

Polyhydroxyalkanoates (PHA) Production using Paper Mill Wastewater as Carbon Source in Comparison with Glucose

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To optimize and investigate conditions using different carbon sources for the production of polyhydroxyalkanoates (PHA). Total of 30 bacterial strains were isolated from environmental samples and screened for bioplastic production using Nile blue dye in agar plates and Sudan black B staining. Four strains were selected and biochemically characterized after which ribotyping was done and were selected for accumulation experiments with two different carbon sources; glucose and papermill wastewater. Wastewater was analysed for different nutrients in it. *Pseudomonas* was selected for fermentation done in a fermentor (BioEngineering 5L fermentor). PHA extraction was done by sodium hypochlorite method and extracted PHA was analyzed by gas chromatography-mass spectrometry. Genomic DNA was isolated to amplify *phaC* gene and amplified products were submitted for sequencing after PCR product purification. The PHA producing strains belong to *Pseudomonas*, *Bacillus* and *Stenotrophomonas* genera. The wastewater contained 19.98 $\mu\text{g/mL}$ of carbohydrates and 0.1315 $\mu\text{g/mL}$ proteins. With glucose as carbon source strain I-2 showed 25% PHA production and I-4 showed 41%. In case of 33.5% wastewater, the PHA production and growth rate were slow in comparison to 50% wastewater and glucose as carbon source. Different waste environmental samples have been used to make the production of bioplastic economically cheap and feasible and the present work concluded that the utilization of wastewater is among one of them.

Keywords: Polyhydroxyalkanoates, Biodegradable, Ribotyping, Fluorescence, Wastewater.

Polyhydroxyalkanoates (PHAs) are biopolymers produced by bacterial species for energy and carbon storage during stress conditions¹. These are the environmentally friendly biodegradable plastics, which can be accumulated to about 90% of cellular dry weight during unfavorable growth conditions in over 300 different microorganisms². PHAs are best alternative to conventional petrochemical based plastics and its demand was about 0.36 million tones worldwide,

which is 0.2% of the total production of conventional plastic³.

Different properties of PHA such as biodegradability, compatibility and piezoelectricity makes their current utilization in industries for different purposes such as packaging materials, for biofuels production⁴, paper industry, as material for medical equipment and as drug carriers etc⁵. Different contaminated environments can serve as a habitat for several PHA accumulating bacterial strains^{6,7,8}. From many literature reviews, it is shown that several types of cheap substrates can be used to produce PHA with varying monomeric compositions⁹. Use of PHA at commercial level is less because of its high production cost. Therefore, we need to investigate low cost substrates and along with it we also need to obtain certain strains

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which are good at producing polymers by the conversion of substrate¹⁰.

Different types of wastewater generated by agro-based industries, paper industry, crude glycerol waste stream, and waste cooking oil can prove to be cheap alternative substrates for PHA production¹¹⁻¹⁴. The activated sludge obtained from wastewater treatment processes has been found to act as a source of biomass for biopolymers production with wastewater as carbon source which contributes towards low cost of PHA^{15, 16}.

Wastewaters are a complex mixture of suspended and dissolved materials¹⁷. Therefore, many types of wastes like food industry wastes, potato starch wastewater, alpechin and wastewater sludge have been used as substrates for bioplastic production. Different waste environmental samples have been used to make the production of bioplastic economically cheap and feasible. The purpose of the present work is to isolate PHA producing strains and then use of wastewater as carbon source by these strains.

EXPERIMENTAL

Sampling and pretreatment of wastewater

Wastewater was collected from a local paper manufacturing factory located on Sheikhpura Road near Lahore, Pakistan. Temperature and pH was noted at the time of sample collection. The wastewater was boiled to remove solids wastes from it then cooled and centrifuged at 4000 rpm for 10 min¹⁸. This clarified wastewater was then autoclaved at 121°C for 15 min to sterilize and used as a growth medium.

Analysis of wastewater

After collecting the wastewater for PHA production, it was used as sample for determination of different nutrients in it. Phenol-sulfuric acid assay was used for carbohydrate analysis¹⁹. Optical density (O.D) was taken at 490 nm. For protein estimation, 100 µL of sample was taken in eppendorf, 1 mL of Bradford reagent (Coomassie Blue) was added to all eppendorfs, incubated for 15 min at 37 °C. Absorbance was monitored at 595 nm²⁰.

Isolation, characterization and ribotyping of bacterial strains

Isolates were taken from contaminated environmental sites to determine if they have PHA

production ability and were purified on N-agar plates. PHA detection agar (PDA) containing 5 µL/mL Nile blue was used for the direct screening of PHA producers. Plates were incubated at 37 °C for 24 hrs. PHA producers show fluorescence when the plates were illuminated with UV light and hence are detected. Heat fixed film of bacterial strains were prepared on a slide and Sudan black B staining was done for 15 min. Dried slides were observed under 100X power lens of light microscope²¹. Four strains named as I-1, I-2, I-4 and I-5 which showed positive results were selected for further experimentation. Genomic DNA was isolated by miniprep method²² and used as template in PCR reaction using thermocycle Primus96 (PeQLab). Following PCR primers were used for *PhaC* gene amplification, 179-L: CCGCAATTGAACAAGTTCTACGT and 179-R: CGGGAGACGCGTGGTGTTCGTTG. PCR purified product was submitted to Center of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan. DNA sequencing was done by dideoxy method (chain termination) using a DNA sequencing system (Applied Biosystem, 3100/gas 3100-1696-013). The isolated 16S rRNA sequence was sent to Macrogen, Korea for detection (<http://www.macrogen.com/eng/sequencing/16s.jsp>).

PHA production using glucose and wastewater as carbon source

Seed culture preparation

Seed culture media was prepared which has enough nitrogen source. Its composition is g/L [Na_2HPO_4 6.36, KH_2PO_4 2.7, $(\text{NH}_4)_2\text{SO}_4$ 4.70, MgSO_4 0.39, N-Broth 1, Glucose 9, trace element solution 1 mL/L, (g/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 10, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 2.25, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1, $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ 0.5, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.0, $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ 0.23, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ 0.1, 35% HCl 10 mL)]. pH of the medium was adjusted to 7.0. The inoculums were prepared in 50 mL flasks and incubated at 37°C for 24 hrs.

PHA screening media

PHA screening media was prepared with the same composition as seed culture media while keeping nitrogen source limited g/L (Na_2HPO_4 6.36, KH_2PO_4 2.7, $(\text{NH}_4)_2\text{SO}_4$ 0.50, MgSO_4 0.39, Glucose 9 and trace elements). Total volume was kept at 400 mL in a 1L conical flask. The inoculum of pre-cultured respective strains was taken so that initial optical density was around 0.5. Flasks were incubated in a shaking incubator at 150 rpm at 37

°C for up to 96 hrs. After incubation 50 mL sample was collected and O.D was taken at 600 nm after every 24 hrs interval starting from 0 hrs to 96 hrs. Mineral solution was prepared and volume was kept at 400 mL in 1L flask. Wastewater was added to act as a carbon source for PHA production in two concentrations; 50% and 33.5%. After incubation 10 mL sample was collected and O.D was taken at 600 nm after every 24 hrs interval starting from 0 hrs to 96 hrs. Biomass was calculated and finally PHA extraction was done using sodium hypochlorite digestion method.

Laboratory scale fed-batch culture was carried out for the I-2 strain at 37 °C in a jar fermenter (2.0 liters) containing 1.6 L of PHA medium, supplemented with 2% glucose and 6% inoculum. The conditions of the reactor were maintained with a pH and O₂ electrode to monitor oxygen demand and pH profiles. pH was controlled at the desired value (7.00 ± 0.1) using 2 mol l⁻¹ HCl or 2 mol l⁻¹ NaOH, temperature was controlled using a water-jacketed thermostat bath. 10 mL culture was collected after every 4 hrs interval for 2 days.

Extraction of PHA

Extraction of PHA was done by Sodium hypochlorite digestion method²³. Cells were collected by centrifugation and lyophilized. In 8g of biomass 100 mL of sodium hypochlorite was added and pellet was suspended by vortexing. 100 mL of chloroform was added in it and incubated at 37°C for 90 min on shaker. Centrifugation was done and upper layer was removed, lower layer containing the chloroform was allowed to evaporate. The weight of PHA was calculated and percentage determined using the formula.

$$\text{PHA}\% = \frac{\text{Weight of PHA}}{\text{Weight of Biomass}} \times 100$$

Analysis of PHA by Gas Chromatography-Mass Spectrometry

Extracted PHA was analyzed for its chemical structure. For this purpose, 8 mg of purified PHA was methanolized by heating at 100 °C for 140 min in a mixture of 1.0 mL chloroform, 0.85 mL methanol and 0.15 mL concentrated sulfuric acid. Sample (1.0 µL) was injected via split injection mode and nitrogen was used as the carrier gas at a flow rate of 3 mL min⁻¹. 3-hydroxyalkanoic acid methyl ester standards (C-4 to C-16) were used to

determine the respective retention times for monomer identification²⁴. Gas chromatography system was used to analyze the monomers produced in this PHA sample.

RESULTS

Analysis of wastewater

Carbohydrate estimation of wastewater was done using phenol-sulfuric acid assay [19] and sample value was obtained by standard curve of known concentration of glucose. From this standard curve, estimated value of carbohydrate content of wastewater was found to be 19.98 µg/ml of sample as indicated in the table 1. Using Bradford reagent method²⁰ bovine serum albumin (BSA) standard curve was obtained. Then protein content of wastewater was estimated and it came out that 0.1315 µg/ml proteins are present in wastewater as shown in table 2.

Isolation and characterization of bacterial strains

From different environmental samples, 30 strains were isolated on N-agar plates. These were further screened for PHA production on PDA media containing 5 µL/mL Nile Blue in it. On Nile blue plates, PHA producing strains showed fluorescence. Only four were positive, which were named as I-1, I-2, I-4 and I-5. PHA production ability of those strains which showed growth on PDA was further confirmed by Sudan Black staining²¹. On staining all PHA positive strains showed black granules as shown in figure 1.

Genomic DNA was successfully isolated²² from all strains and was visualized on agarose gel. *PhaC* gene was amplified successfully from I-2 strain; it showed 99% homology to *phaC* gene of *Pseudomonas aeruginosa*. Amplified *phaC* gene was submitted under NCBI GenBank Submissions grp 3768567. The 16S rRNA sequence was determined by MacroGen using genomic DNA provided and primers, 27F and 1492R for PCR amplification and 518F and 800R for sequencing. NCBI nucleotide BLAST was used to identify bacterial strains; I-2 showed 99% homology to *Pseudomonas aeruginosa* (KF270348) and I-4 strain showed 99% homology to *Stenotrophomonas* sp. (KM234128), while I-1 and I-5 showed homology to *Bacillus* sp.

PHA production in shake flask and fed batch experiment using glucose and wastewater as carbon source

Time profiling for PHA production was done using glucose and wastewater as carbon sources. With glucose as carbon source strain I-2 showed 25% PHA production and I-4 showed 41% while I-1 and I-5 production was low as compared to these two as shown in figure 2. With 50% wastewater, I-1 gave 14%, I-2 gave 21%, I-4 showed 27% and I-5 showed 17% PHA production as shown in figure 3. In case of 33.5%, the PHA production and growth rate were slow in comparison to 50%.

GC-MS

After esterification and gas chromatography-mass spectroscopy analysis (GC-MS) of the extracted PHA, the presence of 3-PHB was confirmed. Chromatogram peak observed at 37.9 was found to be of methyl ester of PHA when

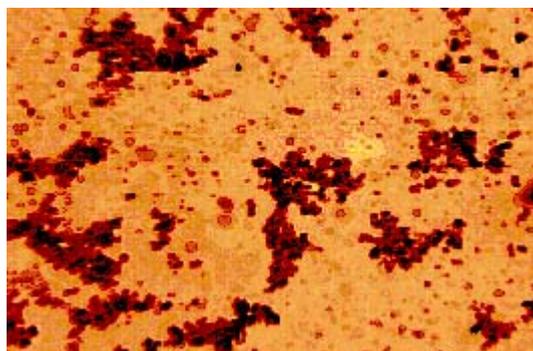


Fig. 1. Sudan Black B staining for strain I-2 showing black granules inside the cells

compared its mass peak from mass spectrometry with the data.

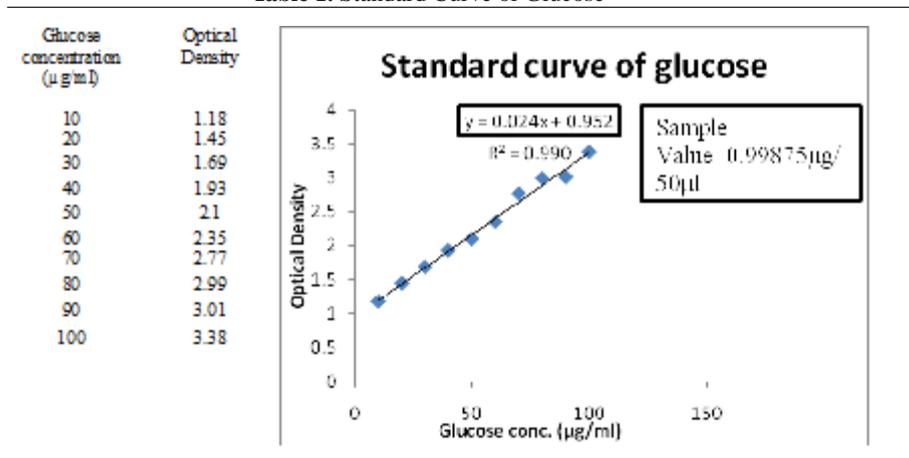
DISCUSSION

In the last few decades, polyhydroxyalkanoates have been replacing petrochemical plastics due to their usage in various fields such as medical devices, carriers, printing and photographic materials etc.^{3,25}, but its application is limited in some fields because of its high cost production. The most important factors for PHA production is the cost required for culture maintenance and accumulation conditions²⁶.

Environmental samples were taken for isolation of PHA producers and grown on nutrient agar plates. Their colonial morphology and microscopic analysis was done. For the isolation of PHA producers, PHA detection agar supplemented with Nile blue dye was used²¹. Nile blue causes the PHA producing strain to fluoresce under UV illuminator. It was less suitable for discriminating between gram positive bacterial strains which are either producing PHA or not such as *Bacillus megaterium* or *Rhodococcus ruber*. Hence to check PHA production ability further, Sudan Black B staining was also carried out. There were a total of 4 strains selected after screening, all were rods; two were Gram negative and two were Gram positive.

Wastewater sample was analyzed for different nutrients such as proteins and carbohydrates. Wastewater obtained from different reservoirs contain enough organic matter that can

Table 1. Standard Curve of Glucose



act as feed for bacteria²⁷. Results showed that wastewater sample is loaded with organic carbon which helped the strains to survive and produce polyhydroxyalkanoates.

Results of strain I-1 showed that PHA production was increasing as biomass increases till 96 hrs when cultured using 2% glucose as carbon source with maximum 18% PHA achieved (Figure 2a). But when strain I-1 was supplemented with 50% wastewater, growth trend seems not to correlate with PHA production as it starts to decrease after 72 hrs despite of increase in biomass till the end of the experiment and maximum PHA obtained was 13% (Figure 3a). Growth trend was even different when 33.5% of wastewater used. In this case, although the biomass was increasing till the end but PHA production was maximum only after 24 hrs after which there was sharp decrease.

Regarding strain I-2, biomass was increasing till 72 hrs but PHA production was

declining after 48 hrs accumulations. This may be an indication that after 48 hrs, bacteria has started utilizing PHA because of depletion of glucose in the medium (fig. 2b). Same trend in biomass and PHA production was seen for this strain when cultured on 50% wastewater till 48 hrs and then both started decreasing (fig. 3b). A bit similar case is observed for this strain when grown with 33.5% wastewater. At start, PHA production is increasing with biomass till 24 hrs and later on both started declining.

Strain I-4 when given with 2% glucose as carbon source growth of this strain increases till 72 hrs of incubation and then showed a sudden decline whereas PHA production increases sharply till 48 hrs that was 41% of cell dry weight and then decreases (fig. 2c). But when this strain was inoculated with 50% wastewater, PHA production increased with increase in biomass till 72 hrs and then both decreased (fig. 3c). In case of 33.5%

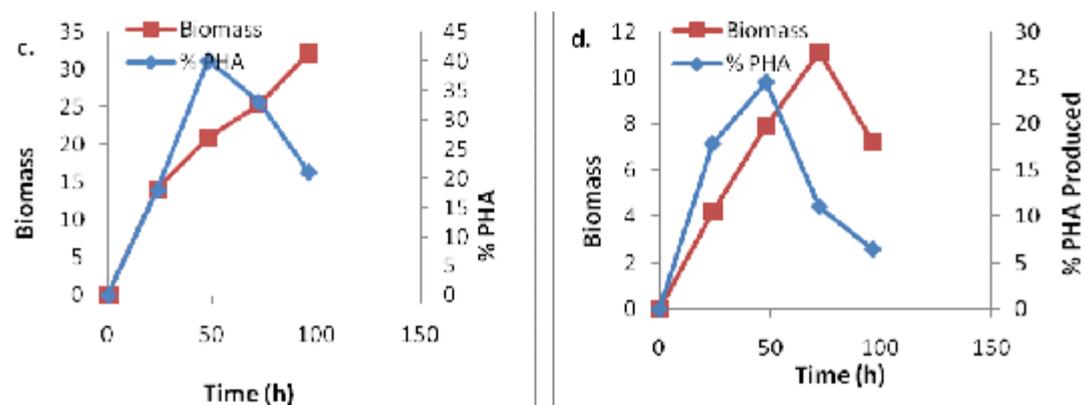
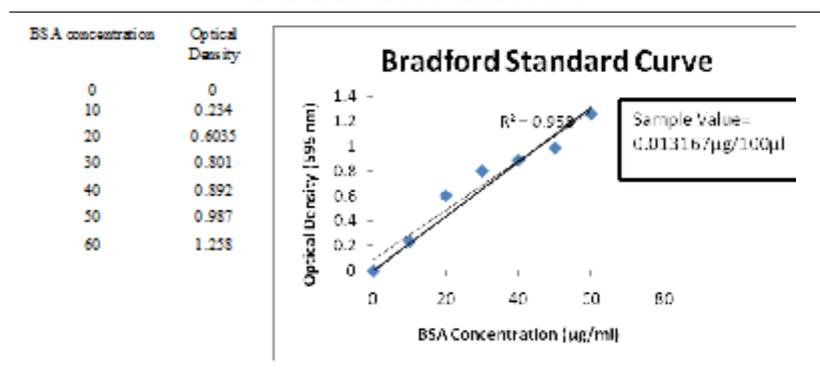


Fig. 2. %PHA and Biomass production with 2% glucose (a. I-1, b. I-2, c. I-4, d. I-5).

Table 2. Bradford Standard Curve



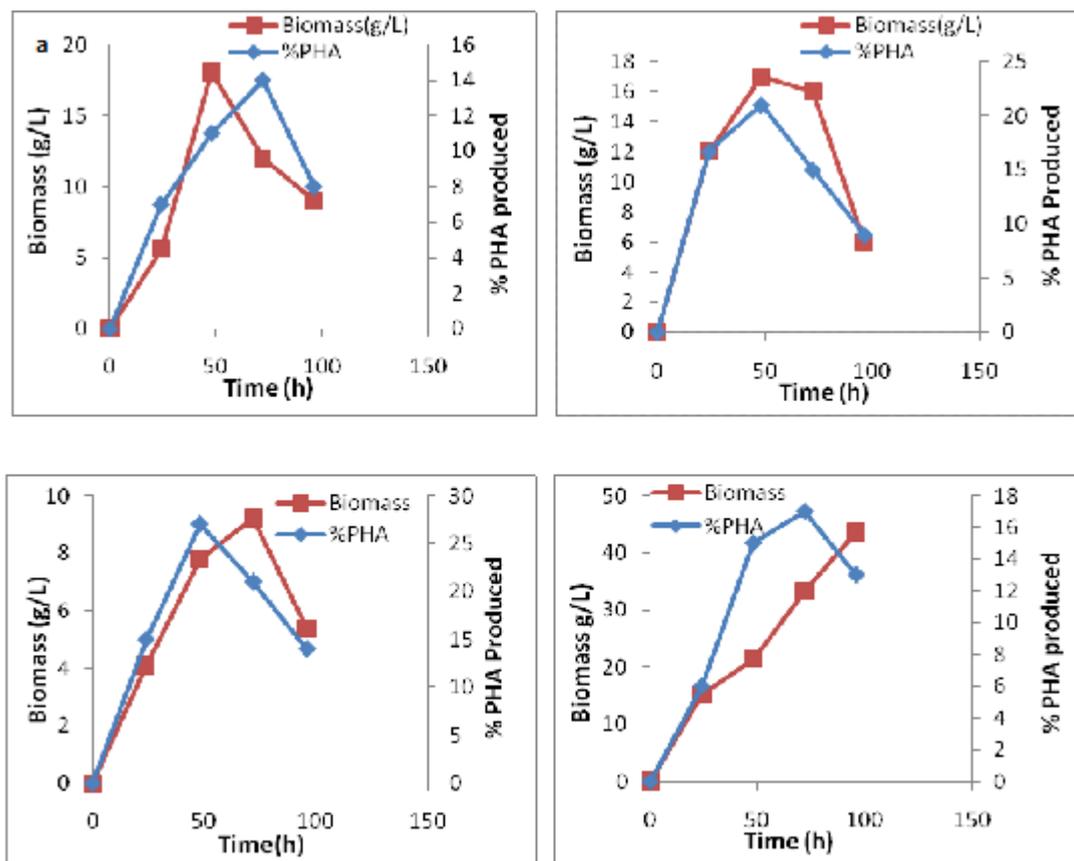


Fig. 3. %PHA and Biomass production with 50% wastewater (a. I-1, b. I-2, c. I-4, d. I-5).

wastewater, strain I-4 showed increase in biomass till 48 hrs of incubation but PHA production is maximum only after 24 hrs.

Strain I-5 biomass increases till 72 hrs when inoculated in 2% glucose after which bacterial cells enter death phase whereas PHA production was maximum at 48 hrs and later on decreased sharply. Same behavior shown by this strain when supplemented with 50% wastewater but here biomass increased till 96 hrs but PHA production was seen after 24 hrs that was 17% of cell dry weight. Growth behavior of strain I-5 on 33.5% wastewater was the same as on glucose. PHA production as well as biomass was increasing only till 24 hrs and then both decreased. The reason for increased growth and PHA production in 50% wastewater as compared to 33.5% is because there are more nutrients in the 50% concentration as indicated previously²⁸. Carbohydrate and protein

concentration (C:N) ratio is very important parameter for PHA production. Polymerase chain reaction based strategy was used to confirm the PHA producers from different *Pseudomonas* sp²⁹.

CONCLUSION

Four strains were analyzed which produced PHA using glucose and wastewater and these can be further investigated to produce much better amount of PHA using different carbon sources. New methods and raw sources are needed for good future of PHA production globally at industrial level are required and utilization of wastewater is among one of them as it not only treats wastewater but also produces polymers of industrial importance.

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