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Polyhydroxyalkanoate production from unexplored sugar substrates
Producción de polihidroxialcanoatos a partir de sustratos azucarados inexplorados

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Abstract
Industrial-scale production of biopolymers is restricted by its elevated production costs in comparison with those associated with synthetic (no-biodegradable and no-biocompatible) polymers. In this study we tested for the first time two low-cost carbon substrates (i.e. carob pulp and fique juice) for lab-scale production of polyhydroxyalkanoate (PHA) with *Bacillus megaterium*. PHA detection and quantification was conducted by gas chromatography/mass spectrometry-selected ion monitoring (GC/MS-SIM). The results suggest that PHA production using carob pulp (from *Hymenaea courbaril*) may be as high as with sugar cane molasses. Moreover, it could serve for the synthesis of the most commercialized type of PHA (i.e. polyhydroxybutyrate; PHB) and/or other varieties (e.g. polyhydroxy-butyrate-co-valerate; PHBV) with different properties and potential applications.

Keywords: polyhydroxyalkanoate (PHA); polyhydroxybutyrate (PHB); carob pulp; fique juice.

Resumen
La producción de biopolímeros a escala industrial es restringida por los elevados costos de producción, en comparación con aquellos asociados a polímeros sintéticos (no biodegradables y no biocompatibles). En este estudio evaluamos por primera vez dos sustratos de carbono de bajo costo (i.e. pulpa de algarrobo y jugo de fique) para la producción a escala de laboratorio de polyhydroxyalcanoato (PHA) con *Bacillus megaterium*. La detección e identificación de PHA se hizo mediante cromatografía de gases con detector selectivo de masas operado en el modo de Monitoreo de Ion Selectivo (GC-MS/SIM). Los resultados sugieren que la producción de PHA a partir de pulpa de algarrobo (de *Hymenaea courbaril*) puede ser tan alta como con melaza de caña. Más aún, puede servir para la síntesis del tipo de PHA más comercializado (i.e. polihidroxibutirato; PHB) y/o de otras variedades (e.g. polihidroxi-butirato-co-valerato; PHBV) con diferentes propiedades y posibles aplicaciones.

Palabras clave: polihidroxialcanoato (PHA); polihidroxibutirato (PHB); pulpa de algarrobo; jugo de fique.

1. Introduction

Polyhydroxyalkanoates (PHA) are organic polyesters produced by a variety of bacterial species to store carbon and energy, especially under environmental/nutritional stress [1]. These biopolymers are a good alternative to replace petroleum-based polymers, because they have similar mechanical properties to conventional polymers such as polypropylene [2], but additionally are biodegradable and can be produced from a wide range of renewable sources. [3,4]. However, industrial-scale PHA production is restricted by its elevated costs in comparison with those associated with traditional nonbiodegradable polymers [2]. One of the most extended approaches to reduce these costs is the use of inexpensive carbon substrates [1,5,6].

Colombia has a great variety of plants with carbon-rich fruit that can potentially serve as substrates for bioplastic production. One of these is the *Hymenaea courbaril* (carob tree), a timber tree that extends from the west coast of central Mexico southward into Bolivia and south central Brazil. It is also found in Spain, Portugal, Arabia, Somalia and the West Indies [7]. The fruit of this tree consist in a woody capsule with hard seeds and a dry pulp rich in carbohydrates (Table 1), that is currently used for medical purposes [8] and for human and animal consumption [7].

Table 1.
Composition of carob pulp.

Component	Content per 100g pulp
Total carbohydrates	75.3 g
Water	14.6 g
Fiber	13.4 g
Proteins	5.9 g
Fat	2.2 g
Phosphorus	143 mg
Calcium	24 mg
Ascorbic acid	11 mg
Niacin	4.1 mg
Iron	3.2 mg
Thiamine	0.24 mg
Riboflavin	0.14 mg

Source: Adapted from [7]

Another plant that produces a potentially valuable carbon substrate is the *Furcraea bedinghausii* (fique).

This plant is highly used in Colombia and other South American countries to produce a natural fiber called *cabuya*. In its production, large amounts of fique juice (which represents the 90% of fique leaves) are discarded in soils and water streams [9]. This juice is composed of sugars (3% total sugars), lignin (1%), proteins (0.96%), calcium (0.24%), potassium (0.03%), magnesium (0.03%), phosphorus (0.02%), and trace amounts of sodium, iron, cooper, and zinc (from a bromatological analysis conducted in our lab).

The purpose of this study was to investigate the production of PHA by *Bacillus megaterium*, using carob pulp and fique juice as the sole carbon sources. Glucose and sugar cane molasses were used as a control and a reference, respectively, of inexpensive carbon substrates. Although the last has been reported as an effective and inexpensive substrate for PHA production [5,10], its extensive use in the biopolymer industry is restricted by the food and biodiesel industries.

2. Materials and methods

Bacterial strain

A strain of *Bacillus megaterium* was isolated from soil in Colombia, and characterized by molecular (16S rDNA sequence similarity), morphological and biochemical techniques [11]. The cultures were maintained on nutrient agar at -4 °C and a stock was stored at -20 ºC in 15% (v/v) glycerol.

2.1. Culture medium and inoculum preparation

The culture medium was comprised of 0.6 g/l Na_2HPO_4, 2.0 g/l KH_2PO_4, 2.0 g/l $(NH_4)_2SO_4$, 0.2 g/l $MgSO_4{\cdot}7H_2O$, 0.02 g/l $CaCl_2$, 0.1 g/l yeast extract, 10 ml/l trace solution ($FeSO_4$ 2 g/l, $MnCl_2$ $4H_2O$ l0.2 g/l, $NiCl_2$ $6H_2O$ 0.02 g/l, $(NH_3)_6$ MoO_7 $4H_2O$ 0.03 g/l y $Na_2B_4O_7{\cdot}10H_2O$ 0.1 g/l) and 20 g/l sugar substrate (glucose, sugar cane molasses, carob pulp or raw fique juice). After homogenization, the medium was centrifuged (2000 g, 10 min) and filtered (0.45 µm). The pH was adjusted to 7.0 with NaOH. Culture media with carob pulp and fique juice was also sterilized by autoclaving (121 ºC, 15 min).

Inoculums were prepared in test tubes containing 5 ml (10% of the total volume) of sterile culture medium. Each test tube was inoculated with a single *B. megaterium* colony and incubated at 30 ºC, 150 rpm for 24 h.

2.3. Fermentation studies

Inoculums (5 ml) were transferred into 250 ml erlenmeyers containing 45 ml of sterile culture medium. Fermentations were conducted at 30 ºC and 150 rpm. Shake flask cultures were harvested and assayed for biomass production and reducing sugar concentration at 0, 36, 72 and 144 h.

2.2. Biomass production

After harvesting, culture media were centrifuged at 5000 g for 15 min. The pellets were resuspended in Tris-HCl 0.01 M (pH 7.0) and frozen at -75 ºC. Finally, the pellets were lyophilized at -50 ºC, 0.05 mBar for 24 h and weighed [12].

2.3. PHA extraction and chromatography analysis

PHA was extracted by digestion with sodium hypochlorite and chloroform [13]. Lyophilized samples were combined with a hypochlorite:chloroform (1:1) solution and shaken at 200 rpm for 1h. This solution was centrifuged at 8000 g for 10 min to isolate the PHA in the organic phase. The biopolymer was precipitated from the chloroform solution with methanol (1:3) added dropwise. The methanol solution remained at 4 ºC for 24 h. The precipitated PHA was purified by washing several times with methanol. Finally, excess methanol was eliminated by evaporation and PHA polymer was identified by Gas Chromatography/Mass Spectrometry – Selected Ion Monitoring (GC/MS-SIM) [3]. The analyses were conducted using a DB-WAX (60 m x 0.25 mm x 0.25 µm) column, and a standard PHB (19.6 g; from Aldrich) as a reference. The injection was conducted in splitless mode (volume of injection = 1 µl).

2.4. Reducing sugar concentration

The dinitrosalicylic (DNS) technique (Miller 1959) was used to measure the reducing sugar concentration throughout each fermentation period. Briefly, after doing a calibration curve with glucose (0 to 2 g/l), 500 µl of supernatant, obtained by centrifugation, was added to 500 µl of the color reagent. These solutions were heated in boiling water for 5 min and immediately transferred to cold water for 5 min. Finally, absorbance was measured at 540 nm.

3. Results

3.1. Biomass production and sugar substrates consumption

The highest biomass production was obtained with glucose at 36 h (Fig. 1, A), followed by those obtained with carob pulp and sugar cane molasses (Fig. 1, B and C, respectively). For fique juice this maximum was at 72 h (Fig. 1, D). Similarly, the largest decrease in reducing sugar concentration for media supplemented with carob pulp and fique juice was between 0 and 36 h (Fig. 1, B and D, respectively). Reducing sugar concentration in sugar cane molasses-supplemented media, increased between 0 h and 36 h and then decreased slowly to 1.06 g/l at 144 h (Fig. 1, C). The sugar concentration in media with glucose was higher than 8 g/l during all the fermentation period.

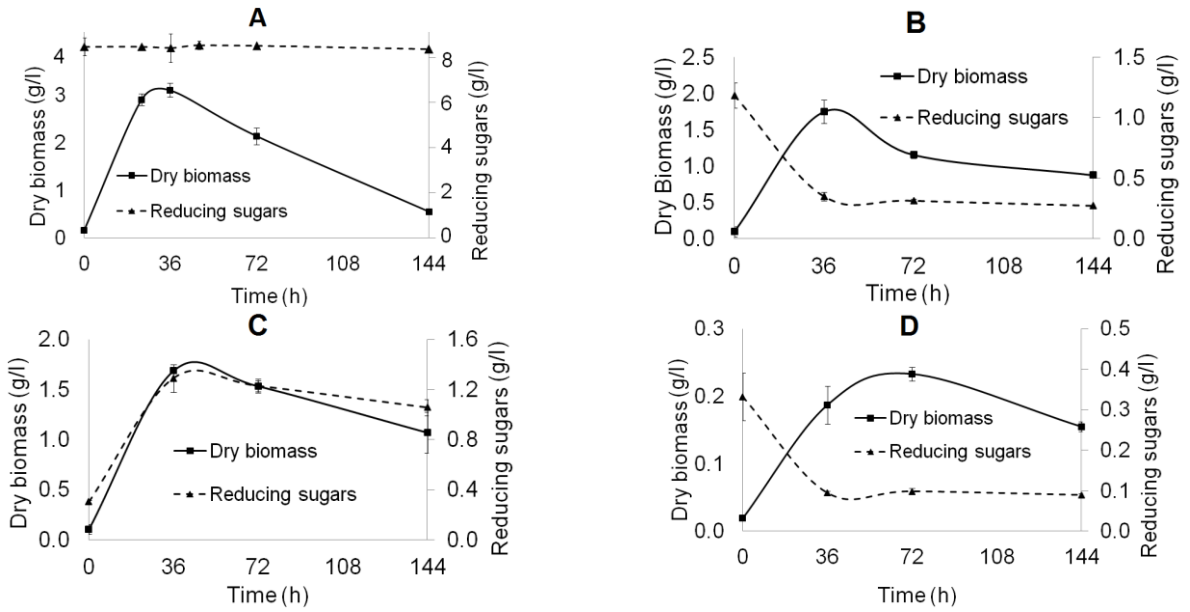

Figure 1. Dry biomass and reducing sugars concentration in media supplmeneted with: **A)** glucose; **B)** carob pulp; **C)** sugar cane molasses and **D)** fique juice. Notice that the scales for dry biomass and reducing sugars are not the same.

Without considering the glucose (due to its expense as a substrate), the highest dry biomass was obtained with carob pulp (1.75 ± 0.16 g/l) and sugar cane molasses (1.69 ± 0.02 g/l) at 36 h. Fique juice-supplemented media shows the maximum production at 72 h (0.23 ± 0.01 g/l), but it is significantly lower than those obtained with the others substrates. After all these maximum points, the biomass production decreased 55% for carob pulp, 30% for sugar cane molasses, and 33% for fique juice, at 144 h.

The highest (1.18 ± 0.10 g/l) and lowest (0.33 ± 0.06 g/l) values of initial reducing sugar were obtained with carob pulp and fique juice, respectively. The highest reducing sugar concentration (1.29 ± 0.11 g/l) with sugar cane was obtained at 36 h. From these maximum points to 144 h, the reducing sugar concentration decreased 77%, 73% and 18% for carob pulp, fique juice, and sugar cane molasses, respectively.

3.2. PHA extraction and characterization

The mass spectrum of the monomers obtained by derivatization of the reference PHB (Fig. 2A) and the produced PHA (Fig. 2, B to E), confirms the presence of hydoxybutyric (HB) monomers in all samples except in those from fique juice (Fig. 2, E). Besides HB monomers, another compound (possibly hydroxyvalerate) was detected when glucose and carob pulp were used as the sole carbon source (peaks at 27 min in Fig. 2, B and C, respectively). The PHA production with glucose, carob pulp, sugar cane molasses and fique juice were 2.5, 0.8, 0.8 and < 0.002 g/l, respectively.

4. Discussion

All substrates tested in this research can be used as the sole carbon source for the growth of *B. megaterium*, which is a common bacteria used for PHA production [1,5]. Nevertheless, there are significant differences between the amount and composition of the biopolymers produced from each substrate. Similar results have been observed when comparing PHA productions from different carbon sources. Valappil et al. (2007) were able to produce PHA (using a strain of *Bacillus cereus*) with 3-HB, 3-HV, and 4-hydroxybityril (4-HB)-like monomer units from structurally unrelated carbon sources, such as fructose, glucose, and gluconate [3]. Similarly, Pijuan et al. (2009) found that different phosphorus-removal microbial communities produced PHA with different compositions [amount of PHB, PHV, and polyhydroxy-2-methylvalerate (PH2MV)], depending on the type of carbon source (i.e. acetate, propionate, butyrate, and glucose) [14]. Therefore, studies focused on novel carbon sources for PHA production (such as this one) have to consider not just the amount but the type of PHA produced with each carbon source.

Biomass and PHA production were related to the availability of reducing sugars. The highest and lowest biomass and biopolymer productions were obtained with glucose and fique juice, which respectively showed the highest and lowest reducing sugar concentrations. Although sugar concentrations are higher in sugar cane molasses than in carob pulp, the PHB production from both substrates were similar. There are two aspects that must be considered in this case: (1) sugar cane molasses are rich in polysaccharides (mainly sucrose) that cannot be detected by the DNS technique (Miller 1959), but as the culture grows these polysaccharides are metabolized and reducing sugars are released to the culture media (Fig. 1,C and 3); (2) carob pulp has volatile compounds as methylpropanoic, methylbutanoic, hexanoic and heptanoic acids [15], that microorganisms can use for PHA synthesis [16]. It is possible that these volatile compounds have compensated for the deficiency of reducing sugars in carob pulp with respect to cane molasses, so that both PHB productions were similar.

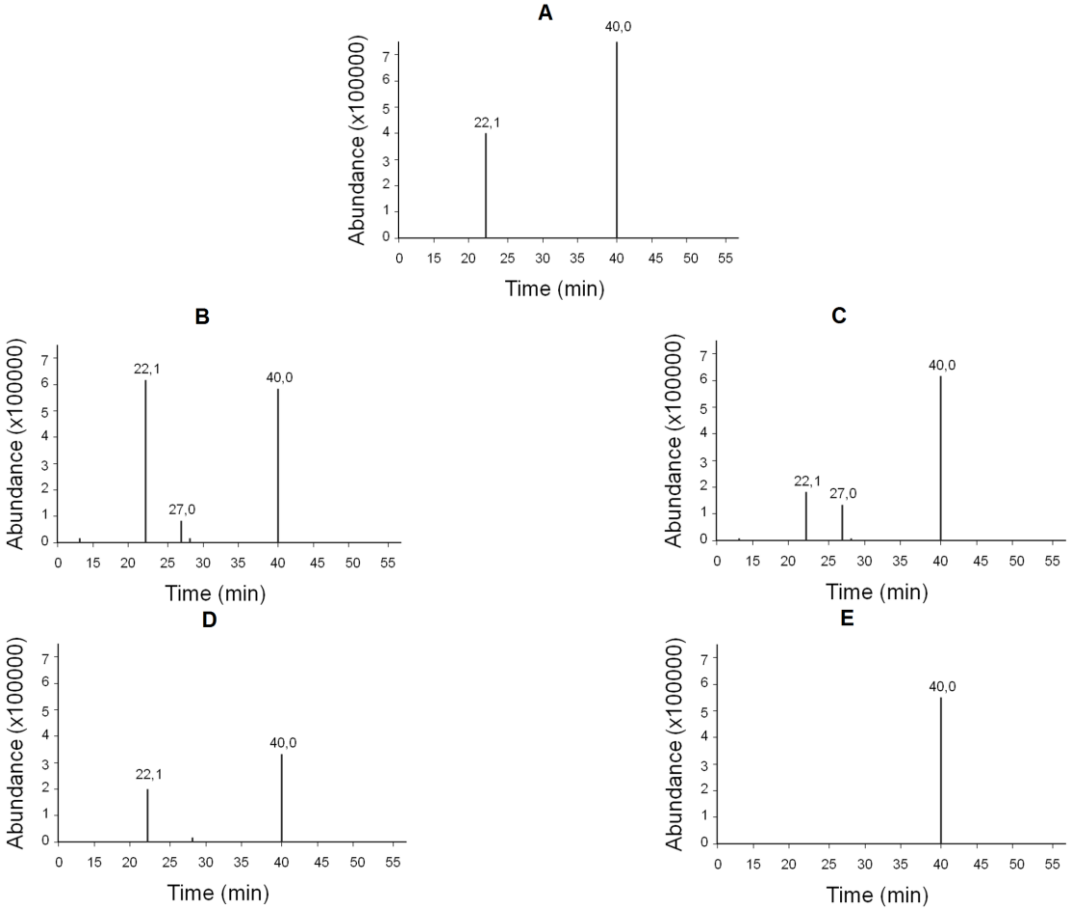

Figure 2. GC/MS-SIM chromatograms of monomers prepared from: A) reference PHB; and from those obtained from media supplemented with B) glucose (PHB at 22.1 and possible PHV at 27.0); C) carob pulp (PHB at 22.1 and possible PHV at 27.0); D) sugar cane molasses (PHB at 22.1);and E) fique juice (undetected).

Besides HB monomers, another compound was detected in the PHA produced from glucose and carob pulp. Based on the results reported by Keum et al. (2008), the peak at 27 min in Fig. 2 B and C could represent the production of hydroxyvalerate (HV) monomers. This suggest that the PHA obtained from glucose and carob pulp is the copolymer poly(hydroxybutyrate-co-valerate) (PHBV) [17]. This biopolymer has different properties than the common PHB, and is used for different biomedical and industrial applications [18,19].

In summary, this is, to our understanding, the first evidence that carob pulp can be used as a carbon source for PHA production. The use of this and other inexpensive carbon substrates, such as beet molasses [20], extruded rice bran [21], and dairy wastes [1], could lead to significant reductions in the production costs of PHA.

5. Conclusions

Carob pulp is a promising carbon source for PHA production. Moreover, it may be used for the production of biopolymers with composition and properties different than those of the traditional PHB. This may be due to the presence of volatile fatty acids in carob pulp. An additional advantage of this novel carbon source is that carob trees are widely spread and their fruits are mostly unexploited.

Contrary to carob pulp, raw fique juice does not seem to be an adequate carbon source for PHA production. However, due to the large amounts of fique juice that are annually wasted in Colombia and other South American countries, it could be economically viable to consider pretreatments (e.g. to increase sugar concentration) to enhance the efficiency of fique juice as a substrate for PHA production.

Although carob pulp represents an opportunity to reduce the production costs of PHA, more research is needed in order to reduce the gap in production costs between petroleum-based and biodegradable polymers.

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