

## Evaluation of Upstream Process parameters influencing the growth associated PHA accumulation in *Bacillus* sp. Ti3

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Batch fermentation of a newly characterized *Bacillus* strain, *Bacillus* sp. Ti3, was investigated for PHA accumulation. Time course study revealed growth associated PHA production, with maximum PHA yield  $0.6 \pm 0.02$  g/L and  $44.1 \pm 0.9$  % PHA accumulation coinciding with the maximum biomass stage of 24 h followed by a time dependent decline due to onset of sporulation. pH 7 supported maximum PHA yield of  $0.86 \pm 0.01$  g/L and PHA accumulation of  $48.0 \pm 0.72$  %. Biomass of  $2.1 \pm 0.1$  g/L and  $0.92 \pm 0.07$  g/L PHA yield was obtained with 4% (v/v) inoculum. Glucose supplemented with casein hydrolysate gave the highest biomass  $1.85 \pm 0.02$  g/L, PHA yield  $0.96 \pm 0.02$  g/L and  $51.6 \pm 0.5$  % PHA accumulation. Fermentation kinetic parameters of the *Bacillus* sp. Ti3 grown in batch cultures with optimized culture conditions accounted for 1.7 and 1.2 fold increase in PHA yield (0.57g/L to 0.96g/L) and PHA accumulation (44.1% to 51.6%). FTIR analysis of the polymer revealed that the strain was a potent polyhydroxybutyrate (PHB) producer. A relatively high yield of polyhydroxybutyrate within 24h, independent of nutrient limitation and precursors in a single growth phase makes this strain a promising option for industrial PHA production.

**Keywords:** Polyhydroxyalkanoates, *Bacillus* sp. Ti3, optimization, one factor approach, FTIR.

### Introduction

Polyhydroxyalkanoates (PHAs) represent a promising group of biodegradable polymers, produced intracellularly in a wide range of bacteria. Their biodegradability, biocompatibility, thermoplasticity and zero-toxicity characteristics, make them suitable for various applications in industry, medicine or agriculture<sup>1,2</sup>. Possessing similar material properties to conventional plastics makes them excellent substitutes for petroleum-derived polymers<sup>3</sup>. Their current production scales account for only a small fraction of the global plastic production, which is mostly due to the comparably high production costs<sup>4,5</sup>. Optimization of the key-process variables and fermentation strategies, do contribute in reducing the production costs. One factor at a time approach for optimization of fermentation conditions has been used to substantially enhance yield and productivity of many bioprocesses. In this view, aim of the present study was to optimize the physical and nutritional parameters for maximization of polymer yield and productivity by *Bacillus* sp. Ti3.

### Experimental Section

#### Microorganism

*Bacillus* sp. Ti3 isolated and identified as a potent PHA producer in the previous study was used<sup>6</sup>. The 16S rRNA gene sequence has been deposited with EMBL-ENA under the accession number HF968632. The strain was maintained on Nutrient agar slants at 4°C.

#### PHA Production: Growth medium and culture conditions

Overnight grown Nutrient Broth culture of *Bacillus* sp. Ti3 was used as inoculum (1% v/v) for PHA production medium (pH 6.8) containing (g/L) 10 glucose, 0.2 MgSO<sub>4</sub>, 0.1 NaCl, 0.5 KH<sub>2</sub>PO<sub>4</sub>, 2.5 peptone, and 2.5 yeast extract<sup>7</sup>. Production studies were carried out in 250ml flasks containing 100 ml culture medium and incubated at 30°C on a rotatory shaker at 120 rpm for 24h. After incubation, samples were analyzed for cell growth and PHA accumulation. Biomass content (DCW: dry cell weight) was evaluated by gravimetry. For this, 10ml of the culture sample was centrifuged (8,000 rpm, 10 min) separately; cell pellet was washed with distilled water and dried to a constant weight at 60°C.

#### Fermentation Optimization using One factor at a time approach

Media used for optimization studies using *Bacillus* sp. Ti3 were the same as described earlier. The effect

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of incubation time, concentration of precursors such as acetate and 3-hydroxybutyrate, pH and inoculum percentage on PHA accumulation was determined. The ratio of Glucose to Yeast extract and Peptone (YE+P) was 2:1. The incubation time was varied from 0 to 120h. Precursors Na-acetate or Na-3-hydroxybutyrate were tested at two different concentrations (2g/L or 3g/L) for two different time periods 24h and 48h respectively. pH of the medium was varied in the range 5.0 to 9.0, overnight grown inoculum percentage was varied from 1 to 10%, and the fermentation was carried as stated before.

#### Influence of Nutritional parameters: carbon and nitrogen sources

Glucose of the  $N_2$  deficient medium was replaced with different carbon sources such as mannose, xylose, arabinose, fructose, maltose, sucrose, starch, carboxy-methyl cellulose, and glycerol. The nitrogen sources Yeast Extract and Peptone both together were replaced with (ammonium nitrate, sodium nitrate, ammonium sulfate, corn steep liquor, beef extract, tryptone, casein, soyabean meal, yeast extract and peptone respectively). The carbon and nitrogen source concentration was maintained as in the production medium (2:1).

#### Extraction and Quantification of PHA

10 ml of 24h grown culture was centrifuged at  $10,000\times g$  for 15 min. 5ml Sodium hypochlorite was added to pellet and incubated at  $30^\circ C$  for 2 h which was then subjected to centrifugation at  $10,000\times g$  for 15 min followed by washing the pellet with distilled water, acetone, methanol and diethyl ether respectively for washing and extraction. Quantification of PHA was performed routinely by gravimetric analysis<sup>8</sup>. The dry cell weight (DCW) was used as a reference for the dried PHA pellet weight.

#### Statistical Analysis

The optimization experiments were carried out in triplicate. The data was analyzed using MS Excel and SPSS-ANOVA. All data sets were expressed along with their mean standard deviation.

#### Ultraviolet (UV) and Fourier transform infrared (FTIR) spectroscopic Analysis

The ultraviolet (UV) absorption spectrum of the polymer was analyzed following its conversion to crotonic acid by treatment with concentrated  $H_2SO_4$ , and the absorbance was scanned between 200 and 350 nm with UV. The presence and characterization

of PHA in dry cell matter was confirmed by Fourier transform infrared spectroscopy (FTIR)<sup>9</sup>. Precipitated dry PHA polymer from *Bacillus* sp. Ti3 was used to prepare KBr discs. Spectra were recorded between 400 and  $4000\text{ cm}^{-1}$  using Nicolet 200 FTIR spectrometer from the Nicolet Instrument Corporation, USA.

## Results and discussion

#### Effect of incubation Time, precursor concentration, pH and inoculum density on PHA production

Bacteria used for the production of PHAs can be divided into two groups based on the culture conditions required for PHA synthesis. The first group of bacteria requires the limitation of an essential nutrient such as N, P, Mg, K, O or S for the efficient synthesis of PHA from an excess carbon source. *Ralstonia eutropha*, most extensively studied industrial PHA producer belongs to this group. The second group of bacteria, do not require nutrient limitation for PHA synthesis and are able to accumulate polymer during growth. *Alcaligenes latus*, a mutant strain of *Azotobacter vinelandii*, *Azotobacter beijerinckii* and recombinant *E. coli* belong to the second group. These characteristics have to be taken into consideration while production of PHA<sup>10,11</sup>. The time-course of aerobic growth and PHA accumulation measured at various time intervals revealed that *Bacillus* sp. Ti3 belongs to the second group as PHA accumulated during the growth phase (Fig.1). Biomass increased steadily, attained its maximum

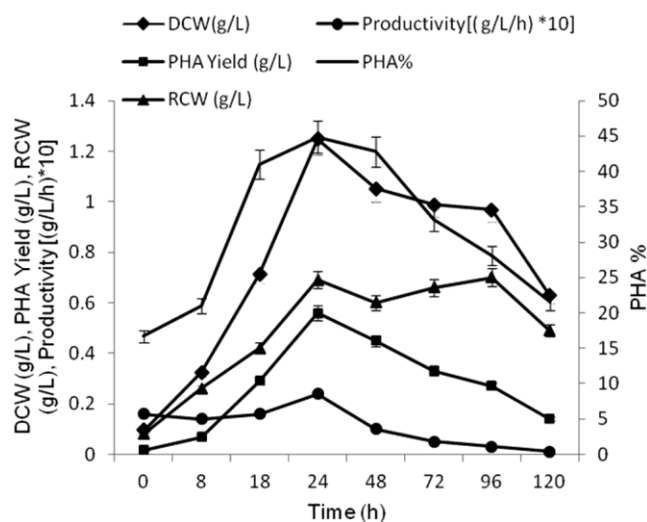


Fig. 1—Effect of incubation time on biomass yield and PHA production by *Bacillus* sp. Ti3. DCW (g/L) = cell dry weight, PHA yield (g/L) = PHA content in the medium, RCW (g/L) = Residual cell dry weight, Productivity [(g/L/h)\*10] = PHA yield per hour, %PHA (wt/wt) = PHA accumulation based on cell dry weight.

1.3  $\pm$  0.05 g/L in 24 h of cultivation and then declined gradually. The maximum PHA yield 0.6  $\pm$  0.02 g/L accounting for 44.1  $\pm$  0.9 % PHA accumulation coincided with the maximum biomass stage of 24 h and then decreased in time dependent manner. There is an interesting relationship between the residual biomass and PHA content. Since PHA is accumulated in the cytoplasm, the residual biomass determines how much PHA can potentially be produced. Cell growth and PHA accumulation need to be balanced, thus avoiding incomplete PHA accumulation at low cell concentration. A high residual biomass with a high PHA content will give the best results but there exists an upper limit of PHA concentration that can be obtained owing to the maximum cell concentration practically achievable in a fermentor. Therefore, it is important to decide when to terminate the fermentation. In most cases, fermentation is stopped when the productivity is highest<sup>10</sup>. In view of this, in the present study, 24 h was selected as the optimum incubation time corresponding to the highest biomass with concomitantly high PHA yield, residual biomass therefore leading to maximum productivity of 0.024 g/L/h (Fig.1). The decrease in PHA accumulation observed after the cessation of cell growth could be attributed to the intracellular consumption of PHA as carbon and energy source for sporulation. Though known for rapid growth and ability to utilize a variety of cost-effective substrates, the large-scale PHA production using *Bacillus* sp. has met limited success due to the interference of sporulation with PHA production<sup>12,13</sup>. *Bacillus* sp. have shown maximum PHA accumulation during the late log phase or early stationary phase, followed by degradation of the accumulated PHA at the onset of sporulation<sup>14,15,16</sup>. In this study also, PHA accumulation had attained its maximum until the late exponential phase of 24 h, following which it decreased due to the onset of sporulation (data not shown). The highest yield attained at 24 h of growth by the strain could be explained by the maximum cell growth at that stage. Hence, PHA accumulation appeared to be growth associated. In contrast to the report of Valappil *et al.*, 2007<sup>17</sup>, the observations of PHA yield, residual pH and sporulation index in the present study were in concordance to previous report on a *Bacillus* sp. where low pH conditions have been shown to inhibit utilization of the polymer as well as spore formation in *B. cereus* T<sup>18</sup>. This observation in the present study is important for the production of PHA using *Bacillus* sp. Ti3. An optimized media favoring maximum

polyhydroxybutyrate production over sporulation was evaluated by<sup>12</sup>, wherein a correlation between the above two stated was demonstrated. Thus, optimization of fermentation process parameters like media composition and pH might enhance and maintain PHA content even at the instance of sporulation. Acetyl-CoA and 3-hydroxybutyryl-CoA are intermediates of the PHA anabolism. Precursors such as Acetate and 3-hydroxybutyrate should be able to increase the concentrations of acetyl-CoA and 3-hydroxybutyryl-CoA, respectively, in the cells and hence facilitate the synthesis of PHA<sup>14</sup>. A marginal positive influence of acetate on accumulation of PHA by *Bacillus* sp. Ti3 was observed. PHA yield had increased from 0.66g/L to 0.76 g/L (2g/L acetate) and 0.85g/L (3g/L acetate) respectively in 24h. Similar levels of influence on PHA accumulation by *Bacillus megaterium* DSM 90 were also observed in presence of 2g/L of acetate and 3-hydroxybutyrate<sup>14</sup>. As the % PHA accumulation by *Bacillus* sp. Ti3 was good enough even without addition of any of the precursors in 24h time period, further optimization studies were carried out without any precursor supplementation. The influence of initial culture pH was investigated for 2:1 G: (Y+P) ratio. Although the growth of culture and PHA accumulation was supported at lower and higher pH values, maximum PHA yield of 0.86  $\pm$  0.01g/L and PHA accumulation of 48.0  $\pm$  0.72 % was observed at pH 7.0. PHA accumulation was observed to decrease slightly beyond pH 7.0. Similar optimum pH values have also been recommended by Borah *et al.*, 2002<sup>19</sup>; Aarthi Narayanan *et al.*, 2012<sup>12</sup>; Tamdogan and Sidal 2011<sup>20</sup>, wherein pH range 7.0 – 7.5 has been identified as optimum for maximum PHA accumulation using different *Bacillus* spp. Studies on the effect of different inoculum concentration showed that biomass and PHA yield increased steadily and attained maximum yield of 2.1  $\pm$  0.1 g/L and 0.92  $\pm$  0.07 g/L, respectively, with a PHA accumulation of 43.5 $\pm$ 0.75 %, with 4% inoculum (v/v) and then declined gradually.

#### Effect of nutritional parameters: Carbon and Nitrogen sources on PHA production

Carbon sources serve three different functions within the organisms: biomass synthesis, cell maintenance, and PHA polymerization. *Bacillus* spp. is known for their metabolic versatility, and therefore the isolate *Bacillus* sp. Ti3 was also able to utilize all ten carbon sources for growth and PHA accumulation

(data not shown). However, the maximum PHA yield  $0.75 \pm 0.02$  g/L corresponding to  $47.8 \pm 0.9$  % of cell dry weight was obtained using glucose as the sole carbon source, followed by sucrose, fructose, maltose, starch, xylose, glycerol, CMC, mannose and arabinose. Glycerol as a sole carbon source was able to support PHA yield  $0.24 \pm 0.01$  g/L accounting to  $29.7 \pm 1.0$  % of cell dry weight, suggesting that the isolate could also be tested for PHA production using crude glycerol (CG), a by-product of biodiesel production<sup>21</sup>. Starch and CMC accounted for  $33.0 \pm 0.9\%$  and  $27.5 \pm 1.0$  % PHA accumulation, respectively. *Bacillus* sp. Ti3 has already been reported to produce an array of hydrolytic enzymes including amylase and cellulase and accumulate PHA utilizing the respective complex substrates<sup>6</sup>. Less PHA accumulation % could be attributed to the complex structure of starch and CMC, requiring more than 24h time period for transforming these complex substrates to simple absorbable metabolites, and making them available for PHA production. Increase of incubation time in case of complex substrates might lead to enhancement of PHA yield and % PHA accumulation by the isolate.

The effect of different nitrogen sources on the growth and PHA production by the isolate in media containing glucose as a carbon sources showed that, casein hydrolysates gave the highest biomass  $1.85 \pm 0.02$  g/L and PHA yield  $0.96 \pm 0.02$  g/L accounting for  $51.6 \pm 0.5$  % PHA accumulation, followed by beef extract, tryptone and yeast extract. The organic nitrogen sources ( $30.7 \pm 0.5$  % to  $51.6 \pm 0.5$  %), supported higher PHA % accumulation than the inorganic nitrogen sources ( $18.4 \pm 0.7$  % to  $42.5 \pm 0.5$  %). Complex nitrogen sources can be supplemented to enhance cell growth as well as polymer accumulation in case of growth associated PHA producers, since polymer synthesis is not dependent on nutrient limitation in such bacteria<sup>10,11</sup>. The enhancement of PHA accumulation may be due to the presence of amino acids and peptides in such organic nitrogen sources, further leading to the channeling of carbon source to PHA biosynthetic pathway. Similar enhancement of PHA accumulation by *Bacillus mycoides* RLJ B-017<sup>19</sup>, *Bacillus mycoides* DFC1<sup>12</sup>, *Bacillus subtilis* 25 and *Bacillus megaterium* 12<sup>16</sup>, *Azotobacter vinelandii* UWD<sup>22</sup>, *Azotobacter chroococcum*<sup>23</sup>, *Azotobacter beijerinckii*<sup>24</sup>, *Ralstonia eutropha*<sup>25</sup>, recombinant *E.coli*<sup>26</sup> has been reported when the organisms were grown in media containing

organic nitrogen sources. In future, several renewable materials, e.g. casein hydrolysates or glutene, known to be available from agriculture as nitrogen sources can be exploited as a low cost substrates for cost-effective production of PHAs<sup>19,27</sup>. Amongst inorganic nitrogen sources ammonium nitrate was able to support  $42.5 \pm 0.5$  % PHA accumulation, this can be advantageous with respect to the industrial applications of the strain, wherein  $\text{NH}_4^+$  containing waste liquids of various industries could be utilized for PHA production<sup>19</sup>. A comparison of the fermentation kinetic parameters of the *Bacillus* sp. Ti3 grown in batch cultures with unoptimized and optimized culture conditions (Table 1), revealed that glucose with replacement of yeast extract and peptone with casein hydrolysate as nitrogen source in the production medium, accounted for 1.7 and 1.2 fold increase in PHA yield (0.57g/L to 0.96g/L) and % PHA accumulation (44.1% to 51.6%). Accordingly, the PHA productivities  $Q_p$  and  $q_p$  also had increased by 1.7 and 1.2 folds respectively. PHA production by the members of genus *Bacillus* has been shown to range from 1.06 to 57.2%<sup>6</sup>. There have also been few studies reporting higher PHA accumulation in various *Bacillus* sp. such as *Bacillus mycoides* DFC1 (3.32g/L, 76.32% in 72h)<sup>12</sup>, *B.mycoides* RLJB-107 (56.6% in 24h)<sup>19</sup>, *Bacillus* sp. 87I (1.9g/L, 70.04% PHB DCW in 32h) and *Bacillus* sp. 112A (1.8g/L, 67.63% PHB DCW in 36h)<sup>28</sup>, respectively. The PHA yield  $0.96 \pm 0.02$  g/L accounting for  $51.6 \pm 0.5$  %

Table 1—Fermentation kinetic parameters of the *Bacillus* sp. Ti3 grown in batch culture's with unoptimized and optimized culture conditions.

| Parameters                  | Unoptimized | Optimized |
|-----------------------------|-------------|-----------|
| PHA yield (g/L)             | 0.57        | 0.96      |
| DCW (g/L)                   | 1.3         | 1.86      |
| $Q_p$ $g_{PHA}/l/h$         | 0.024       | 0.04      |
| $Q_x$ $g_{DCW}/l/h$         | 0.054       | 0.08      |
| $q_p$ $g_{PHA}/g_{DCW}/h$   | 0.018       | 0.022     |
| $Y_{p/s}$ $g_{PHA}/g_s$     | 0.057       | 0.096     |
| $Y_{x/s}$ $g_{DCW}/g_s$     | 0.13        | 0.186     |
| $Y_{p/x}$ $g_{PHA}/g_{DCW}$ | 0.441       | 0.52      |
| PHA(%DCW)                   | 44.1        | 51.6      |

$Q_p$ , Volumetric rate of polyhydroxyalkanoate (PHA) formation ( $g_{PHA}/l/h$ );  
 $Q_x$ , volumetric rate of cell formation ( $g_{DCW}/l/h$ );  
 $q_p$ , specific rate of PHA formation ( $g_{PHA}/g_{DCW}/h$ );  
 $Y_{p/s}$ , PHA yield at the end of fermentation ( $g_{PHA}/g_s$ );  
 $Y_{x/s}$  cell yield at the end of fermentation ( $g_{DCW}/g_s$ );  
 $Y_{p/x}$ , PHA yield based on cells at end of fermentation ( $g_{PHA}/g_{DCW}$ )

PHA accumulation in 24h incubation time, obtained in the present study after optimization, is comparatively higher when compared with other *Bacillus* sp. strains like *Bacillus* sp INT005 (0.14g/L, 35.30 % in 48h)<sup>29</sup>, *Bacillus cereus* SPV (38.0 % in 48h)<sup>30</sup>, *Bacillus cereus* CFR06 (1.0g/L, 50% in 72h)<sup>31</sup>, *Bacillus mycoides* (WSS2) (0.142g/L in 72h)<sup>32</sup>, *Bacillus subtilis* ATCC 6633 (0.01g/L in 24h)<sup>20</sup> reported so far. Selection of microorganism for the industrial scale PHA production is governed by several criteria, such as growth rate, the ability of the cell to utilize an inexpensive carbon source (agricultural and dairy wastes are most recently thought of), cost of medium, polymer synthesis rate and the quality and quantity of PHAs, and the cost of downstream processes. Isolation of potential bacterial strains from natural environment, improvisation of bioprocess parameters or by designing recombinant strains fulfilling one or more of the above criteria has been an area of extensive research in the field of PHA production<sup>33</sup>. In this respect, the newly isolated *Bacillus* sp. Ti3 giving, a relatively high yield of PHA in 24h and also varied hydrolytic enzymes production<sup>6</sup> can be considered as a potential candidate for cost effective industrial scale PHA production.

#### Statistical analysis

ANOVA (one way) was performed for each of the optimization factors affecting the production of PHA by *Bacillus* sp. Ti3 using the one factor approach. P-value was found to be significant for all the factors ( $P \leq 0.05$  level of significance) indicating the significant influence of all the upstream parameters tested.

#### UV and FTIR Analysis

Preliminary analysis of PHA produced, by digesting the polymer with concentrated  $H_2SO_4$  and scan with UV-Vis spectrophotometer revealed absorption maximum at 235 nm, characteristic of crotonic acid indicating the presence of polyhydroxybutyrate type of polyhydroxyalkanoate biopolymer (data not shown). FTIR analysis of the isolated polymer from *Bacillus* sp. Ti3, using glucose as the carbon source, revealed absorption bands at  $1724\text{ cm}^{-1}$ , corresponding to the ester carbonyl group and at  $1280\text{ cm}^{-1}$  corresponding to the  $-CH$  group, characteristic of polyhydroxybutyrate type of polymer<sup>9</sup> (Fig. 2).

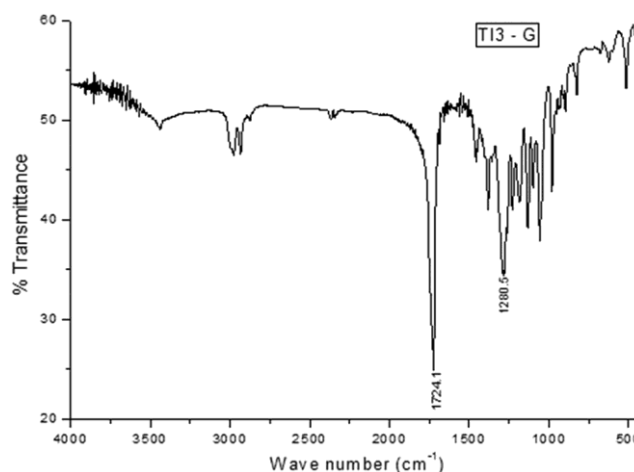


Fig. 2—Fourier transform-infrared (FTIR) absorption spectra of the PHA isolated from *Bacillus* sp. Ti3 when grown on Glucose as the carbon source.

#### Conclusion

Media optimization by one factor approach revealed that a combination of glucose and casein hydrolysate in the ratio of 2:1 produced maximum polyhydroxybutyrate within 24h of growth independent of nutrient limitation or precursors. Further the ability of the strain to accumulate PHAs other than PHB by growth on n-alkanoic acids can also be explored.

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