Bacterial production of poly(3-hydroxybutyrate) from whey

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Abstract

Polyhydroxyalkanoates are 100% biodegradable polymers produced intracellularly by bacteria, which exhibit characteristics similar to petroleum-based polymers and therefore constitute an alternative to recalcitrant polymers. However, its high cost of production can prejudice its application on a large scale. Among the available solutions for reducing costs one of them is the use of a substrate from industrial wastes such as whey, which generated in cheese production. In the present work PHB production tests were carried out with *Alcaligenes latus* bacterium, using whey after acid protein precipitation and subsequent neutralization, comparing the results obtained with those acquired from commercial lactose. Through statistical analysis it was found that the best results for the production of the polymer are obtained by neutralization of the pH using NH_4OH, which provided an average 1.28 $g.L^{-1}$ PHB in 12 hours. The Infrared Fourier Transform Spectroscopy analysis showed that both the default PHB, and the one produced by the *A. latus* bacterium showed the same characteristic bands.

Key words: Poly(3-hydroxybutyrate). *Alcaligenes latus*. Whey.

Theme Area: Environmental technologies.

1 Introduction

Polymers are widely used due to their properties. Because they enable several possibilities in regards to processing and optimization of mechanical and thermal properties, these materials have been used to replace glass, paper and even metal alloys in functions such as packaging, automotive parts, coatings, among others (KHANNA & SRIVASTAVA, 2005). This results in accumulation of wastes, mostly non-biodegradable, which is a serious environmental problem (JACQUEL et al., 2008). The management of these wastes can be performed by heat treatment, recycling, degradation and also optimization of processes for residue reduction at its source. Heat treatment is an expensive and potentially polluting process if carried out without proper control. Recycling becomes limited by the amount of additives and fillers used, and the loss of properties associated with each reprocessing performed (KHANNA & SRIVASTAVA, 2005).

Consequently, it becomes necessary not only to study polymer biodegradation further, but also the production of biodegradable polymers of non-fossil origin (JACQUEL et al., 2008). Polyhydroxyalkanoates (PHAs) are polymers 100% biodegradable arising as an alternative (SIVAKUMAR; AL-BAHRY; AL-BATTASHI, 2013). These are produced by various microorganisms as a carbon reservoir source by the use of organic substrates (LEE & CHOI, 1999). One of the most studied PHAs is poly(3-hydroxybutyrate) (PHB), which is produced by several bacterial species and has characteristics that resemble petroleum-based polymers (FACCIN et al., 2013). However, the PHAs currently have high prices due to the fact that their production process demands high investments (NATH et al., 2008). The substrate used for the growth of the producing bacteria is one of the reasons why production costs are expensive.

Milk whey is obtained indirectly and in high volume from cheese production (8-9 kg of milk whey per each kg of produced cheese) is still treated as a residue in brazilian industry (BALDASSO; BARROS; TESSARO, 2011). When untreated, this effluent can be the cause of serious environmental problems. This occurs due to the high organic matter content, which induces proliferation of microorganisms and algae and consequently reduces the concentration of dissolved oxygen (DO), which makes its degradation slower and affects therein fauna and flora. However, this by-product has high amounts of lactose, a disaccharide which can be used as carbon source for the microorganisms that produce PHAs.

The use of whey as a carbon source for the production of PHAs by bacteria can result in a more affordable price, popularizing the use of these biodegradable polymers, and assisting in the allocation of the liquid effluent.

2 Experimental

2.1 Materials

The carbon sources used were milk whey (powder form) and commercial lactose. Standard PHB used was *Sigma-Aldrich* brand and other reagents were from *Dinâmica* brand. *Alcaligenes latus* bacterium was acquired from Oswaldo Cruz Foundation, from the lineage number DSMZ 1123.

2.2 Polymer production

This experiment was performed using different reagents to neutralize the pH of the cultivation medium prepared with milk whey which had gone that went through citric acid precipitation process. Furthermore, commercial lactose produced medium was also used for comparison.

The commercial lactose cultivation medium was adapted from Faccin et al. (2013) and from Zafar et al. (2012). The medium composition can be observed in Table 1.

Table 1 - Commercial lactose cultivation medium composition

Reagent	Value
Lactose ($g.L^{-1}$)	16
$(NH_4)_2SO_4$ ($g.L^{-1}$)	1
KH_2HPO_4 ($g.L^{-1}$)	1.5
$Na_2HPO_4.12H_2O$ ($g.L^{-1}$)	3.6
$MgSO_4.7H_2O$ ($g.L^{-1}$)	1
$CaCl_2.2H_2O$ ($g.L^{-1}$)	0.01
Anhydrous citric acid ($g.L^{-1}$)	0.1
Trace metals solution ($mL.L^{-1}$)	3

Trace metals solution composition (FACCIN et al., 2013; ZAFAR et al., 2012) can be seen in Table 2. This solution was used in all of the mediums produced in the same amount (3 $mL.L^{-1}$).

Table 2 - Trace metals solution composition

Reagent	Concentration ($g.L^{-1}$)
$FeSO_4.7H_2O$	0.20
$MnCl_2 . 4 H_2O$	0.03
H_3BO_4	0.30
$CuSO_4 . 5 H_2O$	0.01
$CoCl_2 . 6 H_2O$	0.20
$(NH_4)_6Mo_7O_{24} . 4 H_2O$	0.03
$ZnSO_4 . 7 H_2O$	0.03
$NiSO_4 . 7 H_2O$	0.03

The cultivation medium of citric acid precipitated milk whey was produced by diluting the solution until lactose concentration of 16 $g.L^{-1}$ was attained and 3 $mL.L^{-1}$ of metals solution (composition in Table 2) were added. Thereon one of the hydroxides were added for pH neutralization. The used hydroxides were NaOH 10% (w/v), KOH 10% (w/v) and NH_4OH 10% (w/v). Experiments were realized in a benchtop orbital shaker (Marconi, MA832) at 35 °C, 200 rpm, total duration of 12 hours, 200 mL of cultivation medium in 500 mL erlenmeyer flasks, destructive samples in quadruplicate and initial pH of approximately 6.5.

The results were evaluated through analysis of variance (ANOVA ONE WAY - one multilevel factor). Analyzed response variables were substrate consumption ($g.L^{-1}$), dry cell weight ($g.L^{-1}$) and polymer mass ($g.L^{-1}$).

2.3 Milk whey protein precipitation

A 12% (w/v) milk whey solution was prepared. Protein precipitation was realized by adding a 40% (w/v) citric acid solution until the pH was reduced to 3.3 (for every 50 mL of 12% whey, about 4 mL of the acid solution were used). Samples were then centrifuged with 3250 *x*g at -4 °C for 30 minutes (Novatecnica NT825). The supernatant liquid was used in the medium production.

2.4 Inoculum preparation

For sample inoculation, a pre-culture was prepared with the same cultivation medium used in the experiments. To erlenmeyer flasks (500 mL total volume) containing 200 mL of cultivation medium, single colonies from the bacterium were added and then put in a benchtop orbital shaker at 35 °C, 200 rpm for 24 hours. Aiming for the standardization of the initial bacterium cell weight, optical density (OD) of the pre-culture was measured in a spectrophotometer (Biospectro SP 22) at 600 nm, and when necessary, the pre-culture was

diluted until OD became approximately 1. Pre-culture/cultivation medium proportion used was 10% v/v.

2.5 Polymer extraction

The polymer was extracted with an adaptation of the patented method used by Baptist (1962). To approximately 2 g of wet washed centrifuged cell mass 50 mL of acetone were added. After 30 minutes the sample was filtered and again 50 mL of acetone were added. A new filtration was made after 24 hours, and then 15 mL of a dichloromethane-ethanol 5:1 (v/v) solution were added and put in a orbital shaker with 35 °C, 200 rpm for 1 hour. Once again, the suspension was filtered and to the filtered liquid 50 mL of ethyl ether were added. After natural evaporation of the solvents, the dry polymer was obtained.

2.6 Analytical methods

2.6.1 Lactose quantification

Lactose concentration was determined by Miller's method, using 3,5- dinitrosalicylic acid (DNS). This assay was performed to characterize the milk whey and to determine the initial and final concentrations of lactose in the tests. The substrate consumption was determined by subtracting the final concentration from the initial lactose concentration. The analysis consists in adding 0,75 mL of DNS reagent to 0,25 mL of a previously diluted sample. This mixture is then put into a 100 °C water bath for 5 minutes and cooled to room temperature. 4 mL of distilled water are added and the absorbance is read in a spectrophotometer using 540 nm wavenumber.

2.6.2 Protein quantification

Protein quantification was done by Lowry's method, by using a phenolic reagent and copper in alkaline conditions. This assay was performed to characterize the milk whey. In this method, 5 mL of a copper sulphate reagent to 1 mL of a previously diluted sample and after mixing and keeping it in room temperature for 10 minutes, 0,5 mL of diluted Folin Ciocalteu reagent is added. The mixture is mixed again and left in room temperature for 30 minutes. After that, absorbance is read in a spectrophotometer using 750 nm wavenumber.

2.6.3 Dry cell weight quantification

Dry cell weight was determined by the gravimetric method using 10 mL aliquots in 15 mL falcons, centrifuging them with 3250 *x*g, -4 °C for 15 minutes. The supernatant solution is then removed, and water is added for cell washing. The cells are again centrifuged for 30 minutes in the same conditions and then dried for 72 hours at 65 °C. After the quantification of the extracted polymer, the value is subtracted from the total dry weight, thus determining the dry cell weight.

2.6.4 Polymer quantification

Polymer quantification was performed through gas chromatography. First, PHB propanolysis is prepared by using dichloroethane, n-propanol, hydrochloric acid and benzoic acid. The mixture is agitated at 200 rpm, 70 °C for 4 hours. After cooling to room temperature, 4 mL of deionized water are added. The resulting organic phase is injected in a gas chromatograph (Dani Master GC) using a flame ionizing detector (FID) with the Riis and Mai (1988) method. A 30 m x 0,25 mm x 0,25 μm Agilent DB-WAX capillary column and Helium for carrier gas were used.

2.6.5 Infrared Fourier Transform Spectroscopy (FTIR)

FTIR through attenuated total reflectance (ATR) analysis was performed to determine the extracted polymer purification level and the occurrence of sample degradation during the extraction process. Analysis were made in Nicolet IS10 Termo Scientific equipment.

3 Results and Discussion

3.1 Whey characterization

Table 3 shows the obtained results for whey characterization, which were similar to those obtained by other authors (BYLUND, 1995; YADA, 2004; MILLER; JARVIS; MCBEAN, 2000).

Tabela 3 - Whey characterization before and after acid protein precipitation process

Parameter	Milk whey	Acid protein precipitated milk whey
pH	6.2	3.3
Lactose ($g.L^{-1}$)	44.2	86.1
Protein ($g.L^{-1}$)	9.7	10.6
Total dissolved solids ($g.L^{-1}$)	54.0	113.7
Volatile dissolved solids ($g.L^{-1}$)	51.7	108.9
Fixed dissolved solids ($g.L^{-1}$)	2.3	4.1
Electric conductivity ($mS.cm^{-1}$)	2.6	14.9

3.2 Statistical analysis

Figure 1 shows the graphics obtained (lactose consumption and dry cell weight) with standard deviations for each level of the variable factor. Higher substrate consumption was noticed (in the 12 hour period - total experiment duration) in the commercial lactose medium, and similar averages for the other used mediums.

Figure 1 - Results obtained for (a) substrate consumption ($g.L^{-1}$) and (b) dry cell weight ($g.L^{-1}$)

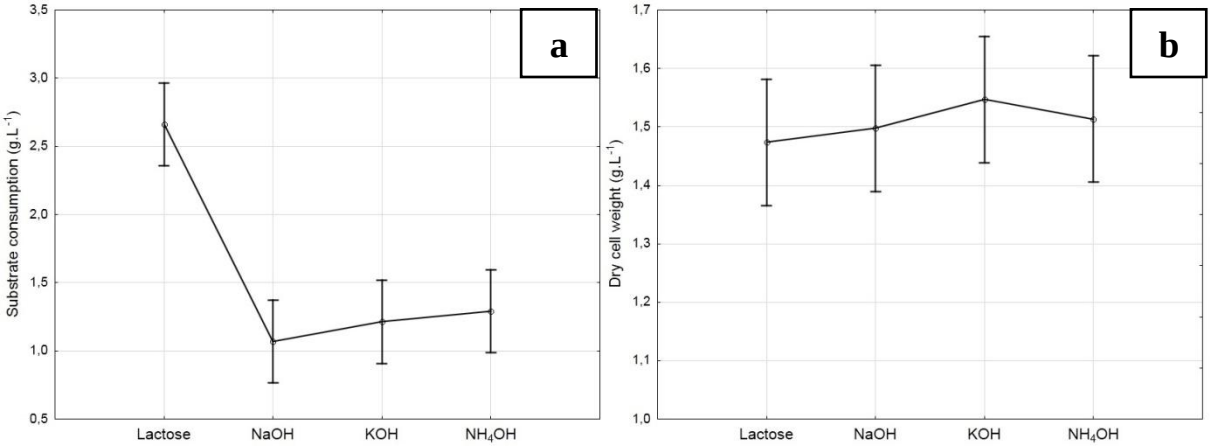

Figure 2 shows the obtained graphic for PHB mass, with standard deviations for each level of the variable factor. The medium which was neutralized with NH_4OH presented the highest PHB concentration after the total time of 12 hours.

Figure 2 - PHB mass results ($g.L^{-1}$)

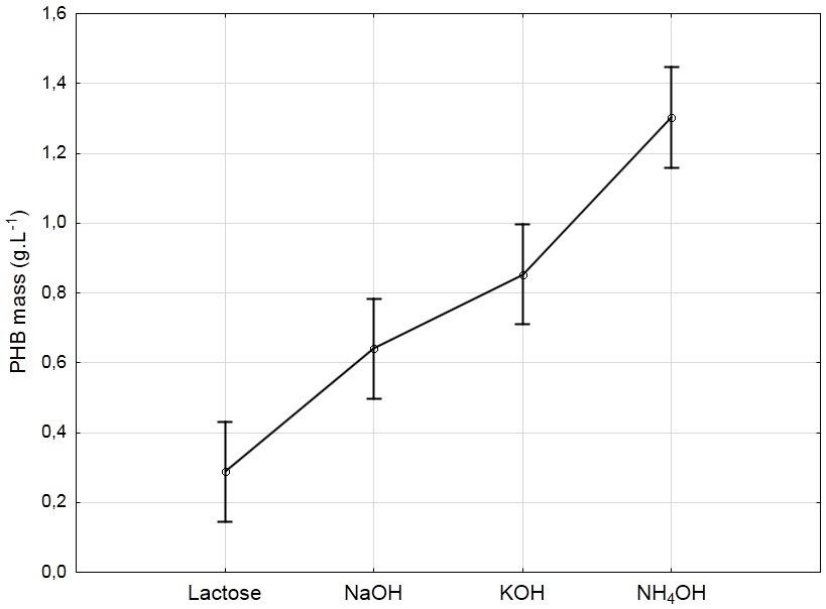

Table 4 shows the results obtained by Duncan's multiple comparison of averages. When dry cell weight results were compared, it was seen that none of the factors rendered significant influence on the results. When each cultivation medium effects over substrate consumption were evaluated it was evident that commercial lactose medium resulted in the highest consumption. The difference in these results was significant when comparing all other mediums. The remaining cultivation mediums did not present any significant difference between them.

Table 4 - Duncan's multiple comparison of averages results

Cultivation medium	**Substrate consumption**				**PHB mass**			
	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
(1)Lactose	-	+	+	+	-	+	+	+
(2)NaOH	+	-	-	-	+	-	+	+
(3)KOH	+	-	-	-	+	+	-	+
(4)NH_4OH	+	-	-	-	+	+	+	-

Note: + Significant difference; - Non-significant difference.

When PHB mass was evaluated, it was seen that the difference between results was significant among all of the tested cultivation mediums. Highest PHB production was obtained with ammonium hydroxide neutralized medium, whilst the lowest PHB production was observed in commercial lactose produced medium.

3.3 FTIR analysis

FTIR analysis was conducted to verify if the produced polymer was in fact PHB, and also to perform a comparison with the standard PHB. Table 5 shows identified bands in both analyzed polymers. Other authors (DOMÍNGUEZ-DÍAZ et al., 2015, FURUKAWA et al., 2007) also verified the same bands in very similar wavenumbers.

Table 5 - FTIR analysis identified bands in *Alcaligenes latus* produced PHB and standard PHB

Wavenumber (cm^{-1})	**Corresponding band**
1719 cm^{-1}*	C=O axial deformation
1700 e 1800 cm^{-1}*	C=O stretching
1000 a 1300 cm^{-1}*	C-O-C and C-C stretching
	CH deformation band
1453 cm^{-1}*	CH_3 asymmetric deformation
1379 cm^{-1}*	CH_3 symmetric deformation
Padrão: 1278 cm^{-1}	C-O-C stretching
Extraído: 1275 cm^{-1}	

Note: * Same wavenumber for both evaluated polymers

4 Conclusion

It was seen that PHB production through *Alcaligenes latus* using lactose as a substrate is possible, even when using milk whey. The acid protein precipitation process and posterior pH neutralization did not affect polymer production, in fact, it rendered higher final PHB concentrations when using ammonium hydroxide. FTIR analysis confirmed that the polymer produced by *A. latus* was PHB, which showed the same spectra pattern seen in standard PHB.

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