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Research Paper

Production and characterization of Polyhydroxybutyrate by *Nocardia* sp. RD13 isolated from agriculture rhizosphere soil

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Abstract

This work was focused on to isolate polyhydroxybutyrate (PHB) producing actinobacteria from agriculture rhizosphere soil. The strain accumulated PHB 1.96 g/L, dry cell weight 66.55% under growth conditions in a nitrogen deficient medium containing galactose, with a maximum production at 72 h. Rice bran, corn starch, jack fruit seed powder, soy bean meal and groundnut husk were used as substrates for PHB production to reduce the cost of producing such biopolymer. Maximum PHB yield was obtained with groundnut husk (0.59 g/L) followed by rice bran (0.58 g/L) as sole nitrogen and carbon sources. The biopolymer was highly soluble in chloroform and peaked sharply at 235 nm upon digestion with concentrated H_2SO_4. Recovered polymers were confirmed by Fourier transform infrared spectroscopy (FTIR), Nuclear magnetic resonance (NMR) and Thin layer chromatography (TLC). The potent isolate was identified as *Nocardia* sp. and designated as strain RD13. This study adds new genera to the members of the PHB producers.

Keywords: Polyhydroxybutyrate – actinobacteria – *Nocardia* – biopolymer.

Introduction

In recent years the interest in biopolymer production has advanced to find non-fossil fuel based polymers. The most common microbiologically produced, biopolymer is polyhydroxybutyrate (PHB) belonging to the member of the polyhydroxyalkanoate (PHA). Microorganisms have the ability to accumulate polymeric substance as carbon and energy storage materials in the form of mobile, amorphous and lipid granules under stressful condition[1]. PHB have attracted increasing attention for their biodegradability, biocompatibility, thermoplastic features, composted or burned without producing toxic byproducts and be used as packaging and biomedical materials[2]. Since 1990s, the discovery of new PHA producers opened a new research field finding of different bacterial genera and species. Some of them showed good potential for industrial production, while others showed the opposite[3-7]. In the present study, *Nocardia* species capable of producing PHB was identified during screening for PHB producing bacteria. Recent research has focused on the use of alternative substrates, novel extraction methods in order to make PHB more commercially attractive. The aim of the research was to determine PHB production by actinobacteria and to test further PHB production in different agro waste as carbon and nitrogen sources.

Materials and Methods

Analytical grade (AR) chemicals, reagents and ready-made media ingredients procured from Hi-Media Laboratories Pvt. Ltd., Mumbai.

Sample collection

Agriculture rhizosphere soil samples were collected from various locations in Naganathi, Vellore, Tamil Nadu (latitude 12°46'0" and longitude 12°46'0"), India. Soil samples were collected at the depth of 10-25 cm in a sterilized container and transferred to the laboratory and stored in refrigerator at 4°C until further processing[8].

Isolation of Actinobacteria

Isolation of actinobacteria was performed on selective media such as Starch Casein agar (SCA). Nystatin (20 µg/ mL) and nalidixic acid (100 µg / mL) were added into the medium as antifungal and antibacterial agents[9]**.** In 99 ml sterile distilled water, 1g of soil sample was mixed. Then, the samples were serially diluted in sterile distilled water followed by plating and incubated at 28°C for 7-14 days. Actinobacteria colony was preliminarily selected based on colony morphology. Purified strains were maintained on SCA agar slant.

Rapid screening of PHB

Two approaches were used for the selection of putative PHB producing actinobacteria. For microscopic selection of potential PHB producer of actinobacteria was done by using lipophilic dye. 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 Fuchsine[10]. For macroscopic selection, the isolates were grown in nitrogen deficient medium. After appropriate growth, an alcoholic solution of Sudan Black B dye (0.02% in 96% ethanol) was added to the plates and after 20 min, blue colonies were observed as PHB producers[11].

Morphological and Biochemical characterization of selected Actinobacteria

Visual observations of both morphological and microscopic characteristics using light microscopy (UNILAB Model RH-85 UXL) Gram-stain reaction, acid-fastness were studied. Identification was according to biochemical tests that are including: Growth in lysozyme broth, hydrolysis of casein, gelatin, tyrosine, starch, esculin, cellulose, adenine, nitrate reduction and assimilation of carbohydrates such as arabinose, rhamnose, mannose, galactose, fructose and sorbitol were also followed for 3-5 days at 28°C by inoculating the selected strain separately in the defined medium.

Production of PHB

Nitrogen deficient medium containing 1% lactose, 0.02% $MgSO_4.7H_2O$, 0.01% NaCl, 0.05% KH_2PO_4, 0.25% peptone and 0.25% yeast extract, inoculated with selected PHB producing isolate and incubated at 28°C for 3 days at 150 rpm on a rotary shaker (ORBITEK SHAKER, Scigenics Biotech).

Recovery of PHB

The recovery of PHB from dried biomass was carried out by digestion method using sodium hypochlorite and chloroform as described[12].

Quantitative analysis of PHB

Dry Cell Weight

Dry weight was estimated from 100 ml of culture broth. The cell suspension was centrifuged at 10,000 rpm for 10 min, washed with warm distilled water several times, transferred to preweighed vials and dried in an oven at 105°C till constant weight. This was calculated to determine the cellular weight and accumulation other than PHB.

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

Crotonic acid Assay

The extracted polymer granule was dissolved in concentrated sulfuric acid (1 mg/ml) and heated at 100°C for 10 min to convert PHB into crotonic acid and absorbance at 235 nm was measured against a concentrated sulfuric acid blank in UV Visible spectrophotometer (Double Beam UV-SpectrophotometerSL164, version: 4.02 PC, ELICO Ltd, India) [13]. By referring to the standard curve, the quantity of produced PHB was determined.

Optimization of culture conditions for PHB production

Different factors affecting the PHB production by the selected strain RD13 was optimized i.e. pH, carbon source and agro waste. For pH optimization, culture was inoculated in nitrogen deficient

medium and incubated at pH viz, 5.0, 7.0 and 9.0. Effect of carbon sources on PHB production was determined by simply replacing the carbon source with other carbon sources (glucose, galactose, mannose, fructose and lactose).

For the production of PHB, various inexpensive agro industrial wastes such as groundnut husk, rice bran, soybean meal, corn starch and jack fruit seed powder were used. Two types of pretreatments were carried out, viz. direct infusion and acid treatment. Direct infusion was carried out by drying and pulverizing the agro 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 sulfuric acid (solid: liquid, 1:10-1:20) and autoclaved at 121^{o}C for 30 min. the hydrolyzed samples were filtered and the supernatants were neutralized using NaOH (6N). Reducing sugar content was measured by DNSA method. Media prepared by using these hydrolysates at a concentration of 10% v/v [14].

Characterization of biopolymer
White dry crystals were collected and crushed into fine powder and used for characterization.

Thin Layer Chromatography
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 solution was vaporized in water bath at 80-100^{o}C. TLC plate was kept over the beaker containing iodine solution for 5-10 min in order 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[15].

FTIR Spectroscopy
The chemical structure of an extracted PHB granule was analyzed by FT-IR (Shimadzu FTIR 8101A) spectroscopy. The biopolymer was dissolved in chloroform and added to KBr pellets and then the solvent was evaporated. The infra spectrum of the extracted polymer was used the spectral range 400 – 4000 cm^{-1} to confirm functional group.

NMR Analysis
Nuclear Magnetic Resonance is a property that magnetic nuclei have magnetic field and applied electromagnetic pulse, which cause the nuclei to absorb energy from the electromagnetic pulse and radiate this energy back out. ^{1}H NMR spectra of the sample was obtained at BRUKER FT NMR 400 MHz.

Results and Discussion

Agriculture soil has rich biodiversity of microbial population. For rapid screening and isolation of PHB producing actinobacteria, 0.02% alcoholic solution of Sudan Black B was used, formation of dark blue color colonies on nitrogen deficient medium and PHB granules were observed microscopically. The morphological characteristics of strain RD13 were mainly as described below in Table 1. It was filamentous Gram positive, partially acid fast bacterium. The chemo taxonomical studies showed the presence of amino acids and sugar patterns of strain RD13, confirming the cell wall chemotype IV. The colonies were (diameters, 0.5-1.2 mm), rough, white cream colored and chalky. The strain was identified as *Nocardia* sp.

The occurrence of PHB has been repoted from wide variety of bacteria, but strains of *Nocardia* sp. have not been investigated for this ability until now. A review of the available literature indicates that this is the first report on PHB production by *Nocardia* sp. However, *Nocardia coralline* demonstrated the capability to produce copolymers such as poly-(-3-hydroxybutyrate-co-hydroxyhexanoate) and poly-(-hydroxyvalerate-co-hydroxyheptanoate) when cultivated on glucose or fructose as sole carbon source[16] not PHB.

The effect of different carbon sources on the production of PHB by actinobacteria was studied, in order to choose the best one. Lactose, glucose, galactose, mannose and fructose were added at 1% concentration to the production medium, one at a time. The amount of PHB and growth rate of the organisms after 3 days was determined.

Table 1: Morphological and Biochemical characteristics of strain *Nocardia* sp. RD13

Characteristics	Strain RD13	Genus *Nocardia*
Gram staining	Gram positive filamentous	+
Acid fast staining	+	+
Reverse side pigment	Yellow	
Aerial spore mass color	White	
Melanoid pigment	Dark brown	
Starch hydrolysis	+	+
Tyrosine degradation	+	+
Esculin hydrolysis	+	+
Gelatin liquefaction	+	+
Nitrate reduction	+	+
Casein hydrolysis	+	+
Growth in lysozyme broth	+	+
Catalase	+	+
Adenine	+	+
Assimilation of carbon sources:		
Arabinose	+	+
Fructose	+	+
Galactose	+	+
Rhamnose	+	+
Mannose	+	+
Sorbitol	+	+

As shown in Figure 1 the best carbon source for the growth of cells was fructose (DCW 3.86 g/L). On the other hand galactose was the best carbon source for PHB production (1.96 g/L, 66.55%), this was followed by glucose (1.18 g/L, 45.66%). Lactose (1.02 g/L, 41.29%) was also a good carbon source for PHB accumulation while fructose and mannose was poorest carbon sources.

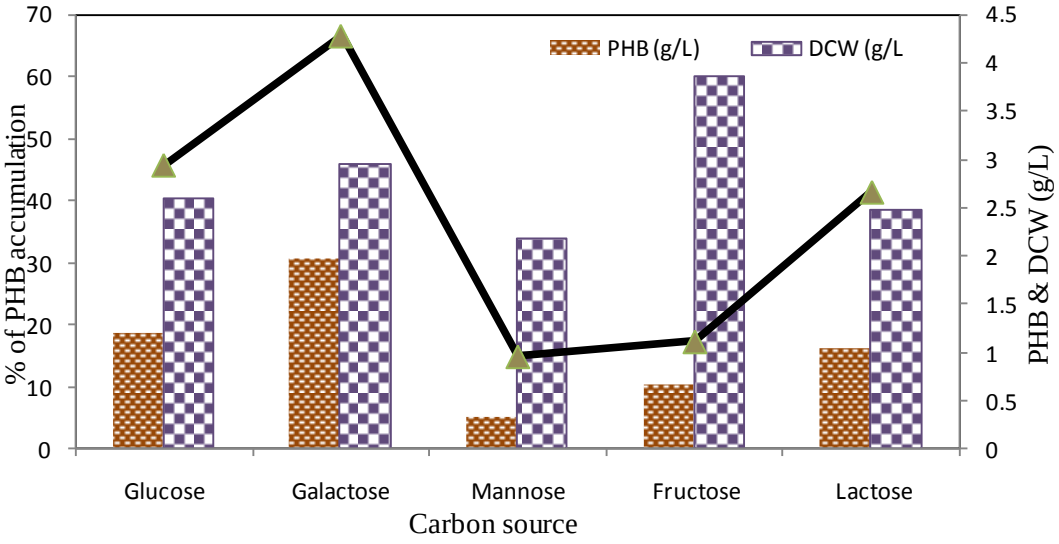

Figure 1: Effect of different carbon sources (1%) on PHB production, Dry cell weight and % of PHB production by the cells of *Nocardia* sp. RD13

The purified polymer was highly soluble in chloroform, but insoluble in water, sodium hypochlorite, diethyl ether, methanol, ethanol and acetone. Digestion of polymer with concentrated H_2SO_4 gave sharp peak at 235 nm, characteristic of crotonic acid.

Maximum production of PHB achieved at pH 7.0 up to 1.02 g/L followed by pH 5.0 and 7.0 (0.72 and 0.43 g/L).

One of the limiting factors in the commercial success of PHB was high cost of substrate used and its downstream processing. Previous research attempts have been made to reduce the cost of production of biopolymer so that its use can be enhanced. In this study, to minimize the cost of producing such biopolymer, various low cost carbon and nitrogen sources (rice bran, corn starch, jack fruit seed powder, soy bean meal and groundnut husk) were used for the production of PHB. As shown in table 2. Maximum PHB yield was obtained with groundnut husk (0.59 g/L) followed by rice bran (0.58 g/L) as sole nitrogen and carbon source.

Table 2: Production of PHB by the strain *Nocardia* sp. RD13 using agro waste

Agro waste	PHB yield (g/L)
Rice Bran	0.58
Groundnut husk	0.59
Soybean meal	0.18
Corn starch	0.22
Jackfruit seed powder	0.34

Green black color band was observed in TLC. R_f value was measured by the subtraction of solvent distance and solute distance and by solvent distance. R_f value was found to 0.34 indicates the presence of homopolymer 3-hydroxybutyrate after compared with standard chart[17].

The functional groups of the extracted PHB granules were identified by FTIR spectroscopy. The IR spectroscopic analysis gave further insight into chemical structure of the polymer and reflects the monomeric units. The FTIR spectrum revealed the presence of marked peaks at wave number 2926 cm^{-1} corresponding to CH_3 group in them. This gave an ester containing group which consisted of PHB. The peaks at 2956 and 2854 cm^{-1} are due to O-H stretching and aliphatic C-H stretching. The peaks obtained at 1450, 1400, 1070, 989 cm^{-1} represented that CH_2, (O=H) tertiary alcohol, C-O stretching and (=C-H) stretching respectively. Peak at 1228 cm^{-1} sensitive to crystallinity characteristic of C-O-C stretching and were identical to PHB.

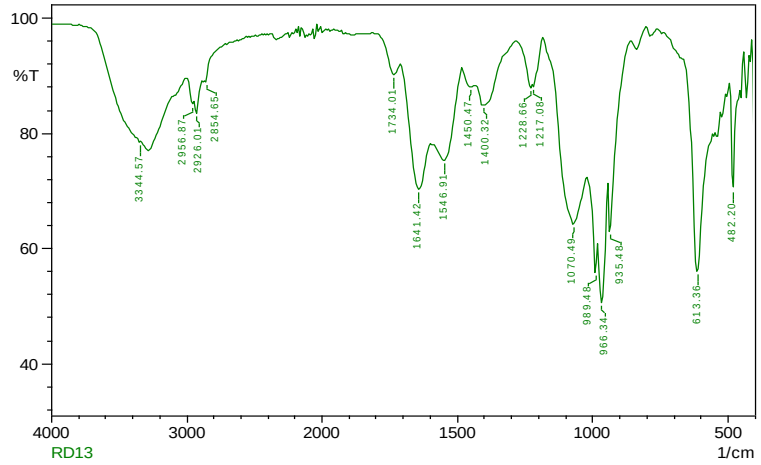

Figure 2: IR spectrum of PHB produced by *Nocardia* sp. RD13

The extracted polymer was dissolved in 1 ml $CDCl_3$ and the structure of polymer was investigated by ^1H NMR spectroscopy (BRUKER FT NMR 400 MHz). ^1H NMR spectra of the extracted PHB from strain RD13 showed three groups of signals characteristic of PHB were seen in the spectrum. A doublet at 1.53 ppm represented the methyl (CH_3) group coupled to one proton. The second signal was doublet of quadruplet at 2.5 ppm attributed to methylene (CH_2) group adjacent to asymmetric carbon bearing single proton and multiplet at 5.2 ppm due to the presence of CH (methyne) group. Another signal at 7.25 ppm attributed to chloroform. Therefore, NMR results obtained with strain RD13 complete agreement with the earlier reports[18]. From these results, RD13 produces PHA exclusively in the form of PHB.

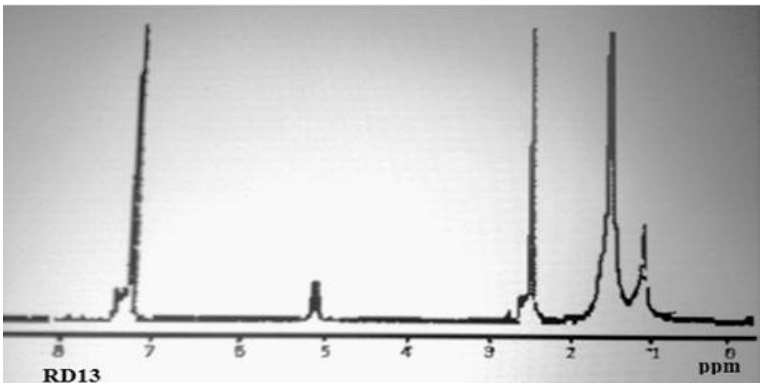

Figure 3: NMR spectrum of PHB produced by *Nocardia* sp. RD13

Conclusion

Rhizoshere soil with its spatial and temporal changes in availability of nutrient is a good source of PHB producing actinobacteria. This is the first report of PHB accumulation in *Nocardia* sp. especially using agro waste as carbon and nitrogen source. PHB granules were observed intracellularly in spores. The addition of new genera to the existing list of PHB producing microorganisms will certainly provide new opportunities for the production of cost-effective biodegradable plastics. Future studies will focus on investigating the ability of this strain to accumulate various other copolymers.

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