CHAPTER 7

Characterization of the polymer produced by the selected isolates when cultured on different structurally related and unrelated substrates
7.1 INTRODUCTION

The genus *Bacillus* has been recognized well for the PHA diversity observed with the strain type and/or physiological conditions mainly feeds used for the polymer production. Besides the homopolymer PHB (most common), a variety copolymer synthesis have been reported by various *Bacillus* species on structurally related substrates (Chen *et al*., 1991; Caballero *et al*., 1995; McCool *et al*., 1996; Łabużek and Radecka, 2001; Tajima *et al*., 2003; Valappil *et al*., 2007b and 2008; Divyashree *et al*., 2009; Mizuno *et al*., 2010; Divyashree and Shamala, 2010; Tay *et al*., 2010; Masood *et al*., 2012b; Ray and Kalia, 2017); pure unrelated substrates (Tajima *et al*., 2003; Valappil *et al*., 2007b; Otari and Ghosh 2009; Shahid *et al*., 2013; Singh *et al*., 2013b; Moorkoth and Nampoothiri 2016) and even on inexpensive unrelated substrates (Anil-Kumar *et al*., 2007; Santimano *et al*., 2009; Sangkharak and Prasertsan, 2012; Nagamani and Mahmood, 2012; Shamala *et al*., 2012; Kumar *et al*., 2014; Kumar *et al*., 2016). Knowledge concerning the optimal physiological state(s) needed to switch from homopolymer to copolymer production by the producer strain is of utmost important.

The present study focused on investigating PHA accumulating ability of *B.megaterium* strain Ti3 under different nutritional conditions: nitrogen sources. The influence of the nitrogen source on the quantity and monomeric composition of PHA was determined in two varied nutritional conditions: organic or inorganic nitrogen supplemented with the same carbon source.

7.2 MATERIALS AND METHODS

7.2.1 PHA production under varied nutritional conditions

The metabolic potential of *B.megaterium* strain Ti3 was explored for the PHA copolymer production using structurally unrelated carbon substrates such as sucrose and starch; and related substrates of different carbon chain length such as propionic acid (C3), valeric acid (C5), hexanoic acid (C6), octanoic acid (C8), decanoic acid (C10), 1,4-butanediol (C4) and ε – caprolactone (C6) respectively. Casein hydrolysate or potassium nitrate was used as nitrogen sources, respectively.
7.2.1.1 PHA production using structurally unrelated carbon substrates

Overnight grown Nutrient broth culture of *B. megaterium* strain Ti3 was used as inoculum (2 % v/v) for PHA production medium (pH 7.0) containing (g/L): 10 carbon substrate (sucrose or starch), 0.2 MgSO$_4$, 0.1 NaCl, 0.5 KH$_2$PO$_4$, 5.0 nitrogen source (casein hydrolysate or KNO$_3$). Production studies in shake flasks were carried out as mentioned in Section 3.6 for 24 h.

7.2.1.2 PHA production using structurally related substrates

- **Preparation of substrates**
  
The substrates were added in the form of sodium salts to the production media. They were purchased as sodium salts if available, or were neutralized using 2N NaOH (Caballero *et al.*, 1995). The solutions were filter sterilized using a 0.2 µm filter prior use (Chai *et al.*, 2009). The respective carbon substrates were added at a concentration of 1-3 g/L to production media (Chen *et al.*, 1991) as sole substrates. [Substrate abbreviations: sodium propionate (SP); sodium valerate (SV); sodium hexanoate (SHx); sodium octanoate (SO); sodium decanoate (SD); 1,4-butanediol (1,4-Btdiol) and ε – caprolactone (ε –Cp)].

- **Screening of related substrates for PHA production: Nile red viable colony staining**
  
The related substrates were screened for PHA production by qualitative Nile red viable colony staining. The production media comprised of (g/L): 0.2 g magnesium sulphate, 0.5 g potassium dihydrogen phosphate, 0.1 g sodium chloride, 20 g agar. Two different nitrogen sources: casein hydrolysate or potassium nitrate (Carbon: Nitrogen:: 1:1) were tested respectively. The production media was sterilized by autoclaving at 121 ºC, 15 lbs pressure for 15 min and cooled to 45 ºC. Filter sterilized respective carbon substrates were added to production media aseptically. Further, Nile red dye solution at a final concentration 0.5 µg/mL (in Acetone) was added to the sterilized and cooled media just before pouring the plates. The inoculated plates were incubated at 30 ºC for 48 h in dark. The colonies were observed for growth and orange fluorescence under UV trans-illuminator at a wavelength of 312 nm (Spiekermann *et al.*, 1999).
PHA production under submerged conditions using related substrates

Production media preparation was same as mentioned in the Nile red viable colony staining. Productions were performed with enrichment (Amirul et al., 2008). For enrichment, the isolate was maintained on agar plates with respective substrates and the same was used for development of overnight grown inoculum [2 % (v/v)] for polymer production at shake flask levels. The production studies were carried out as per Section 3.6 for 48 h.

7.2.2 PHA extraction and quantification

For PHA extraction, fermented culture broth was centrifuged at 8000 g for 15 min. The cell pellet was treated with 40 % (v/v) Sodium hypochlorite (culture broth: hypochlorite :: 2:1) for 15 min at 30°C. The treated biomass was centrifuged at 10,000 g for 20 min. The washings and gravimetric quantification of the polymer pellet obtained was done as per Section 3.8.2.

7.2.3 $^{13}$C and $^{1}$H Nuclear magnetic resonance (NMR) spectroscopy

Structural characterization of the PHA accumulated by B. megaterium Ti3 using the unrelated (starch and sucrose) and related (fatty acids) substrates, respectively was done by $^{13}$C and $^{1}$H nuclear magnetic resonance spectrometry as mentioned in Section 3.9.2. The NMR spectra shown in the upcoming sections are representative of respective carbon substrate with KNO$_3$ as a nitrogen source.

7.3 RESULTS AND DISCUSSION

Various applications need distinctive physical and material properties of polymers. PHAs are known to span a large range of the same owing to their varied monomer content. The properties, improved by varying the monomer composition include melting point (Tm), glass transition temperature (Tg), crystallinity, elastic modulus, tensile strength, elongation etc. to name a few (Singh et al., 2015; Magdouli et al., 2015). Strategies such as selection of appropriate producer, carbon and nitrogen sources, feed type, feed concentration, feeding strategy/regime, production phase and
genetic manipulations are some of the key parameters influencing the overall physiology of PHA production (Kumar et al., 2013; Singh et al., 2015).

7.3.1 PHA production on unrelated substrates

Appreciable levels of PHA yields were observed on the unrelated substrates, sucrose and starch, with casein hydrolysate (0.68± 0.02 and 0.61± 0.03 g/L) and KNO₃ (0.61± 0.01 and 0.53± 0.02 g/L) respectively (Fig 7.1).

The ¹H NMR spectra of the polymers recovered from B. megaterium Ti3 in the presence of unrelated substrates sucrose and starch, respectively exhibited peaks at 5.2 ppm (multiplet), 2.4 – 2.6 ppm (multiplet) and 1.2 ppm doublet), corresponding to one proton bonded to C3 (methine group; -CH group), two protons of C2 (methylene group; -CH₂ group) and three protons of the C4 (methyl group; -CH₃ group), respectively (Fig 7.2 and 7.3). Thus, the monomers present in the polymer were of 3HB type exclusively. The ¹H NMR analysis of the polymer showed the strain B. megaterium Ti3 to accumulate the homopolymer P(3HB) on both the unrelated substrates, sucrose and starch in presence of either of the nitrogen sources tested.
Fig 7.1 PHA production by *B. megaterium* Ti3 in the presence of unrelated substrates with casein hydrolysate (Casein hy.) or KNO₃ as nitrogen source

C: carbon source (sucrose or starch, respectively)
Fig 7.2 $^1$H NMR spectra of the PHA extracted from *B.megaterium* grown on sucrose and KNO$_3$

Fig 7.3 $^1$H NMR spectra of the PHA extracted from *B.megaterium* grown on starch and KNO$_3$
The accumulation of P(3HB) from unrelated carbon sources like sucrose and starch must have been due to their conversion to intermediate acetyl Co-A which further undergoes a step wise conversion to form R-(3)-hydroxybutyryl CoA being polymerized by PHA synthase into homopolymer P(3HB) (Taguchi et al., 2001). In contrast to ours, diverse copolymer synthesis ability of different Bacillus spp. have been observed with unrelated carbon substrates. To quote a few, Bacillus sp. INT005 showed the ability to produce copolymers with 3HB and 3HHx monomer units using glucose as the carbon source (Tajima et al., 2003). Various B.megaterium and B. cereus strains have been reported to accumulate copolymer PHB-co-HV using glucose as sole carbon sources (Reddy et al., 2009a, 2009b; Mizuno et al., 2010; Masood et al., 2012a, 2017). Strains of B.megaterium have also shown copolymer PHB-co-HV synthesis on sucrose and glycerol as sole carbon sources (Otari and Ghosh, 2009; Reddy et al., 2009a). Biosynthesis of copolymer like P (3HB-co-4HB) and tercopolymer like P (3HB-co-3HV-co- 4HB) on unrelated carbon sources sucrose, fructose and gluconate, respectively by B. cereus SPV was reported by Valappil et al., (2007b). PHA with 4-oxopentanoic acid-like monomer units by B. thuringiensis EGU45 from glucose was observed by Singh et al., (2013b). Moorkoth and Nampoothiri, (2016) reported P(3HB-co-3HV) copolymer production by Bacillus sp. MG12 with broad range of carbon sources such as monosaccharides (glucose, lactose), disaccharides (galactose, sucrose, maltose), pentoses (xylose and arabinose), various organic acids (succinic acid) and a lignocellulosic substrate [acid pre-treated liquor (APL) of sugar cane trash]. In concordance with the above cited literature including the current study results clearly highlight strain specific varied PHA production physiology of the genus Bacillus.

7.3.2 PHA production on related substrates

Preliminary screening of structurally related carbon substrates via nile red viable colony staining showed fluorescence for all the substrates, indicative of growth and PHA production on them, qualitatively (Plate 7.1).

0.2 % (w/v) of all fatty acids and 0.05 % (w/v) of octanoate were determined as the optimum concentration for future experiments. Decanoate even at as low a concentration of 0.025 % (w/v) did not support growth, hence wasn’t considered further. Comparatively
better growth and polymer accumulation (higher fluorescence intensity) was observed for the organic nitrogen source casein hydrolysate than that of the inorganic nitrogen (KNO₃).

Similarly at the shake flask level experiments also, higher PHA yields (0.90 to 0.17 g/L) were observed with the use of organic nitrogen casein hydrolysate. Propionate and octanoate supported the minimum and maximum yield, respectively. In the case of inorganic N sources (KNO₃), the PHA yields varied from 0.02 to 0.12 g/L (Fig. 7.4). The complex or organic nitrogen source, casein hydrolysate being a rich source of both peptides as well as amino acids, supported both growth and polymer accumulation in the growth associated PHA producer, *B. megaterium* strain Ti3. The higher polymer yields in presence of casein hydrolysate could be attributed to directing of its bonus carbon from its metabolic pathways to PHA biosynthesis.

The polymer extracted from related substrates was also analyzed using the ¹H and ¹³C NMR (Fig 7.5-7.9). The ¹H and ¹³C NMR analysis of the related carbon sources such as 1,4-butanediol; ε – caprolactone, hexanoic acid and octanoic acid, respectively showed the peak assignments of the both spectras being in concordance with the glucose casein substrate combination observed in the present study earlier, signifying the production of homopolymer of P(3HB) exclusively on all the above mentioned substrates irrespective of the nitrogen source used. The Fig 7.5 -7.8, I and II, represent the ¹H and ¹³C NMR analysis of the related carbon sources: 1,4-butanediol, ε – caprolactone, hexanoic acid and octanoic acid, respectively with the KNO₃ as the nitrogen source.

Interestingly, *B. megaterium* Ti3 was able to accumulate copolymer P(3HB3HV) in the presence of structurally related fatty acid valerate as a sole substrate under non limiting (1X) inorganic nitrogen conditions only. The assignment of the spectral peaks for sodium valerate in presence of potassium nitrate is shown in Fig. 7.9 (I) and (II). The ¹H spectrum showed the presence of five different environments for the protons, and hence the five peaks. The peak at 5.2 ppm was corresponding to protons at the C3 (-CH group), 2.5 ppm to C2 (-CH₂ group), 1.6 ppm to C4 (-CH₂ group of 3HV), 1.2 ppm to C4 (-CH₃ group of 3HB), 0.9 ppm to C5 (-CH₃ group of 3HV). The ¹³C spectrum showed 2 different types of monomers in the polymer. The chemical shift at 169.09 ppm corresponded to the C1 (C=O group), 71.01 and 67.573 ppm to C3 (–CH group of 3HV)
and 3HB), 40.760 and 38.761ppm to C2 (-CH$_2$ group of 3HB and 3HV), 26.8 ppm to C4 (-CH$_2$ of 3HV), 19.720 to C4 (-CH$_3$ of 3HB) and 9.302 to C5 (-CH$_3$ of 3HV). The monomers thus present in the polymer were 3HB (3-hydroxybutyrate) and 3HV (3-hydroxyvalerate). In *B.megaterium* strain Ti3, fatty acid β-oxidation could be the major pathway involved in PHA accumulation from fatty acids of various carbon chain lengths. The fatty acids must have entered the PHA biosynthetic pathway via the intermediates R-(3)-hydroxyacyl CoA monomers. The enzyme (R)-specific enoyl-CoA hydratase (PhaJ), known to catalyze the (R)-specific hydration of intermediate, 2-trans-enoyl-CoA to (R)-3-hydroxyacyl- CoA, functions as a key supplier of monomer units from β-oxidation to PHA synthesis (Kim *et al*., 2007). Table 7.1 represents the summary of PHA production by *B.megaterium* on varied structurally related and unrelated substrates used in the present study.

The easy availability and preferential utilization of the casein hydrolysate as both carbon and nitrogen source by the strain might have channeled the carbon of casein preferably to the P3HB anabolic pathway. Absence of any other external carbon source in media led to shift for copolymer synthesis using only valerate. Substrate specificity of PHA synthases is also known to significantly affect the overall PHA diversity. In general, Class IV synthases of *Bacillus* sp. are known for polymerization of scl-monomer units preferably. Class IV PHA synthases have two subtypes, the IVc (*cereus*) and IVm (*megaterium*) type (McCool and Cannon, 2001; Tsuge *et al*., 2015). An *in vivo* enzyme assay study by Hyakutake *et al*., (2011) indicated the IVc subtype PHA synthases having broader substrate specificity as compared to the IVm subtype. Interestingly, for few *Bacillus* spp. production of PHA copolymers comprising monomers units other than scl, as minor constituents has also been reported by some research groups (Tsuge *et al*., 2015). In the present study, based on the integration values of the $^1$H NMR spectra peaks, the mol % incorporation of the scl monomers (3HB and 3HV) by *B.megaterium* strain Ti3 was determined as P(95 % 3HB-co-5 % 3HV). Also, in contrast to the study of Chen *et al*., (1991) and Kumar *et al*., (2015) in the present study better growth and polymer accumulation was supported by valerate compared to propionate for *B.megaterium* strain Ti3.
Plate 7.1 Nile Red viable colony staining for screening of structurally related substrates for PHA production with Casein hydrolysate (A1 – F1) and KNO₃ (A2 – F2) as nitrogen sources

A1-A2: Sodium propionate; B1-B2: Sodium valerate; C1 – C2: 1,4-Butanediol; D1-D2: ε – caprolactone; E1-E2: Sodium hexanoate; F1 – F2: Sodium octanoate
Fig 7.4 PHA production by in the presence of related substrates with casein hydrolysate (Casein hy.) or KNO₃ as nitrogen source

C: carbon source (structurally related fatty acids, respectively)
Fig 7.5 Nuclear magnetic resonance (NMR) spectra of the PHA extracted from *B. megaterium* on 1,4-butanediol (C4) and KNO₃ (I) $^1$H NMR and (II) $^{13}$C NMR.
Fig 7.6 Nuclear magnetic resonance (NMR) spectra of the PHA extracted from *B. megaterium* on ε – caprolactone (C6) and KNO₃ (I) $^1$H NMR and (II) $^{13}$C NMR
Fig 7.7 Nuclear magnetic resonance (NMR) spectra of the PHA extracted from *B. megaterium* on hexanoic acid (C6) and KNO₃ (I) $^1$H NMR and (II) $^{13}$C NMR
Fig 7.8 Nuclear magnetic resonance (NMR) spectra of the PHA extracted from *B. megaterium* on octanoic acid (C8) and KNO$_3$ (I) $^1$H NMR and (II) $^{13}$C NMR
Fig 7.9 Nuclear magnetic resonance (NMR) spectra of the PHA extracted from *B. megaterium* on sodium valerate and KNO₃ (I) $^1$H NMR and (II) $^{13}$C NMR
Table 7.1 Summary of PHA production by *B. megaterium* Ti3 using various structurally related and unrelated carbon sources

<table>
<thead>
<tr>
<th>Substrate type</th>
<th>Number of carbon atoms in monomer</th>
<th>Concentration (g/L)</th>
<th>PHA yield (g/L)</th>
<th>PHA Monomer composition by $^{13}$C NMR Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>10</td>
<td>0.57± 0.02</td>
<td>3HB</td>
</tr>
<tr>
<td>Starch</td>
<td>-</td>
<td>10</td>
<td>0.53± 0.02</td>
<td>3HB</td>
</tr>
<tr>
<td>Sodium Valerate</td>
<td>5</td>
<td>2</td>
<td>0.12± 0.03</td>
<td>3HB, 3HV (scl PHA)</td>
</tr>
<tr>
<td>Sodium Propionate</td>
<td>3</td>
<td>2</td>
<td>0.02± 0.02</td>
<td>ND</td>
</tr>
<tr>
<td>1,4 - Butanediol</td>
<td>4</td>
<td>2</td>
<td>0.09± 0.05</td>
<td>3HB</td>
</tr>
<tr>
<td>ε - caprolactone</td>
<td>6</td>
<td>2</td>
<td>0.09± 0.02</td>
<td>3HB</td>
</tr>
<tr>
<td>Sodium Octanoate</td>
<td>8</td>
<td>0.5</td>
<td>0.04± 0.03</td>
<td>3HB</td>
</tr>
<tr>
<td>Sodium Hexanoate</td>
<td>6</td>
<td>2</td>
<td>0.07± 0.05</td>
<td>3HB</td>
</tr>
<tr>
<td>Sodium Decanoate</td>
<td>10</td>
<td>1 – 0.25</td>
<td>NG</td>
<td>ND</td>
</tr>
</tbody>
</table>

NG: No growth; ND: Not determined.
Several studies have reported copolymer production by *Bacillus* sp. exclusively in the presence of fatty acids feeds as precursors or sole carbon sources. Eleven different *Bacillus* sp. produced copolymer P(3HB3HV) on addition of related fatty acids such as propionic, valeric and heptanoic acid, respectively, as co-substrates to nitrogen free media (Chen *et al*., 1991). The study reported precursors tested being toxic to *Bacillus* sp. at concentrations above 2 g/L. Similar concentration of fatty acids was tolerated in the present study, wherein above 2 g/L very faint growth was observed for *B. megaterium* Ti3. Caballero *et al*., (1995) reported PHA copolymer production with minor content of HC or HO monomer units by *B. cereus* 14579 on caproate and octanoate as sole carbon sources. In contrast, both the *B. megaterium* strains (14581 and 13632) used in the same study accumulated only P3HB homopolymer on all the respective substrates. *Bacillus cereus* UW85 biosynthesized homopolymer P(3HB) on either glucose alone or glucose supplemented with caprolactone. However, the same strain was able to accumulate tercopolymer with 3HB, 3HV and 6HHx monomer units respectively, when ε-caprolactone was used as a sole carbon substrate (Labuzek and Radecka, 2001). Interestingly, tolerance of higher concentration 3 g/L (ε – caprolactone) by the strain *B. cereus* UW85 (Labuzek and Radecka, 2001), was not observed in *B. megaterium* strain Ti3 of present study. *Bacillus* sp. INT005 exhibited the accumulation of copolymers with 3HB, 3HV, 3HHx, 4HB, and 6HHx in various combinations from six related fatty acids except butyrate. This study reflected the metabolic versatility of *Bacillus* sp. in terms of copolymer production, which could be attributed to *de novo* fatty acid synthesis and β-oxidation pathways (Tajima *et al*., 2003). *B. cereus* SPV accumulated P(3HB3HV) copolymer by the strain on odd chain n-alkanoates such as propionate, heptanoate and nonanoate, respectively (Valappil *et al*., 2007b). In the study of Shahid *et al*., (2013), the two PHA producer strains *B. megaterium* strains (DSM 90 and DSM 509), respectively were quite different in terms of utilization of carbon substrates (sugars, organic or fatty acids) and nature of the polymer produced under similar growth conditions of nitrogen rich (growth phase) and depleted media respectively. *B. megaterium* DSM 90 accumulated P(3HB) exclusively on glucose, glycerol, citrate, and succinate; and failed to grow on related carbon substrates pentanoic and octanoic acid, respectively. Whereas, *B. megaterium* DSM 509, irrespective of the carbon source (related or unrelated)
accumulated PHA of varied monomer composition with nitrogen depletion. An unusual metabolic shift from scl to diverse mcl PHA and scl-mcl copolymer production on nitrogen depletion for unrelated substrates was reported. In contrast to the study of Shahid et al., (2013); copolymer P(3HB-co-3HV) accumulation by B.thuringiensis EGU45 was reported by Kumar et al., (2015), even at higher or non-limiting nitrogen conditions with crude glycerol alone, or supplemented with propionic and valeric acids as feed.

Material property wise the homopolymer P3HB has a high melting point ($T_m$) of 173 – 180 °C and a glass transition temperature ($T_g$) of 4-9 °C. The polymer material is highly crystalline and thus brittle and stiff with low elasticity. The incorporation of 3-hydroxyvalerate (3HV) into P3HB also helps reduce its brittleness. The mechanical properties of PHBV are related with 3HV ratios. As the 3HV ratio increases, the value of Young’s modulus decreases indicating the incorporation of 3HV into P3HB making the material more flexible (Wang and Chen, 2017). The copolymer being more flexible and stronger has also been explored for biomedical applications such as nanoparticle-based drug delivery and tissue engineering (Zhu et al., 2009; Kuppan et al., 2011; Gaudio et al., 2012).

Overall, swapping from homopolymer to copolymer production with varying physiological conditions by Bacillus spp. has increased their significance as PHA producers. Type and optimum concentration of substrates and/or feed for copolymer production is usually strain dependent. This in turn governs the biomass and quality of the PHA produced. B.megaterium Ti3 of the present study exhibited varied profiles of PHA yields and majorly accumulated scl homopolymers and copolymer of C3 and C5 atoms only. The innate ability of this wild-type strain to produce PHA copolymer P(3HB3HV) in the presence of structurally related fatty acid and inorganic nitrogen source gives an option of stable copolymer biosynthetic process in comparison to the genetically engineered strains, thus making it an interesting strain to be worked on. The role of nitrogen source type, in the switch to copolymer synthesis from related substrates could be further investigated. Also, the optimization of cultural conditions and investigation of material properties of the copolymer P(3HB-co-3HV) obtained in the present study would pave a way for testing its application potential in various fields.