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Production and Characterization of Tailor-Made Polyhydroxyalkanoates by *Bacillus cereus* FC11

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Abstract.- Polyhydroxyalkanoates (PHAs) are non-toxic, biocompatible, piezoelectric, and biodegradable thermoplastic. This study was designed to isolate the bacterial strains from trinitrotoluene (TNT)-contaminated soil capable of giving PHAs at high yields with tailor-made compositions. The isolated bacterial strain *B. cereus* FC11 gave PHA yield at 76.40 wt% using 500 mL Erlenmeyer flasks each containing 200 mL synthetic medium in the presence of glucose as a sole carbon source at pH value 7.0, 30°C and 150 rpm after 48 h of incubation under submerged fermentation. Furthermore, tailor-made PHA compositions were synthesized by *B. cereus* FC11 by adding different co-substrates along with glucose in the medium. High performance liquid chromatography analysis revealed that the retention time of crotonic acid (by-product) obtained from digested PHA was approximately 24.97 min which was similar to the retention time of crotonic acid (by-product) obtained from the digestion of commercially available poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (Sigma–Aldrich). Fourier transform infrared spectrum showed the absorption bands at 1719, 1275 and 2932 cm^{-1}, which were attributed to C=O, C-O stretching and C-H vibrations, respectively. The proton and carbon nuclear magnetic resonance analysis confirmed that this indigenously synthesized polymer was composed of 3-hydroxybutyrate (3-HB) and 3-hydroxyvalerate (3-HV) monomers. The number average molar mass (Mn) of copolymer was 37.98 kDa.

Keywords: Biodegradable, polyhydroxyalkanoates, PHAs.

INTRODUCTION

Plastics are indispensable for all industries predominantly due to their versatile qualities such as durability, lightness and recalcitrance to degradation. Consequently, the endless applications of bio-stable plastics in our daily life ultimately led to the environmental pollution (Lazarevic *et al*., 2010). The approximate time required for the degradation of plastics in the environment is 400 years (Preeti and Archana, 2011). The solid waste generated due to the discarded plastic bags is expected to rise considerably in the near future by the year 2020 (Hopewell *et al*., 2009). The discarded bio-stable plastics also have deleterious effects on flora and fauna as well as on the aesthetic persona of the world.

Biodegradable polyesters of hydroxyalkanoic acid (HA) are called as polyhydroxyalkanoates (PHAs) and are considered the excellent substitute of bio-stable synthetic plastics (Jendrossek, 2009; Verlinden *et al*., 2007). PHAs are synthesized by a large number of archea, bacteria, fungi and cyanobacteria as intracellular inclusion bodies in response to a nutrient imbalance, *i.e.*, deficiency of nitrogen, sulfur, or phosphorus and presence of an excess of carbon (Masood *et al*., 2014a; Akaraonye *et al*., 2010). PHAs are readily biodegradable in soil and produce carbon dioxide and water under aerobic conditions. Methane is also produced under anaerobic conditions (Verlinden *et al*., 2007). There are about 150 different monomeric units which are identified as homopolymer or as copolymer of PHAs (Verlinden *et al*., 2007; Ojumu *et al*., 2004; Reddy *et al*., 2003). This diversity of PHAs depends on the substrate specificity of PHA synthases, PHA biosynthetic pathways and a particular type of carbon sources supplied to microorganisms (Valappil *et al*., 2007c). PHAs are water insoluble, heat stable, impermeable to oxygen, and resistant to grease, oil or UV radiation. Poly-3-hydroxybutyrate (PHB) and poly-3-hydroxybutyrate-co-3-

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hydroxyvalerate (PHBV) are the most commonly studied members of PHAs family. However, the high melting point, brittleness and crystallinity of homopolymer PHB made its thermal processing difficult for various industrial applications (Akaraonye *et al*., 2010; Reddy *et al*., 2003). On the contrary, copolymer PHBV has high impact resistance, flexibility, toughness and lower melting temperature. Furthermore, the aforementioned properties can be manipulated easily by supplementing the appropriate fatty acid precursor and co-substrates in the medium in addition to glucose during the fermentation process to obtain tailor-made compositions of PHAs (Valappil *et al*., 2007c). PHAs are widely used on the commercial scale for different applications such as food packaging, paper coatings, biomedical implant materials, and long-term dosage of drugs, hormones, insecticides or herbicides (Masood *et al*., 2014a).

The representatives of the genus *Bacillus* are considered the dark horse in the battle to produce sustainable energy, eco friendly non-fossil fuel-based PHA polymers, and bioactive molecules for use as therapeutics (Kumar *et al*., 2013). The distinctive characteristics of *Bacillus* which made them potential candidate for the production of PHAs on commercial scale include absence of the toxic Lipo-polysaccharides (LPS) secretion (Singh *et al*., 2009; Valappil *et al*., 2007 a), simple and timely recovery of PHAs after its biosynthesis due to expression of self-lysing genes (Kumar *et al*., 2013; Hori *et al*., 2002), inhibition of utilization of the polymer and/or spore formation in *B. cereus* due to the acidic pH of the medium after PHA biosynthesis (Valappil *et al*., 2007b), tolerance to high temperatures (Tajima *et al*., 2003) and/or osmotic pressures and capability to produce variations in monomeric composition of PHAs due to the broad range substrate specificity of its class IV PHA synthases (Valappil *et al*., 2007b; Tajima *et al*., 2003). Class IV PHA synthases are found in *Bacillus* and are responsible for polymerization of 3HB-CoA monomers into PHA (Rehm, 2003). Both PhaC (approx. 40 kDa) and PhaR (approx. 22 kDa) are essential for activity of Class IV PHA synthases. A gas field soil isolate *Bacillus* sp. INT005 was reported to produce copolymers and terpolymers of PHAs with variable monomeric compositions *i.e*., PHB, PHBV, poly-3-hydroxybutyrate-co-3-hydroxyhexanoate (PHBHHx), poly-3-hydroxybutyrate-co-4-hydroxybutyrate-co-3-hydroxyhexanoate (P3HB4HB3HHx) and poly-3-hydroxybutyrate-co-6-hydroxyhexanoate-co-3-hydroxyhexanoate (P3HB6HHx3HHx) in the presence of butyrate, valerate, hexanoate, octanoate, decanoate, and e-caprolactone in the medium via de novo fatty acid synthesis and β-oxidation pathways (Tajima *et al*., 2003). All this signifies the prospective of the genus *Bacillus* for the synthesis of novel tailored made compositions of PHAs along with the range of previously known PHAs based copolymers and tercopolymers. Furthermore, tailored made compositions of PHAs will dictate their potential applications in various sectors of life.

The main obstacle in commercialization of PHAs is their high production cost. Many endeavors have been done to trim down the price of PHA by isolating new microbial strains capable of giving PHAs at high yields from un-explored environmental sites and/or developing better bacterial strains via metabolic engineering, more efficient fermentation and/or more economical recovery processes. The comparison of PHA yields obtained by various *B. cereus* strains isolated from different environmental sites is given in the Table I. The selection of TNT-contaminated soil in this study was based on the finding that the nitro based aromatic compounds especially TNT could not serve as growth substrates for microorganisms under aerobic conditions (Esteve-Núñez *et al*., 2001). Therefore, the non-availability of nutrients might trigger the microorganisms to synthesize secondary metabolite such as in the form of PHA under nutrient imbalance conditions. The PHA metabolism could be an adaptive mechanism by the microorganisms to withstand the nutrient scarcity which is commonly encountered stress factor in TNT-contaminated soil.

In this context, the objectives of this study were the screening of TNT-contaminated soil as a potential source for the isolation of PHA producing *Bacillus* spp.; optimization of physicochemical parameters to attain a high yield of PHA copolymer using a single carbon source on the small scale and also its structural elucidation by HPLC, FTIR, TGA, ^{1}H and ^{13}C-NMR. The number average molar mass

Table I. The comparison of PHA yields obtained by various strains of *B. cereus* (isolated from different environmental sites) using glucose as a sole carbon source in the medium.

Bacterial strains	Source of isolation	Biomass (g/L)	PHA (g/L)	PHA yield (%)	References
B. cereus FA11	TNT-contaminated soil	6.4	3.10	48.43	Masood *et al.* (2012b)
B. cereus FB11		4.15	1.95	46.98	Masood *et al.* (2013)
B. cereus FC11		3.56	2.72	76.40	This study
B. cereus YB-4		6.10	2.14	35.08	Mizuno *et al.* (2010)
B. cereus S10	Sewage sludge	7.90	5.52	69.91	Masood *et al.* (2012a)
B. cereus SPV	UK, culture collection	2.14	0.81	38.00	Valappil *et al.* (2007b)

of copolymer was also determined. Moreover, the tailor-made PHA compositions ranging from pure PHB to PHBV were synthesized during fermentation by adding various co-substrates, *i.e.*, citric acid, propionic acid, butyric acid and valeric acid in the medium.

MATERIALS AND METHODS

Isolation and screening of PHA producing bacterial strains

About one gram of TNT-contaminated soil was vigorously shaken for 3-5 minutes in 10 mL sterilized water for the isolation of PHA producing bacterial strains. The dilutions from 10^{-1} to 10^{-7} were poured on the nutrient agar plates, incubated for 72 h at 30°C and three strains *i.e.*, FA11, FB11 and FC11 were isolated. The screening of isolated bacterial strains for PHA production was done by growing them on PHA-detection agar (PDA) medium [$(NH_4)_2SO_4$, 2 g/L; KH_2PO_4, 13.3 g/L; $MgSO_4.7H_2O$, 1.3 g/L; citric acid, 1.7 g/L; agar, 15 g/L; glucose, 2 g/L; trace element solution, 10 mL/L ($FeSO_4.7H_2O$, 10 g/L; $ZnSO_4.7H_2O$, 2.25 g/L; $CuSO_4.5H_2O$, 1 g/L; $MnSO_4.5H_2O$, 0.5 g/L; $CaCl_2.2H_2O$, 2.0 g/L; $Na_2B_4O_7.10H_2O$, 0.23 g/L; $(NH_4)_6MO_7O_{24}$, 0.1 g/L; HCl,10 mL)] (Lee and Choi, 1999). The Sudan Black B and Nile Blue A staining was used for the detection of PHA granules. The selection of bacterial strains for optimization studies was done on the basis of PHB assay (Yilmaz *et al.*, 2005). Briefly, batch fermentation was carried out in 500 mL Erlenmeyer flasks each containing 200 mL medium containing 2.5 (g/L) yeast extract, 4.0 (g/L) tryptone, 1.25 (g/L) NaCl and 1% (w/v) glucose (Kung *et al.*, 2007). The flasks were inoculated with a 2% (v/v) of 24 h old inoculum and incubated in a shaking incubator at 150 rpm and 30°C for 48 h. Suspensions of cultures were separated by centrifuge (Kokusan model H-251) at 6,000 g for 30 min. The pellets were then dissolved in 5 mL of sterilized distilled water and homogenized for 5 min using ultrasonic water bath (35 KHz, 285 w) at 25°C. About 2 mL of 2 M HCl was added in 2 mL of cell suspension, heated at 100°C for 2 h in a water bath and then centrifuged at 6,000 g for 20 minutes. About 5 mL of chloroform was added to the resulting pellet. The test tubes were placed in a shaking incubator at 28°C and 150 rpm for overnight. The content of test tubes were then centrifuged at 6,000 g for 20 min and extracted with 0.1 mL chloroform. The chloroform extract was dried at 40ºC. The dried extract was treated with 5 mL of concentrated sulfuric acid. The test tubes were placed in a water bath and heated at 100°C for 20 min. After that solution was cooled at room temperature. The absorbance of the solution was measured by a spectrophotometer (model UV-120-01 Shimadzu Scientific Instruments, Kyoto, Japan) at 235 nm wavelength and the sulfuric acid was used as a blank. A standard curve was established with the standard 99% pure crotonic acid (Sigma–Aldrich) for the determination of the quantity of crotonic acid produced by bacterial strains.

Identification of bacterial strain

The selected bacterial strain FC11 was identified on the basis of carbohydrates assimilation tests using the Analytical Profile Index (API 50CHB; bioMérieux, Marcy l'Etoile, France). The genomic DNA of bacterial strain was extracted from 1.5 mL of culture by using the Genomic DNA Extraction kit from Promega (Madison, WI). The 16S rRNA gene sequence of bacterial strain FC11

was amplified using a set of universal primers *i.e.*, 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'ACGGCTACCTTGTTACGACTT3') for its phylogenetic analysis (Weisberg *et al*., 1991). The PCR conditions were as follow; initial denaturation at 98°C for 30 s, denaturation at 98°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 30 s with a total number of 35 cycles. The PCR reaction mixture (50 µL) contained template, 12 µL; primers (0.2 µM) each, 1 µL; 10 mM dNTPs, 1 µL; Phusion DNA Polymerase, 0.5 µL; 5X Phusion HF Buffer, 10 µL; PCR water, 24.5 µL; $MgCl_2$ (50 mM), 1 µL. The amplified PCR product was purified with PCR Product Purification kit (Rapid-Tips Diffinity Genomics, West Henrietta, NY) and was visualized by running the samples on 1.2% agarose gel for 40 min at 50 V. The size of amplified amplicon was compared with 1 kb DNA marker (GeneRuler TM, Fermentas). The purified PCR product was sequenced by dye terminator cycle sequencing method (AAC Genomic Facility, Guleph, ON, Canada). The respective sequence after interpretation was submitted to NCBI Genbank using Sequin (online software), where the partial sequence was assigned by accession number. The resulting amplified nucleotide sequence was analyzed by using the BLAST search tool at the NCBI website against non-redundant databases (Altschul *et al*., 1997). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987).

Optimization of culturing conditions

The effect of various factors such as pH, time, temperature, carbon sources and co-substrates were studied in a detail and PHA yield was determined during the optimization of each parameter. Briefly, the bacterial cells were collected by centrifugation at 10,000 rpm for 20 min after completion of fermentation using synthetic media as described above. A biomass of 0.2 g were suspended in 5 mL of 0.4% sodium hypochlorite solution and incubated for 1 h at 37ºC. PHA pellet was separated by centrifugation at 10,000 rpm for 20 min. Pellet was washed by acetone and then with water. The pellet was purified by dissolving in chloroform, it was allowed to evaporate, and PHA was weighed (Lee and Choi, 1999). The PHA yield was defined as a mass fraction of PHA (g/L) in biomass (g/L). The extracted polymer was purified by soxhlet apparatus at 60°C for 6 h using chloroform as a solvent.

Characterization of PHA

High performance liquid chromatography (HPLC)

HPLC technique was used to determine the purity of the PHA produced by *B. cereus* FC11. About 5 mL samples containing 0.01 to 500 mg PHA was centrifuged after fermentation and digested in 1 mL of concentrated sulfuric acid at 100°C for 30 min in a water bath. The test tubes were then cooled on ice for 5 minutes and diluted with 4 mL volume of 0.014 N H_2SO_4 with rapid mixing. The samples were diluted to an additional 5 to 100 times with 0.014 N H_2SO_4 and filtered using cellulose acetate membrane filters (0.45 µm) before analysis by HPLC (Waters, Maple St., Milford, MA 01757, USA). The injection volume was 10 µL and sample concentration was ranging from 0.2-560 µg/mL. The samples were eluted from an IC-Pak TM Ion-Exclusion 50 A° 7 µm (300x7.8 mm) column preceded by an ion-exclusion guard column of IC-Pak TM Ion-Exclusion using 0.014 N H_2SO_4 at 0.70 mL/min flow rate and 60°C. The amount of crotonic acid produced from PHA was calculated from the regression equation derived from known standard poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-5 mol% 3-hydroxyvalerate, obtained from Sigma-Aldrich which hereafter called as PHB, and PHBV-S, respectively.

Fourier transform infrared (FTIR) spectroscopy

The structural analysis of polymer obtained from bacterial strain FC11 was performed using a FTIR spectrophotometer (Thermo Electron Corporation, USA, Nicolet, 6700) and compared with the IR spectrum of PHBV-S. The polymer sample was placed in a sample holder and the spectra were recorded using an attenuated reflectance technique having diamond crystal. The samples were scanned from 4000-400 cm^{-1} with resolution of 6.0 cm^{-1} and the results were averaged over 200 scans.

Nuclear magnetic resonance (NMR)

For NMR analysis, 20 mg of the polymer was dissolved in chloroform-d ($CDCl_3$) at room temperature and vortexed for 10 min. About 0.75 mL of polymer solution was placed into NMR tubes and ^1H and ^{13}C-NMR was done. The spectra were recorded using an AVANCE 300 B spectrometer (Bruker Daltonics, Billerica, MA). The quantification of the 3-HV content of the copolymer PHBV was done by integrating the area under the signals at 0.9 and 1.25 ppm in the ^1H-NMR spectrum according to the following equation (Bloembergen *et al*., 1986):

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

Thermogravimetry analysis (TGA)

Thermal analysis of PHBV-S and PHA samples was determined on TGA/SDTA851 (Mettler, Toledo, Switzerland) instrument in alumina pans (5 mm ø). About 4-6 mg of sample in the pan was heated under a steady flow of nitrogen (50 mL/min) at a heating rate of 10°C/min in the range of 50-400°C.

Molecular weight determination

The number average molar mass (Mn) of the PHBV-S and PHA sample obtained from bacterial strain FC11 was measured at 30°C using a Cannon Ubbelohde 75 (CD-15 LAUDA) Viscometer. About 20 mg of polymer was dissolved in 15 mL of chloroform and the average molecular weight of sample solution was determined by using following Mark-Houwink equation:

$$\eta = K.Mw\ \alpha$$

where η is the viscosity (Pa s), K is the consistency index 1.18×10^{-4}, *Mw* is the average molecular weight and $\propto$ is the flow behavior index 0.78 (Bandrup and Immergut, 1989).

Statistical analysis

All the experiments were carried out in triplicate and obtained data were evaluated by employing the analysis of variance (ANOVA). The difference of $P<0.01$ was considered significant.

RESULTS AND DISCUSSION

Characteristics of Bacillus cereus *FCII*

The TNT-contaminated soil used in this study was reddish to dark maroon in appearance. Moreover, the TNT was heterogeneously distributed in soil in the form of fine dust, flakes or crystallized chunks. Figure 1 shows the results of initial quantitative screening based on PHB assay and it was found that about 7.84, 7.94 and 5.36 nmol of crotonic acid were produced by the isolated bacterial strains *i.e.*, FA11, FC11 and FB11 after 48 h of fermentation. The *Bacillus* isolate FC11 was acclimatized to hot and drought weather conditions prevailing in our country and was selected for the optimization study as it produced a higher quantity of crotonic acid in comparison to others. The biochemical characteristics of bacterial strain FC11 were given in Table II. The isolated bacterial strain was identified on the basis of 16S rRNA gene sequencing as *Bacillus cereus* FC11 which was submitted in NCBI GenBank under accession number JN593010. The blastn search revealed that *Bacillus cereus* FC11 (JN593010) was closely related to the *Bacillus cereus* strain JR08 (HM989896.1) and *Bacillus cereus* strain P5 (HQ230354.1) with 95% sequence identities and 99% sequence coverage with e-value (0.0). Phylogenetic analysis was conducted in MEGA6 (Tamura *et al*., 2013). The optimal tree with the sum of branch length = 7.25841178 was shown in Figure 2. The confidence probability estimated by using the bootstrap test (500 replicates) was shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and were in the units of the number of base substitutions per site (Tamura *et al*., 2004). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 981 positions in the final dataset.

Light microscopy revealed the strain *B. cereus* FC11 was positive for Sudan Black B staining and PHA granules appeared as blue-black droplet (Fig. 3A). The results were consistent with the previous findings that PHB inclusions appeared as blue-black droplets (Arshad *et al*., 2007).

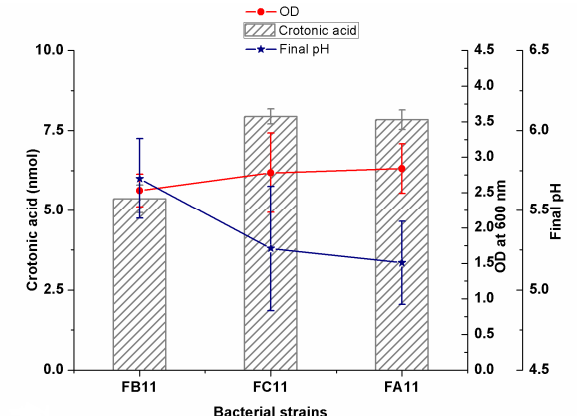

Fig. 1. Quantitative screening of crotonic acid production by three bacterial strains after 48 h of incubation at 30°C, 150 rpm and pH 7.

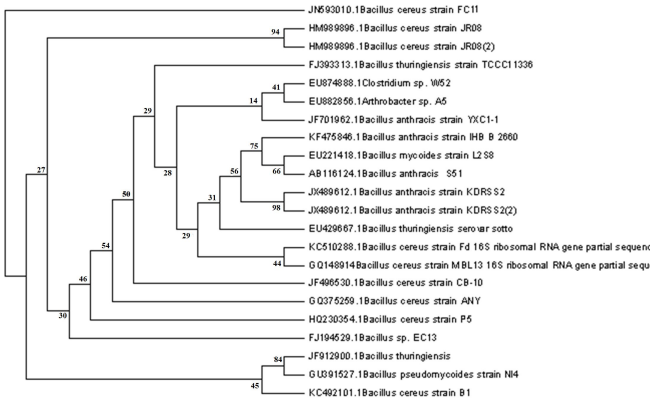

Fig. 2. Phylogenetic tree of isolated bacterial *Bacillus cereus* FC11 strain.

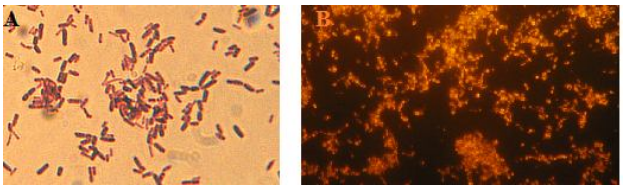

Fig. 3. Light micrograph of strain *B. cereus* FC11 stained with Sudan Black B (100×) *(a).* Fluorescent micrograph (wavelength 460 nm) of strain *B. cereus* FC11 stained with Nile Blue A ***(b).***

Fluorescent microscopy demonstrated that PHA inclusion bodies produced golden yellow fluorescence on staining with Nile Blue A (Fig. 3B). PHB inclusion bodies produced strong orange fluorescence on staining with Nile Blue A (Cortés *et al*., 2008).

Table II. Analytical Profile Index of the *Bacillus cereus* FC11 strain.

Tests	Bacterial strain FC11
Glycerol	-
Erythritol	-
D-Arabinose	-
L-Arabinose	-
D-Ribose	-
D-Xylose	+
L-Xylose	-
D-Adonitol	-
Methyl β-D-xylopyranoside	-
D-Galactose	-
D-Glucose	-
D-Fructose	+
D-Mannose	+
Methyl α-D Glucopyranoside	-
L-Sorbose	-
L-Rhamnose	-
Dulcitol	-
Inositol	-
D-Manitol	-
D-Sorbitol	-
Methyl α-D Methylopyranoside	-
Amygdalin	-
Arbutin	+
N-Acetyl Glucosamine	-
Esculin	+
Salicin	+
D-Cellobiose	-
D-Maltose	-
D-Lactose	+
D-Melibiose	-
D-Sucrose	-
D-Trehalose	-
Inulin	+
D-Melezitose	-
D-Raffinose	-
Starch	-
Glycogen	-
Xylitol	-
Gentibiose	-
Turanose	-
D-Lyxose	-
D-Tagtose	-
D-Fucose	-
L-Fucose54	-
D-Arabitol	-
L-Arabitol	-
Pottasium gluconate	-
Potassium 2-Ketoglutarate	-
Potassium 5-Ketogluconate	-
Identified microorganism	*Bacillus cereus*

Table III. Biomass and PHA yield produced at different pH, temperatures, harvest times, carbon sources and co-substrates by *B. cereus* FC11.

Optimization parameters		Biomass (g/L)*	PHA (g/L)*	PHA yield (%)*
pH	6	4.52±0.87	0.75±1.41	16.59±0.61
	7	5.12±1.23	1.55±0.32	30.27±0.36
	8	3.98±0.65	0.85±0.60	21.36±0.49
Times (h)	24	1.43±0.43	0.22±0.52	15.38±1.38
	48	3.42±0.35	1.39±0.46	40.64±0.45
	72	3.98±0.65	1.58±0.56	39.69±0.21
	96	4.03±0.75	1.60±0.43	39.70±0.43
Temperature (°C)	25	2.86±0.52	0.51±1.09	17.83±0.84
	30	3.15±1.12	1.20±0.78	38.10±0.64
	35	3.50±1.15	1.09±0.35	31.14±0.92
	40	2.15±1.65	0.35±1.26	16.27±1.32
Carbon source (1% w/v)	Glucose	3.56±0.45	2.52±0.49	70.78±1.45
	Molasses	7.23±0.62	2.95±1.01	40.80±0.65
	Sucrose	2.58±1.41	0.65±1.29	25.19±0.39
Co-substrates (0.02% v/v)	Citric acid	2.51±0.65	0.89±0.59	35.40±1.55
	Propionic acid	2.63±1.79	0.50±1.43	18.86±0.85
	Butyric acid	3.21±1.23	0.84±1.78	25.21±1.29
	Valeric acid	1.74±1.48	0.25±0.23	14.42±1.59

* Two-way Anova ± = standard error
**N.D. = Not determined
Results were expressed as mean of three independent experiments.

Optimum culturing conditions of Bacillus cereus FC11

During fermentation the optimization of physicochemical parameters has been done to enhance the yield or efficiency of bioprocess and the results of this study are shown in Table III. Typically, metabolic processes are highly susceptible to even slight changes in pH value and proper control of pH value is critical. Therefore, PHA production by *B. cereus* FC11 was checked at pH value 6, 7 and 8. It was found that the significantly higher ($P<0.01$) biomass (5.12 g/L) and PHA yield (30.27%) obtained by *B. cereus* FC11 at pH value 7, 37°C and 150 rpm in comparison to other TNT-contaminated soil isolate *B. cereus* FB11 which was cultivated under the similar condition in the previous study (Masood *et al*., 2013; Wei *et al*., 2011). This higher PHA yield was due to the better specific activity of crude PHA synthase produced by *Bacillus cereus* FC11 at pH value 7 than *B. cereus* FA11, *B. cereus* FB11and *B. cereus* S10 (un-published results). Prabhu *et al*. (2010) reported that PHA synthase of *Bacillus* sp. was active over narrow pH and temperature range with optima at 7 and 30°C. The biomass production was 4.52 and 3.98 g/L, respectively, at pH value 6 and 8. While, the PHA yield was 16.59 and 21.36% in pH value 6 and 8, respectively. These results suggested that appropriate adjustments of pH value would promote the growth of microorganisms and led to an increase in the polymer storage yield. Similarly, the previous finding reported that pH range of 6.0 to 7.5 was an appropriate for optimum microbial growth and PHA production (Masood *et al*., 2012a,b, 2013; Wei *et al*., 2011; Prabhu *et al*., 2010). For the optimization of incubation time, the PHA yield was investigated after 24, 48, 72 and 96 h. In this study, initially the slower cell growth was observed with low biomass (1.43 g/L) production. Similarly, PHA yield was 15.38% after 24 h of

incubation because the low PHA yield was obtained either prior to the late exponential stage of the growth curve or after onset of PHA hydrolysis. The significantly higher ($P<0.01$) biomass (3.42 g/L) and PHA yield (40.64%) was found by *B. cereus* FC11 after 48 h of incubation at pH value 7, 37°C and 150 rpm in comparison to *B. cereus* FB11 (Masood *et al*., 2013). The biomass production was 3.98 and 4.03 g/L after 72 and 96 h of fermentation, respectively. While, PHA yield was 39.69% and 39.70 after 72 and 96 h of fermentation indicating the lack of utilization of PHA. Previously, it was reported that PHA produced by *B. cereus* SPV, was found to remain at a constant maximal concentration, without any degradation (Valappil *et al*., 2007b). In the present study, PHA yield was checked at different temperatures, *i.e*., 25, 30, 35 and 40°C. The significantly higher ($P<0.01$) biomass (3.15 g/L), and yield (38.10%) was obtained by *B. cereus* FC11 at 30°C, pH value 7 and 150 rpm. The high PHA yield at 30°C was due to the elevated specific activity of crude PHA synthase produced by *B. cereus* FC11 in comparison to the *B. cereus* FA11 and *B. cereus* FB11 (un-published results). *Bacilli* are mesophiles and any increase or decrease in optimum growth temperature decreased the PHA yield. During the process of fermentation, temperature effects dissolved oxygen levels and mass transfer efficiency, which in turn controls the cellular growth and secondary metabolite production (Wei *et al*., 2011). The biomass production was 2.86, 3.50 and 2.15 g/L at 25, 35 and 40°C. The PHA yield was 17.83, 31.14 and 16.27%, respectively, at 25, 35 and 40°C. *B. megaterium* and *R. eutropha* could not accumulate PHA at temperatures higher than 41°C (Tajima *et al*., 2003). To obtain the high PHA yield, the different carbon sources, *i.e*., glucose, molasses and sucrose were supplied in the medium in this study. *B. cereus* FC11 produced the significantly higher ($P<0.01$) biomass (3.56 g/L) and PHA yield (76.40%) using glucose as the sole carbon source after 48 h of incubation at pH value 7, 30°C and 150 rpm. The biomass production was 7.23 and 2.58 g/L in the presence of molasses and sucrose in the medium. The PHA yield in the presence of molasses and sucrose as a carbon source in the medium was 40.81 and 25.19%, respectively. The order of specific activity of crude PHA synthase produced by different *B. cereus* strains using glucose as a sole carbon source in the medium was in the following order S10 > FA11> FC11> FB11 (unpublished results). Thus, it could be concluded that during PHA synthesis the specific activity of crude PHA synthase for the utilization of carbon sources was a strain dependent phenomenon. Previously, the comparative *in vitro* and *in silico* analysis of the phaC subunit of type IV PHA synthases among *B. cereus* FA11, *B. cereus* FC11, and *B. cereus* FS1 was done to determine its structural and functional properties. In *B. cereus* FC11, the PhaC subunit contained α/β hydrolase fold-1 which has catalytic triad *i.e*., cysteine (Cys), histidine (His), and aspartate (Asp) at its active site (Tariq *et al*., 2014). According to them a shorter inter-atomic distance present between the carboxyl (–COO) group of Asp and amino (NH_2) group of His may be responsible toward the broad substrate specificity of type IV PHA synthases. Furthermore, a shorter distance between the sulfhydryl (SH) group of cysteine (Cys) and NH_2 group of His in case of *B. cereus* FC11 leads to a higher enzymatic activity (0.01 U/mg) and maximum PHA yield (49.26%) as compared to *B. cereus* FA11 and *B. cereus* FS1. The low PHA yield obtained using molasses as a sole carbon source in medium was due to the fact that molasses being mixture of fructose and sucrose (not easily available carbohydrates) had not efficiently utilized by microorganisms in PHA synthesis. PHA-accumulating bacteria isolated from municipal sewage sludge could accumulate 64.32% PHB when grown on the glucose supplemented medium (Reddy *et al*., 2008). The significantly higher ($p<0.01$) biomass (2.51 g/L) and PHA yield (35.40%) was obtained by *B. cereus* FC11 at pH 7, 30°C and 150 rpm in the presence of citric acid as a co-substrate after 48 h of fermentation than the presence of other co-substrates in the medium. The PHA yield was 18.86, 25.21, and 14.42% in the presence of propionic, butyric and valeric acid in the medium, respectively. Yu *et al*. (2002) used the acetic, propionic and butyric acids during batch fermentations both individually and in mixtures and stated that the total concentration of acids in the medium had a profound effect on PHA yield.

Characterization of PHA

HPLC analysis

All three samples including commercially available PHB, PHBV-S and PHA (obtained from *B. cereus* FC11) were studied by using HPLC technique and the result is shown in Figure 4. Both PHB and PHBV-S was converted into crotonic acid, a by-product, after digestion in the presence of concentrated H_2SO_4. Similarly, the PHA obtained from *B. cereus* FC11 was also converted into crotonic acid. It was found that a peak of crotonic acid was eluted at around 24.97 min for all three aforementioned samples. A second peak at 7.5 min was due to the solvent elution containing digested cell residue. The appearance of the single peak at same retention time was indicating that the PHA produced in our case is same (Fig. 4). The similar retention time of PHB was found in the literature (Júnior *et al*., 2010).

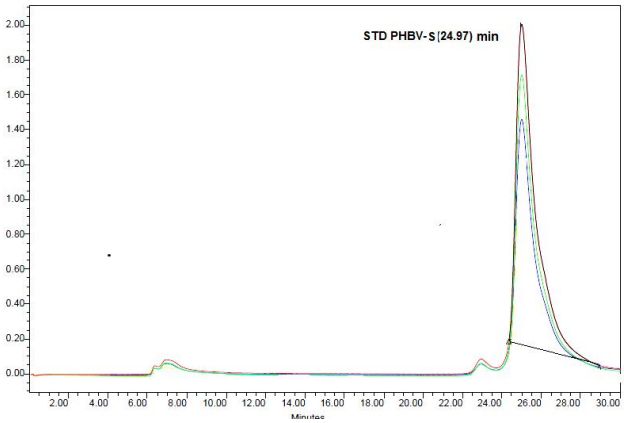

Fig. 4. HPLC chromatogram of PHB (upper), PHBV-S (middle) obtained from Sigma-Aldrich and PHA obtained from *Bacillus cereus* FC11 (lower).

FTIR analysis

The structural analysis of PHA produced by *B. cereus* FC11 was done by FTIR technique. The absorption bands at 1720.2 and 1278.5 cm^{-1} were typical to PHBV and corresponding to C=O and C-O stretching, respectively. Other absorption bands at 1375.6, 1450.7 and 2932.1 cm^{-1} were attributed to C-H vibration form CH_3, CH_2 and CH bonds (Fig.5). The intense absorptions at 1724–1740 cm^{-1} corresponds to the ester functional groups primarily from the lipids, fatty acids and PHAs (Shamala *et al*., 2003).

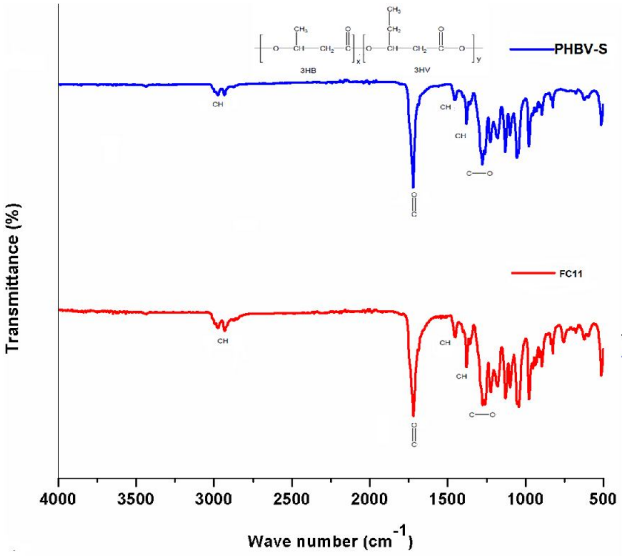

Fig. 5. Fourier transform-infrared absorption spectra of standard PHBV-S (upper) and polymer obtained from *B. cereus* FC11 (lower).

NMR analysis

In this study, ^{1}H-NMR analysis of PHA sample obtained from *B. cereus* FC11 using glucose as a sole carbon source is shown in Figure 6. The characteristic signals were observed at 0.87 (7) and 1.24 (5) ppm and were corresponding to CH_3 groups of 3-hydroxybutyrate (3-HB) and 3-hydroxyvalerate (3-HV), respectively. The two signals (1, 3) appeared at 2.62 ppm were attributed to the CH_2 groups of 3-HB and 3-HV. The signal (6) obtained at 1.62 ppm was corresponding to CH_2 group of 3-HV. Whereas, the signals (2, 4) appeared at 5.24 ppm were representing the CH groups of 3-HV and 3-HB.

When the area under the signals at 0.87 and 1.26 ppm integrated it was found that *B. cereus* FC11 produced a copolymer. The copolymer was made up of two monomeric units, *i.e*., 89 mol% of 3-HB and 11 mol% of 3-HV. The presence of valerate monomer in the polymer is represented by peaks at 0.9, 1.6 and 5.1 ppm corresponding to the triplet, a methylene and methyne (Allen *et al*., 2010; Kumar *et al*., 2007). The composition of PHA copolymer is determined by different factors such as

the type of carbon substrate used (alkanes, alkenes, alcohols, fatty acids, etc.) and specificity of PHA synthases (Galia, 2010). *Bacillus cereus* FC11 was using a beta-fatty acid oxidation pathway to consume the co-substrates present in the medium as reported previously by Tajima *et al*. (2003). The variations in the monomeric composition of the polymer samples produced by *B. cereus* FC11 when grown in the presence of both glucose and co-substrates in the medium are shown in Table IV. The ^{1}H-NMR spectra of the resulting PHA samples are shown in the Supplementary (S1) file. Abdelhad *et al*. (2009) found that *R. eutropha* ATCC 17697 produced a copolymer made up of 87.19 mol% of 3-HB and 12.81 mol% of 3-HV in the presence of glucose and propionic acid in the medium. Furthermore, they found that *A. latus* ATCC 29712 produced a copolymer made up of 83.76 mol% of 3-HB and 16.24 mol% of 3-HV using sucrose and propionic acid after 40 h of fermentation.

Table IV.- Monomer composition of the copolymer samples produced by *B. cereus* FC11, grown in the presence of glucose and co-substrates, as determined by ^{1}H-NMR spectra.

Co-substrates	Monomer composition	
	3-HB (mol%)	3-HV (mol%)
Citric acid	85.55	14.45
Propionic acid	79.42	20.58
Butyric acid	100.00	ND*
Valeric acid	93.51	6.49

The signals identified at 169.12, 67.59, 40.77, 29.67 and 19.74 ppm in the ^{13}C-NMR spectrum were corresponding to the carbonyl carbon (C=O), methine carbon (=CH−) beta to a carbonyl carbon (CH), a carbon alpha to a carbonyl carbon (CH_2), an ethyl carbon (C_2H_5) and a methyl carbon (CH_3), respectively. The signal appeared at 77 ppm was attributed to $CDCl_3$ (Fig. 7). Previously, Kumar *et al*. (2007) obtained signals at 169.10 ppm (carbonyl carbon), 67.92 ppm (methine carbon), 41.10 ppm (methylene carbon alpha to the carbonyl carbon), 30.01 ppm (methylene carbon) and 20.0 ppm (methyl carbon) during characterization of PHBV. In another study, it was found that ^{13}C-NMR spectrum showed characteristic signals at 169.53,

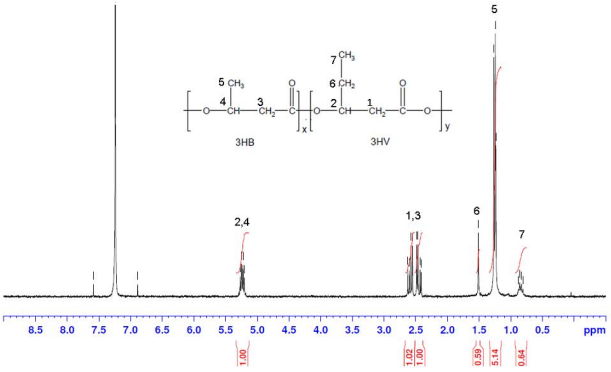

Fig. 6. Proton nuclear magnetic resonance spectrum of poly-(3-HB-co-11 mol% 3HV) polymer obtained from *B. cereus* FC11.

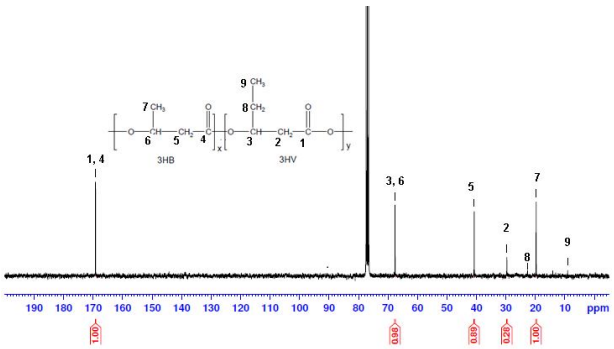

Fig. 7. Carbon nuclear magnetic resonance spectrum of PHBV-11 obtained from *B. cereus* FC11.

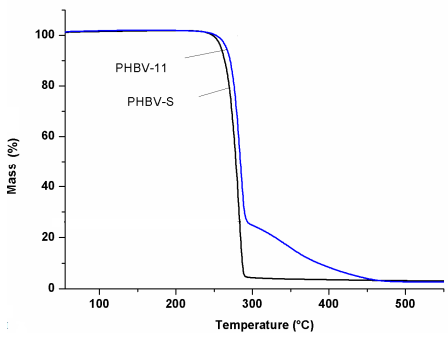

Fig. 8. TG thermogram of PHBV-S and PHBV-11.

71.97, 38.70, 26.96 and 9.33 ppm which were indicating the presence of valerate monomer in homopolymer produced by *P. putida* KTHH08 (Wang *et al*., 2011).

Thermogravimetric analysis

TGA is a standard technique used to determine the thermal stability of the polymer. In this study, the thermal stability of PHA obtained from *B. cereus* FC11 is compared with the PHBV-S and thermogram is shown in Figure 8. This figure shows that thermal decomposition of PHBV-S occurred in one step and the onset of degradation starts from 255°C. The decomposition of PHBV-11 occurred in two steps. The onset of degradation of first step started at 262°C and completed at 292°C. This represents that approximately 75% part of the polymer PHBV-11 has a lower thermostability. In the second step, a gradual mass loss was observed and it was completed at 460°C. Furthermore, 25% part of copolymer PHBV-11 has a higher thermostability.

Molecular weight determination

The molar mass of PHA sample obtained from *B. cereus* FC11 using viscometer was 37.98 kDa. During the incubation of microbial biomass with sodium hypochlorite, a profound decrease, in molecular weight of polymer was reported previously (Valappil *et al*., 2007b). But, in this study the use of chloroform as solvent might slow down the kinetics of polymer degradation. The molar mass of the polymer depends upon a number of factors such as: pH and temperature during fermentation, type and concentration of substrates and type of microbial strains used for PHA production (Lenz and Marchessault 2005).

CONCLUSION

In conclusion, the TNT-contaminated soil is a good source for the isolation of potent PHA producing bacterial strain *B. cereus* FC11 capable of giving PHBV at high yields (76.40% wt) in the presence of single carbon source, which was not utilized, once it was accumulated. Furthermore, the tailoring of PHA composition from pure PHB to PHBV by providing various co-substrates in the medium has given the evidence about the broad range of substrate specificity of class IV PHA synthases. The molar mass of the resulting copolymer was 37.98 kDa. The PHBV-11 showed two step degradation and higher thermal stability as compared to the commercially available PHBV-S.

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