EVALUATION OF POLYESTERS PRODUCTION IN SUBMERGED CULTIVATION OF CUPRIAVIDUS NECATOR AND BURKHOLDERIA CEPACIA STRAINS USING SUNFLOWER SEED AS SUBSTRATE

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Abstract- Microbiologically produced polyesters are known as polyhydroxyalkanoates (PHA) which are an alternative to reduce the amount of non-degradable plastics discarded on the environment. The PHA can be produced from renewable raw materials such as glucose and sucrose, however, these sugars represent a high cost in the production chain and the use of cheaper substrates has been largely studied and broadcasted. In this paper, we proposed the evaluation of polyhydroxyalkanoates production using sunflower seed as an alternative substrate for the submerged cultivation of the strains Cupriavidus necator IPT 026 and 027, and Burkholderia cepacia IPT 119 and 400. All microorganisms tested were able to utilize sunflower seed to synthetize PHA, the highest polymer production was achieved using C. necator IPT 026 which exhibited 4.38 ± 0.17 g l^{-1} of PHA with a concentration of 15 g l^{-1} of substrate in the fermentation medium. All PHA produced showed the bands of the characteristic functional groups of polyesters in the FTIR spectra.

Index Terms- PHA, biopolymer, biosynthesis, sunflower seed, alternative substrate.

I. INTRODUCTION

Synthetic plastics are widely used in daily life due to its low cost, versatility, durability, ease of processing and resistance. The global market for thermoplastic resins is huge, according to Plastics Europe [1], in 2014 were produced worldwide 250 million tons of these materials, of which the Brazilian market contributed with a share of 6.5 million tons. However, the durability associated with the non-biodegradability of the synthetic polymers compromises the environmental sustainability since its accumulation on the planet causes pronounced damage to the ecosystems.

Therefore, the replacement, at competitive costs, of synthetic materials for biodegradable ones produced from renewable sources is an emerging necessity in the current world scenario. The production of bacterial biopolymers is an alternative to this demand, especially the polyhydroxyalkanoates (PHA), which can replace conventional thermoplastics. PHA are a family of polyesters

completely synthesized by microorganisms, with more than 150 different monomers observed, which allows the development of several technological applications [2], [3].

The bacterial production of PHA occurs intracellularly when there is excess carbon source and limitation of at least one nutrient required for cell multiplication such as nitrogen, phosphorous or magnesium [4]. The carbon sources used for industrial production of PHA are usually glucose and sucrose, however, one of the biggest barriers to increase its production is the substrate cost, which can reach up to 50% of the total fabrication expenses [5].

As an attempt to increase economic viability, low cost alternative carbon sources have been investigated for PHA production such as agricultural products and residues, lignocellulosic materials, palm oils and sugar cane molasses [6], [7]. In the present paper sunflower seed was tested for PHA biosynthesis catalyzed by different bacteria to identify promising strains for submerged cultivation.

Sunflower seed is a low cost substrate with good availability in Brazil. Composed mainly of fatty acids it is nutritionally promising as carbon source for a variety of biotechnological applications.

II. METHODS

Bacterial strains

C. necator IPT 026 and IPT 027, and B. cepacia IPT 400 and ITP 119 were provided by the Institute for Technological Research (IPT), in São Paulo, Brazil. Stock cultures were grown at 33°C in Nutrient Broth, maintained on Nutrient Agar (NA) at 4°C and subcultured monthly.

Main carbon source

Sunflower seed was acquired in the trade market of Salvador-BA, Brazil, milled and stored at 4°C for preservation.

PHA production

Culture media

Microorganisms were stored below 5°C in NA consisting of 5.0 g l^{-1} meat peptone, 3.0 g l^{-1} beef extract, and 3.75 g l^{-1} agar. Replating was performed every 15 days, and inoculation was performed in Nutrient Broth (NB, 5.0 g l^{-1} bacteriological peptone, 3.0 g l^{-1} beef extract and distilled water) over a 24h period at 30°C.

PHA were produced using two cultures according to Campos [7]. Mineral media without nitrogen limitation was used as the First Culture (FC), and similar mineral media with nitrogen limitation was used as the Second Culture (SC). The FC and SC were composed of nitrilotriacetic acid (19.1 g l^{-1}), ferrous ammonium citrate (10 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (50 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (5 g l^{-1}), $(NH_4)_2SO_4$ (200 g l^{-1} in FC and 50 g l^{-1} in SC), $Na_2HPO_4 \cdot 12H_2O$ (223.8 g l^{-1}), and the main carbon source (15 g l^{-1}). The pH of the media was adjusted to 7.0 with 10 mol l^{-1} NaOH or 10 mol l^{-1} HCl solutions.

Shakerflask cultivation

Experiments were performed in triplicate in 250 ml Erlenmeyers containing 50 ml of nutrient broth medium and 2 ml of pre-culture inoculum, which was incubated at 30°C in a shaker flask without baffles for 24 h (best cell concentration, 10^{11} UFC/ml) at 150 rpm. Two cultures were prepared (with and without nitrogen limitation) in 250 ml shaker flasks without baffles containing 80 ml of mineral medium and 10% (v/v) of the previous culture at 35°C for 72 h at 150 rpm [7].

Cellular separation from the culture medium

Cell cultures were harvested by centrifugation at 15,700×g for 30 min at 5°C (HITACHI, model CR 22G), washed twice with distilled water, transferred into round bottom flasks (50 ml), and frozen at -80°C for subsequent lyophilization (LIOBRAS model L101) at -42°C for 24 h. PHA extraction from the freeze-dried cells was performed using chloroform at 60°C for 2h with vigorous shaking on a magnetic stirrer plate with heating (model IKA HS 7) [7]. PHA production (obtained after extraction) was calculated using a gravimetric method and expressed as g l^{-1}.

PHA characterization

Characterization of PHA functional groups was determined by Fourier Transform InfraRed spectroscopy (FTIR) using the Perkin Elmer equipment, Model Spectrum 100 (Waltham, Massachusetts, USA) in the range of 4,000 cm^{-1} to 600 cm^{-1} by the technique of Attenuated Total Reflectance (ATR) at 600 cm^{-1} frequency.

Data processing

All experiments were performed in three replicates with standard deviation calculated. Statistical analysis was performed using the tools available in the Statistica 8.0 (STATSOFT Inc., Tulsa) software.

III. RESULTS AND DISCUSSION

Table 1 shows the results obtained for PHA production for each microorganism evaluated. It is possible to see that strain C. necator ITP 026 presented the highest biopolymer production, 4.38 ± 0.17 g l^{-1}, followed by B. cepacia ITP 119 which produced 3.48 ± 0.28 g l^{-1} of PHA. C. necator IPT 027 and B. cepacia IPT 400 showed no statistically difference for polymeric productivity. The variance analysis for the productive performance of each strain studied is displayed in Table 2.

Table 1: Data for PHA production in the fermentation of sunflower seed in a concentration of 15 g L -1 over 72 hours of incubation, pH 7.0 and rotation of 150 rpm.

Microrganism	PHA (g L^{-1})
C. necator IPT 026	4,38±0,17[a]
C. necator IPT 027	2,60±0,40[b]
B. cepacia IPT 119	3,48±0,28[c]
B. cepacia IPT 400	2,53±0,09[b]

Averages followed by different letters, in the upper right show differences between the strains (P<0.05) by Tukey's test.

Table 2: Variance analysis (ANOVA) for PHA production from different bacterial strains.

Source	SS	df	MS	F	P
Treatment	6.8486	3	2.2829	21.97	0.000323
Error	0.8314	8	0.1039		
Total	7.68	11			

Reference [7] reported a production of 0.32 g l^{-1} of PHA in the submerged cultivation of C. necator ITP 026 using glucose as carbon source, under the same process conditions run in this paper, which shows the advantage in using sunflower seed as substrate alternatively to the traditional use of glucose. The substantial difference of polymer production in the use of the different carbon sources can be explained by the more complex nutrient composition of sunflower seed that would have offered better conditions for the C. necator ITP 026's development reflecting in greater cell multiplication and polymer accumulation.

The characterization of the PHA's functional groups executed by spectroscopy in the infrared spectra (FTIR) in the range of 4,000 cm^{-1} to 600 cm^{-1} is displayed in Figure 1 for the polymers obtained from the four strains evaluated.

The characteristic bands of PHA documented in the scientific literature [8]-[10], such as the axial deformation of the carbonyl (C=O) in 1,750 cm^{-1} and the peak assigned to vibration of the carbonyl of the ester group in 950 cm^{-1} are observed. As well as the bands for symmetric angular deformation in the plane of methyl groups in 1250 cm^{-1} and for the stretching vibration of the carbon-hydrogen bond (C-H) in 2,950 cm^{-1} and 2,895 cm^{-1}.

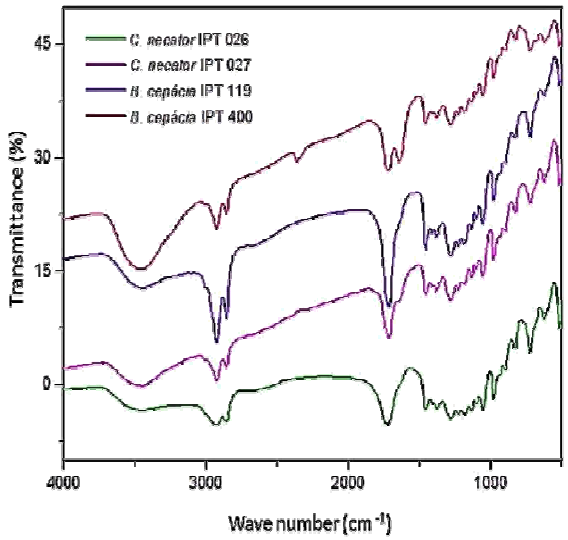


Figure 1: FTIR spectra of PHA produced by C. necator IPT 026 and 027, and B. cepacia IPT 119 and 400 in the fermentation of sunflower seed.

Sunflower seed is a commercially available and inexpensive substrate that can be used by Cupriavidus necator ITP 026 and ITP 027, and Burkholderia cepacia ITP 119 and ITP 400 in the synthesis of PHA.

CONCLUSION

Sunflower seed is a low cost raw material highly available in Brazil and with potential to be used in the fabrication of high-added value products. The strains Cupriavidus necator IPT 026/027 and Burkholderia cepacia IPT 400/119 can assimilate sunflower seed and accumulate PHA within their cells.

Cupriavidus necator IPT 026 presented the highest PHA production among the microorganisms evaluated. The referred production was substantially greater than what is reported in literature for the same process conditions using glucose as carbon source. Therefore, optimization of process variables must be performed to maximize the synthesis of biopolymers and application of the strain in large-scale PHA production.

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