Journal of Bioprocessing & Biotechniques
ISSN: 2155-9821
Tripathi and Srivastava, J Bioprocess Biotech 2015, 5:7
DOI: 10.4172/2155-9821.1000238
Research Article Open Access

Statistical Optimization of Parameters Affecting Polyhydroxybutyrate (PHB) Recovery by Dispersion Method from *Alcaligenes* Cells and Its Characterization

Abhishek Dutt Tripathi[1]* and Suresh Kumar Srivastava[2]

[1]*Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221005, India*
[2]*School of Biochemical Engineering, Indian Institute of Technology, Banaras Hindu University, Varanasi-221005, India*

Abstract

In the present study, efforts have been made to optimize the three process variables i.e., incubation time, temperature of separation and hypochlorite concentration for enhanced polyhydroxybutyrate (PHB) recovery from *Alcaligenes* cells which may serve as precursor for bio-plastic production. Screening of bio-plastic (PHB) producing microorganism was done by TEM (Transmission electron microscopy). Initially, three recovery methods were applied to achieve efficient PHB recovery which included; Alkali treatment, ATPS (Aquas two phase separation (PEG 6000)/ KH_2PO_4) and chloroform-hypochlorite dispersion (1:1, v/v). Maximum PHB recovery of 95 ± 0.5% with 97.0 ± 0.29% purity was obtained by dispersion method. In order to enhance the PHB recovery further, dispersion process was optimized using design expert (DX 8.0.6) software. PHB recovered under optimized physical condition comprising: incubation temperature 37°C, incubation time 28 h and hypochlorite concentration of 30.0, gave maximum PHB recovery of 98.2 ± 0.05% against the predicted yield of 99.0 ± 0.05% with 99.0% purity. Characterization of recovered polymer as PHB was done by FTIR (Fourier transform infrared microscopy) and NMR (Nuclear magnetic resonance spectroscopy).

Keywords: *Alcaligenes cells;* Process variables; Optimization; PHB recovery; PHB characterization

Introduction

Plastic finds tremendous applications in medical, food, pharmaceutical and agricultural commodities. According to Global Industry Analysts Inc. the global consumption of plastic materials will increase to 297.5 million tons by 2015. Polyhydroxyalkanoates (PHAs) are thermoplastic polymers produced by diversified group of microorganisms [1,2], via the metabolic transformation of various carbon sources, under unbalanced growth conditions as intracellular reserve material [3,4]. PHAs can be categorized in three types i.e.; Short Chain Length (SCL), Medium Chain Length (MCL) and Long Chain Length (LCL) depending on the monomer size. SCL PHAs like Poly-3-Hydroxybutyrate (PHB) finds wider application in bio-plastic manufacturing due to their biodegradability and biocompatibility. Physical properties of PHB resemble to petrochemical-derived thermoplastic polypropylene; hence, PHB is considered as a potential replacement of certain traditional thermoplastics [5].

PHB can be synthesized chemically or by microorganisms and finds wider application in food packaging, biomedical and pharmaceutical industry. However, their use is currently limited owing to their high production cost. PHB production cost is dependent on several factors like substrate, chosen strain, cultivation strategy and downstream processing [6-9]. Inexpensive and scalable recovery schemes need to be devised to achieve low-cost production that is competitive with traditional thermoplastics [5]. Therefore, selection of the time-optimal process operating conditions to maximize the biomass production rate and PHA yield, is a problem of significant economic importance in a bioprocess [10]. There are many methods developed by researchers for the extraction, purification and recovery of PHA but the methods are still in consideration because of the high cost, complex process and non-environmental friendly aspects.

The efficient recovery process for PHA production depends on several factors. A high recovery and purity of recovered polymer with no reduction of molecular weight represents the efficiency of the recovery process.

In the present study, three recovery methods viz; alkali treatment, ATPS (aquas two phase separation) and chloroform-hypochlorite dispersion were applied to achieve efficient PHB recovery. In order to further enhance the PHB recovery, factors affecting the best recovery method were optimized and were characterized as PHB by FTIR and NMR.

Materials and Methods

Bacterial strain and growth conditions

Alcaligenes sp. NCIM 5025 was obtained from NCL (National Chemical Laboratory), Pune, India. Production media for PHB accumulation consisted of Mineral Salt Medium (MSM), comprising 40 g/l cane molasses, 0.8 g/l urea, 2.0 g/l KH_2PO_4, 0.6 g/l Na_2HPO_4, 1.0 g/l, $MgSO_4.7H_2O$, 0.1 g/l yeast extract and 1 ml/l of trace element (1.3 g/l $ZnSO_4.7H_2O$, 20.0 g/l $CaCl_2$, 0.2 g/l $FeSO_4.7H_2O$, 0.6 g/l $(NH_4)_6Mo_7O_{24}.4H_2O$ and 0.6 g/l H_3BO_3).The carbon sources were sterilized separately at 121°C for 15 min and then aseptically added into the flask containing the other components at room temperature. The pH of the final culture medium was adjusted to 7 ± 0.5 using 0.1N HCl/0.1N NaOH before bacterial inoculation. Production media

***Corresponding author:** Abhishek Dutt Tripathi, Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221005, India, Tel: +91-945-053-3651; E-mail: abhi_itbhu80@rediffmail.com

Received June 08, 2015; **Accepted** July 07, 2015; **Published** July 13, 2015

Citation: Tripathi AD, Srivastava SK (2015) Statistical Optimization of Parameters Affecting Polyhydroxybutyrate (PHB) Recovery by Dispersion Method from *Alcaligenes* Cells and Its Characterization. J Bioprocess Biotech 5: 238 doi:10.4172/2155-9821.1000238

was prepared in 250 ml conical flask containing 100 ml mineral salt medium. 5 ml of the seed of each bacterial strain inoculums were added into different conical flasks containing 100 ml of production medium and incubated at 150 rpm, 37°C for 48 h. Samples were extracted for analysis at different time interval viz; 24, 48 and 72 h respectively.

Analytical study

Dry cell mass, PHB estimation: 20 ml culture broth was centrifuged at 10,000 rpm for 10 min at 4°C and cell pellet was obtained. The cell pellet was washed with saline water (NaCl, 0.8% (wt/vol)) and then dried in aluminium weighing dish at 90°C for 24 hr. PHB estimation was done by gas chromatography (Neukon 5700, Detector-FID, Column-2 m in length, External diameter-30 mm, internal diameter-2 mm, Porapack OV-101) as reported in our previous research paper [11].

PHB extraction: PHB was extracted by using alkali treatment, ATPS and chloroform-hypochlorite extraction method. Pure PHB was obtained by non solvent precipitation and filtration. The non solvent used was a mixture of methanol and water (7:3, vol/vol). Filtration was done using membrane filters (mesh size, 2 µm, millipore).

PHB recovery by alkali treatment: Dry cell mass obtained after different time interval of cultivation was subjected to alkali treatment. 0.1N sodium hydroxide solution was taken to estimate the recovery and purity of samples at 25°C.

PHB recovery by ATPS (Aquas two Phase separation system): Two Phase system was developed by using 26% w/v polyethylene glycol (PEG 6000) and 20% (w/v) KH_2PO_4. The standard PHB of concentrations 3.0 g/l was used for partitioning in ATPS made by PEG 6000 and KH_2PO_4. ATPS (10 ml) were prepared by taking point at the center of the tie line of bimodal curve. The PEG phase was separated by disposable syringe and transferred to another flask (100 ml) and the flask was kept in rotation at 200 rpm for 10 minutes again. The PHB on the top was separated and analyzed. The salt phase was also separated and analyzed for PHB concentration and partition coefficient K_d was calculated using formulae.

$$Kd = \frac{PHB\ in\ PEG\ phase\,(\mathrm{g/l})}{PHB\ in\ aquas\ phase\,(\mathrm{g/l})}$$

PHB recovery by using dispersions of sodium hypochlorite and chloroform: 10 ml of culture medium was placed in a 15 ml centrifugal tube and cells were harvested by centrifugation at 4500 rpm for 10 min. The harvested cells (1.0 g dry cell weight) were treated with dispersion of 50 ml of chloroform and 50 ml of sodium hypochlorite solution. The concentrations of sodium hypochlorite solution used were 30% (v/v). After that the mixture was agitated at 30°C for 90 min. It was centrifuged at 8,000 rpm for 10 min. The resulting PHB Containing bottom Phase (chloroform Phase) was withdrawn and added into a non-solvent solution (70% methanol in water). The PHB filtered was recovered by filtration (Whatman no. 1 filter paper) and then dried at 70°C for 5 h.

Determination of purity and recovery yield: Recovery yield and purity in different extraction techniques were determined by following formulae

$$\%\ purity = \frac{Amount\ of\ PHB\ after\ recovery}{Amount\ of\ total\ dry\ matter\ after\ recovery} \times 100$$

$$\%\ \mathrm{Recovery} = \frac{Amount\ of\ PHB\ recovered}{Total\ amount\ of\ PHB\ \mathrm{in}\ the\ cell} \times 100$$

Preparation of samples for TEM analysis: Samples were collected after 48 h of fermentation and were fixed with 2.0% glutaraldehyde solution to observe the intracellular granule under electron microscope. Sample preparation, cell fixation, sectioning and visualization were done by method as reported in our previous study [12].

Sample preparation for FTIR analysis: Extracted PHB granules were dissolved in isotonic saline solution (30 kg m^{-3}) and then 20 µL of the solution was deposited on KBr disc. The depositors were then dried and IR spectra was recorded with a Bruker model IFS-55 FTIR spectrometer coupled to a Bruker IR microscope fitted with an IBM compatible PC running OPUS, Version 2.2 software.

H^1 NMR study of the molecular structure of PHB: The 500 MHz ^1H NMR spectrum of PHB was obtained by using a Bruker model AMX-FT NMR spectroscope. A PHB solution was prepared by using $CDCl_3$ at a concentration of 2% (wt/vol). The spectra were recorded at 25°C with a pulse repetition time of 3 s. All ^1H NMR spectra were recorded at 30°C on a Bruker AM300-WB spectrometer (Wissembourg, France) equipped with an Aspect 3,000 computer using a 5 mm $^1H/\ ^{13}C$ dual probe. Proton spectra were recorded at 300.13 MHz with a spectral width of 2,840 Hz over 16 K data points. A 66° pulse angle was used. No apodization was used for ^1H interferograms. The measurements of spin-lattice relaxation time (T_1) were carried out by means of the inversion recovery method (180°-t (variable)-90°-free induction decay). Data were averaged over 32 acquisitions with PHBse cycling and a recycle delay dependent on the internal standard at a concentration of l.0% v/v in $CDCl_3$, to avoid saturation. T, values were calculated after examination of peak intensities using the TlCAL Bruker program. Chemical shifts were referenced to the internal reference TMS.

Result and Discussion

Screening of PHB producing microbe

Intracellular granule biosynthesis in *Alcaligenes sp*. was done by TEM. Figure 1 represents TEM images (magnification 100 nm) at different time interval which showed *Alcaligenes* cell filled with large sized PHB granules attached to membrane vesicle and in the cytoplasm obtained after 48 h of cultivation.

PHB recovery by three extraction methods

PHB granules obtained were by three extraction methods viz; alkali treatment, ATPS method and chloroform-hypochlorite dispersion. Chlorinated solvent extraction (hypochlorite-chloroform dispersion) gave maximum PHB recovery and purity of 95.0 ± 0.5 and 97.0 ± 0.29%, respectively in comparison to alkali treatment and ATPS extraction (Table 1).

Statistical optimization of parameters affecting PHB recovery by chloroform-hypochlorite dispersion

PHB recovery from chloroform and hypochlorite dispersion is dependent on three parameters viz., incubation time, temperature of separation and hypochlorite concentration. In order to enhance PHB recovery, optimization of these parameters viz., incubation time, temperature of separation and hypochlorite concentration was performed by CCRD.

Experimental design

A five-level-three factor Central Composite Rotary Design (CCRD) was obtained by using the software (Minitab 16) to find out the interactive effect of three process variables viz; temperature, incubation

time and hypochlorite concentration on PHB recovery (Table 2). Twenty set of experiment were designed by Minitab 16 software at different levels of three parameters (Table 3)

Recovery Model

Table 3 represents the design matrix in actual terms and experimental results of cell mass and PHB content in the *Alcaligenes sp.* by the CCRD with 8 factorial points, 6 axial points and 6 central points. Maximum PHB recovery of 96.0 ± 0.35% was obtained in the trial no 3, comprising temperature (35°C), hypochlorite concentration (30.0% v/v) and incubation period (26 h) (Table 3). The regression analysis of experimental design demonstrated that the linear model terms (A temperature and incubation time B), quadratic model terms (A^2, B^2 and C^2) and interactive model term (BC) were significant ($p<0.05$) (Table 4). However, the linear model term C and interactive model terms (AB and AC) were found to be insignificant ($P>0.05$) (Table 4).

The experimental data obtained from the design were analyzed by the response surface regression procedure using the following second-order polynomial equation:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x^2_i + \sum \beta_{ij} x_i j_i$$

Where Y_i was the predicted response, $x_i x_j$ were independent variables, β_0 was the offset term, β_i was the linear coefficient, β_{ii} was the quadratic coefficient and β_{ij} was the interaction coefficient. Applying multiple regression analysis, the results were fitted to second order polynomial equation. The mathematical regression model for PHB recovery from *Alcaligenes sp.* fitted in the terms of coded variables was obtained as follows:

Y(PHB recovery)=+45.9942+1.59584 A+0.846288 C+0.638159 B-0.0188385 A^2-0.0148743 B^2-0.0108318 C^2-0.00187500 AB-0.00397727 AC+0.00426136 BC

Where, Y is the response in terms of PHB content. Coded terms A, B and C represents temperature, incubation time and hypochlorite concentration, respectively.

ANOVA

Table 5 represents ANOVA of results of quadratic models. Analysis of variance (ANOVA) was used to estimate the statistical parameters. The second order polynomial equation was used to fit the experimental data. The significance of the model equation and model terms were evaluated by F-test. The quality of the fit of the polynomial equation was expressed by the coefficient of determination (R^2), adjusted and "adequate precision". The model F-value of 21.30 depicts that model was significant. Lack of fit value of 34.88 implies that the lack of fit is not significant relative to pure error. The fit of model was also expressed by the coefficient of determination R^2, which was found to be 0.9505, indicating that the 95.05% of the variability in the response can be explained by the model. The "pred R-squared of 0.8916 is in reasonable agreement with the "Adj R-Squared" of 0.9008. "Adeq Precision" measures signal to noise (S/N) ratio and its value obtained in the present model was found to be 17.25, which indicates an adequate signal (S/N ratio >4 is desirable). The obtained results clearly suggest that quadratic model could be used to navigate the design space.

Interactive effect of process variables on PHB recovery

The fitted polynomial equation was expressed as three dimensional surface plots to visualize the relationship between the responses and the experimental levels of each factor used in the design. Interactive effect of different process variables were studied for PHB recovery by using 2D contour plots and 3D response surface. Figure 2A represents the interactive effect of temperature and incubation time on PHB recovery. It can be clearly deduced from 3D surface and contour plot (Figure 2A and 2B) that increase in temperature and incubation time, enhanced PHB recovery up to 40°C and 30 h, respectively. However, further increase in these two process variables decreased PHB recovery which is in agreement with previous report, where increased temperature (>41°C) decreased the PHB content probably due to polymer degradation and excessive cell lysis at elevated temperature [13]. Figure 3A represents the interactive effect of temperature and hypochlorite concentration on PHB recovery, which showed enhanced PHB recovery at suboptimal level of two process variables (temperature 37°C and hypochlorite concentration 30%). Further increase in temperature and hypochlorite concentration showed decreased PHB recovery which is represented by convergence of curve towards boundary of 3D plot (Figure 3A). Contour plot representing interactive effect of temperature and hypochlorite concentration confirmed the above finding that PHB recovery was maximum at 30% hypochlorite concentration while keeping incubation time at zero level of 26 h (Figure 3B). PHB recovery increased rapidly up to 30% hypochlorite concentration and 37°C temperature and then showed decreasing trend. Previously 91% PHB recovery was obtained from *Alcaligenes eutrophus* using 30% hypochlorite solution under similar condition [14].

Incubation time and hypochlorite concentration showed significant influence on PHB recovery in *Alcaligenes sp.*, when considered as individual process variable ($p<0.05$), However, interactive effect of incubation period and hypochlorite concentration was insignificant ($p>0.05$). Figure 4A and 4B represented interactive effect of two parameters (incubation time and hypochlorite concentration) on PHB recovery which showed that PHB recovery enhanced till 30% hypochlorite concentration. This suggests that chloroform can

S.No	Recovery process	PHB Recovery (%)	PHB purity (%)
1.	Alkali treatment	80.0 ± 1.04	91.0 ± 0.58
2.	Chlorinated solvent extraction (chloroform-hypochlorite dispersion)	95.0 ± 0.5	97.0 ± 0.29
3.	Aquas two phase separation (ATPS)	90.0 ± 1.0	95.0 ± 0.76

Table 1: Comparison of three recovery process based on percentage recovery and purity.

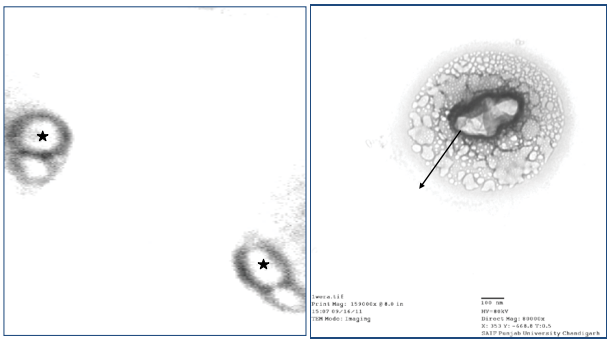


Figure 1: TEM analysis of *Alcaligenes sp.* membrane vesicle after negative staining. TEM analysis of *Alcaligenes sp.* after 48 h of inoculation. Asterisk represents large PHB granules. *Alcaligenes* cell filled with granules. Arrows point to intracellular granules present inside membrane vesicle. Arrow represented the large PHB granule inside the vesicle (magnification 100 nm).

S.No	Variable	Code	Levels				
			-2 (-α)	-1	0	1	2 (+α)
1.	Temperature (°C)	A	18.18	25.00	35.00	45.00	51.82
2.	Incubation time (h)	B	-10.99	4.00	26.00	48.00	62.99
3.	Hypochlorite concentration (%)	C	-3.64	10.00	30.00	50.00	63.64

Table 2: Levels of physical factors used for optimization of three factors affecting PHB recovery

Run Order	Run	Levels	Levels	Temperature (°C)	Hypochlorite Concentration (% v/v)	Incubation period (h)	PHB recovery (%)
17	1	0	1	35.0000	30.0000	26.0000	94.0 ± 0.25
19	2	0	1	35.0000	30.0000	26.0000	95.0 ± 0.21
20	3	0	1	35.0000	30.0000	26.0000	96.0 ± 0.35
5	4	1	1	25.0000	10.0000	48.0000	83.0 ± 0.4
6	5	1	1	45.0000	10.0000	48.0000	88.0 ± 0.15
10	6	-1	1	51.8179	30.0000	26.0000	93.0 ± 0.38
3	7	1	1	25.0000	50.0000	4.0000	78.0 ± 0.2
8	8	1	1	45.0000	50.0000	48.0000	86.0 ± 0.35
7	9	1	1	25.0000	50.0000	48.0000	89.0 ± 0.26
9	10	-1	1	18.1821	30.0000	26.0000	90.0 ± 0.25
11	11	-1	1	35.0000	-3.6359	26.0000	78.0 ± 0.50
4	12	1	1	45.0000	50.0000	4.0000	85.0 ± 0.42
15	13	0	1	35.0000	30.0000	26.0000	95.0 ± 0.38
12	14	-1	1	35.0000	63.6359	26.0000	82.0 ± 0.53
14	15	-1	1	35.0000	30.0000	62.9994	85.0 ± 0.53
13	16	-1	1	35.0000	30.0000	-10.9994	79.0 ± 0.3
16	17	0	1	35.0000	30.0000	26.0000	95.5 ± 0.21
2	18	1	1	45.0000	10.0000	4.0000	88.0 ± 0.25
18	19	0	1	35.0000	30.0000	26.0000	94.0 ± 0.25
1	20	1	1	25.0000	10.0000	4.0000	86.0 ± 0.40
17	1	0	1	35.0000	30.0000	26.0000	95.0 ± 0.32

Table 3: Central composite design of independent variables for the optimization of parameters affecting PHB recovery from *Alcaligenes sp*.

Term	Coef	SE Coef	T	P
Constant	97.7790	0.8921	109.605	0.000
Temperature	1.1749	0.5929	1.985	0.075
Hypochlorite concentration	-0.0200	0.5919	-0.034	0.974
Incubation time	1.3979	0.5919	2.362	0.040
Temperature*Temperature	-1.8838	0.5762	-3.270	0.008
Hypochlorite concentration* Hypochlorite concentration	-5.9497	0.5762	-10.326	0.000
Incubation time*Incubation time	-5.2646	0.5762	-9.099	0.000
Temperature* Hypochlorite concentration	-0.3750	0.7733	-0.485	0.638
Temperature* Incubation time	-0.8750	0.7733	-1.131	0.284
Hypochlorite concentration* Incubation time	1.8750	0.7733	2.425	0.036

Table 4: Estimated regression coefficient for recovery (%).

Variables	Before	After	PHB recovery (% DCW)		
			Before	After	
				Predicted	Experimental
Temperature(°C)	37.00	36.00	96.00	99.0 ± 0.10	98.20 ± 0.05
Incubation time (h)	30.00	28.00			
Hypochlorite concentration (%)	30.00	30.00			

Table 5: Analysis of variance for recovery (%).

protect the PHB molecules from being digested by hypochlorite if a moderate hypochlorite concentration is used. Maximum PHB recovery was obtained below 30 h of incubation, which indicates that PHB degradation is more prominent than PHB release from cell biomass after 30 h of treatment. PHB purity was found to be 99% at 30% hypochlorite concentration which is higher in comparison to previous report [14]. Hann et al. [15] reported 88% and 93% purity in PHB obtained from *Alcaligenes eutrophus* and recombinant *E.coli* using 20% sodium hypochlorite and chloroform dispersion [15]. PHB recovery of 80% with 98.3% purity was achieved after 72 h of extraction

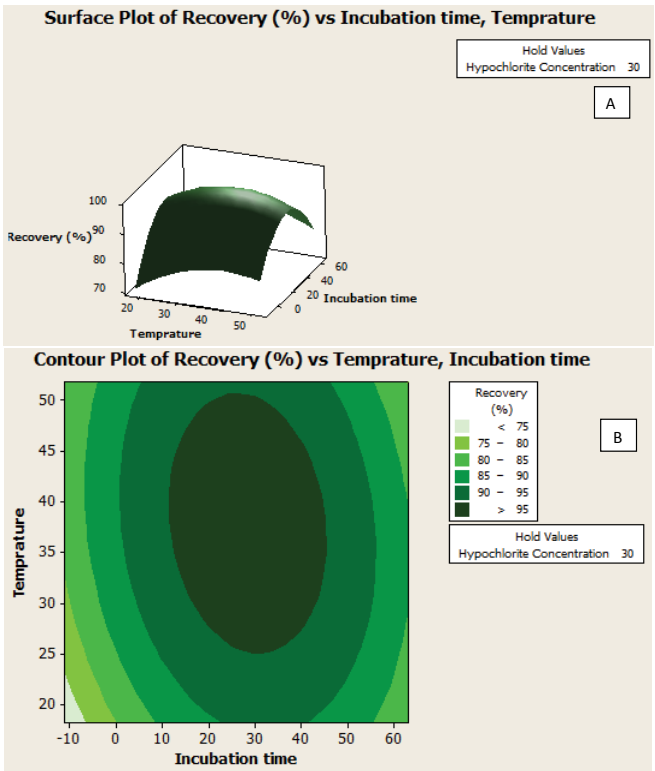


Figure 2: A) Surface plot of recovery between temperature and incubation time. B) Contour plot of recovery between temperature and incubation time.

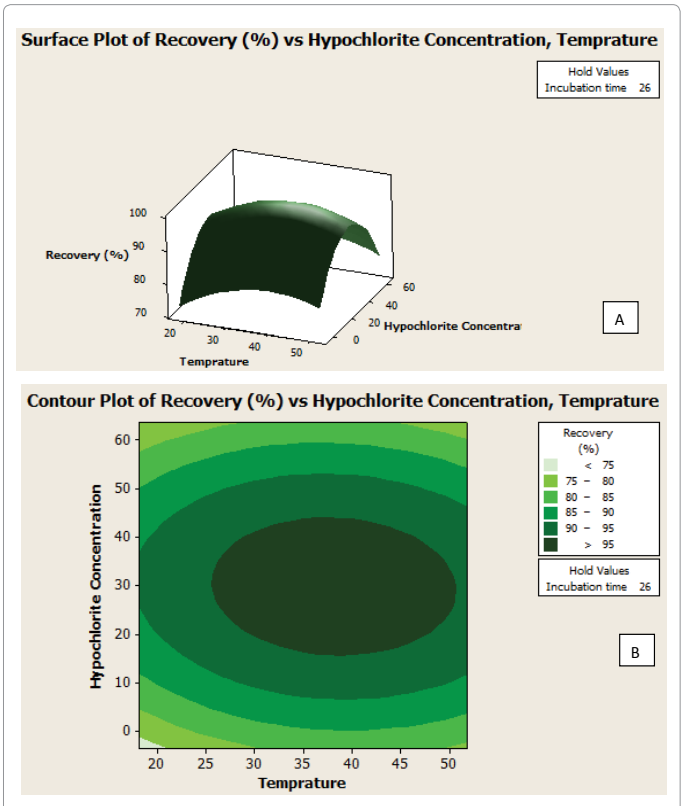


Figure 3: A) Surface plot of recovery between hypochlorite concentration and temperature B) Contour plot of recovery between hypochlorite concentration and temperature.

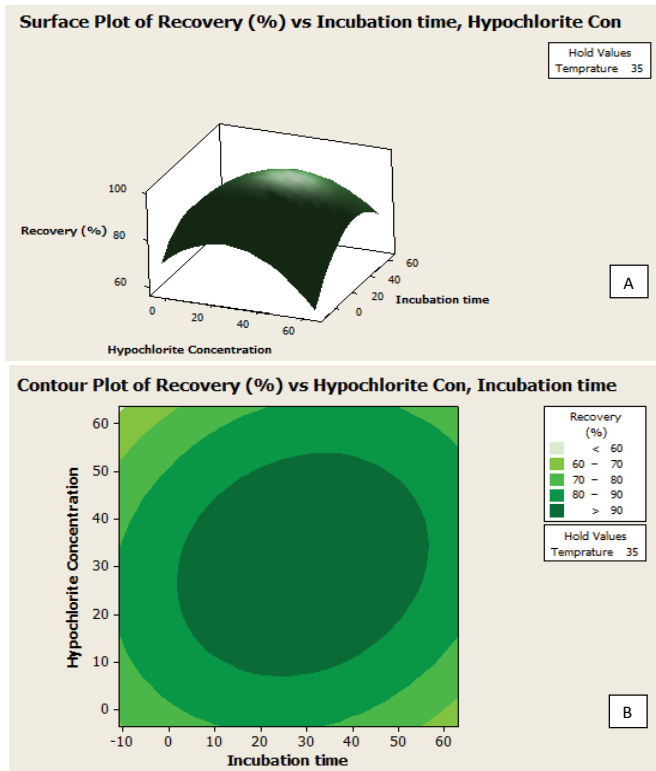


Figure 4: A) Surface plot of recovery between hypochlorite concentration and incubation time. B) Contour plot of recovery between hypochlorite concentration and incubation time.

with chloroform at 30°C [13]. PHB yield of 70% with 90% purity was obtained in solvent extraction with 1,2 dichloroethane with acetone pretreatment for 24 h at 83°C [16].

Model verification

CCRD used for the optimization of the three parameters for enhanced PHB recovery revealed the maximum PHB recovery of 98.20 ± 0.05% recorded against the predicted yield of 99.0 ± 0.10 % at optimum condition comprising: incubation temperature 37°C, incubation time 28 h and hypochlorite concentration of 30%. It can be visualized from Table 5 that the predicted and experimental PHB content after optimization were well in agreement. The desirability was found to be 0.95 which showed 95% validation between observed and experimental value.

Characterization of PHB

Characterization of recovered polymer was done by FTIR and H^1NMR. FTIR spectra predicted the presence of functional groups of PHB i.e. aliphatic C-H, =O stretching, =C-H deformation, =C-H, =CH, =C-O etc. In pure PHB samples the band appeared at 1460 cm^{-1} which correspond to the asymmetrical deformation of C-H bond in CH_2 groups including CH_3 groups at 1379 cm^{-1} [17]. The band at 1726 and 1280 cm^{-1} corresponded to the stretching of the C=O bond, whereas a series of intense bands located at 1000-1300 cm^{-1} reads the stretching of the C-O bond of the ester group. PHB extracted from *Alcaligenes sp.* by ATPS method and chloroform-hypochlorite dispersion (Figure 5A and 5B) resembled commercial PHB because there was a strong adsorption band at 1244 cm^{-1} which is characteristic for ester bonding. Other adsorption bands at 1392, 1486, 2922, 1726 and 3760 cm^{-1} for

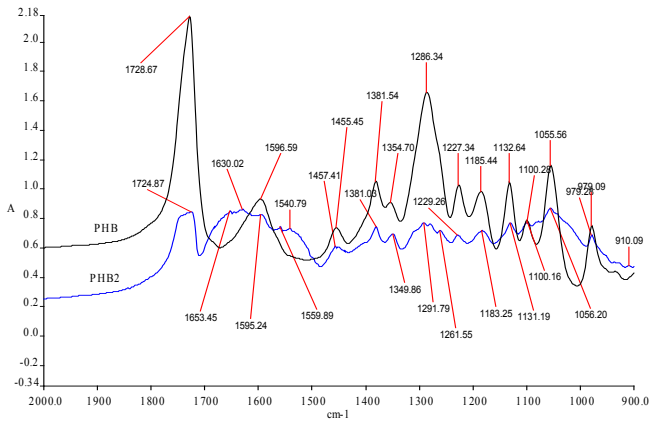


Figure 5a: A. FTIR (Fourier Transform Infrared Spectroscopy) showing PHB produced by *Alcaligenes sp*. NCIM 5085 after 24 h of production phase recovered by ATPS method.

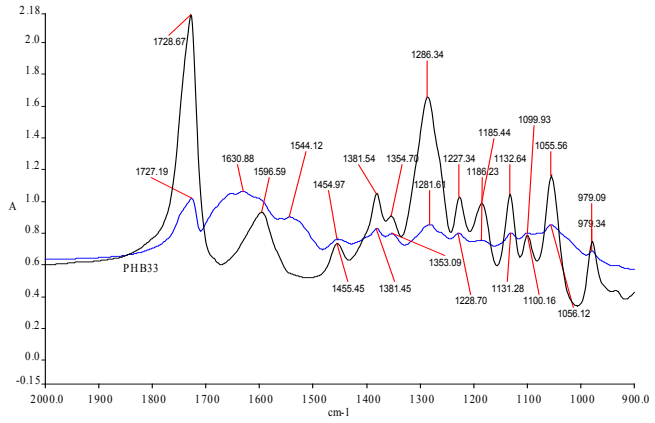
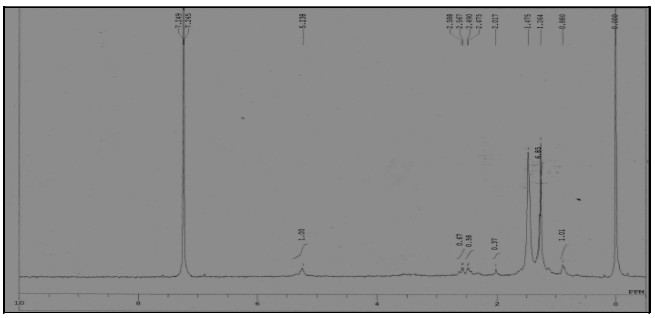


Figure 5a: FTIR (Fourier Transform Infrared Spectroscopy) of PHB produced by *Alcaligenes sp*. NCIM 5085 by hypochlorite-chloroform dispersion method.



δ 5.26 (1H, m, CH), δ 2.52 (2H, d, CH_2), δ 1.274 (3H, s, CH_3)

Figure 6: ^{1}H-NMR (D_2O) of polyhydroxyalkanoate extracted from *Alcaligenes sp*. in optimized medium.

CH_3, -CH_2, -CH, C=O, and O-H groups respectively were similar to the absorption band of commercial PHB. Extracted polymer was characterized as PHB by NMR analysis. H^1 NMR analysis of extracted polymer showed a doublet at 1.53 ppm representing the methyl group (CH_3) coupled to one proton while a doublet of quadruplet at 2.75 ppm resulted from methylene group (CH_2) adjacent to an asymmetric carbon atom bearing a single proton. The third signal was multiplet at 5.52 ppm, which is attributed to methylene group (CH) (Figure 6). From the contribution of various groups to NMR spectra, it may deduced that cane molasses from sugar refinery industry could directly serve as an inexpensive source of biodegradable polymer and that the bacterial biomass produced PHA mainly in the form of PHB when grown under optimized condition.

Conclusion

Dispersion method gave maximum PHB recovery and purity of 95.0 ± 0.5 and 97.0 ± 0.29, respectively in comparison to alkali treatment and ATPS extraction. PHB recovery was further enhanced by optimizing the three variables affecting dispersion separation. Maximum PHB recovery of 98.20 ± 0.05 % with 99.0 ± 0.05% purity was obtained under optimized condition comprising: incubation temperature 37°C, incubation time 28 h and hypochlorite concentration of 30% (v/v). Chloroform-hypochlorite dispersion showed efficient PHB recovery from *Alcaligenes* cells in comparison to other conventional recovery method. Previously, PHB recovery and purity of 80 and 95% respectively were obtained with chloroform extraction [18]. PHB recovered by enzymatic treatment (pancreatin) gave maximum recovery and purity of 93.5 and 91.2%, respectively in *Bukerholdria cepacia* [19]. Enhanced PHB recovery with high purity from chosen strain (*Alcaligenes sp*.), obtained in the present study by dispersion method can minimize the overall downstream processing cost. Current methodology may be adopted for efficient PHB recovery from large cell density harvested from bioreactors at industrial level. PHB recovered by dispersion method suggests their crystalline behavior owing to their resistance towards chloroform, may find application in tissue engineering and food packaging.

Acknowledgements

We would like to pay our sincere thanks to Department of Biotechnology (GOI) for providing financial support in the research work. We would also like to acknowledge Prof. S.B. Roy, Department of Physics, Banaras Hindu University, India for providing FTIR facility. We would be grateful to Central Instrumentation Laboratory, UIET, Punjab University for providing TEM facility.

References

1. Lu JN, Tappel RC, Nomura CT (2009) Mini-review: biosynthesis of poly(hydroxyalkanoates). Polym Rev 49: 226-248.
2. Verlinden RA, Hill DJ, Kenward MA, Williams CD, Piotrowska-Seget Z, et al. (2011) Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. AMB Express 1: 11.
3. Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54: 450-472.
4. Madison LL, Huisman GW (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63: 21-53.
5. Tamer IM, Moo YM, Chisti Y (1998) Optimization of poly (β-hydroxybutyric acid) recovery from *Alcaligenes latus*: Combined mechanical and chemical treatments. Bioprocess Eng 19: 459-468.
6. Dias JM, Lemos PC, Serafim LS, Oliveira C, Eiroa M, et al. (2006) Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: from the substrate to the final product. Macromol Biosci 6: 885-906.
7. Johnson K, van Geest J, Kleerebezem R, van Loosdrecht MC (2010) Short- and long-term temperature effects on aerobic polyhydroxybutyrate producing mixed cultures. Water Res 44: 1689-1700.
8. Khanna S, Srivastava AK (2005) Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. Process Biochem 4: 2173-2182.
9. Philip S, Keshavarz T, Roy I (2007) Polyhydroxyalkanoates: biodegradable polymers with a range of applications. J Chem Technol Biotechnol 82: 233-247.
10. Chen GQ (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Rev 38: 2434-2446.

11. Tripathi AD, Srivastava SK (2011) Novel approach for optimization of fermentative condition for polyhydroxybutyrate (PHB) production by *Alcaligenes* sp. using Taguchi (DOE) methodology. Afr J Biotechnol 10: 7219-7224.

12. Tripathi AD, Srivastava SK (2011) Kinetic Study of Biopolymer (PHB) synthesis in *Alcaligenes* sp. in submerged fermentation process using TEM. J Pol Sci Environ19: 732-738.

13. Heinrich D, Madkour MH, Al-Ghamdi MA, Shabbaj II, Steinbüchel A (2012) Large scale extraction of poly(3-hydroxybutyrate) from *Ralstonia eutropha* H16 using sodium hypochlorite. AMB Express 2: 59.

14. Hahn SK, Chang YK, Kim BS, Chang HN (1994) Optimization of microbial poly(3-hydroxybutyrate) recover using dispersions of sodium hypochlorite solution and chloroform. Biotechnol Bioeng 44: 256-261.

15. Hahn SK, Chang YK, Lee SY (1995) Recovery and characterization of poly(3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus* and recombinant *Escherichia coli*. Appl Environ Microbiol 61: 34-39.

16. Ramsay JA, Berger E, Ramsay BA, Chavarie C (1990) Recovery of poly-3-hydroxyalkanoic acid granules by a surfactant-hypochlorite treatment. Biotech Technol 4: 221-26.

17. Kansiz M, Billman-Jacobe H, McNaughton D (2000) Quantitative determination of the biodegradable polymer Poly(beta-hydroxybutyrate) in a recombinant *Escherichia coli* strain by use of mid-infrared spectroscopy and multivariative statistics. Appl Environ Microbiol 66: 3415-3420.

18. Mahapatra K, Suresh Kumar M, Chakrabarti T (2007) Production and recovery process of polyhydroxybutyrate (PHB) from waste activated sludge. J Environ Sci Eng 49: 164-169.

19. Wang Y, Zhou XH, Hu JJ, Zhang QG (2012) Study on Extraction and Identification of PHB from H_2 Production Fermentation Residue by Photosynthetic Bacteria. Adv Mater Res 518-523: 108-115.