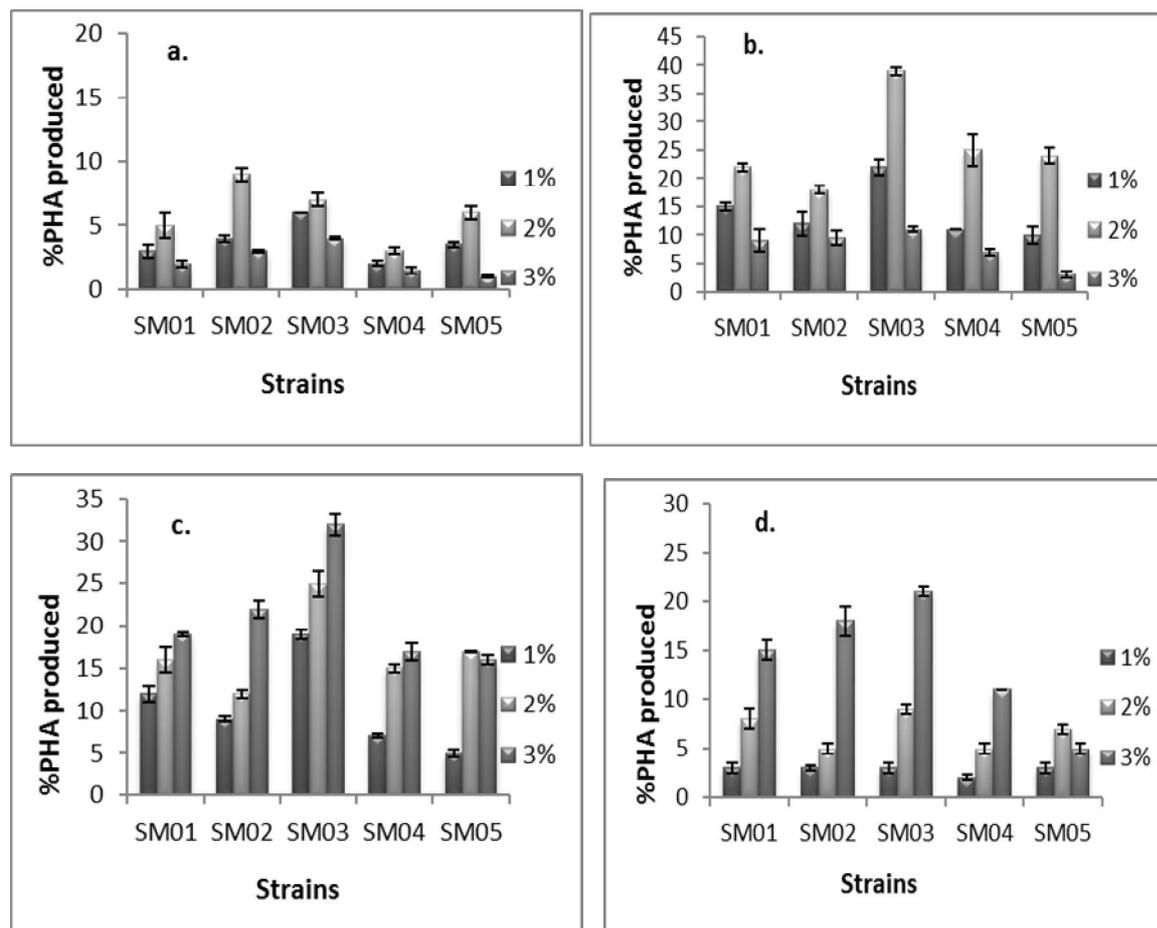


Fig 2: PHA production by bacteria at various concentrations of glucose; (a). after 24 h, (b). after 48 h, (c). after 72 h, and (d) after 96 h

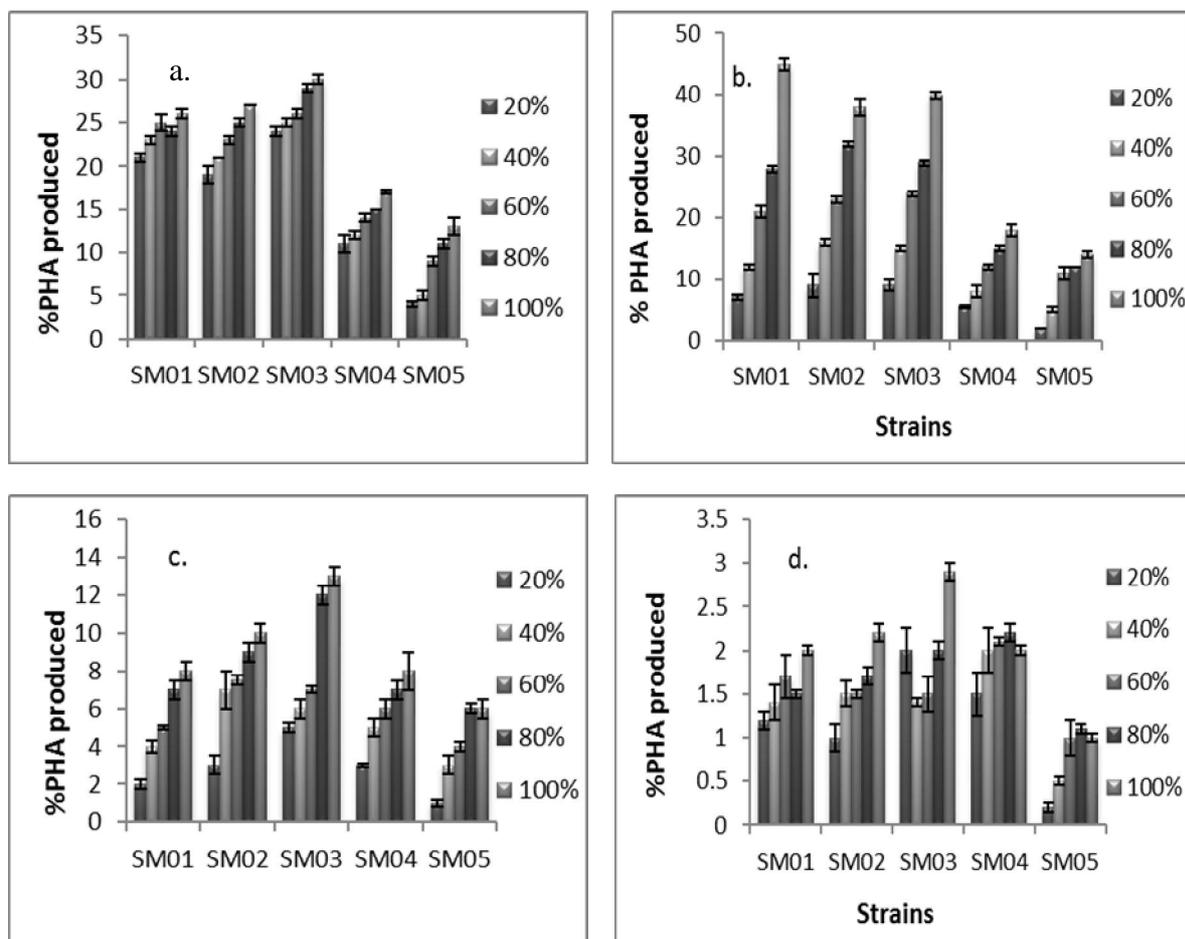


Fig 3: PHA production by bacteria at different concentrations of wastewater; (a). after 24 h, (b). after 48 h, (c). after 72 h, and (d) after 96 h

FTIR and GC

When biomass was tested for presence of PHA, the bands were observed at $1730\text{--}1740\text{ cm}^{-1}$. According to Jarute *et al* [19] and Kansiz *et al* [20] the FTIR spectra peaks at about 1730 cm^{-1} , and 1200 cm^{-1} to 900 cm^{-1} are considered as of PHA in pure cultures. The bands in the spectra which are regarded as PHA and other molecules such as cellular protein can be clearly distinguished from each other. With the help of GC, the PHA content was identified as either HB or HV. In case of glucose as a carbon source, bacteria produced 3PHB while copolymers were produced in case of wastewater such as P (3HB-co-3HV).

DISCUSSION

Production of PHA at industrial level is limited due to its high production cost. The major hurdle is due to the availability of proper and cheap substrate. Therefore previous studies on production of PHA have focused to find suitable strategies for culture and media formulation. In

this work, industrial wastewater and glucose were used to check the efficiency of the strains to produce PHA. We mainly focused at optimizing the polymer production with respect to substrate utilization with different concentrations. Results showed that wastewater sample is loaded with different nutrients as shown in Table 1 which helped the strains to survive and produce polyhydroxyalkanoates. Raw wastewater produced by industries and domestic uses is considered to be composed of organic material major part of which consists of organic carbon and also contains some compounds that are synthetic in nature and non-biodegradable [25].

The results for different strains with glucose showed that biomass increased till 48 h when the glucose was in abundance and there was less amount of PHA produced. Glucose was used efficiently at 2 % concentration and after 48 h, the biomass production was not increasing but the strains tend to accumulate PHA. The highest PHA ($41.00 \pm 0.22\%$) obtained for strain SM03 indicates that this organism has the potential to use glucose more efficiently as compared to other strains and the environment from which it

was isolated seems to contain carbohydrates present in the industrial effluents. This leads to the conclusion that when most of the glucose was consumed, the bacteria was in stress condition which enhanced the storage of limited substrate available into PHA granules as described previously [26,27]. In case of 1 % glucose, the glucose was consumed within 24 h and accordingly no significant PHA production was observed after that. In case of 3 % glucose, the biomass and PHA production was slow due to high carbon load in the media.

When wastewater was used as a substrate for PHA production, all the strains showed growth and PHA production. Initially biomass production increased up to 24 h on 20 % concentration, following which an increase in PHA content was observed. At 40, 60, 80 and 100 % concentration of wastewater biomass increased until 48 h, after which bacteria began to accumulate PHA. The maximum PHA production was observed with 100 % wastewater by strain SM01 (*Pseudomonas*) up to 45.00 ± 0.32 % PHA of CDW as indicated by the Figure 2, indicating that there is much amount of carbon available for the bacteria [26].

The gas chromatography results indicated that there are PHB granules produced by the bacteria with glucose as a carbon source. In the case of wastewater, a random copolymer of HB and HV units was confirmed by GC. The reason is that wastewater contains organic acids such as acetate, propionate, and butyrate which convert into copolymers with varying properties. Based on these results, it can be assumed that mixing of different types of carbon sources can lead to the production of biopolymers with desirable properties.

This indicates that mixed culture PHA production from wastewater may be a profitable avenue for the development of low cost, biodegradable polymers. Highly loaded wastewater reservoirs can potentially be further exploited for better PHA recovery and different types of polymers.

CONCLUSION

Using glucose and wastewater as carbon sources for PHA production is feasible. Utilization of wastewater will not only reduce pollution load of the environment but also lead to the production of polymers that have industrial importance. Various environmental wastes should be investigated to determine their suitability for the production of PHA and reducing its cost of production.

ACKNOWLEDGEMENT

The authors would like to thank the University of the Punjab, Lahore and Higher Education Commission of Pakistan.

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