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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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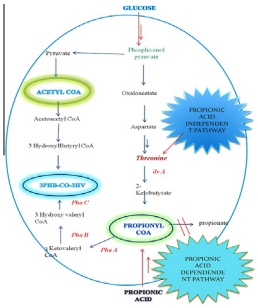
HIGHLIGHTS

- 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.

1 mg mL^{-1} of PHBV standard sample and 1 mg mL^{-1} methyl benzoate in chloroform was added to a mixture of 2 mL 15% sulphuric acid in methanol (ratio 1:1) at 100 °C for 6 h in a reflux. After the reaction, the tubes were cooled on ice for 5 min, 1.0 mL distilled water was added and the tubes were vortexed for 1 min, and centrifuged the samples at 10,000 rpm for 15 min for the phase separation. The organic phase was collected and dried over anhydrous sodium Sulphate. The analysis was performed using a Shimadzu GC-2014 gas chromatograph, using a 0.32 mm inner diameter stabil wax Restek column. Injection volumes was 1 µl, and was done in the split injection ratio: 1:100. The injector temperature was 240 °C and the carrier gas was nitrogen at an approximate velocity of 5 ml/min. The analysis started at 40 °C for 1 min, whereupon it was increased to 240 °C at the rate of 4 °C/min. After reaching 240 °C, the temperature was held stable for 5 min before the analysis was terminated. Column temperature was 140 °C. FID detector was used.

2.5. Extraction of P(3HB) and P(3HB-co-3HV) from fermented broth

Lyophilised cell pellets obtained from individual flasks were treated with 50 ml chloroform and 50 ml 30% sodium hypochlorite at 30 °C for 1 h at 250 rpm and mixture was centrifuged at 5000*g* for 15 min. Lower chloroform containing phase was concentrated, and nonchloroform phase was discarded. Both P(3HB-co-3HV) and P(3HB) was recovered by non-solvent precipitation with cold methanol. Methanol was evaporated, and the polymer was obtained. Precipitation is repeated 2–3 times to get a pure polymer (Althuri et al., 2013).

2.6. Characterization of extracted polymer

Characterization of polymer was done by GPC, HNMR, FTIR, DSC.

2.7. ^1H NMR

The P(3HB) and P(3HB-co-3HV) samples were characterized by proton nuclear magnetic resonance spectroscopy (^1H NMR) (Bruker Avance III 500 MHz). About 10 mg of the polymer was dissolved in chloroform-d (CDCl3) and sonicated for 10 min. (Sonics, Vibra Cell, USA). The extracted poly (3HB-co-3HV) absorbance peaks was compared with 9-mol% P(3HB-co-3HV) obtained from Sigma–Aldrich was used as the standard.

2.7.1. GPC analysis

The number-average molecular weight of the P(3HB-co-3HV) copolymers was determined by gel permeation chromatography (Shimadzu, Japan) equipped with a Phenomenex gel column (300 × 7.8 mm) and a RI detector. P(3HB-co-3HV) copolymer was analyzed by GPC. Polystyrene with low polydispersity was used as standard. The mobile phase was THF (Tetrahydrofuran) at a flow rate of 1 ml/min with an injection volume of 20 µl.

2.7.2. FTIR

The infrared spectra were recorded using a Fourier transform infrared (FTIR) spectrometer (Perkin Elmer, USA). The copolymer P(3HB-co-3HV) (1 mg) was mixed with KBr sample to form KBr plate for the FTIR (Perkin Elmer, USA) analysis. Poly (3HB-co-3HV) was compared with that of pure P(3HB-co-3HV) (9% HV) obtained from Sigma–Aldrich. The structural analysis was performed using an FTIR spectrophotometer.

2.7.3. DSC (differential scanning calorimetry)

The glass transition temperature (Tg), melting temperature (Tm), and crystallization temperature (Tc) was determined via differential scanning calorimetry (DSC200F3, NETZSCH, Germany). The melting point (Tm), the heat of fusion (ΔHm), and the decomposition temperature (Td) were determined from the DSC spectrogram.

2.8. Preparation of P(3HB-co-3HV) nanoparticles by nanoprecipitation

P(3HB-co-3HV) NPs of desired size were prepared as it is reported for P(3HB) by our group (Moorkoth and Nampoothiri, 2014) by nanoprecipitation method. 100 mg of P(3HB-co-3HV) was dissolved in DMSO or chloroform (8 ml) and subjected to ultrasonication for 10 min (50 s ON and 10 s OFF cycle). It was then dropped to a dispersing phase by inserting a syringe needle directly to water (40 ml) which was continuously stirred with a magnetic stirrer. Suspension of nanoparticles was further obtained by centrifugation using 3 kDa Millipore filter for 15 min at 5000*g*.

2.8.1. Dynamic light scattering

NPs size and zeta potential (surface charge) was measured by DLS using a Zeta Nano sizer 90 (Malvern Instruments Zeta sizer 90, UK) as per the manual instructions.

3. Results and discussion

3.1. Identification of the PHA-producing culture

Soil isolates when subjected to Nile Blue A analysis, two of them showed orange, red fluorescence (360 nm) due to the presence of poly(3-hydroxybutyrate) inclusion bodies. From that, the one with more fluorescence was selected for further studies. By morphology and biochemical characterization analysis mentioned in Bergey's manual, it appeared as a *Bacillus* culture and named as *Bacillus* sp. MG12. For species identification, 16S rRNA sequencing (790 bp) was done. A comparative search for the obtained sequence (NCBI Genbank accession No. KP635259) in a BLAST search showed 99% homology to *Bacillus thuringiensis*. When the phylogenetic tree is constructed (Fig. 1), the culture MG12 grouped closely to *B. cereus* group comprising *B. anthracis*, *B. cereus* and *B. thuringiensis*. One clade, formed by *Bacillus anthracis*, *B. cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *B. thuringiensis*, and *Bacillus weihenstephanensis* under current classification standards, should be a single species (within 97% 16S identity), but due to medical reasons, they are considered separate species due to pathogenicity caused by *B. anthracis* and *B. mycoides* (Okstad and Kolsto, 2011). Hence, for further confirmation of *B. thuringiensis* a full-length sequencing, and the presence or absence of parasporal bodies has to be carried out and were in progress.

The biochemical characterization of the culture revealed that it is having many significant features like tolerance to high glucose level (up to 20%), capable of utilizing pentose sugars like xylose and arabinose etc. Being a mangrove isolate, the culture tolerated salinity (NaCl) up to 9% w/v.

3.2. P(3HB-co-3HV) production from different carbon sources

Table 1 summarizes the P(3HB-co-3HV) production by this isolate when wide-ranging carbon sources were used. The strain produced 88.4 wt% P(3HB-co-3HV) at pH 6.2 after 48 h of incubation using xylose (2% w/v) as sole carbon source at 30 °C with the addition of 10 mM of propionic acid during log phase by propionic acid-dependent pathway. However, in this case, the biomass was not very impressive. It means the culture could have been in a kind of catabolic repression due to this pentose uptake and also stress from propionic acid supplementation to induce the P(3HB-co-3HV) production. The growth was maximum with sucrose, but

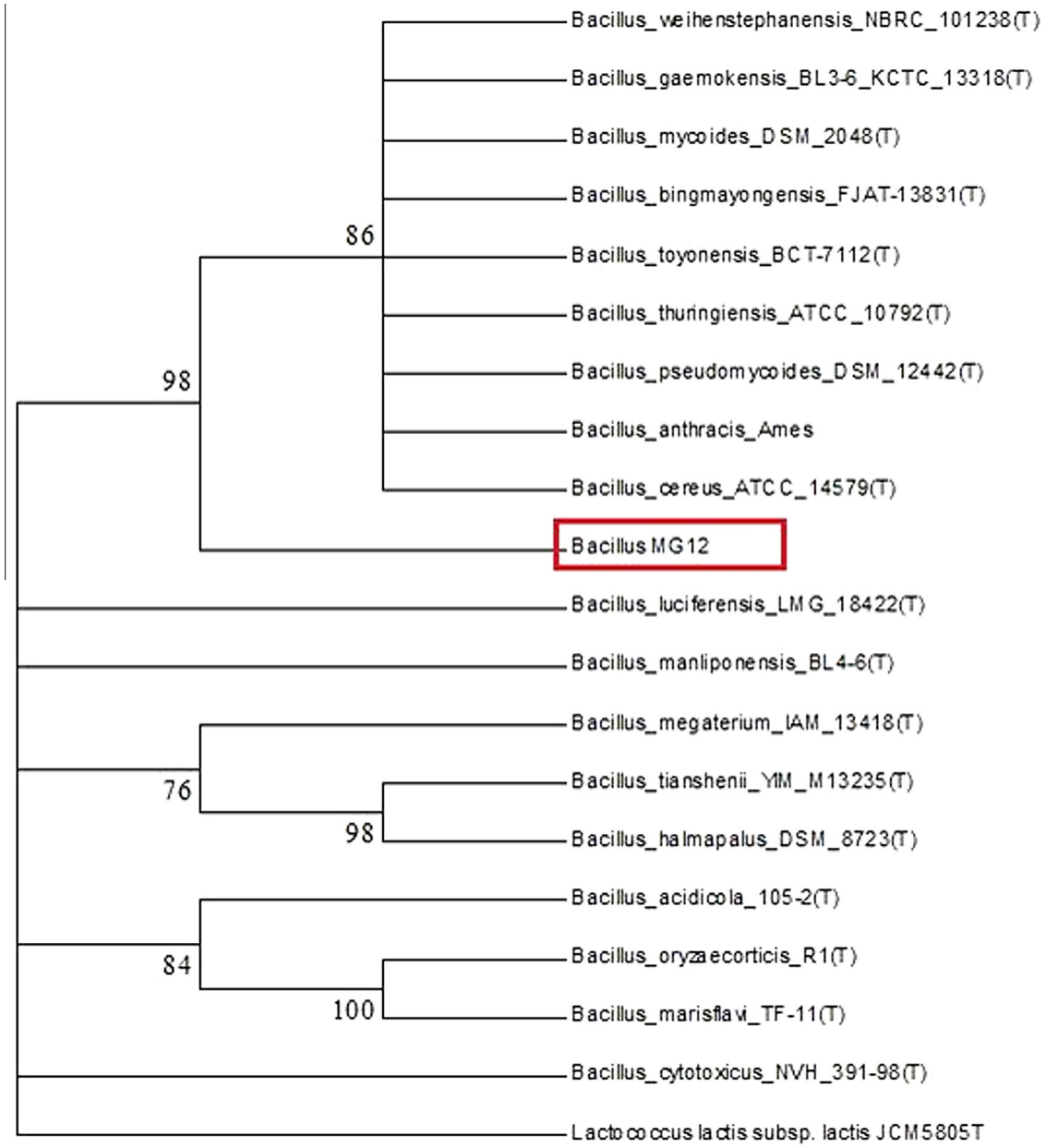

Fig. 1. Phylogenetic tree for the isolate, *Bacillus* MG 12. A phylogenetic tree based on 16S rDNA sequence similarities between the type species of the *Bacillus* and the newly identified strain halophilic strain *Bacillus* MG12 to other members of the Firmicutes phylum (*Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*) and to other *Bacillus* group members like *Bacillus megaterium*. The tree was constructed using multiple sequence alignment and Neighbour-Joining method (Tamura and Nei, 1993; Saitou and Nei, 1987) and rooted with *lactococcus lactis subsp lactis* as an outgroup organism. Bootstrap values are shown at node points. Scale bar represents 20 nucleotide substitutions. The analysis involved 20 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 473 positions in the final dataset.

the polymer production was only 20%. Glucose and glycerol shared reasonably good biomass and P(3HB-co-3HV) productions were 73 and 52 wt%). Further, the experiments were done with 2% glucose, and it has been found that pH, incubation temperature and incubation period influenced the production of P(3HB-co-3HV) (Table 2). A previous report by the strain of *B. thuringiensis* DSM 2046 sp. accumulated 3.6 mol% HV when supplemented with 1.5 g/L of propionic acid (Chen et al., 1991).

3.3. P(3HB-co-3HV) production from unrelated carbon sources

After exploring possible metabolic pathways leading to the formation of propionyl-CoA from propionic acid and glucose in *Bacillus* MG12, we checked the derivation of this biopolymer from an amino acid or vitamin in a propionate independent route. It has been noticed that supplementation of the acetic acid produces polyhydroxy butyrate only (Table 3). When a combination of acetic acid and threonine was added, a maximum production of 34% P (3HB-co-3HV) was obtained and the production was only 25%, when threonine alone was used. Even though, the production was detected it was significantly reduced to 15% when acetic acid and cyanocobalamin combination was attempted.

3.4. P(3HB-co-3HV) production from acid pre-treated liquor (APL) of sugarcane trash

In order to develop a feasible bioprocess based on a biorefinery concept, P(3HB-co-3HV) production was tested in acid pre-treated

Table 1
The effect of different carbon sources on P(3HB-co-3HV) production (g/1L), Biomass yield (g/1L) and on the HV content (mol%) by *Bacillus* sp. MG12. Fermentation for 48 h at 30 °C with an agitation of 200 rpm.

Carbon source (2% w/v)	Dry biomass (g/L)	P(3HB-co-3HV) purified (g/L)	3HV mol%	3HB mol%	% P(3HB-co-3HV)
Glycerol	2.44 ± 0.01	1.27 ± 0.034	12	88	52
Sucrose	3.30 ± 0.05	0.65 ± 0.02	28	72	20
Maltose	2.78 ± 0.05	0.5 ± 0.023	20	80	18
Galactose	1.58 ± 0.067	0.67 ± 0.03	51	49	42
Xylose	1.04 ± 0.27	0.92 ± 0.02	26	74	88.4
Arabinose	1.26 ± 0.02	0.56 ± 0.04	47	53	44
Lactose	0.95 ± 0.03	0.23 ± 0.05	17	83	24.2
Glucose	2.77 ± 0.528	2.02 ± 0.076	48	52	73
Succinic acid	2.63 ± 0.08	1.03 ± 0.034	27	73	39
APL	2.89 ± 0.03	0.236 ± 0.05	42	58	8

APL- acid pre-treated liquor of sugar trash.

Table 2
Parametric screening of P(3HB-co-3HV) production and biomass yield by *Bacillus* sp. MG12. Checked the effect of different pH; incubation temperature and harvest time. In all cases 2% w/v glucose was used as carbon source.

Optimization parameters	Propionic acid conc.	Biomass (g/L)	P(3HB-co-3HV) yield purified (g/L)	% P(3HB-co-3HV)
pH (30 °C and 48 h)				
7	10 mM	3.30 ± 0.6	0.65 ± 0.02	20
8	10 mM	2.78 ± 0.5	0.5 ± 0.03	18
Temperature (°C) pH 6.2 and 48 h				
30	10 mM	2.44 ± 0.34	1.67 ± 0.34	68
37	10 mM	3.32 ± 0.05	0.85 ± 0.04	26
Time (h) (pH 6.2 and 30 °C)				
36 h	10 mM	2.07 ± 0.04	1.108 ± 0.021	54
48 h	10 mM	2.79 ± 0.03	2.02 ± 0.023	73

liquor of sugarcane trash that has been used for lignocellulosic based bio fuel generation. Acid pre-treated liquor containing ~20 mg/ml of total sugars (13 mg/ml xylose, 5 mg/ml glucose and about 2 mg/ml arabinose) was used for this. It has been noticed that the culture was capable of growing on it, even though in a slow growth rate. APL contains many inhibitors such as furfural, acetic acid, formic acid, etc. which may affect the growth. A maximum dry biomass of 2.89 g/1L was achieved in 48 h with P(3HB-co-3HV) production (g/1 L) of 0.236. However, % production of P(3HB-co-3HV) was only 8%. Valerate incorporation was confirmed by ^{1}H NMR.

3.5. Characterization of copolymer

Molecular weight of P(3HB-co-3HV) by Gel permeation chromatography (GPC) was found to be 206.22 kDa. Number average molecular weight (M_N) of polymer was found to be 31.2 kDa (0.031265×10^6) and weight average molecular weight (M_W) of polymer was found to be 51.1 kDa. (0.051197×10^6) polydispersity index (M_W/M_N) was found to be 1.63 for the extracted polymer.

3.6. ^{1}H NMR

The extracted polymer P(3HB-co-3HV) was subjected to ^{1}H NMR, according to the characteristic peaks at 0.87 and 1.24 ppm were assigned to the resonance absorption of methyl (CH3) from the hydroxy valerate unit and methyl (CH3) from the hydroxybutyrate unit respectively (Liu et al., 2010). The peaks at 0.875, 1.244, 1.623, 2.626 and 5.249 ppm were identified as the –CH3 (HV side group), –CH3 (HB side group), –CH2 (HV side group) and –CH2 (for both HV and HB). The characteristic peaks obtained for HV content were 0.9 and 1.25 ppm in the proton NMR spectrum (Supplementary Fig. 1) and that can be used to determine the mole % of HV composition in the poly (3HB-co-3HV) sample by the following equation.

$$\text{HV mol}\% = \frac{\text{Area CH3 (HV)}}{\text{Area CH3 (HV)} + \text{Area CH3 (HB)}} \times 100$$

and it has been found that HV composition is 48%.

Table 3
Propionate independent P(3HB-co-3HV) production (48 h). P(3HB-co-3HV) production induced by the addition of threonine alone, a combination of (10 mM acetic acid + 4 mM threonine) and with (1 mM cyanocobalamin (CNB12) + 4 mM threonine) combination.

Source	Dry biomass (g/L)	PHB (g/L)	P(3HB-co-3HV) g/L	3HB %	3HV %	P(3HB-co-3HV) yield %
10 mM acetic acid	2.06 ± 0.4	0.50	–	100	0	0
10 mM acetic acid + 4 mM threonine	1.87 ± 0.02	–	0.64 ± 0.03	61	39	34
10 mM acetic acid + 1 mM CNB-12 + 4 mM threonine	2.75 ± 0.057	–	0.43 ± 0.02	86	14	15.6
4 mM threonine	3.42 ± 0.06	–	0.86 ± 0.04	72	28	25.1

3.7. FTIR

In the FTIR spectrum of P(3HB-co-3HV) (Supplementary Fig. 2) the strong absorption peak at approximately 1277 cm^{-1} was associated with the saturated ester linkage of C–O groups. The absorption peak at 1376 and 1464 cm^{-1} corresponded to the respective stretching and bending mode of the vibration of methyl (–CH3) group (Pramanik et al., 2014). The strong absorption peaks at 2935, 1736 and 3647 cm^{-1} were the respective characteristic peaks of methine (–CH), –carbonyl (C=O) and hydroxyl (–OH) groups (Bayari and Severcan, 2005).

3.8. Differential scanning calorimetry (DSC)

DSC spectrum analysis provided (data not shown) the thermal characteristics of the P(3HB-co-3HV) produced by *Bacillus* MG12. The melting point (Tm), the heat of fusion (ΔHm), and the decomposition temperature (Td) of the P(3HB-co-3HV) from *Bacillus* MG12 were 162.8 °C, 47.28 J g^{-1}, and 203.42 °C respectively.

Following the characterization of the PHBV polymer produced, further confirmation and quantification was undertaken using GC analysis (Supplementary Fig. 3). Glucose and propionic acid when used as a carbon source (20% w/v) in M9 medium resulted in the production of a copolymer containing 3HB, 3HV monomers, as revealed by GC analysis. It shows the chromatogram for the methanolysis products of the PHA produced when methyl benzoate added as internal standard. Methyl-3-hydroxybutyrate (Rt 2.90) and methyl-3-hydroxyvalerate (Rt 3.40) peaks showed exceptional similarity to the respective mass spectra of standard P(-3HB –co-9 mol% 3HV) obtained from Sigma Aldrich.

3.9. P(3HB-co-3HV) nanoparticle synthesis

P(3HB-co-3HV) nanoparticles were synthesized by nanoprecipitation and were compared with P(3HB) nanoparticles that were synthesized previously (Moorkoth and Nampoothiri, 2014). NPs were characterized by DLS in terms of size, polydispersity index and zeta potential. P(3HB-co-3HV) NPs showed a smaller size and good zeta potential than the P(3HB) one. P(3HB-co-3HV) nanoparticles size was confirmed by DLS (164 nm) and scanning electron microscopy (SEM).

B. megaterium was first discovered to synthesize PHAs and cultivated in a variety of carbon sources. In a recent work (Kang et al., 2011) a method describes for producing a high purity polyhydroxy valerate (PHV), from *Bacillus* using succinate as a carbon source and the recovery of the PHV is ~95%. When P(3HB-co-3HV) was produced from *R. eutropha*, a natural PHA accumulator, glucose and propionate were supplied to a fed-batch fermentation. Adjusting the ratio of these carbon sources in the feed controlled the copolymer composition as well.

Mangrove ecosystems are, in general, rich in organic matter, but deficient in nutrients such as nitrogen and phosphorus. Moreover, the particular mangrove area in the Kerala is in the coastal areas, whereby the niche is constantly in contact with salts coming from the sea. Bacteria that succeeded in such ecosystems should have adapted their metabolisms to variations in salt concentrations, excess in carbon sources, and limited amounts of other essential nutrients such as nitrogen or phosphorous. Halophiles (salt loving) are categorized as slight, moderate, or extreme, by the extent of their halo tolerance. Slight halophiles prefer 0.3–0.8 M (1.8–4.7%), moderate halophiles 0.8–3.4 M (4.7–20%), and extreme halophiles 3.4–5.1 M (20–30%) (Ollivier et al., 1994). The *Bacillus* MG12, the present isolate is found to be a moderate salt tolerator, up to 9%. A similar report on the production of polyesters by halophilic and halotolerant bacterial species isolated from mangrove soil samples in Northern Vietnam (Van-Thuoc et al., 2012). The application of halophiles for PHA production is that they can reduce the cost of fermentation and recovery processes due to the following reasons. First, a high salt concentration prevents contamination by non-halophiles, hence reduces the expense, energy and process complexity for sterilization. Cell lysis is the principal component that increase cost for PHA recovery. But for some halophiles, that can be reduced by using hypo-osmotic shock treatment (Quillaguamán et al., 2010).

Bacilli are mesophiles with temperature optima between 30 and 45 °C and the shift in optimum growth temperature affect the PHA production like the decrease at 37 °C in this study. The *B. megaterium* and *R. eutropha* could not accumulate PHA at temperatures higher than 41 °C (Masood et al., 2012). In the present study, optimum P(3HB-co-3HV) production found in stationary phase, i.e., 48 h when the biomass also reached to the maximum under the given conditions.

An important factor in the prohibitively high price of this biopolymer was that of propionate, which was activated to form the propionyl-CoA precursor of HV, which is expensive to produce industrially and is considerably more costly than glucose. A more economical alternative is to produce propionyl-CoA from an inexpensive, unrelated carbon source (Poirier et al., 1995). Based on analysis of the P(3HB-co-3HV) production pathways, we found it is possible to produce P(3HB-co-3HV) with a high 3HV fraction from unrelated carbon sources through the threonine biosynthetic pathway as it was possible to vary the average HV fraction in the copolymer by adjusting the propionic acid or cyanocobalamin (precursor of coenzyme B12) concentration in the medium. Genetic evidence for the presence of Threonine dehydratase (www.uniprot.org) coded by an *ilvA* gene for threonine biosynthesis was identified in *Bacillus cereus* group is likely to be present in *Bacillus* MG12 since it shows similarity to *B. thuringiensis*. Threonine dehydratase (TD) converts threonine to α-ketobutyrate; Pyruvate dehydrogenase converts α-ketobutyrate to propionyl-CoA. TD is required for HV to get incorporated into PHA (Han et al., 2013) Propionate is taken up from the medium, is ligated to CoA, and is incorporated into P (3HB-co-3HV) via reactions catalyzed by the same enzymes as those responsible for P(3HB) biosynthesis. In the recent study (Yang et al., 2014) by the incorporation of citramalate pathway in *E. coli* led to the biosynthesis of P(3HB-co-3HV) with 1.9 mol% 3HV fraction, which is similar to that obtained in our study. The PHA obtained in this study comprise more than 45% poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV).

Amino acids are attractive as potential precursors for propionyl-CoA because usually they are present in relatively high concentrations in bacteria. Over expression of the *ilvA* gene and rapid conversion of threonine to ketobutyrate in recombinant *E. coli* was reported in a study by Chen et al. (2011) 3 HV (hydroxy valerate) incorporation took place on supplementation of CN-B12 (cyanocobalamin) as well along with the acetic acid. What is important here, is P(3HB-co-3HV) production can be induced in this culture in the absence of propionate. Thus, it has the remarkable property of having propionate dependent and independent pathways for P(3HB-co-3HV) production.

PHAs synthesised by this *Bacillus* belong to the type of scl-PHAs, Which include different compositions of P(3HB), P(3HB-co-4HB), and P(3HB-co-3HV) confirmed by ^{1}H NMR. *A. eutrophus* have produced copolyesters of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) with supplementation of butyric and pentanoic acids. When pentanoic acid was used as the sole carbon source, a copolyester with an unusually high 3HV fraction of 90 mol% was produced (Doi et al., 1988). For P(3HB) synthesis, acetyl-CoA produced from glycolysis of glucose is converted to acetoacetyl-CoA through the catalysis of 3-ketothiolase, followed by reduction through acetoacetyl-CoA reductase to form

(R)-3-hydroxy butyryl-CoA. Finally, short chain length P(3HB) is produced through the polymerization of P(3HB) polymerase. *Bacillus* strain isolated in our laboratory utilize propionate with greater than 65% efficiency, tolerate propionate concentration of up to 20 mM in the growth medium, accumulate polymer up to 40% of the cellular dry weight. It alters the 3-HB-3-HV ratio in the final polymer in response to propionate concentrations in the medium and do not require specific nutrient limitations (except for nitrogen limitation) for polymer production.

Coming to the utilization of acid pre-treated liquor of lignocellulosic biomass, this is a proof of concept for simultaneous use of C6 and C5 sugars in APL for P(3HB-co-3HV) synthesis by this culture for its remarkable ability to survive in the APL where many growth inhibitory components are there. Once this bioprocess is reasonably developed, PHA production can be a kind of value addition for the global biofuel programe from lignocellulosic biomass.

At present, most of the PHA-producing bacteria are Gram-negative and, as a result, frequent contamination of Lipopolysaccharides (LPS) occurs during the purification process. LPS is an endotoxin, which can induce allergic responses easily (Valappil et al., 2007) .Thus, the isolated Gram-positive *Bacillus* has the innate potential to make a bioprocess for this biopolymer production. Many strategies have been examined to control 3HV monomer composition of P(3HB-co-3HV) by engineering of P(3HB-co-3HV) metabolic pathways involved in PHA synthesis and optimizing fermentation strategies (Chen et al., 2011; Wong et al., 2008). In this case, the wild type culture itself provided a high 3-hydroxyvalerate fraction of 48 mol% without any metabolic engineering.

The propionyl-CoA, the precursor for PHBV production, can be generated through MCC pathway (methyl malonyl-CoA pathway) when propionic acid is supplemented with glucose or succinic acid (TCA cycle intermediates). This culture can also explore the the aspartate/2-oxobutyrate pathway when threonine and cyanocobalamin (propionate independent) is supplemented. The PHBV production was further substantiated by the presence of the genes involved in metabolic pathway for polyhydroxy alkanoate production in *B. thuringiensis*. The genes, *Pha R*, *Pha B* and *Pha C* are reported in the *Bacillus* species [accession No. Q6HLN0_BACHK (Pha R), Q52JK0_BACTU (Pha B), Q6HLM8_BACHK (Pha C)].

4. Conclusion

Engineering robust microbes for the biotech industry typically requires high-level, genetically stable expression of heterologous genes and pathways. Although plasmids have been used for this task, fundamental issues concerning their genetic stability have not been adequately addressed. Hence, it would be more appropriate to use a wild-type strain for a stable bioprocess. The innate ability of this halophilic isolate to produce PHA with 3HB and 3HV monomer content and its metabolic capacity to efficiently convert these monomers to short chain and medium chain length polyhydroxy alkanoates makes it an interesting organism to pursue further.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.11.046.

References

Althuri, A., Mathew, J., Sindhu, R., Banerjee, R., Pandey, A., Binod, P., 2013. Microbial synthesis of poly-3-hydroxybutyrate and its application as targeted drug delivery vehicle. Bioresour. Technol. 145, 290–296.

Alvarez, H.M., Kalscheuer, R., Steinbuchel, A., 2000. Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126. Appl. Microbiol. Biotechnol. 54, 218–223.

Bayari, S., Severcan, F., 2005. FTIR study of biodegradable biopolymers: P(3HB), P (3HB-co-4HB) and P(3HB-co-3HV). J. Mol. Struct. 744, 529–534.

Chen, G.Q., Wu, Q., 2005. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 26, 6565–6578.

Chen, G.Q., Konig, K.H., Lafferty, R.M., 1991. Occurrence of poly-D(−)-3-hydroxyalkanoates in the genus *Bacillus*. FEMS Microbiol. Lett. 84, 173–176.

Chen, Q., Wang, Q., Wei, G.Q., Liang, Q.F., Qi, Q.S., 2011. Production in *Escherichia coli* of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) with differing monomer compositions from unrelated carbon sources. Appl. Environ. Microbiol. 77 (14), 4886–4893.

Choi, J.I., Lee, S.Y., 1999. High-level production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by fed-batch culture of recombinant *Escherichia coli*. Appl. Environ. Microbiol. 65, 4363–4368.

Doi, Y., Tamaki, A., Kunioka, M., Soga, K., 1988. Production of copolyesters of 3-hydroxybutyrate and 3-hydroxyvalerate by *Alcaligenes eutrophus* from butyric and pentanoic acids. Appl. Microbiol. Biotechnol. 28, 330–334.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Han, J., Jing, H., et al., 2013. Multiple propionyl coenzyme A-supplying pathways for production of the bioplastic poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in *Haloferax mediterranei*. Appl. Environ. Microbiol. 79 (9), 2922–2931.

Huijberts, G.N.M., Wal, H.V., Wilkinson, C., Eggink, G., 1994. Gas-chromatographic analysis of poly(3-hydroxyalkanoates) in bacteria. Biotechnol. Tech. 8, 187–192.

Kang et al., 2011. Method for production of high purity polyhydroxyalkanonate (PHAs). U.S. patent. 8,053,219.

Lee, S.Y., J-i, Choi., Han, K., Song, J.Y., 1999. Removal of endotoxin during purification of poly(3-hydroxybutyrate) from gram-negative bacteria. Appl. Environ. Microbiol. 65, 2762–2764.

Liu, H., Pancholi, M., Stubbs III, J., Raghavan, D., 2010. Influence of hydroxyvalerate composition of poly hydroxybutyrate valerate (P(3HB)V) copolymer on bone cell viability and in vitro degradation. J. Appl. Polym. Sci. 116 (6), 3225–3231.

Masood, F., Hasan, F., Ahmed, S., Hameed, A., 2011. Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from *Bacillus cereus* FA11 isolated from TNT-contaminated soil. Ann. Microbiol. 62, 1377–1384.

Masood, F., Hasan, F., Ahmed, S., Hameed, P., Chen, 2012. Biosynthesis and Characterization of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) from *Bacillus cereus* S10. J. Polym. Environ. 20, 865–871.

Mokhtari, H., Zahra, B., et al., 2009. Statistical media optimization for growth and PHB production from methanol by a methylotrophic bacterium. Bioresour. Technol. 100 (8), 2436–2443.

Moorkoth, D., Nampoothiri, K.M., 2014. Synthesis, colloidal properties and cytotoxicity of biopolymer nanoparticles. Appl. Biochem. Biotechnol. 174, 2181–2194.

Okstad, O.A., Kolsto, A., 2011. Genomics of *Bacillus* species. In: Wiedmann, M., Zhang, W. (Eds.), Genomics of Foodborne Bacterial Pathogens, 29 Food Microbiology and Food Safety. Springer Science + Business Media, LLC. http://dx.doi.org/10.1007/978-1-4419-7686-4_2.

Ollivier, B., Caumette, P., Garcia, J.-L., Mah, R., 1994. Anaerobic bacteria from hypersaline environments. Microbiol. Rev. 58, 27–38.

Ostle, A.G., Holt, J.G., 1982. Nile blue a as a fluorescent stain for poly-β-hydroxybutyrate. Appl. Environ. Microbiol. 44, 238–241.

Peña, C., Castillo, T., García, A., Millán, M., Segura, D., 2014. Biotechnological strategies to improve production of microbial poly-(3-hydroxybutyrate): a review of recent research work. Microbiol. Biotechnol., 278–279

Poirier, Y., Nawrath, C., Somerville, C., 1995. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacterial and plant. Biotechnol 13, 142–150.

Pramanik, N., Das, R., Rath, T., Kundu, P.P., 2014. Microbial degradation of linseed oil-based elastomer and subsequent accumulation of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer. Appl. Biochem. Biotechnol. 174 (4), 1613–1630.

Quillaguamán, J., Guzmán, H., Van-Thuoc, D., Hatti-Kaul, R., 2010. Synthesis and production of polyhydroxyalkanoates by halophiles: current potential and future prospects. Appl. Microbiol. Biotechnol. 85, 1687–1696.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Slater, S.T., Gallaher, D., Dennis, 1992. Production of poly-(3-hydroxy- butyrate-co-3-hydroxyvalerate) in a recombinant *Escherichia coli* strain. Appl. Environ. Microbiol. 58, 1089–1094.

Steinbuchel, A., Pieper, U., 1992. Production of a copolyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from single unrelated carbon sources by a mutant of *Alcaligens eutrophus*. Appl. Environ. Microbiol. 37, 1–6.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Valappil, S.P., Boccaccini, A.R., Bucke, C., Roy, I., 2007. Polyhydroxyalkanoates in gram-positive bacteria: insights from the genera *Bacillus* and *Streptomyces*. Antonie Van Leeuwenhoek 91, 1–17.

Van-Thuoc, D., Huu-Phong, T., Thi-Binh, N., Thi-Tho, N., Minh-Lam, D., Quillaguamán, J., 2012. Polyester production by halophilic and halotolerant bacterial strains obtained from mangrove soil samples located in Northern Vietnam. Microbiol. Open 1 (4), 395–406.

Wong, M.S., Causey, T., Mantzaris, N., Bennett, G.N., San, K.Y., 2008. Engineering poly (3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer composition in *E. coli*. Biotechnol. Bioeng. 99 (4), 919–928.

Yang, J., Choi, Y., et al., 2014. Metabolic engineering of *Escherichia coli* for biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from glucose. Appl. Microbiol. Biotechnol. 98 (1), 95–104. http://dx.doi.org/10.1007/s00253-013-5285-z.