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Purification of Poly (3-hydroxybutyrate) Produced by Fatty Acid Fermentation Using Organic Polar Solvents

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Poly (3-hydroxybutyrate) or (P3HB) produced by the fermentation of fatty acids with the mutant strain *Burkholderia cepacia B27* was extracted and purified using different techniques including washing and precipitation of the biomass, and the polymeric materials with organic polar solvents such as ethanol (96%) or methanol (99,7%). The materials obtained after the purification processes were thermally characterized (using differential scanning calorimetry and thermo gravimetric analysis). The polymeric samples treated with organic polar solvents showed improved melting point passing from 169,2°C; for the chloroform purified samples to 174,9°C for the methanol purified samples, also, appearance and odor of the films was improved.

1. Introduction

Synthetic polymers are composed by monomeric units, which came from fossil hydrocarbons; these materials are widely used around the world due to its low production costs and good mechanical properties. One of the most common application of these materials is packaging, which represents a short useful life in most cases. Usually, many packages are used one, or a couple of times before they are discarded, this and the non-biodegradable behavior of most synthetic polymers used for packaging caused a continuous growth of plastics in landfills from 1% in 1960 to 10% in 2005, and the accumulation of millions of tons of plastic wastes in water bodies all around the world (Geyer et al., 2017).

That is why in recent years, the interest in the synthesis of materials able to replace the conventional polymers but with high biodegradation rates and low toxicity has grown. Polyhydroxialkanoates (PHAs) are thermoplastic polyesters synthetized by a large group of bacteria under stress conditions (limitation of nutrients), to store energy as intracellular fat deposit; they are biodegradable and non-toxic, and some of them have interesting mechanical properties that allow them to be used in the packaging industry. Poly (3-hydroxibutyrate) or P3HB is a linear homochiral, and crystalline biopolymer that belongs to a group of short chain PHAs (Nayak, 1999). It's widely studied due to its mechanical properties that are similar to those of polypropylene (Parra et al., 2007), but with a better oxygen barrier. It is also resistant to hydrolytic degradation, which is a feature that many other biopolymers like PLA or starch-based polymers lack. However, it's necessary to improve some of its weakest characteristics like the high brittleness, the production cost and the narrow processing window. These improvements can be achieved using different extraction, purification and modification technics that result in materials with different physicochemical characteristics and production costs (Cyras et al., 2000)

There are several research works, which analyze the influence of downstream activities into the characteristic of the polymer obtained. Nevertheless, most of them increase the cost per kilogram of the biopolymer obtained due to the complexity of the procedures evaluated. Additionally, when the carbon source is vegetal oil, the separation processes are complicated. For this reason the analysis of solvents like the methanol or ethanol in order to facilitate the biomass precipitation and the polymer purification could be useful in the optimization of the extraction and purification of the polymer after fermentation (Koller et al., 2013).

In the Biotechnology institute of the National University of Colombia research works have been developed in the production of the biopolymer Poly (3-hydroxybutyrate) from mutant *Burkholderia cepacia B27* (Mendez,

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2016). Also, studies to optimize the production process were carried out, evaluating and optimizing operational conditions in 7L, 100L and 2000L fermentations(Viloria et al., 2017). In this work, different purification processes were evaluated in order to obtain a high purity polymer. Initially, the polymer was extracted and separated from the surrounding PHA hyper-productive mutant bacteria *Burkholderia cepacia B27* biomass, using chemical digestion with SDS, centrifugation and solvent precipitation with ethanol. Then the polymer was purified to remove protein and oil residues from the fermentation, for this the performance of different solvents such as ethanol and methanol was tested under different operation conditions.

2. Materials and Methods

2.1 Fermentation Process – Polymer Extraction and Purification

The polymer was produced by the mutant strain *Burkholderia cepacia* in 7L, 20L and 100L bioreactors using a commercial vegetal oil as carbon source, the fermentations were carried out according to previous research (Mendez et al., 2016). The process was carried out at 32°C and pH 6 for 72 hours. Also, a commercial Poly [(R)-3hydroxybutyric acid] Sigma Aldrich was used for the analysis.

After the fermentation, the culture broth was sterilized at 121°C for 40 min to deactivate the biomass and stop all the metabolic processes. Then, chemical digestion of the bacteria cell membrane was made using a SDS (sodium dodecyl sulfate) solution as it is described by Mendez et al. (2016). Multiple washes were carried out to remove waste salts, residual SDS and other contaminants from the fermented culture broth. The washes were made in a batch centrifuge at 8000 rpm for 15 minutes each one. Then, the supernatant was removed and the solids (biomass and polymer) where re-suspended to the initial volume with tap water. The process is repeated until the supernatant is clear and colorless. Finally, the solids were re-suspended in 1/6 of the initial volume with tap water and stored for drying. The concentrated solution of polymer and biomass was served in metal trays and left to dry in a 60°C oven for 24 hours. Then, the dried product was ground in a coffee mill and the powder obtained was stored.

2.2 Chloroform purification and solvent casting

Solubilization of the powder (polymer and biomass) in chloroform was performed to separate the PHB from the residual biomass. Solutions of 10 ml gr^{-1} were stirred at 500 rpm for 1 hour and then vacuum filtered using a 0.22-micron membrane. The solid phase retained in the membrane was the biomass, and the liquid phase was the chloroform – PHB solution. The solution was served in petri dishes and left at room temperature until the chloroform was complete evaporated and a thin polymeric film was formed. The efficiency of the purification was evaluated using the following equation.

$$Efficiency(\%) = \frac{P3HB\ Film\ Weigth}{Initial\ Solid\ Material\ Weigth} x100 \qquad [1]$$

2.3 Ethanol Precipitation – Flushing with Methanol

Ethanol precipitation was proposed and evaluated as a possible method for the separation of the biomass and the polymer from the culture medium instead of centrifugation. Mixtures of the fermented broth after the sterilization and ethanol (96% of purity) were made at different concentrations (as shown in Table 1) and left at 4°C for 24 hours in 250 ml glass bottles to be able to see the content. After that the polymer was dried to remove the residual ethanol and purified to evaluate the polymer quality.

Table 1: Experimental design for the ethanol precipitation of the culture broth solids.

EXP	1	2	3	4	5	6	7	8	9	10
%EtOH (v/v)	0	0	0	0	0	10	30	50	70	90
%Water (v/v)	10	30	50	70	90	0	0	0	0	0
% PHB broth (v/v)	90	70	50	30	10	90	70	50	30	10

2.4 Flushing with Methanol

Flushing of the solid material with Methanol were performed to evaluate the removal of residues that caused a yellowish color and a strong odor to the material, and that couldn't be eliminated using the chloroform purification.

The solid material after drying and milling was diluted in the solvent in a 10 ml gr^{-1} relation. The mixture was stirred at 500 rpm for 1 hour and then vacuum filtered with a 0.22 micron membrane. The liquid phase was the

solvent with residual oil from the fermentation, the solid phase was dried at room temperature for 24 hours, gravimetric test were performed to determine the mass lost after this purification process, and finally the dry powder was purified with chloroform and characterized.

2.5 Thermal Characterization

Thermo-gravimetric (TGA) essays were performed to evaluate the thermal stability of the polymers obtained. Samples between 5 and 10 mg were heated from room temperature to 500°C at 20°C min^{-1} with a nitrogen flow rate of 50 mL min^{-1} in a Mettler Toledo 3 TGA system. Then the sample mass loss was plotted against the temperature, and degradation temperatures of the polymer were determined. Also, the purity of the polymer can be measured indirectly evaluating the residual mass after the purification process. On the other hand, Differential scanning calorimetry (DSC) was performed using a Mettler Toledo DSC analyzer on samples of about 5mg. First, the sample was heated at a rate of 20°C min^{-1} to 200°C to erase the thermal history, then cooled at a rate of 50°C min^{-1} to -30°C, then heated again at 10°C min^{-1} to 200°C and finally cooled at a rate of 20°C min^{-1} under a nitrogen atmosphere with 50 ml min^{-1} flow rate. Melting temperature (T_m), crystallization temperature (T_c), melting enthalpy(ΔH_m), crystallization enthalpy(ΔH_c), and cristallinity degree (X_c) were calculated. The X_c was calculated according to the following equation (Uzun & Aydemir, 2016):

$$X_c = \frac{\Delta H_f}{\omega \Delta H_f^0} \times 100 \qquad [2]$$

Where ΔH_f is the heat of fusion of the polymer, $\Delta H_f^0 = 146\, J\, g^{-1}$ is the heat of fusion of 100% crystalline PHB (Uzun & Aydemir, 2016) and ω is the PHB mass fraction of the material, in this case is 1 for all of them.

3. Results and Discussion

3.1 Ethanol Precipitation

When an organic polar solvent such as ethanol is added to an aqueous solution of proteins (in this case the fermented culture medium), the macromolecules tend to agglomerate and subsequently to precipitate. This phenomenon is mainly due to the fact that organic polar solvents present a dielectric constant lower than the one of water, which generates an increase in the attraction between opposite charges and a decrease in the degree of ionization of the protein radicals and consequently a decrease in the solubility of proteins in the medium (Tejeda et al., 2011). The bacterial cell membrane is rich in proteins, so the precipitation of the cells and the polymer is possible using this kind of systems. Different solvent-polymer ratios were evaluated as shown in table and the percentage of precipitated solid was calculated taking in to account that the solid content of the broth measured by dry weight was 11.7 g/L as shown in Table 2. Additionally, water-polymer mixtures were made as blank. The best results were obtained for mixtures with alcohol concentrations higher that 70% (v/v) as shown in Figure 1.

Table 2: Results of biomass and PHA ethanol precipitation

EXP	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
%EtOH (v/v)	0	0	0	0	0	10	30	50	70	90
%Water (v/v)	10	30	50	70	90	0	0	0	0	0
% PHB broth (v/v)	90	70	50	30	10	90	70	50	30	10
Dry Solids (g)	0.139	0.084	0.194	0.125	0.058	0.196	0.246	0.626	0.558	0.202
Precipitated Solids (%)	6.64	5.12	16.6	17.9	24.8	9.3	15.0	53.5	79.5	86.3

When the precipitation step was performed after the digestion step, a new phase was generated, a solid supernatant as shown in the right photo of the Figure 1.

Chloroform extraction was performed to obtain polymer from the solid with no success because this solid phase is lysed biomass without polymer that is separated by gravity from the rest due to its low molecular weight.

The yellowish color of the liquid phase is generated due to residual oil and secondary metabolites solubilization in the alcohol. The ethanol used was recovered through a distillation process and 11L of ethanol with a purity degree of 88% were recovered from the initial 14L of azeotropic ethanol used for all the essays.

3.1 Flushing with Methanol

The materials obtained from chloroform purification had poor mechanical properties, bad appearance and were yellowish and has a strong odor generated by the remaining oil, biomass and secondary metabolites in the polymeric matrix. These characteristics of the material obtained were due to the capacity of chloroform to solubilize the lipid part of non-PHA cell mass (NPCM) and the non-consumed carbon source instead of only the polymer.

Polymeric films with high content of remaining oil had bad mechanic properties and appearance. The reason for this is that as the polymer, the oil is soluble in chloroform, and during the vacuum extraction the polymer was separated from the biomass but no from the residual oil. The washes with organic polar solvents like methanol are a good way to avoid this problem because the oil is soluble in this kind of solvents (Zhou et al., 2006) but the polymer is not. The polymeric films made with the material that was flushed with methanol where colorless and odorless.

Also, a gravimetric evaluation of the mass loss during the process was performed to determine the mass percentage removed from the powder after the wash and before the chloroform purification. The results showed that the solvent remove between 30% and 32% of the initial mass, it means that around 30% of the initial mass of the powder was remaining oil from the fermentation.

3.2 DSC and TGA Results

In Figure 2b TGA curves for polymer purified with chloroform, polymer flushed with methanol, polymer precipitated with ethanol and the commercial sample are represented, the curve of the material purified with chloroform has a significant decrease in the slope at 300°C because everything that had to be degraded at that temperature (i.e the polymer) was already degraded. It means that there is an important residual mass at this temperature (around 10%) that was not P3HB, but oil or even residual biomass. The sample precipitated with ethanol show a similar behavior, but the residual mass is lower at any point of the graphic because a significative part of residual oil and impurities were solubilized and removed during the ethanol precipitation.

a) b)

Figure 1: a) precipitation essays after 24 h. b) 3 phase separation with ethanol precipitation after the SDS digestion,

The other two samples do not have this behavior, it can be seen clearly how the mass decreases to its minimum point between 250°C and 320°C for the commercial sample and the organic polar solvent treatments while the sample purified with chloroform reaches it minimum point at almost 450°C. The table 3 show the thermal characteristics of each sample, T_{90} is the temperature in which the 90% of the sample mass has been degraded, Res is the final residual mass.

It can be seen that the final residual mass for the chloroform sample is also higher than the other ones, however, the final residual mass of the commercial sample is much lower than the ones of the samples produced by fermentation, it means that is possible to purify the polymer even more to remove that residual mass that must be some fermentation remainder. Also, the thermal behavior of the samples treated with

ethanol and methanol is closer to the commercial sample than the one treated with chloroform, confirming the previously discussed.

The DSC technique measures the energy required to raise the temperature of the sample, in this way is possible to detect latent heat points. The latent heat points are energy peaks; this means that a high amount of energy was invested to raise the temperature of the sample at this point because the energy was being used for a phase change instead of heating the sample. The peaks shown in figure 2a around 170°C represent the melting point if the polymer, the area under the peak corresponds to phase-change enthalpy, in this case the fusion enthalpy ΔH_m.

Table 3 shows the fusion point T_m, the fusion enthalpy ΔH_m and the crystallinity degree X_c of each sample. It can be seen that the T_m of the commercial sample and the on threated with methanol are very similar, while the T_m of the chloroform purified sample was slightly lower. All the treatments evaluated lead to different crystallinity degrees, suggesting that the crystallization behavior of the PHB granules depends on the extraction technique used. This result agrees with other works that suggest that the crystallinity of PHB can change with modifications in the conditions of the surrounding medium (Lauzier et al., 1992). According to these, the treatment with mild polar organic solvents like ethanol or methanol tends to increase the crystallinity degree of the polymer.

Table 3: Thermal characterization of the samples

Sample	$T_m[°C]$	$\Delta Hm[J\ g^{-1}]$	$X_c[\%]$	$T_{90}[\%]$	Res. [%]
P3HB(METOH)	174.9	78	53.4	281.3	2.07
P3HB(ETOH)	169.4	70	47.9	300.3	1.91
P3HB($CHCl_3$)	169.2	66	45.2	300.6	3.11
P3HB(SIGMA ALDRICH)	174.8	65	44.4	305.5	0.03

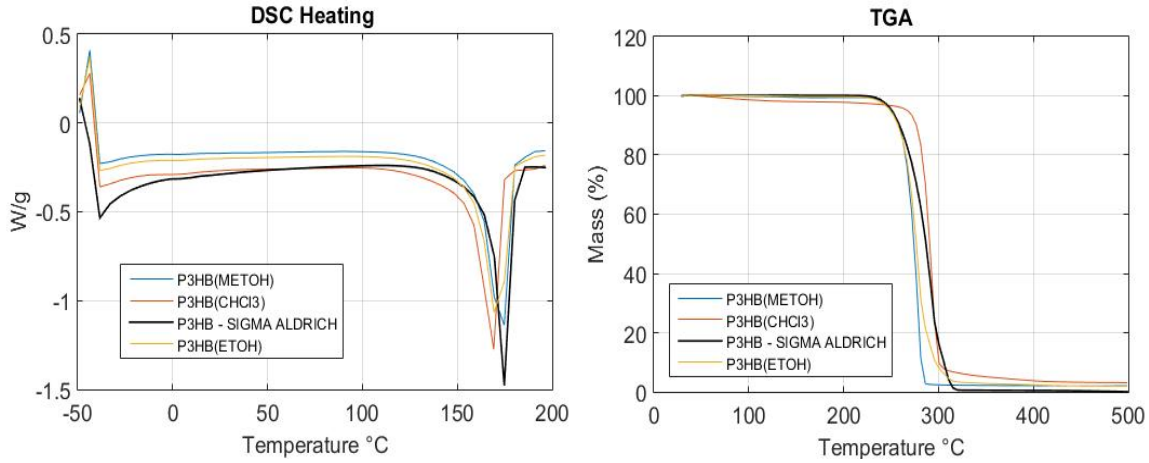

Figure 2: a) precipitation essays after 24 h. b) 3 phase separation with ethanol precipitation after the SDS digestion.

4. Conclusions

The use of organic polar solvents instead of chloroform in downstream treatment of PHB production is a viable option, which allows a large amount of fermentation waste removal. Additionally, the solvents can be easily recovered by distillation with relatively low energy expenditure due to the low boiling points. However, the use of chloroform in the process has to be removed because it antagonizes the character of P3HB biosynthesis as “green” technology. On the other hand, the thermal behavior of the polymer is strongly linked to the extraction and purification processes used. The different treatments evaluated in this work resulted in polymers with different melting points, degradation temperatures and crystallinity indexes, even though all the treatments started from exactly the same material.

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