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Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Halogeometricum borinquense* strain E3

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ABSTRACT

Polyhydroxyalkanoates (PHA's) can be a key solution for pollution problems caused by plastics derived from petrochemical sources. Extremely halophilic archaeon *Halogeometricum borinquense* strain E3 showed maximum PHA accumulation of 73.51% ±1.7 of cell dry weight (CDW) with 2% glucose. The crotonic acid assay, XRD, FT-IR and ^{1}H NMR analysis revealed that the polymer was a co-polymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)] comprising of 21.47% HV units. This is the first report on P(HB-*co*-HV) production by an extremely halophilic archaeon *Hgm. borinquense* strain E3.

1. Introduction

High usage of conventional plastics viz. polyethylene, polypropylene and poly(vinyl alcohol), derived from non-renewable petro-chemical resources have various environmental problems [1]. The need of sustainable plastics, obtained from renewable resources like starch, protein and cellulose is the need of the hour [2]. Plastics made from these materials are either used directly or in combination with conventional plastics, still resulting in pollution because of their partial degradation. Therefore, there is a continuous search for biodegradable plastics, which can efficiently replace conventional plastics. Presently, polyhydroxyalkanoates (PHAs) are the only 100% biodegradable microbial plastics [3], accumulated intracellularly by numerous microorganisms [3]. In spite of its huge potential, PHAs are more expensive than synthetic plastics due to the high production cost [4].

PHAs are accumulated in response to stress by many genera of bacteria such as the Gram-positive genera *Bacillus*, *Streptomyces*, etc. and Gram negative genera *Cupriavidus*, *Halomonas*, *Pseudomonas*, etc. and a few members of archaea viz. halophilic archaea [5]. Gram-negative bacteria have limitations due to the co-extraction of lipopolysaccharide (LPS) endotoxin along with the PHA polymer. Such a polymer is highly unsuitable for biomedical applications as LPS can elicit strong inflammatory responses in individuals. In spite of the existing methods on the removal of LPS, the treatment process itself changes the polymer properties resulting in an overall increase in the production cost. Lack of LPS in Gram-positive bacteria gives them an advantage over their Gram-negative counterparts however the relative production of PHAs is lesser [6]. These issues make haloarchaea interesting candidates to look for biodegradable polymers.

Archaea are a huge untapped resource of potential industrially important metabolic products with interesting environmental applications. Among the members of archaea, halophilic archaea have gained much attention due to easier handling and culturing techniques. Halophilic archaea are commonly inhabit hypersaline environments like salt lakes and solar salterns. Employing haloarchaea for PHA production have added advantages, as they are grown in hyperosmotic environments, intracellular accumulated PHA can be easily harvested from the cells by suspending them into low osmalarity solutions including water [7]. Use of haloarchaeal strains requiring 20–25% (w/v) salt for its growth can cut down media sterilization costs as well [8,9].

As of November 2014, the family *Halobacteriaceae* is reported to have 49 genera and 182 species [10–12], among which only few genera are reported to accumulate PHA. The haloarchaeon, *Haloferax mediterranei* is the most widely studied representative of

Abbreviations: Hfx., *Haloferax*; Har., *Haloarcula*; Hgm., *Halogeometricum*; PHA, polyhydroxyalkanoates; PHB, polyhydroxybutyrate; NaClO, sodium hypochlorite; CDW, cell dry weight; P(HB-*co*-HV), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate); HB, hydroxybutyrate; HV, hydroxyvalerate.

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the family *Halobacteriaceae*, and is reported to synthesize PHBV naturally, unlike most of the bacteria which require expensive/cellular toxic precursors such as propionic/valeric acid as media supplements for PHBV synthesis [13,14]. Very little information is available on the other haloarchaeal members as PHA accumulators and the kind of PHA synthesized.

In this study, the ability of extremely halophilic archaeon *Halogeometricum borinquense* strain E3 to synthesis PHA was investigated. To the best of our knowledge, this is the first report on genus *Halogeometricum* as an accumulator of copolymer [P(HB-co-HV)].

2. Materials and methods

2.1. Extremely halophilic archaea and media used

The extremely halophilic archaeon, strain E3 was isolated from solar salterns of Marakkanam in Tamil Nadu, India and maintained on extremely halophilic medium (EHM) ingredients (gL^{-1}) NaCl 250; $MgSO_4{\cdot}7H_2O$ 20.0; KCl 2.0; $CaCl_2{\cdot}2H_2O$ 0.36; $NaHCO_3$ 0.06; NaBr 0.23; Peptone 5.0; yeast extract 10.0; $FeCl_3{\cdot}6H_2O$ 0.005 [15]. Four PHA accumulating strains, namely *Halococcus* strain BK6, *Haloferax* strain BBK2, *Haloarcula* strain BS2, and *Halogeometricum* strain TN9 obtained from our previous study [16] were also used in this study and maintained on NaCl tryptone yeast extract (NTYE) and/or NaCl trisodium citrate (NT). The production medium was NaCl synthetic medium (NSM) ingredients (gL^{-1}) NaCl 200; $MgCl_2{\cdot}6H_2O$ 13.0; KCl 4.0; $CaCl_2{\cdot}2H_2O$ 1.0; $NaHCO_3$ 0.2; NH_4Cl 2.0; KH_2PO4 0.5; $FeCl_3{\cdot}6H_2O$ 0.005; yeast extract 1.0. Glucose was added separately (20% w/v stock) after sterilization to the medium to a final concentration of 2% and referred to as NGSM. The pH of the medium was adjusted to 7.0–7.2 with 1 N NaOH.

2.2. Screening of haloarchaeal strains for PHA accumulation and quantification

The strain E3 was screened for PHA accumulation, on NGSM agar plates with 2% (w/v) glucose as substrate as described by Salgaonkar et al., 2013a [16]. Briefly, the NGSM plates were prepared by adding 2% agar-agar (w/v) to the medium followed by autoclaving and while still molten 50 μl of Nile Red stain was added such that the final concentration is 0.5 μg ml^{-1} medium [stock of 0.001% (w/v) Nile Red in DMSO]. The haloarchaeon strain E3 was streaked on the NGSM agar plates and incubated at 37 °C for 6–7 days. After every 2 days interval, the plates were exposed to Ultra-Violet (UV) light on a Gel documentation system (BIO-RAD Laboratories CA, USA) [16]. Sudan black B and Nile Red stains were used for staining of cells for lipid and PHA granules, respectively [16–18]. The cells were observed under oil immersion lens (100×) of phase contrast microscope (Olympus BX41, Tokyo, Japan) and fluorescence microscope using propidium iodide (PI) filter (Nikon Eclipse TS100, Tokyo, Japan).

2.3. Characterization of the promising haloarchaeal strain E3

2.3.1. Morphological and biochemical characterization

Characterization of the haloarchaeal strain E3 was done in accordance with the proposed minimal standards for family *Halobacteriaceae* [19,20]. The colony characteristic were determined on EHM agar medium. Cell morphology was determined by Gram staining and scanning electron microscopy (SEM) (JEOL-5800 LV SEM, Japan) as described by Salgaonkar et al., 2012 and Mani et al., 2012 [21,22]. The biochemical tests like production of acid from various carbohydrates, screening for various hydrolytic enzymes and other tests were carried using Norberg and Hofstein media as the basal medium for growth [19,23].

2.3.2. Molecular charcaterization

Genomic DNA of the strain E3 was extracted according to the modified protocol described by Salgaonkar et al. (2013a) [16]. The 16S rRNA gene fragment of the extracted genomic DNA was amplified using polymerase chain reaction (PCR) with universal archaeal primers A109 (F) AC(G/T)GCTCAGTAACACGT and 1510(R) GGTTACCTTGTTACGACTT [22]. An automated DNA sequencer (Applied Biosystems, USA) was used to sequence the purified, amplified PCR products and the results obtained were subjected to similarity search using BLAST tool. Multiple sequence alignment was performed with MUSCLE and the phylogenetic tree was constructed by the neighbor-joining method of MEGA 5.0 with bootstrapping values calculated for 1000 and displayed for 100.

2.4. The growth kinetics and polymer quantification for Hgm. borinquense strain E3

Growth rate and intracellular PHA content was determined as follows. Strain E3 grown in EHM medium for 2–3 days (mid-log phase) was used as starter culture. Two percent inoculum of strain E3 was used to inoculate the production medium [NGSM supplemented with 2% (w/v) glucose]. The flasks were incubated at 37 °C on a rotary shaker at 110 rpm. After regular intervals of 24 h the following parameters were analyzed, (i) absorbance at 600 nm, (ii) cell dry weight (CDW), (iii) pH of media, (iv) reducing sugar in media and (v) PHA content in the cells. For determining the CDW, pellet obtained from 2 ml of the culture broth was washed twice with distilled water, centrifuged at high speed (10,000 rpm for 15 min) and dried at 70 °C until constant weight. Reducing sugar was estimated by DNSA method [24]. The polymer content in the cells was determined by acid hydrolysis method in which the cell pellet was hydrolyzed in conc. sulphuric acid to convert the intracellular polymer to crotonic acid and the absorbance was recorded at 235 nm using a UV–visible spectrophotometer (UV-2450, Shimadzu, Japan) [25].

2.5. Extraction of PHA

Cells of *Hgm. borinquense* strain E3 were harvested at stationary phase, by centrifuging the culture broth at 10,000 rpm for 10 min. The cell pellet obtained was dried at 60 °C after briefly rinsing with distilled water. The dried cells were ground using a motor and pestle and the polymer was recovered by extracting with chloroform using soxhlet extractor, for 12–20 h at 60–65 °C. Up to 95% of the chloroform was collected by distillation on a rotary evaporator under vacuum at 60 °C and the remaining 5% of the chloroform containing polymer was poured in a clean glass petri dish and kept undisturbed for total evaporation to obtain a polymer film.

2.6. Polymer characterization

The polymer was characterized using crotonic acid assay, X-ray diffraction (XRD), thermal analysis, Fourier transform infra red (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy, respectively.

2.6.1. Crotonic acid assay

The polymer (approx. 100 μg) was acid hydrolyzed with conc. H_2SO_4 at 100 °C for 10 min in a boiling water bath. The hydrolyzed solution was screened from 190–600 nm using a UV–visible spectrophotometer (UV-2450, Shimadzu, Japan) against sulphuric acid as blank. Commercial poly[(R)-3-hydroxybutyric acid)] natural origin (Sigma–Aldrich) was treated in similar way for comparison.

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