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Evaluation of short-chain-length polyhydroxyalkanoate accumulation in *Bacillus aryabhattai*

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ABSTRACT

This study was focused on the polyhydroxybutyrate (PHB) accumulation property of *Bacillus aryabhattai* isolated from environment. Twenty-four polyhydroxyalkanoate (PHA) producers were screened out from sixty-two environmental bacterial isolates based on Sudan Black B colony staining. Based on their PHA accumulation property, six promising isolates were further screened out. The most productive isolate PHB10 was identified as *B. aryabhattai* PHB10. The polymer production maxima were 3.264 g/L, 2.181 g/L, 1.47 g/L, 1.742 g/L and 1.786 g/L in glucose, fructose, maltose, starch and glycerol respectively. The bacterial culture reached its stationary and declining phases at 18 h and 21 h respectively and indicated growth-associated PHB production. ^{1}H NMR and ^{13}C NMR spectra confirmed the material as PHB. The material has thermal stability between 30 and 140 °C, melting point at 170 °C and maximum thermal degradation at 287 °C. The molecular weight and poly dispersion index of the polymer were found as 199.7 kDa and 2.67 respectively. The bacterium *B. aryabhattai* accumulating PHB up to 75% of cell dry mass utilizing various carbon sources is a potential candidate for large scale production of bacterial polyhydroxybutyrate.

Introduction

Polyhydroxyalkanoates (PHAs) are reserves of carbon and energy found in bacteria in the form of intracellular inclusions. They are synthesized and deposited when bacterial cells are cultured in a medium containing surplus amount of carbon source with inadequate supply of other nutrients.[1] These are biodegradable-biocompatible thermoplastics, non-toxic, hydrophobic, impermeable to gases, piezoelectric, enantiomerically pure and show a high degree of polymerization with molecular weights of 20,000 to 30 million Daltons.[2–4]

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PHAs have attracted a great deal of attention because of their bio-degradability and thermoplastic properties.[5] They show physical and material properties which make them suitable for applications in various fields such as manufacturing of packaging materials, as biomedical implant materials, as drug delivery carriers, as biofuels, as water resistant coatings on cardboard or paper, as additives in cosmetics and in food processing industries.[2,6–8] PHAs can be produced from renewable resources and they are considered as an alternative to non-biodegradable plastics produced from fossil oils.[9] Commercial production of PHA is limited by the high cost of production compared to conventional plastics. The main focus on the biopolymer research is to develop economically feasible methods for the large scale production of good quality biopolymer.

Most PHAs have been produced by prokaryotic microorganisms, including bacteria and archaea, although transgenic plants were reported to produce PHAs.[10] In prokaryotes PHA accumulation property is broadly distributed among the Gram-negative organisms such as *Cupriavidus*,[11] *Pseudomonas*,[12] etc., Gram-positive organisms such as *Bacillus*,[12] *Clostridium*,[13] *Corynebacterium*,[14] *Nocardia*, *Rhodococcus*,[15] *Streptomyces*,[16] *Staphylococcus*,[17] etc. and certain archaeal strains of *Halobacterium*,[18] *Haloarcula*,[19] *Haloquadratum*[20] and *Haloferax*.[21] *Bacillus* spp. are well known for their ability to accumulate poly-3-hydroxybutyrate (PHB) which is the most common and simplest form of PHA found in bacteria.[22–25] PHB is the first discovered and the most extensively studied biopolymer.[26,27]

Bacillus aryabhattai was first isolated from cryotubes used for collecting air from upper atmosphere.[28] The PHB accumulating property of this strain was reported by Van-Thuoc et al.[29] In this study we isolated a *B. aryabhattai* strain from soil and its molecular characterization was done. We also investigated the polyhydroxybutyrate biosynthetic property of the strain, variations in polymer accumulation in response to change in carbon source, time course analysis of polymer accumulation and its polymer characteristics.

Materials and methods

Sampling, isolation and maintenance of bacterial strains

Soil samples were collected from paddy fields, forests, riverbeds, sewerage system and estuaries of Kerala, India. Bacterial strains were isolated on nutrient agar medium (5 g of peptone, 5 g of sodium chloride, 1.5 g of beef extract, 1.5 g of yeast extract and 15 g of agar per liter at pH 7.4) (Himedia Laboratories, Mumbai, India) from serially diluted samples and incubated overnight. Since these are environmental bacterial isolates, incubation in initial experiments were done at room temperature (30 °C). The colonies were streaked several times for making them as pure cultures. The isolates were labeled as PHB series (PHB1-62) and were maintained on nutrient agar slants and stored at 4 °C.[30,31]

Preparation of seed inoculum

One loop full of the culture from slant was inoculated in 5 mL of sterile nutrient broth (5 g of peptone, 5 g of sodium chloride, 1.5 g of beef extract and 1.5 g of yeast extract per liter at pH 7.4) (Himedia Laboratories, Mumbai, India). After incubation for 24 h at room temperature, 1% (v/v) of culture having 10^8 cells/mL was aseptically transferred into 50 mL sterile nutrient broth and incubated for 18 h at room temperature. From this, inoculum was added at 1% level in all the polymer quantification experiments.

Screening of isolates for PHA production

Bulk screening of isolates was done by colony staining on half strength nutrient agar (2.5 g of peptone, 2.5 g of sodium chloride, 0.75 g of beef extract, 0.75 g of yeast extract and 15 g agar per liter at pH 7.4) supplemented with 20 g/L glucose, after 48 h of incubation at room temperature.[32] *E. coli* colony was used as negative control. The bacterial colonies on Petri plates were flooded with Sudan Black B solution (0.05% in ethanol) and kept undisturbed for 30 min. The excess stain was washed out by sterile saline and the dark blue colored colonies were identified as PHA positive.

Staining for PHA accumulation and microscopy

48 h old bacterial cells grown in basal medium (1.5 g of peptone, 1.5 g of yeast extract, 1 g of Na_2HPO_4 and 0.2 g of $MgSO_4{\cdot}7H_2O$ per liter, pH 7.2) supplemented with 20 g/L glucose at room temperature were taken for staining and microscopic analysis. Sudan Black B stained smear was observed under 100× oil immersion objective lens of light microscope Nikon YS100 (Nikon Corporation, Tokyo, Japan).[33] Nile Red stained cell suspension was taken on a glass slide and was covered by a coverslip.[34–36] The cells were imaged on a Nikon A1R-Si laser scanning confocal spectral microscope with 50× magnification (Nikon Corporation, Tokyo, Japan) excited at 561 nm.

Scanning Electron Microscopy (SEM) analysis was performed according to Soo-Hwan et al. with some modifications.[37] Polymer accumulated bacterial cells were harvested, washed in phosphate buffered saline (PBS) and fixed overnight in 3% gluteraldehyde solution. The fixed cells were again washed in PBS to remove excess gluteraldehyde and successively dehydrated in 30%, 50%, 70%, 80% and 100% ethanol. 5 μL of this cell suspension was sputter coated with gold and analyzed in a Scanning Electron Microscope JEOL Model JSM – 6390LV (JEOL USA, Inc., MA, USA).

Evaluation of bacterial strains for PHA production

From the isolates, six cultures producing considerable amount of PHA granules were inoculated in 1000 mL basal medium with 20 g/L of glucose and incubated at room temperature for 48 h at agitation rate of 150 rpm. The experiment was done in triplicate and the cells harvested were washed with sterile normal saline. The biomass obtained was lyophilized and the cell dry mass (CDM) was calculated. Polymer was extracted from the lyophilized cells, weighed and estimated the yield in percentage (w/w).[34,38]

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