

Production of PHB (bioplastics) using bio-effluent as substrate by *Alcaligenes eutrophus*

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Alcaligenes eutrophus MTCC 1285 produced bioplastics (PHB) in N₂ deficient medium containing carbon (glucose concentration 2%). Using sago effluent, thippi and molasses as substrates and at different pH (6.9-8.0) the production of PHB was recorded; higher production of the biopolymer was obtained at pH 8.0 in the molasses based production media. The amount of PHB produced at pH 6.9 was 0.8, 0.5, 0.4, and 0.73 (g/mL⁻¹) and 1.1, 0.65, 0.55 and 1.0 (g/mL⁻¹), respectively in glucose, sago, thippi, molasses substrate based media. The present investigation revealed that bio effluents could be used for the production of PHB so as to decrease the pollution caused by them.

Keywords: PHB, *Alcaligenes eutrophus*, bio-effluent, bioplastics
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Introduction

Poly-β-hydroxy-butyrate (PHB) is a biodegradable and biocompatible thermoplastic produced by various microorganisms. It can be made into films, fibres, sheets, even molded to the shape of a bag and bottle. PHB and poly hydroxyvaleric acid (PHV) are being developed for a variety of applications¹.

Alcaligenes eutrophus H16 is a facultative autotroph capable of rapid growth in simple mineral medium. To synthesize PHB, the organism requires anaerobic conditions with CO₂ and N source².

Molecular structure of PHB does not depend on the features of the strain and condition of carbon nutrition of microorganisms producing PHB³. PHB was originally shown to be a constituent of lipid inclusions in the cells of *Bacillus*⁴. It can be extracted with hypochlorite solution, which is a highly degradative procedure and decreases the molecular weight of the isolated granules presumably by the removal of their outer layer⁵.

A complex enzyme obtained from the extracts of *Rhodospirillum rubrum* cells hydrolyses PHB contained in the native PHB granules isolated from *Bacillus megaterium*. The native PHB granules possess definite structural features, disruption of which results in decreased susceptibility of the

polymer to enzymatic hydrolysis and their crystal structure could be revealed by IR absorption, and under electron microscope⁶.

Materials and Methods

PHB Production Strain

Alcaligenes eutrophus MTCC 1285 was obtained from the Microbial Type Culture Collection (MTCC) and Gene Bank of Institute of Microbial Technology, Chandigarh for the production of PHB.

Production of PHB using Glucose Substrate

A. eutrophus was grown in the Nutrient broth medium (Hi-Media) at 37°C for 24 h. The broth culture was centrifuged at 5000 rpm for 10 min. The culture pellet was transferred to N₂ deficient medium containing MgSO₄, NaCl, KH₂PO₄, citric acid, glucose; trace element solution or yeast extract (Hi-Media) used as N₂ deficient medium. To make a solid medium, 1.5% agar was added to the broth^{7,8}.

Production of PHB using Bioindustrial Waste Substrate Medium

Sago effluent, Sago thippi and Sugar molasses used as substrates were collected from the Sago Industries located in Attur, Salem District and Sugar factory in Namakkal District of Tamil Nadu for the production of PHB. In the N₂ deficient medium, glucose was also used as standard bioindustrial wastes (60%) considering it as a carbon source.

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Optimization of pH

Bioindustrial waste based N₂ deficient medium was prepared at pH 6.9 and 8.0 and inoculated by *A. eutrophus* and incubated at 37°C for 48 h.

Extraction of PHB

- After 48 h incubation at 37°C, 10 mL of culture was taken and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the pellet was treated with 10 mL of sodium hypochlorite (Loba) and the mixture was incubated at 30°C for 2 h. After incubation, the mixture was centrifuged at 5000 rpm for 15 min and then washed with distilled water, acetone, methanol respectively for washing and extraction.
- After washing, the pellet was dissolved in 5 mL of boiling chloroform (Loba) and was evaporated the chloroform by pouring the solution on sterile glass tray and kept at 4°C. After evaporation the powder was collected for further analysis⁹.

Determination of PHB

Fluorescence Staining Method (Acridine Orange)

10 µL of 48 h old culture *A. eutrophus* was taken from the production medium and transferred to the Eppendorf tube, containing 50 µL of acridine orange (Hi-Media) and incubated for 30 min at 30°C. After the incubation period, the culture was centrifuged at 4000 rpm for 5 min. The pellet was collected and re-suspended in distilled water. Smear prepared on a clean microscopic slide observed under the Fluorescence Microscope (De Winter).

TLC

About 50 µL sample was loaded on the TLC plate and allowed to run in the solvent system consisting of ethyl acetate and benzene (SRL) (1:1) mixture for 40 min. For staining, 50 mL of iodine (Hi-Media) solution was vapourized in water bath at 80-100°C. TLC plate was kept over the beaker containing iodine solution for 5-10 min in order for it to get saturated with iodine vapour. After 10 min green-black colour spots indicated the presence of PHB. The R_f (Retardation factor) value was measured and compared with the standard chart⁹.

C¹³ NMR Spectral Analysis

The sample was dissolved in cadmium chloride and tetra methyl saline was added as internal standard to

deduce the chemical shift of carbon atoms. The measurement was taken in GSX 400 NMR-JEOL spectrometer operating at 400 MHz of magnetic field⁹.

IR Analysis

One mg of sample was ground well with 10 mg of spectral pure anhydrous potassium bromide crystals. The powder was made into a pellet for IR analysis. The relative intensity of transmitted light energy was measured against the wavelength of absorption on the region 400-4000 cm⁻¹ using JEOL-FT IR-4000 plus double beam spectrometer. IR spectra of the samples were measured at ambient conditions.

Results

The acridine orange stained slide was examined under Fluorescence Microscope. The production of PHB was identified in cells, which became enlarged in size and the granules were observed with fluorescence. The production amount was also approximately determined and compared with the control (Table 1).

Evaporation of chloroform extracts resulted in the formation of a thin layer. This indicated the production of PHB by *A. eutrophus*. In the absence of PHB production, such a film was not formed. It was dry weighed. The amount of PHB produced varied depending upon the type of substrate used. Molasses based medium produced 1.0 g/100 mL PHB, whereas thippi, and sago effluent based media produced 0.55 g/100 mL and 0.65 g/100 mL PHB, respectively (Table 2).

Green-black colour bands were observed in TLC for all the cases and R_f value was measured and calculated as 0.71, which indicated the presence of PHB in the production medium.

Of the substrates used for PHB production, molasses and sago effluent based media produced highly pure PHB, while the thippi based medium produced impure PHB. The IR analysis showed the presence of CH₂-CH₃-C=O methyl ester groups in the extract. These methyl ester groups confirmed that the extract was PHB.

IR spectrum of the compound was recorded in the range of 100-4000 cm⁻¹ and it showed characteristic bands for the groups CH, C=O and C-O (Tables 3 & 4). The methine (CH) group gave a strong band in range of 1360-1416 and 2914-3097. These frequency values were higher than the normal values because of polymerization. The carbonyl group

Table 1—PHB observed under fluorescence microscope

Medium	pH 6.9	pH 8.0
Glucose	++	+++
Sago	+	+
Thippi	+	+
Molasses	++	+++

+++ PHB Granule clearly observed; ++ PHB Granules were observed; + PHB Granules were observed in low intensity when compared with other production medium; (-) Absence of PHB granules

Table 2—Weight of granules of PHB extracted from different substrate based media

Medium	pH 6.9 g/100 mL	pH 8.0 g/100 mL
Glucose	0.8	1.1
Sago	0.5	0.65
Thippi	0.4	0.55
Molasses	0.73	1.0

Table 3—IR peak region of various bio effluents at pH 6.9 & 8.0

Bio effluents	Peak region	Comments
Thippi	3578.59	Intramolecular H bond
	3097.39	CH ₂ Stretching
	1635.51	C=O
	1366.48	O-H tertiary alcohol
	1089.40	C-O stretching
Sago effluent	3447.74	Intramolecular H bond.
	3415.06	H bond
	2948.81	CH ₂ Stretching
	1636.33	C=O
Molasses	1069.51	C-O stretching
	3330.13	Intramolecular H bond.
	3417.70	H bond
	2928.30	CH ₂ Stretching
	1673.37	C=O
	1076.60	C-O stretching

Table 4—IR peak region of various bio effluents at pH 8.0

Bio effluents	Peak region	Comments
Thippi	3533.24	Intramolecular H bond.
	3055.67	CH ₂ Stretching
	1657.81	C=O
	1081.36	C-O stretching
Sago effluent	3443.33	Intramolecular H bond.
	3310.20	H bond
	2913.88	CH ₂ Stretching
	1666.31	C=O
	1060.43	C-O stretching
Molasses	3334.19	Intramolecular H bond
	3401.77	H bond
	2920.10	CH ₂ Stretching
	1698.31	C=O
	1047.25	C-O stretching

(C=O) gave a strong band in the range of 1636-1673. These frequency values were lower than the normal values because of polymerization. The (C-O) group showed strong and broad absorption in the range of 1047-1089.

¹³C-NMR spectrum of the compound was recorded in the range of (0-200) ppm. There were four peaks in the 26.69-67.00 ppm range. This showed that there are four different types of carbon atoms. The four peaks appeared at 26.69, 63.82, 63.82 and 67.03 ppm due to the presence of methyl (CH₃), methine (CH), methylene (CH₂), carbonyl (C=O) groups, respectively.

Discussion

PHB granules exhibited a strong orange fluorescence when stained with Nile Blue A¹⁰. The cells, in which PHB was produced, became enlarged, while the normal cells remained unenlarged. As per the field count of enlarged cells and granules of PHB stained with fluorescence dyes, the amount of PHB production was determined.

PHB was obtained from corn oil acids and the mixture of glucose (15 g L⁻¹) and acetic acid (2.5 g L⁻¹)¹¹. *A. eutrophus* produces PHB homopolymer when fed exclusively with glucose under appropriate conditions. Carbon source other than the glucose such as agricultural by-products (molasses and sugar beets) did not produce PHB¹².

The production of PHB was noticed by the formation of a thin layer after extraction with chloroform, which may be due to the presence of PHB granules that interlinked with each other and formed a thin layer.

When the organism was grown at pH 8.0 more PHB was produced as compared to its production at pH 6.9 (Tables 3 & 4).

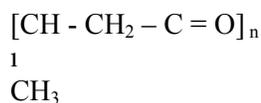
Green-black colour chromatography band indicated the presence of PHB in the extract, which correlated with the study of Rawate and Mavinkurve⁹.

The peak at 1.735 as could be assigned to ester carbonyl stretching. The peaks at 1.180 and 1,300 cm⁻¹ were difficult to assign. The former band could be assigned to C-O stretching, in alkyl amines or various skeletal carbon stretching and the P=O stretching, O-H bending, or CH₂ scissors vibrations. The IR spectra of PHA film and the purified methyl ester are identical⁹. The IR analysis of molasses at two different pH showed that the peak values matched with that of the standard chart as well as with that of the references.

High purity of PHB production was pronounced in the media containing molasses and sago effluent, while thippi showed small variation in the peak for CH₂ group. It was concluded that molasses or sago effluent could be used as a substrate for PHB production. However, the thippi needs to be hydrolyzed further before it can be used.

High resolution C¹³-NMR spectroscopy of live cells has been used to show that PHB is predominantly in a mobile state within *A. eutrophus* and *Methylobacterium* sp. Comparison of chemical and NMR analysis of PHB indicates that about 70% of the polymer in *A. eutrophus* gives sharp observable resonance. The peak stretching indicates the presence of the carbonyl group¹.

In this present study, both IR and C¹³-NMR spectra of the compound confirms the polymer was the following structure:



The above structure explains the four types of carbon such as methyl, methine, methylene and carbonyl.

There are a number of sago industries in Salem and Namakkal Districts. The effluents discharged from these industries are spoiling the agricultural land and aquatic organisms, thereby affecting the ecosystem around them. The sago effluent utilization will be a boon for Agriculturist and Industrialist to get rid of the problems. This study revealed that sago effluent can be used as substrate to produce PHB.

References

- 1 Barnard G N & Sanders J K M, The Poly β-hydroxybutyrate granules in *in-vivo*, *J Biol Chem*, 264 (1989) 3286-3291.
- 2 Schlegal H G, Gottshalk & Bartha R V, Formation and utilization of poly hydroxyl butyric acid by knallgas bacteria (*Hydrigenomonas*), *Nature (Lond)*, 191 (1961) 463-465.
- 3 Volova T G, Vasil'ev A D, Zeer E P, Petrakovskaia E A & Falaieev O V, Study of themolecular structure of poly hydroxybutyrate-A thermoplastic and degradable biopolymer, *Biofizika*, 45 (2000) 445-451.
- 4 Lemoigne M, Grelet N & Croson M, Origin of β-hydroxybutyric lipids formed by bacterial process, *Bull Soc Chim Biol*, 32 (1950) 719-721.
- 5 Winfered F D & Robards A W, Ultra structural study of poly β-hydroxybutyrate granules from *Bacillus cereus*, *J Bacteriol*, 114 (1973) 1271-1280.
- 6 Merrick J M, Lundgren D G & Pfister R M, Morphological changes in poly β-hydroxybutyrate granules associated with decreased susceptibility of enzymatic hydrolysis, *J Bacteriol*, 89 (1965) 234-239.
- 7 Stockdale H R & Dawes E A, Occurrence of poly β-hydroxybutyrate in the Azatobacteriaceae, *J Bacteriol*, 95 (1968) 1798-1803.
- 8 Suzuki S, Yamane & Shimizu S, Mass production of poly -β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding, *Appl Microbiol Biotechnol*, 24 (1986) 370-374.
- 9 Rawate T & Mavinkurve S, Chacterization of polyhydroxy alkonates-Biodegradable plastics from marine bacteria, *Curr Sci*, 83 (2002) 562-564.
- 10 Ostle G A & Holt J G, Nile-blue as a fluorescent stain for poly β-hydroxybutyrate, *Appl Environ Microbiol*, 44 (1982) 238-241.
- 11 Kocer H, Borcakli M, Dermirel S & Hazer B, Production of polysters from various new substrates by *Alcaligenes eutrophus* and *Pseudomonas olevorans*, *Turk J Chem*, 27 (2003) 365-373.
- 12 Luzier L D, Materials derived from biomass/biodegradable materials, *Proc Natl Acad Sci USA*, 89 (1992) 839-842.