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Characterization of Biopolymer Produced by *Streptomyces* Sp. RDD

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Abstract
The present investigation was designed to evaluate the biopolymer production by filamentous bacteria isolated from the soil in Naganathi of India, identified as *Streptomyces* sp., and designated as strain RDD. The organism accumulates poly-β-hydroxybutyrate (PHB) granules in their cells when it was cultivated under limited nutrients with excess carbon rich medium. Purified polymers from cells were determined as PHB by thin layer chromatography, Fourier transform infrared and Nuclear magnetic resonance spectroscopy. While using galactose as carbon source, PHB content was up to 62.74% and the productivity was 1.91 g/L. One of the limiting factors in the commercialization of biopolymer production is the cost of substrate used and its downstream processing. To increase the cell density and production of homopolymer PHB by filamentous bacteria, less expensive agro waste as alternative carbon and nitrogen source were used.

Keywords
Biopolymer; Poly-β- hydroxybutyrate; *Streptomyces* sp. RDD; Nuclear magnetic resonance; Agro waste

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INTRODUCTION
The production of polyhydroxyalkanoates (PHA) has been investigated for more than 80 years but recently the increase in the price of crude oil and public awareness of the environmental issues have forced for extended research on biopolymers (TajalliKeshavarz and Ipsita Roy, 2010). Polyhydtoxybutyrate belonging to the family of PHAs was first discovered by Maurice Lemoigne in 1926 in *Bacillus megaterium*. This polymer is formed as intracellular inclusions under unbalanced growth conditions, i.e. in the presence of excess carbon or energy source and a limiting nutrient (N, P, O, S) or trace elements (Mg, Ca, Fe) (Lee, 1996; Khanna and Srivastava, 2005).

The limiting factors in the commercialization of biopolymer production are the cost of substrate used and its downstream processing. The occurrence and formation of PHB by actinobacteria have not been investigated to a significant extent. In this study, the production of homopolymer PHB by filamentous bacteria, less expensive agro waste used as alternative carbon and nitrogen source. Therefore, use of agro waste could be a good approach for cost effective production of PHB. The natural biopolymer possesses three excellent features: biodegradability, biocompatibility and thermoplastic properties. In addition, PHB shows material properties similar to that of petrochemical derived plastics. It exhibits good

barrier properties resembling PVC and PET (Sharma and Ray, 1995). Hence, the replacement of non-biodegradable plastic with biodegradable from organic environment contributes to sustainable development (Spiekermann *et al.,* 1999).

MATERIALS AND METHODS

Actinobacterial strain

Streptomyces sp. RDD, which was isolated from soil in India (latitude 12°46'0'' and longitude 12°46'0''), and identified in our laboratory (morphological, biochemical characteristics) was used in this study. The stock culture was maintained on starch casein agar slants overlaid with 20% (v/v) glycerol and kept at 4°C.

Chemicals

The chemicals used in the present study were of highest quality obtained mainly from the local suppliers. Ready-made media ingredients were obtained from Hi media Laboratories.

Culture media

In the laboratory, formulated starch casein agar (SCA) and Nitrogen deficient medium were used in this study.

Rapid Screening of PHB producing actinobacteria

Two approaches were used for the selection of PHB producing actinobacteria.

Viable colony method/ macroscopic selection: For the putative selection of PHB producing actinobacteria, plate containing nitrogen deficient medium was divided into 6 equal parts and in each part, the isolate was spotted. Then the plates were incubated at 28°C for 7 days. An alcoholic solution of Sudan Black B dye (0.02% in 96% ethanol) was spread over the colonies and the plates were kept undisturbed for 30 min. They were washed with 96% ethanol to remove excess stain from the colonies. The dark blue colored colonies were taken as positive for PHB production (Schlegel *et al.* 1970).

Sudan Black B staining/ microscopic selection: For microscopic selection of potential PHB producer of actinobacteria lipophilic dye was used for staining. The culture was heat fixed on a glass slide and immersed in alcoholic solution of 0.3% (w/v) Sudan Black B dye in 70% ethanol (v/v) for 10 min. The excess stain was taken out with chloroform. Counter stained with Fuchsin acts as cell wall dye (Greenspan *et al,* 1985).

Characterization of the selected actinobacteria

The morphological and physiological properties of the actinobacterium were investigated by performing Gram's staining. Various biochemical tests namely, hydrolysis of starch, casein, esculin, cellulose utilization, tyrosine degradation, gelatin liquefaction, and nitrate reduction were performed for their biochemical characterization. The utilization of various carbohydrates (arabinose, rhamnose, mannose, fructose, sorbitol, glucose) was tested by inoculating the selected isolate separately in the defined medium to which various sugars followed by 3-5 days incubation at 28°C.

Poly-β-hydroxybutyrate production and extraction

Streptomyces sp. RDD was incubated at 28°C for 5 days at 150 rpm in nitrogen deficient medium contained 1% lactose, 0.02% $MgSO_4.7H_2O$, 0.01% NaCl, 0.05% KH_2PO_4, 0.25% peptone, and 0.25% yeast extract. After (5 days), PHB was extracted by solvent chloroform method (Santhanam and Sasidharan 2010). Culture broth was centrifuged at 5,000 rpm for 10 min at room temperature. The pellet dissolved in 2.5 ml of sodium hypochlorite was incubated at 30°C for 1-2 h for complete digestion of cell components except PHA, where by lipids and proteins were degraded. The cell extract obtained was centrifuged at 1,500 rpm for 10 min and then sediment 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. After washing, the polymer granules was dissolved in chloroform and was evaporated by air drying at 30°C, to yield dry powder of PHB.

Quantitative analysis of PHB

Both dry cell weight (DCW) and PHB quantification were determined gravimetrically.

Crotonic acid assay: The extracted granules were dissolved in concentrated sulfuric acid (1mg/ml) and kept in water bath at 100°C for 10 min. The PHB is converted into crotonic acid, which gives absorbance maximum at 235 nm in UV visible spectrophotometer against a concentrated sulphuric acid as blank (Chen, 2009).

Dry cell weight: The culture broth was centrifuged at 6000 rpm for 10 min. The pellets (cells) were harvested and washed twice with distilled water by resuspension and centrifugation as above, and then transferred to

preweighed dish. The cells were dried at 80°C until for a constant weight.

$$\text{PHB accumulation (\%)} = \frac{\text{Dry weight of extracted PHB } (\frac{g}{l})}{\text{DCW } (\frac{g}{l})} \times 100\%$$

Optimization of culture conditions

50 ml of the production medium was inoculated with the culture and incubated at 28°C for 5 days in shaker incubator under different conditions of growth. To optimize the culture conditions, different carbon sources, pH and agrowaste were used for the PHB production. Among the carbon sources, glucose, lactose, galactose, maltose and mannose were tested at a fixed concentration 1%. The pH gradients evaluated were 5, 7, 9.

1% of Rice bran, groundnut husk, jack fruit seed powder, soy bean meal and corn starch were used among the agro waste. Two different types of pretreatments were carried out, viz. direct infusion and acid treatment. Direct infusion was carried out by drying and pulverizing the agricultural waste. The powdered substrates were used as the sole carbon source. In acid treatment, agricultural waste substrates were hydrolyzed with 0.5-5.0% v/v sulphuric acid (solid: liquid, 1:10-1:20) and autoclaved at 121°C for 30 min. The hydrolyzed samples were filtered, and the supernatants were neutralized using sodium hydroxide (6N). The reducing sugar content was measured by DNSA method. Media prepared by using these hydrolysates at a concentration of 10% (v/v). After autoclaving, 24 h old culture was inoculated in the modified media and incubated (Kannan and Rehacek 1970; Ramadas *et al.* 2009).

Characterization of Biopolymer

Thin Layer Chromatography (TLC)

About 50 µl of 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 vaporized 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 vapor. After 10 min green-black color spots indicated the presence of PHB. The R_f (Retardation factor) value was measured and compared with standard chart (Rawte and Mavinkurve 2002).

Fourier Transform Infrared Spectroscopy (FTIR)

The presence of different functional groups in PHB was analyzed by FTIR. Extracted PHB (1 mg) was dissolved in 1 ml chloroform and added on KBr disc. After evaporation of chloroform, PHB polymer film was subjected to FTIR analysis (Shimadzu FTIR) in the range of 400-4000 cm^{-1}.

Nuclear magnetic resonance spectroscopy (NMR)

The nuclear magnetic resonance spectrum was recorded at on a Bruker FT NMR 400 MHz spectrometer equipped with ^1H probe to study the structural elucidation. Proton spectra were recorded at 300.13 MHz with a spectral width of 2,840 Hz over 16 K data points. 10 mg of the extracted PHB was dissolved in $CDCl_3$ and subjected to analysis. The structure of PHB in the sample was investigated by the 400 MHz ^1H NMR spectra recorded at 30°C on a $CDCl_3$ solution of polymer (10 mg/ml). Peak areas were determined by spectrometer integration by using tetramethylsilane as an internal standard.

RESULTS AND DISCUSSION

Isolation and purification of actinobacteria

Actinobacteria used in this study were isolated from different sources including forest, wood waste, garden and agriculture rhizosphere soil samples. The samples were collected from different localities in Naganathi, India. Eighteen isolates of actinobacteria were obtained on starch casein agar media. All the isolate was screened for PHB production. A large proportion of tested actinobacteria, was able to produce PHB. The growth of the actinobacteria was measured by recording the dry weight g/l. the percentage of PHB accumulation ranged from of their dry weight of cells. The most potent isolate was RDD from forest soil on starch casein agar.

Identification of the selected isolate RDD:

Based on the screening data, the most potent actinobacteria in PHB production was the isolate RDD from forest soil sample collected from Thellai Forest, Naganathi, India. Morphological and biochemical characteristics of selected isolate RDD were examined (Table 1). Based on the results the isolate was identified to be *Streptomyces* sp. RDD.

Table 1. Morphological, physiological and biochemical characteristics of selected isolate RDD.

Character	Reaction
Gram's staining	Gram positive filamentous
Colony morphology on SCA	Medium, powdry deposited cream colored
Reverse side pigment	Violet
Aerial spore mass color	Creamish pink
Spore chain morphology	
Melanin pigment production	Spiral spira
Tyrosine degradation	+
Starch hydrolysis	+
Casein hydrolysis	+
Esculin hydrolysis	
Gelatin liquefaction	
Nitrate reduction	+
Cellulose utilization	+
Adenine utilization	+
Assimilation of carbon sources:	
Arabinose	+
Rhamnose	+
Mannose	+
Fructose	+
Sorbitol	+

+: positive,

According to the description of the genus *Streptomyces* (Deepa *et al.,* 2015), it has the following determinative phenotypic characteristics: as shown in the table, taxonomic properties of the strain were consistent with those distinguishing characteristics of *Streptomyces* and the strain should therefore be assigned to that genus.

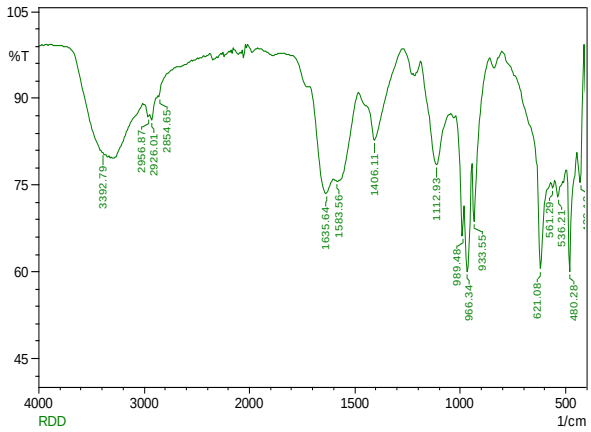
Digestion of polymer with concentrated H_2SO_4 gave a sharp peak at 235 nm, characteristic of crotonic acid. The amount of PHB produced estimated to be 190µg/ml. The identity and purity of the PHB obtained from *Streptomyces* sp. RDD were confirmed by solubility properties and UV absorption spectra.

When tested on the various carbon sources, the strain exhibited nutritional versatility in terms of varied growth and PHB formation. The highest level of PHB accumulation was observed in the N_2-deficient medium with galactose (PHB yield 1.91 g/l, DCW 62.74%), followed by the media featuring lactose (1.07 g/l, 45.12%), glucose (0.60 g/l, 29.51%), mannose (1.09 g/l, 16.69%) and fructose (0.78 g/l, 15.11%).Maximum accumulation of PHB achieved at pH 7.0 up to 1.07 g/l. Several factors influence the economics of biopolymer production. These factors include substrate cost and the ability to produce biopolymer from inexpensive or renewable substrates. Of the various agro wastes tested, rice bran (0.47 g/l), jack fruit seed powder (0.46 g/l), corn starch (0.36 g/l), ground nut husk (0.32 g/l) and soy bean meal (0.15 g/l), although PHB yield was not appreciably high. Several renewable materials, including casein hydrolysate, gluten is known to be available from agriculture sector as nitrogen source that can be used as cheap substrate.

Green black color band was observed in TLC. Rf value (0.27) was measured (Rawte and Marvinkruve, 2002) indicates the presence of homopolymer poly-3-hydroxybutyrate as component.

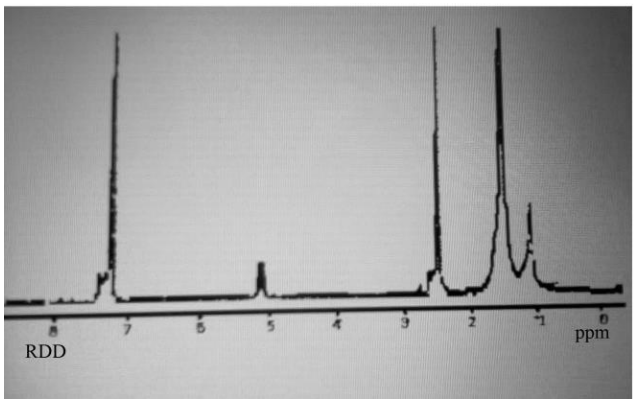
The functional groups of the extracted PHB granules were identified by FTIR spectroscopy (Fig). The IR spectroscopic analysis gave further insights into chemical structure of the polymer and reflects the monomeric units. The FTIR spectrum analysis of the PHB clearly revealed its purity. The absorbance peak values obtained were compared with the available literature values and confirmed the products as PHB. The peak values obtained in this study coincide with previous results (Rajesh *et al.,* 2014; Marjadi and Dharaiya, 2014; Samantaray *et al.,* 2014; Rajendran *et*

al., 2013). The FTIR spectrum revealed the presence of marked peaks at wave number 2926 cm^{-1} corresponding to the methyl group in them. This gave ester containing group which consisted of PHB. The peak obtained at 2956 indicated the presence of (O-H) stretching. Peak at 2854 indicates aliphatic C-H stretching. The peaks obtained at 1635, 1112, 989 represented that carbonyl group (C=O), (O=H), C-O stretching, (=C-H) stretching respectively and were identical to PHB.



NMR Analysis

The ^{1}H NMR spectra obtained from extracted PHB of the strain RDD and the results obtained were compared with the previous literature. The peaks observed in the spectra coincide, corresponding to the different types of carbon atoms in the PHB structure. A doublet at 1.53 represented the CH_3 (methyl) group coupled to one proton. The second signal at 2.5 ppm assigned to CH_2 (methylene) group, adjacent to an asymmetric carbon bearing s single proton and multiplet at 5.2 ppm indicates the presence of CH (methyne) group. Another one at 7.25 ppm attributed to the solvent used (chloroform). An NMR result obtained with RDD is complete agreement with the earlier reports of Chakrabarti *et al.,* 2007; Indra Arulselvi *et al.,* 2012.



CONCLUSION

Biopolymers are likely to play a significant role in building an ecofriendly environment by replacing the widely used non-biodegradable synthetic plastics. The development of PHB into a branch of bulk chemical industry will address at least three issues: shortage of petroleum for petroplastics, reduction of carbon dioxide emissions and environmental protection. Actinobacteria have peculiar characteristics and broad biotechnological applications also have a special role in bioplastic production. Bioplastics are compounds with an extremely promising future. The future trend is to focus on the development of more efficient and economical processes for PHB production, isolation, purification and improvement of PHB material properties.

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