

Media engineering for production of poly- β -hydroxybutyrate by *Bacillus firmus* NII 0830

Sreelatha K Deepthi, Parameswaran Binod*, Raveendran Sindhu and Ashok Pandey

Biotechnology Division, National Institute for Interdisciplinary Science & Technology (NIIST), CSIR, Trivandrum 695 019, India

Received 18 February 2011; revised 30 August 2011; accepted 02 September 2011

This study evaluates biodiesel industry generated crude glycerol as sole carbon source for the production of poly- β -hydroxybutyrate (PHB) using *Bacillus firmus* NII 0830. Pretreatment of crude glycerol from biodiesel industry was tried using strong acids. Biomass yield and PHB accumulation by *B. firmus* NII 0830 in submerged fermentation was compared using crude as well as pretreated glycerol as sole carbon source. It was observed that crude glycerol from biodiesel industry, without any pretreatment, can be used for PHB production. The organism when grown in mineral salt media containing crude glycerol as carbon source gave a maximum yield of biomass (3 g/l) and PHB (53% of cell dry wt). Response surface methodology (RSM) showed that presence of nitrogen and phosphate in fermentation media favored biomass production but reduced PHB production. Optimized crude glycerol media for maximum PHB accumulation contains ammonium sulphate (4 g/l) and K_2HPO_4 (0.8 g/l).

Keywords: Biodiesel industry, Biopolymer, Glycerol, Poly- β -hydroxybutyrate (PHB), Response Surface Methodology (RSM)

Introduction

Biopolymers originate from renewable sources and, being biodegradable, have a less negative impact on environment compared to petroleum based materials¹. Poly- β -hydroxybutyrate (PHB) is a most common type of polyhydroxyalkanoate (PHA), synthesized by many bacterial species as intracellular carbon and energy source when grown under suboptimal cultural conditions. PHB is an eco-friendly substitute and an ideal candidate for making biodegradable plastics^{2,3}. Important applications of PHB include development of tissue engineered cardiovascular products⁴, PHB based films as matrices for pesticides⁵, treatment of osteomyelitis⁶ and food packaging⁷. However, PHB has not been commercialized for large scale applications due to high cost of production and less efficient recovery methods⁸. Efforts are being made to use less expensive carbon sources to reduce cost of PHA. Use of various carbon sources (malt, soya⁹, corn steep liquor¹⁰, crude glycerol from biodiesel industry^{11,12}, jackfruit seed powder¹³, date syrup¹⁴, molasses^{15,16} etc.) are reported for the production of PHA using various bacteria. Because of increasing

demand for biodiesel, large amounts of glycerol would be accumulated as a byproduct and can serve as a promising cheap carbon source. For every 3 mol of methyl esters produced, 1 mol of glycerol is obtained, equivalent to about 10% of total product¹⁷. Crude glycerol (CG) contains glycerol (30-60 wt%), besides soaps, remaining catalyst, water, and esters formed during transesterification process¹⁸. Utilization of glycerol for PHB production offers a potentially cheap substrate for the production of biodegradable plastics and also adds value to bio-diesel production economics. Glycerol is the most energetically favorable substrate for the formation of acetyl-CoA, the precursor for PHB synthesis. A number of *Bacillus* species are reported to accumulate 9-67% cell dry weight PHA^{16,19-22}.

This study utilizes biodiesel industry generated CG as carbon source for PHB production by *B. firmus* NII 0830 under variations in fermentation conditions.

Experimental Section

Microorganism, Maintenance and Inoculum Preparation

B. firmus NII 0830 was obtained from NII Microbial Culture Collection (NIICC), NIIST, Trivandrum, India. Strain was maintained on nutrient agar slants. Inoculums were prepared in LB media and incubated at 30°C for 16 h on a rotary shaker at 250 rpm. Seed cultures

*Author for correspondence
Tel: 91-471-2515361; Fax 91-471-2495949
E-mail: binodkannur@gmail.com

(2% v/v) were transferred to 100 ml of fermentation media as and when required.

Pretreatment of Biodiesel Industry Generated Glycerol

Biodiesel industry generated CG may contain lot of other substances (soaps, remaining catalyst, water and esters), which may inhibit microbial growth. Hence, CG was pretreated by neutralization method using concentrated acids¹⁸. CG (50 g) was diluted using methanol (15 g), pH adjusted to 4 using $\text{H}_3\text{PO}_4/\text{H}_2\text{SO}_4/\text{HCl}$, mixed well for 15 min and centrifuged at 8000xg for 15 min. Upper fatty acid-ester phase and salt precipitate were removed. Methanol was removed from glycerol phase by distillation using rotavapor. Pretreated and untreated glycerols were used for PHB production by *B. firmus* NII 0830.

Production of PHB using Crude Glycerol

Fermentation trials were carried out in 250 ml Erlenmeyer flasks. Inoculum (2%) containing 5×10^7 spores/ml was used to inoculate phosphate limited production media containing biodiesel industry generated CG, obtained from Naturo Bioenergy, Hyderabad. Composition of media was as follows: CG, 20 ml/l; $(\text{NH}_4)_2\text{SO}_4$, 4 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 g/l; citric acid, 1.7 g/l; trace element solution, 10 ml/l; pH, 7. Composition of trace element solution was: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.25; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 7\text{H}_2\text{O}$, 0.23; and $(\text{NH}_4)_8\text{Mo}_7\text{O}_{24}$, 0.1 g/l and 35% HCl, 10 ml/l. It was then incubated at 30°C, 200 rpm for 48 h. Cell pellet was recovered by centrifugation at 8000xg for 15 min, lyophilized and analyzed for PHB.

Optimization of Fermentation Media by Response Surface Methodology (RSM)

Media optimization was carried out by RSM design using software Minitab 15 (Minitab Inc., USA). The design was employed by selecting varying concentrations of glycerol, ammonium sulphate, K_2HPO_4 and KH_2PO_4 . RSM consisted of 30 runs with five levels (Table 1).

PHB Assay

PHB containing cell pellet was digested with H_2SO_4 (conc., 1 ml) at 90°C for 30 min. Tubes were cooled in ice, and then 0.014 N H_2SO_4 (4 ml) was added and mixed. Diluted samples were eluted with 0.014 N H_2SO_4 at a flow rate of 0.6 ml/min from an Aminex HPX- 87H ion-exclusion column for organic acids. Absorbance of

crotonic acid was measured at 210 nm using UV detector. Amount of crotonic acid produced was calculated from regression equation derived from known crotonic acid standards.

PHB Extraction and Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Extraction of PHB was carried out by sodium hypochlorite-chloroform method²³. Lyophilized biomass of *B. firmus* NII 0830 (1 g) was treated with 50 ml each of chloroform and sodium hypochlorite (30%) at 30°C for 1 h. Then mixture was centrifuged at 8000X g for 15 min. Upper hypochlorite and middle non-PHB cell materials phases were removed. Lower chloroform phase containing PHB was concentrated using a rotavapor and PHB was recovered by non-solvent precipitation using ten times volume of ice cold methanol. Precipitated PHB was taken and air dried.

Presence of different functional groups in PHB was checked by FTIR (Shimadzu, Japan). Extracted PHB (2 mg) and standard PHB (2 mg) from Sigma-Aldrich, India were dissolved in chloroform (500 μl) and layered on NaCl crystal. After evaporation of chloroform, PHB film was subjected to FTIR.

Results and Discussion

Pretreatment of Biodiesel Industry Generated Glycerol

Three different pretreatment methods were evaluated for biodiesel industry generated glycerol for its utilization in fermentation. In all method, major difference is in the type of acid (H_3PO_4 , H_2SO_4 and HCl) used for adjusting pH (4) after treating CG with methanol. After adding required amount of acids, solution was mixed using a magnetic stirrer for 15 min, centrifuged at 8000 x g for 15 min and glycerol layer was separated. Effective fatty acid layer separation and salt precipitation was obtained by neutralization. Salt precipitation was more when concentrated H_2SO_4 was used. Pretreated glycerol along with untreated as well as standard glycerol (Sigma-Aldrich, India) was used in fermentation media.

Comparison of PHB Production by *B. firmus* NII 0830 using Pretreated and Untreated Glycerol

When CG was pretreated with methanol and different acids (Fig. 1), amount of biomass formed and PHB accumulation of biomass, respectively, were as follows: H_3PO_4 , 3.75, 44; HCl, 2.3, 54; and H_2SO_4 , 2.4 g/l, 51%. But when CG was used without any pretreatment, biomass yield was 3.0 g/l and PHB yield

Table 1—Response surface design for media optimization

Run	Glycerol ml/l	Ammonium sulphate, g/l	KH_2PO_4 g/l	K_2HPO_4 g/l	Biomass g/100 ml	PHB g/g dry biomass
1	10	2	0.3	0.3	0.37	0.08
2	20	4	0.65	0.65	0.4	0
3	20	8	0.65	0.65	0.31	0.07
4	10	2	1	1	0.4	0.08
5	10	6	0.3	0.3	0.46	0.06
6	20	0	0.65	0.65	0.03	0.01
7	30	2	0.3	1	0.26	0.09
8	20	4	0.65	0.65	0.1	0.25
9	30	6	1	0.3	0.34	0.04
10	20	4	0	0.65	0.09	0.09
11	30	6	1	1	0.48	0.03
12	10	6	1	0.3	0.53	0.04
13	10	2	1	0.3	0.35	0.07
14	10	6	0.3	1	0.4	0.07
15	30	2	0.3	0.3	0.29	0.06
16	20	4	0.65	1.35	0.32	0.06
17	30	6	0.3	0.3	0.46	0.04
18	20	4	0.65	0.65	0.09	0.11
19	0	4	0.65	0.65	0.1	0.01
20	10	2	0.3	1	0.33	0.08
21	20	4	0.65	0.65	0.34	0.01
22	20	4	1.35	0.65	0.35	0.05
23	30	2	1	0.3	0.09	0
24	30	2	1	1	0.36	0
25	10	6	1	1	0.36	0
26	20	4	0.65	0	0.37	0
27	40	4	0.65	0.65	0.37	0.05
28	30	6	0.3	1	0.4	0.03
29	20	4	0.65	0.65	0.44	0.02
30	20	4	0.65	0.65	0.3	0.7

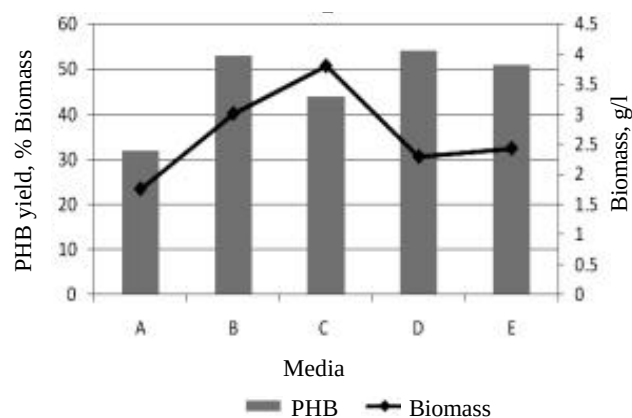


Fig. 1—Biomass and PHB yield in different pretreated media (A, Standard glycerol; B, Biodiesel industry generated glycerol (Untreated); C, Pretreatment with methanol and H_3PO_4 ; D, Pretreatment with methanol and HCl; E, Pretreatment with methanol and H_2SO_4)

was 53% of biomass, indicating that there is not much difference in biomass yield and PHB production by pretreatment. Hence CG without any pretreatment was used for further studies. CG samples of different manufacturers differ mainly in their contamination with salts (NaCl or K_2SO_4), methanol, fatty acids and pH due to the applied process¹¹. Comparing biomass yield from pure glycerol and CG, it was observed that CG enhances bacterial growth as well as PHB accumulation, may be because CG used might be containing less contaminants and salts present in it may enhance growth of *B. firmus* NII 0830.

PHA production from glycerol has been investigated in earlier studies²⁴⁻²⁷. In a recent study¹¹, CG from different biodiesel manufacturers was examined for suitability as a substrate for PHB production. However,

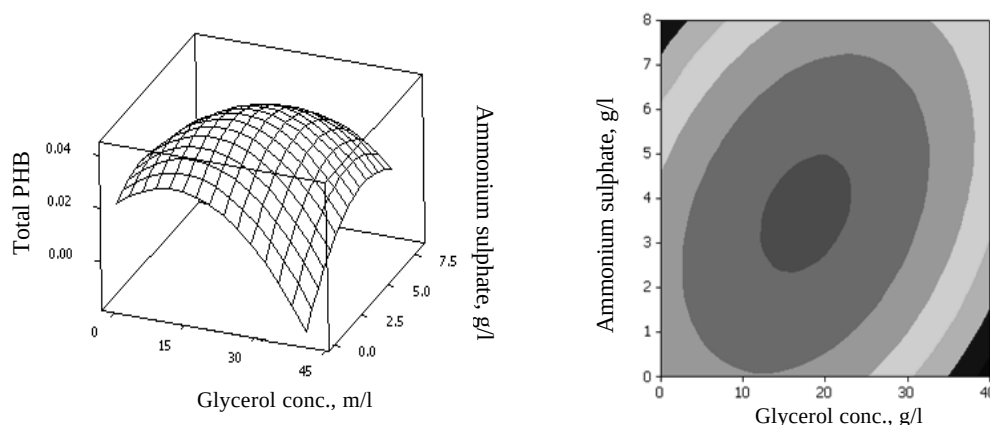


Fig. 2—Surface and contour plot of PHB yield vs glycerol and ammonium sulphate

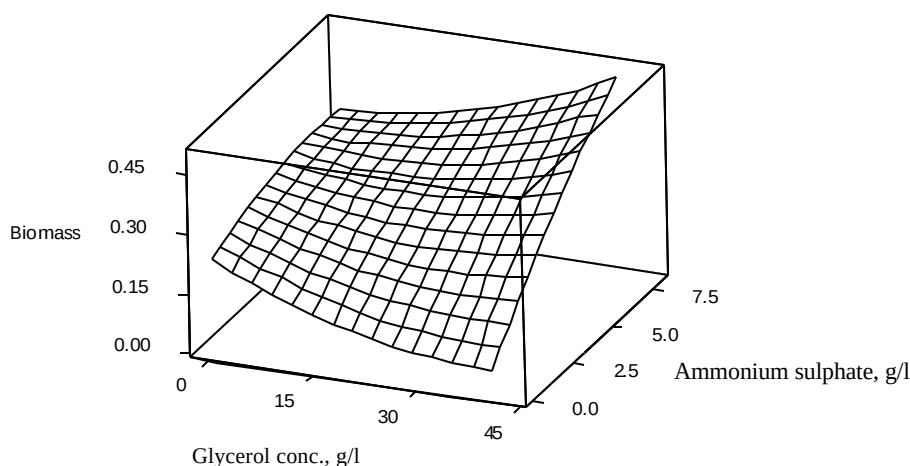


Fig. 3—Surface plot of biomass yield vs ammonium sulphate and glycerol

significant decrease in PHB productivity and product yields were recorded when NaCl-contaminated CG was used. Scale-up studies on fed-batch fermentation by *Zobellella denitrificans* MW1 using glycerol shows higher cell density (81.2 g/l) and PHB (54.3 g/l)²⁸. Several flask-scale cultivations were carried out with a variety of bacterial strains, such as a recombinant *Escherichia coli* strain harboring PHB synthesis genes from *Streptomyces aureofaciens*^{29,30} and different *Pseudomonas* strains^{31,32}. Significant decrease in PHB content (from 70 to 80%, w/w) were reported for PHB production by *Cupriavidus necator* JMP 134 using NaCl-contaminated CG, might be related to increased osmotic pressure caused by NaCl accumulation during glycerol feeding¹¹.

Media Optimization

Conventional fermentation approach, optimized by implementing the variation of one component at a time,

is time consuming, assumes that the process variables do not interact and that process response is function of a single parameter, which is varied. In recent years, this approach has been replaced with statistical optimization methods that take into account the interaction of variables in generating the response. In present study, effect of concentration of ammonium and phosphate sources on the production of biomass and PHB were evaluated, as these nutrients are main stress inducer for PHB accumulation. So it is necessary to choose a media, which can induce high cell density as well as PHB production. RSM results that determine optimal level and interaction of media components indicate that CG content of 2% is optimum for PHB production (Fig. 2). PHB formation is negatively affected if glycerol concentration is increased beyond 2%. Increase in ammonium sulphate concentration favored biomass formation. Carbon sources serve three different functions in *Bacillus* such as biomass synthesis, as energy source for biosynthesis

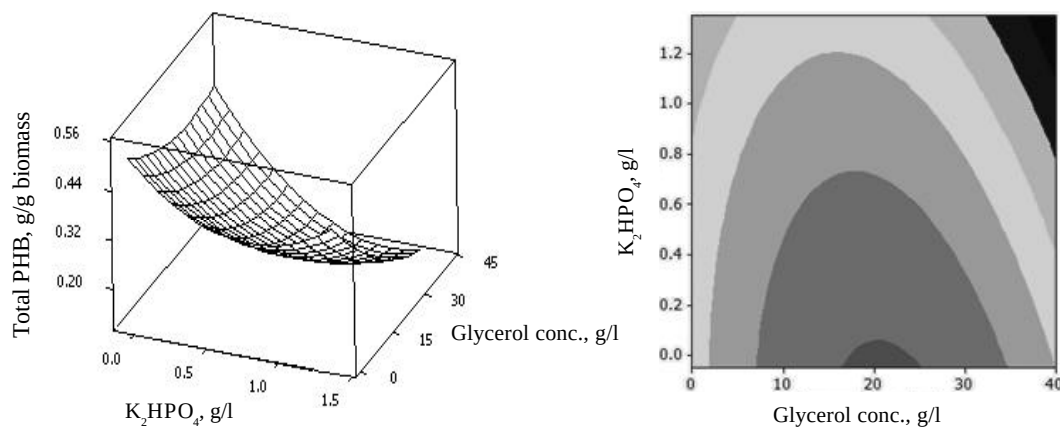


Fig. 4—Surface and contour plot of production of PHB vs glycerol and KH_2PO_4

and cell maintenance and a carbon source for PHB polymerization²².

Increased addition of ammonium sulphate favors growth in CG medium (Fig. 3). Addition of ammonium sulphate (6 g/l) produced 5 g/l biomass. Maximum PHB accumulation was observed when ammonium sulphate concentration was 4 g/l. It is reported that ammonium limitation efficiently encouraged PHB accumulation in organism such as *Rhodopseudomonas palustris* KU 003³³. Higher level of ammonium ion present necessitates synthesis of more protein carrier, resulting in faster uptake rate by the organism. Kleiner³⁴ reported that ammonium uptake in bacteria under ammonium-limited condition is an active process using specific carrier. The rate of this process is believed to be the limiting step, which determines the growth rate of some cells³⁵. This carrier is synthesized under ammonium limited condition and its synthesis is repressed when ammonium concentration is excess in the medium.

Two types of phosphate sources (KH_2PO_4 & K_2HPO_4) were used in fermentation medium for the production of PHB. There was significant reduction in PHB formation with increased concentration of KH_2PO_4 (Fig. 4). It was observed that increase in phosphate content favored bacterial growth but reduced PHB accumulation. PHB accumulation was more in the absence of KH_2PO_4 in fermentation medium. Similarly, amount of K_2HPO_4 was also directly affecting PHB production. Optimum K_2HPO_4 in fermentation medium was 0.8 g/l (Fig. 5). Increased or decreased concentration of this nutrient drastically reduced PHB yield. This corroborates the findings that PHB synthesis occurs

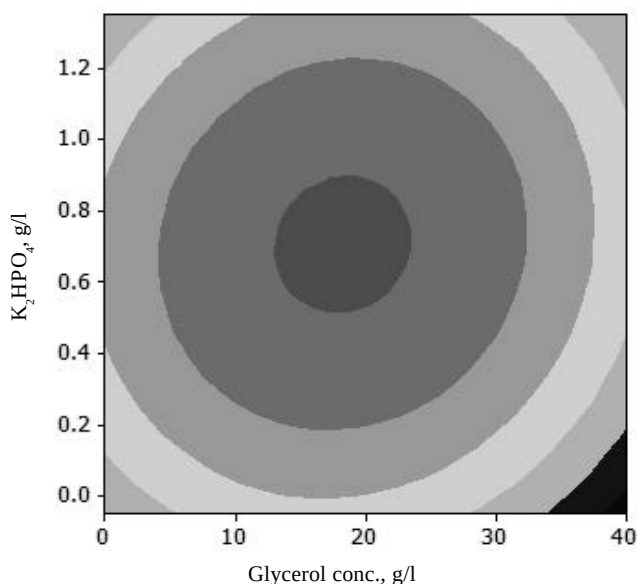


Fig. 5—Contour plot of glycerol and K_2HPO_4 on PHB production

when growth is restricted by the availability of phosphorus³⁶. When excess phosphate is present in the medium, bacteria will utilize for its own growth. When one of the nutrients is limited, it changes its metabolism to accumulate PHB as energy reserve. Phosphate limitations have a significant effect on PHB production.

Bacteria able to synthesize PHB can be divided into two groups: i) that accumulate PHB during stationary phase; and ii) that accumulate PHB during growth phase. Those bacterial strains, which accumulate PHB during stationary phase require limited nutrients (nitrogen, phosphorus or magnesium) and an excess of carbon source³⁷. *Bacillus* strain comes under this group as

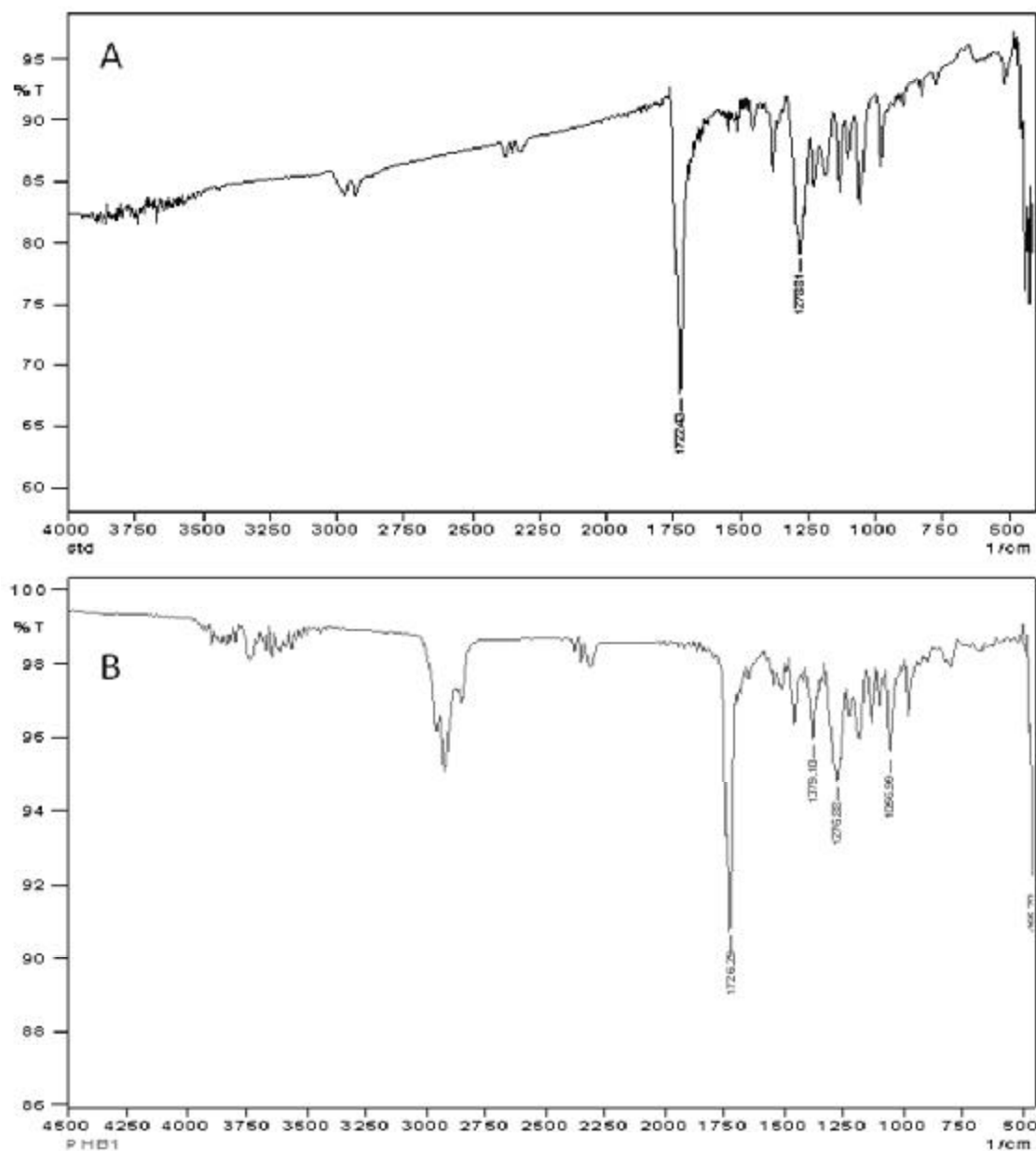


Fig. 6—FTIR analysis of: A) standard PHB (Sigma-Aldrich); and B) PHB extracted from *Bacillus firmus* NII 0830

accumulation of PHB occurs during stationary phase and phosphorus source has been identified as limiting substrate. Hence, overall growth and PHB accumulation of this strain is in biphasic condition, where first phase is required for development of biomass and PHB accumulation takes place during second phase. As biomass production is directly linked with the availability of nutrients in medium, first phase requires all nutrients in proper amount and when bacteria shift to stationary phase, one of the nutrients become limited. In this study,

K_2HPO_4 (0.8 g/l) available in initial medium is utilized for the growth and while bacteria shifts towards stationary phase, this amount become limited, and may induce accumulation of PHB.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of extracted polymer show peaks at 1726.29 cm^{-1} and 1276.88 cm^{-1} corresponding to specific rotations around carbon atoms specific to certain functional groups (Fig. 6). Peak at 1726.29 cm^{-1} corresponds to C=O

stretch of ester group present in molecular chain of highly ordered crystalline structure³⁸. Peak at 1276.88 cm⁻¹ corresponds to –CH group. These peaks are corresponding to the peaks obtained for standard PHB (Sigma) at 1722 cm⁻¹ and 1278 cm⁻¹, confirming that extracted polymer is PHB.

Conclusions

Among various methods used for CG pretreatment, neutralization using concentrated acid was found to be the most effective. Comparison of fermentation trials using pretreated glycerol and CG revealed that CG without any pretreatment can be used for PHB production by *B. firmus* NII 0830. Intracellular PHB produced by organism was extracted by sodium hypochlorite-chloroform dispersion method. Media optimization by RSM reveals that CG (conc., 20 ml/l) was optimum for PHB production. KH₂PO₄ had a negative effect on PHB production. Increased amount of ammonium sulphate favored bacterial growth but it reduced PHB production. Nitrogen and phosphate concentration in optimum media was found to be: ammonium sulphate, 4; and K₂HPO₄, 0.8 g/l. The study shows potential of CG from biodiesel industry, without any preprocessing, for the production of PHB by *B. firmus* NII 0830.

Acknowledgement

Authors thank DBT, Government of India for financial support.

References

- Petersson L & Oksman K, Biopolymer based nanocomposites: Comparing layered silicates and microcrystalline cellulose as reinforcement, *Comp Sci Technol*, **66** (2006) 2187-2196.
- Arun A, Murrugappan, R, Ravindran, A D D, Veeramanikandan, V & Balaji, S, Utilization of various industrial wastes for the production of poly-beta-hydroxybutyrate (PHB) by *Alcaligenes eutrophus*, *African J Biotechnol*, **5**, (2006) 1524-1527.
- Sayyed R Z & Chincholkar S B, Production of PHB from *Alcaligenes faecalis*, *Indian J Microbiol*, **44** (2004) 269-272.
- Williams S F & Martin D P, Applications of PHAs in medicine and pharmacy, in Series of Biopolymers, **vol 4**, edited by A Steinbüchel (Wiley/VCH Verlag, Weinheim, Germany) 2002, 91-121.
- Savenkova L, Gercberga Z, Muter O, Nikolaeva V, Dzene A *et al*, PHB based films as matrices for pesticides, *Process Biochem*, **37** (2002) 719-722.
- Fuson T & Zeynep F, Biosynthesis of PHB and its copolymer and their use in control drug release, *Turkish J Med Sci*, **30** (2000) 535-541.
- Sorrentino A, Gorrasi G & Vittoria V, Potential perspectives of bio nanocomposites for food packaging applications, *Food Sci Technol*, **18** (2007) 84-95.
- Sayyed R Z & Gangurde N S, PHB production by *Pseudomonas* species RZS 1 under aerobic and semi aerobic condition, *Indian J Exp Biol*, **48** (2010) 942-947.
- Arun A, Murrugappan R M, Ravindran A D D, Veeramanikandan V & Balaji S, Utilization of various industrial wastes for the production of poly-β-hydroxybutyrate (PHB) by *Alcaligenes eutrophus*, *African J Biotechnol*, **5** (2006) 1524-1527.
- Vijayendra S V N, Rastogi N K, Shamala T R, Kumar P K A, Kshama L *et al*, Optimization of polyhydroxybutyrate production by *Bacillus* sp. CFR 256 with corn steep liquor as a nitrogen source, *Indian J Microbiol*, **47** (2007) 170-175.
- Mothes G, Schnorpfel C & Ackermann J U, Production of PHB from crude glycerol, *Eng Life Sci*, **7** (2007) 475-479.
- Yoshikazu K & Sei-ichi A, Poly (3-hydroxybutyrate) production by isolated *Halomonas* sp. KM-1 using waste glycerol, *Biosci. Biotechnol Biochem*, **74** (2010) 175-177.
- Ramadas N V, Singh, S K, Soccol, C R & Pandey A, Polyhydroxybutyrate production using agro-industrial residue as substrate by *Bacillus sphaericus* NCIM 5149, *Brazilian Arc Biol Technol*, **52** (2009) 17-23.
- Omar S, Rayes A & Equaab A, Optimization of cell growth and poly (3- hydroxybutyrate) accumulation on date syrup by a *Bacillus megaterium* strain, *Biotechnol Lett*, **23** (2001) 1119-1123.
- Chaijamrus S & Udpuay, N, Production and Characterization of polyhydroxybutyrate from molasses and corn steep liquor produced by *Bacillus megaterium* ATCC 6748, *Agric Engg Int: CIGR E J Manuscript FP 07 030*. vol X (2008).
- Wu Q, Huang H, Hu G, Chen J, Ho K P *et al*, Production of PHB by *Bacillus* species JMA 5 cultivated in molasses media, *Antonie Van Leeuwenhoek*, **80** (2001) 1111-1118.
- Eda Cüelik, Nalan Ozbay, Nuray Oktar & Cüelik P C, Use of biodiesel byproduct crude glycerol as the carbon source for fermentation processes by recombinant *Pichia pastoris*, *Ind. Eng Chem Res*, **47** (2008) 2985-2990.
- Hajek M & Skopal F, Treatment of glycerol phase formed by biodiesel production, *Biores Technol*, **101** (2010) 3242-3245.
- Hori K, Kaneko M, Tanji Y, Xing X & Unnu H, Construction of self disruptive *B. megaterium* in response of substrate exhaustion for PHB production, *Appl Microbiol Biotechnol*, **59** (2002) 211-216.
- Mercan N, Aslim Z, Yuksekdogan N & Beyatli Y, Production of PHB by some Rhizobium bacteria, *Turkish J Biol*, **26** (2002) 215-219.
- Rohini D, Phadnis S & Rawal S K, Synthesis and characterization of PHB from *Bacillus thuringiensis* R1, *Indian J Biotechnol*, **5** (2006) 276-283.
- Borah B, Thakur P S & Nigam J N, The influence of nutritional and environmental conditions on the accumulation of poly-β-hydroxybutyrate in *Bacillus mycoides* RLJB-017, *J Appl Microbiol*, **92** (2002) 776-783.
- Hahn S K, Chang, Y K & Lee, S Y, Recovery and characterization of poly (3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus* and recombinant *Escherichia coli*, *Appl Environ Microbiol*, **61** (1995) 34-39.
- de Almeida A, Nikel P I, Giordano A M & Pettinari M J, Effects of granule-associated protein PhaP on glycerol-

- dependent growth and polymer production in poly (3-hydroxybutyrate)-producing *Escherichia coli*, *Appl Environ Microbiol*, **73** (2007) 7912-7916.
- 25 Koller M, Bona R, Braunegg G, Hermann C, Horvat P *et al*, Production of polyhydroxy-alkanoates from agricultural waste and surplus materials, *Biomacromol*, **6** (2005) 561-565.
 - 26 Nikel P I, Pettinari, M J, Galvagno, M A & Me´ndez B S, Poly (3-hydroxybutyrate) synthesis from glycerol by a recombinant *Escherichia coli* arcA mutant in fed-batch microaerobic cultures, *Appl Microbiol Biotechnol*, **77** (2008) 1337-1343.
 - 27 Solaiman D K Y, Ashby R D, Foglia T A & Marmer, W N, Conversion of agricultural feedstock and co-products into poly(hydroxyalkanoates), *Appl Microbiol Biotechnol*, **71** (2006) 783-789.
 - 28 Ibrahim M H A & Steinbuchel A, Poly (3-hydroxybutyrate) production from glycerol by *Zobellella denitrificans* MW1 via high-cell-density fed-batch fermentation and simplified solvent extraction, *Appl Environ Microbiol*, **75** (2009) 6222-6231.
 - 29 Mahishi L H, Tripathi, G & Rawal, S K, Poly (3-hydroxybutyrate) (PHB) synthesis by recombinant *Escherichia coli* harbouring *Streptomyces aureofaciens* PHB biosynthesis genes: effect of various carbon and nitrogen sources, *Microbiol Res*, **158** (2003) 19-27.
 - 30 Ramachander T V N, Rohini, D, Belhekar, A & Rawal, S K, Synthesis of PHB by recombinant *E. coli* harboring an approximately 5 kb genomic DNA fragment from *Streptomyces aureofaciens* NRRL2209, *Int J Biol Macromol*, **31** (2002) 63-69.
 - 31 Ashby R D, Solaiman, D K Y & Foglia T A, Bacterial poly(hydroxyalkanoate) polymer production from the biodiesel co-product stream, *J Polym Environ*, **12** (2004) 105-112.
 - 32 Ashby R D, Solaiman D K Y & Foglia T A, Synthesis of short-/medium-chain-length poly(hydroxyalkanoate) blends by mixed culture fermentation of glycerol, *Biomacromol*, **6** (2005) 2106-2112.
 - 33 Ramachander M, Girisham S & Reddy S M, Production of PHB (polyhydroxybutyrate) by *Rhodopseudomonas palustris* KU 003 under nitrogen limitation, *Int J Appl Biol Pharm Tech*, **1** (2010) 676 -678.
 - 34 Kleiner D, Bacterial ammonium transport, *FEMS Microbiol Rev*, **32** (1985) 87-100.
 - 35 Annuar M S M, Tan, I K P, Ibrahim, S & Ramachandran, K B, Ammonium uptake and growth kinetics of *Pseudomonas putida* PGA1, *Asia Pacific J Molecular Biol Biotechnol*, **14** (2006) 1-10.
 - 36 Kluttermann K, Tauchert K & Kleber H, Synthesis of poly - β -hydroxybutyrate by *Agrobacterium radiobacter* after growth on D- carnitine, *Acta Biotechnol*, **22** (2002) 261-269.
 - 37 Pal A, Prabhu A, Kumar A A, Rajagopal B, Dadhe K *et al*, Optimization of process parameters for maximum poly(- β -)hydroxybutyrate (PHB) production by *Bacillus thuringiensis* IAM 12077, *Polish J Microbiol*, **28** (2009) 149-154.
 - 38 Padermshoke A, Katsumoto Y, Sato H, Ekgasit S, Noda I *et al*, Surface melting and crystallization behavior of polyhydroxyalkanoates studied by attenuated total reflection infrared spectroscopy, *Polymer*, **45** (2004) 6547-6554.