academicJournals

Vol. 13(40), pp. 4019-4027, 1 October, 2014
DOI: 10.5897/AJB2014.14035
Article Number: F4B852747963
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Sugar cane juice for polyhydroxyalkanoate (PHA) production by batch fermentation

Serna-Cock Liliana* and Parrado-Saboya Darly Silvana

Facultad de Ingeniería y Administración, Universidad Nacional de Colombia Sede Palmira, Colombia.

Received 9 July, 2014; Accepted 3 September, 2014

Clarified sugar cane juice was evaluated as an alternative substrate for the batch production of polyhydroxyalkanoates (PHAs) by *Alcaligenes latus*, and a mineral salt broth was used as the control. The study included the physicochemical characterization of the juice, measurement of the fermentation kinetic parameters and identification of the polymer type by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. Batch-type aerobic fermentations were performed (33°C at 200 rpm and pH 6.5-7 for 60 h) and set at 20 gL^{-1} of fermentable sugar and a carbon/nitrogen ratio of 28.3/1. A 10% v/v ratio of inoculum/substrate was used. The alternative substrate presented a greater concentration of magnesium and micronutrients such as Fe, B, Zn, Mg and Cu compared to the control. The biomass yield ($Y_{P/X}$) of the juice was 1.27 $g.g^{-1}$ (0.414 $g.g^{-1}$ in control medium), which was 69% more than the control medium; the product-substrate yield ($Y_{P/S}$) was 0.10 $g.g^{-1}$, which was similar to the control medium (0.15 $g.g^{-1}$ control medium). The production of PHB was of 1.3 gL^{-1}, less than half of the concentration obtained in commercial substrate. Infrared spectroscopy indicated that the polymer obtained in the evaluated substrates was polyhydroxybutyrate (PHB). The clarified juice of sugar cane, without the addition of nutritional supplements can be used for the production of biomass of *A. latus*, first step in the production of PHB.

Key words: Alternative substrate, *Alcaligenes latus,* polymer, polyhydroxybutyrate (PBH), fermentation.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are lipidic materials that are accumulated by a great variety of microorganisms in the presence of an excess of carbon. The assimilated carbon sources are biochemically transformed into units of hydroxyalkanoates, which are polymerized and stored in the form of insoluble inclusions in the cell cytoplasm (Brito et al., 2011). PHA is a natural polyester that is biocompatible and 100% biodegradable under aerobic

*Corresponding author. E-mail: lserna@unal.edu.co. Tel: 2868888 ext. 35720.

Abbreviations: PHA, Polyhydroxyalkanoate; **PHB,** polyhydroxybutyrate; $Y_{P/X}$:, biomass yield; $Y_{P/S}$, substrate yield; $Y_{X/S}$, biomass-substrate yield; **ATR-FTIR,** attenuated total reflectance Fourier transform infrared spectroscopy; **%w/v,** weight/volume percentage; **Ppm,** parts per million; **OD,** optical density; **DCW,** dry cell weight; **FS,** final concentration of fermentable sugars.

and anaerobic conditions (Verlinden et al., 2011), and it has characteristics similar to polyethylene and polypropylene, which is why it has been considered as a substitute of conventional plastics.

PHAs are produced by at least 75 different bacterial genera including Gram-positive and Gram-negative bacteria, such as *Cupriavidus necator (Ralstonia eutropha), Alcaligenes latus, Bacillus megaterium, Klebsiella aerogenes, Pseudomonas putida, Pseudomonas oleovorans,* and *Sphaerotilus natans.* When these polymers are intracellularly stored under stress conditions, such as phosphorous and nitrogen stress, limited oxygen, and excess carbon, they can represent up to 80% of the dry weight of the biomass obtained in the fermentation (Gonzalez et al., 2013). Depending on the carbonate substrate and metabolism of the microorganism, different monomers, polymers and co-polymers can be obtained (Gonzalez et al., 2013).

To date, a satisfactory PHA production process has not been achieved. One of the major problems faced by the industrial-scale production of this polymer is the high cost of production, especially the high prices of raw materials (for example, carbon and energy sources for growth of microorganisms), which make them uncompetitive compared to petrochemical (Chen, 2009). In addition, the selection of an adequate carbon source is an important criterion because it determines not only the PHA content but also the polymeric composition, which affects the final properties of the polymer. Fifty percent of the final cost of the materials corresponds to the carbon source (Brigham et al., 2011).

According to Ntaikou et al. (2009), cited by Sharifzadeh et al. (2009), an economy source for the fermentation includes a culture medium that contains sugar cane honey, corn steep liquor and effluents of palm oil and olive oil. Cane molasses contains vitamins and minerals and is a source of calcium, magnesium, potassium and iron, which are considered as impurities in no refined sugar (Akaraonye et al., 2010), therefore, this raw material can be used for the production of PHAs. Albuquerque et al. (2007) obtained 30% of P (3HB-co-3HV) and 3.5 gL^{-1} of biomass concentration, from a mixed bacterial culture using sugar cane molasses. Chaijamrus and Udpuay (2008), obtained 43% w/w of *B. megaterium*, (after 45 h of growth), when 4% of molasses was used. Gouda et al. (2001), cited by Bello et al. (2009) found that the greater accumulation of PHB with respect to cell dry weight, was obtained with substrate supplemented with molasses 0.5% (w/v). Similar results were obtained by Waranya et al. (2011), which evaluated the juice of sugar cane to produce PHB with *A. eutrophus.* Oehmen et al. (2014) evaluated the effect of pH control on the volumetric productivity of PHA, using molasses, and a mixture of PHA-producing microorganisms. They found that controlling the pH of the fermentation to 8, the volumetric productivity is increased.

A. latus (*Azohydromonas australica*) is a microorganism producer of intracellular PHB (Wang et al., 2012) and is known for its ability to use sucrose as a carbon source (Yezza et al., 2007; Zafar et al., 2012); therefore, the goal of this work was to evaluate sugar cane juice as an alternative substrate for the production of PHA by *A. latus* in batch fermentation.

MATERIALS AND METHODS

Microorganism and maintenance

A. latus obtained from the American Type Culture Collection was used as the lyophilized culture. The strain was reconstituted and preserved in vials with a mineral salt broth at -20°C. The work was performed by keeping the culture in a slant with the mineral salt medium at 4°C (Grothe and Chisti, 2000).

Fermentation substrates

Two fermentation substrates were used: sugar cane juice and mineral salt medium. The cane juice was selected as a substrate because it provides a good source of carbon, vitamins and minerals, which are required for the growth of *A. latus*, and can be used as a low-cost substrate for the growth of bacteria and production of PHA.

The sugar cane juice was obtained in a Colombian sugar mill at an industrial scale, and sugar cane variety CC8592 was used. The cane was weighed (Fletcher, USA), and to facilitate the juice extraction and improve its efficiency, the cane was shredded in a shredding machine (Dedini, Brazil) and milled in four-roller mills (Fulton mill, USA).

The juice extracted in the mills was weighed (Fletcher, USA) and then neutralized with lime (pH 6.5 +/- 0.5) to help in the separation of the solids. The juice was heated to 105°C in order to accelerate the separation of the non-sugar solids, and the clarified and sediment-free juice was used.

The cane juice was characterized based on the content of macronutrients (nitrogen, sodium, potassium and magnesium) and micronutrients (iron, boron, zinc, manganese, and copper). The clarified juice samples were subjected to acid digestion (500 ml of HNO_3 and 250 ml of $HClO_4$ solution in a 2:1 proportion) and then stirred with a stir plate (Dari, Cali Colombia) at 360°C for 28 min. The sample was filtered in Teflon filters and diluted to approximately 50 ml with distilled water. The macronutrients were determined by atomic absorption (AOAC 985.35) in an atomic absorption spectrophotometer (Perkin Elmer, model 2380, USA); the phosphorous content was analyzed by visible-light colorimetry (AOAC 995.11). The results were expressed in weight/volume percentages (%w/v) for macronutrients and in parts per million (ppm) for micronutrients.

To formulate the substrate, the cane juice was adjusted to 20 ($g.l^{-1}$) of fermentable sugar, and ammonium sulfate was added as a nitrogen source to maintain a carbon-to-nitrogen ratio of 28.3/1 (C:N 28.3/1), which is the commercial substrate ratio. The substrate was adjusted to pH 6.5 +/- 0.5 with 2N NaOH/2N HCl.

The mineral salt substrate was used as a commercial substrate (control substrate), which is composed of (in $g.l^{-1}$) sucrose 20; $(NH4)_2SO_4$ 1.4; KH_2PO_4 1.5; $Na_2HPO_4.12H_2O$ 3.6; and $MgSO_4.7H_2O$ 0.2. In addition, 1 ml/lof trace element solution (TES) was included, and the TES is composed of (in $g.l^{-1}$) ammonium ferric citrate 60; $CaCl_2.2H_2O$ 10; H_3BO_3 0.3; $CoCl_2.6H_2O$ 0.2; $ZnSO_4.7\ H_2O$ 0.1; $MnCl_2.4\ H_2O$ 0.03; $Na_2MoO_4.2H_2O$ 0.03; $NiSO_4.7H_2O$ 0.02 and $CuSO_4.5H_2O$ 0.01 (Grothe and Chisti, 2000). The pH of the medium was adjusted to 6.5 +/- 0.5 with 2 N NaOH/2 N HCl.

Inoculum preparation

For the inoculum preparation, 800 ml of mineral salt broth was prepared in a 2000 ml Erlenmeyer flask. The broth was inoculated under sterile conditions with four slants of *A. latus* (from the described workbench). The Erlenmeyer flask was incubated under aerobic conditions in a rotary shaker (Innova 44, USA) at a temperature of 33°C and rpm of 200 for 48 h (Grothe and Chisti, 2000). The broth was then used as inoculum for the fermentations of the sugar cane juice and mineral salt medium.

Fermentation

Six batch fermentations were performed in 2000 ml Erlenmeyer flasks, which had an effective working volume of 1000 ml (three fermentations per substrate). The Erlenmeyer flasks remained stirring at 200 rpm, without aeration, in the rotary shaker for 60 h at 33°C. The pH of the substrate was measured and adjusted every two hours, to 6.5-7.0 using 2 N NaOH. The procedure was performed in laminar flow cabin, in order to ensure sterility. The substrate fermentation were inoculated with the *A. latus* inoculum (described above), maintaining a 10% v/v ratio with respect to the substrate volume. Dissolved oxygen was not controlled during fermentation. The flasks were opened every 2 h for pH control, therefore, the headspace oxygen was renewed every 2 h.

For each substrate, at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60 h of fermentation, 30 ml of fermented media was aseptically collected in the laminar flow to measure the fermentation kinetic parameters (time 0 corresponded to the initial conditions of each substrate) and by Gram stain purity monitoring fermentation every 6 h.

Determination of kinetic parameters

The following parameters were determined: biomass concentration (X), substrate consumption (carbon source), nitrogen consumption, biomass-substrate yield ($Y_{X/S}$), product-substrate yield ($Y_{P/S}$), product-biomass yield ($Y_{P/X}$) and PHB productivity. The biomass concentration was determined by a previously constructed optical density (OD) vs. dry biomass weight (DCW) calibration curve which replaced the optical density value in the equation of the described curve.

To construct the calibration curve, an *A. latus* culture was created; 400 ml of mineral salt broth was inoculated with two slants of *A. latus* and incubated at 33°C (Binder, USA) for 48 h at 200 rpm in a rotary shaker (Grothe and Chisti, 2000). After finalization of the growth time, 90 ml of the cell suspension was distributed in nine glass test tubes with known initial weight. The tests tubes with 10 ml of cell suspension were placed in an oven (Binder, USA) at 105°C until reaching a constant weight and then cooled in a vacuum desiccator for one hour. Subsequently, they were weighed in an analytical balance (Mettler Toledo xs-204, USA). The values obtained from the nine tubes were averaged, and the dry biomass weight concentration in the initial culture (g/10 ml) was obtained.

With the remaining culture, dilutions in sterile distilled water were prepared (1/5, 1/6, 1/7, 1/9, 1/15, 1/20 and 1/30), and the absorbance of each dilution was measured at 600 nm (Spectronic Genesys 2PC, USA) using distilled water as the blank. The calibration curve was constructed with these results, and the absorbance and grams of dry biomass per liter ($g.l^{-1}$) were correlated (Grothe et al., 1999; Patwardhan and Srivastava, 2004).

The substrate consumption (carbon source) was calculated with Equation 1, using the concentration data of fermentable sugars collected at each of the times mentioned above. The concentration of sugars was measured by high-performance liquid chromatography (HPLC - Shimadzu, USA; LC-10ADvp pump, RID-10A refraction index detector, SCL-10Avp controller, SILC-10AFautosampler, Zstar software,and a Hi-PlexCa column at 300 × 7.7 mm coupled to a YOYO pre-column with WAT015209 insert (Sugar-Pak) USA). The operation conditions were as follows: 75°C column temperature; deionized reverse osmosis water (deionizer, Simplicity, USA) mobile phase; 0.6 ml/min mobile phase flow; and 10 µl injection volume of samples and standards. The samples were filtered (EMD Millipore Durapore PVDF filters, 0.22 µM pore, 47 mm diameter), and the filtrate was injected into a HPLC system. The calibration standards with sucrose concentrations of 0.09, 0.1, and 0.11 $g.l^{-1}$ and glucose and fructose concentrations of 0.009, 0.01 and 0.011 gL^{-1} were prepared in the HPLC-grade water.

The nitrogen consumption as the free amino nitrogen (FAN) (in ppm) was calculated using equation 2. The concentration of ammonia was determined semi-quantitatively by the ammonium (NH_4^+) test by Merck, which is a colorimetric method where the ammonium ions form a yellow-brown compound with the NeBler reactant. The reaction region of the test strip was compared with the regions of a calorimetric scale (10-30-60-100-200-400 mg/l of NH_4^+). The values for $Y_{X/S}$, $Y_{P/S}$ and $Y_{X/P}$ were determined by Equations 3, 4 and 5, respectively.

$$Substrate\ consumption = \frac{\mathrm{FS}}{\mathrm{FS_0}} * 100\% \quad (1)$$

Where FS is the final concentration of fermentable sugars ($g.l^{-1}$) and FS_0 is the initial concentration of fermentable sugars ($g.l^{-1}$) (Serna et al., 2010).

$$FAN\left(\frac{mg}{l}\right) = \frac{ml NaOH\ 0.1\ N\ required\ in\ the\ titration * 1.4 * 1000}{100\ ml\ of\ collected\ sample} \quad (2)$$

The basic ratio used for the calculations is 1 ml of NaOH 0.1 N= 1.4 mg of nitrogen.

$$Y_{X/S} = \frac{X - X_0}{So - S} = g.g^{-1} \quad (3)$$

$$Y_{P/S} = \frac{P}{\mathrm{So - S}} = g.g^{-1} \quad (4)$$

$$Y_{P/X} = \frac{P}{Xo - X} = g.g^{-1} \quad (5)$$

Where, S is the concentration of fermentable sugars ($g.l^{-1}$) at each fermentation time; X is the final concentration of biomass (gL^{-1}); X_0 is the initial concentration of biomass (gL^{-1}); S_0 is the initial concentration of fermentable sugars ($g.l^{-1}$); S is the final concentration of fermentable sugars (gL^{-1}); and P is the final concentration of PHA (gL^{-1}). The volumetric productivity was calculated as the grams of PHB produced per liter per hour of culture time ($gL^{-1}h^{-1}$) (Wang and Lee, 1997).

Extraction and quantification of the PHB

The extraction and quantification of PHB was performed by a modification of the technique proposed by Kim et al. (1994) as follows: a mixture of 12.5 ml of 30% sodium hypochlorite (v/v) and 12.5 ml of chloroform was prepared; this solution was then mixed with 1 g of wet biomass and centrifuged in 50 ml Eppendorf tubes and then placed in a bain-marie at 30°C for 90 min and centrifuged for 15 min at 4000 rpm. Three phases were formed: hypochlorite in the upper phase, cell debris in the middle phase, and PHB with chloroform in the bottom phase. The PHB was extracted with a pipette. For the separation of the chloroform and PHB, the PHB was precipitated using 7:3 methanol and water (v/v) and then filtered (0.45 µ). The mixture was placed in an exhaust hood for 48 h to volatilize the methanol excess. Finally, the PHB pellet was

Table 1. Physicochemical characterization of the sugar cane juice (pure).

Nutrient	Parameter	Value
General chemical and nutritional properties	°Brix (%)	14.94
	Purity (%)	86.4
	Fermentable sugar (%)	13.54
	Volatile Acidity (ppm)	306
	Lactic Acidity (ppm)	100
	pH	6.78
	Free Amino nitrogen (FAN) (ppm)	45
Macronutrients	N- Total%	0.033
	P_2O_5%	0.023
	CaO%	0.41
	MgO%	0.38
	K_2O%	0.37
	Na_2O%	0.07
	S%	0.15
Micronutrients	B (ppm)	14.72
	Cu (ppm)	5.39
	Fe (ppm)	105.5
	Mn (ppm)	16.86
	Zn (ppm)	12.36

weighed (Mettler Toledo xs-204, USA), and the biomass yield was calculated by Equation 6.

$$\%\ of\ accumulation = \frac{Final\ polymer}{Final\ dry\ biomass} * 100 \quad (6)$$

Analysis of biopolymers using ATR-FTIR spectroscopy

The biopolymer samples obtained after fermentation in the evaluated substrates, clarified juice and mineral salt commercial medium were analyzed using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy.

A small amount of each of the biopolymer samples was collected for the ATR-FTIR analysis and directly placed on the germanium crystal of the sample carrier (*Thermo Scientific iS10*, USA). The ATR-FTIR spectra were collected in a spectrophotometer (*Thermo Scientific iS10*, USA), and the results of the functional group analysis was compared with the functional groups of the polyhydroxybutyrate (PHB) standard reported in the literature.

Experimental design

A completely random unifactorial design was used, and the measurements were repeated 11 times by triplicate. The unifactorial design was composed of the type of substrate with two levels: (1) sugar cane juice and (2) commercial substrate.

The response variables were measured at times of 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60 h of fermentation (time 0 corresponded to the initial fermentations value). The response variables were biomass concentration (X), carbon source consumption (FS), and nitrogen source consumption. The determined parameters were PHB productivity, biomass yield($Y_{X/S}$), product yield ($Y_{P/S}$), product-biomass yield, and volumetric productivity, which were calculated after 60 h of fermentation.

The results were analyzed by a variance analysis using the mixed procedure of the SAS (Statistical Analysis System) statistical package, version 9.3. To test the hypothesis of the differences between treatments, the treatment time and treatment type according to the interaction period, structure of the compound symmetry covariance and first-order autoregressive covariance were used based on the characteristics of the repeated measurements in the experiment. In addition, the averages of the treatments, treatment time and treatment type according to the interaction period were compared by a least squares analysis using the LSMEANS statement used in the previous procedure.

RESULTS AND DISCUSSION

Characterization of the sugar cane juice

Table 1 presents the characterization of the sugar cane juice before its use in the formulation of the fermentation substrate. As observed in Table 1, the sugar cane juice presents an adequate content of essential macronutrients and micronutrients, which can influence the growth of bacteria. When comparing these values with those of the commercial substrate, the alternative substrate presents a greater concentration of all micronutrients and of the Mg macronutrient. Mg is necessary for the proper functioning of most metabolism enzymes and is an activator of glycolytic enzymes, stimulator of fatty acid synthesis, and participant with K in slowing phosphate penetration and it is involved in the structure of ribosomes, cellular membranes and nucleic acids (Bouix, 2000). Therefore, because it has a greater amount of magnesium, the clarified juice provides a metabolic advantage to the microorganisms that are cultured in this substrate. In the studies performed by Gahlawat and Srivastava (2012), the substrate nutrients were optimized to increase the production of PHB by *A. latus*. In that research, the Plackett - Burman protocol and surface response methodology were evaluated, and the Mg, P and micronutrients were found to have an important effect on the production of biomass and PHB. This result can be explained by the important role that mineral nutrients (Mg, K and P) play in sustaining the buffer capacity of the culture medium, which is required for bacterial growth and PHB production. In addition, the sugar cane juice is an economical and available carbon source for use in the scaling of the PHA production that decreases the production costs of bioplastics and provides an alternative use of this raw material derived from the production of sugar.

Fermentation kinetic parameters

Figures 1, 2 and 3 show the kinetics of the biomass production, substrate consumption (carbon source) and

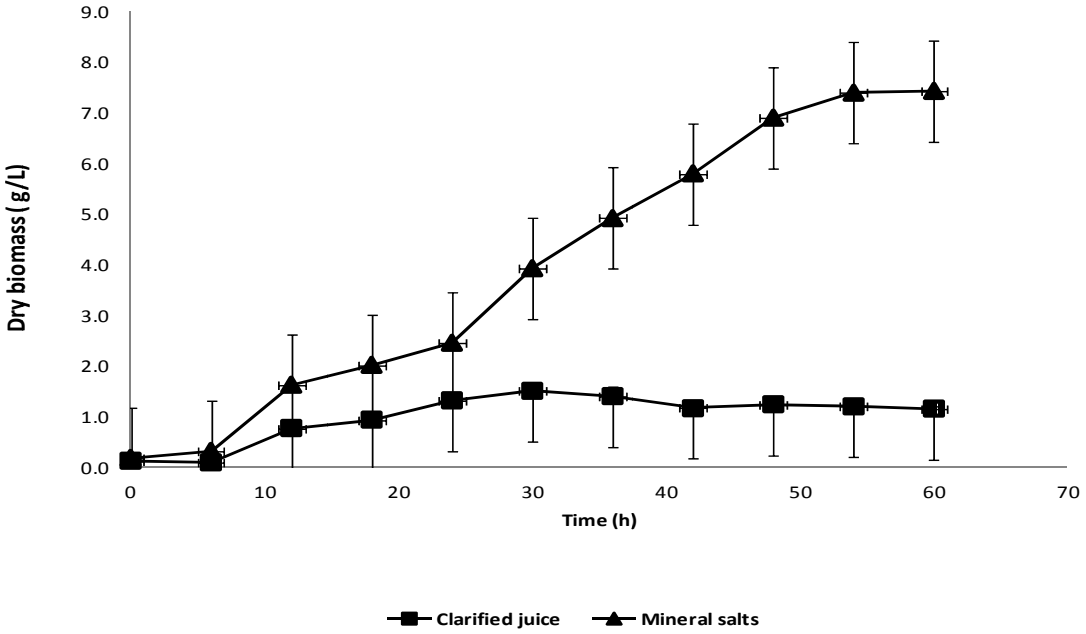

Figure 1. Biomass concentration of *Alcaligenes latus* obtained in mineral salt broth (commercial substrate) and clarified sugar cane juice.

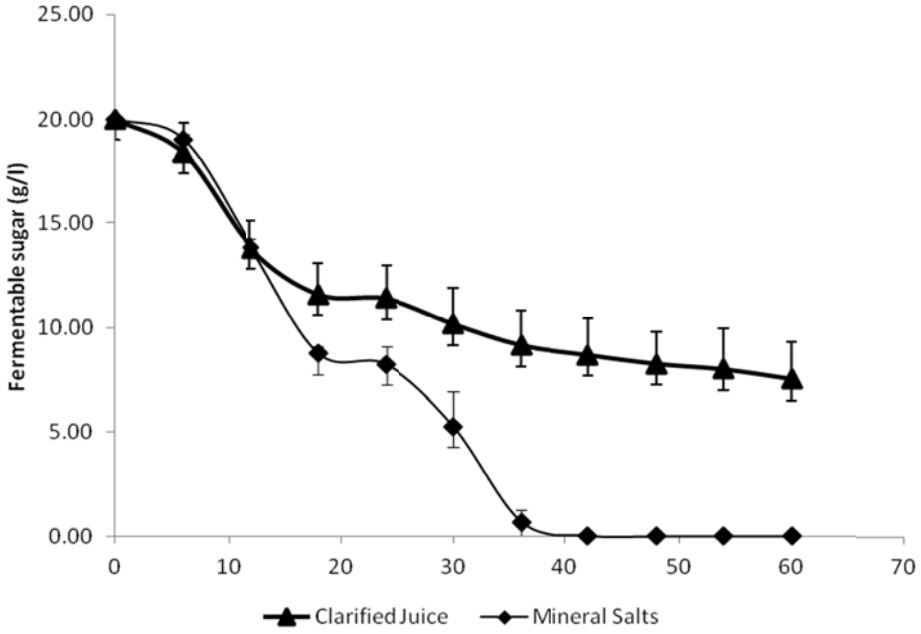

Figure 2. Substrate consumption (carbon source) of *Alcaligenes latus* in mineral salt substrate (commercial substrate) and in sugar cane juice during batch fermentation.

nitrogen source consumption, which were obtained in batch fermentations of the sugar cane juice substrate and mineral salt commercial substrate. The greatest biomass production occurred at 30 h of fermentation for the sugar cane juice (1.50 gL^{-1}) and 60 h of fermentation for the mineral salt substrate (7.42 gL^{-1}) (Figure 1). This result was obtained because the mineral salt medium presented an adequate nutritional balance (essential nutrients) for the reproduction and accumulation of PHA by the microorganism. In the clarified juice substrate was

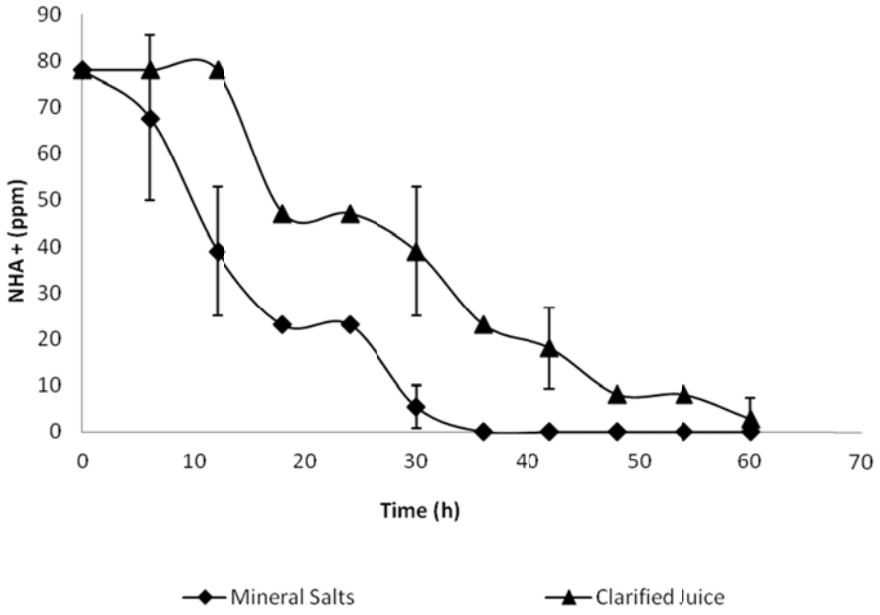

Figure 3. Consumption of the nitrogen source (ammonium sulfate) by *Alcaligenes latus* in the mineral salt substrate (commercial substrate) and sugar cane juice during batch fermentation.

not added any type of nutritional supplementation. In the work performed by Waranya et al. (2011) in which sugar cane juice was evaluated as a substrate for the production of PHA using *A. latus* bacteria, the greatest biomass production occurred at 36 h, which was relatively low. In addition, this result was corroborated by the preference of the bacteria in consuming glucose instead of sucrose as the main carbon source. The statistical analysis demonstrated that there was a significant difference (P< 0.1) in favor of the mineral salt substrate.

Similarly, the greatest substrate consumption (fermentable sugar) occurred in the commercial substrate, which was 100% at 48 h of fermentation. In the sugar cane juice broth, 62.45% of the substrate was consumed at 60 h of fermentation, and this result can be associated with the high temperatures to which the alternate substrate is subjected; these temperatures generate a browning that indicates a thermal decomposition of the sucrose, glucose, and fructose content and reaction of these hydrocarbons with amino-nitrogenated compounds to produce colored polymers, such as melanoidins, and furfurals, such as 5-(hydroxymethyl)-2-furaldehyde,which indicate the growth of bacteria and possible accumulation of PHA (Waranya et al., 2011; Rein, 2012). The greatest nitrogen consumption occurred in the commercial substrate, which was 100% at 36 h of fermentation, and sugar cane juice broth, which showed 96% nitrogen consumption at 60 h. The statistical analysis showed a significant difference from hour 12 to 36 h of fermentation (P< 0.1) in favor of the mineral salt substrate medium. Grothe et al. (1999), found that the standards of sucrose and nitrogen consumption were consistent with the standards of lag-exponential-stationary growth of biomass; in addition, different nitrogen sources, such as urea and ammonium sulfate, were evaluated, and they confirmed that *A. latus* presents a higher affinity towards ammonium sulfate. The rapid depletion of ammonium sulfate in the commercial substrate is primarily a result of the balance of essential macronutrients and micronutrients for bacterial reproduction in the medium.

Table 2 shows the performance kinetic parameters obtained in the evaluated substrates. The polymer accumulation obtained in the commercial substrate was 14% greater than that reported by Grothe and Chisti (2000), who evaluated PHA production in the mineral salt medium with *A. latus* and obtained a PHA accumulation of 63% in 93 h. Yezza et al. (2007) showed that *A. latus* accumulated up to 77% (weight-weight) of PHB in an evaluation of maple sap as an economic carbon source, which contains 10 - 30 $g.l^{-1}$ of sucrose under nitrogen limitation. The kinetic parameters and yields obtained in the sugar cane juice alternative substrate were superior to the results obtained in the work performed by Waranya et al. (2011) except for the $Y_{X/S}$ value and productivity, for which different sugar concentrations were evaluated for the production of PHA in batch fermentation using sugar cane juice as a substrate ($Y_{X/S}$=0.163 $g.g^{-1}$, $Y_{P/S}$=0.05 $g.g^{-1}$, $Y_{P/X}$=0.306 $g.g^{-1}$, and productivity= 0.031 $gL^{-1}.h^{-1}$).The greatest biomass product yield was obtained in the

Table 2. Kinetic parameters obtained in batch fermentations using the following substrates: mineral salt broth (commercial substrate) and substrate formulated with clarified sugar cane juice.

Parameter	Commercial substrate	Clarified juice substrate
$Y_{X/S}$ ($g.g^{-1}$)	0.362	0.082
$Y_{P/X}$ ($g.g^{-1}$)	0.414	1.27
$Y_{P/S}$ ($g.g^{-1}$)	0.15	0.104
Productivity ($g.l^{-1}.h^{-1}$)	0.05	0.022
Polymer production ($g.l^{-1}$)	3.0	1.3
% of polymer accumulation	74	63

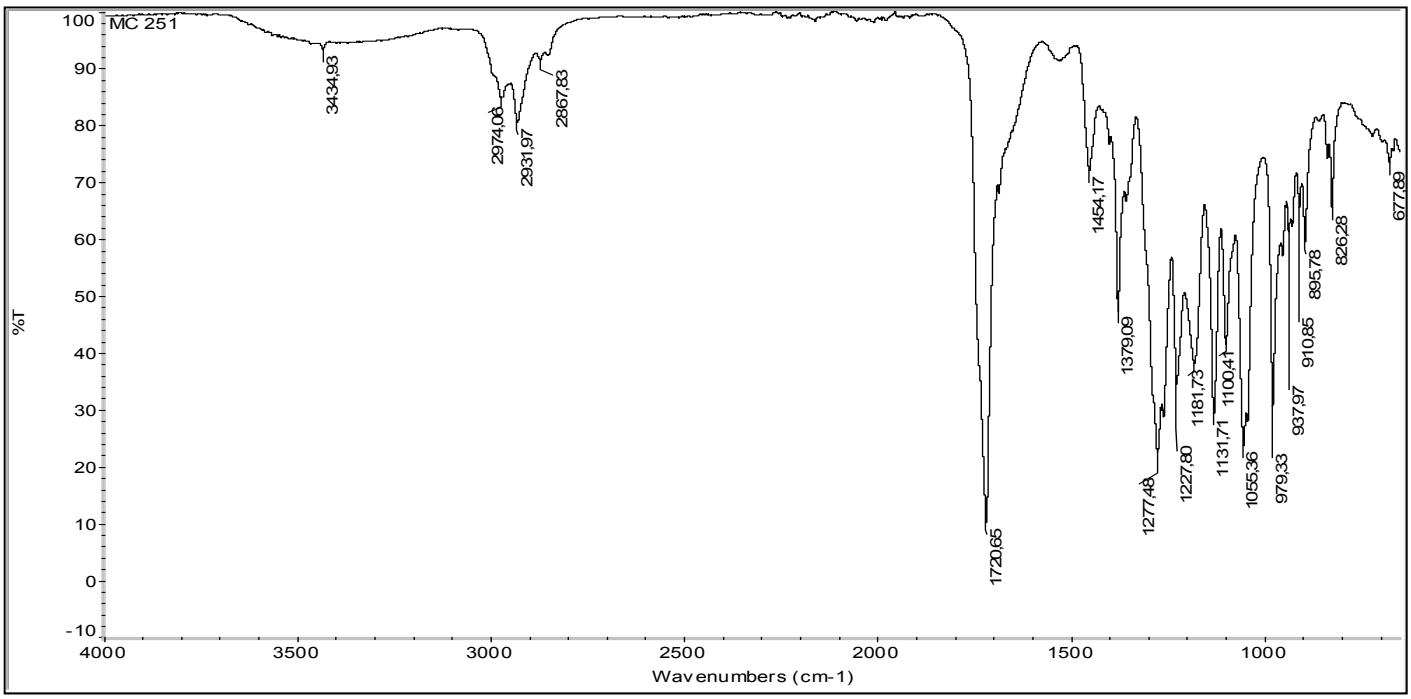

Figure 4. Infrared spectroscopy of the polymer produced by *A. latus* in the substrate formulated with clarified sugar cane juice.

sugar cane juice substrate, which was 69% greater than that obtained for the commercial substrate. The sugar cane juice substrate presented a greater PHB accumulation, greater $Y_{P/S}$, and greater volumetric productivity than in the studies of (Kumalaningsih et al., 2011), who used soybean curd waste as the alternative substrate with *A. latus* and obtained PHB of 0.68 gL^{-1}, $Y_{P/S}$v of 0.39 $g.g^{-1}$ and 0.0125 $gL^{-1}h^{-1}$. This result may have been primarily because cane juice is rich in amino acids such as aspartic (0.11%), glutamic (0.05%), alanine (0.06%), valine (0.03%), gamma-aminobutyric (0.03%), threonine (0.02%), isoleucine (0.01%), and glycine (0.01%) and group B vitamins (Chen, 1991). These amino acids are normally used as growth factors for microorganisms, and although they are required in small amounts, they may be vital for the optimal growth and performance of the microorganisms (Madigan et al., 2000).

Identification of the polymer produced by *A. latus*

By means of infrared spectroscopy (Figure 4), the polymer produced by *A. latus*in both the commercial substrate and clarified sugar cane juice-formulated substrate was a polyester-type polymer. This polymer was identified in the presence of the carbonyl group band (1720 cm^{-1}), aliphatic chain methyl bands (2995 to 2975 cm^{-1}), aliphatic chain methylene bands (2865 to 2855 cm^{-1}), and ester C-O stretching band (1279 cm^{-1}) (Table 3). The obtained results coincide with those reported by

Table 3. Signals for the PHB sample obtained in clarified juice.

Signal (cm^{-1})	Assignment
2995-2975 range	C-H methyl asymmetric stretch
2933	C-H methylene stretch
2865-2855 range	C-H methyl symmetric stretch
1720	C=O carbonyl stretch
1378	Methyl symmetric bend
1279	C-O ester asymmetric stretch
1228	C-O ester symmetric stretch

Hong et al. (1999) and Bayari and Severcan (2005) and were cited by Liuet al. (2011), who revealed the presence of intense absorption bands at 1724 and 1281 cm^{-1} that correspond to the ester carbonyl (C=O) group and the –CH group, respectively.

The identification performed by FT-IR suggests a 90% agreement with the spectrum reported in the scientific literature for poly(3-hydroxybutyrate). This consistency occurs when the position and intensity of the signals presented in the FT-IR spectra are observed, and these results are consistent with those reported by Kansiz et al. (2000).

Conclusions

The present study shows that *A. latus* can grow and produce polyhydroxyalkanoate in a clarified sugar cane juice substrate at a concentration of 20 gL^{-1} of fermentable sugars and C:N ratio of 28.3:1; in addition, the biomass yield was superior to those obtained with the commercial substrate. Therefore the clarified juice of sugar cane, without the addition of nutritional supplements can be used for the production of biomass of *A. latus*, first step in the production of PHB. The results of the physicochemical analyses showed that the composition in macronutrients and micronutrients of the clarified sugar cane juice is comparable with the composition of the mineral salt broth. The infrared spectroscopy results showed that the polymer obtained from the fermentation of the clarified sugar cane juice was of a PHB type. The sugarcane juice is a commercial alternative for the PHB bacteria reproduction, and for the production of PHB, however, the process must be optimized. Additionally, the use of sugar cane juice is an option for product diversification in the sugar mills.

Conflict of Interests

The author(s) have not declared any conflict of interest.

REFERENCES

Akaraonye E, Keshavarz T, Roy I (2010). Production of polyhydroxyalkanoates: The future green materials of choice. J. Chem. Technol. Biotechnol. 85(6):732-743.

Albuquerque MG, Eiroa M, Torres C, Nunes BR, Reis MA (2007). Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. J. Biotechnol. 130(4):411-421.

Bayari S, Severcan F (2005). FTIR study of biodegradable biopolymers: P(3HB), P(3HB-co-4HB) and P(3HB-co-3HV). J. Mol. Struct. 744-747(3):529-534.

Bello D, Otero MA, Ortega G, Carrera, E (2009). State of the art in the microbiological production of polyhydroxyalkanoates. Redalyc. 43(2):3-13.

Brigham CJ, Kurosawa K, Rha C, Sinskey AJ (2011). Bacterial carbon storage to added products. Microb. Biochem. Technol. 3: 1-13.

Brito G, Agrawal P, Araujo E, Mélo T (2011). Biopolimeros, polimeros biodegradáveis e polímeros verdes. Revista Eletrônica de Materiais e Processos 6: 127-139.

Chaijamrus S, Udpuay (2008). Production and characterization of polyhydroxybutyrate from molasses and corn steep liquor produced by *Bacillus megaterium* ATCC 6748. Agric. Eng. Int. 10: 1-12.

Chen GQ (2009). A microbial Polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem. Soc. Rev. 38(8): 2434-2446.

Chen, JC (1991). Sugar Cane Manual. Editorial Limusa. 1-1200.

Gahlawat G, Srivastava AK (2012). Estimation of fundamental kinetic parameters of polyhydroxybutyrate fermentation process of *Azohydromonas australica* using statistical approach of media optimization. Appl. Biochem. Biotechnol. 68(5): 1051-1064.

González Y, Cordová J, González O, Meza J (2013). Synthesis and biodegradation of polyhydroxyalkanoates: bacterially produced plastics. Rev. Int. de Cont. Amb. 29(1): 77-115.

Gouda, MK, Swellam AE, Omar SH (2001). Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiol. Res. 156(3): 201-207.

Grothe E, Chisti Y (2000). Poly(β-hydroxybutyric acid) thermoplastic production by *Alcaligenes latus*: Behavior of fed-batch cultures. Bioprocess Biosyst. Eng. 22: 441-449.

Grothe E, Murray MY, Chisti Y (1999). Fermentation optimization for the production of poly(β-hydroxybutyric acid) microbial thermoplastic. Enzyme Microb. Technol. 25: 132-141.

Hong K, Sun S, Tian W, Chen GQ, Huang W (1999). A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. Appl. Microbiol. Biotechnol. 51(4):523-526.

Kansiz M, Billman JH, McNaughton D (2000). Quantitative determination of the biodegradable polymer poly(beta-hydroxybutyrate) in a recombinant *Escherichia coli* strain by use of mid-infrared spectroscopy and multivariative statistics. Appl. Environ. Microbiol. 66(8):3415-3420.

Kim BS, Lee SC, Lee SY, Chang HN, Chang YK, Woo SI (1994). Production of poly(3-hydroxybutyric-co-3-hydroxyvaleric acid) by fed-batch culture of *Alcaligenes eutrophus* with substrate control using on-line glucose analyzer. Enzyme Microb.Technol. 16(7): 556-561.

Kumalaningsih S, Hidayat N, Aini N (2011). Optimization of polyhydroxyalkanoates (PHA) production from liquid bean curd waste by *Alcaligenes latus* bacteria. J. Agric. Food Technol. 1(5): 63-67.

Liu Y, Huang S, Zhang Y, Xu F (2014). Isolation and characterization of a thermophilic *Bacillus shackletonii* K5 from a biotrickling filter for the production of polyhydroxybutyrate. J. Environ. Sci. 26(7):1453-1462.

Madigan MT, Martinko J, Parker J (2000). Biology of Microorganisms. Pearson Education. 10: 1-1096.

C, Koutrouli EC, Stamatelatou K, Zampraka A, Kornaros M, Lyberatos G (2009). Exploitation of olive oil mill wastewater for combined biohydrogen and biopolymers production. Bioresour. Technol. 100(15): 3724-3730.

Oehmen A, Pinto F, Silva V, Albuquerque M, Reis M (2014). The impact of pH control on the volumetric productivity of mixed culture PHA production from fermented molasses. Eng. Life Sci. 14(2): 143-152.

Patwardhan PR, Srivastava AK (2004). Model-based fed-batch cultivation of *R. eutropha* for enhanced biopolymer production. Biochem. Eng. J. 20(1): 21-28.

Rein P (2012). Engineering of sugarcane. Bartens 1: 828:831.

Serna L, Valencia LJ, Campos R (2010). Kinetic of fermentation and

antimicrobial activity of *Weissella confusa* against *Staphylococcus aureus* and *Streptococcus agalactiae*. Rev. Fac. Ing. Univ. Antioquia 55:55-65.

Sharifzadeh B, Najafpour G, Younesi H, Tabandeh F, Eisazadeh H (2009). Poly(3-hydroxybutyrate) synthesis by *Cupriavidus necator* DSMZ 545 utilizing various carbon source. World Appl. Sci. J. 7(2):157-161.

Verlinden RA, Hill DJ, Kenward MA, Williams CD, Piotrowska-Seget Z, Radecka IK (2011). Production of Polyhydroxyalkanoates from waste frying oil by C*upriavidus necator*. AMB Express 1(1):1-8.

Wang B, Sharma-Shivappa RR, Olson JW, Khan SA (2012). Upstream process optimization of polyhydroxybutyrate (PHB) by *Alcaligenes latus* using two-stage batch and fed-batch fermentation strategies. Bioprocess Biosyst. Eng. 35(9):1591-1602.

Wang FL, Lee SY (1997). Poly(3-hydroxybutyrate) production with high productivity and high polymer content by a fed-batch culture of *Alcaligenes latus* under nitrogen limitation. Appl. Env. Microbiol. 67:3703-3706.

Waranya S, Samart M, Pakawadee K (2011). Yields of polyhydroxyalkanoates (PHAs) during batch fermentation of sugar cane juice by *Alcaligenes latus* and *Alcaligenes eutrophus*. J. Life Sci. 5(11):960-966.

Yezza A, Halasz A, Levadoux W, Hawari J (2007). Production of poly-beta-hydroxybutyrate (PHB) by *Alcaligenes latus* from maple sap. Appl. Microbiol. Biotechnol. 77(2):269-274.

Zafar M, Kumar S, Kumar Su, Dhiman AK (2012). Optimization of polyhydroxybutyrate (PHB) production by *Azohydromonas lata* MTCC 2311 by using genetic algorithm based on artificial neural network and response surface methodology. Biocatal. Agric. Biotechnol. 1(1):70-79.