

## Mixed Cultures Fermentation for the Production of Poly- $\beta$ -hydroxybutyrate

Thomas Shalin<sup>1</sup>, Raveendran Sindhu<sup>1</sup>, Parameswaran Binod<sup>1</sup>, Carlos Ricardo Soccol<sup>2</sup> and Ashok Pandey<sup>1\*</sup>

<sup>1</sup>Biotechnology Division; National Institute for Interdisciplinary Science and Technology; CSIR, Trivandrum-India.

<sup>2</sup>Departamento de Engenharia Química; Divisão de Engenharia de Bioprocessos e Biotecnologia; Universidade Federal do Paraná; Curitiba - PR - Brasil

### ABSTRACT

*Poly- $\beta$ -hydroxybutyrate (PHB) is a biodegradable intracellular microbial product produced by many bacteria and it is comparable to some of the petrochemical derived thermoplastics such as polypropylene. One of the main barriers for the commercial exploitation is the high cost of the substrate for the production of biopolymer. The utilization of mixed microbial cultures facilitates the use of complex substrates thereby reducing the cost of PHB production. In the present study, mixed culture systems were evaluated for PHB production. *Bacillus firmus* NII 0830 was used for the production of PHB since it accumulates a large amount of PHB and a second organism *Lactobacillus delbrueckii* NII 0925 was used to provide lactic acid. FTIR and <sup>1</sup>H NMR analyses revealed that the PHB extracted from pure culture and mixed culture showed exact match to that of standard PHB. Biodegradation studies of the PHB blends showed 87% degradation. It was also found that a consortium of organisms degraded the films faster than a single organism.*

**Key words:** Poly-3-hydroxybutyrate, Biopolymer, Mixed culture, *Bacillus firmus*, *Lactobacillus delbrueckii*, Biodegradation

### INTRODUCTION

Poly- $\beta$ -hydroxybutyric acid or PHB is a bacterial polymer whose biocompatibility, biodegradability and versatile properties make it an eco-friendly substitute for synthetic polymers. Microorganism can accumulate large amount of PHB inside the cell in response to the limitation of an essential nutrient.

The substrate is one of the main cost factors of PHB production (Lee 1996). Most processes for PHB production are based on pure cultures grown in well-defined nutrient-deficient synthetic media and single substrates. The use of renewable sources obtained from waste organic carbon and

mixed cultures could significantly decrease the price of PHB.

Alternative strategies for PHA production based on mixed cultures have been proposed as a means of lowering production costs (Dias et al. 2006). Mixed culture PHA production relies on a selection pressure that favors organisms with PHA storage capacity. In this case no reactor sterilization is necessary and the culture can adapt to various complex waste feed stocks (Albuquerque et al. 2007; Bengtsson et al. 2008; Serafim et al. 2008), leading to potential environmental and economical advantages compared to both pure culture PHA production. Fermentations employing two or more microorganisms offer benefits over single culture

\*Author for correspondence: pandey@niist.res.in

fermentations either by commensalism or by neutralism or by conversion of substrate to a metabolite by first microorganism which is utilized by the second microorganism to produce the product (Kondo et al. 1996; Shimizu et al. 1999).

Mixed or co-culture systems have been recognized to be effective for certain fermentations. Mixed bacterial culture production has recently started to attract a lot of attention. It has the potential to produce large amounts of PHAs with seemingly lower costs due to lower sterility, equipment and control requirements and the ability to utilize a wide range of cheap substrates including industrial and agricultural wastes (Reis et al. 2003; Rhu et al. 2003). Exploiting mixed culture system for PHB production where sugars were converted to lactate by lactate-producing bacteria and the lactate was converted to poly  $\beta$ -hydroxybutyrate (PHB) by the second organism.

The objective of the present study was to evaluate PHB produced by mixed culture, its characterization by FTIR and  $^1\text{H}$  NMR and biodegradation of blends using pure culture and microbial consortium.

## MATERIALS AND METHODS

### Chemicals and reagents

Crotonic acid was procured from Sigma-Aldrich, India. All other chemicals were procured from local vendors.

### Microorganisms

*Bacillus firmus* NII 0830, *B. sphaericus* NII 0838 and *Lactobacillus delbrueckii* NII 0925 were obtained from the NII culture collection at NIIST, Trivandrum. The stock cultures were maintained on nutrient agar slants.

### Culture media and inoculums preparation

LB media was used for preparing seed culture for *B. firmus* NII 0830 and *B. sphaericus* NII 0838 and MRS media was used for preparing seed culture for *Lactobacillus delbrueckii* NII 0925. Seed culture media in LB was prepared for *B. firmus* NII 0830 and *B. sphaericus* NII 0838 so as to make 18 h culture ready before inoculation in each experiment.

For *L. delbrueckii* NII 0925 seed culture was prepared in MRS media to make 18 h culture ready before inoculation. Mineral salt media

(MSM) was used for PHB production. The different carbon sources like glucose, xylose, glycerol, lactose, fructose and sucrose were used at 20 g/L concentration. The pH of the medium was adjusted to 7.0 before bacterial inoculation. The media were autoclaved at 121°C for 20 min.

### The effect of different carbon sources on growth and PHB production by mixed cultures

The effect of different carbon sources on growth and PHB production of mixed culture of *B. firmus* NII 0830 and *B. sphaericus* NII 0838, *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 and *B. sphaericus* NII 0838 and *L. delbrueckii* NII 0925 were carried out using MSM media supplemented with different carbon sources at 20 g/L. 1: 1 ratio of inoculums concentration were added into 100 mL of production medium and incubated at 200 rpm, 30°C for 48 h.

### Effect of incubation time on PHB production by mixed cultures

The effect of different incubation time on growth and PHB production of mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 was carried out using MSM media supplemented with 2% glucose. 1: 1 ratio of inoculums concentration were added into 100 mL of production medium and incubated at 200 rpm, 30°C for different time points (18 – 66 h).

### Effect of inoculums ratio on PHB production by mixed cultures

To study the effect of inoculums ratio on growth and PHB production of mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 was carried out using MSM media supplemented with 2% glucose.

Different ratios of inoculums were added into 100 mL of production medium and incubated at 200 rpm, 30°C for 42 h.

### PHB assay

PHB assay was carried out by the method of Law and Slepecky (1961).

### Characterization of PHB

The extracted PHB from pure culture of *B. firmus* NII 0830 and mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 were subjected to FTIR and  $^1\text{H}$  NMR studies and were compared to standard PHB from Sigma.

### Fourier Transform Infrared Spectroscopy (FTIR)

The presence of different functional groups in PHB was checked by FTIR. Extracted PHB (2 mg) and pure PHB obtained from Sigma (2 mg) were dissolved in 500  $\mu$ L chloroform and layered on NaCl crystal. After evaporation of chloroform, PHB polymer film was subjected to FTIR.

### $^1\text{H}$ NMR studies

The  $^1\text{H}$  NMR spectra was recorded at room temperature with a Bruker Advance II 500 MHz spectrometer to study the structural elucidation. The standard PHB sample from Sigma (5 mg) and the extracted polymer sample (5 mg) were weighed and transferred to NMR tubes. The samples were dissolved in  $\text{CDCl}_3$  before testing.

### PHB blend preparation

**PHB film:** PHB (Sigma) film was prepared by dissolving 50 mg PHB in chloroform by sonication and was left overnight for film formation.

**PHB-PLA:** PHB-PLA blend was prepared by dissolving 50 mg each of PHB and polylactic acid (PLA) in chloroform by sonication and was left overnight for film formation.

**PHB-TS:** Thermoplastic starch (TS) was prepared by mixing starch powder, water and glycerol in the ratio 50:15:35 respectively. The contents were mixed for 15-30 min to obtain a paste. The paste was transformed into TS by heating at  $100^\circ\text{C}$  in water bath with continuous stirring for 15 min. TS were mixed with PHB in the ratio 50:50 and solvent cast films were obtained from chloroform.

**PHB-PLA-TS:** PHB and PLA were dissolved in chloroform in the ratio 50:50 by sonication. TS was mixed with this in the ratio 50:50 and solvent cast films were obtained.

**PHB-PEG-TS:** PHB and poly ethylene glycol (PEG) were dissolved in chloroform in the ratio 50:50 by sonication. TS was mixed with this in the ratio 50:50 and solvent cast films were obtained.

### Biodegradation of PHB and blends using pure and consortium

Minimal media was prepared and inoculated with 2% dairy industry sludge obtained from Milma (Ambalathara, Trivandrum, Kerala, India) and pure cultures of *L. delbrueckii* NII 0925 and consortium from dairy industry sludge. Previously weighed films of PHB and its blends were put into the media after sterilizing by washing in sodium hypochlorite for 10 seconds and repeatedly

washing in distilled water. It was incubated for 7 days at  $30^\circ\text{C}$ . Samples were collected and film retention was calculated.

### Biodegradation of PHB at different time points using mixed and pure cultures

Previously weighed amounts of sterilized PHB-PEG-TS blends were put into 10 flasks with minimal media. Five flasks were inoculated with 2 mL dairy industry sludge (Ambalathara, Trivandrum, Kerala, India) and other five were inoculated with pure culture of *Bacillus firmus* NII 0830 and *Paracoccus denitrificans* NII 0925. They were incubated at  $30^\circ\text{C}$ . Samples were collected at an interval of 5 days (5-35 days) and retention was calculated.

### SEM analysis of native and biodegraded PHB-PEG-TS blends

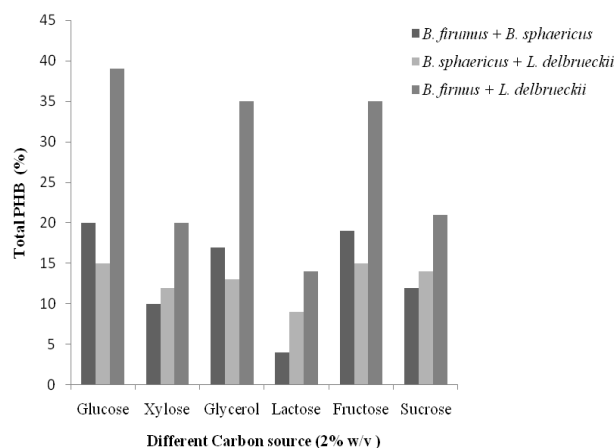
SEM images were taken at magnification  $\times 3000$  for both native and biodegraded PHB-PEG-TS blends at different time points using pure culture and consortium (sludge) using a JEOL JSM-5600 scanning electron microscope (SEM). The samples were mounted on a double sided conductive tape on precut brass sample stubs and the sputter coated with gold palladium using a JEOL JFC -1200 fine coater. The images of native and biodegraded PHB-PEG-TS blends were acquired with a 10 to 15 kV accelerating voltage.

## RESULTS AND DISCUSSION

### The effect of different carbon sources on growth and PHB production by mixed cultures

The results presented in Figure 1 indicates that among the three different combinations of mixed cultures checked for PHB production, maximum PHB production was observed with *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 followed by *B. sphaericus* NII 0838 and *L. delbrueckii* NII 0925. Among the different carbon sources screened for PHB production - glucose, glycerol and fructose gave almost similar type of productivity followed by sucrose, xylose and lactose. In this study glucose was selected as carbon source for further studies. There were several reports on PHB production using mixed cultures (Tohyama et al. 2000; Johnson et al. 2010). In the present study glucose may be converted into lactate by *L. delbrueckii* NII 0925 and the lactate was in turn

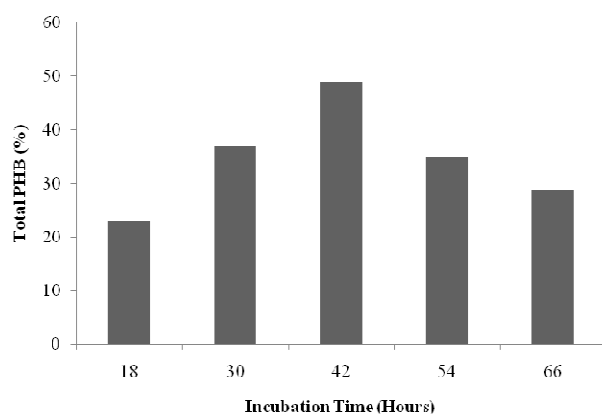
converted to PHB by *B. sphaericus* NII 0838 as an alternative.



**Figure 1** - Effect of different carbon source and different mixed cultures on PHB production.

#### Effect of incubation time on PHB production by mixed cultures

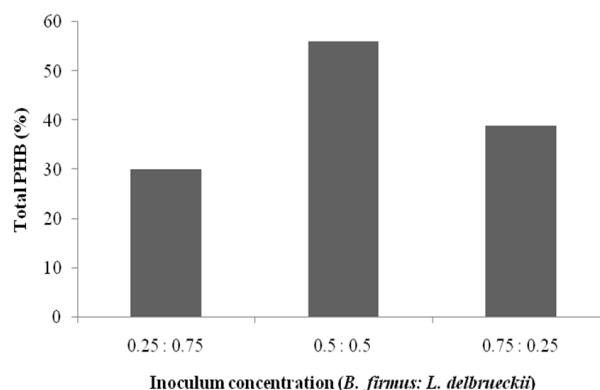
The result presented in Figure 2 indicates that maximum PHB production was observed at 42 h, the PHB productivity at 30 h and 54 h were comparable. In this study 42 h of incubation time was selected for further studies. Jiang et al. 2011 reported two mixed culture systems for PHB system where the first mixed culture enriched on lactate was dominated by a gamma proteobacterium can accumulate 90% of PHB after 6 h of incubation while the second mixed culture consisting of *Plasticicumulans acidivorans* and *Thauera selenatis* can accumulate 84% PHB within 8 h.



**Figure 2** - Effect of incubation time on PHB production by mixed culture of *Bacillus firmus* NII 0830 and *Lactobacillus delbrueckii* NII 0925.

#### Effect of inoculums ratio on PHB production by mixed cultures

The results presented in Figure 3 indicates that maximum PHB production (56%) was observed when the inoculums concentration of *B. firmus* NII 0830: *L. delbrueckii* NII 0925 was kept in a 1: 1 ratio, the PHB production was low when the inoculums concentration was kept at 1: 3 and 3: 1 ratios of *B. firmus*: *L. delbrueckii* (30 and 39% respectively).



**Figure 3** - Effect of inoculums concentration on PHB production by mixed culture of *Bacillus firmus* NII 0830 and *Lactobacillus delbrueckii* NII 0925.

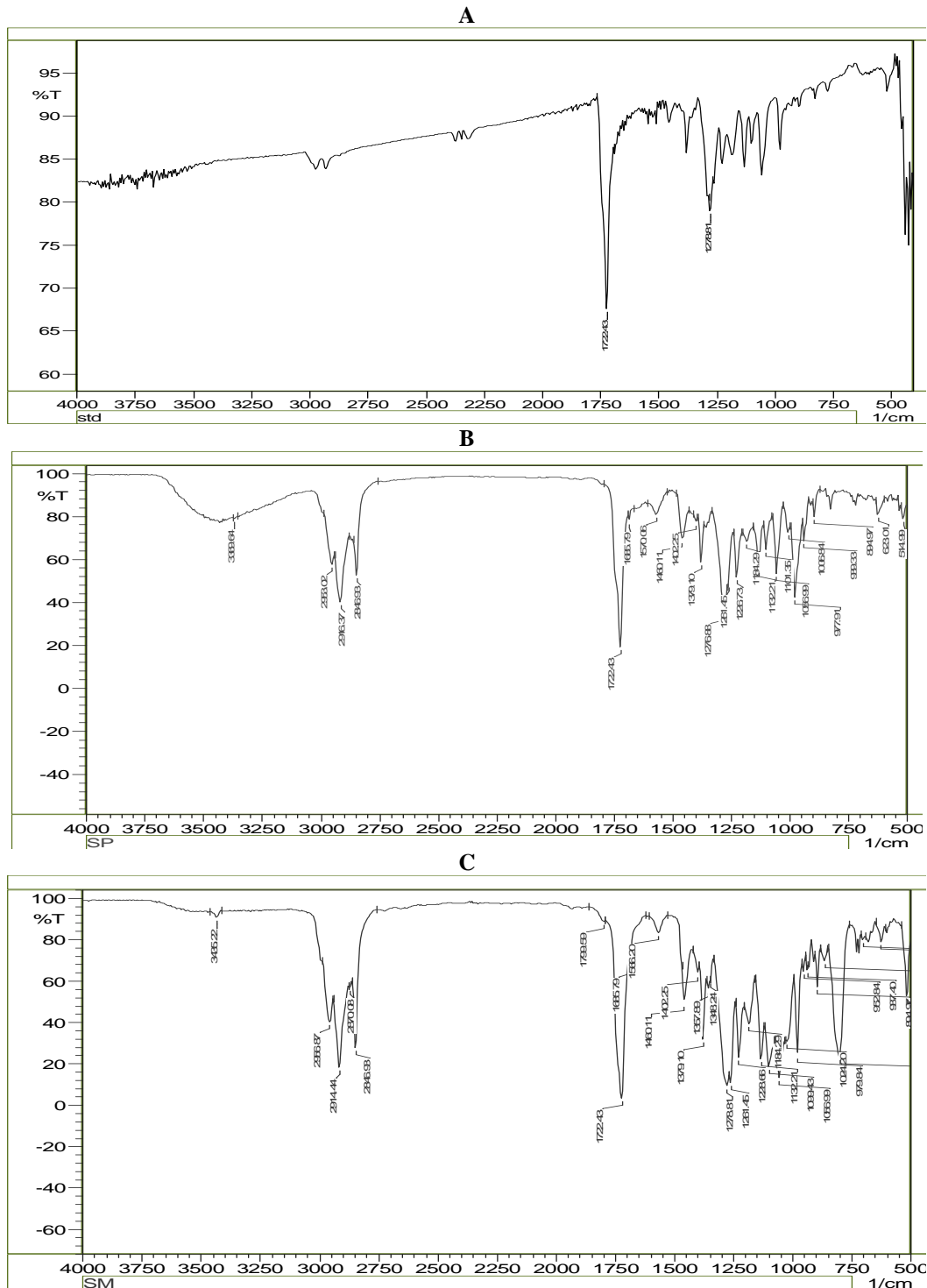
#### Characterization of PHB

##### Fourier Transform Infrared Spectroscopy (FTIR)

Extracted PHB after submerged fermentation by using glucose as carbon source by *B. firmus* and mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 was characterized with FTIR. Figure 4 illustrates the FTIR spectrum of PHB (Sigma-Aldrich Chemicals, USA), extracted PHB from *B. firmus* NII 0830 and extracted PHB from mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925.

The FTIR spectra of standard PHB (Sigma-Aldrich Chemicals, USA) showed peaks at 1728  $\text{cm}^{-1}$  and 1282  $\text{cm}^{-1}$  corresponding to specific rotations around carbon atoms specific to certain functional groups. Peaks at 1728  $\text{cm}^{-1}$  corresponds to ester carbonyl group and at 1282  $\text{cm}^{-1}$  corresponds to  $-\text{CH}$  group (Sindhu et al. 2011). FTIR spectra of the extracted polymer from *B. firmus* NII 0830 shows peaks at 1722.43 $\text{cm}^{-1}$  and 1276.88 $\text{cm}^{-1}$ . The extracted polymer from mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 showed peaks at 1722.43  $\text{cm}^{-1}$  and

1278.81  $\text{cm}^{-1}$  respectively. These peaks are corresponding to the peaks obtained for the standard PHB (Sigma) at 1728  $\text{cm}^{-1}$  and 1282  $\text{cm}^{-1}$  confirming that the extracted polymer is PHB.

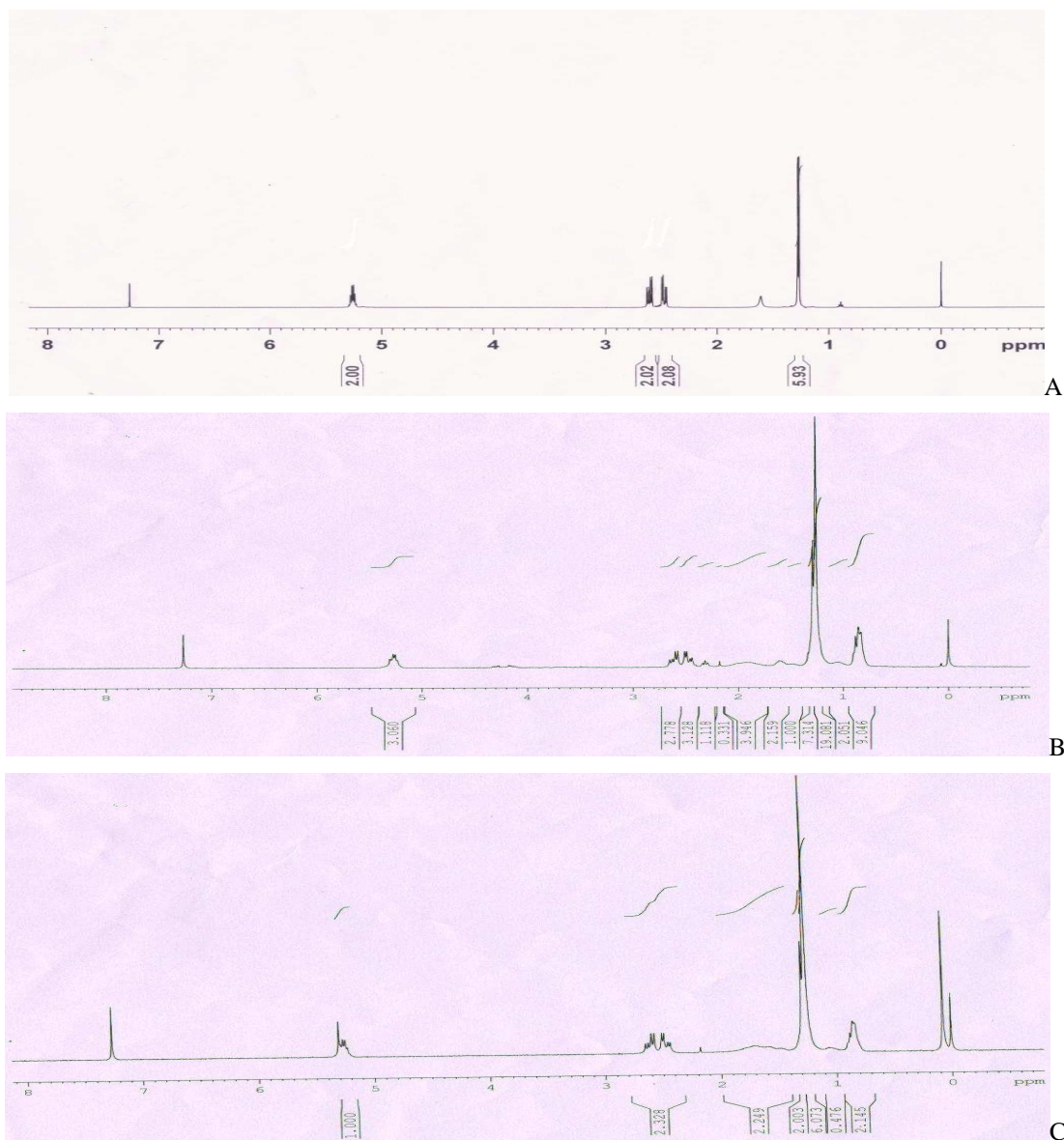


**Figure 4** - FTIR spectra of A) PHB (Sigma) B) extracted PHB from *Bacillus firmus* NII 0830 and C) extracted PHB from mixed culture of *Bacillus firmus* NII 0830 and *Lactobacillus delbrueckii* NII 0925.

**$^1\text{H}$  NMR studies**

Figure 5 illustrates the  $^1\text{H}$  NMR spectra obtained from PHB samples extracted from *B. firmus* NII 0830 and mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925, is compared with the commercial PHB (Sigma-Aldrich Chemicals,

USA). Both spectra were found to match with each other. The spectrum shows a doublet at 1.29 ppm which is attributed to the methyl group coupled to one proton. The doublet of quadruplet at 2.57 ppm is attributed to the methylene group adjacent to an asymmetric carbon atom bearing a single atom.



**Figure 5** -  $^1\text{H}$  NMR spectra of PHB (Sigma) B) extracted PHB from *Bacillus firmus* NII 0830 and C) extracted PHB from mixed culture of *Bacillus firmus* NII 0830 and *Lactobacillus delbrueckii* NII 0925.

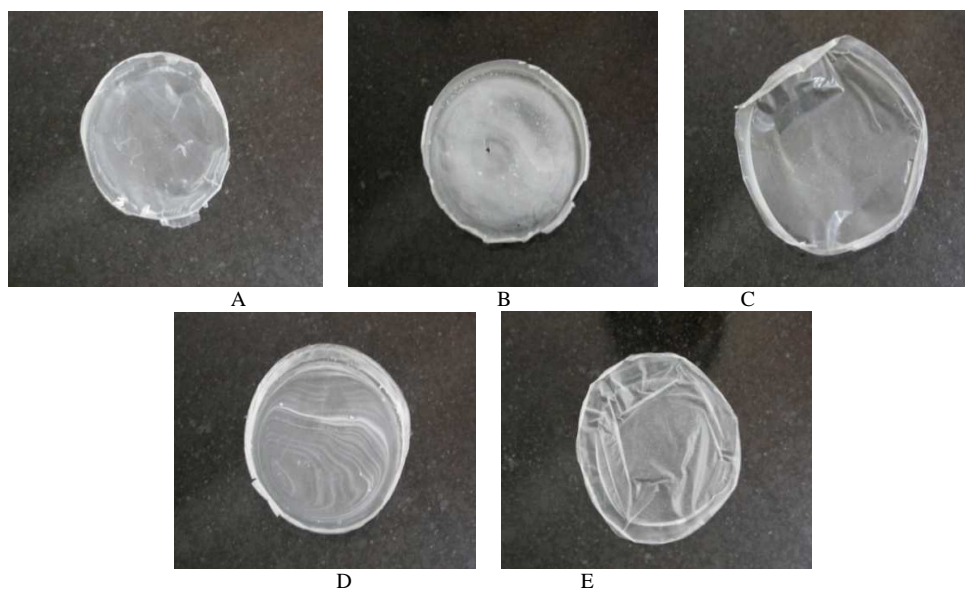
The multiplet at 5.27 ppm is characteristic of methylene group. Two other signals are observed, a broad one at 1.56 ppm which is due to water and another one at 7.25 ppm attributed to the solvent used i.e. chloroform. Identical observations were earlier reported by several researchers (Bonthron

et al. 1992; Jan et al. 1996 and Sindhu et al. 2011). From these results it can be concluded that pure culture of *B. firmus* NII 0830 and mixed culture of *B. firmus* NII 0830 and *L. delbrueckii* NII 0925 when grown on glucose as carbon source produce PHB.

### PHB blend preparation

One of the approaches being pursued to improve the performance of bio-based materials in engineering applications is the blending of biopolymers with biodegradable synthetic polyesters. The goal of this approach is to develop biodegradable materials with acceptable material

properties from blends comprising mostly biopolymers. The PHB was found to be brittle and breaks easily. Therefore blends with different polymers were prepared to improve their physical characteristics. The Figure 6 shows blends prepared with different polymers.



**Figure 6** - Different PHB blends made from A) PHB sigma B) PHB-PLA C) PHB-TS D) PHB-PLA-TS E) PHB-PEG-TS.

### Biodegradation of PHB blends using pure culture and consortium

After 7 days of incubation with sludge and pure cultures PHB-PEG-TS showed maximum degradation followed by PHB-PLA-TS. Pure PHB film showed no degradation at all. The results presented in Table 1 showed that PHB blends were degraded better than pure PHB film, PHB blend showed more degradation and further studies were carried out using PHB-PEG-TS. An identical observation was earlier reported by Rychter et al. 2006 for environmental degradation of polyester blends containing PHB.

**Table 1** - Biodegradation profile of different PHB blends by pure culture and consortium.

Blend	Film retention (%)	
	Pure culture (mg)	Consortium (mg)
PHB Sigma	100	94
PHB-PLA	97.6	83
PHB-PLA-TS	94.8	78
PHB-PEG-TS	94	56
PHB-TS	96	85

### Biodegradation of PHB-PEG-TS blend at different time points using mixed and pure cultures

A comparison of the PHB degradation capabilities of the consortium and pure culture of *B. firmus* NII 0830 and *Paracoccus denitrificans* was made. The results presented in Table 2 showed that after 35 days of incubation, the PHB film incubated with consortium showed 20% retention whereas the one incubated with the pure culture showed 71% retention. Solubility of the polymer in water plays an important role in biodegradation.

**Table 2** - Effect of incubation time on biodegradation of PHB – PEG-TS blend by pure culture and consortium.

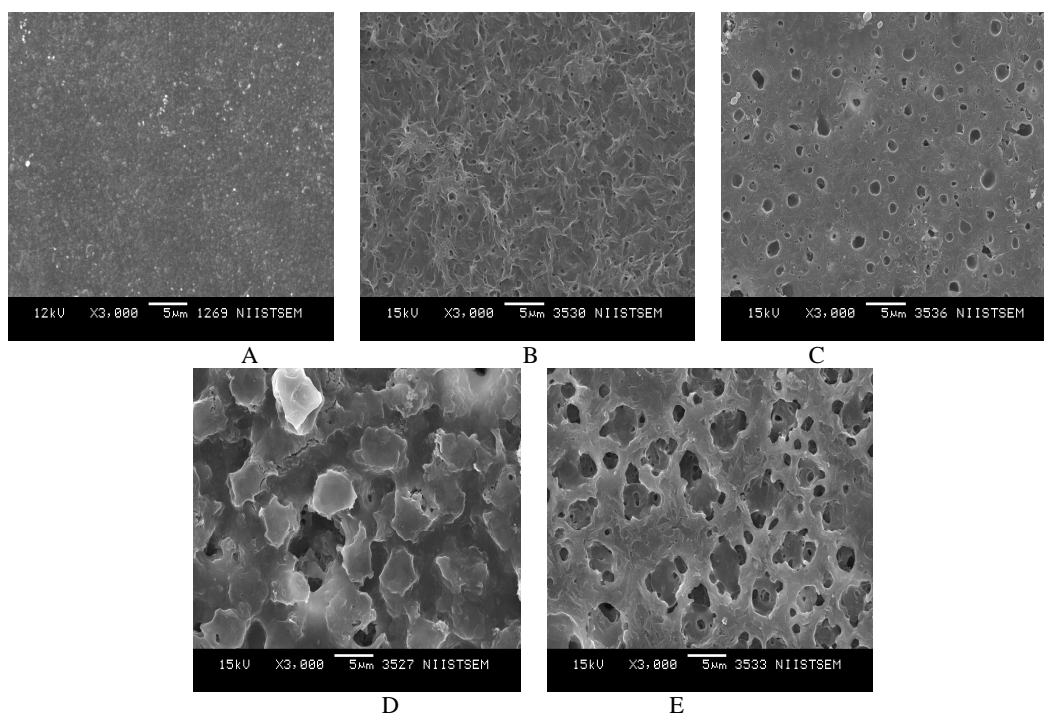
Incubation Time (days)	Film retention (%)	
	Pure culture	Consortium
5	94	56
10	90	51
15	88	46
20	85	43
25	80	39
30	76	33
35	71	20

### SEM analysis of native and biodegraded PHB-PEG-TS blends

Scanning electron micrographs of native and biodegraded PHB-PEG-TS blends using pure and consortiums were taken at magnification 3000X. Figure 7 showed that native blend showed a smooth and uniform surface.

The biodegraded PHB-PEG-TS blend has a rough surface and showed several pores. This indicates

that biodegradation removed external polymers and became rough and porous with a number of visible fibrous pores like structures were formed. Our results indicate that biodegradation using consortium was found to be more effective when compared to pure culture. Identical observations were earlier reported by Bonartseva et al. 2002 using a soil model community.



**Figure 7** - Scanning electron micrographs of PHB-PEG-TS blend after different periods of incubation A) Control B) after 5 days C) after 10 days D) after 15 days and E) after 20 days of incubation with mixed culture of *Bacillus firmus* NII 0830 and *Lactobacillus delbrueckii* NII 0925.

## CONCLUSION

One of the main disadvantages of using pure culture for the production of PHB include the high costs for the pure substrates utilized and the costs for sterile pre-cultivation of the bacteria utilized as well as the sterile operation of the final production process. The present study revealed that mixed culture of *Bacillus firmus* NII 0830 and *Lactobacillus delbrueckii* NII 0925 can be used for the production of PHB and characterized by FTIR and  $^1\text{H}$  NMR. PHB blend preparation as well as biodegradation studies were performed and it was revealed that degradation using consortium was found to be more effective when compared to pure cultures.

## ACKNOWLEDGEMENTS

Authors are grateful to the Department of Biotechnology, Govt of India, New Delhi for financial support for the project. Thanks are also due to the Chemical Sciences Division of NIIST for NMR and FTIR analyses.

## REFERENCES

- Albuquerque MGE, Eiroa M, Torres C, Nunes BR, Reis MAM. Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. *J Biotechnol.* 2007; 130(4): 411-442.



- Bengtsson S, Werker A, Christensson M, Welander T. Production of polyhydroxyalkanoates by activated sludge treating paper mill waste water. *Bioresour Technol.* 2008; 99(3): 509-516.
- Bonartseva GA, Myshkina VL, Nikolaeva DA, Rebrov AV, Gerasin VA, Makhin TK. The biodegradation of poly  $\beta$  - hydroxybutyrate (PHB) by a model soil community: the effect of cultivation conditions on the degradation rate and the physicochemical characteristics of PHB. *Microbiol.* 2002; 71(2): 221-226.
- Bonthrone KM, Clauss J, Horowitz DM, Hunter BK, Sanders JKM. The biological and physical chemistry of polyhydroxyalkanoates as seen by NMR spectroscopy. *FEMS Microbiol Rev.* 1992; 103: 269-278.
- Dias JML, Lemos PC, Serafim LS, Oliveira C, Eiroa M, Albuquerque MGE, Ramos AM, Oliveira R, Reis MAM. Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures from the substrates to the final product. *Macromol Biosci.* 2006; 6(11): 885-906.
- Jan S, Roblot C, Courtois J, Courtois B, Barbotin JN, Shguin JP. <sup>1</sup>H NMR spectroscopic determination of poly 3-hydroxybutyrate extracted from microbial biomass. *Enzyme Microb Technol.* 1996; 18(3): 195-201.
- Jiang Y, Marang L, Kleerebezem R, Muyzer G, Loosdrecht MC. Polyhydroxybutyrate production from lactate using a mixed microbial culture. *Biotechnol Bioeng.* 2011; 108(9): 2022-2035.
- Johnson K, Geest J, Kleerebezem R, Loosdrecht MC. Short- and long-term temperature effects on aerobic polyhydroxybutyrate producing mixed cultures. *Water Res.* 2010; 44(6): 1689-1700.
- Kondo T, Kondo M. Efficient production of acetic acid from glucose in a mixed culture of *Zymomonas mobilis* and *Acetobacter* sp. *J Ferment Bioeng.* 1996; 81(1): 42-46.
- Law JH, Slepecky RA. Assay of poly- $\beta$ -hydroxybutyric acid. *J Bacteriol.* 1961; 82(1): 33-36.
- Lee SY. Bacterial Polyhydroxyalkanoates. *Biotechnol Bioeng.* 1996; 49(1): 1-14.
- Reis MAM, Serafim LS, Lemos PC, Ramos AM, Aguiar FR, Van Loosdrecht MCM. Production of polyhydroxyalkanoates by mixed microbial cultures. *Bioproc Biosystems Eng.* 2003; 25: 377-385.
- Rhu DH, Lee WH, Kim JY, Choi E. Polyhydroxyalkanoate (PHA) production from waste. *Water Sci Technol.* 2003; 48(8): 221-228.
- Rychter P, Biczak R, Herman B, Smylla A, Kurcok P, Kowalczyk M. Environmental degradation of polyester blends containing atactic poly (3-hydroxybutyrate). Biodegradation in soil and ecotoxicological impact. *Biomacromolecules.* 2006; 7(11): 3125-3131.
- Serafim LS, Lemos PC, Albuquerque MGE, Reis MAM. Strategies for PHA production by mixed cultures and renewable waste materials. *Appl Microbiol Biotechnol.* 2008; 81: 615-628.
- Shimizu H, Mizuguchi T, Tanak E, Shioya S. Nisin production by a mixed culture system consisting of *L. lactis* and *K. marxianus*. *Appl Environ Microbiol.* 1999; 65(7): 3134-3141.
- Sindhu R, Ammu B, Binod P, Deepthi KS, Ramachandran KB, Soccol CR, Pandey A. Production and characterization of poly -3-hydroxybutyrate from crude glycerol by *Bacillus sphaericus* NII 0838 and improving its thermal properties by blending with other polymers. *Braz Arch Biol Technol.* 2011; 54(4): 783-794.
- Tohyama M, Takagi S, Shimizu K. Effect of controlling lactate concentration and periodic change in DO concentration on fermentation characteristics of a mixed culture of *Lactobacillus delbrueckii* and *Ralstonia eutropha* for PHB production. *J Biosci Bioeng.* 2000; 89(4): 323-328.

Received: May 28, 2013;  
Accepted: October 31, 2013.