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EVALUATION OF THE PHB PRODUCTION USING MILK WHEY AS FEEDSTOCK

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ABSTRACT

Oil is an important fuel stock that is used mainly in the production of energy and polymers, which are non-biodegradable and cause a major environmental problem. Hence, natural polymers or those originated from microorganisms, such as polyhydroxyalkanoates have increased their industrial value. Currently, the high cost of raw materials and the energy consumed in its production are two of the main problems associated to the industrial production of biopolymers like Polyhydroxybutyrate (PHB). One alternative to solve these problems is the use of different agroindustrial wastes as substrate (such as whey and lignocellulosic material). Whey is a co-product (in some cases is considered as a liquidwaste) that is abundantly generated in the cheese processing factories and could be used for the generation of value-added compounds. The feasibility of PHB production at laboratory scale using *Bacillus megaterium* as the producing microorganism and whey as substrate was tested.An initial pretreatment step to remove nitrogen from the whey medium was implemented. The *B. megaterium* growth curves were carried out using whey as substrate and a PHB accumulation of 1.5 g/L was observed after 26 h. The techno-ecomomic evaluation was performed using Aspen Plus 8.0, for the production and purification of PHB and demonstrated that the production costs are around 4.0 U.S./kg PHB. This high price obtained here is mainly relatedto the pre-treatment process applied to whey.

KEYWORDS:

Whey; PHB; Waste Valorization

1. INTRODUCTION

Oil has been considered an essential raw material in many industrial processes. The production and consumption of petrochemically-derived plastics or polymers have grown rapidly in recent decades[1]. However, these polymers show serious pollution problems due to their low biodegradability and large landfills required for its final disposal hence the necessity to find environmentally friendly substitutes[2].

One option is the use of biodegradable plastics derived from natural sources or originated from microorganisms such as polyhydroxyalkanoates (PHA). PHA are homo or heteropolyesters that can be synthesized and intracellularly stored by many bacteria in the form of granules as a carbon reservoir and can account for up to 80% of the total bacterial dry weight[3].PHA can be accumulated in response to unfavorable growth conditions (deprivation of limiting nutrient as oxygen, nitrogen, phosphorus and sulfur) and an excess of carbon source [4].Poly-3-hydroxybutyrate (PHB), (the most common representative of the PHA[5]) is an attractive substitute biomaterialwhich have similar physical propertiesto conventional plastics like polypropylene or polyethylene. Some applications of this biopolymer are as pharmaceuticals, razor, handles and bottles[1].One of the most important characteristic is based on its complete biodegradability in a natural environment [6]. A drawback for PHB commercialization is the high price compared to petrochemical plastic materials. This high cost is related to the cost of raw materials and the energy consumed in its production[7].Renewable resources such as biomass and carbohydraterich agricultural wastes(such aswhey and lignocellulosic material) are widely used as raw materials for the production through a fermentative process of value-added products like PHB and bioethanol [8].

Cheese whey is the remaining liquid fraction following the precipitation and removal of milk casein duringcheese-making.Approximately 90% of the milk used in the cheese industry is discarded as whey where 55% of milk constituents are retained[9]. Whey is mainly considered as a pollutant due to its high biologicaloxygen demand and its high disposal costs[10]. Whey composition may vary depending on the type of cheese and the process used to produce it. Lactose is the main component (4.5% w/v), followed by protein (0.8% w/v), and lipids (0.5% w/v). [11].In 2014, according to the National Federation of Livestock, the Colombian milk whey production was approximately 3,600 tonnes[12]. Only 55% of the whey produced is used in the production of value-added products of which 45% is transformed into milk drinks, 30% in whey powder and 15% in lactose for food applications[13]. Althoughthere are somederivedproductsfromwhey, theamountproduced causes a greatenvironmentalproblemtothedairyindustryhencethenecessitytofindother uses and a more environmentallyfriendly final disposal.

The aim of this study was to adapt the bacterium*Bacillus megaterium* to a growth medium using milk whey as substrate. A pre-treatment process to achieve the necessary conditions of carbon and nitrogen concentrationswas applied. The obtained yields of PHB and biomass cultured in whey medium were used as

basis for a techno-economic analysis of the potential formilk whey to be used as raw material for the production of value-added products.

2. MATHERIALS AND METHODS

2.1 Milk whey pre-treatment

In order to adequate milk whey as substrate for bacterial growth, a series of pre-treatment steps were performed as follows: i) Sterilization: whey was sterilized at 115°C for 15 min. In this step a greater fraction of the whey protein was precipitated. ii) Ultrafiltration: The supernatant from the sterilization step was ultrafiltrated using a cellulose-acetate hollow fiber membrane. iii) Since whey has a pHbetween 3.5-4.0, a neutralization step to pH 7.0 using NaOH 12 N was required for the optimal bacterial growth. In this step a portion of the whey protein and salts were further precipitated. vi) Centrifugation: after neutralization, milk whey was centrifuged at 6000 rpm for 10 minutes and the supernatant was collected and further used in the preparation of the growth medium.

2.2. Bacterial strains

The *Bacillus megaterium* used in this work is a wild strain isolated from superficial sediments of the Bahia Blanca Estuary (Buenos Aires, Argentina) and characterized as PHB producer in the presence of an excess carbon source and nitrogen restriction [21]. The stock culture adapted to whey as carbon source was maintained at 4°C after growth on a formulated agar medium.

2.3. Culture media

The seeding medium was prepared with the following concentrations:KH_2PO_4, 1.5 g/l; Na_2HPO_4 , 9 g/l; $MgSO_4 \cdot 7H_2O$, 0.2g/l; and 1 ml/l of a trace element solution composed by: $FeSO_4 \cdot 7H_2O$, 10 g/l; $ZnSO_4 \cdot 7H_2O$, 2.25 g/l; $CuSO_4 \cdot 5\ H_2O$, 1g/l; $MnSO_4 \cdot 4H_2O$, 0.5 g/l; $CaCl_2 \cdot 2H_2O$, 2 g/l;H_3BO_4 , 0.23 g/l; $(NH_4)_2Mo_7O_{24}$, 0.2 g/l; and35%HCl, 10ml. The carbon source and the $MgSO_4 .7H_2O$ were autoclaved separately and added aseptically to the medium after cooling. Nitrogen was provided by the remaining amounts of whey protein.

2.4. Batch cultivations

The fermentations to produce PHB were carried out for 46 h in a 1.5 liters fermenter with a 1.2 liters working volume at 32°C, 200 rpm and 5 l/min as the aeration flow rate. 10% v/v of a grown formulated medium was used as seed. .

2.5. Analytical Methods

2.5.1. Biomass

Biomass was measured using the dry weight technique. Briefly, 1 ml samples were collected in previously dried and weighed microfuge tubes and centrifuged at 12.000 rpm for 10 min. Then, the resulting supernatant was discarded and the pellet was washed with distilled water, centrifuged again and the excess of water discarded. The final biomass was weighed after drying for 48 h at 60ºC.

2.5.2. PHB quantification

Dried biomass is used for methanolysis of monomers according to the method described by Braunegg*et al.*[14]and modified by Lageveen*et. al*. [15]. Approximately 10 mg of cells mass was reacted in a small screw-cap test tube with a solution containing 1 ml of chloroform, 0.85 ml of methanol, 0.15 ml of sulfuric acid and 0.2 ml of internal standard (benzoic acid in methanol) [16]for 140 min at 100 °C. After reaction, 0.5 ml of distilled water was added and the test tube was shaken vigorously for 1 min. After phase separation, the organic phase (bottom layer) was removed and transferred to a small screw-cap glass vial. 50µl from this organic phase were taken and added to a test tube and injected in the Gas Chromatographer-Mass Spectrometer. An Agilent Technologies 6850 series II gas chromatographer was used. The gas chromatographer was equipped with a HP-5MS capillary column (25 m length, 0.32 mm internal diameter). Helium (velocity at 5cm/min) was used as the carrier gas. The injector and detector were operated at 230 °C and 275 °C, respectively. A temperature program was used for efficient separation of the esters (120 °C for 5min, temperature ramp of 8 °C per min, 180 °C during 12 min). An Agilent Technologies 5975B mass spectrometer was used for the identification and quantification of derivatized PHB.

2.5.3. Lactose quantification

Lactose concentrationswere determined off-line by HPLC (Hitachi LaChrom Elite) equipped with an auto sampler (Hitachi LaChrom Elite L-2200), anORH-801 Column, a column oven (Hitachi LaChrom Elite L-2300), a HPLC pump (Hitachi LaChrom Elite L-2130) and a Hitachi LaChrom Elite L-2490 refraction index detector. Injection volume was 20 µl. The column was kept at 35°C and the pump was operated at a flow rate of 0.6 ml/min.

2.5.4. Protein quantification

The protein concentration in the initial culture broth was measured using the Kjeldahl method by distillation [17].

2.6Simulation procedure

The Aspen Plus v8.0 software (Aspen Technology, Inc.) was used to simulate the global process of, PHB production using milk whey as substrate and *Bacillus megaterium*as PHB producing bacteria. The physicochemical properties of components not included in the Aspen tech databases (i.e., biological compounds) were obtained from the National Institute of Standards of Technology[18], and calculated from the group-contribution method proposed by Marrero and Gani[19]. Mass and energy balances were calculated by simulation. The economic evaluation was performed with Aspen Plus Economic Analyzer v 8.0 using specific parameters of some Colombian conditions such as raw materials cost, income tax (33%), labor salaries, and interest rate (25 %) among others in order to calculate the production cost per unit of product at the Colombian conditions. For feedstock prices, the international reports from ICIS pricing were employed. Electricity, potable water, low and high pressure costs were 0.1 USD/kWh, 1.52 USD/$m^3$and 8.18 USD/tonne respectively.

In this work, the production of value-added compounds such as PHB was proposed taking into consideration the experimental yields obtained at laboratory scale.Figure 1 and Table 1 show the global process and the technologies used in the PHB production process, respectively. Whey was ultrafiltrated, the resulting liquor was neutralized using a concentrated solution of 12 MNaOH andcentrifuged to remove precipitated salts.Then, the supernatantwas sterilized and fermented using *Bacillus megaterium* as the PHB producing microorganism. A previous pre-culture stage for biomass growth was necessary. The biomass produced in the pre-culture step was inoculated in the fermenter at 32°C,200 rpm, aeration of 5 l/ minand a controlled pH of 7. The produced liquor was then centrifuged to separate the fermented medium from the biomass. Biomass was heated and an enzymatic hydrolysis to release the PHB in the cells was applied. The residual biomass was

centrifuged and then a purification step with hydrogen peroxide was performed. Finally, a spray dry process was applied to obtain the PHB pellets with a 99% purity.

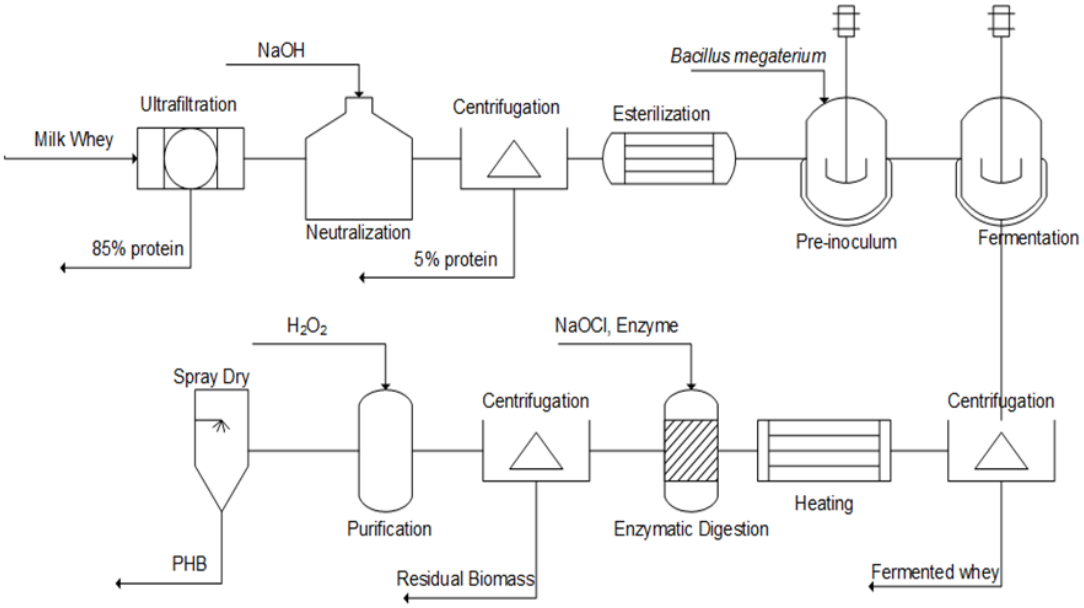

Fig1 PHB production process for the techno-economic evaluation.

Table 1 Technologies and conditions used in the techno-economic assessment of PHB.

Technology	
Ultrafiltration	Cellulose- acetate membrane
Fermentation	Using *Bacillus megaterium*without genetic modification
Enzymatic Digestion	Enzymatic treatment using 2% w/w of *Burkholdeirasp* PTV
Purification	Using H_2O_2 1,2% v/v

Input basis: 100kg/h milk whey.

3. RESULTS AND DISCUSSION

3.1. Milk whey characterization

Table 2 presents the chemical characterization of the milk whey provided by Normady Inc. This industry is locate in the Caldas, Colombian region. It should be noticed that some factors such as the type of bovines, feed and region are determinant factors in the chemical composition of milk whey.

Table 2. Chemical characterization of milk whey.

Compound	**Concentration (g/l)**
Lactose	94.5
Glucose	1.3
Galactose	24.88
Protein	7.49

After the pre-treatment method was applied, a 96% of protein removal was achieved with a final concentration of 0.3 g/l remaining protein.

3.2. Growth curve and PHB accumulation

Figure 2 shows the production of PHB using milk whey as substrate. The highest accumulation was presented at 26hr of fermentation with a value of 1.5 g PHB/ l, a biomass concentration of 5.6 g/l, an accumulation percentage of 73 % and a Lactose consumption of 80 g/l.

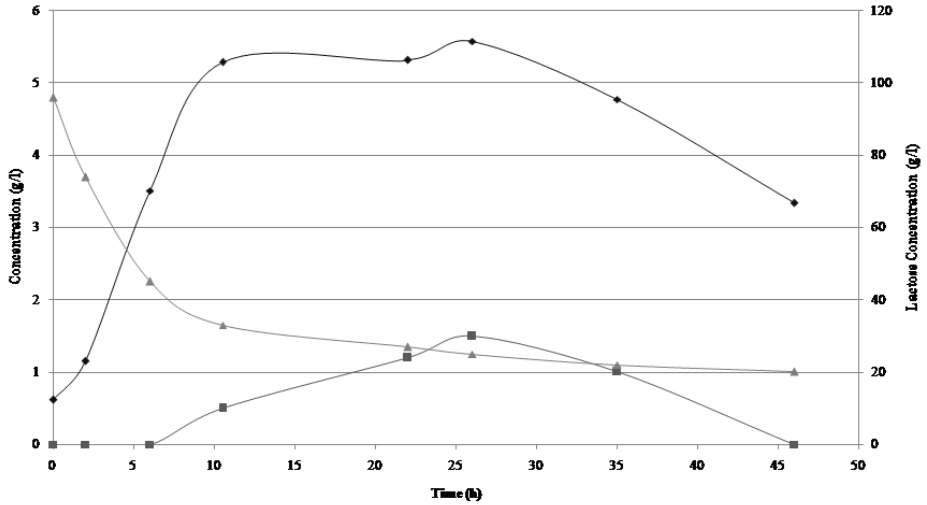

Fig 2 Growth curve of *B. megaterium* using milk whey as substrate. ◆Total Biomass (g/l), ■ PHB concentration (g/l), ▲ Lactose concentration (g/l).

Some studies have shown a PHB accumulation percentage using whey. Duke et al., 2014 shows a 26 wt% accumulation of PHB using a mixed culture of microorganisms[20]. Paris et al., 2013 used a recombinant strain of *E. coli* CML3-1 with accumulation rates of 21 wt%[21]. In the same study, they analyzed a strain of *E.coli* P8-X8 GM where the accumulation rates reached38.65wt% [21]. In the study of Obruca et al., 2011 where a strain of *B. megaterium* CCM-2037 was used, a maximum accumulation of 27.36wt%PHB under normal growing conditions is obtained[22].The PHB concentration obtained in this study (73wt%) is similar to those obtained for other substrates such as glycerol (62wt%) and glucose (70wt%). This result shows the possibility of using whey ultrafiltrate as a viable substrate for PHB production using *B. megaterium*. The application of whey as culture medium in this type of process, allows a reduction of the final pollution load disposed into water sources or soil. This residue presents an alternative option for the production of other value-added products (such as PHB).

Pantazaki et al., 2011 and Obruca et al., 2011 showlactose consumptions of25 g /l[24] and 15 g / l[22] using *Thermusthermophilus* and *B. megaterium* respectively., In this study 80g/l lactose consumption by native *B. megaterium*is shown. This value is higher than those reported by Pantazaki et al and Obruca et al thus indicating that the native strain can use lactose form the ultrafiltrated milk whey as carbon source for the production of PHB.

3.3 Techno- economic Assessment

Figure 3 shows the distribution of the PHBtotal production cost(4 USD/kg). As seen in figure 3, the services used for heating and cooling the different main lines represent the highest share (35%) in the cost distribution. On the other hand,the raw material presents the lowest share (4%).This fact could bedue to the use of a residue namely milk whey as raw material, which currently has a low commercial value.

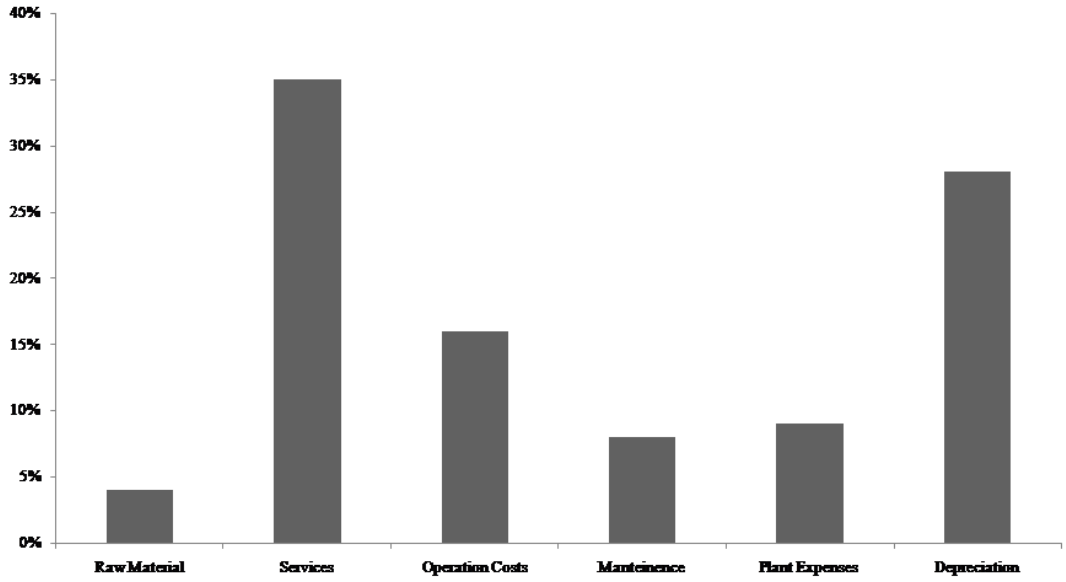

Fig3 Distribution of the PHB total production cost

The high PHBproduction cost obtainedcould be a consequence of the pre-treatment method which requires high amounts of energy and work, including the pressure used for ultrafiltration, and the energy required in the sterilization of the culture medium. The PHB price is far from the production costs of synthetic plastics (around 0.50 $ USD/ kg). The market price of PHB is between 2-3 USD/kg. Technological developments of the PHB production process using milk whey could help to reduce the production costs and make this product more competitive.

4. CONCLUSIONS

The application of renewable and biodegradable sources for the production of novel biopolymers has increased and has led to propose the use of different residues to obtain PHB as an alternative to synthetic polymers.It is possible to obtain PHB using milk whey as raw materialbut it is necessary to invest and develop new cheaper and more efficientpre-treatment technologies.The PHB production could be assessed within a biorefinery scheme where other products are obtained, thereby reducing production costs and reach similar values to synthetic polymers.

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