Statistical optimization of physical process variables for bio-plastic (PHB) production by *Alcaligenes* sp.

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ABSTRACT

In the present study, efforts have been made to optimize the three physical process variables viz; pH, temperature and agitation speed for enhanced polyhydroxybutyrate (PHB) production in batch cultivation by *Alcaligenes* sp. which serves as precursor for bio-plastic (PHB) production. Strain selection was done by viable staining method using nile blue A dye. Agro-industrial by products; cane molasses and urea were used as carbon and nitrogen source for PHB production. Optimization of physical process variables was done by central composite rotatable design (CCRD) using design expert (DX 8.0.6) software. Shake flask cultivation performed under optimum physical condition viz; 34.5 °C temperature, 6.54 pH and agitation speed of 3.13 Hz, gave PHB mass fraction yield of 76.80% on dry molasses substrate and showed 98.0% resemblance with the predicted percentage yield of 77.78%. Batch cultivation further performed in 7.5 L lab scale bioreactor (working volume: 5.6 L) under optimized condition gave maximum cell biomass of 11 ± 0.5 g L^{-1} with a PHB content of 8.8 ± 0.4 g L^{-1} after 48.0 h of fermentation. Scale up study on bioreactor gave maximum PHB yield ($Y_{P/x}$) and productivity of 0.78 and 0.19 g L^{-1} h, which are higher than previous reports under similar condition. Characterization of PHB was done by FTIR.

1. Introduction

In today's modern era of science and technology plastics have become one of the most widely used materials all over the world. Plastic finds enormous application in agriculture, medicine, pharmaceutical, cosmetic and food industry. Although, plastic finds enormous applications in various sectors, its disposal poses serious threat to the environment due to its non degradable behavior. In order to overcome this problem, researchers are now focusing on development of completely biodegradable plastic such as, Polyhydroxyalkanoates (PHAs) [1]. PHAs are water-insoluble, biodegradable storage polymers

produced by microorganisms present in environments utilizing different renewable carbon sources [2]. The most common PHA is polyhydroxybutyrate (PHB) which can accumulate up to 90% of the cellular dry weight in few gram negative bacteria [3]. PHB have received increased attention because of its thermoplastic and elastomeric properties, which resemble those of petroleum-based plastics, yet they are completely biodegradable [4]. PHB has shown enormous applications in bone plates, nails, screws and treatment of oesteomyelitis [5].

Currently, the main concern associated with the widespread application of PHB and its copolymers is its relatively high cost compared to polypropylene. Raw materials cost and downstream processing makes PHB expensive in comparison to other petroleum derived plastics. Optimization of fermentation process and gene cloning has been extensively used for minimizing PHB production cost [6]. Earlier studies revealed that PHB yield is a function of various nutritional and cultural conditions. Process optimization for PHB yield in various microorganisms has been done by taking into account various media components like sucrose, urea and KH_2PO_4 [7]. The concentration of fructose, KH_2PO_4, Na_2HPO_4 and $MgSO_4.7H_2O$ were optimized in *Wausteria eutropha* using RSM for enhanced PHB production [8]. Process optimization of cultural condition to enhance PHB production using two stage fermentation strategies showed marked increase in PHB accumulation in *Cupriavidus taiwanensis* 184 [9].

Physical factors like temperature, pH and agitation speed play significant role in PHB accumulation in various bacterial strains using cheaper carbon source [10]. However, only few reports are available which focused on the effect of physical process variables on PHB production [11]. Present study, therefore envisaged to optimize the physical process variables for enhanced PHB production.

In our previous study media components were optimized to enhance the PHB yield [12]. The present research work was carried out to optimize physical process variables viz; temperature, pH and agitation speed for enhanced PHB production under submerged fermentation by *Alcaligenes* sp. NCIM 5085 using cane molasses and urea as potent carbon and nitrogen source. Shake flask cultivation was further scale up in 7.5 L bioreactor in order to study the growth kinetics in batch mode under optimized physical conditions.

3. Results

3.1. Screening of PHB producing activity

Alcaligenes sp. cultivated in growth media containing nile blue A solution ($5 \, g \, m^{-3}$ in $10 \, mL \, L^{-1}$ DMSO solution) and cane molasses ($1 \, kg \, m^{-3}$) showed fluorescence under ultraviolet light (342 nm) (Fig. 1A). However PHB^- cell colonies (control) showed no fluorescence under similar condition (Fig. 1B). This clearly suggests that bacterial colonies cultivated on nile blue A incorporated agar plate produced intracellular PHB granules which gave fluorescence upon treatment with UV light. Nile blue A dye solution (dissolved in DMSO) diffuses through the cytoplasmic membranes of bacterial cell and form complex with intracellular PHB granules which gives fluorescence in UV light [14].

3.2. Design of experiment

Response surface methodology was employed to check the best operating conditions for enhanced PHB content. Three process variables viz; pH, temperature and agitation speed were chosen at five levels and then twenty set of experiments were designed by design expert 8.0.6 software tool using central composite rotatable design (CCRD) (Table A.1).

3.3. Model fitting

Design matrix in coded terms with 8 factorial points, 6 axial points and 5 central points are presented in the Table A.2. Table 1 represented the experimental results of PHB content in the *Alcaligenes* sp. by the CCRD. Maximum and minimum PHB content of 72.14 ± 0.6 and $34.36 \pm 0.08\%$ were obtained in the trial no 14 and 10, respectively at different combinations of temperature (26.59–43.41 °C), pH (5.15–9.35) and agitation speed (0.83–5.3 Hz)

Table 1 – CCRD matrix response of PHB yield.

Run	Type	Factor 1	Factor 2	Factor 3	PHB content (% DCW[a])
		Temperature (°C)	pH	Agitation speed (Hz)	
1	Center	0.000	0.000	0.000	71.56 ± 0.32
2	Factorial	−1.000	1.000	1.000	48.67 ± 0.17
3	Factorial	−1.000	−1.000	1.000	64.78 ± 0.06
4	Factorial	1.000	1.000	1.000	45.34 ± 0.08
5	Factorial	1.000	−1.000	−1.000	45.67 ± 0.13
6	Factorial	1.000	−1.000	1.000	45.78 ± 0.1
7	Center	0.000	0.000	0.000	70.41 ± 0.35
8	Axial	0.000	0.000	−1.682	45.65 ± 0.12
9	Axial	0.000	−1.682	0.000	66.75 ± 0.09
10	Factorial	1.000	1.000	−1.000	34.36 ± 0.08
11	Factorial	−1.000	−1.000	−1.000	58.96 ± 0.07
12	Center	0.000	0.000	0.000	70.96 ± 0.15
13	Axial	0.000	1.682	0.000	46.72 ± 0.09
14	Center	0.000	0.000	0.000	72.14 ± 0.16
15	Axial	0.000	0.000	1.682	56.48 ± 0.21
16	Factorial	−1.000	1.000	−1.000	43.89 ± 0.12
17	Axial	1.682	0.000	0.000	45.98 ± 0.10
18	Center	0.000	0.000	0.000	71.96 ± 0.12
19	Axial	−1.682	0.000	0.000	58.67 ± 0.29

a DCW Dry cell weight.

(Table 1). The regression analysis of experimental design demonstrated that the linear model (A, B and C) and quadratic model (A^2, B^2 and C^2) were significant ($p < 0.05$) (Table A.3).

Applying multiple regression analysis, the results were fitted to second order polynomial equation. PHB content fitted in the terms of coded variables was obtained as follows:

$$Y \, (\text{PHB}) = +76.67 - 4.87 * A - 5.61 * B + 2.96 * C + 2.43$$
$$* A * B + 0.061 * A * C + 1.23 * B * C$$
$$- 9.3 *^2 - 7.76 * B^2 - 9.72 * C^2$$

Where, Y is the response in terms of PHB content. Coded terms A, B and C represents temperature, pH and agitation speed, respectively. ANOVA of results of quadratic models are represented in Table A.3. The model F-value of 39.64 depicts that model was significant. Lack of fit value of 3.82 implies that the lack of fit was 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 0.9754, indicating that the 97.54% of the variability in the response can be explained by the model. The "pred R-squared" of 0.8316 is in reasonable agreement with the "Adj R-Squared" of 0.9508. "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.

3.4. Interactive effect of physical factors on PHB content

Table 1 represents the interactive effect of different physical process variables on PHB content. Fig. 2A represents the interactive effect of pH and temperature on PHB content. It can be clearly deduced from 3D surface and contour plot (Fig. 2A and Fig. 2B) that increase in pH and temperature, enhanced PHB content upto 7.50 and 37 °C, respectively. However, further increase in these two process variables decreased PHB content.

Fig. 2C represents the interactive effect of temperature and agitation speed on PHB concentration, which showed enhanced intracellular granule synthesis at suboptimal level of two process variables (temperature 35 °C and agitation speed 3.3 Hz). Further increase in temperature and agitation speed showed decreased PHB accumulation which is represented by convergence of curve towards boundary of 3D plot (Fig. 2C). Contour plot representing interactive effect of temperature and agitation speed confirmed the above finding that PHB content was maximum at an agitation speed of 3.3 Hz at zero level of pH (7.5) (Fig. 2D). PHB accumulation increased rapidly upto 3.3 Hz and 35 °C and then showed decreasing trend. Previous findings suggests that induction temperature of 34 °C enhanced PHB content in shorter duration in recombinant E. coli strain due to induced PHB synthase activity [15]. However no PHB granule synthesis occurred at temperature above 40 °C due to reduced PHB synthase induction.

pH and agitation speed showed significant influence on PHB accumulation in Alcaligenes sp., when considered as individual process variable ($p < 0.05$). However, interactive effect of pH and agitation speed was insignificant ($p > 0.05$). Fig. 2E and F represented the interactive effect of two parameters which showed similar behavior as reported in previous findings [5]. PHB accumulation enhanced upto pH 7.25 and agitation speed of 3.3 Hz at intermediate level of temperature (35 °C). This clearly suggests that PHB synthase activity decreases at alkaline and acidic pH. PHB yield was found to be maximum at pH 8.5 in Synechocystis sp. PCC 6803 [16]. These results were in agreement with previous finding, which deduced a pH range of 6.0–7.5 for enhanced microbial growth and PHB production [17].

Previously, a PHB yield of 92.0% was obtained at an agitation speed of 3.3 Hz in Ralstonia eutropha [11]. Agitation speed higher than 3.3 Hz showed negative influence on PHB content. This may be attributed to the fact that the oxygen limitation is one of the most probable cause of PHB accumulation during growth phase. The key feature of this control is the fate of acetyl-CoA,
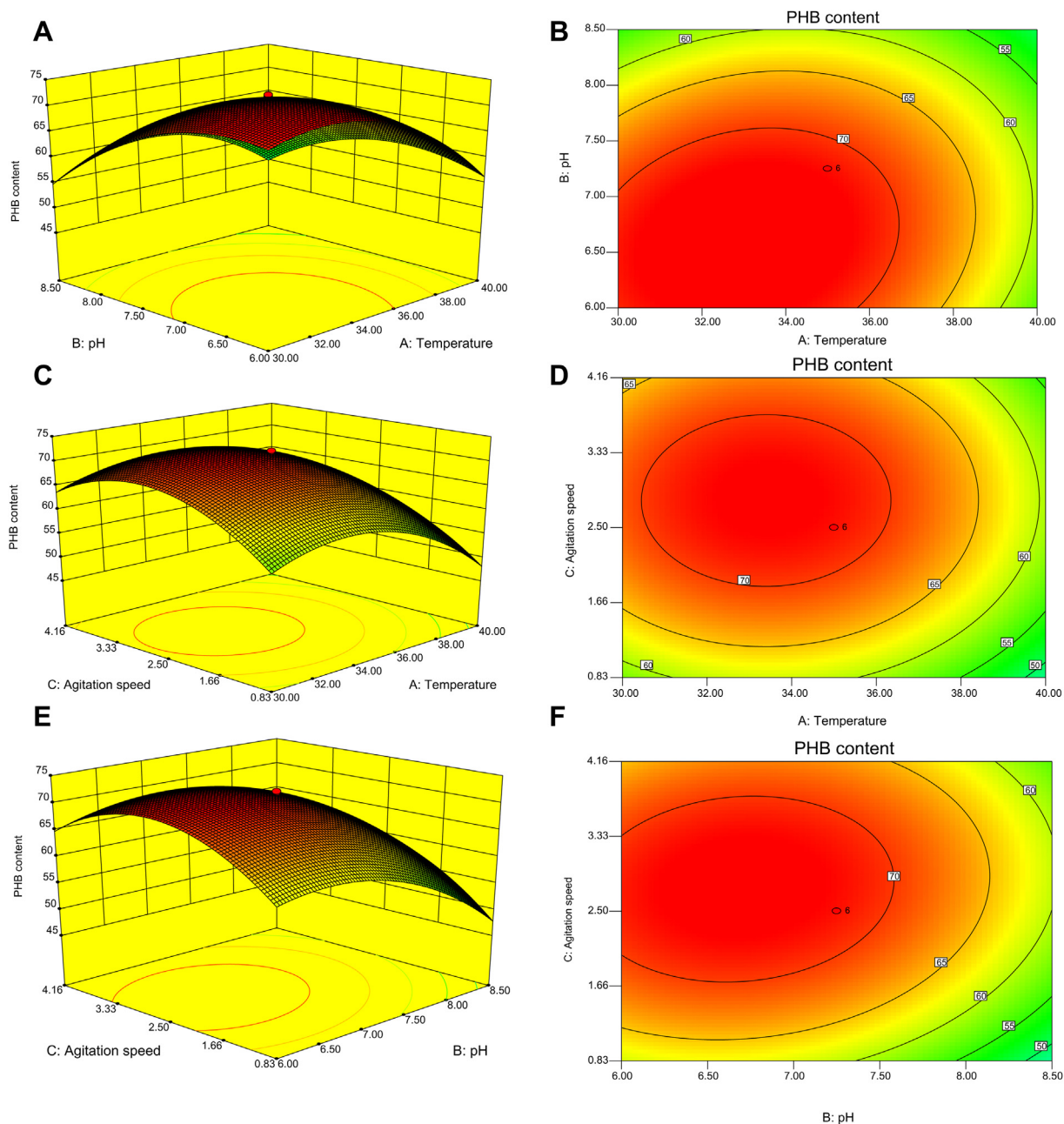
Fig. 2 — A — 3D surface showing the interactive effect of temperature and pH on PHB content after 48.0 h of cultivation. B — Contour plot showing the interactive effect of temperature and pH on PHB content after 48.0 h of cultivation. C — 3D surface showing the effect of temperature and agitation speed on PHB content after 48.0 h of cultivation. D — Contour plot showing interactive effect of temperature and agitation speed on PHB content after 48.0 h of cultivation. E — 3D surface showing the effect of pH and agitation speed on PHB content after 48.0 of cultivation. F — Contour plots showing the effect of pH and agitation speed on PHB content after 48.0 h of cultivation.

which may be oxidized via tricarboxilic acid (TCA) cycle or can serve as a substrate for PHB synthesis. In oxygen limitation, when NADH/NAD ratio increases, citrate synthase and isocitrate dehydrogenase are inhibited by NADH, and in consequence, acetyl-CoA doesn't enter the TCA cycle at the same rate and it is converted to acetoacetyl CoA by 3-ketothiolase (the first enzyme of PHB biosynthesis) which is inhibited by CoA [18].

After knowing the possible direction for maximizing PHB production, the optimization was done by "Point prediction"

technique. A maximum PHB content of 77.78% was predicted at temperature 34.54 °C, pH 6.54 and agitation speed 3.11 Hz (Table 2).

3.5. Model verification

CCRD used for the optimization of physical parameters for enhanced accumulation of PHB content revealed the effect of interaction of these physical variables on intracellular granule

| Table 2 – PHB content before and after optimization.					
Variables	Before	After	PHB yield (% DCW[a])		
			Before	After	
				Predicted	Experimental
Temperature (°C)	35	34.54	72.14	77.78	76.80
pH	7.50	6.54			
Agitation speed (Hz)	3.33	3.13			
a DCW Dry cell weight.					

synthesis. Model verification was done by performing experiment in triplicate under the optimized condition. In the current study, cane molasses and urea were used as sole carbon and nitrogen source to reduce the raw material cost. A PHB yield of 76.80% was recorded against the predicted yield of 77.78%. It can be visualized from Table 2 that the predicted and experimental PHB yields after optimization were well in agreement.

3.6. Scale up in 7.5 L bioreactor

Shake flask study was then scaled up to a lab scale bioreactor. The culture was grown in a 7.5 L Bentchtop bioreactor (BioFlo/Celligen 115, New Brunswick, USA) to study PHB production in batch cultivation. Working volume of bioreactor was kept at 5.6 L. Batch cultivation study was carried out to understand the kinetics of PHB production under controlled condition of temperature, pH, agitation and aeration.

Fig. 3 represents the PHB production under optimized condition by *Alcaligenes* sp. utilizing cane molasses and urea as carbon and nitrogen sources at initial concentrations of 40.0 g L^{-1} (total sugar concentration) and 1.0 g L^{-1}, respectively. pH was kept at 6.54 ± 0.1 throughout the production process and DO was maintained at 30% saturation value with agitation speed of 3.13 Hz. Agitation speed and DO cascade was done by setting minimum and maximum agitation speed at 1.6 and 5.0 Hz, respectively in order to maintain the desired dissolved oxygen concentration. Aeration rate during PHB production was kept at 1.5 L min^{-1}. Fig. 3 clearly depicts that after a lag phase of 12.0 h biomass increased to 11.0 ± 0.5 g L^{-1} at 48.0 h. Maximum PHB yield was found to be 8.58 ± 0.4 g L^{-1} after 48.0 h of fermentative production which is in correlation with previous finding where maximum PHB production of
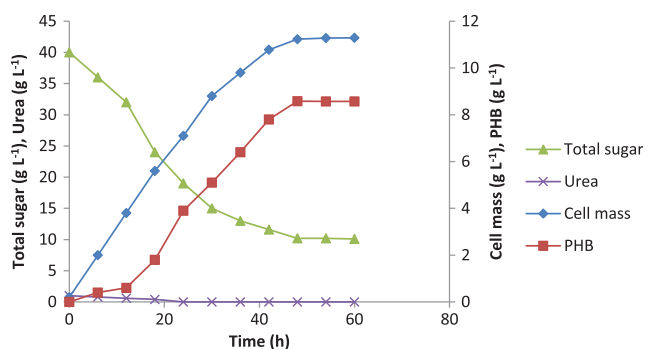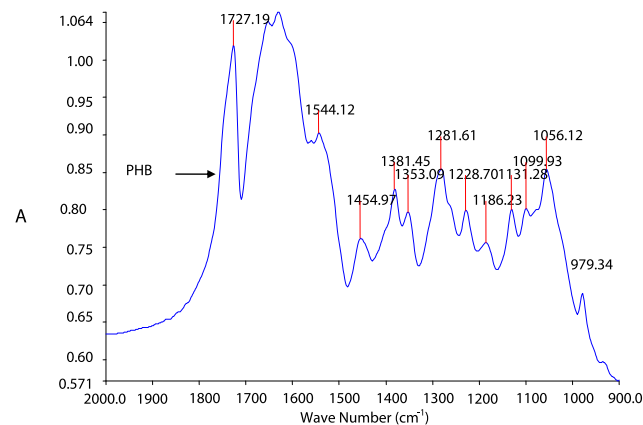
11.32 g L^{-1} was reported by utilizing *paneer* whey as substrate [19]. Total sugar concentration decreased to 10.2 g L^{-1} at the end of production phase in comparison to initial concentration of 40.0 g L^{-1}. Cane molasses composition analysis reveals that it contains approximately 50% total sugar (reducing sugar 10.5%, sucrose 33.9%, fructose 3.6% and glucose 2.0%). Cane molasses contains essential growth factor (calcium, phosphorus, biotin, niacin and riboflavin) in trace amount (ppm) but it lacks essential minerals like cobalt and selenium which enhances PHB synthase activity.

Nitrogen source also depleted after 24.0 h of cultivation which leads to enhanced PHB production. PHB yield ($Y_{P/x}$) in terms of cell biomass produced was found to be 0.78 which is higher than previous yield of 0.64 on dry cell weight, in same strain when fructose and ammonium sulfate were used as carbon and nitrogen source, respectively [17]. PHB yield of 0.42 was reported in *Bacillus megaterium* BA-019 under similar physical conditions [20].

In the present study productivity was found to be 0.19 g L^{-1} h which is higher in comparison to previous studies [21]. Growth factors (organic acids, vitamin and minerals) present in cane molasses induced enhanced biomass and PHB production. Higher cell mass and increased PHB productivity clearly indicates that *Alcaligenes* sp. assimilated nitrogen from urea efficiently and uptake rate of urea across cell membrane may be higher due to its non ionic form and less pH dependency during transport across membrane [22]. Cane molasses and urea are inexpensive substrates which minimize the overall biodegradable plastic production cost and are better replacement for petroleum derived (polypropylene based) plastic.

Fig. 3 – Cell biomass, PHB, cane molasses, urea varying concentration at different time in 7.5 L bioreactor in batch cultivation process.

Fig. 4 – FTIR spectra of PHB granules produced by *Alcaligenes* sp. after 48.0 h of batch fermentation.

3.7. Characterization of PHB granules

Characterization of PHB was done by FTIR (Fourier Transform Infrared Spectroscopy). Fig. 4 represented the spectra showing the presence of functional group of PHB viz, aliphatic C–H, =O stretching, =C–H deformation, =C–O etc. In pure PHB granule, asymmetrical deformation of C–H bond in CH_2 groups and CH_3 groups, C=O bond stretching and C–O ester bond are represented by wave numbers 1460, 1379, 1726 and 1150 cm^{-1}, respectively. Fig. 4 represents sharp peaks at wave number 1727, 1455 and 1381 cm^{-1} which corresponds to C=O bond stretching, C–O ester bond and asymmetrical deformation of C–H bond in CH_2 and CH_3 groups of PHB are similar to IR spectra of pure PHB [23].

4. Discussion

Current study showed marked increase in PHB yield under optimum cultural conditions in comparison to our previous finding [21]. The optimum physical condition for enhanced PHB production comprised: temperature 34.54 °C, pH 6.54 and agitation speed 3.13 Hz, respectively. Shake flask cultivation studies depicted 76.80% PHB content (on dry cell mass basis) under optimized physical variables, which is higher than the previous report under similar condition due to induced PHB synthase activity and reduced PHB depolymerase activity. However, at elevated temperature (>40 °C) and pH (>7.5), PHB content decreased due to PHB depolymerase activity which is in conformity with previous findings [24]. *Streptomyces* sp. KJ 72 and *Streptomyces exfoliates* K10 [25] showed temperature optima of 40 °C for PHB depolymerase activity which is higher than the current finding.

pH and agitation speed showed significant effect on PHB content. Although PHB production can be controlled by precisely manipulating pH, the experimental data indicated that pH values other than 6.54 affect PHB production, which clearly suggested that PHB production is sensitive to the pH of cultivation media. Maximum PHB accumulation occurred at an agitation speed of 3.13 Hz which is less than previously reported agitation speed of 3.33 Hz. This may be due to excessive shear force produced at elevated agitation speed. Reduced agitation speed favors decrease in input cost due to less electricity consumption.

A significantly higher biomass of 11.0 ± 0.5 g L^{-1} with a PHB content of 8.8 ± 0.4 g L^{-1} was obtained when batch cultivation was conducted in 7.5 L lab scale bioreactor giving a yield of 0.78 g PHB produced per gram cane molasses consumed. PHB concentration of 6.0 g L^{-1} was obtained in case of *C. taiwanensis* 184 using two stage fermentation strategy on a 5 L bioreactor [9]. Previously, maximum PHB accumulation of 23.0% on dry cell mass basis utilizing cane molasses as carbon source was deduced under similar condition [26]. FTIR analysis revealed the chemical nature of PHB produced which is in correlation with previous finding [23].

5. Conclusions

Batch cultivation performed in 7.5 L lab scale bioreactor under optimized condition gave PHB yield ($Y_{P/x}$) and productivity of

78.0% and 0.19 g L^{-1} h, respectively. Current finding suggests that PHB synthesis enhanced under optimized cultural condition due to increase in PHB synthase activity and stability at suitable pH, temperature and agitation speed. The data obtained by batch kinetics would be useful for metabolic flux analysis of PHB synthesis and development of mathematical model in different cultivation strategies (continuous/fed batch) for over production of PHB utilizing cheaper substrates. Recombinant *E. coli* containing PHB synthase from the chosen strain may further enhance the PHB production and its physicochemical properties.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biombioe.2013.02.017.

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