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Regular Article

Microbial production of polyhydroxyalkanoate (PHA) utilizing fruit waste as a substrate

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Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates synthesized by various bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells. PHA producing bacteria from soil were isolated, characterized and screened by Nile blue staining method. Screened organisms were subjected to fermentation with glucose as carbon source and low-cost raw material like jambul seed (S*yzygium cumini*). The strain SPY-1 showed higher amount of PHA accumulation when compared to the other strains and was comparable with that of the reference strain *Ralstonia eutropha.* The extracted PHA granules were analyzed with Fourier transform infrared (FTIR) spectroscopy and the peak was in the regions of 1650.9, 1728-1288 and 1725-1277, correspond to the stretching of the C=O bond which was similar to the standard Polyhydroxybutyrate (PHB).

Keywords: Polyhydroxybutyrate, Microorganisms, Jambul seed powder, FTIR.

Polyhydroxyalkanoates (PHA) are biodegradable plastics that have been identified as an alternative to petroleum-based synthetic plastics. The use of biodegradable plastics will reduce non-biodegradable solid wastes [Sudesh and Iwata 2008]. PHAs are a type of polyester polymer produced by many bacteria [Shamala et al., 2003]. It accumulates as discrete granules to levels as high as 90% of cell dry weight as a response to environmental stress and nutrient imbalance (i.e. nitrogen and phosphorus limiting conditions) and plays a role as a sink for carbon and energy. These water insoluble storage polymers are biodegradable, exhibit thermoplastic properties and can be produced from different renewable carbon sources [Brandl et al., 1990]. PHAs are high molecular mass polymers with properties similar to conventional plastics such as polypropylene [Reddy et al., 2003]. Therefore, they have a wide range of applications, such as in the manufacture of bottles, packaging materials; films for agriculture and also in medical applications [Oliveira et al., 2004, Khanna and Srivastava 2005].

The main advantage is that, the biodegradable polymers are completely degraded to water, carbon dioxide and methane by anaerobic microorganisms in various environments such as soil, sea, lake water and sewage and hence, is easily disposable without harm to the environment [Brandl et al., 1988]. To realize this potential, however, there must be a several-fold reduction in the cost of PHA production. The cost of carbon feedstocks can significantly affect

the PHA production cost. Therefore, the identification of alternative cost-effective substrates for the production of PHA has become an important objective for the commercialization of bioplastics.

Microorganisms are able to incorporate up to 60 different type's monomer into their storage polymer and a series of PHAs with different monomeric composition (i.e. different physical and chemical properties) can be produced [Steinbuchel, 1991, Du and Yu, 2002]. It was first discovered in 1926 as constituent of the bacterium *Bacillus megaterium* [Lemoigne, 1926]. Several investigations of natural isolates of Actinobacillus, Azotobacter, Agrobacterium, Rhodobacter, and Sphaerotilius have focused on converting organic waste to bacterial PHA. The bacterium *Alcaligens eutrophus* [Jian, 2001], and *Alcaligens latus* [Yamane et al., 1996] are well known for their ability to produce PHA.

The stored PHA can be degraded by intracellular depolymerases and metabolized as carbon and energy source as soon as the supply of the limiting nutrient is restored. The majority of PHAs are composed of R(-)-3-hydroxyalkanoic acid monomers ranging from C3 to C14 carbon atoms with variety of saturated or unsaturated and straight- or branched chain containing aliphatic or aromatic side groups [Doi et al., 1992, DeSmet et al., 1983].The granules appear as highly refractive inclusion under electron microscopic observation. The Microorganisms accumulating PHA are easily identified by staining with Sudan black or Nile blue [Ostle and Holt, 1982, Schlegel et al., 1970].

In this study, several strains of PHA accumulating bacteria from soil was isolated and characterized for their morphological properties. In addition, comparison of PHA production by the selected strain of SPY and *Ralstonia eutropha* was done in Mineral Salt Media with jambul seed as a carbon source. Jambul fruits are seasonal cropping fruits, were easily available. The jambul seed are usually discarded, thus this study of its use as a substrate for PHA production by the isolated organism and the reference strain *Ralstonia eutropha*. The purity of the extracted polymer was identified using FTIR spectroscopy [Oliveira *et al*., 2007 and Pandian *et al*., 2010].

MATERIALS AND METHODS

Isolation of bacteria from soil

Soil sample was taken from Kumaraguru College of Technology (KCT) campus and used for isolation of the bacteria. Around 1.0 g of sample was serially diluted in sterile distilled water and plated onto nutrient agar plates and incubated at 30˚C for 24 hours. Various colonies of different morphologies were individually picked and were screened for PHA production; potential isolates were sub cultured on nutrient agar plates [Aarthi and Ramana, 2011]. The original cultures were maintained as glycerol stock at –20°C for further use.

Reference strain

The reference strain for PHB production *Ralstonia eutropha* MTCC1472 was collected from the Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH) Chandigarh. All the experiments were carried out using this reference strain for comparison.

Rapid screening of native bacterial isolates for PHB production

All the bacterial isolates were qualitatively tested for PHB production by Nile blue (as a fluorescent) staining method [Ostle and Holt, 1982]. For rapid screening of PHB producers,

bacterial isolates were spread into nutrient agar plate and the plates were incubated at 30°C for 24 hours. Acetone solution of Nile blue (0.5µg/ml) was spread over the colonies and the plates kept undisturbed for 15 minutes.

Characterization of PHB producing bacterial isolates

The selected, most efficient PHB producing bacterial isolates were subjected to morphological test for the purpose of identification. The six potent PHB accumulating strains SP1, SP2, SP5, SP6, SPY-1 and SPG-1 were examined for their gram reaction as per the standard procedures given by Barthalomew and Mittewer (1950).

Substrates for PHB production

Jambul seed (*syzygium cumini*) was collected from a local juice shop and dried in an oven at 60°C to reduce moisture content and was milled into fine particles. The following parameters such as reducing sugar, starch and cellulose content in the raw seed were characterized.

Hydrolysis of seed

Substrate was pretreated with acid-alkali method and the cellulose was hydrolyzed into glucose without dergradation of glucose by zinc chloride method where the end product was used as substrate [Chen *et al.*, 1984]. The glucose content in the hydrolyzed seed was estimated by the DNS method with a standard glucose solution.

Bacterial Growth in Defined Media

Pure culture of *Ralstonia eutropha* and the isolated culture was revived in nutrient broth initially and then grown in a defined Mineral Salt Media (MSM), comprised of the following; 10 g Hydrolyzed Seed, 5 g Glucose, 5 g Sodium Chloride, 5 g Di-Potassium Hydrogen Phosphate, 1 g Potassium Chloride, 1 g Magnesium Sulphate, and 1 g Ammonium Sulphate in 1 L of distilled water. The pH of the media was maintained to be 7.5±0.5. The culture flask was kept in shaker at 150 rpm at 35 °C for two days [Amirul *et al*., 2008, Du *et al*., 2001 and Yamanka *et al*., 2010].

Extraction of PHA

The PHA was directly extracted using the solvent chloroform. The bacterial cultures were harvested by centrifugation at 5000 rpm for 10 min. The cell pellet was suspended in sodium hypochlorite solution and incubated at 37°C for 1 - 2 h for complete digestion of cell components except PHA, where by lipids and proteins were degraded. The mixture was centrifuged to collect PHA granules and the supernatant was discarded. The sediment was washed twice with 10 ml of distilled water and centrifuged. The PHA granules in the sediment were washed twice with acetone, methanol and diethyl ether (1:1:1) respectively. The polymer granule was dissolved with boiling chloroform and was evaporated by air drying, to yield dry powder of PHA [Santhanum and Sasidharan, 2010].

Identification of PHA granules

The bacterial cells were stained with Nile blue stain and visualized under UV trans-illuminator and that gives a bright orange fluorescence at a wavelength of 460nm. The accumulation of PHA in the form of granules would be identified from the fluorescing cells [Amirul *et al*., 2008].

Quantification of PHA

The bacterial culture was centrifuged at 6000 rpm to obtain the cell pellet and dried to estimate the dry cell weight (DCW) in units of g/L [Du *et al*., 2001]. Residual biomass was estimated as the difference between dry cell weight and dry weight of extracted PHA [Zakaria *et al*., 2010]. This was calculated to determine the cellular weight and accumulation other than PHAs. The percentage of intracellular PHA accumulation is estimated as the percentage composition of PHA present in the dry cell weight.

Residual biomass (g/L) = DCW (g/L) – Dry weight of extracted PHA (g/L)

$$\text{PHA accumulation (\%)} = \frac{\text{Dry weight of extracted PHA (g/L)} \times 100\%}{\text{DCW (g/L)}}$$

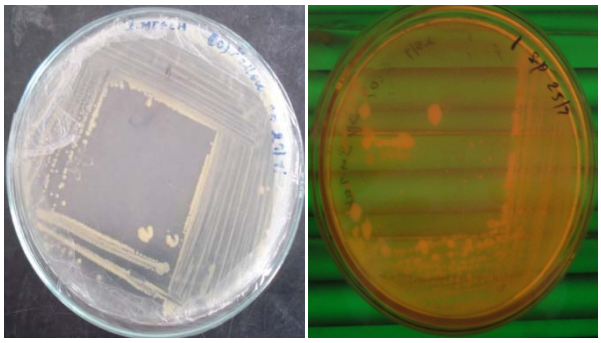
Fourier Transform Infra Red Spectroscopy (FTIR)

The extracted PHA samples were added with KBr and then evaporated. This sample was subjected to FTIR, to analyze the PHA structure and purity. The peaks were observed from 4000-400 cm^{-1} (Oliveira *et al*., 2007 and Pandiyan *et al*., 2010).

RESULTS AND DISCUSSION

Isolation and screening of bacterial isolates for PHA production

Several bacteria were isolated from soil sample by serial dilution method. From this, nine isolates were selected for PHA production as shown in figure 1a. Based on the intensity of the fluorescence were observed in the Nile blue staining method (Ostle and Holt, 1982), six potential PHA producers were screened out of nine isolates. The granules showed Orange fluorescence under UV trans-illuminator at a wavelength of 460nm as shown in figure 1b and the results obtained was similar to Cortes *et al*., 2008.



a) b)

Figure 1a) Isolated organisms from soil and b) Fluorescence of PHA producing microorganism using Nile blue staining

Characterization of PHA producing bacterial isolates

Screened bacterial strains were characterized by Gram staining as shown in table 1. All the six isolates were Gram negative bacteria.

Characterization of substrates

The amount of reducing sugar, starch and cellulose present in the substrate was found to be 430, 375, 80µg/ml.

Hydrolysis of seed

The amount of glucose present in the pellet is 3675 µg/ml and the supernant is 4150 µg/ml. The amount of glucose liberated from the hydrolysed seed sample was in comparison with those stated by Chen *et al*., (1984).

Table 1 Characterization of bacterial isolates

Isolated organism	Gram staining	Shape
SP1	Gram negative	cocci
SP2	Gram negative	Small rod
SP5	Gram positive	cocci
SP6	Gram negative	Dispersed rods
SPY-1	Gram negative	Small rod
SPG-1	Gram positive	Small rod

Extraction of PHA

From the screened organism, dry weight of PHA is high in SPY-1, compared to other isolates, when it utilizes glucose in the production medium and used for further studies as shown in figure 2. *Ralstonia eutropha* and SPY-1 was grown in Mineral Salt Medium with a combination of Glucose and Hydrolyzed Seed, and Glucose alone. The extracted PHA was an ivory white coloured powder. It was found to be sparingly soluble in water. The extracted PHA is shown in figure 3a.

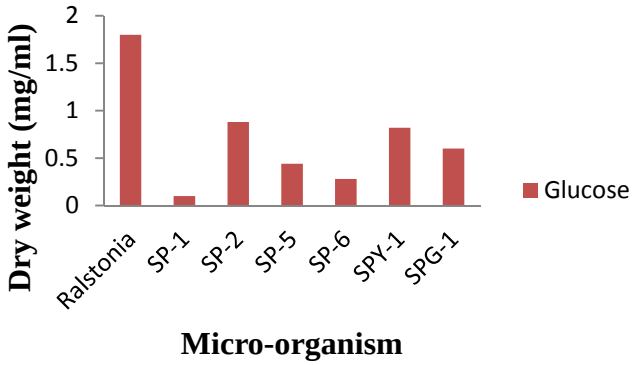
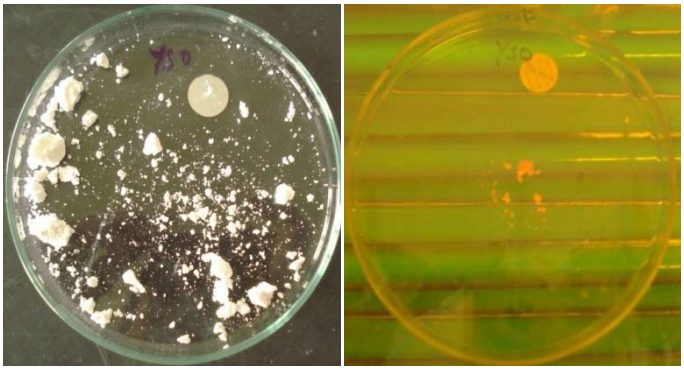


Figure 2 Comparison of extracted PHA weight from the isolates



a) **b)**

Figure 3a) Extraction of PHA from SPY-1 and b) Identification of PHA granules

Identification of PHA granules

The accumulation of PHA in the form of granules would be identified from the fluorescing cells as shown in figure 3b. Similar kind of studies was done earlier by Amirul *et al*., (2008) using fluorescence microscopy to visualize regions of intracellular PHA accumulation.

Quantification of PHA

Extracted PHA was quantified and compared its effeciencies with *Ralstonia eutropha* and isolated organism. From the results it was found that PHA accumulation was in proportion to the Dry cell weight which was similar to the studies of Du *et al*., (2001) and Zakaria *et al*., (2010). The maximum level of PHA accumulation was observed, 41.7% in *Ralstonia eutropha* and 42.2% in SPY-1, when it utilizes the hydrolyzed seed alone compared to combination of glucose and hydrolyzed seed in Mineral Salt Medium as shown in table 2. Similar results have been showed by Yang *et al*., (2010), where PHA accumulation was higher with higher carbon supplementation in the growth media.

Table 2 Quantification of PHA obtained from *Ralstonia eutropha* and SPY-1

Micro-Organism	Media composition	Dry weight of Extracted PHA		Cell Dry weight (g/ml)	Residual Biomass (g/ml)	% PHA Accumulation
		(g/50ml)	(g/ml)			
Ralstonia eutropha	G+HS	0.124	0.003	0.104	0.10	2.38
	HS	2.185	0.044	0.105	0.061	41.77
SPY-1	G+HS	0.119	0.002	0.025	0.023	9.52
	HS	3.001	0.06	0.142	0.082	42.26

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum of the extracted PHA sample was compared with that of the Standard Polyhydroxy-3-Butyric acid. From the spectra, sample bands were observed similar with the standard spectra and according to the results obtained by Oliveira *et al*., (2007) in the regions of 1650.9, 1728-1288 and 1725-1277, correspond to the stretching of the C=O bond, whereas a series of intense bands located at 1103.21 cm -1, which was found exactly similar with the standard spectra and correspond to the stretching of the C–O bond of the ester group. The sample bands were observed similar with the standard spectra and according to the results obtained by Pandiyan *et al*., (2010) in the regions of 509.17-632.62, 516.89-624.89 and 561-603.61, correspond to the presence of the C=O bond. Two peaks in the region of 1103.21 and 2931.60 were exactly the same in the Standard PHB, confirming the presence of PHB in the extracted PHA sample.

Also bands of minor relevance, such as those found a t 3355.91 cm -1, originated by terminal OH groups or to water adsorption onto the sample, are found in sample spectra and more or less similar results obtained by Oliveira *et al*., (2007). The FTIR spectra of the Standard PHB and the extracted PHA sample are given in figures 4 and 5.

CONCLUSION

PHA producing bacteria from soil SPY-1, produced PHA better with the hydrolyzed seed thus the seed can be utilized as a cheap carbon source, for the screened bacteria to grow and accumulate PHA in the production medium. The strain had the capacity to accumulate 80% of the dry cell weight as PHA. The production of PHB was found to increase both in Reference

strain and SPY-1, when the organism utilized hydrolyzed seed alone as a carbon source in the production medium, PHA accumulation was found to be 41.7% in *Ralstonia eutropha* and 42.2% in SPY-1. The methods adopted for seed hydrolysis are cheap and less time consuming than the costlier and elongated enzymatic methods. Further studies are required to optimize the growth media to improve the PHB yield and its degradation capacity.

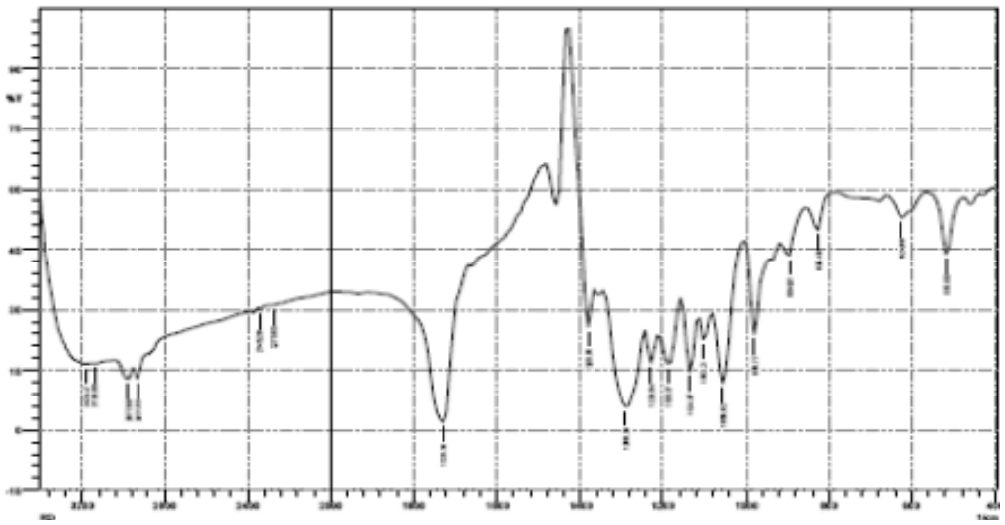


Figure 4 FTIR spectra of Standard Polyhydroxy-3-Butyric acid

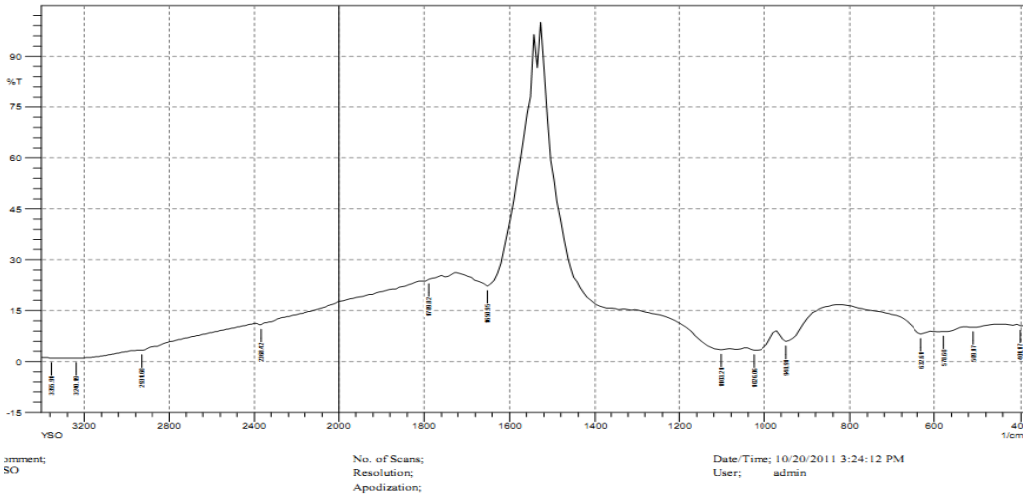


Figure 5 FTIR of extracted PHA sample

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