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Production and characterization of poly(3-hydroxy butyrate-co-3 hydroxyvalerate) (PHBV) by a novel halotolerant mangrove isolate

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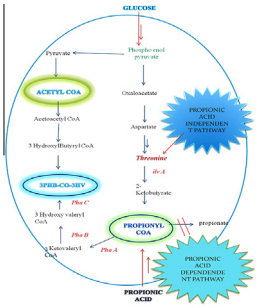
HIGHLIGHTS

- A mangrove isolate produces polyhydroxy butyrate-co-valerate, P(3HB-co-3HV).
- The *Bacillus* sp., is capable of utilizing varying carbon sources for polymer production.
- Culture has the ability to produce P(3HB-co-3HV) by propionate dependent and independent routes.
- The PHBV had a high 3-hydroxyvalerate fraction of 48 mol%.
- The culture can utilize the acid pretreated liquor of lignocellulosic biomass for PHBV production.

GRAPHICAL ABSTRACT

Metabolic pathways involved in the synthesis of P(3HB-co-3HV) copolymer.



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ABSTRACT

A halophilic mangrove isolate identified by 16S rRNA sequence as a *Bacillus* spp. was found to be capable of using a broad range of carbon sources including monosaccharides (glucose and fructose), disaccharides (sucrose), pentoses (xylose and arabinose), various organic acids (acetic acid, propionic acid and octanoic acid) and even the acid pre-treated liquor (APL) of sugarcane trash, a lignocellulosic biomass, for growth and the production of polyhydroxyalkanoates (PHAs) such as poly(3-hydroxybutyrate, P3HB), poly(3-hydroxybutyrate-co-3-hydroxyvalerate, PHBV), and 4-hydroxyhexanoate, 4HHX). The study describes the innate ability of a wild-type culture for PHBV production by both propionate dependent and propionate independent pathways. The biopolymer was extracted and characterized physico-chemically. The PHBV yield from glucose was estimated to be 73% of biomass weight with a high 3-hydroxyvalerate fraction of 48 mol%. Thereafter, spherical homogenous PHBV nanoparticles of ~164 nm size were prepared for future applications.

1. Introduction

Polyhydroxyalkanoates (PHAs) are intracellular carbon and energy reserve materials that are accumulated by a variety of microorganisms under physiological stress (Choi and Lee, 1999). The polymer is primarily a product of carbon assimilation (from glucose or other C5 or C6 carbon sources) and is employed by microorganisms as a form of energy storage molecule in conditions such as limited availability of phosphorus or nitrogen. Polymers of polyhydroxyalkanoates have the useful properties of biodegradability, thermo plasticity, and elasticity (Moorkoth and Nampoothiri, 2014). Poly(3-hydroxybutyrate) [P(3HB)], the best characterized PHA, (Peña et al., 2014) has been found to be accumulated by several microorganisms such as *Bacillus megaterium*,

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Ralstonia eutropha, *Alcaligenes latus*, *Azotobacter vinelandii*, and *methylotrophs* (Mokhtari et al., 2009). However, it is a highly crystalline and brittle homopolymer, which restricts its use to a limited range of applications (Choi and Lee, 1999). Poly(3hydroxybutyrate-co-3hydroxyvalerate) [P(3HB-co-3HV)] was found to be very useful for biomedical applications like nanoparticle-based drug delivery and tissue engineering as they are more flexible and stronger. The production of P(3HB-co-3HV) has been found to be much costlier than traditional oil-derived plastics, so this has hindered its widespread use.

The formation of P(3HB-co-3HV), the copolymer of poly(3-hydroxybutyrate) can be achieved by the microbial fermentation using the co-substrates such as propionic acid, valeric acid etc. (Masood et al., 2011). Most bacteria require the addition of propionate in the media to produce P(3HB-co-3HV). Earlier, researchers found that P(3HB-co-3HV) can be produced from propionate-independent substrates as well by a mutant *Alcaligenes eutrophus* (Steinbuchel and Pieper, 1992) and recombinant *Escherichia coli*. (Slater et al., 1992). To our information, only methyl malonyl CoA pathway has been revealed to provide the majority of the propionyl CoA, the hydroxyl valerate content in this bacteria is regulated by the addition of propionic acid or valeric acid (propionic acid-dependent pathway) and by either acetic acid, cyanocobalamin or threonine (propionic acid-independent pathway) for production of P(3HB-co-3HV).

P(3HB-co-3HV) is produced on a relatively large scale by fed-batch cultures of Gram-negative bacteria like *Cupriavidus necator*, *Pseudomonas oleovorans* and recombinant *E. coli* from glucose and propionic acid. However, the presence of a Pyrogenic outer lipopolysaccharide (LPS) in Gram-negative bacteria induces a strong immunogenic reaction and is, therefore, undesirable for the bio-medical application of the PHAs (Chen and Wu, 2005). Due to the absence of LPS in Gram-positive bacteria, the genera of *Corynebacterium*, *Nocardia*, *Bacillus* and *Rhodococcus* were later widely used to synthesize the commercially important copolymer P(3HB-co-3HV), naturally from simple carbon sources such as glucose (Alvarez et al., 2000; Lee et al., 1999). The *Bacillus* spp. is among the very few wild-type bacteria that synthesize and accumulate different chain length polyhydroxyalkanoates and hence is found to be an ideal host for synthesizing polyhydroxyalkanoate.

PHA biosynthesis by the genera *Bacillus* has unique elements that need further investigation. A very versatile *Bacillus* spp. capable of utilizing a wide spectrum carbon sources for polymer production was reported in this study. It also demonstrate the potential of this culture for the production of copolymer poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by propionic acid-independent pathway from totally unrelated carbon sources.

2. Methods

2.1. Isolation, identification and maintenance of the culture

Soil samples from a mangrove area located in Kannur district of North Kerala, India were collected and subjected to serial dilution and the pure isolates were screened for P(3HB) production by Nile blue staining (Ostle and Holt, 1982). The cultures were routinely propagated in Luria-Bertani medium or nutrient agar medium. One of the P(3HB) producing strains, MG12 appeared as a *Bacillus* culture on the basis of morphological characteristics according to "Bergey's Manual of Determinative Bacteriology". For molecular confirmation, genomic DNA was isolated using a genomic DNA purification kit (Thermo Scientific, USA). A set of universal primers [8F(5′AGAGTTTGATCCTGGCTCAG3′); AFs1492R (5′TACGGTTACCTT GTTACGACTT 3′] was used to amplify the 16S rRNA sequences of the isolated bacterial strain. The homology search for the resulting nucleotide sequence of the PCR product was performed using the BLAST tool of the National Center for Biotechnology Information's (NCBI) website to identify the strain at the molecular level (http://www.ncbi.nml.nih.gov).

2.2. Phylogenetic tree analysis of the Isolate

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.31510592 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

2.3. Biopolymer production (P(3HB) and P(3HB-co-3HV))

For biopolymer production, modified M9 medium (Chen et al., 2011) with the composition (g/L): 17.1 $Na_2HPO_4{\cdot}12H_2O$, 3 KH_2PO_4, 1 NH_4Cl, 2 yeast extract, 0.5 NaCl and 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$, was selected. Unless otherwise mentioned, 2% glucose was added as the carbon source. Other hexose and pentose sugars were also checked as carbon source as indicated in Table 2. Fermentation was carried out in 250-mL Erlenmeyer flasks with a working volume of 100 mL and it was inoculated with 18 h old 2% v/v inoculums and incubated with shaking of 200 rpm at 30 °C for desired interval of time. For P(3HB-co-3HV) production, 10 mM of propionic acid was added to the medium when the culture reached an OD of 0.8 at 600 nm and the pH was adjusted to 6.2. Cell growth was monitored by spectrophotometry (UV-1800 Shimadzu, Japan). After 48 h, the cells were harvested by centrifugation and the cell pellet was resuspended in 5 ml of sterile 0.85% (w/v) NaCl.

In order to check the P(3HB-co-3HV) synthesis in the bacteria by propionic independent pathway, the medium was supplemented with either acetic acid or threonine or both in combination with vitamin B12 (cyanocobalamin). For that, 10 mM acetic acid, 1 mM cyanocobalamin and 4 mM threonine were used and were added after 18 h of growth. Fermentation was carried as per the above mentioned conditions.

2.4. PHBV analysis

2.4.1. Polymer analysis

Cell growth was monitored by measuring the absorbance at 600 nm with (UV-1800 Shimadzu, Japan). The cell concentration, defined as the dry weight of cells per liter of culture broth, was determined by weighing lyophilized dry cells. PHA concentrations were determined by HPLC using crotonic acid as the internal standard. The HPLC conditions were oven temperature at 50 °C using 0.01 N H_2SO_4 as the mobile phase at flow rate 0.6 mL/min. Extraction of the polymer was made by solvents, by this method we could obtain above 98% purity in the polymer. PHA containing cell pellet was digested with 1 mL of concentrated sulphuric acid. The PHA content was defined as the percent ratio of PHA concentration to cell mass. The concentration of propionic acid in the culture medium was measured by high-performance liquid chromatography by using a fast column for Rezex (phenomenex)-ROA-organic acid column (300 × 4.6 mm), milli-Q water was used as the mobile phase.

2.4.2. Gas chromatography (GC)

For the identification of the PHA, a slight modification of the gas chromatographic method of (Huijberts et al., 1994), was employed.