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Effect of nutritional supplements on bio-plastics (PHB) production utilizing sugar refinery waste with potential application in food packaging

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are intracellular carbon and energy storage reserve material stored by gram-negative bacteria under nutrient limitation. PHAs are best alternative biodegradable plastics (bio-plastics) due to their resemblance to conventional synthetic plastic. The present study investigated the synergistic effect of nutritional supplements (amino acid and vitamin) on the PHA production by *Alcaligenes* sp. NCIM 5085 utilizing a sugar refinery waste (cane molasses) under submerged fermentation process. Initially, the effect of individual factor on PHA yield was studied by supplementing amino acids (cysteine, isoleucine, and methionine), vitamin (thiamin), and cane molasses at varying concentration in the production medium. Further, the cultivation medium was optimized by varying the levels of cane molasses, methionine and thiamin using response surface methodology to enhance the PHA yield. The maximum PHA yield of 70.89% was obtained under the optimized condition, which was then scaled up on 7.5 L-bioreactor. Batch cultivation in 7.5 L-bioreactor under the optimized condition gave a maximum PHA yield and productivity of 79.26% and 0.312 gL^{-1} h^{-1}, respectively. The PHA produced was subsequently characterized as PHB by FTIR. PHB extracted was of relatively high molecular weight and crystallinity index. DSC analysis gave T_g, T_m, and X_c of 4.2, 179 °C and 66%, respectively. TGA analysis showed thermal stability with maximized degradation occurring at 302 °C, which is above the melting temperature (179 °C) of the purified polymer. The extracted polymer, therefore, possessed desirable material properties to be used in food packaging.

KEYWORDS

Polyhydroxyalkanoate (PHA); bioplastics; sugar refinery waste; nutritional supplements; polymer characterization; food packaging

Introduction

In today's modern era of science and technology, plastics have become one of the most widely used materials all over the world. Plastics are derived from inorganic and organic raw materials such as carbon, silicon, hydrogen, nitrogen, oxygen, and chloride. Plastics find enormous applications in agriculture, medicine, pharmaceutical, cosmetic, and food industry. However, plastics produced from conventional petroleum source such as polypropylene (PP) are non-biodegradable. Biodegradable polymers like polyhydroxyalkanoate (PHA), polylactic acid (PLA), polyvinyl alcohol (PVA) derived from microbial origin are getting more attention in plastic market due to their similarity in physicochemical properties with conventional PP-derived plastic.[1,2]

Of the different biopolymers used for bio-plastic synthesis, PHA finds major application in different sectors. PHAs are accumulated as intracellular carbon and energy reserves in microbes under nutrient limiting conditions, namely nitrogen, sulfur, and magnesium deficiency.[3] PHA may be categorized into several groups depending on their chain lengths. Nowadays, a short chain length hydroxyalkanoates (e.g., PHB) is being used as a replacement for PP-derived plastic at commercial level. PHB shares many properties with PP.[4] For examples, PHB exhibits similar thermal and tensile properties. PHB is completely biodegradable. However, PHB production is expensive owing to its raw material cost and tedious downstream process during its recovery.[5] The production cost of PHA is approximately 5–10 times more expensive than the petrochemical packaging materials.[6] The production cost can be possibly reduced by the application of agro-based waste products rich in carbon source. The nature and quality of PHA depend on types of carbon sources used in the production. Nutrient supplements such as amino acids, vitamins, and fatty acid can improve the fermentative product yield since biosynthesis of amino acids is energy intensive and can be a burden to the cell during the overproduction of proteins. Similarly, the bacterial growth and PHA production can also

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be enhanced by supplementing certain vitamins and amino acids.

Physicochemical properties of PHB, namely tensile strength, molecular weight, crystallinity, and melting temperature decide its purity and its similarity with conventional plastic. Type of raw material, selected strain, and recovery process decide the material properties of PHB and its industrial applications.[7] Considerable emphasis has been laid on the development of strategy for enhanced PHB production utilizing agro-industrial waste.[8] The commercial production of PHB on soy cake, whey, molasses, and wheat hydrolysate have been previously investigated.[9–11] However, very few reports are available focusing on material properties of PHB extracted from different strains. Hence, the present study was carried out to study the effect of amino acids, vitamin, and carbon sources on the production rate of PHA. Initially, the screening of potential PHA-producing microbial strains was done using the agro-based waste products to reduce the cost of production and the extracted polymer was characterized as PHB using FTIR, GC-MS, and NMR. Physicochemical properties of extracted polymer were further studied to find its probable industrial application.

Materials and methods

Bacterial strains

In the present study, three bacterial strains were chosen based on their PHA producing potential. *Alcaligenes* sp. NCIM No. 5085 was obtained from National Chemistry Laboratory (NCL), Pune, India. *Bacillus megaterium* MTCC No. 8075 and *Pseudomonas aeruginosa* MTCC 2474 were obtained from MTCC (Microbial Type Culture Collection and Gene Bank), Chandigarh, India.

Growth and production media

The growth medium used was mineral salt medium (MSM) which contained (g/L): fructose 10.0, urea 0.8, KH_2PO_4 2.0, Na_2HPO_4 0.6, $MgSO_4$.$7H_2O$ 1.0, yeast extract 0.1 and 1 mL/L of trace element ($ZnSO_4$.$7H_2O$ 1.3 mg/L, $CaCl_2$ 20.0 mg/L, $FeSO_4$.$7H_2O$ 0.2 mg/L, and H_3BO_3 0.6 mg/L) solution. Carbon source, yeast extract, and salt solution were sterilized separately at 121 °C for 15 min and were combined aseptically into the culture flask at room temperature. The pH of the final culture medium was adjusted to 7.0 ± 0.5 with 0.1 N HCl/NaOH prior to inoculation. Similarly, production medium was also prepared in conical flask containing 100 mL MSM keeping the composition same as the growth medium except the concentration of carbon source was increased to 40 g/L. Carbon sources investigated in this study included sucrose, fructose, maltose, glucose, cane molasses, wheat bran husk, rice bran husk, and orange peel powder. Five milliliters of the seed of each bacterial strain was added into different conical flask containing 100 mL of production medium and incubated at 150 rpm, 37 °C for 48 hr. Samples were extracted for PHB analysis at different time intervals, namely 3 h, 7 h, 9 h, 12 h, and 24 h, respectively.

Effect of carbon source on PHA yield and cell mass

To study the effect of different carbon sources on PHB yield and biomass production, 10 mL of samples were taken from different trial supplied with different carbon sources after 24 h of cultivation and then the trial were taken for spectrophotometric analysis to determine the PHB yield by Law and Splecky method, 1960.

Amino acid supplementation

Three amino acids (methionine, cysteine, and isoleucine) were added as nutritional supplements in the production media at a concentration of 10 mg/L. All the experiments were performed in triplicates.

Vitamin supplementation

Thiamin was used as source of vitamin supplement at a concentration of 10 mg/L.

Optimization study

In order to enhance the PHB yield, the cultivation medium was optimized by varying the concentration of cane molasses, methionine and thiamin using response surface methodology (RSM) by Minitab 17 software. The range given was; 20–50 g/L for cane molasses, 10–50 mg/L for methionine, and 10–20 mg/L for thiamin (Table 1).

Bioreactor study

The shake flask study was scaled up in lab scale 7.5 L-bioreactor (BioFlo/Celligen 115, New Brunswick, USA) to study the PHA production in batch cultivation. Working volume of batch cultivation was kept at 3.0 L under controlled condition of temperature, pH, agitation, and aeration. Samples were extracted for analysis after 48 h of cultivation. pH was

Table 1. Effect of different amino acid supplementation on cell mass and PHB yield.

S. N	Amino acids & Vitamin	Cell mass (g/L)	PHB yield (g/L)	%PHB recovery
1.	Control (without amino acid supplementation)	2.29 ± 0.028	1.15 ± 0.054	50.20
2..	Methionine	3.29 ± 0.028	2.02 ± 0.061	62.51
3.	Cysteine	3.14 ± 0.034	1.69 ± 0.067	54.03
4.	Isoleucine	3.07 ± 0.066	1.59 ± 0.054	51.54
5.	Thiamin	2.39 ± 0.050	1.17 ± 0.054	51.56

kept at 7.00 ± 0.5 throughout the production process and DO was maintained at 30% saturation value with an agitation speed of 350 rpm.

Analytical study

Dry cell mass

Twenty milliliters of cultural broth was centrifuged at 10,000 rpm for 15 min at 4 °C. The cell pellet was collected by removing the supernatant and washed with saline water (NaCl, 0.8% w/v) and dried in aluminum weighing dishes at 90 °C for 24 hrs. The obtained dry cell mass was further used for PHB extraction and estimation.

PHB extraction. For PHB extraction, chloroform-hypochlorite extraction method was used in which pure PHB was obtained by non-solvent precipitation (five times the volume of chloroform) and filtration. Non-solvent used here was a mixture of methanol and water (7:3, w/v). Filtration was performed using membrane filters (2 micrometer, Millipore).

PHB estimation. Samples for spectrophotometer analysis were prepared as previously reported (Splecky and Law 1960). Analysis was performed by Shimadzu UV-1800 UV-spectrophotometer taking reading at 235 nm wavelength against concentrated sulfuric acid as blank. One gm of dried cell mass was treated with dispersion containing 50 mL of chloroform and 50 mL of 20% diluted sodium hypochloride solution. The mixture containing two immiscible solvents was centrifuged at 4000 rpm for 10 min at 4 °C temperature and bottom phase of chloroform containing PHB was extracted. The amount of crotonic acid was calculated from the molar extinction coefficient of 1.55×10^4.

Molecular weight determination

The average molecular weight (M_n) and Polydispersity index (M_w/M_n) of the extracted polymer by sodium hypochlorite (30.0% v/v) and chloroform dispersion were determined by GPC (gel permeation chromatography), using a pump (Wayers 715 Ultra WISP, Canada), a differential refractometer detector at 30 °C (Waters 410, Canada), and a 60 cm PL gel 5 L styragel column with a linear range of molecular weight of 200–2,000,000 mol/g at 25 °C. Monodisperse polystyrene was used as the molecular weight standard. A 100 mL portion of a 0.1% w/v PHB solution was injected for each analysis. Chloroform was used as the mobile phase at a flow rate of 1.0 mL/min.

GC-MS analysis

Samples for GC analysis were prepared by esterification of PHB. PHB was extracted in 10 mL chloroform by solvent extraction and then 1 mL, 1% acidified methanol was added in 1 mL chloroform containing PHB. The reaction mixture was heated in sealed tubes for 30 min. Esterified sample was then used for sample analysis. Analysis was performed on Shimadzu, Model: QP-5000 using DB wax column (polar, 30 m, 0.32 µm, 0.25 µm thickness). Carrier gas was nitrogen (50 psi), with a splitless injection (80:1). FID (Flame ionization detector) was used in GC analysis. Injector temperature was 250 °C, oven temperature was set at 50 °C which rose to 200 °C with temperature increase at the rate of 15 °C/min. Benzoic acid was used as internal standard.

Determination of crystallinity and melting temperature

To investigate the morphological state of PHB granules, the melting temperature and the enthalpy of fusion of extracted PHB were measured with a DuPont model 2000 differential scanning calorimeter (DSC). The enthalpy of fusion of a 100% crystalline (theoretical) sample was assumed to be 146 J/g. The crystallinity of PHB was estimated from the enthalpy of fusion.

Thermo gravimetric analysis of PHB

The thermal stability of native PHB granules was examined with a DuPont model 2000 thermo gravimetric analyzer (TGA) operated at a nitrogen flow rate of 20 mL/min and a scanning rate of 10 °C/min.

FTIR study of PHB

Extracted PHB granules were dissolved in isotonic saline solution (30 kg m^{-3}) and then 20 mL 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.

Result and discussion

Screening of potential PHB-producing microbe and carbon source

Screening of potential microbe having higher PHA production capability was done by cultivating these microbes on production media at 37 °C for 48.0 h on incubator shaker (Sigma, USA) at an agitation speed of 150 rpm. Microbes were grown on various carbon sources such as agro-industrial residues (cane molasses, rice bran, wheat bran, and orange peel powder), glucose, maltose, fructose, and sucrose at equimolar concentration. 20.0 mL of samples were withdrawn from each conical flask after 48.0 h of cultivation and cell mass and PHA yield was determined. *Alcaligenes* sp NCIM 5085 and *Pseudomonas aeruginosa* NCIM 2025 gave maximum cell mass of 2.96 ± 0.2g/L and 2.37 ± 0.2 g/L, respectively (Fig. 1a). The maximum PHA productions were 1.40 ± 0.1 g/L (for *Alcaligenes* sp.) and 1.12 ± 0.3 g/L (for *P. aeruginosa*) (Fig. 1b). PHA yields ($Y_{P/X}$) of 54.68% and 47.25% were obtained in *Alcaligenes* sp. and *P. aeruginosa*, respectively.

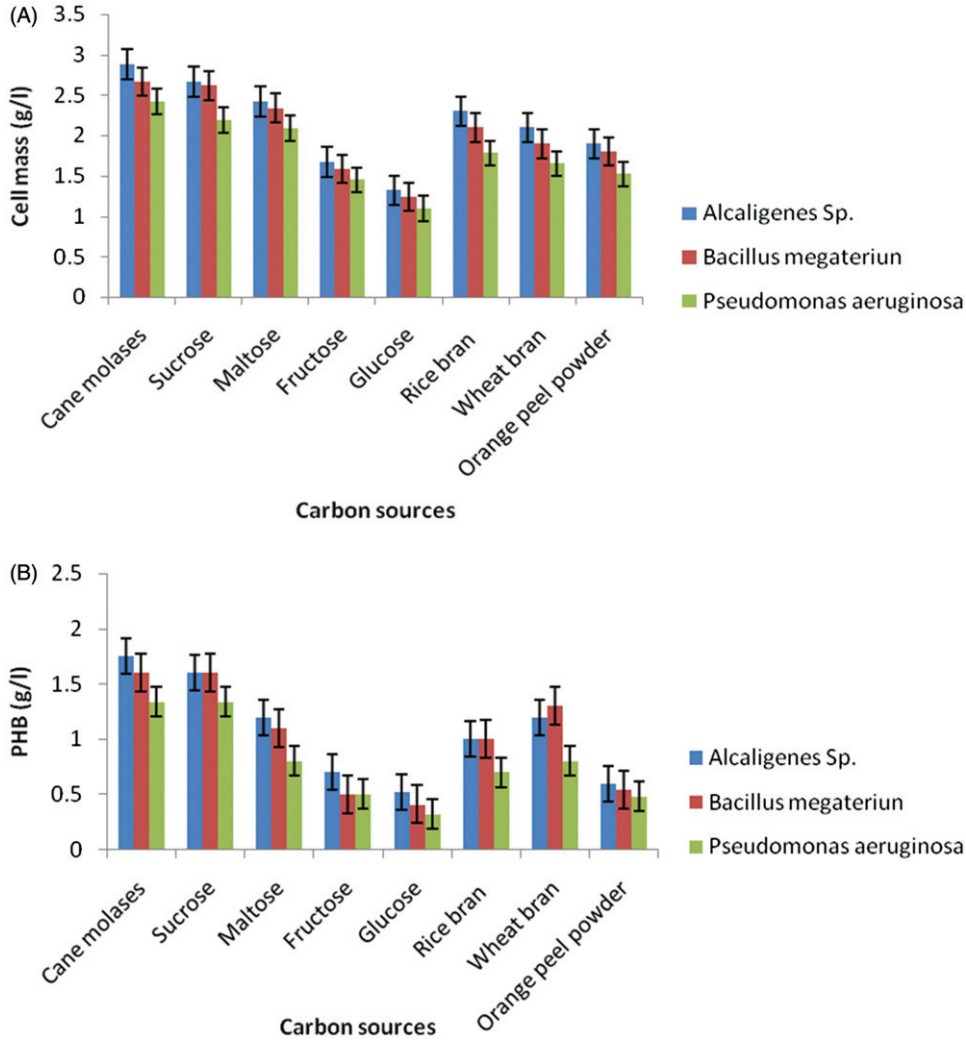

Figure 1. Effect of different carbon sources on the cell mass (a) and PHB yield (b) produced by three different bacterial strains under similar conditions (SD ± 0.5, $n = 3$).

Maximum biomass and PHB yield of 2.93 ± 0.06 and 1.73 ± 0.028 g/L were obtained in *Alcaligenes* sp. NCIM No. 5085 using cane molasses as carbon source and other nutritional supplements which is in correlation to the previous finding of Prasertsan et al. 2008 obtained in *Enterobacter cloacae* under similar cultivation condition.[12]

Effect of amino acids and vitamin (thiamin) supplementation on PHB production

Effects of amino acids and vitamin supplementation on PHB production and its composition were studied by collecting 10 mL of sample from different trial supplemented with different amino acids and vitamin after 24 h of cultivation. The PHB yield of 62.51%, 54.03%, 51.54%, and 51.56% was obtained by the addition of methionine, cysteine, isoleucine, and thiamin, respectively (Table 1). The PHB yield obtained in the present study is higher in comparison to previously reported PHB yield of 61%, 60%, and 45% by methionine, cysteine, and isoleucine addition under similar cultivation condition in a recombinant *E. coli* strain.[13] As maximum PHB yield was obtained on methionine supplementation, further study was carried out on methionine. This may be attributed to the fact that methionine biosynthesis requires more ATP as compared to other amino acids.

In addition to amino acid (methionine), thiamin (vitamin) was also added (10–20 mg/100 mL) in production media. The maximum PHB yield was obtained by supplementing 15 mg/L of thiamin in production media (Table 2). Thiamin showed more significant effect on cell mass as compared to PHB yield which is in resemblance with previous finding.[14] Similarly, maximum PHB yield was observed at 30 mg/L methionine supplementation in the production media.

Optimization study

ANOVA and model fitting

Twenty trials were designed by RSM with varying concentration of cane molasses, methionine, and thiamin (Table 2). Maximum biomass and PHB yield of 3.78 ± 0.34 g/L and 2.68 ± 0.15 g/L, respectively were obtained in trial no. 17 comprising; cane molasses 35 g/L, methionine 30 mg/L, and thiamin 15 mg/L (Table 2). Analysis of variance (ANOVA) was done to predict the significant model for cell mass and

Table 2. Design of experiment for cell mass and PHB production.

Std order	Run order	Cane molasses concentration (g/L)	Amino acid concentration (mg/L)	Vitamin concentration (mg/1)	Cell mass (g/L)	PHB (g/L)
5	1	20	10	20	2.06 ± 0.02	1.08 ± 0.03
12	2	35	63.63	15	2.15 ± 0.03	1.21 ± 0.02
7	3	20	50	20	1.65 ± 0.04	0.7 ± 0.04
18	4	35	30	15	3.74 ± 0.02	2.66 ± 0.14
4	5	50	50	10	3.41 ± 0.01	2.53 ± 0.24
15	6	35	30	15	3.71 ± 0.03	2.67 ± 0.16
17	7	35	30	15	3.78 ± 0.34	2.68 ± 0.15
2	8	50	10	10	3.52 ± 0.12	2.65 ± 0.07
3	9	20	50	10	1.92 ± 0.23	0.82 ± 0.09
20	10	35	30	15	3.75 ± 0.24	2.66 ± 0.11
6	11	50	10	20	2.97 ± 0.16	2.25 ± 0.03
1	12	20	10	10	2.26 ± 0.04	1.19 ± 0.02
9	13	9.77	30	15	1.31 ± 0.07	0.71 ± 0.24
16	14	35	30	15	3.77 ± 0.08	2.65 ± 0.34
11	15	35	−3.63	15	3.35 ± 0.04	2.28 ± 0.56
13	16	35	30	6.591	3.19 ± 0.01	2.11 ± 0.21
19	17	35	30	15	3.75 ± 0.04	2.65 ± 0.17
14	18	35	30	23.409	2.67 ± 0.02	1.81 ± 0.24
10	19	60.22	30	15	3.69 ± 0.12	2.67 ± 0.14
8	20	50	50	20	2.89 ± 0.24	1.95 ± 0.23

PHB production (Tables S1 and S2). The ANOVA of the regression model demonstrates that the model is highly significant for studying the interactive effect of variables on PHB synthesis, as it is evident from the Fisher's *F*-test where F_{model} value is found to be 54.96 ($F_{model} > 1$, significant) (Table S2). The fisher variance ratio, *F* value is a statistically valid measure of how well the factors describe the variation in the data about its mean. The greater the *F*-value is from unity, the more certain it is that the factors explain the adequately the variation in the data around its mean, and the estimated factors effects are real. The goodness of fit with this quadratic model was confirmed by the determination coefficient (R^2). In this case, the value of the determination coefficient ($R^2 = 0.9802$) indicates that 98.02% variability in the response could be explained by the model.

Similarly, ANOVA of the regression model demonstrates that the model is highly significant for studying the interactive effect of variables on PHB yield, as it is evident from the Fisher's *F*-test where F_{model} value is found to be 61.87 ($F_{model} > 1$, significant). The goodness of fit with this quadratic model was confirmed by the determination coefficient (R^2). In this case, the value of the determination coefficient ($R^2 = 0.9824$) indicates that 98.24% variability in the response could be explained by the model (Table S3).

Interactive effect of process variables on biomass and PHB yield

Cane molasses and methionine showed positive interactive effect on cell mass and PHB yield. Cell mass and PHB yield enhanced with increase in cane molasses and methionine concentration up to 40 g/L and 30 mg/L, respectively. However, further rise in cane molasses and amino acid concentration showed no synergistic effect on cell mass and PHB yield. This may be attributed to substrate inhibition of PHB synthase enzyme (Fig. 2). Cane molasses and thiamin showed significant interactive effect on cell mass than PHB yield (Fig. 2). This may be due to the fact that cane molasses contains vitamins like riboflavin and biotin, which are required for cell growth. Similarly, amino acid and vitamins showed significant effect on cell mass and PHB yield. Cell mass and PHB yield enhanced with increase in methionine and thiamin concentration up to 25 mg/L and 15 mg/l, respectively (Fig. 2). This may be attributed to the fact that 7 ATPs and NADPHs are required for synthesis of methionine and exogenous supplementation of amino acid will facilitate the extra ATP and NADPH to be utilized in PHB biosynthesis.[15]

Model validation

CCRD used for the optimization of process variables for enhanced accumulation of PHB content revealed the effect of interaction of these variables on intracellular granule 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 79.26 ± 0.03% was recorded against the predicted yield of 79.91 ± 0.03%. It can be visualized from Table 3 that the predicted and experimental PHB yields after optimization were well in agreement.

Scale up in 7.5-L bioreactor

Fig. 3 represented the time profile of PHB synthesis in a cultivation media comprising 24.90 mg/L of methionine and 13.04 mg/L of thiamin. Cane molasses and urea were supplied initially as a source of carbon and nitrogen, respectively at the concentration of 40 and 0.8 g/L, respectively. pH of the media was maintained at 7.00 ± 0.05 throughout the complete production process and DO was maintained at 30% saturation value. Moreover, the agitation speed and aeration rate were maintained at 350 rpm and 2.0 L/min with

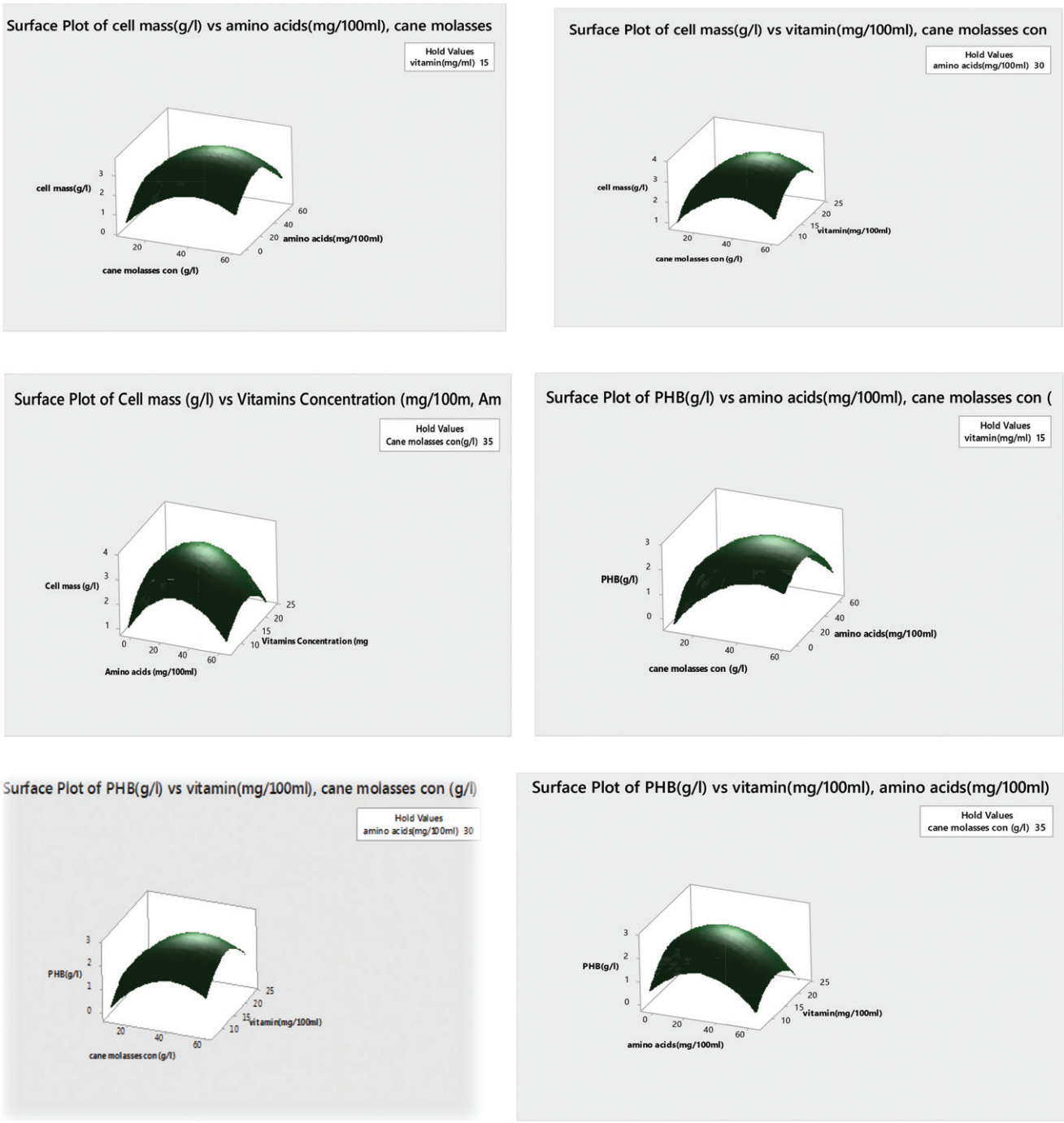

Figure 2. Surface plot showing relationship among cane molasses, methionine and thiamin on biomass and PHB yield.

Table 3. Optimum level of variables with highest PHB production.

			PHB yield (% DCW)		
				After	
Variables	Before optimization	After optimization	Before	Predicted	Experimental
Cane molasses (A)	35 g/L	35 g/L	70.89 ± 0.02	79.26 ± 0.03	79.91 ± 0.03
Methionine (B)	30 mg/L	24.90 mg/L			
Thiamin(C)	15 mg/L	13.04 mg/L			

37 °C cultivation temperature. Maximum PHB yield was found to be 3.63 g/L after 12 h of cultivation. However, total sugar concentration decreased to 16.66 g/L after 48 h of production in comparison to initial concentration of 40 g/L with productivity of 0.312 g/L/h (Fig. 3). PHB yield ($Y_{p/x}$) in terms of cell biomass produced and substrate consumed were found to be 0.76 (g/g cell mass) and 0.15 (g/g substrate consumed).

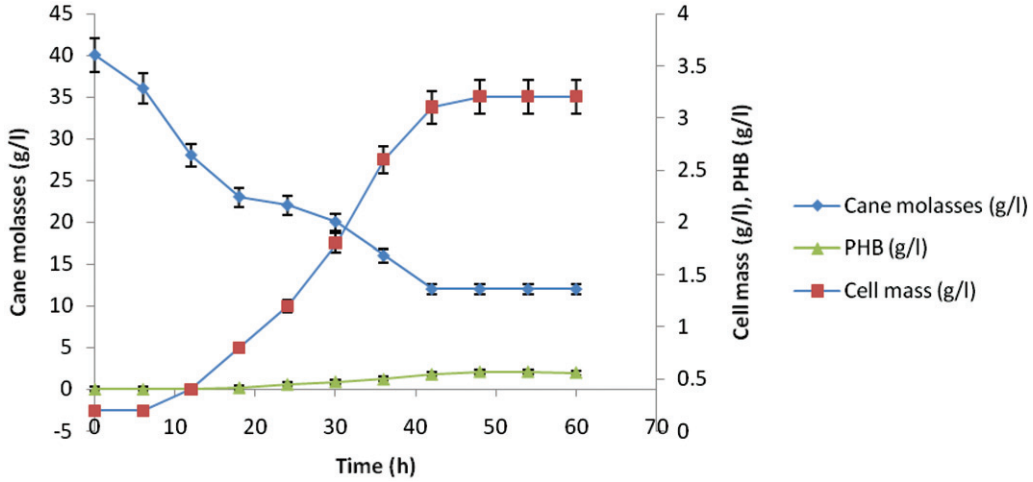

Figure 3. Time profile of cell mass, PHB, and sugar concentration during cultivation on bioreactor (SD ± 0.5, $n = 3$).

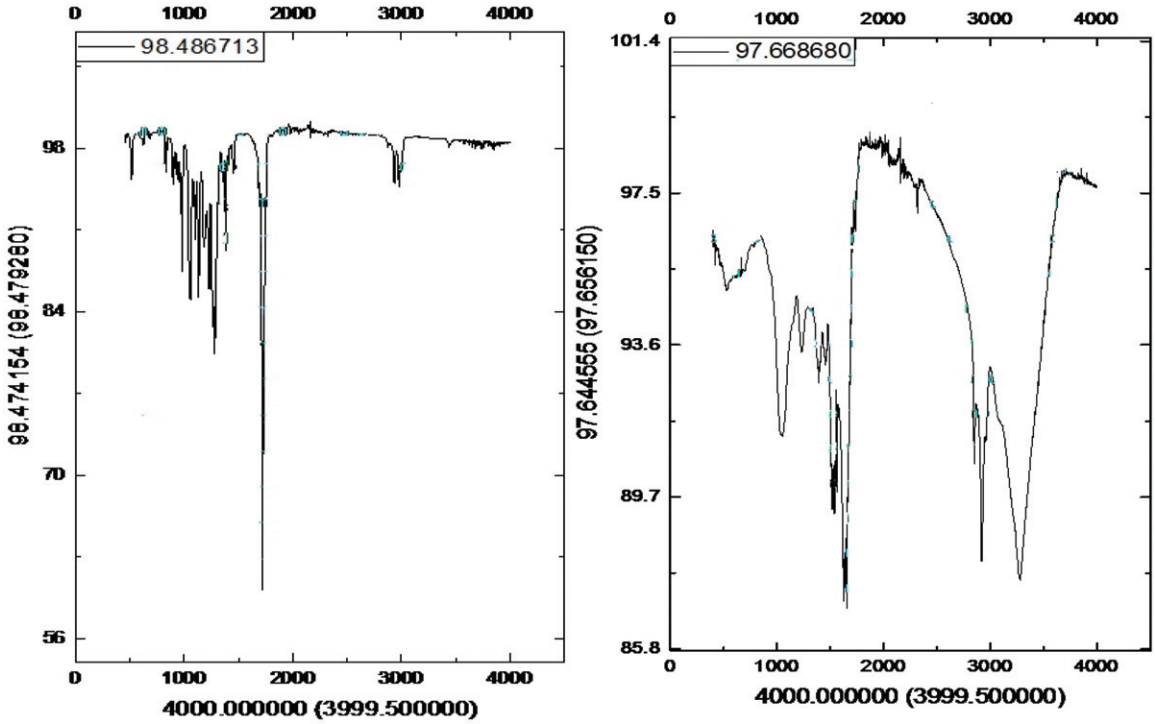

Figure 4. FTIR analysis of *Alcaligenes* sp. and pure PHB showing resemblance in peaks related to functional groups C=O, C–H, etc.

PHB characterization

PHB extracted from *Alcaligenes* sp. by hypochlorite and chloroform dispersion, 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 CH_3, $-CH_2$, $-CH$, C=O, and O–H groups, respectively were similar to the absorption band of commercial PHB (Fig. 4).

Physicochemical properties

The GPC chromatogram of PHB elution through styragel HT column deduced that elution volume 25–40 ml gave maximum RI value in the range of 0.0040 mv (Fig. 5a). The extracted sample contained a homogenous polymer population (single peak) with polydispersity index (M_w/M_n) of 2.29 (Fig. 5b). GPC analysis showed that PHB extracted by dispersion method gave polymer of relatively high molecular weight ($M_w = 2.82 \times 10^5$) and average molecular weight ($M_n = 1.23 \times 10^5$) compared to previous report where PHB produced by *Bacillus* sp. INT005 grown on glucose-containing medium showed M_w and M_n value of 0.5×10^5 and 0.28×10^5, respectively.[16] Polydispersity index (PI) of 2.29 obtained in the present study is higher in comparison to previous finding of Valappil et al.[17] who reported PI of 1.79 in polymer extracted from *Bacillus cereus* SPV under similar condition. The average molecular weight of PHB obtained by *Alcaligenes* sp. utilizing cane molasses as carbon source was higher in comparison to average molecular weight (M_w/M_n) of PHB obtained from other strains in previous studies (Table 4). High molecular weight will enhance

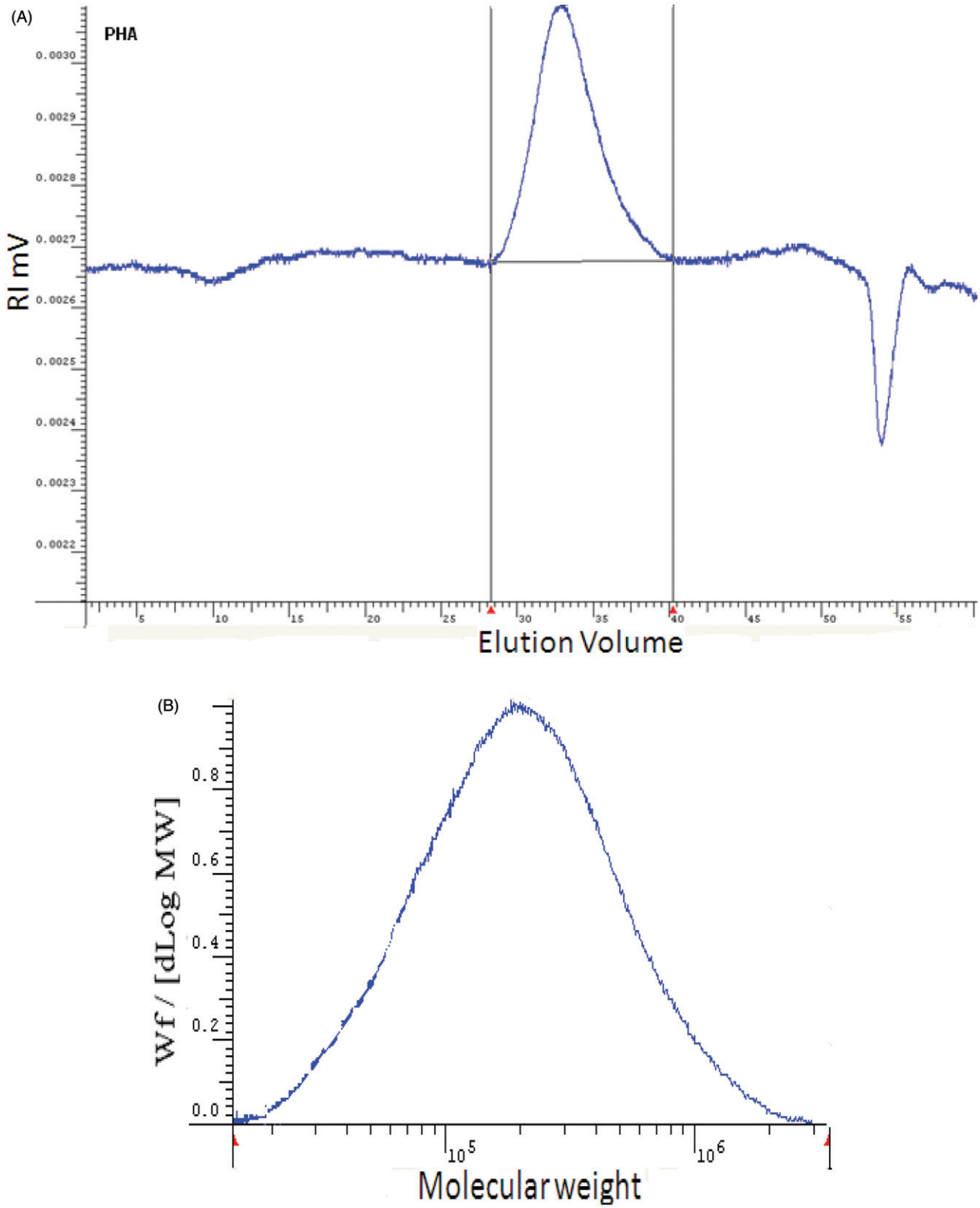

Figure 5. (a) GPC analysis of PHB; Columns: 5 μ-Styragel HT from 2–6 Mobile Phase: Chloroform Calibration: Polystyrene Conn: 0.2% solution in m.p. Flow Rate: 1 ml/min Injection volume: 100 μL. (b) Molecular weight distribution of PHB extracted from *Alcaligenes* sp. obtained after 48.0 h of fermentation.

the tensile strength of the polymer and this may be attributed to lesser PHB degradation during hypochlorite and chloroform extraction. Crystallinity index (CI) of the polymer was determined by FTIR. The crystallinity index is defined as the ratio of band intensity at 1382 cm^{-1} (CH_3), which is insensitive to the degree of crystallinity, to that at 1185 cm^{-1} (C–O–C) which is sensitive to amorphous state. Crystallinity of the PHB isolated by dispersion method was found to be 0.948 which was similar to the previously reported value of 0.949 for PHB.[18] Crystallinity of a polymer is known to play a major role in the degradation of a polymer.[19] The amorphous region of polymer degrade

Table 4. Comparison of molecular weight of PHB extracted from different microbes on bioreactor using agro-industrial residues in SMF.

Compound	Mn	Mw	MW/Mn	Viscosity (*n*)	References
PHB extracted from *Alcaligenes*sp.	1.23 × 105	2.82 × 105	2.29	9.8 × 101	This study
PHB (*A. latus*)	3.13 × 105	5.28 × 105	1.69	9.4 × 101	(Hawari et al., 2007)[31]
PHB (commercial)	0.91 × 105	1.77 × 105	1.95	9.1 × 101	(Oliveira et al., 2007)[32]
PHB (*P.cepacia*)	1.37 × 105	5.37 × 105	3.90	8.1 × 101	(Aremu et al., 2010)[33]
PHB (*B. thuringenesis*R 1)	5.85 × 104	1.04 × 105	1.77	ND*	(Rohini et al., 2006)[34]
PHB (*Bacillus megaterium* ATCC 6748)	0.91 × 10 5	1.77 × 105	1.47	ND*	(Chaijamrus and Udpuay, 2008)[35]

ND*: Not defined.

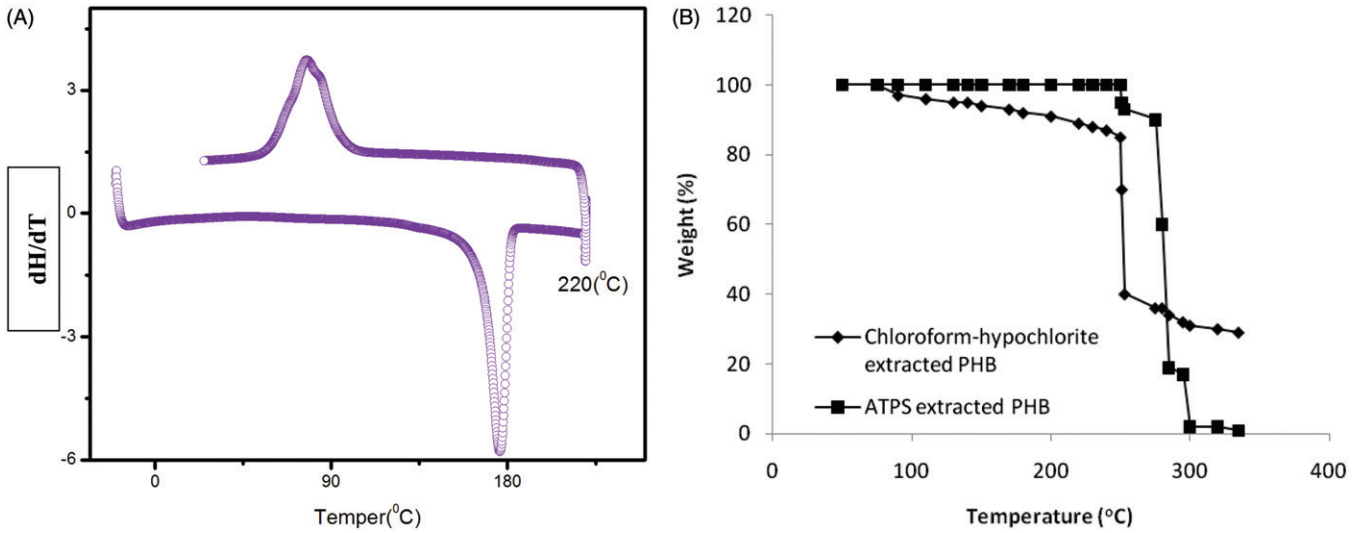

Figure 6. (a) Differential Scanning Calorimetry (DSC) analysis of purified PHB (Polyhydroxybutyrate), where dH/dt represents rate of enthalapy change with temperature. (b) Thermograivemtic analysis (TGA) of PHB by dispersion method and ATPS method.

Table 5. Physical characteristics of PHB obtained by solvent extraction from different microbes.

Compound	Tg (oC)	Tm (oC)	ΔH (j/g)	Xc(%)	References
PHB (Alcaligenessp.)	4.3	178.5	94.0	66.2	This study
Pure PHB	4.8	179.5	142.0	100.0	(Chaijamrus and Udpuay, 2008)
PHB (A. latus)	4.5	177.0	77.2	52.9	(Hawariet al., 2007)

faster in comparison to crystalline state thus the obtained polymer may serve as potent biodegradable polymer in comparison to other polymers.

The thermal properties of polymer such as glass transition temperature (T_g) and melting temperature (T_m) are crucial for polymer processing. The melting temperature (T_m) obtained in the current study by chloroform-hypochlorite extraction was found to be 179 °C (Fig. 6a). Previous reports[20–22] suggested that T_m of the extracted PHB from different microbe and under different extraction condition was reported to be in the range in of 173–180 °C, which is in conformity with current finding (Table 5). The crystallinity of PHB was estimated from the enthalpy of fusion. The percentage crystallinity of the extracted polymer was also determined by measuring the enthalpy of fusion (ΔH_{fusion}) for the extracted polymer by calculating the area under the melting curve. The enthalpy of fusion of a 100% crystalline (theoretical) sample was assumed to be 146 J/g.[23] Percentage crystallinity (% X_c) of the extracted polymer was calculated by comparing the ΔH_{fusion} of the extracted polymer with the pure PHB having ΔH_{fusion} of 146 J/g. The high crystallinity of the extracted PHB may be attributed to solvent treatment employed during recovery. ΔH_{fusion} of extracted PHB polymer by sodium hypochlorite-chloroform dispersion was found to be 94.0 J/g with % X_c of 66.2. Crystallinity of PHB polymer obtained in the current study is higher in comparison to the previous studies where maximum X_c of extracted polymer was found to be in the range of 55–65.0% through solvent extraction or any other method.[24] This clearly suggests that dispersion method was efficient extraction method.

Fig. 6b represented the TGA profile of the purified PHB sample. PHB recovered by dispersion method showed rapid thermal degradation between 275 and 310 °C with a peak at 302 °C. Thermal degradation pattern obtained in the current study is similar to previous finding of Hawari *et al.* (2007) who reported maximum thermal degradation of PHB obtained from *Alcaligenes latus* in the range of 302-308 °C.[25] TGA profile of PHB produced by *Alcaligenes* sp. showed thermal stability with maximized degradation occurring at 302 °C in comparison to ATPS extracted PHB, which is above the melting temperature (179 °C) of the purified polymer. The difference between the decomposition of the polymer and melting temperature of the PHB produced was

high enough to permit the processing of the biopolymer. Polymer derived from *Alcaligenes* sp. may be used for joint suture, bone plate formation owing to its thermal properties.

In the present study, agro-industrial residue (cane molasses) and urea were used as potent carbon and nitrogen source. The chemical composition of sugar cane molasses was determined to assess the nutritional potential of the waste material as substrates. Cane molasses used in the present study contained 54.8% total sugars, 27.1% reducing sugars, and 27.7% non-reducing sugars. PHB yield of 79.0% on dry cell weight basis with 0.19 g/L/h productivity was obtained utilizing 40.0 g/L cane molasses (total sugar) and 0.8 g/L urea by *Alcaligenes* sp. in 7.5-L bioreactor under batch cultivation. PHB extracted from *Alcaligenes* sp. by dispersion method (Chloroform and 30% (v/v) hypochlorite solution dispersion in 1:1 ratio) was of high molecular weight ($M_w = 2.82 \times 10^5$) in comparison to previously extracted PHB from sucrose-fed media. The difference in M_w under similar media may be attributed to difference in growth condition, extraction and purification strategy.[26,27] The molecular weight of polymers influences the physicochemical and mechanical strength. The high molecular weight PHB extracted from *Alcaligenes* sp. shows high degree of polymerization which enhances its industrial application and commercialization. PHB was characterized using FTIR, GC-MS, and NMR. The extracted sample contained a homogenous polymer population (single peak) with polydispersity index of 2.29 and showed thermal stability with maximum degradation above the melting temperature (179 °C) of the purified polymer. The significant temperature difference between polymer decomposition and its melting point, suggests that PHB extracted from *Alcaligenes* cells utilizing cane molasses as substrate may be processed for commercial application. The high enthalpy of fusion and the relatively high M_w of the recovered PHB from cane molasses suggested a high crystallinity and a degree of polymerization which make it applicable in food packaging, medical applications, and tissue engineering. PHA nanoparticles having similar physicochemical properties have been used in food packaging to certain extent.[28] The PHB produced in the present study due to its high crystallinity and CI shows its unique properties such as flexibility and processibility which may be used in short term food packaging.[29] This also provides excellent gas barrier properties to PHB which is required to maintain the freshness of food products. PHA based packaging materials are not affected by changing humidity as compared to conventional PP based packaging material which makes it suitable for food packaging.[30] Bucci et al. investigated the application of PHB in food packaging, compared to PP. The reports of Bucci et al. clearly revealed that the deformation value of PHB was about 50% lower than that of PP. PHB was found to be more rigid and less flexible than PP. The performance of PHB tends to be lower than those of PP under normal freezing conditions. However, at higher temperatures, PHB performed better than PP.[29] The present work has clearly demonstrated the successful use of cane molasses and urea as a carbon and nitrogen source for *Alcaligenes* sp., which in turn biotransforms excess carbon into PHB (bio-plastic). PHB obtained from chosen strain under controlled fermentative condition exhibited similar physicochemical and spectroscopic properties to that obtained from commercial sucrose. The present work may facilitate the utilization of this renewable feedstock (cane molasses) that is abundant in certain temperate countries such as India, Pakistan, and Thailand. This works also emphasizes the importance of research in the development of PHB blends with improved thermal, physicochemical, and biodegradable properties for wide industrial application.

Conclusions

Alcaligenes sp. gave maximum PHB yield using cane molasses in comparison to other expensive sources of carbon and agro-based waste products. In addition to cane molasses as carbon source, methionine and thiamin enhanced the PHB yield. Optimized condition for PHB synthesis comprised: cane molasses 35 g/L, methionine 30 mg/Land thiamine15 mg/L. Under optimized condition, PHB yield increased from 62.0% to 79.26% after 12 h of cultivation in a 7.5-L bioreactor. PHB yield enhanced with amino acid and vitamin supplementation with less fermentation time possessing desired physicochemical properties which makes it suitable for food packaging application. Utilization of sugar refinery waste would minimize the production cost and production kinetics can be utilized to reveal the metabolic flux of PHB biosynthesis in fed-batch and continuous mode. The utilization of sugar refinery waste for PHB biosynthesis will minimize the production cost by approximately 50% and making the overall production process economical and industrially feasible.

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