



## A kinetic study of polyhydroxybutyrate production on nitrogen limited medium using *Bacillus subtilis* MTCC 9763 through a two stage cultivation strategy

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### Abstract

In this study, a two-stage cultivation strategy was adopted for the production of Poly-β-hydroxy butyrate (PHB), using *Bacillus subtilis* MTCC 9763. The culture conditions for PHB production by *Bacillus subtilis* MTCC 9763 were optimized with different carbon, nitrogen sources, temperature, pH and incubation time. Glucose showed a maximum of 73 mg l<sup>-1</sup> (12.56%) yield out of the carbon sources used, whereas peptone gave 81 mg l<sup>-1</sup> PHB (14.72%) for nitrogen source. The optimized condition observed for PHB production was 42 hr of incubation time with a temperature of 37 °C and pH 7. A two stage cultivation strategy was approached. Nitrogen source was limited in the production medium with various concentrations (25%, 50%, 75% and 100%) for enhanced production of PHB. Product yield was doubled when the nitrogen concentration was completely limited as compared with 75% of limitation with an yield of 93 mg l<sup>-1</sup> (20.26%). PHB was characterized using Infra Red spectroscopy. A kinetic model that describes microbial growth, cell concentration and biopolymer production was used to predict the performance of batch fermentation of *Bacillus subtilis* MTCC 9763. The experimental data was also fitted with the logistic equation that could provide adequate description for PHB synthesis. The present investigation revealed that industrial effluents could also be used for effective production of PHB so as to greatly reduce the risk of production cost.

### Key words

Industrial effluents, Kinetic model, Logistic equation, Poly-β-hydroxybutyrate, Sodium hypochlorite

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### Introduction

Plastic materials which have made entry in every sphere of human life are now causing serious environmental problems due to their non biodegradability. The intrinsic qualities of durability and resistance to degradation, over the last two decades, have been increasingly regarded as a source of environmental and waste management problem emanating from plastic materials (Ojumu *et al.*, 2004). One option is to produce truly biodegradable polymers, which may be used in the same applications as the existing synthetic polymers. These materials, however, must be processible, impervious to water and capable of retaining their integrity during normal use but readily degradable in a biologically rich environment.

Biodegradable materials under development include polylactides, polyglycolic acids, polyhydroxyalkanoates (PHAs), aliphatic polyesters, polysaccharides and their co-polymers and/or blends (Desouky and Haleem, 2009). Among these, PHAs are of particular interest because they possess thermoplastic characteristics and resemble synthetic polymers to a larger extent.

Plastics produced from PHAs have been reported to be truly biodegradable in both aerobic and anaerobic environments (Yilmaz *et al.*, 2005). PHAs are composed mainly of poly-beta-hydroxybutyric acid (PHB) and poly-beta hydroxyvaleric acid (PHV), although other forms are possible. More than 90 different forms of PHAs have been detected in bacteria (Wang *et al.*, 2013).

PHA accumulation can be stimulated under unbalanced growth conditions, *i.e.*, when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C: N ratio of the feed substrate is higher (Nandini *et al.*, 2011). PHB is accumulated by numerous microorganisms and is the best characterized PHA (Madison and Huisman, 1999). PHB was first discovered in bacteria (Ojumu *et al.*, 2004). It is a unique intracellular polymer, accumulated under stress conditions but, with excess carbon source. During starvation, PHB serves as carbon and energy source and is rapidly oxidized, thereby retarding the degradation of cellular components, combating the adverse conditions as in rhizosphere (Kadouri *et al.*, 2002). *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) has been the subject of much published research work because it can accumulate PHAs upto 80 % dry weight (Yilmaz *et al.*, 2005).

The production of biodegradable plastics on a large scale is limited because of relative expense of the substrate and low polymer production. According to Ojumu *et al.* (2004), higher production costs, especially raw material costs, make it difficult for PHA plastics to compete with conventional petroleum-based plastics in commercial market place. Hence, alternative strategies for PHB production are being investigated. PHB production costs could be reduced by several means such as by using cheaper substrates like agro-industrial residues (Pandey *et al.*, 2009) or enhancing of product yield *e.g.*, by using recombinant *E. coli* (Heshiki, 2013 ). There have been some investigations on the possibility of producing PHB in transgenic plants also (Wang *et al.*, 2013).

PHBs are natural thermoplastic polyesters, that can be produced from inexpensive substrates such as whey and starch (Kim, 2000). The success in biodegradable plastic strategy largely depends on the usage of different agricultural and industrial effluents as an alternative source of carbon. In the recent past, researches have been focused on two-stage fermentation strategy for enhanced production rate of PHB (Wei *et al.*, 2011; Sun *et al.*, 2007; Hartmann *et al.*, 2010).

In view of the above, the objective of the current work was to adopt a two stage approach for batch production of Poly- $\beta$ -hydroxy butyrate, supplemented with various limited concentrations of nitrogen source. Cell growth concentration and PHB production kinetics were analyzed with Ludeking-piret model and logistic model, where the experimental results were fitted with predicted models.

### Materials and Methods

**Microorganism :** *Bacillus subtilis* MTCC 9763 was obtained from Microbial Type Culture Collection (MTCC) Chandigarh, India and maintained on agar slants. The primary inoculum was prepared in Nutrient Broth medium in a 250 ml Erlenmeyer flask, containing

100 ml of sterile medium (autoclaved at 121°C for 15 min.) and inoculated from stock culture. After 16 hr of incubation at 37°C and 200 rpm, 2 ml of culture was taken to inoculate the flask containing 100 ml of sterile cultivation medium, which contained: Glucose, 0.5%; Yeast extract, 0.3%; NaCl, 0.5%; Peptone, 0.5%. The flasks were incubated at 37 °C and 200 rpm in an incubator shaker for 48 hrs.

Lipophilic stain, Sudan black was used for the detection of PHB production by *Bacillus subtilis* MTCC 9763. Sudan black stain was prepared as a 0.3% solution (w/v) in 60% ethanol. Smears of PHB producing bacteria were prepared on glass slides and heat fixed. Samples were stained for 10 min with Sudan black solution, rinsed with water and counter-stained with 0.5% safranin for 5 sec. Stained samples were observed under oil immersion at 400x magnification with direct bright-field illumination, using a Light microscope.

**Optimization :** Different factors affecting PHB production by *Bacillus subtilis* MTCC 9763 were optimized *viz.*, temperature, carbon source, nitrogen source, pH and incubation time.

Poly- $\beta$ -Hydroxy Butyrate (PHB) production on various incubation time (6-48hr) was studied. *Bacillus subtilis* MTCC 9763 was grown in 250 ml conical flasks containing 100 ml nutrient broth and kept for incubation in a rotary shaker (200 rpm) at 37 °C and pH 7. PHB production was quantified by taking culture samples from 6-48hr at an interval of 6hr. Different temperature ranges such as 28°C, 32°C, 37°C and 42°C were maintained in the medium and incubated for 48 hr at pH 7. PHB produced by *Bacillus subtilis* MTCC 9763 was quantified spectro photometrically.

Production media was then incubated at different pH, ranging from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, in a rotary shaker (200 rpm) kept at 37 °C and pH 7. After 48 hrs of incubation, PHB produced was quantified. Different carbon sources such as glucose, fructose, mannitol, and maltose were used in the medium at 5 g l<sup>-1</sup> concentration and incubated for 48 hr to study their effect on PHB production. Also, different nitrogen sources of peptone, ammonium phosphate, potassium nitrate and glycine were used at 5 g l<sup>-1</sup> concentration to study their influence on PHB production. After 48 hr, PHB yield was quantified.

**Two stage approach on PHB production :** *B. subtilis* MTCC 9763 was grown in four 250 ml conical flasks of 100 ml nutrient broth containing optimized media composition and kept for 24 hr of incubation (Stage 1). After 24 hrs, the culture sample was centrifuged and biomass was transferred in to four different optimized media (Stage 2) containing various limited nitrogen concentrations (3.75 , 2.5 , 1.25 and 0 g l<sup>-1</sup>) with excess carbon, incubated in a rotary shaker (200 rpm) kept at 37 °C and pH 7. After 24 hrs of incubation, cells were harvested and quantified, using UV spectrophotometer at 235 nm.

**Determination of PHB:** Biomass was collected by centrifugation of culture sample (10 ml) at 8000 rpm for 15 min. The pellet was digested with 30% sodium hypochlorite solution at 37 °C for 1 hr. Spectrophotometric assay was done as described by Law and Slepecky (1960). The residue was collected by centrifugation at 8000 rpm for 15 min and a series of washing steps were performed using water, acetone and finally ethanol. The polymer was dissolved in hot chloroform and kept for evaporation. Then 4.5 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added to 0.5 ml chloroform-polymer mixture and heated for 10 min at 80 °C in a water bath. The resultant crotonic acid was measured at 235 nm against H<sub>2</sub>SO<sub>4</sub> as blank in an Ultra Violet (UV) spectrophotometer.

Ten mg of the extracted sample of PHB (from 100% nitrogen source limited medium) was subjected to Infra Red analysis. The relative intensity of transmitted light energy was measured against the wavelength of absorption on the region ranging from 400-4000 cm<sup>-1</sup>, using a JEOL-FT IR-4000 plus double beam spectrometer. Infrared spectrum of the sample was measured at ambient conditions.

**Growth kinetic studies of the culture:** The medium containing glucose and peptone was used for kinetic studies as per the kinetic model. Certain important features of PHB production kinetics by *Aspergillus niger* were incorporated in the present study (Fatmawati *et al.*, 2009). In the present study, unstructured fermentation model was used to obtain kinetic parameters such as  $\mu_m$ ,  $\alpha$ ,  $\beta$ ,  $a$  and  $b$ .

## Results and Discussion

PHB present in *B. subtilis* MTCC 9763 was determined using Sudan Black B stain (Fig. 1). Under oil immersion microscope, PHB granules appeared blackish in color while cell cytoplasmic contents appeared as pink due counter staining of 0.5% safranin.

Different temperature ranges showed noticeable increase in PHB production between 32 °C and 37 °C rather than at 28 °C and 42 °C. Maximum yield of 71 mg l<sup>-1</sup> (10.28%) was obtained at 37 °C. Maximum production of PHB was recorded at a temperature of 33 °C, as reported by Grothe *et al.* (in the range of 25 °C-37 °C). Out of different carbon sources used, medium containing glucose showed highest production of PHB with 73 mg l<sup>-1</sup> (12.56%) yield. While maltose showed the second highest PHB yield of 5.38% (45 mg l<sup>-1</sup>), followed by mannose with 43 mg l<sup>-1</sup> of PHB and sucrose with 36 mg l<sup>-1</sup> of yield. Since glucose is a simple monosaccharide involved in EMP pathway of PHB production, it is readily utilized by microbes. In *Bacillus megaterium*, PHB content in the cell reached maximum level (39.9%) after growing in glucose as the sole carbon source (Borah *et al.*, 2002).

Peptone containing medium showed maximum absorbance with a yield percentage of 14.72% and PHB concentration of 81 mg l<sup>-1</sup>. Parshad *et al.* (2001) utilized peptone

for growth and PHB production by *Azotobacter chroococcum* and our study also reported the maximum PHB production using peptone as nitrogen supplement. Of the various pH ranges maintained, *Bacillus subtilis* MTCC 9763 showed highest PHB produced at pH 7 with 83 mg l<sup>-1</sup> (10.64%) concentration, since pH 7 is the optimal value for the enzymes involved in biosynthesis of PHB. At pH 5 and 6, due to change in pH from the optimum, enzyme activity decreased which in turn resulted in decreased production rate of PHB (Kulprecha *et al.*, 2009). PHB Yield was increased slowly for every 6 hr until 24 hr. PHB concentration of 31 mg l<sup>-1</sup> was obtained at 24 hr and increased to a maximum of 50 mg l<sup>-1</sup> at 42 hr. Yield percentage was decreased to 5.94% (44 mg l<sup>-1</sup>) at 48 hr due to utilization of PHB storage granules by the organism while entering the sporulation cycle (Pandey *et al.*, 2009) (Fig. 2a).

Two-stage approach was developed since higher production rate of PHB is essentially needed. PHB production was 73 mg l<sup>-1</sup> (7.89%) at first stage until *Bacillus subtilis* MTCC 9763 was grown on nutrient broth medium for 24 hrs. After 24 hrs at second stage, due to sudden change of environment to low nitrogen concentration medium (75, 50, 25 and 0% respectively), production of PHB showed some remarkable increase. Increased biomass at first stage (24 hr) showed maximum utilization of the substrate to get higher biomass. The nitrogen limited medium at second stage (24hr to 48 hr) provided an environment for the organism to synthesize PHB to the maximum level. PHB production showed a slow increase for 25, 50 and 75% limited concentrations with yield percentage of 8.16, 10.17 and 11.22% respectively. Highest PHB production was observed at 100% limited nitrogen source with PHB concentration of 93 mg l<sup>-1</sup> (20.26%) (Fig. 2b). Previous reports recorded an increased PHB content from 52 to 83.9% using *A. latus* in a nitrogen limited medium (Valappil *et al.*, 2008). In the current study, an increase of PHB from 11.22 to 20.26% was recorded and it is in line with the above report.

Similar reports using two stage cultivation was discussed by Verma *et al.* (2012). PHB production was improved using two-stage fermentation by *Azotobacter vinelandii* UWD. At first stage, cell growth was promoted by supplementing a high aeration rate. In the subsequent second stage, PHB production was enhanced by lowering the aeration rate and the final PHB concentration of 36 g l<sup>-1</sup> was reported. Also, investigations carried out by Verma *et al.* (2012) showed significant importance of two stage approach in enhancing PHB productivity.

A maximum cell and PHB concentration was obtained when the nitrogen source was completely limited (0 g l<sup>-1</sup>) in the production medium. This concentration level was taken for the kinetic study in order to analyze the feasibility of obtained result.

IR analysis of the extracted sample showed the presence of CH<sub>2</sub>-CH<sub>3</sub>-C=O methyl ester groups which confirmed PHB. IR

spectrum of the compound was recorded in the range of 400-4000 $\text{cm}^{-1}$  and it showed characteristic bonds for the groups CH, C=O and C-O. Methine (CH group) gave a strong bond in the range of 1456.2. This frequency value was higher than the normal



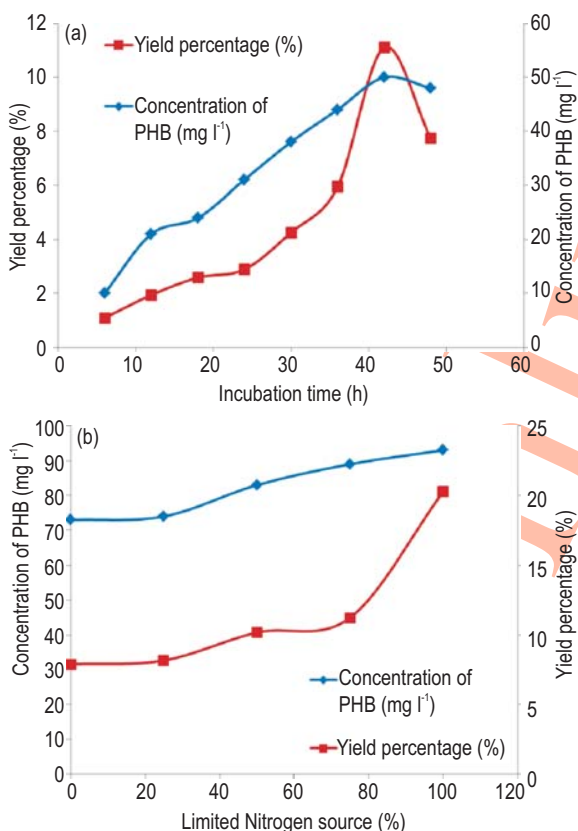
**Fig. 1 :** Sudan black B stain of PHB granules (stained black) by *Bacillus subtilis* MTCC 9763 observed under 100X oil immersion microscope

values because of polymerization. The carbonyl group (C=O) gave a strong bond in the region of 1634.7. This frequency value was lower than the normal values because of polymerization. C-O group showed strong absorption range of 1058.5.

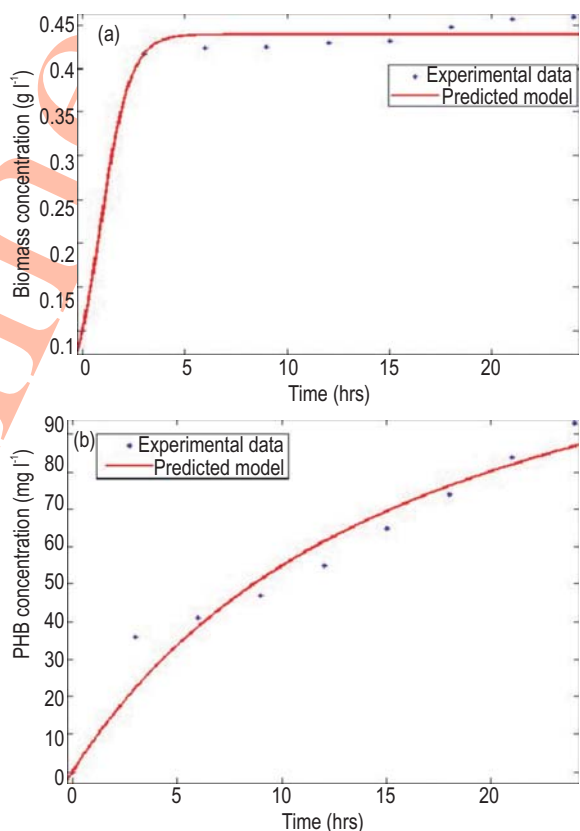
IR spectra of PHA film and the purified methyl ester are identical (Rawate and Mavinkurve, 2002). IR analysis of 100% limited nitrogen source medium was compared with the standard PHB chart as well as with that of the references (Senthilkumar and Prabhakaran, 2006).

Cell concentration profile of 100% limited nitrogen source concentration is shown in Fig. 3a and it showed that the model described by Fatmawati *et al.* (2009) fitted well with the experimental data obtained. By employing the kinetic equations, maximum cell concentration of 0.46  $\text{g l}^{-1}$  was obtained at 24 hr of second stage. This experimental value was found to be well fitted with the predicted results and forms good agreement.

Similarly, PHB production at 100% limited nitrogen source medium (0  $\text{g l}^{-1}$ ) showed a two fold increase in yield and showed 93  $\text{mg l}^{-1}$  PHB. This result of maximal PHB yield (20.26%) enhanced



**Fig. 2 :** PHB yield percentage and concentration on (a) different time intervals and (b) on different nitrogen limited concentration medium



**Fig. 3 :** Experimental (a) Cell and (b) PHB concentration profile and model obtained from 100% limited nitrogen source medium



**Table 1** : Values of kinetic parameters obtained from the Luedeking-Piret model

Parameter	Values for 100% limited nitrogen source
$X_0$ (g l <sup>-1</sup> )	0.1
$X_m$ (g l <sup>-1</sup> )	0.439
$\mu_m$ (h <sup>-1</sup> )	1.378
$\hat{a}$	2.163
$\hat{a}$ (L h <sup>-1</sup> )	1.12
$S_0$ (g l <sup>-1</sup> )	5.00
$a$ (mg l <sup>-1</sup> )	148.8
$b$ (h)	17.1

$X_m$ -maximum cell concentration (g l<sup>-1</sup>),  $X_0$ -initial cell concentration (g l<sup>-1</sup>), P-Product concentration (g l<sup>-1</sup>),  $\alpha$ -growth associated product formation constant,  $\beta$ -Non-growth associated product formation constant (L h<sup>-1</sup>),  $a$ -maximum product concentration (mg l<sup>-1</sup>),  $b$ -Time constant (h)

in the second stage of fermentation is reported for the first time with a 2 fold increase in PHB content using *B. subtilis* MTCC 9763 than that of 75% limited source. Also, the kinetic model studied for PHB production by supplementing the best produced limited nitrogen source concentration (100% limited) was fitted well with the predicted values that yielded a valuable result.

Results of the kinetic parameters obtained by applying the model equations are presented in Table 1. Maximum specific growth rate ( $\mu_m$ ) was observed to be 1.378 g l<sup>-1</sup> and maximum biomass concentration was found to be 0.439 g l<sup>-1</sup>. It was also observed that higher initial substrate concentration resulted in higher  $X_m$ ,  $X_0$ , and  $\mu_m$  values. These parameters obtained by applying the kinetic equations using Matlab software is inline with the results of Fatmawati *et al.* (2009).

Khanna and Srivatsava (2008) studied the growth of *A. eutrophus* and intracellular PHB storage kinetics. A modified kinetic model for PHB synthesis was proposed with different fed batch feed strategies (including constant nitrogen feed).

A similar study on PHB production kinetics was investigated by Pirouz *et al.* (2011). A kinetic model was conducted for both growth and production of PHB, with different carbon sources. Three models such as Logistic equation, Malthus model and Luedeking-piret model were used for determining kinetic parameters to yield maximum PHB productivity. In a similar way, by utilizing whey as a carbon source, maximum yield was obtained with the combination of all the models.

Therefore, such a kinetic model-based two stage approach can be implemented in real time fermentations that will ultimately enhance the productivity rate of PHB in the resulting fermentation.

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