AGRO PRODUCTIVIDAD

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Polyhydroxyalkanoates from pear (*Pyrus communis* L) waste by *Bacillus subtilis*

Hernández-Manjarrez Cynthia Estefania[1]; Cortés-Sánchez Alejandro De Jesús[2]; Fabela-Morón, Miriam F.[3]; Rayas-Amor Adolfo A.[3]; Díaz-Ramírez Mayra[3*]

[1,3] Universidad Autónoma Metropolitana, Unidad Lerma, Departamento de Ciencias de la Alimentación. División de Ciencias Biológicas y de la Salud, Lerma de Villada, Estado de México, México.

[2] Consejo Nacional de Ciencia y Tecnología (CONACyT). Departamento de Ciencias de la Alimentación, División de Ciencias Biológicas y de la Salud, Universidad Autónoma Metropolitana, Unidad Lerma. Lerma de Villada, Estado de México, México

* Correspondence: m.diaz@correo.ler.uam.mx

ABSTRACT

Objective: Analyze the production of polyhydroxyalkanoates (PHA) by *Bacillus subtilis* sp., *subtilis* in submerged culture using pear residues as a carbon source.

Design/methodology/approach: The culture consisted of pear residue flour concentrations of 5 and 15% for 72 h. Reducing sugars, biomass, pH, protein, and pHA extraction and quantification were analyzed during submerging cultivation of *Bacillus subtilis* sp., *subtilis.*

Results: The results showed that *Bacillus subtilis* sp., *subtilis* can grow and generate PHA, having its maximum proportion in PHA extract of 0.094 g/L at 72h of culture, at a substrate concentration of 5%.

Study limitations/implications: More studies related to the optimization of culture conditions for PHA production are required and prior treatment of pear waste flour will be considered.

Findings/Conclusions: The synthesis of these biopolymers is important to promote their large-scale production using agro-industrial waste, thus contributing to reduce the environmental impact.

Keywords: *Bacillus*, pear, biodegradable plastic, polyhydroxyalkanoates.

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INTRODUCTION

Plastics are called, conventionally, those polymeric products of the petrochemical industry, which are widely used at an industrial level, becoming the basis of most common consumer products due to their elasticity and flexibility properties, easy molding, and resistance (Lemos and Mina, 2015). Nevertheless, these compounds can last before, during and after their use in nature for long periods, due to their chemical properties, making them recalcitrant materials and resistant to biodegradation, accumulating and negatively affecting the environment (González-García *et al.*, 2013). For several years, bioplastics have emerged as potentially substitute materials for petroleum-derived plastics, which can be produced from renewable, biodegradable, and environmentally compatible sources, being the polyhydroxyalkanoates (PHA) among the bioplastics studied (Lemos and Mina, 2015).

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PHAs are polymers of hydroxyalkanoic acids that different microorganisms, such as Gram positive and negative bacteria; they synthesize and accumulate intracellularly as reserve material under adverse culture conditions, to later use it as a carbon and energy source (González-García *et al.*, 2013). They have physical characteristics like those of petroleum-derived plastics, but they can be degraded to carbon dioxide and water under aerobic conditions or to methane in anaerobic conditions in the environment (Lemos and Mina, 2015). For several years, studies on these bio-compounds have focused on the search for substrates, as well as economic production and extraction strategies that allow them to be considered substitutes for chemical plastics, widely spread in the market, and of a polluting nature (Lemos and Mina, 2015). During the processes of production, industrialization and consumption of food, waste and losses are generated, which constitute a worldwide problem, and where the fruit sector (apple, pear, orange, pineapple, etc.) is the most affected (Cervilla *et al.*, 2019) generating air, soil, and water pollution, as well as pests and disease vectors. It has been proposed through various investigations around the world that agro-industrial fruit residues can be exploited and used, through microbial activity, to obtain different value-added products such as biofuels, enzymes, plant growth stimulators, formulation of prebiotics for animal feed (Costa *et al.*, 2009; Serrat *et al.*, 2016). Therefore, the objective of this research is to analyze the production of polyhydroxyalkanoates (PHA) by *Bacillus subtilis* sp., *subtilis* from agro-industrial pear residues (*Pyrus communis* L) in submerged culture.

MATERIALS AND METHODS

Materials. Agro-industrial residue of pear (*Pyrus communis* L) was obtained from the supply center of the city of Toluca, State of Mexico, Mexico. All reagents used were reagent grade.

Pear waste flour. Once the pear (*Pyrus communis* L) waste was collected, it was washed and disinfected, and stored frozen at −18 °C. After, the pear residues were ground in a blender (Oster, México) and was subsequently dried in an oven (Thermo Scientific, USA) at 60 °C/6 days; after, the dry mixture was placed in a blade mill (High-speed multifunction Grinder-Maya 70 -300) and sieved to obtain fine particles. Finally, the sample was stored in a desiccator until use (Aguirre *et al.*, 2018).

Biological material propagation and conservation. The bacteria were inoculated in Tryptic Soy Broth and incubated at 37 °C/24h. Subsequently, 0.5 mL of culture in tubes in Tryptic Soy Broth and 0.5 mL of previously sterilized 50% glycerol were transferred to sterile vials, with an operating volume of 1.5 mL; the content was homogenized and stored at −80 °C.

Starter culture. The starter culture was prepared by adding, at room temperature, the contents of a vial with *B. subtilis* sp., *subtilis* stored at −80 °C, to a flask with culture medium corresponding to Tryptic Soy Broth and incubated at 37 °C/24h.

Preparation of culture medium for PHA production. The culture medium to produce PHA was prepared from the one reported by Du *et al.* (2001), modified with the incorporation of 2 different concentrations of pear waste flour (5% and 15%). Each culture medium was sterilized at 121 °C/15 min prior to use.

Submerged cultivation. 250 mL Erlenmeyer flasks, with an operating volume of 100 mL of culture medium, were used for PHA production. Submerged culture conditions were 35 °C/200 rpm/72h using a shaking incubator (Thermo Scientific, Mod. MAXQ 4000). The pH was adjusted to 7 prior to adding the starter culture of *Bacillus subtilis* sp., *subtilis*, corresponding to 2% of the operation volume, and samples were taken every 24 h for 3 days for the analysis of biomass, pH, reducing sugars, protein, extraction, and PHA quantification.

Reducing sugars; 5 mL of sample were placed in conical tubes and centrifuged at 5000 rpm/15 min; then, the supernatant was separated, and 3 mL of 3,5-dinitro salicylic reagent (Sigma-Aldrich) were added; afterwards, the mixture was heated to 90 °C between 5-15 min and 1 mL of 40% sodium potassium tartrate was added; finally, the reading at 575nm was recorded in a spectrophotometer (Shimadzu UV spectrophotometer UV-1800) (Bello *et al.*, 2006).

Biomass determination. For biomass determination, 10 mL of the medium were placed in conical tubes and centrifuged at 4400 rpm for 10 min at 25 °C. The supernatant was separated for subsequent analyses and the pellet was washed with distilled water, shaken, and centrifuged 3 times. The sediment was placed in aluminum pans at constant weight (90 °C/24 h) and oven-dried at 105 °C/24 h. Biomass was determined by weight difference (Harris, 2001).

pH determination. The pH determination was performed directly using a potentiometer (Thermo Scientific, Orion Star A211).

Protein determination. To quantify the protein, a sample was taken from the supernatant of the biomass determination. This sample was centrifuged at 4400 rpm/10 min and 10 μL were placed in a 96-well microplate and 100 μL of Bradford reagent and the absorbance at 595 nm was recorded in a spectrophotometer (Thermo Scientific, Multiskan GO) (Lozano *et al.*, 2011).

PHA extraction and quantification. The extraction and quantification of PHA consisted of taking 10 mL of culture and placing it in test tubes at constant weight, centrifuging at 4700 rpm for 20 min and discarding the supernatant; then, adding 3 mL of concentrated NaClO to the sediment and allowing it to settle at 37 °C/2h. After that, it is centrifuged again at 4700 rpm/20 min and the pellet obtained is suspended in $CHCl_3$; finally, it is placed in the oven at 60 °C/15 h for the volatilization of the solvent. For quantification, the sample is cooled and the PHA amount was determined by weight difference (Grados, 2020).

Analysis of results. The experiments were done in triplicate. MS-Excel 2014 software was used for data analysis.

RESULTS AND DISCUSSION

Parameters evaluated during submerged culture

Reducing sugars. Figure 1 shows the percentage of consumption of reducing sugars by the microorganism at the end of the bioprocess. The highest consumption of reducing sugars was in concentrations of 5% of the substrate with 89.9% and, finally, 64.8% of consumption of sugars in concentrations of 15% of substrate, identifying that in the lower

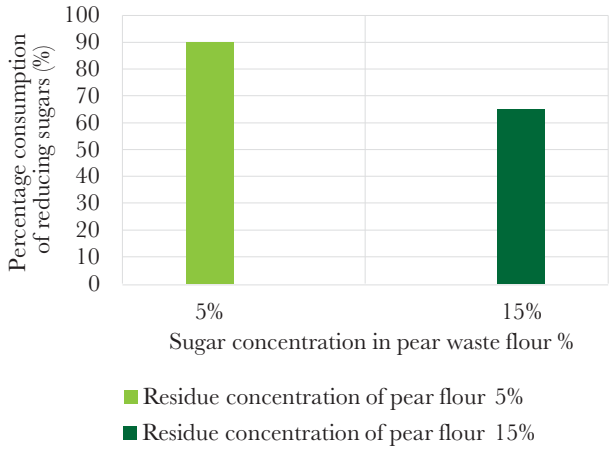

Figure 1. Percentage of reducing sugars consumption at the end of the submerged culture of *Bacillus subtilis* sp., *subtilis* to two different concentrations of pear waste flour (*Pyrus communis* L).

concentration of substrate, the consumption of reducing sugars by the microorganism was greater for the growth, production and accumulation of PHA.

The difference in carbon source consumption may be due to adaptation to culture conditions and substrate proportion by the microbial strain. The carbohydrates supplied by the pear waste flour are the main source of carbon in the culture medium, so the microorganism during the culture establishes a balance between the generated bacteria and non-viable cells. The remaining bacteria tend to consume the source of carbon for survival and generate reserve compounds such as PHA. Growth conditions have been reported to influence PHA formation (Jia *et al.*, 2013), where the metabolic process to produce short-chain PHA is through derivatives of acetyl-CoA. The carbon source is initially converted into thioesters of coenzyme A, with the activity of enzymes 3-ketothiolase (acetyl-CoA acetyltransferase, acetoacetyl-CoA reductase, hydroxybutyryl-CoA dehydrogenase, and poly(3-hydroxybutyrate) synthetase (Akiyama *et al.*, 2003).

Biomass determination. Figure 2 shows the highest growth of *Bacillus subtilis* sp., *subtilis* using the 2 different concentrations of pear waste flour, at 24h of cultivation, at a concentration of 5% pear waste flour with 41±3 g/L to subsequently decrease to 15.5 g/L at 72h. Meanwhile, there was a concentration of 15% of pear residue flour at 24h, which presented an adaptation phase in the first 24h of cultivation, with a decrease in biomass with respect to the initial value to later increase, reaching a maximum at 48h with 25±3g/L.

In related studies, Anjali *et al.* (2014) reported *B. subtilis* AMN1 in culture supplemented with sugarcane molasses from 10 to 100% as carbon source at 30 °C and 150 rpm for 48 h biomass yield dry weight of 2.09 at 1.07 g/100 mL. Meanwhile, Sanabria and Sarmiento (2017), and using potato starch residues at a concentration of 10 and 15g/L for the growth of *B. subtilis* at 35 °C, 15.75 rad/s, pH 7 for 72 h, reported a biomass yield of 0.01 to 0.07 g/ml, at a substrate concentration of 10g/L and from 0.06 to 0.07 g/ml of biomass at a concentration of 15g/L of substrate. The biomass proportions obtained in this study are lower than those reported in related studies. However, it should be noted that the

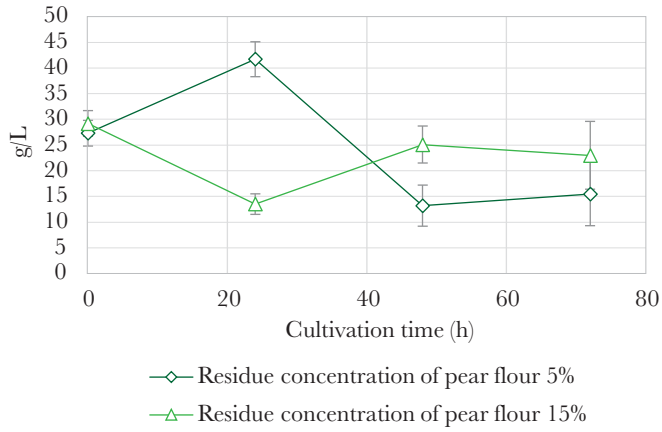

Figure 2. Biomass (g/L) during the growth of *Bacillus subtilis* sp., *subtillis* in submerged culture with different concentrations of pear waste flour (*Pyrus communis* L).

culture conditions (temperature, time, agitation, inoculum, type of carbon and nitrogen source, and treatment) influence the growth and production of PHA by microorganisms (Mohapatra *et al*., 2017).

pH determination. pH is an important factor that must be evaluated during microbial growth in culture. Microorganisms, in the growth phase, assimilate the carbon source consisting mainly of carbohydrates, generate and release various acidic metabolites into the medium, causing a change in the pH of the medium. In the present study, there was a decrease in pH in the 2 study concentrations, where a concentration of 5% of pear waste flour had the greatest decrease with pH 6.27 at 24h and later had an increase to pH 6.9 at 72h; at the same time, at a concentration of 15% it had its lowest decrease at 48h with pH 6.35 to later rise to 6.67 at 72h (Figure 3). The largest decreases in pH at a concentration of 5% at 24h and 15% at 48h are related to the highest proportions of biomass obtained in similar culture times (Figure 2).

Protein determination. Figure 4 shows the protein concentration during the growth of *Bacillus subtillis* sp., *subtillis*, using pear waste flour as substrate. The highest proportions

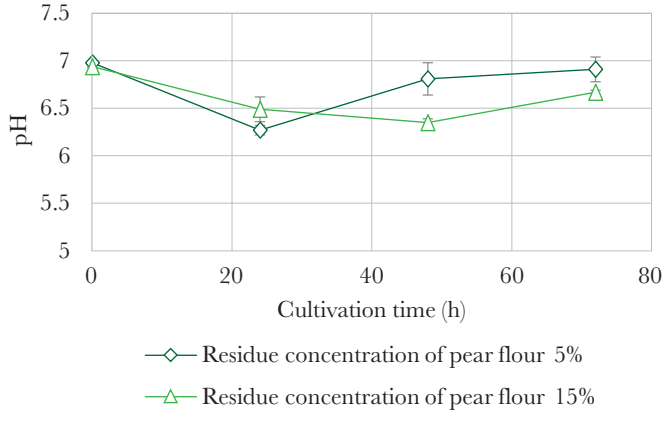

Figure 3. pH of the submerged culture of *Bacillus subtilis* sp., *subtillis* in different concentrations of pear waste flour (*Pyrus communis* L).

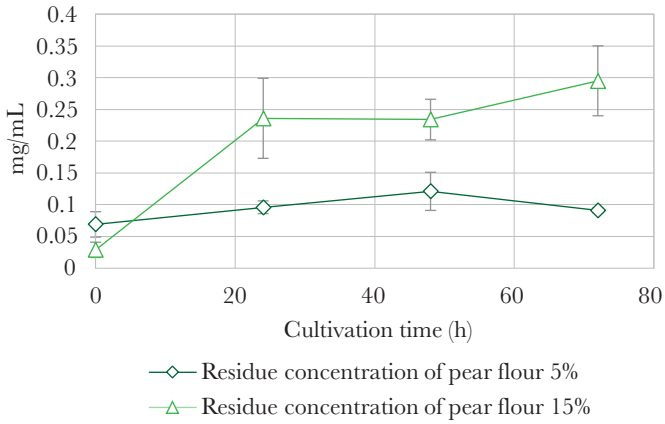

Figure 4. Protein (mg/mL) in submerged culture of *Bacillus subtilis* sp., *subtilis* at different concentrations of pear waste flour (*Pyrus communis* L).

were presented at concentrations of 15% with 0.23 and 0.29 mg/mL at 48 and 72h of culture, followed by 0.096 and 0.121 mg/mL at 24 and 48h, respectively, for the 5% concentration of flour from residues of pear. The protein content in the culture medium may be related to the assimilation of the carbon source for microbial growth and the synthesis of enzymes in the metabolism of the carbon source. *Bacillus* species can secrete a variety of extracellular enzymes, including α-amylases and proteases. Besides, *Bacillus* can use polysaccharides and polypeptides for cell growth and accumulation of PHA directly (Tsuge *et al.*, 2015).

Extraction and quantification of PHA. In the extraction and quantification of PHA during cultivation, there was an increase with respect to time at a substrate concentration of 5% pear residue flour, reaching its maximum at 72 h with 0.094 g/L. Meanwhile, at a concentration of 15%, it presented its maximum proportion of PHA extracted with 0.039 g/L at 24 h to subsequently decrease to 0.023 g/L at 72 h (Figure 5). Between the concentrations of 5 and 15% of pear residue flour, the highest proportion of PHA extracted was at 24 h for a concentration of 5% with 0.094g/L of PHA.

In related studies, Sanabria and Sarmiento (2017), reported the production of PHA by *B. subtilis* in submerged culture for 72 h using potato starch as substrate without prior

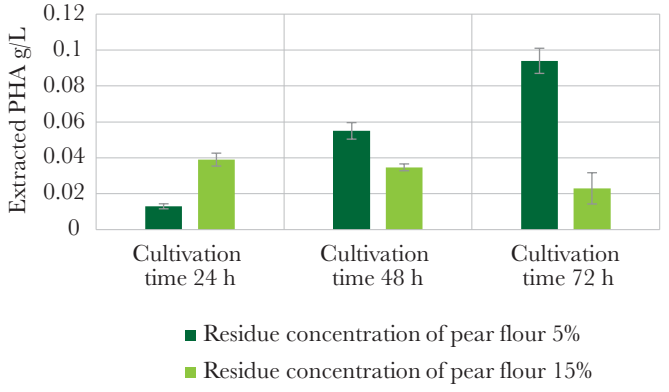

Figure 5. PHA production (g/L) during the growth of *Bacillus subtilis* sp., subtilis in submerged culture from pear waste flour (*Pyrus communis* L) at different concentrations.

hydrolysis treatment of the substrate at concentrations of 5 and 15 g/L, obtaining PHA extracts using extraction method with NaCl, NaOH and ethanol at 24 and 72h from 0.006 to 0.058 g, respectively, in substrate concentration of 5 g/L and 0.43 and 0.12g of PHA at 24 and 72h, respectively, for substrate concentration of 15g/L.

Likewise, the accumulation of intracellular PHA during the submerged culture indicated the tendency to increase as the culture time elapsed at a concentration of 5% substrate, having its maximum of 0.6% at 72h, while at a concentration of 15% substrate the trend was inverse, decreasing during the culture, reaching a minimum of 0.1% at 72h of culture. Between both concentrations of 5 and 15% of substrate, the highest percentage of PHA accumulation occurred at 5% at 72h of culture with 0.6% (Figure 6).

Most bacteria can accumulate PHA to around 30-50% dry cell weight, while *Ralstonia eutropha*, a known PHA producer, is able to accumulate PHA to more than 90% dry cell weight (Tsuge *et al.*, 2015). Several, but not all, species of the genus *Bacillus* sp., can accumulate PHA under unbalanced growth conditions. The species reported as native PHA producers are *B. amyloliquefaciens*, *B. anthracis*, *B. aryabhattai*, *B. badius*, *B. brevis*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. flexus*, *B. laterosporus*, *B. lentus*, *B. licheniformis*, *B. macerans*, *B. megaterium*, *B. mycoides*, *B. odyssey*, *B. pasteurii*, *B. pumilus*, *B. sphaericus*, *B. subtilis*, *B. thuringiensis*, and some isolates of unidentified species (Tsuge *et al.*, 2015).

Bacillus spp. can synthesize PHA in the stationary and exponential growth phases. The accumulation of PHA may, or may not, be associated with growth compared to other producing microorganisms; therefore, when it is not associated with growth, the synthesis occurs in the stationary phase of growth with limitation of N, P, Mg and oxygen and excess carbon source, unlike the production of PHA associated with growth that takes place in balanced conditions (Mohapatra *et al.*, 2017).

For species of the genus *Bacillus* spp., PHA accumulation percentages ranging between 6.43 and 48.2% are generally reported (Santimano *et al.*, 2009). A wide range of cheap carbon sources assimilable by *Bacillus* strains useful to produce PHA has been reported, such as oligosaccharides from soybean molasses, sugarcane molasses, sugarcane bagasse, date syrup, palm oil and fruit bunch hydrolyzate (Tsuge *et al.*, 2015). In this study, accumulation

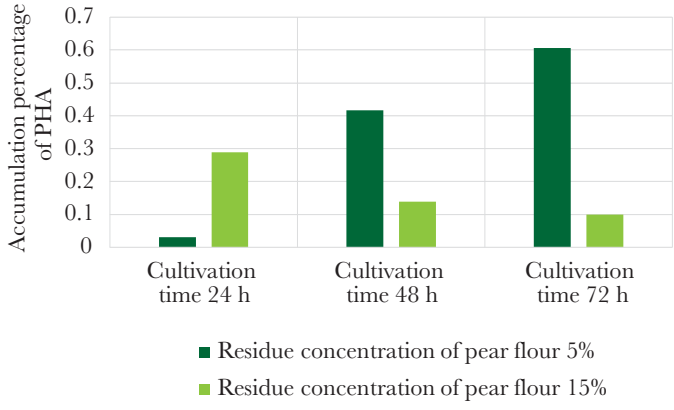

Figure 6. Percentage of accumulation of PHA by *Bacillus subtilis* sp., *subtilis* in submerged culture from pear waste flour (*Pyrus communis* L) at different concentrations.

percentages lower than those reported by other researchers were achieved. It should be noted that for the production of PHA, the use of economic carbon sources has been proposed, including agro-industrial residues (of fibrous and cellulosic composition) and it has been reported that a hydrolysis treatment prior to the culture with microorganisms (which was not the case in this study) for the production of PHA favors the availability and release of metabolizable sugars, and therefore the production of PHA (Rojas *et al.*, 2016) even with respect to common substrates such as starch in strains such as *Bacillus* sp. (Santimano *et al.*, 2009).

CONCLUSIONS

The growth and production of PHA by *B. subtilis* sp., *subtilis* was present in the two concentrations of pear waste flour (*Pyrus communis* L). The use of pear waste flour (*Pyrus communis* L) may help reduce the cost of the substrate and in turn provide value to the waste generated by the fruit industry. However, further studies are required focused on prior treatment of pear waste flour for its use as a carbon source, as well as the optimization of culture conditions to improve production, PHA extraction and characterization.

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