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Bacteria from industrial waste: potential producers of polyhydroxyalkanoates (PHAs) in Manizales, Colombia

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Abstract Polymers are currently used in the industry as raw material, yet they are rapidly eliminated and largely contaminate the environment. To address this issue, there is a special interest in biodegradable polymers, namely, polyhydroxyalkanoates (PHAs), produced by microorganisms. This study identifies PHA-producing bacteria from two industrial wastewaters of Manizales, Colombia. The samples were cultured in mineral salt medium with glucose as the carbon source in the presence of Nile red stain. The fluorescent colonies were independently transferred to another medium and assessed through fluorescence microscopy with Nile blue stain. The fluorescent strains under Nile blue staining were purified in Nutrient Agar, and their morphological and microbiological characteristics were determined. The bacteria positive for red-orange fluorescence were purified in Nutrient Agar medium, and molecular analyses were performed by PCR amplification of a 650-bp fragment of the 16S ribosomal DNA gene. The bacteria were also assessed in terms of PHA production. We confirmed the identity of 12 out of 14 PHA-positive strains, which belonged to the following genera: *Bacillus*, *Lactococcus*, *Citrobacter*, *Enterobacter*, and *Acinetobacter*. Five of the isolates (*Enterobacter cloacae*, *Enterobacter* sp., *Enterobacter ludwigii*, *Bacillus thuringiensis*, and *Bacillus safensis*) are promising strains for PHA production, with production values ranging from 0.360 to 0.9960 g/L. Bacteria that produce more than 0.3 g/L are considered useful for the industrial manufacture of bioplastic. We recommend performing large-scale studies on these strains to assess their use for the industrial production of biopolymers, allowing to generate high-impact bioconversion processes of industrial interest.

Keywords Wastewater · Strains · Biopolymers · Plastic · Wastewater treatment plants

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Introduction

The expanding population, urbanization, and industrialization have led to the production of an excessive amount of wastewater that generates high contamination loads (Bengtsson et al. 2008). Industrial wastewater can produce waste material that is used by microorganisms (Bitton 2005). In particular, wastewater can be a culture

medium that allows the growth of certain microbial populations. These microorganisms can interact and perform metabolic reactions that degrade the organic matter and remove nutrients from the wastewater (Bravo et al. 2005).

Industries rely on wastewater treatment plants to remove organic loads and deposit solid wastes through aerobic and anaerobic processes (Harding et al. 2007). These biological processes produce mixed microbial consortia (MMC), which are a potential raw material for biopolymer production (Reddy and Mohan 2012). These consortia contain microorganisms such as bacteria, yeast, and fungi that are capable of synthesizing biopolymers in the presence of an excess carbon source and limited nitrogen or phosphorous in the growth medium (Kumar et al. 2016).

Biopolymers, commonly known as polyhydroxyalkanoates (PHAs), were discovered in the early twentieth century in *Bacillus megaterium* (Keshavarz and Roy 2010). Over 300 species of microorganisms accumulate PHAs, among them halophiles can reach yields above 70% (Guzmán et al. 2017). PHAs are a group of biodegradable and biocompatible polyesters of natural origin that are synthesized by a wide range of Gram-negative and Gram-positive bacteria as carbon reserves (Neira and Pardo 2010). Some of these bacteria are industrially useful due to their efficient substrate transformation processes and the final concentration of biopolymer inside the cells (Barbosa et al. 2005). Gram-negative bacteria that efficiently produce PHAs are *Cupriavidus necator* (formally *Alcaligenes eutrophus*), *Alcaligenes latus*, *Pseudomona putida*, *Pseudomona oleovorans*, and *Azotobacter vinelandii*, as well as the recombinant strain of *Escherichia coli* that contains the PHA biosynthesis operon from *C. necator* (Barbosa et al. 2005). Similarly, some Gram-positive bacteria are also PHA producers, including several species of *Bacillus* (e.g., *Bacillus megaterium* and *Bacillus cereus*). In addition, fungi of the genus *Streptomyces* (Actinomycetes) are also known to produce PHAs (Yilmaz and Beyatli 2005; Franco et al. 2009; Reddy and Mohan 2012).

Due to their lipidic nature, the detection and isolation of PHA-producing bacteria are based on the use of lipophilic stains such as Sudan black (Schlegel et al. 1970), Nile blue (Ostle and Holt 1982), fluorescent oxazone, and Nile red (Spiekermann et al. 1999). However, the detection of PHA-producing bacteria is supported by confirmatory PCR-based molecular assays to determine the genus and species of the bacteria as well as the presence of one or several PHA biosynthesis genes (Solaiman et al. 2000; Ciesielski et al. 2006). In this regard, the 16S rDNA gene provides high-grade resolution for the taxonomical determination; therefore, if two strains share less than a 97% identity in their 16S rDNA sequence it can be inferred that these do not belong to the same species (Rosselló-Mora and Amann 2001).

This study aimed to characterize PHA-producing bacteria using microbiological and molecular techniques to search for native strains useful for the industrial production of biopolymers. The identification of these bacteria can promote the use of an economical substrate derived from wastewater treatment plants, which are a primary source of activated sludge containing polymers for bioplastic production. These microorganisms can be used as an alternative for wastewater treatment since industrial wastes can represent settings for biological degradation and the production of bioplastics, therefore, promoting biotechnological development in the region.

Materials and methods

Study area

The study area comprised wastewater treatment plants (WWTP) from two food-processing factories, namely, *Super de Alimentos* (5° 36′ 25″ N, 75° 45′ and 62″ N 75° 45′ 63″ W) and *Industrias Lácteas Normandy* (5° 34′ 97″ N and 75° 45′ 39″ W), located in the city of Manizales, Caldas, Colombia. We monitored three sites at each treatment plant: (a) entrance to the wastewater treatment plant (percolator filter), (b) sedimentation tank (activated sludge chamber), and (c) exit tank (decanting chamber) (Fig. 1).

Sample collection and selection of isolates with PHA-producing capacity

Sample collection was conducted on a normal production day at each wastewater treatment plant. The samples were collected at 1-h intervals during 4 continuous hours. The samples were taken at a depth of 1.2 m and stored in clean and clear wide-mouth 250-mL glass jars, carefully closed and labeled, according to the recommendation of the IDEAM (Guía para el Monitoreo de

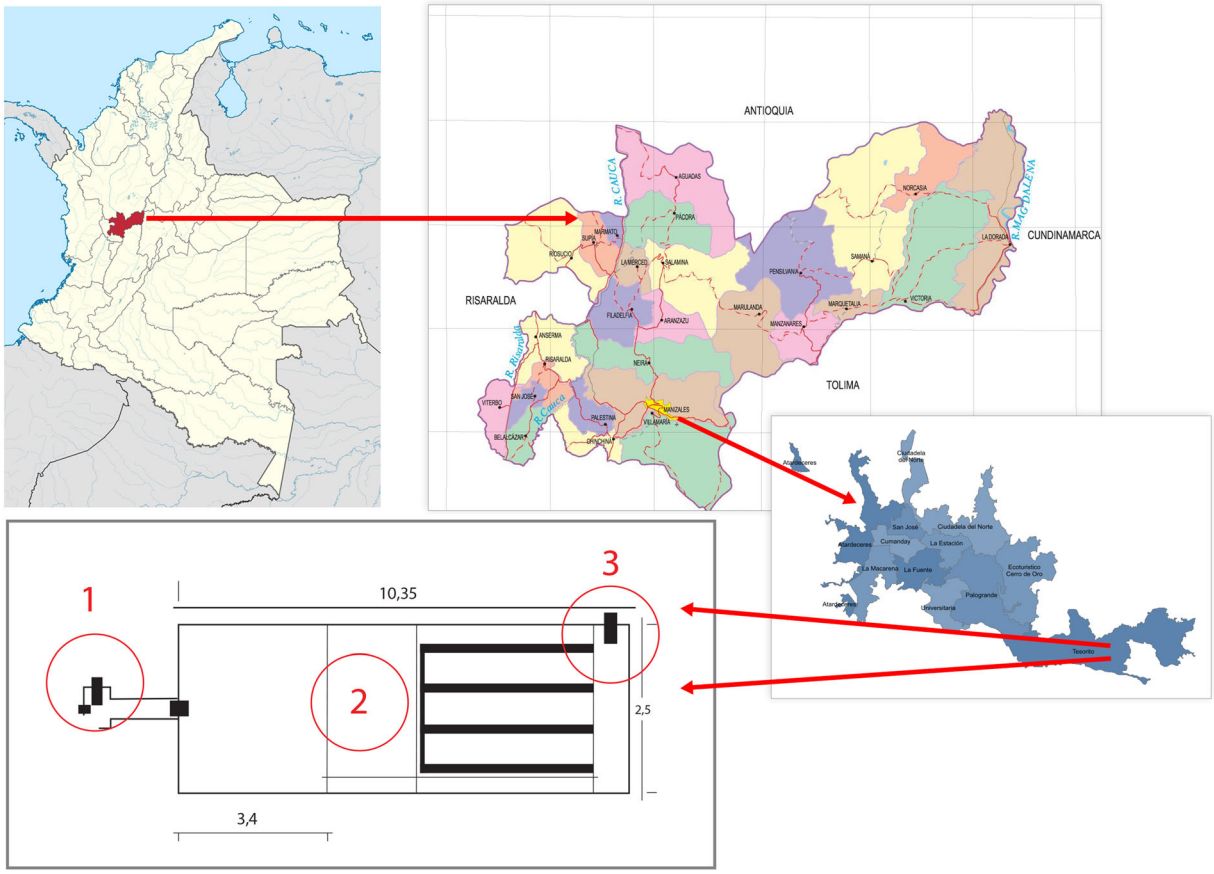

Fig. 1 Study area and monitoring sites at the wastewater treatment plants: (1) entrance to the percolator filter, (2) activated sludge chamber, and (3) exit to the decanting chamber

Vertimientos, Aguas Superficiales y Subterráneas 2003). The samples were taken to the biotechnology laboratory of *Tecnoparque SENA*—Manizales, Colombia, for processing and analyses.

The samples were cultured by spreading 1 mL of the sample on Nutrient Agar medium. After, the bacterial morphotypes were isolated by the streak plate method on a solid mineral salt medium (MSM), pH 7.0, supplemented with 2% glucose, 0.2% yeast extract, and 1 mL/L 0.1% Nile red stain in acetone solution to detect PHA-producing colonies under an ultraviolet transilluminator at a wavelength of 340 nm (Spiekermann et al. 1999).

The cultures were incubated at 30 °C and monitored every 24 h under ultraviolet light for 72 h. After, the fluorescent colonies under Nile red staining were further isolated through streak-plating on Nutrient Agar medium. A confirmatory test was performed using Nile blue stain, according to Ostle and Holt (1982), using a Nikon *CI-S INTENSILIGHT* fluorescent microscope at 450 nm and × 100. The red-orange fluorescent bacteria indicated a possible production of PHAs, and these were purified in Nutrient Agar (Fernández et al. 2005).

Morphological and molecular identification of PHA-producing strains

Each isolate *presumably* capable of producing biopolymers was assessed by morphology based on the macroscopic and microscopic characteristics proposed by Bou et al. (2011). Finally, the isolates were Gram-stained and stored in cryovials with 15% glycerol at − 80 °C (Capuccino and Sherman 2007).

For the molecular analyses, DNA was extracted with the UltraCleanTM Microbial DNA Isolation kit (Vidal et al. 2007) and an ~ 650-bp fragment of the V1–V6 regions of the 16S rDNA gene was amplified by PCR using primers 27F 5′AGAGTTTGATCMTGGCTCAG 3′ and 104R 5′CGGTGTGTACAAGACCC 3′ (Kuske

et al. 1997). The amplicons were visualized through horizontal electrophoresis on 1% agarose gels with 1X TBE pH 8.0 at 110 v/50 mA. SYBR Safe® dye was used to visualize the DNA bands using a GelDoc-It®2 310 Imager (UVP) photodocumentor device. The PCR products were purified using the QIAquick PCR purification (Qiagen®) kit, following the manufacturer's recommendations, and sequenced at Macrogen (South Korea). The sequences were viewed and edited using Geneious Trial v8.14 (Drummond et al. 2009) and BioEdit 7.2.6. The sequences were searched against the public databases (i.e., GenBank) with MegaBLAST to retrieve similar sequences.

Assessment of polyhydroxyalkanoate-producing strains

We assessed the capacity of the isolates to produce biopolymers using a modified synthetic culture medium proposed by Fernández et al. (2005), enriched with glucose to facilitate the measurement of biopolymer production. For the assessment, we inoculated 9×10^8 cells mL/L (McFarland nephelometric scale) of the isolates in the synthetic substrate in batch bioreactors. We recovered the precipitate at 24, 48, and 72 h of incubation by centrifugation and resuspension in 5% sodium hypochlorite and 10 mM EDTA. After, we incubated the suspension at 60 °C for 1.5 h, then, we centrifuged and washed the pellet with distilled water. The precipitate was resuspended in acetone, centrifuged, and the supernatant was removed. Then, we diluted the pellet in cold methanol, centrifuged once more, recovered the precipitate, and left it to dry at room temperature for 12 h. Finally, the resulting biomass was weighed, and this value was used to determine the total PHA content per dry mass based on the volume and dry weight of the sample (Fernández et al. 2005). As positive control we included *Bacillus megaterium* (cepa ATCC 14581), and as negative control we used the modified synthetic culture medium proposed by Fernández et al. (2005).

We determined which strains from the dairy (*Industrias Lácteas Normandy*) or candy (*Super de Alimentos*) factories showed the highest biopolymer production for 24, 48, and 72 h by performing a bootstrap test (1000 replicas) according to Crawley (2005). In addition, we also used bootstrapping (1000 replicas) to compare the production of biopolymers between the dairy and candy factories. These bootstrap analyses were conducted due to the small sample sizes (diary $n = 8$ and candy $n = 6$) and non-normal distribution of the data. Finally, we compared PHA median production of strains by Kruskal-Wallis test (H). The statistical analyses were performed in R version 3.3.3 (R Core Team 2017).

Results

Selection of the isolates with PHA-producing capacity

We identified 86 bacterial morphotypes growing on Nutrient Agar medium. These morphotypes grouped 106 strains; among them 31 were fluorescent under Nile red staining (Table 1). The confirmatory test (Nile blue staining) allowed identifying 14 strains capable of producing PHAs (Table 1).

Morphological and molecular identification of PHA-producing strains

The 14 strains screened for PHA-production displayed macroscopic features including spherical, circular, and irregular shapes; creamy textures; and smooth and rough surfaces, among others. Microscopically, we observed bacilli and cocci, including 4 Gram-positive and 10 Gram-negative strains.

The amplification of the 650-bp fragment of the 16S rDNA gene confirmed the identity of 12 strains (Table 1). The 16S partial sequences showed 97% and 99% identity to reported sequences for *Bacillus*, *Lactococcus*, *Citrobacter*, *Enterobacter*, and *Acinetobacter* (Table 2). The GenBank accession numbers for the 16 rDNA nucleotide sequences obtained in this study are [MN013897, MN013898, MN013899, MN013902, MN013903, MN013904, MN013905, MN013906, MN013907, MN013908, and MN013909].

Assessment of the PHA-producing strains

We determined the identity of 12 PHA-positive bacteria, including 8 strains isolated from *Industrias Lácteas Normandy*, namely *Bacillus thuringiensis* that showed the highest PHA production (960.67 mg/L), followed by *Bacillus safensis* (330.33 mg/L) (Fig. 2). These two strains showed a higher biopolymer production than the other six strains at 24, 48, and 72 h ($P < 0.025$). The PHA production differ among trains in *Industrias Lácteas Normandy* ($H = 18.01$; $P = 0.012$). Furthermore, 6 of the 12 PHA-positive strains were obtained from *Super de Alimentos*. Of these, we identified four

Table 1 PHA-producing strains isolated from wastewaters of *Industrias Lácteas Normandy* and *Super de Alimentos* (Manizales, Colombia)

	Number or strains	Positive Nile red	Positive Nile blue
Morphotypes 14 (IN)	1	1	1
Morphotypes 26 (IN)	3	3	1
Morphotypes 56 (IN)	1	1	1
Morphotypes 69 (IN)	1	1	1
Morphotypes 67 (IN)	1	1	1
Morphotypes 20 IN)	2	2	0
Morphotypes 75 (IN)	1	1	1
Morphotypes 46(IN)	4	4	1
Morphotypes 58 (IN)	1	1	1
Morphotypes 8 (IS)	1	1	1
Morphotypes 12 (IS)	2	2	0
Morphotypes 28 (IS)	1	1	1
Morphotypes 60 (IS)	1	1	1
Morphotypes 83 (IS)	1	1	1
Morphotypes 65 (IS)	4	4	1
Morphotypes 54 (IS)	2	2	1
Morphotypes 55 (IS)	4	4	0
Total	31	31	14

IN Industrias Normandy, Manizales, Colombia; *IS* Industria Super de alimentos, Manizales, Colombia

strains, namely, *Enterobacter cloacae* and *Enterobacter ludwigii*, which showed a high PHA production capacity (730.33 mg/L and 800 mg/L, respectively) (Fig. 3). *Enterobacter cloacae* (Manizales) showed a higher biopolymer production at 24 h ($P < 0.025$), while *E. ludwigii* (Manizales) showed a higher production at 48 and 72 h ($P < 0.025$). The PHA production differ among trains in *Super de Alimentos* ($H = 14.30$, $P = 0.014$). Finally, no significant differences in PHA-production were found between the dairy and candy industries at 24 h ($P = 0.516$), 48 h ($P = 0.515$), or 72 h ($P = 0.487$). However, future research involving these PHA-positive bacteria must assess the purity of the PHA, as described in Amaro et al. (2019).

Discussion

We identified PHA-producing bacterial strains isolated from wastewater treatment plants of dairy (*Industrias Lácteas Normandy*) and candy (*Industria Super de Alimentos*) factories in Manizales, Colombia. The data provide new evidence that supports the PHA-producing bacterial isolates in wastewater treatment plants and the use of these beneficial microorganisms for the industrial production of bioplastic.

The morphological and molecular identifications of the 12 PHA-producing isolates showed that these belonged to the genera *Bacillus*, *Lactococcus*, *Citrobacter*, *Enterobacter*, and *Acinetobacter*, which have been reported to produce PHAs (Cardona-Echavarria et al. 2013; González-García et al. 2013; Malagón-micán et al. 2017). Cardona-Echavarria et al. (2013) reported 38 PHA-producing bacterial strains isolated from the wastewaters of the dairy industry. González-García et al. (2013) found five *Bacillus* strains and one *Enterobacter* strain in wastes derived from the industrial production of saccharose and molasses, which showed a high capacity for PHA production.

Among the bacterial strains reported here, we found that *B. thuringiensis* and *B. safensis* (from *Industrias Lácteas Normandy*) showed the highest levels of production for this factory at a laboratory scale (960.67 mg/L and 330.33 mg/L, respectively). Otero-Ramírez and Fernández (2013) reported production values of 0.3 to 2 g/L for *Bacillus* strains. Similarly, Porwal et al. (2008) reported a production of 0.19 g/L in *Bacillus* grown on a medium supplemented with glucose as a carbon source, while Tam-Dogan and Sidal (2011) found a production

Table 2 Strains and GenBank accession numbers of the 16S rDNA gene sequences used for molecular identification

Species or Genus—Locality	Closest GenBank identity (gene: accession number)	The Genbank accession numbers for sequences obtained in this study
Lactococcus lactis (Industrias Normandy, Manizales-Colombia-14)	*Lactococcus lactis* subsp. lactis strain PMC32-57 [MK333792] 99.29%	[MN013897]
Citrobacter amalonaticus (Industrias Normandy, Manizales-Colombia-26)	*Citrobacter amalonaticus* strain TB10 [MH085466] 98.74%	[MN013898]
Enterobacter sp. (Industria Super de alimentos, Manizales-Colombia)	*Enterobacter tabaci* strain TBMAX92 [MK834730] 99.61% *Enterobacter asburiae* strain PB1 [MK422613] 99.61%	[MN013899]
Enterobacter sp. (Industria Super de alimentos, Manizales-Colombia-28)	*Enterobacter cloacae* [MK414959] 99.7% *Enterobacter* sp. [MH725605] 99.7%	[MN013902]
Enterobacter sp. (Industrias Normandy, Manizales-Colombia-56)	*Enterobacter* sp. [MH725605] 99.8% [MF289158] 99.8%	[MN013903]
Enterobacter ludwigii (Industria Super de alimentos, Manizales-Colombia-60)	*Enterobacter ludwigii* [MK855343] 99.9% [MK422616] 99.9%	[MN013904]
Enterobacter sp. (Industria Super de alimentos, Manizales-Colombia-65)	*Enterobacter* sp. [FN997607] 99.9% [LN848744] 99.8%	N.D
Acinetobacter sp. (Industria Super de alimentos, Manizales-Colombia-83)	*Acinetobacter* sp. [KY400652] 97.5% [MK346044] 97.5%	[MN013905]
Bacillus safensis (Industrias Normandy, Manizales-Colombia-58)	*Bacillus safensis* [MK414964]	[MN013906]
Bacillus aerius (Industrias Normandy, Manizales-Colombia-69)	*Bacillus aerius* [MK875178] 99.9% [MK287636] 99.9%	[MN013907]
Bacillus thuringiensis (Industrias Normandy, Manizeles-Colombia-67)	*Bacillus thuringiensis* 99.9%	[MN013908]
Citrobacter amalonaticus (Industrias Normandy, Manizales-Colombia-8)	*Citrobacter amalonaticus* strain TB10 [MH085466] 98.74%	[MN013909]

N.D not done

value of 0.01 g/L for *Bacillus* using manitol as a carbon source.

The strains of *Enterobacter cloacae* and *E. ludwigii* from wastewaters of *Super de Alimentos* showed the highest production levels for this factory (730.33 and 800 mg/L, respectively). For *E. cloacae*, González-García et al. (2013) report a PHA production equal to over 94% of its dry weight and Malagón-Micán et al. (2017) found PHA production values close to 0.6 g/L, similar to those found in this study. Regarding

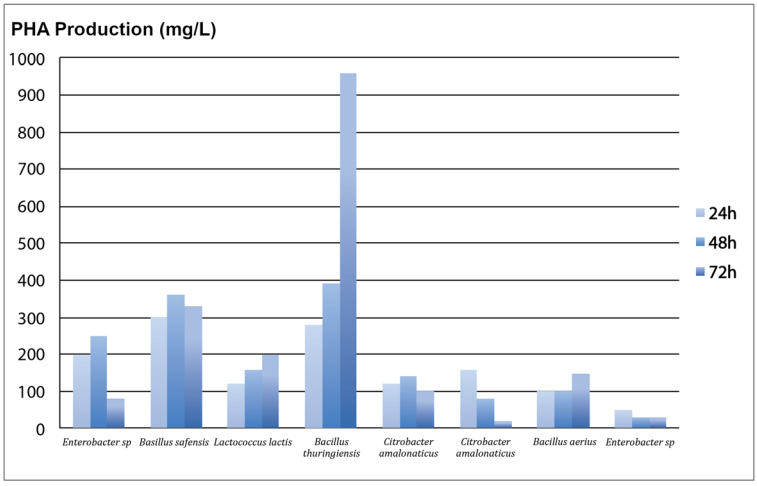

Fig. 2 PHA production (mg/L) at 24, 48, and 72 h ($n = 3$). The eight isolates are isolated from wastewaters of *Industrias Lácteas Normandy, Manizales, Colombia*

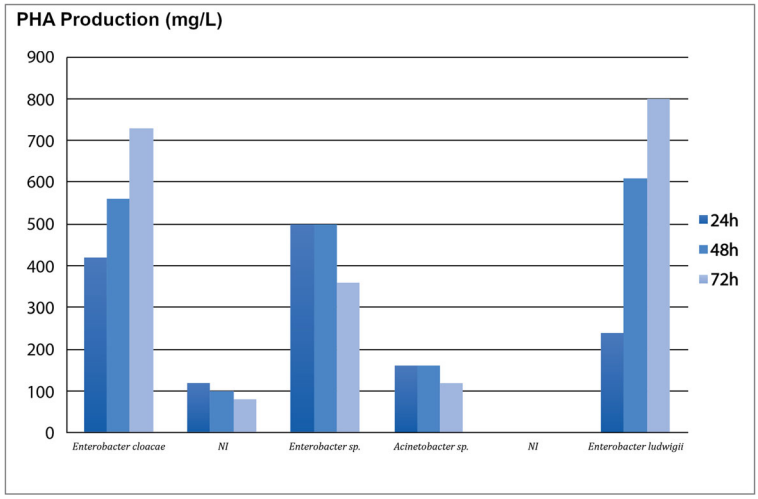

Fig. 3 PHA production (mg/L) at 24, 48, and 72 h ($n = 3$). The six isolates are isolated from wastewaters of *Super de Alimentos, Manizales, Colombia*

E. ludwigii, we found that this bacterium had the highest polymer production capacity (800 mg/L). Mora et al. (2017) found that *E. ludwigii*, isolated from sub-products of sugarcane, is capable of producing PHA from various carbon sources. Finally, the genus *Enterobacter*, found here in wastewater from the candy factory, has been widely studied (Koller et al. 2012) and isolated from diverse agro-industrial wastes (Naheed et al. 2012).

Fernández et al. (2005) report that bacteria capable of producing more than 0.3 g/L of PHAs are considered promising for the industrial manufacture of bioplastic. Of the 14 strains found here, 5 are promising for PHA production (*Enterobacter cloacae*, *Enterobacter* sp., *Enterobacter ludwigii*, *Bacillus thuringiensis*, and *Bacillus safensis*), showing values between 0.360 and 0.9960 g/L. Based on our findings, we recommend conducting large-scale assays and evaluating the industrial production of biopolymers using these strains. This will allow generating high-impact bioconversion processes to allow recovering industrially useful metabolites, such as PHAs, from contaminating sub-products. Although solvent-based methods are economical and show high PHA yields, we must assess and implement more environmentally friendly extraction methods, such as the use of enzymes and detergents that remove cellular components while leaving PHAs intact. These strategies are currently more suitable for the environment but normally result in lower PHA yields (Suriyamongkol et al. 2007; Amaro et al. 2019).

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