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Production of polyhydroxybutyrate (PHB) by *Alcaligenes latus* using sugarbeet juice

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ABSTRACT

The practicality of using sugarbeet juice as medium to grow *Alcaligenes latus* (ATCC 29714) for production of polyhydroxybutyrate (PHB), a biodegradable plastic, was explored in this study. Dilute sugarbeet juice, sugarbeet juice with partial and complete addition of nutrients other than sugar were used as culture media. Media with partial nutrient addition was shown to be optimal for PHB production, with final dry cell weight (DCW) 10.30 ± 1.01 g/L, PHB concentration 4.01 ± 0.95 g/L, PHB content 38.66 ± 7.28%, $Y_{p/x}$ (g PHB produced per g dry cell weight) 0.39 ± 0.07 and a maximum PHB productivity of 0.22 ± 0.01 g/L h. The melting temperature of PHB extracted from sugarbeet juice-grown cells supplemented with partial nutrients was measured to be 151.46 °C with crystallinity of 43.12% and the corresponding crystallinity temperature of 45.42 °C. Thermal degradation of extracted PHB occurred from 255.14 to 283.69 °C with the degradation peak at 273.86 °C.

1. Introduction

The use of plastics has increased significantly in recent years and its application has permeated most aspects of human life and industrial production (Yu et al., 1998). The dominant raw material for current plastic production is petroleum (Yezza et al., 2007), which is nonrenewable and also the main source of energy for the world. Limited future availability of petroleum, increasing price of fossil fuel and environmental and waste management concerns due to non-biodegradability of conventional plastics have thus driven various entities to look for more sustainable alternatives such as polyhydryalkanoates (PHAs), polylactides (PLA), aliphatic polyesters, polysaccharides, blends of starch and polypropylene and other copolymers (Lee, 1996) to replace petro-derived plastics (Yu et al., 1999).

Of the various biodegradable plastics being investigated to replace petroleum-derived plastics, polyhydroxyalkanoates (PHA), especially polyhydroxybutyrate (PHB), have received special attention due to some mechanical properties that make them comparable to commercialized plastics such as polypropylene (PP) and polyethylene (PE) (Yu, 2001). PHB is well-known for its environmental friendliness and complete decomposition to water and carbon dioxide by aerobic microorganisms existing in sewage, sea or soil (Lee, 1996). Key applications for PHB include packaging materials, bags, containers, disposable items like one-time use cups and diapers (Lee, 1996). It also has medical applications, either in surgical materials or as a slow-release carrier for long-term drug delivery (Patwardhan and Srivastava, 2004).The main obstacle limiting wide spread commercialization of PHB is its high production cost, which at present is approximately 10 times higher than conventional synthetic polymers (Wegen et al., 1998) and accounts for up to 50% of the processing cost (Choi and Lee, 1999). Corn is a common feedstock currently used by various companies like Cargill Dow Polymers, LLC for biopolymer production (Lunt, 2000). Besides being a major food and feed source in many regions of the world, corn is priced at $269/metric ton (USDA, 2011a,b). Thus, using cheaper feedstocks is the key to reducing production cost of PHB (Khanna and Srivastava, 2005).

Soy wastes from soy milk processing facilities, malt wastes from beer breweries, agro-industrial waste water, extruded rice bran, hydrolyzed corn starch and whey from dairy processing are some resources that have been investigated for sustainable production of biodegradable plastics (Gomez et al., 1996; Huang et al., 2006; Khardenavis et al., 2007; Yang et al., 1994; Yezza et al., 2007; Yu et al., 1999). The potential of sugarbeet, a sucrose-rich and sustainable biomass that is cultivated globally, has also been investigated (Page, 1989, 1992). Sugarbeet acreage in the US has increased steadily and estimated at 1.25 million acres (NASS,

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2010) with the world-wide annual production of sugarbeets estimated at 227 million tons in 2009 (FAO Statistics, 2009). The leading sugarbeet producing countries include France, USA, Germany, and Russia (FAO Statistics, 2009). Sucrose content of dry sugarbeet powders can reach up to 80% and it contains nutrients such as N, P, K and Na which makes it highly suitable for microbial cultivation (OECD, 2002). Page (1989, 1992) investigated downstream products of beet sugar industry such as molasses and pulps for PHB production using *Azotobacter vinelandii* strain UWD. PHB concentration using molasses was however lower than (2.74 g/L vs. 7.04 g/L) that reported using sucrose-based synthetic media with fermentation by *Alcaligenes latus* (El-Sayed et al., 2009) and thus calls for further investigation. Utilization of sugarbeet juice as a medium for PHB production offers a significant cost advantage ($61/ton sugarbeet (USDA, 2011a,b)) especially when compared to pure sugars or currently used starch based feedstocks. However, there is no literature on strain selection and media optimization as well as process development in order to improve PHB production using sugarbeets to make them suitable for industrial applications. Therefore, this research focused on investigating the potential of sugarbeet juice based media, with varying supplemental nutrient concentrations, for PHB production in a two-stage batch fermentation process involving *A. latus*. Fermentation process time was optimized by monitoring PHB production parameters like yield, content and concentration. *A. latus* is a growth associated PHB producer that has been reported to use sucrose as carbon source to produce PHB and was selected due to its ability to accumulate up to 80% of dry cell mass as PHB (Braunegg and Bogensberger, 1985).

2. Materials and methods

2.1. Microorganisms

An intracellular PHB producing microorganism, *A. latus* (ATCC 29714), purchased from American Type Culture Collection (ATCC) was used in this study. Selection of the strain was based on results of a previous study by Wang et al. (2012).

2.2. Media preparation

A variety of media types were used at various stages in this study.

Med. 1: Difco™ Nutrient agar (Becton, Dickinson and Company, MD, USA) was used to maintain *A. latus* (El-Sayed et al., 2009).

Med. 2: Media to prepare *A. latus* inoculum for subsequent PHB production using sugarbeet juice contained the following components (El-Sayed et al., 2009): sucrose 20 g/L, $(NH_4)_2SO_4$ 2.0 g/L, KH_2PO_4 1.5 g/L, $Na_2HPO_4{\cdot}12H_2O$ 9 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, $FeCl_2{\cdot}H_2O$ 60 mg/L, $CaCl_2{\cdot}2H_2O$ 10 mg/L, and 1 mL of filter sterilized trace elements solution. Each liter of trace element solution contained H_3BO_3 0.3 g, $CoCl_2{\cdot}6H_2O$ 0.2 g, $ZnSO_4{\cdot}7H_2O$ 0.1 g, $MnC1_2{\cdot}4H_2O$ 30 mg, $Na_2MoO_4{\cdot}2H_2O$ 30 mg, $N_iSO_4{\cdot}7H_2O$ 28 mg and $CuSO_4{\cdot}5H_2O$ 10 mg. The pH of formulated media was 7.0 (unadjusted) as reported by El-Sayed et al. (2009).

Med. 3 (no nutrient addition): sugarbeet juice obtained by extraction of sugarbeets under conditions identified as optimum in this study was diluted with deionized water to adjust the sucrose concentration to 20 g/L. The same media was used both at stage 1 and stage 2. The pH of this media was 7.03 ± 0.05 (unadjusted).

Med. 4 (*complete nutrient addition*): sugarbeet juice with sucrose concentration adjusted to 20 g/L (*Med. 3*) was supplemented with all the nutrients identified in *Med. 2* (except sucrose) per liter of diluted sugarbeet juice for stage 1 fermentation. For the 2nd stage, only 0.2 g/L $(NH_4)_2SO_4$, which was equivalent to 10% nitrogen of 1st stage, was added. The pH of this media was 7.11 ± 0.04 (unadjusted) for first stage and 7.08 ± 0.04 (unadjusted) for second stage.

Table 1
Elemental composition analysis of sugarbeet juice.

Elements/chemicals	Diluted sugarbeet juice (*Med. 3*) (mg/L)[a]	*Med. 2* (mg/L)	Nutrients added (*Med. 5*) (mg/L)
N	5.50 ± 2.52		
$(NH_4)_2SO_4$	25.93 ± 11.90	2000.00[b] 200.00[c]	1974.07[b] 174.07[c]
Ca	3.75 ± 0.04		
$CaCl_2{\cdot}2H_2O$	13.68 ± 0.13	10.00	
Co	<0.05		
$CoCl_2{\cdot}6H_2O$	<0.05	0.20	0.20
Cu	<0.05		
$CuSO_4{\cdot}5H_2O$	<0.05	0.01	0.01
K	161.33 ± 1.53		
KH_2PO_4	562.60 ± 5.33	1500.00	937.40
Mg	27.03 ± 0.95		
$MgSO_4{\cdot}7H_2O$	277.09 ± 9.74	200.00	
Mn	0.31 ± 0.01		
$MnCl_2{\cdot}4H_2O$	1.10 ± 0.02	0.03	
Na	51.63 ± 1.27		
$Na_2HPO_4{\cdot}12H_2O$	401.84 ± 9.89	9000.00	8598.16
Ni	<0.05		
$Ni_2S0_4{\cdot}7H_2O$	<0.05	0.03	0.03
Zn	0.09 ± 0.02		
$ZnSO_4{\cdot}7H_2O$	0.40 ± 0.08	0.10	
Fe	0.83 ± 0.27		
$FeCl_2{\cdot}H_2O$	2.16 ± 0.70	60.00	57.84

[a] The sugar concentration of sugarbeet juice was first adjusted to 20 g/L. All the elemental analysis was done in triplicates on individual elements, not chemicals.
[b] Nitrogen rich media for first stage.
[c] Nitrogen limited media for second stage.

Med. 5 (*partial nutrient addition*): sugarbeet juice with sucrose concentration adjusted to 20 g/L (*Med. 3*) was supplemented with: $(NH_4)_2SO_4$ 1.97 g/L (nitrogen rich media for first stage) or 0.17 g/L (nitrogen limited media for second stage); $Na_2HPO_4{\cdot}12H_2O$ 8.6 g/L; KH_2PO_4 0.94 g/L; $FeCl_2{\cdot}H_2O$ 57.84 mg/L and 1 mL of filter sterilized trace elements solution containing H_3BO_3 0.3 g/L; $CoCl_2{\cdot}6H_2O$ 0.2 g/L; $Na_2MoO_4{\cdot}2H_2O$ 0.03 g/L; $NiSO_4{\cdot}7H_2O$ 0.03 g/L; $CuSO_4{\cdot}5H_2O$ 0.01 g/L per 1 L sugarbeet juice. The level of nutrients to be added was based on elemental analysis of *Med. 3* (Table 1). When levels of inherent components were higher in *Med. 3*, the corresponding salt was not added. In other cases the difference between amount of salts added in *Med. 2* and those present in *Med. 3* was used to estimate the amount needed for partial addition. The pH of this media was 7.22 ± 0.04 (unadjusted) for first stage and 7.24 ± 0.04 (unadjusted) for second stage.

2.3. Extraction of sugar from sugarbeets

Fresh sugarbeets harvested from Custer area of Montana were sliced and dried in a convection oven (Fisher Scientific) at 60 °C for 25 h. The dried slices were stored at 4 °C in a temperature controlled chamber during the course of the study. The slices were ground to pass through a 2 mm sieve using a Wiley Mill for extraction. Sugarbeet powder and deionized water were mixed at a solid loading of 1:15 (sugarbeet (w):water (w)), and placed in a shaking water bath (Precision, Thermo Scientific) at 60 °C/150 rpm for 1 h to generate 4000 mL sugarbeet juice extract for use during fermentation studies. Extraction conditions were identified as optimal based on preliminary studies (data was not shown) looking at solid: water ratio, extraction time, and sugarbeet preparation (fresh vs. oven dry) for samples harvested from Plymouth, NC in September, 2009. After vacuum filtration, the sugarbeet juice based media was obtained.

Sugar concentration was measured in °Brix by a refractometer (Alla France). Brix values were converted to grams of sugar, using Eq. (1) (Bruce, 1995):

$$SC_g = SC \times SG \times 10 \quad (1)$$

where, SC was sugar concentration in °Brix and SC_g represented sugar concentration in g/L. Specific gravity (SG) under different °Brix was obtained from the sucrose conversion table (USDA, 1981) and 10 was the conversion factor.

2.4. Inoculum preparation

Thirty mL of *Med. 2* in three 250 mL flasks was inoculated with 1.5 mL thawed *A. latus* ATCC 29714 glycerol (20%) stock and incubated at 33 °C for 24 h, for propagation of frozen stocks prior to starting each stage of the study. Two and a half mL of inoculum (Santhanam and Sasidharan, 2010) was added to 100 mL sugarbeet media for the actual fermentation process.

2.5. Fermentation of sugarbeet juice for PHB production

Based on the results of a previous study (Wang et al., 2012), two-stage batch fermentation with introduction of N limitation at 16 h by replacing spent N rich sucrose based media with fresh N limited media (10% N of original) was identified as optimal for this study. Sugarbeet juice prepared as described above had an average sucrose concentration of approx. 50 g/L, which was much higher than that normally used for *A. latus* cultivation (Yamane et al., 1996; Grothe et al., 1999). Thus, the sugarbeet juice was diluted as needed with deionized water to the desired concentration (20 g/L).

Fermentation studies were conducted with no additional nutrients (*Med. 3*), complete nutrient addition (*Med. 4*) and partial nutrient addition (*Med. 5*) to determine PHB production potential of the juice. Hundred mL of desired media (*Med. 3*, *Med. 4* or *Med. 5*) was added to 250 mL polypropylene centrifuge bottles with plastic foam stoppers (3 media types × 3 replicates × 12 sample points). Each bottle was inoculated with 2.5 mL *A. latus* inoculum and incubated at 33 °C in the incubator shaker (Series 25; New Brunswick Scientific Co., Inc., Edison, New Jersey, USA) at 200 rpm. Three bottles each were drawn at 4, 8, 12 and 16 h. After 16 h of the first stage, the remaining bottles were centrifuged at 4000 rpm/30 °C for 10 min and residual media decanted. For N limited stage (stage 2), 100 mL of *Med. 3* was added to the no additional nutrient set, *Med. 4* with 10% of N compared to 1st stage was added to the complete nutrient addition set and *Med. 5* with 10% of N was added to the partial nutrient addition set. After changing to new media, the remaining bottles were incubated at 33 °C, 200 rpm in an incubator shaker for up to 28 h. As during the first stage, three bottles each were taken out at 4, 8, 12, 20, 24, 26, 27, 28 h from start of second stage for subsequent analyses.

2.6. PHB extraction and quantification

The method used for PHB extraction and quantification was adapted from the gravimetric method of Kim et al. (1994). A mixture of sodium hypochlorite (12.5 mL, 30% (v/v)) and chloroform (12.5 mL) with 1 g dry cell mass in 50 mL centrifuge bottles was vortexed and kept in a water bath at 30 °C for 90 min. It was then centrifuged for 15 min, 4000 rpm (Eppendorf 5810 R, Eppendorf AG, Hamburg, Germany) at 30 °C. Of the three layers formed, top (aqueous hypochlorite solution), middle (cells, other biological matter), and bottom (PHB rich chloroform), the chloroform phase was pipetted out carefully and PHB was recovered by non-solvent precipitation using a mixture of methanol:water (7:3 (v/v), 1.25 mL/g dry cell mass) and filtration. The extraction mixture in uncapped tubes was left in a fume hood for 48 h to volatilize excess solvent. The final PHB pellet obtained was weighed and the yield of PHB estimated.

2.7. Analytical procedures

2.7.1. Optical density (OD) and dry cell weight (DCW)

Optical density (OD) of the suitably diluted cell suspension was measured by a spectrophotometer (Shimadzu UV-1700, Suzhou Instruments Manufacturing Co., Ltd., Suzhou, China) at 600 nm against a media blank. Dry cell weight was evaluated by a gravimetric method in which fermentation samples were centrifuged (4000 rpm, 15 min, 4 °C), decanted and dried in an oven (90 °C) until constant weight was reached. The samples were then cooled in a desiccator and weighed to determine DCW.

2.7.2. Elemental analysis

Elemental analysis including determination of N, Ca, Co, Cu, K, Mg, Mn, Ni and Zn in sugarbeet juice was performed by soil analysis lab in the department of Soil Science at North Carolina State University and Iron (II) measurement was done by environmental analysis lab in the department of Biological and Agricultural Engineering. Iron (II) was measured by nitric acid digestion followed by direct aspiration atomic absorption spectroscopy based on the standard method 3111-B of American Public Health Association, American Water Works Association and Water Environment Federation (Clesceri et al., 1998). For inorganic nitrogen measurement, standard method 4500-NH3H provided by Greenberg et al. (2005) was applied. Standard method 3120 was used for the detection of other metals (Greenberg et al., 2005).

2.7.3. Sugar analysis using ion chromatography (IC)

An IC (Dionex ICS-5000, California, USA) equipped with a pulsed electrochemical detector (Dionex ICS-5000) was used to measure the sugar concentration of fermentation broth samples. The column used was CarboPac PA1 (4 × 250 mm) with eluent (18 mM potassium hydroxide) at a flow rate of 0.9 mL/min and working temperature 18 °C.

Samples in 250 mL bottles were centrifuged and the supernatant obtained was diluted 50 times before filtering through 0.22 μm syringe filters into vials with pre-split caps. Concentrations of sucrose, glucose and fructose were recorded for each sample. A stock solution containing 0.5 g each of sucrose, fructose and glucose in 200 mL HPLC grade water was prepared in a volumetric flask. The standards were prepared by gradient dilution to obtain sugar concentrations of 0.5 g/L, 0.25 g/L, 0.125 and 0.0625 g/L.

2.7.4. Confocal imaging of inclusion bodies of A. latus ATCC 29714

Five μl each of broth from fermentation runs resulting in enhanced PHB production by *A. latus* ATCC 29714 in synthetic and sugarbeet juice media were placed on a slide and dried in an incubator overnight at 33 °C (Ostle and Holt, 1982). The slide was then immersed in 1% Nile blue A aqueous solution in a shallow container and stained for 40 min by placing in a shaking incubator (55 °C, 50 rpm). The slide was dried again before 5 μl of water was put on it and a cover slip placed over it.

A confocal microscope (LSM 710, Carl Zeiss MicroImaging LLC, NY, USA) with Zeiss Axio Observer Z1 inverted microscope and Zeiss Plan Apochromat 63× objectives (NA 1.4 oil immersion) was used to obtain images of inclusion bodies of *A. latus* at the Cellular and Molecular Imaging Facility (CMIF) in the Department of Plant Biology. The inclusion bodies were excited at 488 nm (argon laser) and fluorescence emission was determined from 492 nm to 625 nm.

Table 2
Growth and PHB production of *A. latus* (ATCC 29714) using sugarbeet juice with three different nutrient addition strategies during two-stage batch fermentation with introduction of N limited media at 16 h.[a]

Nutrient addition strategies	Time (h)	Dry cell weight (g/L)	$Y_{p/x}$[b]	PHB productivity (g/L h)[c]
No nutrient addition (*Med. 3*)	4	3.47 ± 0.05	0.14 ± 0.10	0.10 ± 0.11
	8	2.46 ± 0.07	0.25 ± 0.10	0.13 ± 0.06
	12	4.58 ± 0.12	0.26 ± 0.02	0.13 ± 0.01
	26	6.60 ± 0.08	0.38 ± 0.09	0.13 ± 0.02
	27	8.89 ± 0.08	0.39 ± 0.07	0.13 ± 0.02
	28	9.23 ± 0.06	0.39 ± 0.14	0.12 ± 0.03
Complete nutrient addition (*Med. 4*)	4	3.74 ± 1.36	0.13 ± 0.07	0.09 ± 0.05
	8	4.41 ± 0.46	0.13 ± 0.10	0.06 ± 0.07
	12	5.51 ± 0.35	0.15 ± 0.10	0.05 ± 0.04
	26	7.71 ± 0.94	0.30 ± 0.13	0.08 ± 0.03
	27	8.05 ± 1.02	0.27 ± 0.18	0.07 ± 0.04
	28	7.88 ± 0.76	0.31 ± 0.12	0.08 ± 0.03
Partial nutrient addition (*Med. 5*)	4	6.46 ± 0.71	0.19 ± 0.08	0.16 ± 0.16
	8	7.88 ± 2.28	0.34 ± 0.22	0.22 ± 0.09
	12	8.28 ± 1.51	0.39 ± 0.07	0.22 ± 0.01
	26	9.87 ± 0.84	0.38 ± 0.10	0.12 ± 0.03
	27	8.96 ± 1.02	0.42 ± 0.04	0.12 ± 0.02
	28	10.30 ± 1.01	0.39 ± 0.07	0.12 ± 0.03

[a] Each value is an average of triplicates.
[b] Gram PHB produced per gram dry cell weight.
[c] Gram PHB produced per liter media per hour culture time.

2.7.5. PHB identification using gas chromatography–mass spectrometry (GC–MS)

Samples for GC analysis were prepared as described by Riis and Mai (1988). Forty mg each of dry cell mass obtained from fermentation using synthetic media (Wang et al., 2012) and sugarbeet juice media, both resulting in optimized PHB production were placed in 50 mL sealable glass centrifuge tubes. Two mL 1,2-dichloroethane (DCE), 2 mL propanol mixed solution (1 volume hydrochloric acid and 4 volume propanol) and 200 μl of internal standard solution (prepared by adding 2.0 g benzoic acid in 50 mL propanol) were added to each tube. The tubes were incubated in a convection oven for 2 h at 100 °C and shaken once every 30 min. After cooling the tubes to room temperature, 4 mL DI water was added and the tubes were shaken for 30 s before allowing to gravity settle. The bottom organic phase was directly injected into the GC–MS. Standards were prepared by dissolving 200 mg PHB, extracted from dry cell mass of the optimized synthetic media fermentation conditions (Wang et al., 2012), in DCE in a 10 mL volumetric flask placed at 100 °C for 2 h. Upon cooling, the solution was made up to 10 mL by adding DCE. Two hundred μl, 400 μl, 600 μl and 800 μl of this mixture were taken and treated in the same way with propanol and benzoic acid as mentioned above.

GC analysis for confirming the presence of PHB in fermentation broth was conducted at mass spectrometry laboratory in the department of Chemistry at NCSU. An Agilent 5975 GC–MS in electron ionization (EI) mode equipped with a HP-5MS 30 m × 250 μm × 0.25 μm column with helium at a flow of 1 mL/min was used. Initial temperature of 50 °C was held for 3 min before ramping to 325 °C at 15 °C/min and holding for 5 min. A 3 min solvent delay was used.

2.7.6. Rheological analysis of PHB

Rheological properties of PHB samples obtained from fermentation of sugarbeet juice were analyzed. Sample discs for rheological analysis were prepared using a Carver® Press (CH-4386, Wabash, IN, USA) in the department of Chemical and Biomolecular Engineering. A 25 mm die wrapped with polyimide film to prevent sticking was filled with 0.7 g extracted PHB and pressed twice at room temperature, molded at 140 °C by sequentially pressing the dies under 1–9 tons and releasing pressure/wt immediately after each application. In the end pressure was held for 2 min before cooling to room temperature.

Dynamic rheological experiments were conducted using an AR2000 Advanced Rheometer (TA Instruments, DE, USA) fitted with 25 mm ETC flat plate geometry. First, stress sweep experiment was performed to evaluate the maximum stress within the linear viscoelastic (LVE) region. Then frequency sweep was conducted in the predetermined LVE region. All experiments were done at 180 °C.

2.7.7. Differential scanning calorimeter (DSC) and thermogravimetric analysis (TG)

The melting temperature and crystallinity of PHB obtained from fermentation using sugarbeet juice media resulting in enhanced production was determined in the department of Textile Engineering, Chemistry and Science by Diamond DSC (PERKIN ELMER, Inc., USA) equipped with Intracooler 2P. Two heating and cooling scan cycles were performed within the temperature range from −20 to 220 °C at a scanning rate of 10 °C/min. The information on thermal history of PHB samples was obtained from the first cycle and the melting temperature (T_m), crystallinity temperature (T_c) and enthalpy of fusion (ΔH) were determined from the second cycle. The crystallinity of PHB samples was calculated as the ratio of ΔH from this study to the ΔH corresponding with 100% crystallinity. The ΔH corresponding to 100% crystallinty of PHB was assumed to be 146 J/g based on that reported by Barham et al. (1984).

TG analysis was conduct using Pyris 1 TGA (PERKIN ELMER, Inc., USA) with a temperature scanning rate of 20 °C/min from 0 to 700 °C under nitrogen (flow rate of 60 mL/min), to further examine the degradation process of PHB samples (Yezza et al., 2007).

2.8. Process parameters and statistical analysis

All experiments and analyses were performed in triplicate. Optical density (OD), dry cell weight (DCW), PHB yield coefficient relative to cell dry weight ($Y_{p/x}$, g/g, defined as gram PHB produced per gram dry cell mass produced) (Grothe et al., 1999), PHB content (g/g, defined as the ratio of PHB concentration to dry cell concentration) and PHB productivity (g/L h, defined as gram PHB produced per liter per hour) (Wang and Lee, 1997) were measured and calculated accordingly for comparison of different media performances after completion of the fermentation process. SAS® procedure MIXED (version 9.1.3 SP4, SAS Inc., Cary, NC) was applied to fit the experimental data into a general linear model. Sampling time, media type and their interaction were

treated as fixed effects in the model. Separate residual variances were estimated for each sampling point to account for heterogeneity of residual variances. The three repetitions for media type and sampling time combination were seen as random effects. Pairwise Student-*t* test ($\alpha = 0.05$) was used to further test null hypothesis of no differences among groups. Test of hypothesis for main and interaction effects were conducted at 0.05 significance level.

3. Results

3.1. Elemental analysis of sugarbeet juice

Elemental analysis data for synthetic media (*Med. 2*) used in PHB production by *A. latus* and sugarbeet juice based media (*Med. 3*) is presented in Table 1. The concentration of elements was converted to that of chemicals to make it more convenient to determine the level of nutrient addition required for partial nutrient addition based media (*Med. 5*). The results indicated that for trace elements such as Co, Cu and Ni, the amount was below the measurement range (<0.05 mg/L) of methods used. Although N, Na and Fe were present in sugarbeet juice, the amounts were not enough compared to those in synthetic media, e.g., 25.93 ± 11.90 mg/L equivalent of $(NH_4)_2SO_4$ was present in *Med. 3* compared to 2000 mg/L required for nitrogen rich and 200 mg/L for nitrogen limited *Med. 2*. The differences (1974.07 mg/L for nitrogen rich media and 174.07 mg/L for nitrogen limited media) were the amount of corresponding salt added in *Med. 5*. Nutrients like Ca, Mg and Mn were in excess amounts in the natural media and hence did not need to be added during fermentation with partial salt addition.

3.2. Effect of different nutrient supplementation strategies on PHB production from sugarbeet juice

Fig. 1 presents the growth curves of *A. latus* (ATCC 29714) during the first stage (16 h) for the three media tested. *A. latus* showed exponential growth under all three nutrient addition strategies. Media with partial addition resulted in the highest DCW of 7.95 ± 1.25 g/L, while the corresponding DCWs with no nutrient addition media and complete addition media were 4.75 ± 0.05 g/L and 4.07 ± 0.23 g/L, respectively.

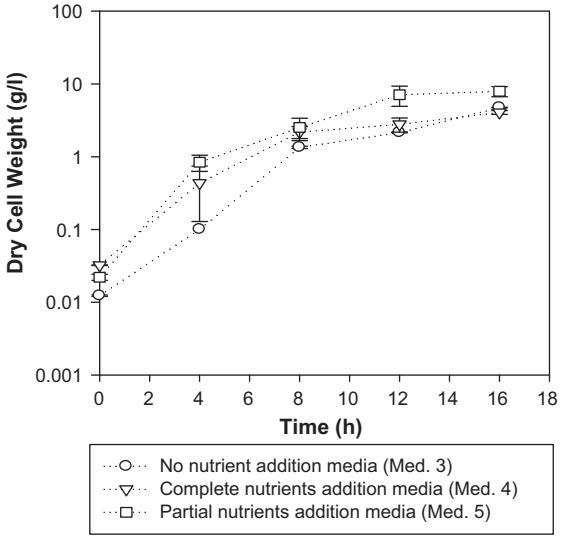

Fig. 1. Dry cell weight of *A. latus* (ATCC 29714) obtained from the first stage of two-stage cultures using sugarbeet juice with different nutrient addition strategies. Dotted lines only represent the trend.

The results for DCW, PHB yield coefficient based on DCW ($Y_{p/x}$) and PHB productivity during 28 h second stage cultivation are shown in Table 2. DCW data indicated that the exponential growth phase was extended for all three nutrient supplementation runs. The run using partial nutrient addition ended at the highest DCW of 10.30 ± 1.01 g/L while DCWs were 9.23 ± 0.06 g/L for no nutrient addition and 7.88 ± 0.76 g/L for complete addition fermentations. $Y_{p/x}$ was similar ($P > 0.05$) with partial addition and no nutrient addition, at 0.39 ± 0.07 g/L and 0.39 ± 0.14 g/L, respectively, and 0.31 ± 0.12 g/L with complete nutrient addition. PHB productivity ranged from 0.10 to 0.13 g/L h, 0.05 to 0.09 g/L h and 0.12 to 0.22 g/L h for no nutrient addition, complete nutrient addition and partial nutrient addition, respectively. Fig. 2a and b, respectively, illustrate the change in PHB concentration and PHB content during 28 h second stage cultivation. Both parameters showed an increasing trend with ending values generally within 2.39 ± 0.78 g/L and $31.01 \pm 11.81\%$ for complete nutrient addition and 4.01 ± 0.95 g/L and $38.66 \pm 7.28\%$ for partial nutrient addition, relative to PHB concentration and content, respectively.

Sucrose consumption was 8.10 ± 6.60 g/L, 9.58 ± 0.60 g/L and 5.96 ± 1.18 g/L for no nutrient addition, complete addition and partial addition fermentations, respectively. During the second stage, corresponding PHB yield coefficients based on sugar consumed were 0.24 ± 0.08, 0.25 ± 0.10 and 0.71 ± 0.28, respectively.

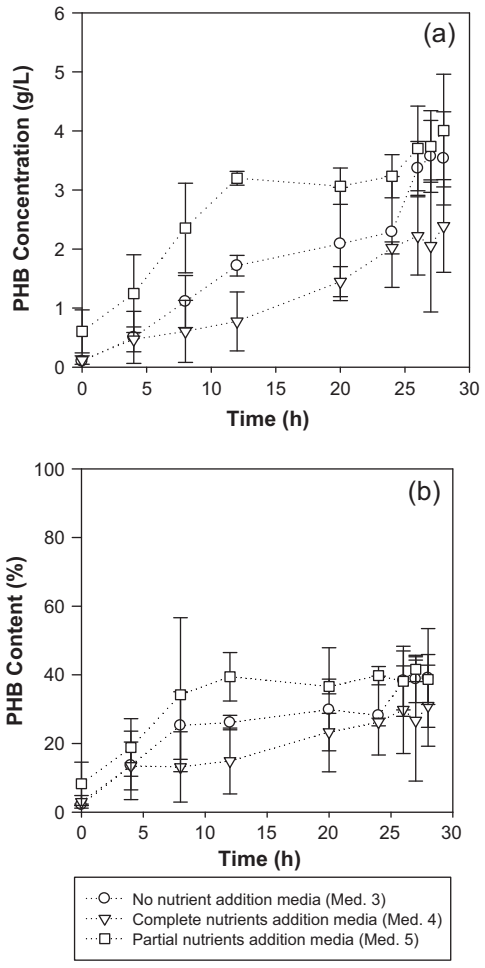

Fig. 2. PHB concentration and PHB content obtained from second stage of two-stage batch fermentation of sugarbeet juice with different nutrient addition strategies by *A. latus* (ATCC 29714). Dotted lines only represent the trend.

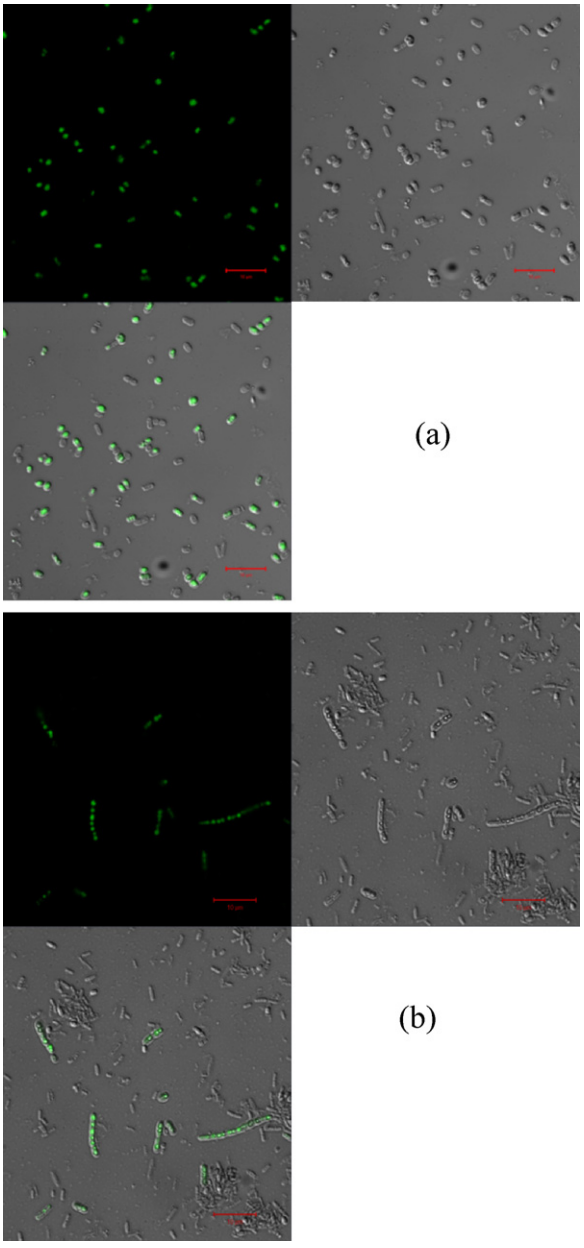

Fig. 3. PHB inclusion bodies in *A. latus* ATCC 29714 obtained from (a) synthetic media and (b) sugarbeet juice media, stained with Nile blue A (top left), digital image correlation (top right) and overlay of both (bottom left) observed with a confocal microscope. Scale bar, 10.0 μm.

3.3. Identification of PHB using confocal microscope and gas chromatography–mass spectrometry (GC–MS)

Inclusion bodies in cells from fermentation broth of synthetic media and sugarbeet juice media with partial nutrient addition were visually examined using confocal microscopy. PHB inclusion bodies can be distinguished in all the cells which appear as green fluorescence (Fig. 3). The images in this study were similar to the ones taken by Ostle and Holt (1982), for PHB granules in *A. chroococcum* stained with Nile blue A. The size (~2 μm in diameter and 2.5 μm in length) and shape of *A. latus* is consistent with that observed by Palleroni and Palleroni (1978) and Holt et al. (1994). Visual estimation of the percentage of cells with PHB inclusion bodies is approximately 50% for synthetic media and 40% for sugarbeet juice media.

PHB obtained by both synthetic and sugarbeet juice media with partial nutrient addition was confirmed by GC–MS analysis. In the chromatograms shown in Fig. 4, three main peaks with similar retention times can be seen. As identified by comparing molecules in the GC database, the first peak represents the solvent used during sample preparation (DCE and propanol), the second peak denotes hydroxybutyric acid propyl ester and the third represents benzoic acid propyl ester. The sequence of components represented by the 3 peaks in the chromatographs is in accordance with that reported by Riis and Mai (1988). Based on the peak area, PHB content in dry cell mass was determined to be 80.15% and 65.60% for synthetic and sugarbeet juice media, respectively.

3.4. Rheology of PHB produced by sugarbeet juice fermentation

Rheological properties of PHB samples were examined in terms of their dynamic elastic (G') and (G'') viscous moduli. In Fig. 5a, we examine the frequency dependence of G' and G'' of a representative sample (from partial nutrient addition fermentation), as the relative shape and magnitude of the moduli provide a signature of the molecular interactions in the system (Raghavan et al., 2000). This experiment was carried out at a stress within the linear viscoelastic region (3 Pa) of the sample obtained from stress sweep experiment (Fig. 5b) (Gunasekaran and Mehmet, 2003). Fig. 5b shows the effect of increasing stress on the two moduli. This experiment was carried out at a constant frequency of $\omega = 1$ rad/s, and was done to examine the effect of stress on microstructural breakdown.

3.5. Thermal properties of PHB produced by sugarbeet juice fermentation

PHB samples extracted from *A. latus* cells obtained from fermentation of sugarbeet juice with partial nutrient addition were analyzed for thermal properties. Based on results of differential scanning colorimetery, the peak representing melting point of PHB in the first scan appeared at 165.15 °C and the corresponding crystallization temperature was 66.28 °C. The enthalpy of fusion was 61.161 J/g and crystallinity during melting in the first cycle was calculated as 41.89%. In the second scan, the melting point peak appeared at 151.46 °C with the crystallization peak at 45.42 °C. The corresponding enthalpy of fusion was 62.962 J/g and crystallinity during melting was calculated as 43.12%.

According to thermogravimetric analysis (Fig. 6), the temperature range for rapid thermal degradation of PHB was from 255.14 to 283.69 °C with the degradation peaking at 273.86 °C (the only large inverted peak). The total weight loss within this temperature range was 95.39%.

4. Discussion

Elemental analysis of sugarbeet juice indicated that in its natural form it is a highly complex mixture of nutrients suitable for PHB fermentation. Investigation on exploring inhibition/promotion effect and optimal nutrient addition strategies was thus essential to establish the foundation for the future application and development of sugarbeet juice in PHB production.

4.1. Effect of different nutrient supplementation strategies on PHB production from sugarbeet juice

Based on statistical analysis (ANOVA GLM with $\alpha = 0.05$), DCW data obtained from the end of first stage (16 h) for each nutrient addition strategy showed no significant difference ($P > 0.05$) between no addition and complete addition fermentation. However, partial nutrient addition resulted in significantly higher ($P \leq 0.05$) DCW. When growth of *A. latus* within 24 h (data not

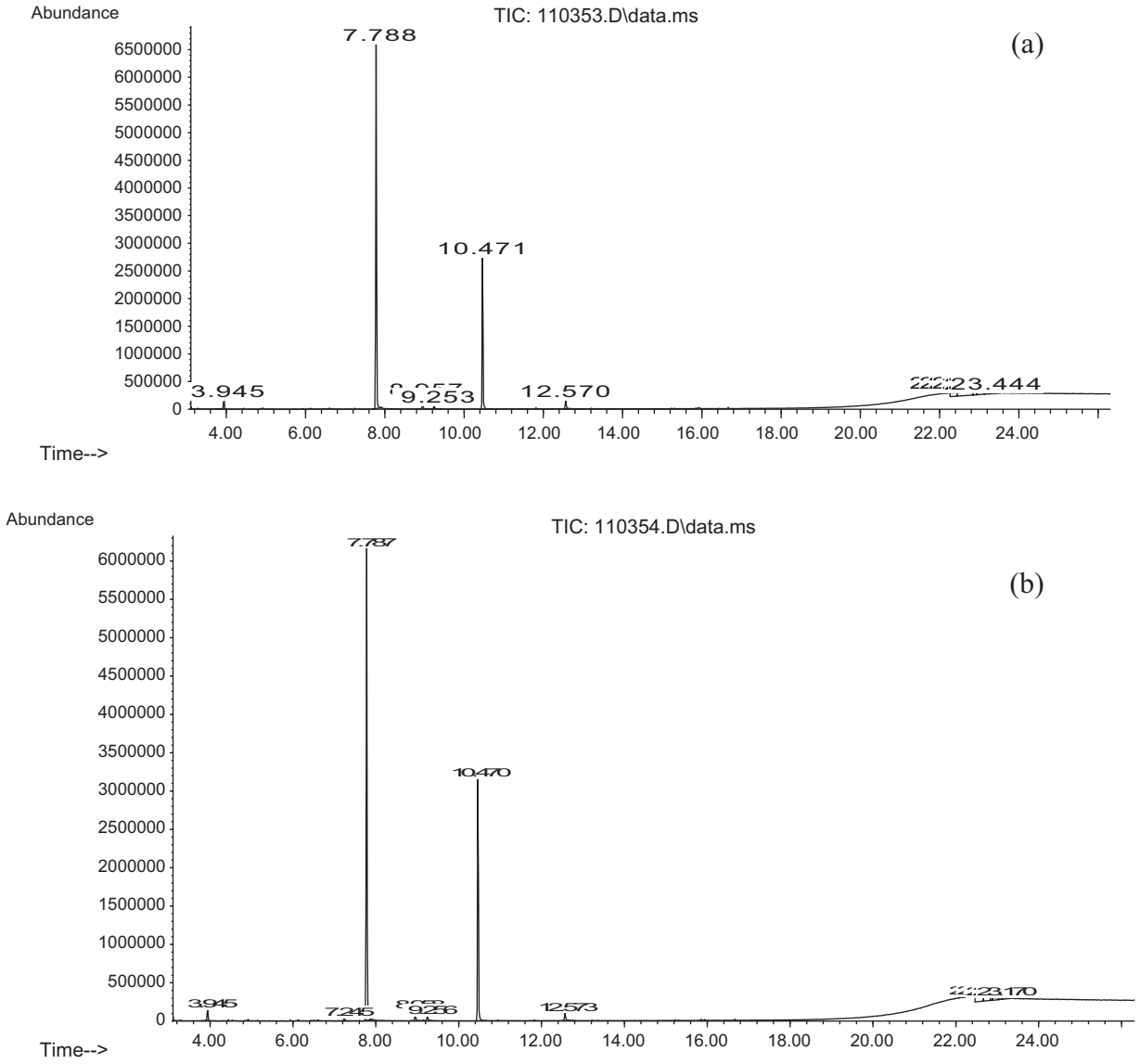

Fig. 4. Chromatogram of sample from (a) synthetic media and (b) sugarbeet juice with partial nutrient addition. The peak at 3.945 min represents solvents (DCE and propanol), at 7.787 ± 0.001 min represents hydroxybutyric acid propyl ester and at 10.470 ± 0.001 min represents benzoic acid propyl ester.

Table 3
Two-way ANOVA (Type 3 Test of Fixed Effects) for sampling time points (Time), three different types of media used (Treatment) and their interaction (Treatment × Time) during two-stage fermentation of sugarbeet juice.

Dependent variable	Effect	Num DF	Den DF	*F* Value	Pr > *F*
PHB concentration	Treatment	2	28.8	31.65	<0.0001
	Time	6	14.1	56.01	<0.0001
	Treatment × Time	12	15.7	3.30	0.0147
Dry cell weight	Treatment	2	42	37.18	<0.0001
	Time	6	42	29.93	<0.0001
	Treatment × Time	12	42	1.55	0.1432
PHB content	Treatment	2	30.3	7.76	0.0019
	Time	6	14	26.43	<0.0001
	Treatment × Time	12	15.6	0.86	0.5955
PHB yield based on dry cell weight	Treatment	2	30	7.63	0.0021
	Time	6	14	26.72	<0.0001
	Treatment × Time	12	15.6	0.90	0.5625
PHB productivity	Treatment	2	26.2	9.11	0.0010
	Time	6	33.4	5.95	0.0003
	Treatment × Time	12	29.9	0.95	0.5138

shown) was compared among the three nutrient addition strategies, although DCW from fermentation of media with complete nutrient addition was low in the initial stage, all three media types showed no significant ($P > 0.05$) difference in DCW at the end of the growth period. It was observed that they all entered stationary growth after 16 h.

According to statistical analysis using mixed analysis (Type 3 Tests of Fixed Effects, $\alpha = 0.05$), when data for all the parameters (DCW, PHB concentration, PHB content, $Y_{p/x}$, PHB productivity) and sampling time points (4, 8, 12, 20, 24, 26, 27, 28 h after changing the media) for second stage was included, different nutrient addition strategies had a significant effect ($P \leq 0.05$) on PHB production, as shown in Table 3.

Mixed analysis ($\alpha = 0.05$) conducted to see the differences among three nutrient addition strategies showed the same result for DCW and PHB content such that there was no significant difference ($P > 0.05$) between no addition and partial addition samples. However, they were both significantly higher than fermentations with complete addition. For PHB concentration and productivity, fermentations with all three nutrient addition strategies gave significantly different ($P \leq 0.05$) results. The highest value was obtained from partial nutrient addition followed by no nutrient addition. It is believed that complete nutrient addition may have had an inhibitory effect during fermentation due to excess nutrients.

Partial nutrient addition gave the overall best performance on PHB production among the three media types. For fermentation with partial nutrient addition, there was no significant difference in the values of various parameters (DCW, PHB concentration, PHB content, $Y_{p/x}$ and PHB productivity) among the samples taken at 26 h, 27 h and 28 h during second stage but a significant jump in PHB concentration was observed between 24 h and 26 h. Therefore, it can be inferred that the optimized two-stage fermentation strategy for using sugarbeet juice to produce PHB involves using partial nutrient addition media (*Med. 5*) with the second stage ending at 26 h. It is noteworthy that PHB content and productivity were comparable while DCW and PHB concentration obtained with fermentation of sugarbeet juice supplemented partially with nutrients were higher than those from the fermentation using synthetic media under optimized fermentation conditions (Wang et al., 2012).

Results of PHB production obtained from partial nutrient addition were comparable with other renewable feedstocks and even enhanced in spite of the smaller scale and shorter overall processing time investigated in this study. Compared to the research by Yezza et al. (2007), which used maple sap as media for PHB production in 100 mL shake flasks over 27 h incubation, higher PHB concentration (4.01 g/L vs. 3.41 g/L) and PHB yield coefficient based on sugar consumed (0.71 ± 0.28 vs. 0.34) were obtained. In a fermentation study on sweet sorghum juice media for PHB production in a bioreactor with 2 L working volume, Tanamool et al. (2009) obtained much lower PHB concentration of 0.68 g/L and DCW of 1.73 g/L after 54 h. Another research conducted by Aremu et al. (2010) using cassava starch for PHB production in 4.2 L working volume for 84 h also resulted in much lower PHB concentration (1.25 g/L) and DCW (1.75 g/L). Compared to studies using chemically defined sucrose based synthetic media, PHB concentration and content obtained from fermentation of sugarbeet juice media were observed to be comparable (Grothe et al., 1999; El-Sayed et al., 2009).

4.2. Properties of PHB obtained through fermentation of sugarbeet juice

The frequency spectrum of G' and G'' in Fig. 5a shows both moduli to be similar in magnitude, and relatively independent of frequency, particularly at low frequencies. Such behavior is analogous to that of weak-gel like materials (Bonino et al., 2011). Fig. 5b is consistent with this notion of a weak gel wherein the microstructure to break with increasing stress can be seen. Initially G' is slightly larger than G'', but with increasing stress there is structural breakdown and G' decreases rapidly and becomes lower than that of G''. The melting point and crystallinity from two DSC scans were lower than those from Yezza et al. (2007) whose work focused on exploring thermal properties of PHB produced from maple sap by *A. latus* and El-Hadi et al. (2002) whose work focused on investigating effect of melt processing on crystallization behavior and rheology of poly (3-hydroxybutyrate) (PHB) produced by

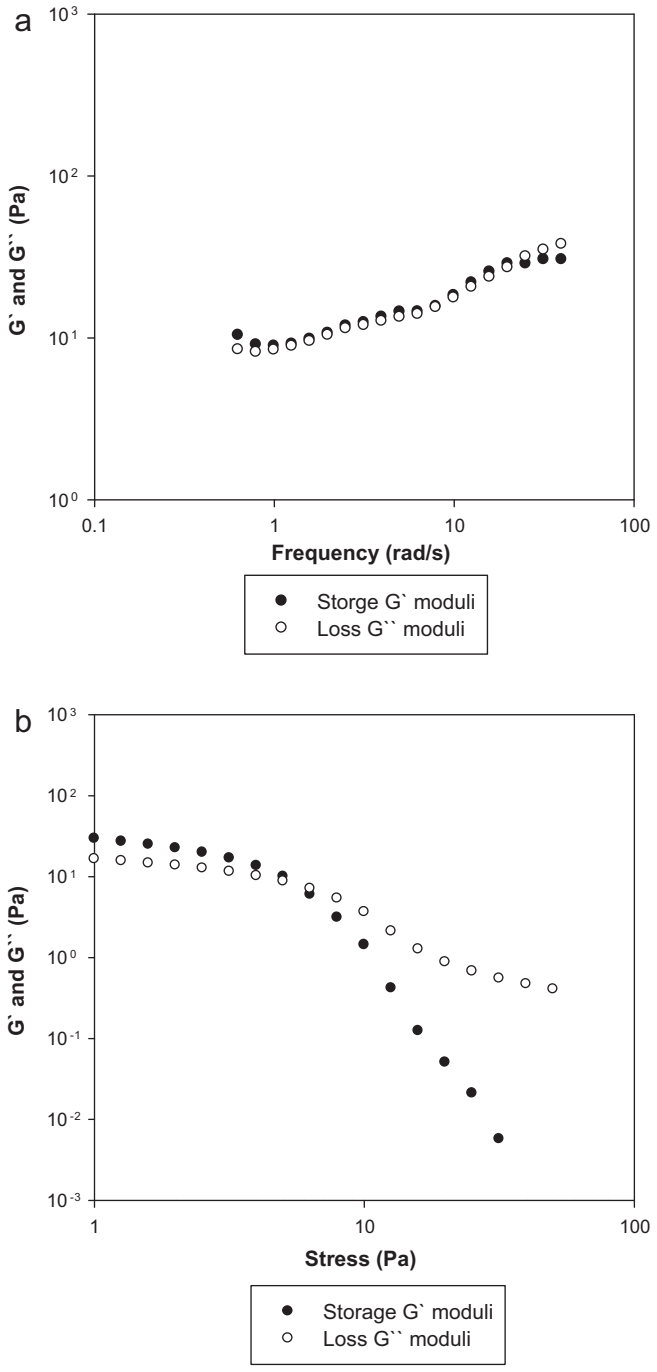

Fig. 5. Rheological analysis of PHB samples from fermentation of sugarbeet juice with partial nutrient addition. (a) Storage G' and loss G'' moduli measured during frequency sweep analysis at 180 °C; (b) storage G' and loss G'' moduli measured at different stresses (Pa) at 180 °C.

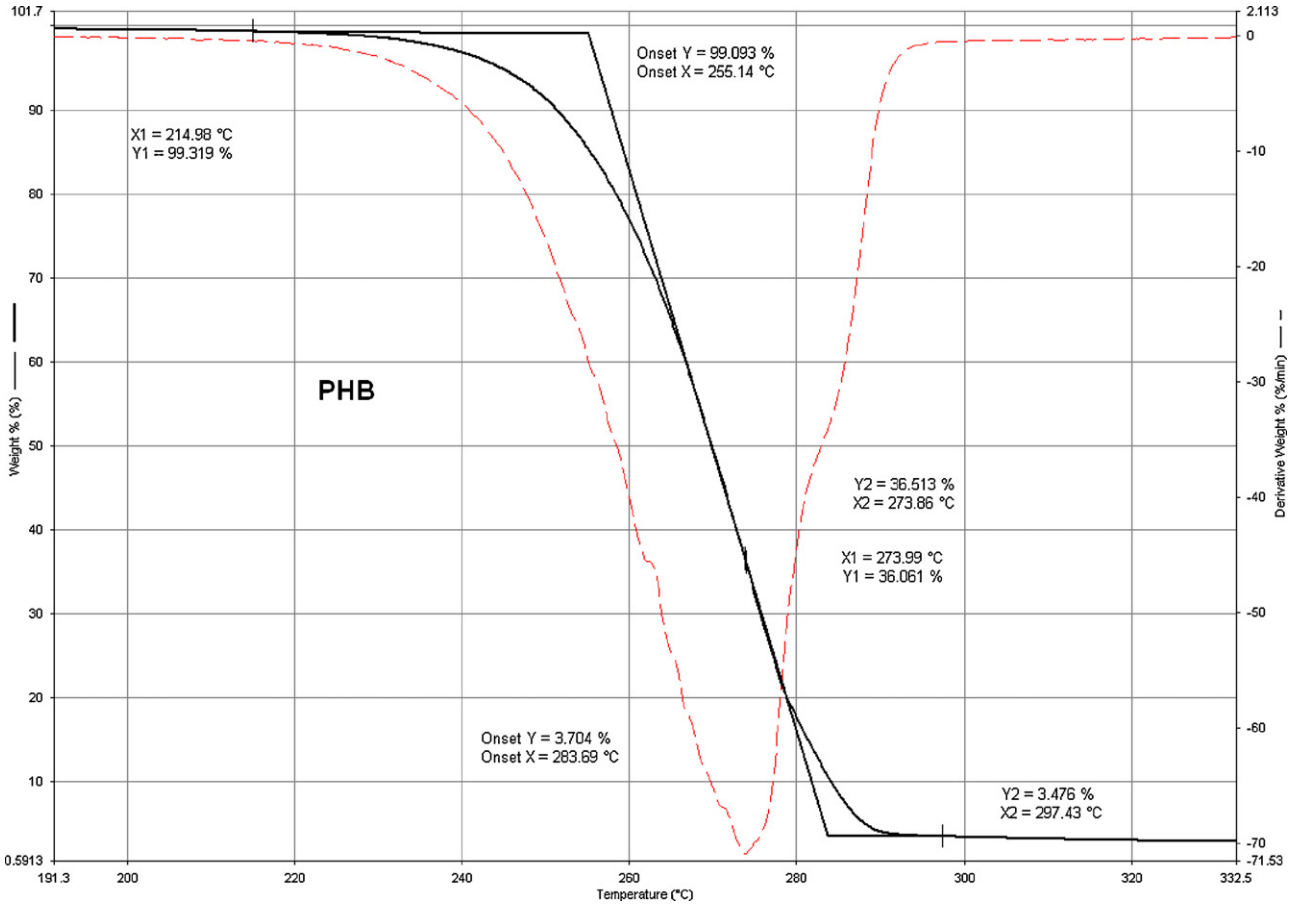

Fig. 6. Thermogravimetric analysis of PHB samples from fermentation of sugarbeet juice with partial nutrient addition.

the fermentation of molasses by *A. euthrophus*. The values obtained by them were 177.0 °C and 175.0 °C, respectively. The degradation temperature (273.86 °C) of PHB obtained from TG analysis was close to that (292 °C) obtained by El-Hadi et al. (2002).

5. Conclusions

The potential of sugarbeet juice based media for production of PHB by *A. latus* was highlighted in this study. Dilute sugarbeet juice, partially augmented with nutrients, is optimal for achieving high PHB productivity and concentration. Although promising at the small scale, scaled-up fermentation studies with better controlled conditions (mainly pH and dissolved oxygen) can provide further insight into the functional feasibility of PHB production from a media source that is sustainable and economically competitive. Though sugarbeet is a key feedstock for sugar production in the United States, with 55% of all sugar being produced from it (USDA, 2009), this research highlights the potential of this feedstock for conversion to another value added product, PHB. It is expected that results of this study can subsequently be applied to enhance the conversion of waste from sugar industry (like sugarbeet molasses and pulps) for PHB as well as copolymer (such as PHBV and P(3HB-4HB)) production, thus increasing market potential and cost effectiveness.

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