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Short Communication

Microbial production of poly-β-hydroxybutyrate by marine microbes isolated from various marine environments

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ABSTRACT

Considering the industrial interest of Poly-β-hydroxybutyrate (PHB), bacteria isolated from the various marine arenas were screened for their ability to accumulate PHB and were compared with *Wausteria eutropha* (MTCC-1285). Among the 42 isolates, four strains showed the accumulation of PHB. The maximum PHB producer *Vibrio* sp. (MK4) was further studied in detail. To increase the productivity, steps were taken to evaluate the effect of carbon sources, nitrogen sources, pH and sodium chloride concentration on PHB productivity by MK4. The optimized conditions were further used for the batch fermentation over a period of 72 h. Significantly higher maximum biomass of 9.1 g/L with a PHB content of 4.223 g/L was obtained in a laboratory-scale bioreactor at 64 h, thus giving a productivity of 0.065 g/L/h. The extracted polymer was compared with the authentic PHB and was confirmed to be PHB using FTIR analysis and ^{1}H NMR analysis. Thus, the study highlights the potential of the use of *Vibrio* sp (MK4) in the commercial production of PHB.

1. Introduction

Increasing awareness of environmental pollution has generated a resurgence of interest in biological methods for the production of biodegradable polymers (Patnaik, 2006). Poly-β-hydroxybutyrate (PHB) is the simplest and most commonly known polyhydroxyalkanoates (PHAs) and can be used as a substitute for the synthetic polymer. Both PHAs and synthetic plastics are thermoplastics, moldable and can be tailor-made for numerous applications (Azehar et al., 2003; Fusun and Zeynep, 2000). The quality that sets PHAs apart from conventional plastics is their complete biodegradability in the environment both aerobically and anaerobically (Hankermeyer and Jieerde, 1998; Sudesh et al., 2000).Until now, there are only few reports on marine PHAs-producing microorganisms (Chien et al., 2007; Mavinkurve and Rawte, 2002; Weiner, 1997).

In this study, we isolated and identified several strains of PHB-accumulating bacteria from various marine arenas in South India. All the potential PHB-producing bacterial strains were characterized by microbial characterization studies and 16S rRNA sequencing analysis. In addition, comparison of PHB production by the selected marine strains was done in nitrogen limited minimal synthetic broth medium (NLMSB). The major problem associated with the industrial PHB production is its cultural optimization studies. Hence, different carbon sources, nitrogen sources, pH and salt concentration were tested to analyze the effect on PHB production by the higher yielding PHB producer. Thereafter, the same strain was further used for scale up in fermentation studies. The PHB inclusion in the marine microbe was identified to be PHB from FTIR analysis and ^{1}H NMR analysis by comparing with the authentic PHB.

2. Methods

2.1. Collection of samples from marine arena

Samples were collected from different marine regions of south India such as soil samples of Mandapam (MM) at 9°17′0″N, 79°7′0″E, soil samples of Katch island (MK), marine mangrove sediments of Portonovo region near Chidambaram (MP) in the east–west direction where the tides meet, back water samples of Cochin region (MC) at 76.25°E and 10.02°N, and sea water samples from Rameshwaram region (MR) at 79.29°E and 9.15°N.

2.2. Isolation and cultivation of marine bacteria

Primary isolation of marine bacteria was done by serial dilution technique using Zobell marine agar medium obtained from Himedia, India. The pH of the medium was about 7.6 and the media were sterilized before use. The plates were maintained in an aerobic condition at 30 °C for 48 h.

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2.3. Pure culture

The bacterial strain used in this study was *Wausteria eutropha* (MTCC 1285) and was obtained from the IMTECH, Chandigarh, India. It was maintained on nutrient agar slants at 4 °C and was subcultured every 15 days to maintain its viability.

2.4. Isolation, identification and cultivation of PHB-producing microorganisms

Screening for the marine bacterial PHB producers was done in nitrogen limited minimal synthetic broth medium (NLMSB) (Arun et al., 2006). The culture flasks were incubated at 30 °C for 64 h. The PHB producing capabilities of the organisms were confirmed by Sudan Black staining method (Kitamura and Doi, 1994).

Pure isolates containing lipophilic inclusions were identified based on their microscopic, morphological and biochemical characters (Holt et al., 1994) and by sequencing partial sequences of their 16S rRNA. The isolation of DNA was done according to Janardhanan and Vincent (2007). The partial 16S rRNA was amplified according to Rochelle et al. (1995). Partial DNA obtained from PCR was sent to Bioserve, India, for sequencing service. The sequences of the partial 16S rRNA were compared with the 16S rRNA sequence available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) by using its World Wide Web site (http://www.ncbi.nlm.nih.gov), and the BLAST (basic local alignment search tool) algorithm.

2.5. Production of PHB in shake flask cultures

The PHB production by the selected marine isolates and that by *W. eutropha* were compared by growing them in NLMSB. The media were inoculated and incubated at 30 °C for 64 h in a shaker.

2.6. Extraction and estimation of PHB

PHB produced was extracted as described by the method of Ramsay et al. (1994). The amount of PHB in the extracted sample was determined spectrophotometrically at 235 nm (Lee and Choi, 1999) using HITACHI 2000 UV Spectrophotometer, Japan.

2.7. Optimization studies

Marine microorganism with higher PHB production were further used for the optimization and fermentation studies. The optimization studies were carried out by shake flask culture method. The media used for these experiments were same as described earlier. But, for comparison, different carbon sources (arabinose, glucose, glycerol, lactose, lactic acid, mannitol, sodium acetate, starch and sucrose at a level of 20 g/L), nitrogen sources (ammonium chloride, ammonium sulphate, glycine, potassium nitrate, protease peptone and urea at a level of 20 g/L), pH (2, 3, 4, 5, 6, 7, 8 and 9) and salt concentrations (5%, 10%, 15%, 20%, 25% and 30%) were taken. Inoculum was prepared in NLMSB and after 24 h of incubation, 0.5 ml of the culture was added to 50 ml of the production medium. The production medium in each erlenmeyer flasks consisted of NLMSB and different carbon sources, nitrogen sources, pH levels and salt concentrations. The flasks were incubated at 30 °C. PHB production was estimated in the culture broth after 64 h as described earlier.

2.8. Fermentation studies

Marine microorganism with higher PHB production were further used for the fermentative production of PHB in 3 L fermentor and the above-mentioned optimized conditions were used for media formulation.

Batch fermentation was carried out in a 3 L fermentor (Lark-Hygene plus, India) with a working volume of 2 L, and the duration of each run was 72 h. The culture conditions were same as reported by Patwardhan and Srivastava (2008). Samples were taken at every 8 h interval and were used for analytical methods.

2.8.1. Analytical methods

Cell growth was monitored by measuring the absorbance of the culture broth at 600 nm on a HITACHI 2000 UV Spectrophotometer. The cell pellet obtained after centrifugation was dried at 90 °C till a constant weight was obtained. The supernatant that was obtained by centrifugation of the culture broth at 10,000 rpm for 10 min at 4 °C was used for residual substrate analysis. Residual glucose was determined by dinitrosalicyclic acid (DNS) method (Palanivelu, 2001). The content of residual ammonia–nitrogen were done according to Atlas et al. (1998). Extraction and estimation of PHB was determined as described earlier. The concentration of residual biomass was calculated as a difference between the total biomass and PHB concentration (Shahhosseini, 2004). The overall PHB productivity at the end of the fermentation was calculated as described by Khanna and Srivastava (2006).

The pure form of PHB was collected (Ramsay et al., 1994), and was then compared qualitatively with the PHB extracted from *W. eutropha* and PHB obtained from Sigma.

2.9. Qualitative analysis of PHB

The qualitative analysis of PHB was done for the selected marine bacterial PHB producer and *W. eutropha.* The samples extracted in the pure form were qualitatively analyzed for PHB using Infra Red analysis (Silverstein et al., 1991) and ^{1}H NMR analysis (Chakrabarti et al., 2007). The spectrum obtained was compared with that of the commercially available PHB (Sigma).

3. Results and discussion

3.1. Strains isolation and identification

Forty-two phenotypically different colonies were selected from Zobell marine agar plates. Of these, four isolates showing positive results for the presence of lipophilic inclusions were chosen for further characterization and studies.

Based on the macroscopic, microscopic and biochemical characters, the two isolates were identified as *Vibrio* sp., (we designated them as strain MK3 and MK4, respectively). The sequences of the partial 16S rRNA of the other two bacteria (481 bp and 461 bp) were compared against those available in the public databases. They were closely related to those of *Bacillus cereus* (100% homology) and *Bacillus mycoides* (100% homology). The bacteria were thus identified as *B. cereus* and *B. mycoides* (we designated them as MC1 and MR7, respectively).These nucleotide sequences (MC1 and MR7) have been deposited in the GenBank database under accession number EU735070 and EU735071, respectively.

3.2. PHB production in shake flask cultures

In shake flask cultures, the PHB production by the selected strains, such as MC1, MK3, MK4 and MR7, was tested and compared with that by *W. eutropha*. The PHB production in NLMSB by MC1, MK3, MK4, MR7, and *W. eutropha* was 3.791, 3.923, 4.078, 2.839, and 4.724 g/L, respectively. *W. eutropha* and MK4 produced a relatively higher amount of PHB.