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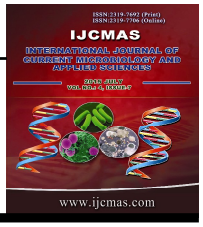

Original Research Article

Production and Characterization of Polymer from *Bacillus* OU73T from In-expensive Carbon Sources

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Keywords

PHA, Bacillus, Inexpensive carbon sources, FTIR, NMR

A B S T R A C T

Bacillus OU73^T is an indigenous isolate and is able to produce PHA when grown in unbalanced nutrient conditions. In this study the strain was subjected to different cheap carbon sources to synthesize PHA was examined. Strain was isolated from polluted water, found to produce higher and quicker yield compared to other wild types amongst the E_2 mineral medium studied. 2% rice bran was successfully utilized by the strain and produced the PHA % (g/L) was 56.76, containing 92.50% HB units and 7.50% HV units. Whey, lactose containing dairy industry waste was used to produce 40.00%PHA (g/L) containing 82.06% HB units and 17.94% HV units. The potency of the organism to hydrolyze starch due to the intrinsic amylase activity was considered and starch was used as the sole carbon source for growth and produced the PHA% was 60.03 % (g/L) containing 95.00% HB units and 5.0% HV units. Strain OU73^T was found to utilize the bagasse very well and produced the PHA % (g/L) 53.62 containing 94.00% HB units and 6.0% HV units. The purified polymer sample from cells was confirmed as PHA by FTIR and ^{1}HNMR.

Introduction

Biodegradable polymers or polyhydroxyalkanoates (PHA) are polyesters synthesized by many bacterial isolates. PHA is a biopolymer which can be fully biodegraded into water and carbon dioxide and can be synthesized from sustainable raw materials. The PHA producing bacteria are found at various locations particularly contaminated sites have a large diversity of them (Lopez-Cortes *et al.*, 2010). The most common polymer among these polyesters is poly (3 hydroxy butyrate), which was the first to be discovered in *Bacillus megaterium* by Lemoigne 1926. Recent investigations emphasize the fact that PHA synthesis from pure substrates can be considered as optimized to a high degree. The overall production cost of biodegradable polymer production depends greatly on the cost of the carbon source. So it is required to enhance economics of PHA production by

substituting pure substrates by cheaper and inexpensive carbon sources (Koller *et al.*, 2008; Nagamani *et al.*, 2013). The development of copolymer production or blending PHA with other monomers has widened their applications. PHA and their derivatives are now used in the field of agricultural, food and biomedical materials, which has been recently reviewed by Chen *et al.* There are almost 250 organisms known to produce PHA, but only a few species can produce PHA at a high concentration. The best PHA accumulating bacterial species should have several properties like the high growth rate, capable of utilizing cheap inexpensive carbon sources and have high accumulation percentage (Lopez-Cortes *et al.*, 2010). PHB can be accumulated up to 80% of the cell dry weight from various carbon sources by *Ralstonia eutropha* (Dawes and Senior, 1973) and near 90% in recombinant *E. coli* (Pringsheim and Wiessner, 1963).

Several heterotrophic and autotrophic aerobic bacteria synthesize and accumulate PHA as carbon and energy storage materials under the condition of limiting nutrients in the presence of excess carbon source. *Bacillus* species were studied by number of researchers for their ability to synthesize PHA. In this study, *Bacillus cereus*OU73was studied and has the ability to produce appreciable amount of PHA.

Materials and Methods

Bacterial strain and cultural conditions

Polyhydroxyalkanoate accumulating strain *Bacillus* OU73^{T} which was isolated from the polluted water was used in this study. E2 mineral medium (Carr. 1996) was used for PHA production. Aerobic conditions were maintained by shaking the inoculated Erlenmeyer flasks at 300 C for 28 h. Pure cultures were isolated on agar containing Nile blue .Cultures were directly monitored for the fluorescent colonies by exposing to ultraviolet light to detect accumulation of lipid storage compounds including PHA.

Estimation of viable cell count

To estimate viable cell count, the samples were serially diluted with sterile saline solution (1% w/v NaCl). The diluted samples (0.1 ml) were plated in triplicate, on nutrient agar plates and incubated at 30 °C for 24 h to form fully developed colonies.

Fluorescence microscopy

The presence of cytoplasmic PHA inclusions was evidenced by Nile blue staining and observing the cells under the fluorescence microscope. The observation was made using a Zeiss Axio Imager M1 upright wide-field fluorescence microscope. For image processing, the Zeiss Axiovision 4.5 software was used.

PHA production using Agro-industrial byproducts

Raw materials

Rice bran, sugarcane bagasse, starch were used as cellulose containing raw materials along with whey for production of polymer.

Culture conditions

2% of each raw material residue was taken in a conical flask containing 200ml of E_2 mineral medium. The conical flasks were plugged with cotton and sterilized at 15 lbs for 20 minutes. Each flask was inoculated with selected isolates. These flasks were incubated at room temperature for 48 hrs, at 30±2 on an Orbital shaker. According to the optimum incubation period of each isolate, polymer was quantified and analyzed.

Analytical methods

Microbial growth was monitored by measuring the cell density of the culture at 600 nm after suitable dilution with distilled water. PHA quantification was quantitated according to the method of Law and Slepecky (1961), whereby the dried pellets containing intracellular PHA were hydrolysed using concentrated sulfuric acid for 1 h to obtain crotonic acid, which was quantified by measuring absorbance at 235 nm. Analysis was performed in triplicates for shake flask samples. Cell dry weight (cdw) was measured by lyophilizing harvested cells from 3 ml culture broth. PHA content and its composition were determined by gas chromatography using PHA standards. Cell concentration was defined as cell dry weight per litre of the culture medium. The PHA content was defined as the ratio of PHA concentration to cell concentration given as percentage.

Determination of total sugar content

The carbohydrate content of raw materials in the culture broth was measured by phenol sulphuric acid method (Dubios *et al.,* 1956) using standard graph.

Production of PHA from rice bran

20 gms of finely powdered rice bran (2%) was suspended in 1 liter of water. pH was adjusted to 7.0 and heated to 80^0 C for 30 min. The content was filtered and the volume was made up to 1 liter. This was used directly with the E_2 mineral medium.

Production of PHA from starch

2% Starch was used and hydrolyzed with 5% conc. Hcl before adding to the E_2 mineral media. The experiments were done with all other optimal conditions and in triplicates. The means of the results of experiments conducted in triplicates.

Production of PHA from bagasse

Bagasse is the fiber left over after the juice has been squeezed out of sugarcane stalks.2% dried, finely powdered bagasse hydrolyzed with 1% conc, Hcl was added to E_2 mineral medium. These materials have favourable compositions with rather low amounts of lignin in the range of 10% (w/w) and a high percentage of carbohydrates.

Production of PHA from whey

Whey or Milk Serum is the liquid remaining after milk has been curdled and strained. It is a by-product of the manufacture of cheese or casein and has several commercial uses. The media containing lactose as sole carbon source directly prepared from cheese whey. Whey obtained from local dairy (Vijaya Dairy, Hyderabad), was acidified to pH 4.5 with 1N HCl, heated to 74°C for 15 min, cooled and centrifuged at 10,000 g for 15 min at 4°C (Vellore and Desai, 1998). The supernatant obtained after the above treatment (whey supernatant) was used in the experiments after adjusting the pH to 7.0 with alkali.

Analytical methods

After incubation, each sample was used for the determination of the cell dry weight (cdw) and PHA content in the culture supernatant fluid. The cell concentration was determined by measuring cdw as follows: 5 ml culture broth were centrifuged, pellet obtained was washed and dried at 105°C until the weight did not decrease further. PHA estimation was carried out according to Law and Slepecky (1961). PHA (%) was defined as the percentage of the ratio of PHA to cdw.

Extraction of PHA

The polymer was extracted from the bacterial pellet by using the hypochlorite method as described previously. For the production of polymer films the polymer was washed with methanol and acetone consecutively and centrifuged at 8000 rpm for 20 min. The polymer was dissolved in hot chloroform (60° C) and the solution poured onto glass trays. The chloroform was allowed to evaporate slowly at 4° C by placing the trays in the cold room, because uniform films of the polymer could not be obtained at room temperature, due to rapid evaporation of chloroform. On evaporation of chloroform, the film of the polymer was obtained, which was used to study the physical properties of the polymer.

Chemical properties

For FT-IR analysis, the PHB was precipitated from the chloroform using cold ethanol. The precipitated polymer was used to prepare KBr discs (sample: KBr, 1:100). An FT-IR spectrum 1720X spectrometer (Perkin Elmer, USA) was used under the following conditions: spectral range, 4,000–400 cm^{-1}; window material, CsI; 16 scans; resolution 4 cm^{-1}; the detector was a temperature-stabilized, coated FR-DTGS detector.

The ^{1}H NMR analysis of the polyester samples was carried out on Varian-300 spectrometer (USA). The 300 MHz ^{1}H NMR spectra were recorded at 24°C in $CDCl_3$ solution of polyester (50 mg/ml) with a acquisition time of 2.0480 seconds, sweep width of 4000 Hz.

Tetra methyl silane was used as an internal chemical shift standard. The spectra was recorded for commercial PHA (Sigma-Aldrich, USA) and for the polymer extracted from test strains.

Results and Discussion

Florascence microscopy revealed the presence of polymer granules. Polymer was extracted and used for the analytical methods.

Composition of raw materials

Total sugars, reducing sugars, non-reducing sugars, organic carbon, nitrogen, total solids, moisture content of each raw material was determined. The initial composition of the raw materials used in the study was shown in Table 1.

In the rice bran the amount of organic carbon was highest and in the sugar cane bagasse total sugars were highest. As observed in a number of previous studies, the size of the raw material is very much important for heat transfer and enzymatic hydrolysis.

Production of PHA from cheap carbon sources

2% rice bran was successfully utilized by the strain OU73 and produced the PHA % (g/L) was 56.76, containing 92.50% HB units and 7.50% HV units. Whey, lactose containing dairy industry waste was used to produce 40.00%PHA (g/L) containing 82.06% HB units and 17.94% HV units.

The potency of the organism to hydrolyze starch due to the intrinsic amylase activity was considered and starch was used as the sole carbon source for growth and produced the PHA% was 60.03 % (g/L) containing 95.00% HB units and 5.0% HV units. Strain OU73 was found to utilize the bagasse very well and produced the PHA % (g/L) 53.62 containing 94.00% HB units and 6.0% HV units (Table 2).

Table.1 The initial composition of raw materials

Raw materials	Total sugars (mg/g)	Reducing sugars (mg/g)	Moisture (%)	Total Solids (%)	Organic carbon (%)	Nitrogen (%)
Rice bran	0.0120	0.6780	6.723	93.27	39.2	0.13
Bagasse	0.0145	0.4731	2.943	97.06	35.3	0.43
Starch	0.0171	0.5461	3.041	96.94	36.4	0.72
Whey	0.0192	0.3825	96.015	3.05	14.2	0.64

Table.2 PHA % (g/L) content and composition of polymer accumulated by Bacillus OU73T grown in different cheap carbon sources

Carbon source	OD 620 nm	CDW wt(g/L,W/V)	Monomer composition HB%	HV%	PHA%
Starch	2.84	2.4±0.001	95.00	5.00	42.53
Whey	1.05	3.08±0.0024	82.06	17.94	40.00
Rice bran	2.96	3.5±0.0013	92.50	7.50	56.76
Bagasse	2.78	3.4±0.0031	94.00	6.00	53.62

Fig.1 Scanning electron micrograph of extracted polymer from *Bacillus*OU73^{T}

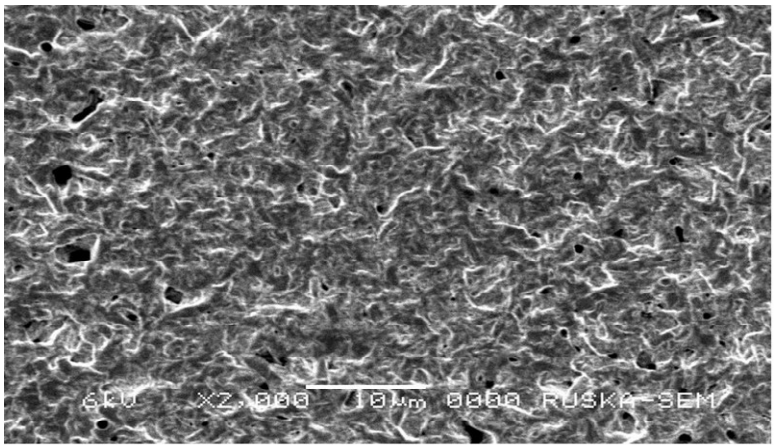


Fig.2 IR spectra of polymer from *Bacillus*OU73^{T}, grown on rice bran

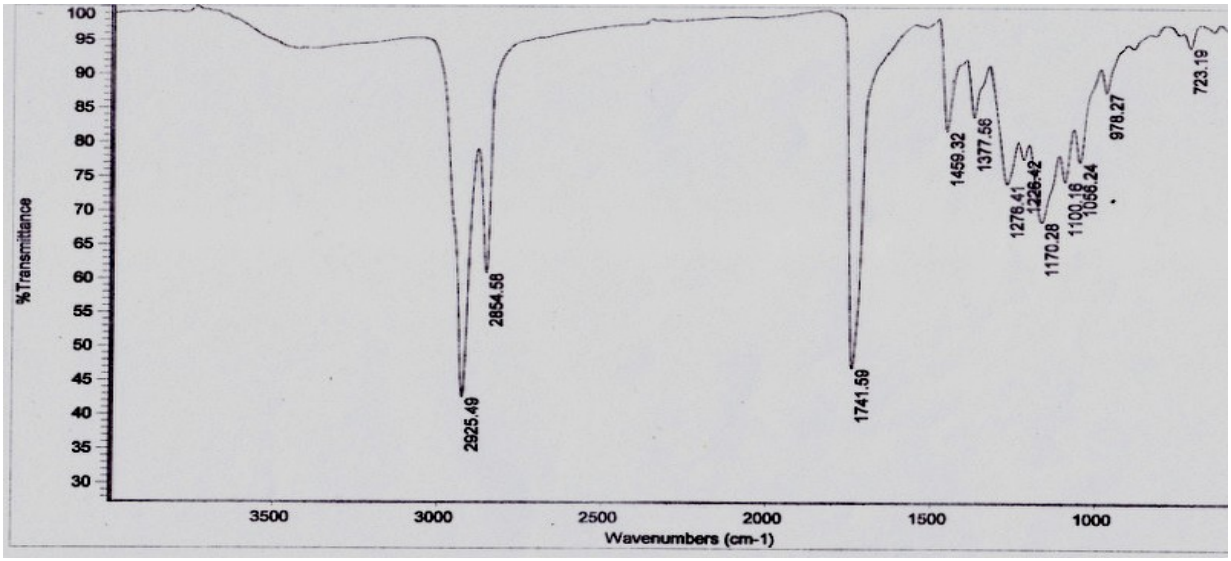
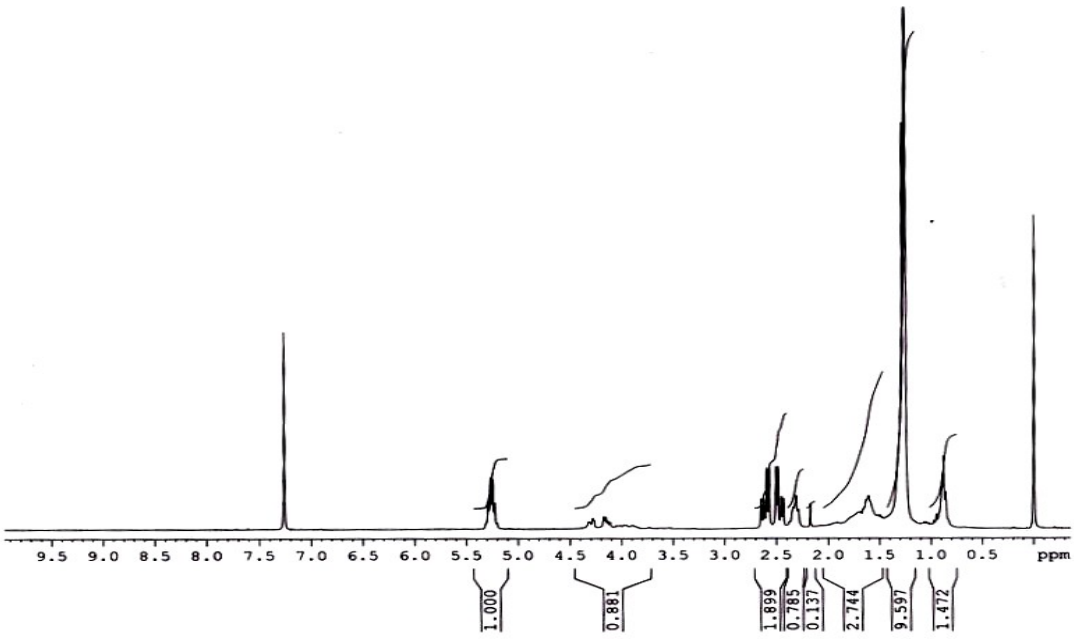


Fig.3 H^{1} NMR of polymer from *Bacillus*OU73^{T} when rice bran used as carbon substrate



SEM of polymer

The SEM image of PHA surface showed that films made of PHB, and other copolymers had different surface roughness and topology (Figure 1). PHA films, especially PHAHD film, possessed the most notable roughness and unique topology while PHA film showed smooth surface among all the tested materials. A porous structure with highly interconnected spaces is a desirable property for scaffold implants; as such property will provide an interconnecting mechanic support for cell

attachment and migration, and hence may promote proliferation. (Xian *et al*., 2010).The SEM studies on PHA film showed that these PHA matrices were made of continuous fibrous networks that constituted highly interconnected porous Structures.

FTIR

Spectra were recorded for the polymers dissolved in chloroform. Spectra (Figuer 2) showed two

Intense absorption bands at 1,741.59 and 1,276.41 cm-1, corresponding to C = O and C–O stretching groups, respectively. Other absorption bands at 1,377, 1,459, 2,925 and 2,854 cm- 1corresponding to -CH3, -CH2, -CH and O–H

^{1}H NMR

^{1}H NMR analysis confirmed the presence of different monomer signals (Figuer 3). The resonance, as observed at 1.0715, 1.2559, 1.6135, 2.4870, 5.2700 and 7.2637 ppm by 1H-NMR analysis were, respectively, for CH3 (3HV, and 3HOD sidegroup), CH3 (3HB side group), CH2 (3HV, 3HHD and 3HOD side group), CH2 (3HV, 3HB, 3HHD and 3HOD) bulk structures), CH (3HV, 3HB,3HHD and HHD bulk structures) of theCDCl3 -soluble fraction of the polymer confirmed the presence of the copolymer consisting of 3HB, 3HV, 3HHD and 3HOD units.

Raw material cost is one of the major reasons for the high price of PHA.. In this paper we tried various cheap carbon sources could be used for the PHA production. In terms of the PHA properties, there is no significantly difference between fermentation using pure substrate and using industrial or agriculture by-products. In this work, *Bacillus cereus*OU73was studied and has the ability to produce appreciable amount of PHA.

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