Potentialities of polyhydroxyalkanoate (PHA)-producing bacteria isolated from *Asparagus officinalis* L.

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**Abstract**

The objective was to determine the production of polyhydroxyalkanoates produced by potentially nitrogen-fixing bacteria isolated from *Asparagus officinalis* L., in 2017. The method used was through samples of previously disinfected rhizospheric soil, roots, and stems, they were cultured in nitrogen-free media to isolate rhizospheric and endophytic fixing bacteria, respectively. The production of PHA granules by these bacteria was studied in fermentation broth with glycerol as a carbon source, coloring them with Sudan Black B after 24 and 48 h. The two bacterial cultures in which the largest number of cells with PHA granules was observed were fermented with glycerol in the balanced and unbalanced media for 36 h, determining the biomass. Some of the results were 51.1% of the fixing bacteria were rhizospheric and 48.9% endophytic. PHA granules were detected in 56.67% of the bacteria. The $Y_p/x$ yield was 0.57855 g g$^{-1}$ (*Azospirillum* sp.155) and 0.22543 g g$^{-1}$ (*Azospirillum* sp. 130) in the balanced medium and 0.76718 g g$^{-1}$ (*Azospirillum* sp. 155) and 0.26229 g g$^{-1}$ (*Azospirillum* sp. 130) in the unbalanced medium, after 32 h of fermentation. In conclusion, the production of PHAs by nitrogen-fixing bacteria isolated from asparagus was demonstrated.

**Keywords:** *Azospirillum*, *Burkholderia*, *Herbaspirillum*, polyhydroxyalkanoates.

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Introduction

Polyhydroxyalkanoates (PHAs) are gaining increasing attention in the biodegradable polymer market due to their promising properties, such as high biodegradability in different environments, not only in composting plants, and processing versatility (Bugnicourt et al., 2014). Polyhydroxyalkanoates (PHAs) are synthesized by numerous bacteria as carbon and energy storage compounds and are good candidates for biodegradable plastic material (Lee, 1996). Polyhydroxyalkanoates (PHAs) comprise a wide class of polyesters that are synthesized by many bacteria as an intracellular compound of carbon and energy (Lemos et al., 1998; Zinn et al., 2001; Quillaguamán et al., 2005).

In fact, among biopolymers, these biogenic polyesters represent a potential sustainable replacement for fossil fuel-based thermoplastics (Lee, 1996). Most commercially available PHAs are obtained from pure microbial cultures grown on renewable feedstocks; that is, glucose, under sterile conditions, but recent research studies focus on the use of waste as growth media (Choi and Lee, 1999).

PHA can be extracted from the bacterial cell and then formulated and processed by extrusion for the production of rigid and flexible plastic (Jendrossek, 2009), suitable not only for the most evaluated medical applications, but also for applications including containers, molded products, paper coatings, nonwoven fabrics, adhesives, films, and high-performance additives (Lee, 1996). Bacterial polyhydroxyalkanoates (PHAs) are isotactic polymers that play a fundamental role in central metabolism, as they act as dynamic carbon reservoirs and reducing equivalents.

These polymers have a number of technical applications as they exhibit thermoplastic and elastomeric properties, making them attractive as a replacement for petroleum-derived materials (López et al., 2015). They are produced and accumulated as intracellular granules by different bacterial genera (Días et al., 2006) when, in the medium, there is an abundance of carbon (Dai et al., 2007) and a deficit of elements such as nitrogen, phosphorus or magnesium, among others (Volova, 2004).

PHA-producing bacteria have a versatile metabolism with cellulolytic activity (Grados et al., 2008), hydrolysis of polymers such as starch (Guzmán et al.2017) and diazotrophy or atmospheric nitrogen fixation (Rubio and Perla, 2012; Rojas et al., 2016). PHAs accumulate under conditions of nutritional imbalance (usually an excess of carbon source with respect to a limiting nutrient, such as nitrogen or phosphorus) (López et al., 2015).

Polyhydroxyalkanoate (PHA) producers have been reported to reside in several ecological niches that are naturally or accidentally exposed to high organic matter or limited growing conditions, such as dairy waste, hydrocarbon-contaminated sites, waste from pulp and paper mills, agricultural waste, activated-sludge treatment plants, rhizosphere, and industrial effluents (Singh et al., 2014).
Bacteria are thought to use PHA accumulation to increase survival and stress tolerance in changing environments and in competitive environments where carbon and energy sources may be limited, such as those found in soil and the rhizosphere (Kadouri et al., 2005). In agricultural crops such as *Asparagus officinalis* L. ‘asparagus’, there is a diversity of nitrogen-fixing bacteria (López et al., 1998), which have not yet been studied, with the prospect of obtaining strains with higher yield in the polymer and that can be used in commercial production at low cost.

The cycle of PHA synthesis and degradation has been recognized as an important physiological characteristic when these biochemical pathways were originally described, but their role in bacterial processes as diverse as global regulation and cell survival is only beginning to be fully appreciated (López et al., 2015). Bioremediation and plant growth promotion appear as examples of environmental applications in which PHA accumulation has been successfully exploited (López et al., 2015). The objective of this research was to determine the yield of polyhydroxyalkanoates produced by nitrogen-fixing bacteria isolated from *Asparagus officinalis* L.

**Materials and methods**

The study population was constituted by the plants of *Asparagus officinalis* (asparagus) of the Josymar farm (50 ha) located in Virú, Trujillo, Peru and a non-probabilistic sample of 96 plants of *Asparagus officinalis* collected during November to December 2017 was studied, the sample size of the asparagus plants was not calculated, and this was due to the logistical and processing capacity of the researchers. The population consisted of isolates of nitrogen-fixing, PHA-producing bacteria and from which ten selected bacterial cultures were selected, which was calculated considering a prevalence of 90%.

The 96 samples of asparagus roots, stems and rhizospheric soil were collected at the Josymar farm, located in lot 10.6 of sector IV, Chavimochic Special Project in the province of Virú, Peru. In the asparagus field, every five furrows the most vigorous plant was selected and approximately 50 g of roots with rhizospheric soil adhered was extracted, deposited in properly identified polyethylene bags and immediately transported in a thermal box (10 ±1 °C) to the Laboratory of Microbiology and Parasitology: Microbial Biotechnology of the Faculty of Biological Sciences, Pedro Ruiz Gallo National University in Lambayeque.

**Isolation of potential nitrogen-fixing endophytic bacteria**

Microaerophilic nitrogen-fixing endophytic bacteria were isolated from roots and stems previously disinfected with 10% bleach, in which they were immersed for 2 min and then rinsed with distilled water. The tissue was deposited on blotting paper to remove excess moisture and then was taken to 15 x 16 cm polyethylene bags, where it was macerated in 1 L of distilled water. One milliliter was extracted with a syringe, immediately sowing one drop by double puncture in the semisolid culture media without nitrogen: NFb and LGI for *Azospirillum* spp., JNFb for *Herbaspirillum* spp., LGI-P for *Gluconacetobacter* spp., and JMV for *Burkholderia* spp.
After incubation at 30 ± 2 °C for 7 days, the media where a whitish film and the change of the pH indicator were observed were selected. Subsequently, subcultures were carried out in similar media twice consecutively. For the isolation of microaerophilic bacteria, suspensions were made from the bacterial films using sterile saline solution and were sown in the respective solid culture media, incubating at 30 ± 2 °C for 48 hours. The various morphotypes of the isolated bacteria were cultured again in semisolid medium (third subculture) and subsequently in the respective solid medium.

**Isolation of potential nitrogen-fixing rhizospheric bacteria**

Aerobic and microaerophilic nitrogen-fixing rhizospheric bacteria were isolated from roots with rhizospheric soil, previously dehydrated under shade, at room temperature of 28 °C, for 72 h. The roots were fragmented with a scalpel (5 cm), 10 g of previously cut roots was randomly taken, together with the adhered soil and deposited in bottles of 500 ml capacity, containing 90 ml of sterilized saline solution (NaCl 0.85% w/v).

After stirring manually for 10 min, aliquots (one drop) were taken and sown by the technique of depletion and streak on the surface of solid media without nitrogen, to isolate aerobic bacteria and by the technique of puncture in semisolid media without nitrogen, to isolate microaerophilic bacteria. Aerobic bacteria were isolated in solid culture media, LG for Azotobacter, LGD for Dersxia and BEIJ for Beijerinckia spp. After incubation at 30 ± 2 °C for 2 days, suspensions in sterilized saline were obtained with the morphotypes of the representative bacteria and were sown in the respective solid media.

Microaerophilic bacteria were isolated in a manner similar to that explained in endophytic bacteria. The colonies of endophytic and rhizospheric nitrogen-fixing bacteria were cultured on nutritious agar (yeast extract, peptones, agar-agar, sodium chloride and distilled water at pH 6.8) at 30 °C, for 24 h, Gram stains were performed, and pure cultures were obtained.

The identification of the genus of rhizospheric and endophytic nitrogen-fixing bacteria was carried out according to morphological and physiological characteristics based on Bergey’s Manual of Systematic Bacteriology. Catalase, oxidase, and motility tests were performed with all bacteria. For aerobic bacteria of the genus Azotobacter, the tests were nitrate reduction and acidification of glucose, sucrose, maltose, and fructose. For the genera Dersxia and Beijerinckia, the tests were production of indole, use of citrate as a carbon source, growth in 1% of peptone and acidification of glucose, sucrose, and mannitol.

**Detection, preselection, and identification of PHA granule-producing bacteria**

The isolates with potential capacity to fix atmospheric nitrogen were cultured in triplicate in 5 mL of broth for the production of PHA: fermentation broth with glycerol as a carbon source, at 30 °C in aerobiosis, with constant stirring (125 rpm). At 24 and 48 h, aliquots were taken from the cultures, smears were made, they were colored with Sudan Black B, heating for 30 s until before the emission of vapors. After 15 min they were discolored with xylol (20 s), dried at room temperature (25 to 28 °C), and stained with safranin for 1 mn.
The presence of grayish granules inside the pink bacterial cells was considered positive for the detection of PHA granules. The number of cells with PHA granules was quantified in five microscopic fields per bacterial culture and the ten cultures with the highest number of cells with PHA granules per microscopic field at 100x were preselected and identified. These bacteria were cultured in the fermentation broth with glycerol as a carbon source, at 30 °C, for 24 h, with constant stirring (125 rpm) and inoculated (10% v/v) in flasks with 180 ml of broth.

The incubation was carried out under similar conditions and at 12, 24, 36, 48 and 60 h, aliquots were taken to color them with Sudan Black B and determine the time required to reach the largest number of cells with PHA granules, two cultures of bacteria with the shortest time were selected for the next experimental phase of the research. Two trials were performed: one to select the most suitable bacterial culture and biomass and PHA production medium, and the other to determine the kinetics of biomass and PHA production with the selected bacterial culture and culture medium.

The first trial was conducted under a completely randomized design (CRD), with 2x2+3 factorial arrangement, examining two independent variables: corresponding to the bacterial culture (A), with two levels 1 and 2 and culture medium (B), with two levels: balanced and unbalanced. The treatments were four with three repetitions, totaling 12 experimental units.

**Obtaining the standard curve for the quantification of biomass and PHA**

The selected PHA granule-producing bacteria were cultured in triplicate in the balanced and unbalanced fermentation media for 36 h. According to (Becerra, 2013), assuming a yield (Yx/s) in biomass of 40% from glycerol (5 g L⁻¹), the following equation was proposed: 

\[ C_3H_3O_3 + 2.225O_2 + 0.3NH_3 + 1.2CH_3O0.5N0.5 + 3.25H_2O + 1.8CO_2 \]

Equation 1. Balanced fermentation of producing bacteria

Where, for each mole of carbon, 0.1 moles of nitrogen are needed in a balanced medium. Thus, in the balanced medium the C/N ratio was 10 with (NH4)2SO4: 1.08 g L⁻¹ and in the unbalanced medium the C/N ratio was 20 with (NH4)2SO4: 0.54 g L⁻¹. To obtain the inoculum, each bacterium was cultured in 2 ml of the corresponding medium at 30 °C, for 24 h and then in 70 ml of the same medium at 30 °C, for 24 h (Guzmán et al., 2017).

The inoculum (10%: 20 ml) was deposited in flasks of 250 ml capacity, containing 180 ml of the corresponding broth, and incubated at 30 °C, for 36 h with manual stirring for 10 minutes, every 4 h. Then, samples of 2 ml were taken per triplicate, in order to determine the absorbance in the visible light spectrophotometer (Model Tenso Med NV-203) at λ= 600 nm (tube 1) and to perform decimal dilutions up to 10⁻³ (tubes 2, 3, 4), to which the absorbance was also determined.

The remaining 194 ml of each of the culture broths were centrifuged (Model GT 119-200) at 3 000 rpm, for 15 min (Guzmán et al., 2017). The sediment or biomass was washed once with sterilized saline solution (NaCl 0.85% w/v), centrifuged at 3 000 rpm for 15 min and dehydrated in oven (Hinotek GRX-9023a Model) at 45 °C, until obtaining a constant weight (Baca et al., 2010). The dehydrated biomass was weighed, and the value obtained was expressed in grams per liter (g L⁻¹).
corresponding to the absorbance of tube 1. Likewise, the biomass value was divided by 10, 100, 1,000, equivalent to the absorbance of tubes 2, 3 and 4 (10-1, 10-2, 10-3). With the values obtained, the regression equation was determined to calculate the biomass of each bacterial culture.

**Fermentation process**

In the fermentation process (Becerra, 2013), the inoculum (10%: 20 ml) of the studied bacteria was taken in triplicate to flasks of 250 ml in 180 ml of the corresponding culture broth, incubating at 30 °C, for 36 h, with manual stirring, for 10 min, every 4 h. From the moment of inoculation (0 h) and every 4 h, up to 36 h, samples of 1 ml were taken in triplicate, to determine the absorbance in the visible light spectrophotometer (Model Tenso Med NV-203), at 600 nm and the biomass values were calculated in the regression equation of the standard curve between absorbance and biomass that was previously obtained.

To quantify the PHA, the remaining 190 ml of the broth cultured at 36 h was centrifuged at 3,000 rpm for 15 min. The cell biomass obtained was washed once with saline solution (NaCl 0.85% w/v), was centrifuged, and dehydrated in an oven at 45 °C, until obtaining a constant weight, which was determined with a precision balance. With the biomass, the PHA produced by each bacterium was recovered. According to the protocol of Law and Slepecky (1961), described by Cholula (2005), 1 ml of 5% sodium hypochlorite was added to the tube with the dehydrated biomass, to weaken the cell membrane and facilitate the extraction process.

After 2 h, 1 ml of chloroform was added to separate the biomass from the polymer. After 20 min, the tubes were centrifuged at 3,500 rpm, for 5 min and the chloroform with the polymer was extracted, deposited in a tube of 10 x 100 mm, previously weighed (Iw) on a precision balance and taken to the oven at 40 °C, for enough time for the evaporation of chloroform. The tube containing the PHA was weighed (Fw) and the difference between this weight minus the initial weight (Iw) constituted the PHA obtained.

To verify the nature of the polymer obtained, the samples were diluted in 1.5 ml of 80% H2SO4, were heated for 30 min at 90 °C in a water bath, cooled to room temperature (28 °C), deposited in quartz cells, and scanned in the UNICO S-2150 ultraviolet light spectrophotometer, in the range of 220 to 250 nm. The absorbance of a maximum peak at 235 nm was considered positive for PHA. Next, the absorbance of each PHA sample examined was read at 235 nm (Guzmán et al., 2017).

The Yp/x is the coefficient of yield of the product in relation to the biomass or quantity of the product obtained by amount of biomass formed (g g⁻¹) and was calculated by dividing the grams of PHA by the grams of biomass (Doran, 1998): the bacterial culture and the culture medium with which the highest Y(p/x) yield was achieved were selected to determine the kinetics of the production.

**Kinetics of biomass and PHA production**

The fermentation process to determine the kinetics of the production of biomass and PHA by the bacterial culture in the selected culture medium was carried out in a similar way to what was explained above, but samples of 40 ml were collected, in triplicate at 0, 8, 16, 24 and 32 h to quantify the biomass and PHA obtained and subsequently the Yp/x yield was calculated.
Statistical analysis

The values of the concentration of biomass, PHA and yield of the bacteria were ordered in tables to perform the tests of normality and homogeneity of variances. The analysis of variance determined the differences between the treatments and Tukey’s multiple comparisons test the significance between them. The statistical software SPSS version 15.0 was used.

Results

Potentially nitrogen-fixing or diazotrophic bacteria from asparagus roots, stems and rhizospheric soil were isolated in nitrogen-free culture media, resulting in 427 pure cultures. The 51.1% of diazotrophic bacteria were rhizospheric and 48.9% endophytic. The 57.6% of nitrogen-fixing bacteria developed in the fermentation broth, showing turbidity from 24 h, the 65.9% corresponded to Gram-negative bacteria. In 98.4% of the bacterial cultures that developed in the fermentation broth, PHA granules stained with Sudan Black B were observed, between 24 and 48 h (Figure 1), such that 56.67% of nitrogen-fixing bacteria were recognized as producers of PHA granules.

![Figure 1. PHA granules in Gram-negative bacteria.](image)

The range in the number of cells with PHA granules per microscopic field in Gram-negative bacteria was from 1 to 248 at 24 h and from 1 to 247 at 48 h. In Gram-positive bacteria, the range was from 1 to 183 at 24 h and 1 to 173 at 48 h. In the phenotypic identification, 50% of the preselected bacteria corresponded to the genus Azospirillum sp., 30% to Herbaspirillum sp., 10% to Azotobacter sp., and 10% to Burkholderia sp., quantifying 170 to 248 PHA granules at 24 h and 190 to 247 PHA granules at 48 h.

The time in which the highest number of cells with PHA granules was quantified or optimal incubation time (Table 1) was from 24 h (Azospirillum sp., 350; Herbaspirillum spp., 151, 152 and Burkholderia sp., 238) to 36 h (Azospirillum spp., 155, 130, 149, 102; Herbaspirillum spp., 135 and Azotobacter sp., 86). After this time, the number of cells with PHA granules decreased. The cultures of Azospirillum spp., 155 and 130 were selected to obtain the biomass and PHA, because they showed the highest number of cells with PHA granules. With the absorbance values obtained in triplicate with different concentrations of biomass of Azospirillum spp., 130 and 155 in the balanced and unbalanced culture media, 12 equations were obtained, among which the 4 that presented the highest values in the coefficient of determination were selected, maintaining as reference standard $R^2 > 0.9$. 
### Table 1. Number of bacterial cells with PHA granules at different incubation times.

<table>
<thead>
<tr>
<th>Nitrogen</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azospirillum</em> sp. 155</td>
<td>40</td>
<td>185</td>
<td>300</td>
<td>240</td>
<td>100</td>
</tr>
<tr>
<td><em>Azospirillum</em> sp. 130</td>
<td>38</td>
<td>170</td>
<td>290</td>
<td>235</td>
<td>110</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> sp. 135</td>
<td>95</td>
<td>180</td>
<td>284</td>
<td>232</td>
<td>108</td>
</tr>
<tr>
<td><em>Azospirillum</em> sp. 149</td>
<td>39</td>
<td>183</td>
<td>250</td>
<td>210</td>
<td>90</td>
</tr>
<tr>
<td><em>Azotobacter</em> sp. 86</td>
<td>21</td>
<td>160</td>
<td>270</td>
<td>205</td>
<td>80</td>
</tr>
<tr>
<td><em>Azospirillum</em> sp. 102</td>
<td>13</td>
<td>185</td>
<td>245</td>
<td>190</td>
<td>23</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> sp. 151</td>
<td>93</td>
<td>230</td>
<td>200</td>
<td>185</td>
<td>49</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. 238</td>
<td>15</td>
<td>239</td>
<td>215</td>
<td>182</td>
<td>30</td>
</tr>
<tr>
<td><em>Azospirillum</em> sp. 350</td>
<td>21</td>
<td>245</td>
<td>200</td>
<td>175</td>
<td>82</td>
</tr>
<tr>
<td><em>Herbaspirillum</em> sp. 152</td>
<td>52</td>
<td>225</td>
<td>190</td>
<td>150</td>
<td>20</td>
</tr>
</tbody>
</table>

In the fermentation process, the absorbance of the biomass of *Azospirillum* sp., 130 ranged from 0.06 to 0.1 in the balanced medium, and from 0.07 to 0.13 in the unbalanced medium, values corresponding to 0.03 to 0.23 g L\(^{-1}\) and 0.06 to 0.3 g L\(^{-1}\), respectively. The absorbance of the biomass of *Azospirillum* sp., 155 ranged from 0.09 to 0.12 in the balanced medium, and from 0.1 to 0.169 in the unbalanced medium, values corresponding to 0.14505 to 0.23379 g L\(^{-1}\) and 0.19554 to 0.49738 g L\(^{-1}\), respectively.

The concentration of PHA produced by *Azospirillum* can be seen in Table 2. The analysis of variance of the Yp/x yield of factorial 22 demonstrated high significance for bacterial culture (factor A), culture medium (factor B) and AxB interaction. Regarding the bacterial culture (A), the highest yield corresponded to *Azospirillum* sp., 155, as for the culture medium (B), the highest yield was reached with the unbalanced medium. In the AxB interaction, the highest yield was obtained with *Azospirillum* sp., 155 unbalanced medium. *Azospirillum* sp., 155 in glycerol as a carbon source reached a Yp/x yield of 0.77908 g g\(^{-1}\) or 77.9% after 32 h of fermentation (Table 3).

### Table 2. Coefficient of yield of the product according to the biomass by *Azospirillum* spp. 155 and 130 cultured for 32 h in balanced and unbalanced media.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>X Biomass (g L(^{-1}))</th>
<th>P PHA (g L(^{-1}))</th>
<th>Yp/x Yield (g g(^{-1}))</th>
<th>Sign</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azospirillum</em> sp. 155 - Unbalanced</td>
<td>0.49738</td>
<td>0.38158</td>
<td>0.76718</td>
<td>A</td>
</tr>
<tr>
<td><em>Azospirillum</em> sp. 155 - Balanced</td>
<td>0.23379</td>
<td>0.13526</td>
<td>0.57855</td>
<td>B</td>
</tr>
<tr>
<td><em>Azospirillum</em> sp. 130 - Unbalanced</td>
<td>0.301</td>
<td>0.07895</td>
<td>0.26229</td>
<td>C</td>
</tr>
<tr>
<td><em>Azospirillum</em> sp. 130 - Balanced</td>
<td>0.23346</td>
<td>0.05263</td>
<td>0.22543</td>
<td>D</td>
</tr>
</tbody>
</table>
Table 3. Kinetics of biomass and PHA production by *Azospirillum* sp. 155 cultured in unbalanced medium for 32 h.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>X Biomass (g L⁻¹)</th>
<th>P Pha (g L⁻¹)</th>
<th>Yp/x (g g⁻¹) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.27865</td>
<td>0.0075</td>
<td>0.02692 2.69</td>
</tr>
<tr>
<td>16</td>
<td>0.37927</td>
<td>0.0175</td>
<td>0.0574 5.74</td>
</tr>
<tr>
<td>24</td>
<td>0.40114</td>
<td>0.1475</td>
<td>0.3677 36.8</td>
</tr>
<tr>
<td>32</td>
<td>049738</td>
<td>0.3875</td>
<td>0.77908 77.9</td>
</tr>
</tbody>
</table>

**Discussion**

Polyhydroxyalkanoates are energy reserve polymers produced by bacteria to survive periods of starvation in natural habitats (Gasser *et al*., 2009). Bacteria are thought to use PHA accumulation to increase survival and stress tolerance in changing environments and in competitive environments where carbon and energy sources may be limited, such as those found in the soil and the rhizosphere (Kadouri *et al*., 2005).

In nitrogen-fixing bacteria, PHA serves as protection to the nitrogenase complex, acts as an oxidizable compound, and is also a constituent of the cytoplasmic membrane (Anderson and Dawes, 1990). These bacteria accumulate PHA as a strategy of survival and regulation of energy metabolism, both in symbiosis and in free life (Guzmán *et al*., 2017). The presence of PHA granules in isolates of potentially nitrogen-fixing *Azospirillum* spp. coincides with reports of several studies in which it was reported (Tal and Okon, 1985; Itzigsohn *et al*., 1995; Okon *et al*., 1995; Yaacov and Itzigsohn, 1995).

PHA fixation has been identified as a key physiological property in *Azospirillum brasilense*, which contributes to rhizosphere adaptation and plant growth promotion abilities (Fibach-Paldi *et al*., 2012). *Azospirillum* is also considered in agriculture for its nitrogen-fixing potential (Tikhonovich *et al*., 2012). In the study, *Burkholderia* sp. was isolated as a producer of PHA, this was reported in other studies in which it was isolated from cane bagasse (Lopes *et al*., 2014; Chee *et al*., 2010; Lau *et al*., 2011; Pan *et al*., 2012). *Burkholderia terricola* was reported by Gasser *et al*. (2009) as a producer of PHA (Gasser *et al*., 2009) and also as a growth promoter (Pan *et al*., 2012).

With respect to the isolation of *Herbaspirillum* sp., it was observed that it produced PHA, this is indicated in several studies in which similar results were obtained (Catalán *et al*., 2007), this same genus is indicated as a growth promoter (Batista *et al*., 2016). PHA was observed in the form of granules stained with Sudan Black B. PHAs are polymers of hydroxyalkanoic acids or polyesters of (R)-3-hydroxy carboxylic (R)-HA acids that some species of bacteria accumulate intracellularly (Bugnicourt *et al*., 2014), in the form of granules, surrounded by a monolayer of phospholipids containing polymerases and depolymerases (López *et al*., 2015).

The lipid membrane of the granules of hydrophobic nature facilitates staining with Sudan Black B. This lipophilic stain allows the *in vivo* detection of PHAs, evidencing the lipid nature of the granules, which acquire a color from gray to black, in contrast to the pink of vegetative cells. The
genera of nitrogen-fixing bacteria with the highest number of cells with PHA granules were previously reported as producers of the polymer by Baca et al. (2010) (Azospirillum brasilense and A. lipoferum), the decrease in the number of cells with PHA granules after reaching the maximum was also observed by Guzmán et al. (2017).

This decrease is a consequence of intracellular degradation, as an endogenous response of bacteria to hydrolyze their carbon reserves accumulated in PHA forms, with the participation of depolymerases located on the surface of the granules. The higher PHA yield achieved in the unbalanced medium is attributed to the fact that, under unfavorable conditions, bacteria accumulate the polymer, as a strategy to regulate carbon concentration and consume reducing power (Kadouri et al., 2005). The synthesis of PHA granules is favored in culture media with excess carbon (Baca et al., 2010) and deficit of aeration, nitrogen, phosphorus, magnesium, and sulfur.

Conclusions

Azospirillum sp., 155 reached a Yp/x yield of 0.77908 g g⁻¹, which means that 77.91% of the weight of the biomass corresponded to the PHA, the profitability in the production of PHA requires that the bacterial strain accumulates at least 60% of cell mass with PHA. In this context, Azospirillum sp., 155 is considered to have industrial potential, whose yield could increase with the optimization of the culture medium, growing conditions and product separation. In the roots, stems and rhizospheric soil of asparagus, potentially nitrogen-fixing bacteria were isolated, which proved to be producers of PHA, with Azospirillum sp. standing out with potential for the production of biopolymers.

Cited literature


