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Production of Polyhydroxyalkanoates using Volatile Fatty Acids from Municipal Wastewater Treatment Plant Sludge

Jeniffer Gracia[a*], Carlos Montenegro[a], Nubia Moreno[b], Iván Cabeza[c*]

[a] Universidad Distrital Francisco José de Caldas, Bogotá, Carrera 7 No. 40B-53, Colombia
[b] Instituto de Biotecnología, Universidad Nacional de Colombia, Bogotá, Carrera 45 N° 26-85, Colombia
[c] Facultad de ingeniería, Departamento de procesos químicos y biotecnológicos, Universidad de la Sabana, Km. 7, Autopista Norte de Bogotá. Chía, Cundinamarca
jpgraciar@udistrital.edu.co; ivan.cabeza@unisabana.edu.co

The massive and global use of plastic elements causes serious environmental, economic and social problems for aquatic fauna, fishery, and tourism. This makes it necessary to consider alternatives to replace conventional plastics for easily biodegradable bioplastics made from renewable raw materials. However, the cost of production of so-called green plastics is high compared to the conventional plastics industry, causing the implementation and development of this type of material to be limited. This work aims to develop a sustainable process for the production of polyhydroxyalkanoates (PHAs) using as carbon source volatile fatty acids (VFAs) from sludge from wastewater treatment plants of WWTP El Salitre. The concentrations to complete the 1 g/L VFAs condition were: acetic acid 0.722 g/L; propionic acid 0.208 g/L and butyric acid 0.098 g/L; for 0.7 g/L VFAs: acetic acid 0. 505 g/L; propionic acid 0.145 g/L and butyric acid 0.068 g/L and 0.5 g/L VFAs: acetic acid 0.361 g/L; propionic acid 0.104 g/L and butyric acid 0.049 g/L. The percentages used in each mixture were: 69% acetic acid, 21% propionic acid, and 10% butyric acid respectively. The fermentations were carried out in a shaker incubator at 30°C. The tests were carried out at a flask scale and the biomass and PHA concentration were determined. The best percentage of polyhydroxyalkanoates accumulation concerning the *Pseudomonas Burkholderia cepacia 2G-57* Biomass was 91%, using 0.7 g/L of volatile fatty acids as a carbon source, and using mixed cultures from the WWTP was 86% with the same concentration of VFAs.
Keywords: Polyhydroxyalkanoates, Volatile fatty acids, Sewage sludge.

1. Introduction

Environmental pollution from plastics is a global concern. This environmental problem stemming from the accumulation of non-biodegradable plastic waste has reached 25 million metric tons per year (Murray & King, 2012; Somleva et al., 2013). The high volume of production and the long permanence in the environment due to the lack of naturally occurring enzymes that allow its efficient degradation, makes the final disposal of this type of material difficult, which is why it has become an increasingly alarming pollution problem (Méndez et al., 2016; Sudesh et al., 2011). Plastic pollution in the oceans threatens marine species, tourism, and fisheries, increasing carbon footprint as well as having economic and social impacts (Chow et al., 2017).
Due to the above, the generation of biodegradable and biocompatible bioplastics, some made from renewable raw materials, stands out as an alternative solution. Some biopolymers are bio-based polymeric materials generated by the biomass of microorganisms (Jha & Kumar, 2019). Polyhydroxyalkanoates PHAs, a family of bacterial polyesters, have been developed by the industry to replace synthetic polymers (Hao et al., 2018). To produce polyhydroxyalkanoates, it is common to use methods of isolating pure bacterial strains to accumulate these polyesters, which are thermoplastics and are produced by numerous microorganisms under different culture conditions (Lai et al., 2013). However, the high production costs of polyhydroxyalkanoates compared to petroleum-based plastics, resulting from the use of sterile equipment and substrates that guarantee the purity of the fermentations, have limited the development of technology for the industrial production of PHAs (Choi & Lee, 1999).

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Then, considerable attention has been devoted to cost reduction of the process by using mixed microbial cultures and carbon-rich waste substrates (Albuquerque et al., 2010; Hanson et al., 2016; Huang et al., 2017; Tao et al., 2016). A promising strategy for reducing the cost of producing polyhydroxyalkanoates is to use mixed microbial cultures such as sludge from wastewater treatment plants (Morgan-Sagastume et al., 2014). Accordingly, it is important to consider sustainable feedstocks in the production of polyhydroxyalkanoates. An eco-efficient feedstock should be renewable biomass, economical with low cost, carbon-rich and close to the plant. Therefore, selecting feedstock based on sustainability criteria favours a more efficient process, with low environmental impact and with an excellent yield in the production of polyhydroxyalkanoates (Yukesh Kannah et al, 2022). The purpose of this research was to the production of polyhydroxyalkanoates using as a carbon source the bio-produced volatile fatty acids by anaerobic fermentations using primary sludge from the wastewater treatment plant El Salitre-Bogotá, Colombia, and to establish the comparisons of the production yields of PHAs using mixed cultures of the activated sludge from the WWTP Salitre and the pure strain of the bacterium *Pseudomonas Burkholderia 2G-57.*

2. Material and methods

2.1 Fermentation

For the fermentations, a culture medium was prepared, mainly composed of inorganic compounds necessary for the micro-organisms to grow. The culture medium was prepared with (g/L) KH_2PO_4 2.65, Na_2HPO_4 3.39, $(NH4)_2SO_4$ 2.8, $MgSO_4$ 0.3 and micronutrients containing (g/L) $FeSO_4$ 2, $CaCl_2$ 2, $CoCl_2$-$6H_2O$ 0,2, $CuCl_2$-2H2O 0,01, $NiCl_3$-$6H_2O$ 0,2, $MnCl_2$-$4H_2O$ 0,03, $ZnSO_4$-$7H_2O$ 0,1, H_3BO_3 0,3, $NaMoO_4$. $2H_2O$ 0.03, dissolved in 1N HCl was added at 0.2% (v/v) to the culture medium and sterilized before inoculation (Mendez et al., 2016). The entire culture medium was adjusted to pH 7 (± 0,1) with NaOH solution 1N. The synthetic volatile fatty acids: acetic acid, propionic acid and butyric acid at 99% purity in three different concentrations were used as carbon sources, as shown in Table 1 since inhibition of microbial growth was identified at a concentration of 1.0 g/L and higher. These were the main VFAs obtained in the acidogenic fermentation of digested and primary sludge from WWTP El Salitre (Bogotá-Colombia) in previous experiments by Gracia et al, (2020) and identified by Agilent 7890A Gas Chromatography, in proportions of 70% acetic acid, 30% propionic acid and 10% butyric acid.

Table 1: Conditions of VFAs as a carbon source for biomass and PHAs production

Concentration Carbon Source	Volatile Fatty Acids VFAs	mgCOD VFAs/L
1.0 g/L	Acetic Acid	0.74
	Propionic Acid	0.32
	Butyric Acid	0.19
0.7 g/L	Acetic Acid	0.37
	Propionic Acid	0.16
	Butyric Acid	0.09
0.5 g/L	Acetic Acid	0.52
	Propionic Acid	0.22
	Butyric Acid	0.13

2.2 Selection of mixed micro-organisms

From the activated sludge sample from the WWTP, serial dilutions up to 10^8 were made, then inoculated on seven selective media, named: 1. MacConkey, 2. Cetrimide, 3. Nutrient Agar, 4. Potato Dextrose Agar for Microbiology - PDA, 5. Brilliant Green, 6. Salmonella Shiguella SS and 7. Nutrient medium for PHAs (described in paragraph 2.1). The boxes were incubated at 30°C for 5 days, taking into account the morphology of the colonies in the different media, these were isolated and inoculated again in Tryptone Soy Agar TSA medium and PHA growth medium with 0.5 g/L VFAs and incubated for 5 days at 30°C. Twenty colonies were obtained in the PHA medium using VFAs as a carbon source and were used to prepare the inoculum stock.

To avoid genetic mutations and to ensure homogeneous biomass concentration during inoculum preparation, a working stock was prepared in 100 Eppendorf tubes each containing 2 mL. The bacteria were propagated in an LB medium with glycerol as a cryo-converter and frozen at a temperature of 3°C. This was done for the bacterium *Pseudomonas Burkholderia 2G57* and in the case of the mixed micro-organisms selected from the activated sludge of the WWTP, a stock of 5 tubes of each microorganism was made, for a total of 100.

2.3 Inoculum train

A factorial design was determined, with the independent variable being carbon source and the response variables biomass and PHA concentration, to complete three combinations in triplicate. Additionally, positive control was maintained using sucrose as a carbon source, for a total of 12 flasks. An inoculum train was performed to ensure that the inoculation in the final fermentation volume was adequate for the adaptation and development of the microorganisms in their exponential phase. The train was started in 200 mL flasks with a working volume of 100 mL with LB medium in which 100 µL of the microorganism *P. Burkholderia 2G-57* were placed for the experiments with the pure strain and the mixed cultures, 100 µL of the 20 microorganisms selected as indicated in section 2.2, were incubated under agitation at 150 rpm and a temperature of 32°C in an Orbital Shaker HD 3000 for 24h. After 24 hours, 50 mL of the fermented broth in LB medium was taken and transferred to 1 L flasks, with a volume of 500 mL of the culture medium described in section 2.1 with the addition of 10g/L sucrose as carbon source and incubated for 24 hours on the Orbital Shaker at 150 rpm and 32°C. Finally, 100 mL of the fermented broth was taken from the sucrose-containing culture medium and placed in 2 L flasks, with a volume of 1 L of the culture medium using the VFAs as a carbon source at the three concentrations described in Table 1. Fermentation was carried out for 72 hours at 32°C with constant shaking at 150 rpm and samples were taken in duplicate every 12 hours to show the evolution over time. The procedure was the same for both case studies.

2.4 Biomass and PHA quantification.

To quantify biomass, samples of 3 mL of the fermented broth were taken every 12 hours and centrifuged at 5000 rpm for 10 min in a Thermo Scientific Sovall centrifuge. The precipitate was washed twice with 3 mL of distilled water, collected and dried at 80°C until it reached a stable weight (Yang et al. 2006).
PHA quantification was developed through the gravimetric method (Mendez et al, 2016). Two samples of 3 mL of the fermented broth, were centrifuged at 5000 rpm for 10 min. The precipitate was resuspended, washed and centrifuged with 3 mL of distilled water twice, and then 0.33 mL of sodium dodecyl sulphate (SDS) per biomass was added at 20 % concentration for digestion at 80°C for one hour, the mixture was resuspended, washed and centrifuged with 5 mL of distilled water twice, collected, dried at 80 °C to constant weight.

3. Results and Discussion

3.1 Biomass and PHAs production results using *Pseudomonas Burkholderia 2G-57*

The graphs represented in Figures 1a, b, c and d show the trend of biomass and polyhydroxyalkanoates accumulation at sampling times of 12, 24, 36, 48 and 72 hours. It can be seen that there is an increasing trend from the second day of fermentation onwards and that *Pseudomonas Burkholderia cepacia 2G-57* accumulates PHAs from the biomass. The results reported are the average production in flasks in triplicate and the samples taken in duplicate, additionally, purity tests were performed on TSA agar at each sampling time which guarantees that the production results correspond to *Pseudomonas B2G-57.*

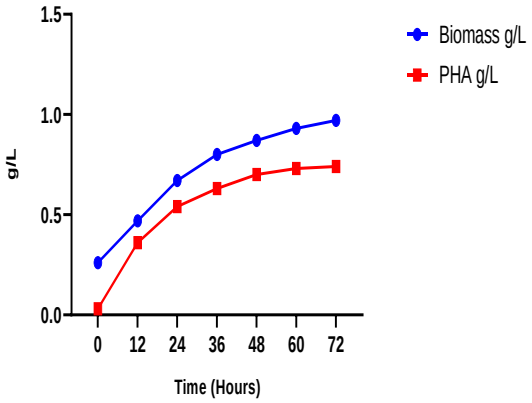

Figure 1a: Biomass and PHAs production in B2G57 using 1.0 g/L VFAs

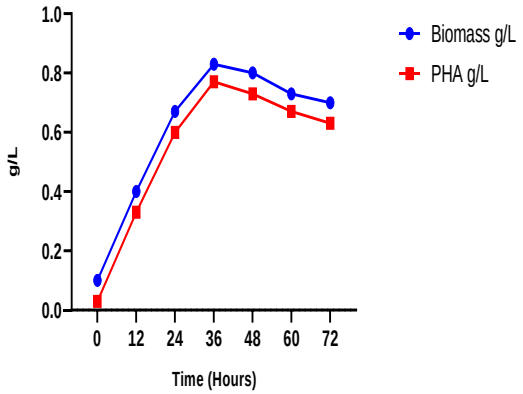

Figure 1b: Biomass and PHAs production in B2G57 using 0.7 g/L VFAs

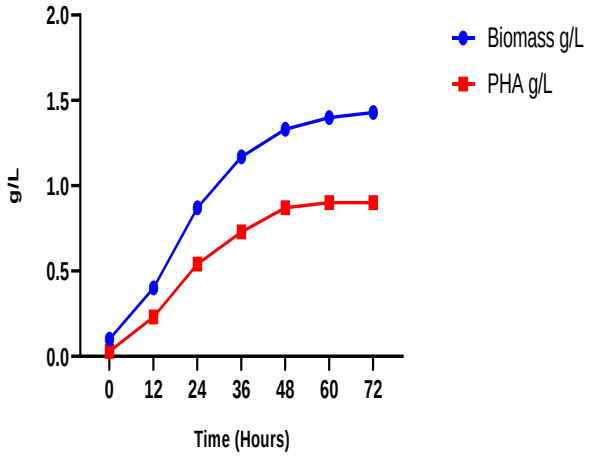

Figure 1c: Biomass and PHAs production in B2G57 using 0.5 g/L VFAs

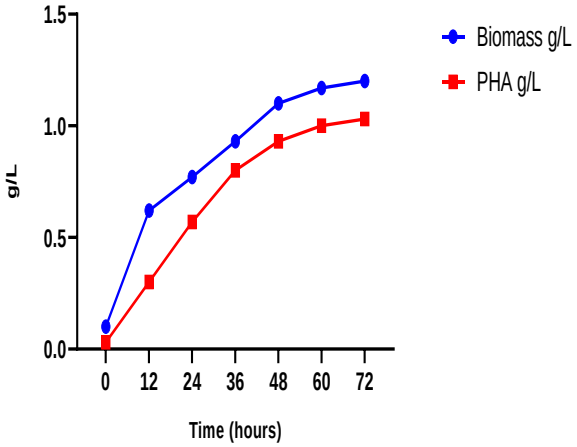

Figure 1d: Biomass and PHAs production in B2G57 using Sucrose

In the polyhydroxyalkanoates accumulation experiments using *Pseudomonas Burkholderia* 2G-57 the best percentage accumulation of PHAs concerning Biomass was 91% and occurred when using 0.7g/L volatile fatty acids as a carbon source, which agrees with the accumulation rates reported by Johnson et al., 2010. On the other hand, when using 1 g/L and 0.5 g/L VFAs as carbon sources, accumulation percentages of 77% and 63% were obtained, respectively, similar to that reported by Albuquerque et al., 2010 and Argiz et al., 2020. Regarding the test using sucrose as a carbon source, 86% was obtained, indicating that VFAs are a viable carbon source for the intercellular accumulation of the biopolymer in *P. Burkholderia cepacia 2G-57*.

3.2 Biomass and PHAs production using mixed cultures from the activated sludge of the Bogotá wastewater treatment plant

Once it was proven that volatile fatty acids types observed in fermentations using primary and digested sewage sludge as the substrate is suitable as a carbon source to obtain polyhydroxyalkanoates, the results of biomass and PHAs production were obtained using the selected mixed cultures from the activated sludge of the WWTP El Salitre - Bogota. Figures 2a, b, c and d show the propensity of accumulation of biomass and polyhydroxyalkanoates from mixed cultures of the activated sludge from WWTP el Salitre during 12, 24, 36, 48 and 72 hours. It is observed that as with *Psudomona Bulkholderia 2G-57*, there is an increasing trend from the second day of fermentation and that the mixed cultures accumulate PHAs from the biomass.

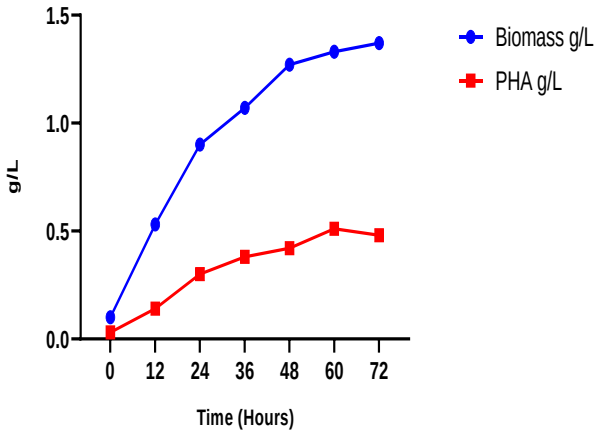

Figure 2a: Biomass and PHAs production in mixed crops using 1.0 g/L VFAs

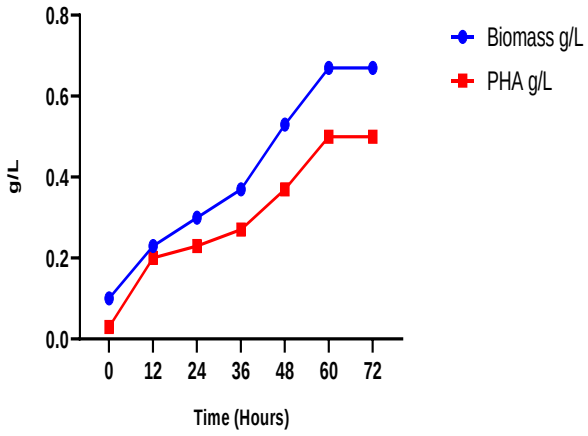

Figure 2b: Biomass and PHAs production in mixed crops using 0.7 g/L VFAs

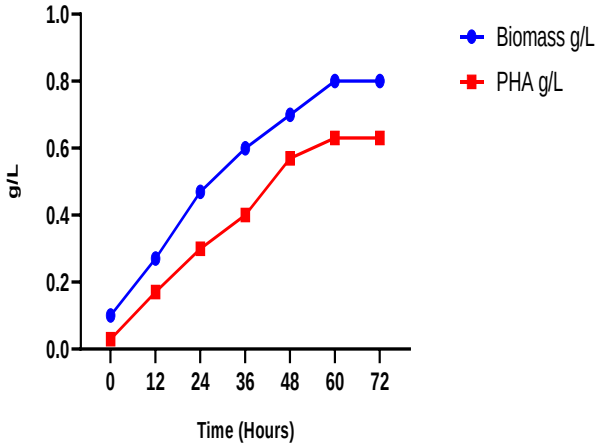

Figure 2c: Biomass and PHAs production in mixed crops using 0.5 g/L VFAs

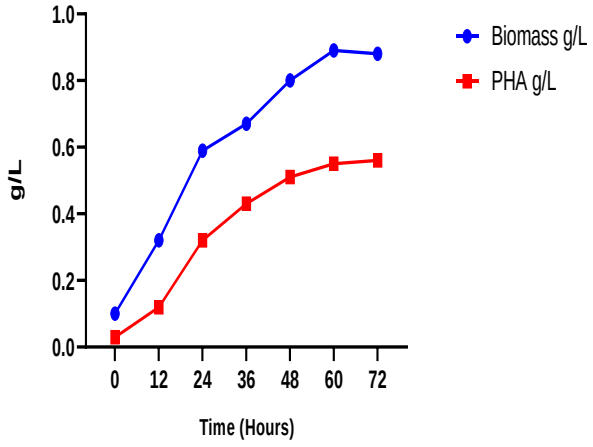

Figure 2d: Biomass and PHAs production in mixed crops using Sucrose

In the experiments of polyhydroxyalkanoates accumulation using mixed cultures from the activated sludge of the wastewater treatment plant El Salitre, the best percentage of PHAs accumulation concerning biomass was 86 % and occurred when 0.7 g/L of volatile fatty acids was used as a carbon source, which coincides with the accumulation percentages reported by Albuquerque et al., 2010, who also used VFAs as carbon source. On the other hand, when using 1 g/L and 0.5 g/L VFAs as carbon sources, accumulation percentages of 38% and 81% were obtained, respectively, similar to those reported by Zhang et al., 2019, Albuquerque et al., 2007; Dionisi et al., 2005 and Chua et al., 2003. For the test using sucrose as a carbon source, 64% was obtained, similar to the results of Jia et al., 2013. This indicates that the 0.7 g/L VFAs as a carbon source better favoured the accumulation of PHAs in the mixed cultures and this coincides with the production of the biopolymer using *Pseudomonas Burkholderia cepacia 2G-57.*

4. Conclusions

The volatile fatty acids obtained from the bio-fermentation of primary and digested sludge from the El Salitre wastewater treatment plant favour the intracellular accumulation of polyhydroxyalkanoate biopolymers when used as a carbon source. The accumulation of PHAs from the activated sludge of El Salitre WWTP ranged from 28% to 86% using mixed cultures. According to the results obtained in the experiments carried out with *Pseudomonas Burkholderia cepacia 2G57* and the mixed cultures, using a concentration of 0.7 g/L maximises the percentage of PHA accumulation concerning the biomass, obtaining between 91% and 86% respectively. Further experiments on the accumulation of PHAs using sustainable bio-based raw materials as carbon sources such as VFAs should be continued, which can decrease the cost of production of bioplastics, therefore, further experiments should be carried out on a larger scale.

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