Production, efficient recovery and partial characterization of biodegradable polymer produced by soil Streptomyces sp.

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In the present paper, we report the production of poly-β-hydroxybutyrate (PHB) by local soil isolates of Streptomyces sp. under submerged fermentation in nitrogen deficient medium. Streptomyces sp. was isolated on glycerol asparagine agar from soil samples collected from the campus of PSGVP Mandal, Shahada (Maharashtra) India. Of the seven isolates of Streptomyces sp., two isolates, namely, RZS 1 and RZS 2, accumulated 1.7 g/L (4.52 g/L biomass) and 1.5 g/L (4.11 g/L biomass) PHB, respectively on the basis of dry wt. Further increase in intracellular level of PHB was observed during the growth of these isolates in carbon rich medium with pH 7 at 30°C. We also report the use of modified solvent system consisting of 1:1 mixture of acetone and ethanol as a more specific and efficient recovery method vis-à-vis traditionally used sodium hypochlorite system.

Keywords: Poly-β-hydroxybutyrate (PHB), Streptomyces sp.

Liberal use of large amount of non-biodegradable synthetic polymers has created frightening scenario for the environment. These non-degradable petrochemical plastics accumulate in the environment at the rate of more than 25 million tons per year. To combat this environmental problem, replacement of non-biodegradable polymers by biodegradable and eco-friendly polymers like poly-β-hydroxybutyrate (PHB) has become the need of the time to protect the environment. PHB is a biodegradable polyester synthesized and accumulated intracellularly under nitrogen limiting condition by a large variety of Gram positive and Gram negative bacteria. However, there are only few reports on PHB accumulation by Streptomyces sp. The aim of the present study was to isolate Streptomyces sp. capable of accumulating intracellular PHB granules and to screen the most potent PHB producing Streptomyces sp.

Soil samples were collected from different depths (5-15 cm) of garden soil from the campus of PSGVP Mandal, Shahada (Maharashtra), India. All samples were transported in plastic bags to the laboratory and spread on paper sheet until air-dried. A serial dilution of the fine soil powder was prepared and 10⁶ dilution was selected for plating on glycerol asparagine agar containing: asparagine, 1 g/L; glycerol, 10 mL/L; K$_2$HPO$_4$, 1 g/L; agar, 20 g/L; trace salt solution (1 mL/L) containing FeSO$_4$, 0.1 g/100 mL, MnCl$_2$, 0.1 g/100 mL, ZnSO$_4$, 0.1 g/100 mL. The plates were incubated at 30°C for 7-10 d. The purified colonies were maintained on glycerol asparagine agar.

Pure colonies from glycerol asparagine agar were subjected for identification on the basis of cultural characteristics, Gram reaction, their ability to produce oxidase and catalase enzymes and the presence of characteristic branched, aerial and vegetative mycelia bearing sporophores in chain.

A smear of biomass grown in nitrogen deficient minimal medium (NDMM) was stained for 15 min with 0.3% (w/v) Sudan Black B solution prepared in 70% (v/v) ethyl alcohol. It was then de-stained with 50% (v/v) alcohol and counterstained with 0.5% (w/v) safranin solution. The slides were then washed, air dried and observed under bright field microscope.

The Streptomyces isolates were used for PHB production in two phases. In the first phase, organism was grown in carbon rich medium at 28±2°C for 24 h, with constant shaking on rotary shaker at 120 rpm. After 24 h, cell mass obtained by centrifugation (10,000 rpm for 10 min) was aseptically transferred to nitrogen deficient minimal medium (NDMM). The pH of the medium was adjusted to 7.0 with KOH and grown at 28±2°C for 48 h on rotary shaker at 120 rpm. Following the incubation, broth was centrifuged to obtain cell mass. This cell mass was used for the extraction of PHB. Streptomyces sp. accumulating higher amounts of PHB was selected as potent PHB producing isolates.

PHB is soluble in organic solvents and this property leads to the effective and higher PHB extraction from cells. Although traditionally used sodium hypochlorite causes the digestion of non-PHB cell mass (NPCM) and gives PHB. It also affects the sudanophilic properties and mol wt of the polymer granules.
Therefore, some other solvent system needs to be explored for efficient recovery of PHB while maintaining its original properties. The solvent system consisting of 1:1 mixture of ethanol and acetone was checked for efficient recovery of PHB that causes specific lyses of NPCM without affecting PHB.

Spectrophotometric assay for detection and estimation of PHB was done as per Henneke et al. In this assay, a sample containing 10-100 µg of polymer in chloroform was transferred into clean test tube. The chloroform was allowed to evaporate, followed by addition of 10 mL of concentrated sulfuric acid. Tubes were heated in boiling water bath at 100°C for 10 min, cooled at room temperature and the amount of PHB present in sample was determined at 235 nm on UV-Vis spectrophotometer (Model 1240 Shimadzu, Japan). The PHB extract was scanned between 190-1100 nm to get the absorption maxima. The concentration of crotonic acid formed was detected at 235 nm from a set of standard prepared with crotonic acid in the range of 100 to 1000 µg/mL.

From local garden soil samples, seven isolates of *Streptomyces* sp. were obtained on glycerol asparagine agar medium. The *Streptomyces* sp. isolates formed irregular, wrinkled, grayish-white and reddish-white colonies. Isolates were Gram positive, non-motile, oxidase and catalase positive. The isolate had branched, aerial and vegetative mycelia. The aerial mycelia produced sporophores of different morphological forms bearing spores in chain. Pridham and Lyons reported similar cultural characteristics of *Streptomyces* sp. grown on glycerol asparagine agar medium. The colonies also showed characteristics vegetative aerial hyphae bearing sporophores in chain. Based on this the isolates were identified as *Streptomyces* sp. and were further used for the production of biodegradable plastic.

Under bright field microscope, Sudan Black B stained smear of all seven isolates of *Streptomyces* sp. revealed the presence of varying level of blue colour PHB granules against pink colour cytoplasm. Of the seven isolates, two isolates showed the abundance of intracellular PHB granules and these were named as *Streptomyces* sp. RZS1 and RZS2.

During submerged growth, the biomass obtained from *Streptomyces* sp. RZS1 was 3.51 g/L and accumulation of PHB was 1.2 g/L. Whereas *Streptomyces* sp. RZS2 produced 3.11 g/L biomass and yielded 1.0 g/L of PHB. Optimization of growth conditions resulted in further increase in PHB productivity to 1.7 g/L (4.52 g/L biomass) and 1.5 g/L (4.11 g/L biomass) by RZS1 and RZS2, respectively. Figs 1 and 2 show the PHB accumulation profile in isolates RZS1 and RZS2, respectively and their respective biomass yield.

The acetone-alcohol (1:1 v/v) solvent system was proved more useful and specific since it lysed non-PHB cell mass without affecting PHB. This solvent system gave PHB recovery of 140.12 µg/mg from *Streptomyces* sp. RZS1 and 123.64 µg/mg from *Streptomyces* sp RZS 2. Spectrophotometric analysis revealed the presence of two peaks in the range of 248-365 nm. These peaks resembled very well with the characteristic absorption maxima of PHB and gave the confirmation that the given isolates produced PHB.

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References