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# Polyhydroxyalkanoate (PHA) production by *Lynisibacillus* sp. strain UEA-20.171

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**PHA is a biodegradable and biocompatible natural thermoplastic produced from renewable bioresources and is hence attracting attention as a plastic material for use in the environment and medical fields. In the present study, the *Lynisibacillus* sp. strain UEA-20.171 was selected for production of polyhydroxyalkanoate (PHA) in bioreactor. The accumulation of polymer in the bacterium occurs when there is an imbalance in the concentration of nitrogen in relation to carbon (glucose). After the period of adaptation in mineral medium from 12 to 24 h, the cell division occurred from 12 to 24 h, and the formation of biopolymers from 36 to 48 h. It was observed that the polymer produced by the isolated UEA-20.171, was P(3HB).**

**Key words:** Polyhydroxyalkanoates, polyhydroxybutyrate, bioplastics, *Lysinibacillus.*

## INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a class of biopolymers that are an alternative to petrochemical polymers in some applications (Lee et al, 2012; Poblete-Castro et al, 2012; Kawashima et al, 2012). They are polyesters synthesized by microorganisms and are completely biodegradable. The diversity of the PHA, allows a wide range of properties and can exhibit features that range from thermoplastics, elastomers and have potentiality for use in many applications (Khanna and Srivastava, 2005; Silva et al., 2007). In addition, the PHAs are obtained from renewable sources, in general, from agriculture (Thomson et al., 2013).

Due to its biocompatibility, the PHAs currently have the largest market in the medical field, a market which is willing to pay a higher price due to the excellent application characteristics (Chen et al, 2011; Shrivastav et al., 2013). In addition to biocompatibility, the PHAs have a biodegradability which allows reabsorption of the body by the material allowing applications such as sutures, implants and controlled release of drugs (Gumel

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et al., 2012; Shrivastav et al., 2013).

PHA accumulation is stimulated under unbalanced growth conditions such as nitrogen- or phosphorus-source deficiency; most bacteria are able to accumulate PHA at around 30 to 50 wt% of dry cell (Tsuge et al., 2015). Members of the genus *Bacillus*, as well as several other microorganisms that produce PHA also produce copolymers containing 3HB and 3HV units when grown on different substrates such as glucose, among others (Gomes, 2013; Tsuge et al., 2015).

In general, the greatest obstacle to the use of PHA is still the cost of production (Gamal et al., 2013; Brito et al., 201; Falcone et al., 2007). Therefore, the aim of this study was to search for new microorganisms with biopolymer production potential of commercial importance in the industry.

## MATERIALS AND METHODS

### Microorganism

The microorganism used in this study (UEA-20.171) was isolated from the Madeira River, affluent of the Amazon River – Brazil. The tests were conducted in the laboratory of the Amazonas State University, Brazil. This bacterium was isolated and purified on nutrient agar (NA- Himedia), composed of 5 g/L peptone; 1 g/L meat extract; 5 g/L sodium chloride; 2 g/L yeast extract; agar 15 g/L and incubated at 28°C for 24 h.

### Means for producing polyhydroxyalkanoate

For production of biopolymer bacteria were grown on mineral medium (MM) consisting of 40 g/L glucose; 1.5 g/L of $KH_2PO_4$; 3.50 g/L $Na_2HPO_4$; 0.2 g/L $MgSO_4.7H_2O$; 0.01 g/L $CaCl_2.2H_2O$; 0.06 g/L ferric ammonium citrate; 0.05 g/L $(NH_4)_2SO_4$ and 1 mL of trace elements solution prepared according to the method described by Ramsay et al. (1990) and pH 7.2. For solid medium, the composition was identical to the liquid media, plus 15 g /L of agar and subsequently autoclaved for 20 min at 121°C and 1 atm. After growth in mineral medium the bacteria were stained with safranin solution and observed under an optical microscope.

### Growth in bioreactor

The UEA-171 strain was grown in 1 L liquid nutrient medium under agitation (150 rpm) at 30°C for 24 h; after growing, the bacteria were centrifuged at 4000 g for 20 min at 4°C. The cell mass was transferred to the bioreactor (Bioflo 110) containing 3 L of mineral medium (Figure 1), being agitated at 50 rpm at 30°C and 3 $m^3$ of air per hour. Every 12 h, samples were collected for reading the spectrophotometer at 600 nm (FEMTO 600 plus) and for viable cell count (colony forming unit-CFU / ml) on nutrient agar.

### Extraction and purification of biopolymers

About 20 mg of lyophilized bacterial mass were used for the extraction of the biopolymer, and 2 mL of chloroform were added. The tubes were closed tightly, and the samples agitated and heated for 3 h at 100°C. Then the samples were filtered through a cellulose filter with pore 0.4 µm (Millipore) to remove cell debris. The biopolymers were agitating them vigorously for 30 s and precipitated in methanol-chloroform 5:1 v/v, and then dried at 30°C and separated (Jamil et al., 2007).

### Polymer analysis: Gas chromatography

The sample biopolymers were dissolved in 2 ml of acidified methanol (3% (v/v) $H_2SO_4$), and 1 mL of chloroform. The samples were maintained at 100°C for 60 min. After cooling to room temperature, 1 mL of sterile Mili Q water was added to the whole sample, and then stirred for 10 min for phase formation (Braunegg et al., 1978).

After separation, the supernatant (upper) was discarded and the organic phase (bottom) was used for analysis. A volume of 1 ml of this organic phase was analyzed after fractionation of the sample ("split" 1:20) by gas chromatography coupled to mass spectrophotometer, Model QP2010, Shimadzu, with helium carrier gas purity and 5.0.

### Identification of isolate

The identification of isolated strain was performed according to Bergey's Manual of Determinative Bacteriology (Brown and Howard 1939). Genomic DNA was extracted by a bead beating lysis method with 10% sodium dodecyl sulfate and phenol-chloroform. The 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-GAGAGTTTGATCCTGGCT CAG-3') and 1401R (5'-CGGTGTGTACAAGGCCCGGGAACG-3') according to Procópio et al. (2012). The PCR conditions were: 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 s, and a final step at 72°C for 10 min. Subsequently, the DNA amplification product was purified (GFX PCR kit, Amersham Pharmacia Biotech) and sequenced. The partial 16S rDNA sequence obtained was submitted to GenBank for BLAST searching, and phylogenetic analyses were conducted using MEGA5 (Tamura et al., 2011).

## RESULTS

### Biopolymers accumulation

The assimilated carbon sources are biochemically transformed into hydroxyalkanoates units, polymerized and stored in the form of inclusions insoluble in water in the cell cytoplasm (Brito et al., 2011), as illustrated in Figure 2, to the Isolated UEA-20.171 of *Lysinibacillus* sp. where isolates were grown on mineral medium containing glucose, after 10 days of incubation and stained with Safranin which the biopolymers can be viewed under an optical microscope in clearer structures

### Identification and phylogeny

The molecular identification technique using amplification of the 16S ribosomal gene allowed the identification of isolated UEA-20.171, compared with sequences in the public database Genbank using the BLASTn program

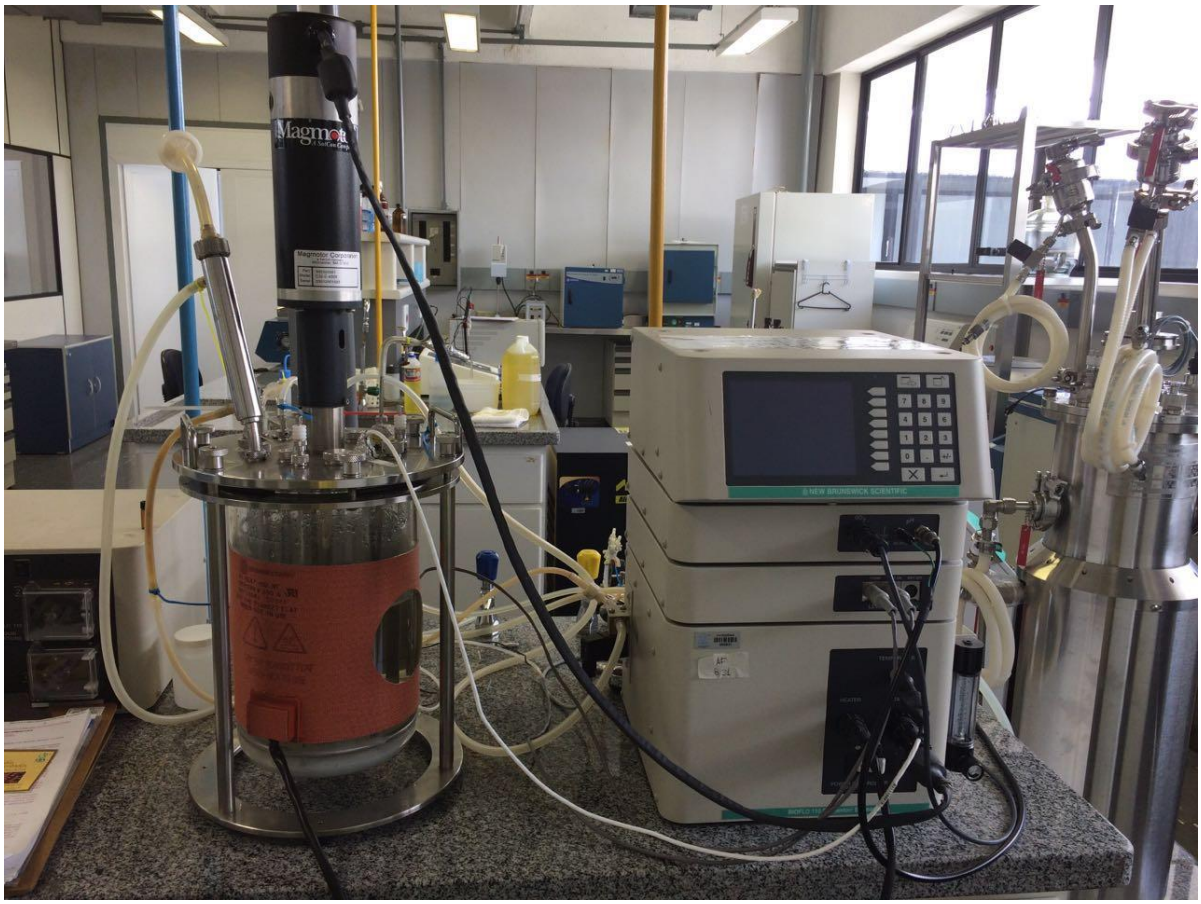
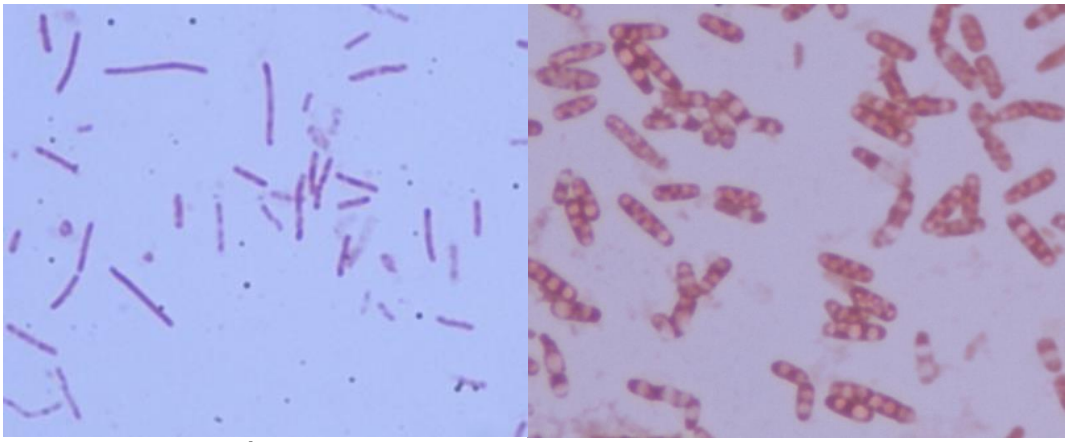


**Figure 1.** Autoclavable 3 L, BioFlo 110 bioreactor (New Brunswick Scientific).



A B

**Figure 2.** Isolated UEA -20.171 of *Lysinibacillus* sp. stained with Safranin and viewed in optical microscope 1000x. A- medium NA and B- medium MM.

(www.ncbi.nih.gov) showing similarity to strains of the genus *Lysinibacillus*.

Currently, RNAs genes can be considered one of the most important molecules for study of microbial ecology

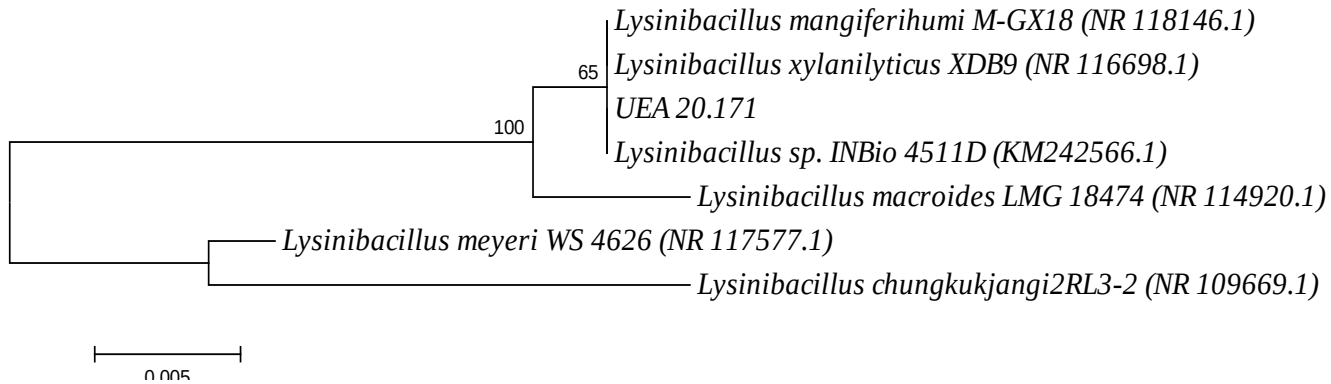



**Figure 3.** Phylogenetic relationship of isolate UEA 20.171 with closely related members of *Lysinibacillus* based on 16S rRNA gene sequence analysis.

and phylogeny because they allow an evaluation of the microbial biodiversity in different ecological niches (Heyndrickx et al., 1996; Muyzer and Smalla, 1998). Besides the identification of microorganisms of the genus level and possibly at the level of species, can also allow correlations between genotype and the environment studied (Chèneby et al., 2000). Despite the UEA-20171 isolated belong with great similarity to *Lysinobacillus* genus; it was not possible to define the specie as shown in Figure 3. *Lysinobacillus* is a genus of Gram-positive bacteria and a member of the phylum Firmicutes and class Bacilli.

### Analysis of biopolymer

The determination of biopolymer was performed using gas chromatograph coupled to mass spectrometer. The methanolizaded samples were injected (1 uL) in the machine using helium as a carrier gas and purity 5.0. As indicated by Figure 4, it was observed that the polymer produced by the isolated UAS-20171, was P(3HB), with peaks at a retention time of approximately 12 and 15 min, similar to the pattern of PHB/HV.

### Growth bioreactor

The accumulation of polymer in the bacterium occurs when there is an imbalance in the concentration of nitrogen in relation to carbon (glucose). After the period of adaptation in mineral medium from 0 to 12 h, the isolated UEA-20.171 significantly increased the cell number (CFU), possibly due to accumulated nitrogen during growth in nutrient medium. Only after 36 h there was an increase in the OD possibly due to the accumulation of biopolymer in the cell, as a consequence of the lack of nitrogen. Cell division was primarily 12 to 24 h, and the formation of biopolymers occurred from 36 to 48 h as shown in Figure 5.

## DISCUSSION

Polyhydroxyalkanoates (PHAs) power reserve and carbon (Tajima et al., 2012; Chen et al., 2011; Roa et al., 2010) accumulated by a wide variety of microorganisms, such as inclusions polyesters insoluble (hydrophobic) as intracellular granules, which may correspond to 90% of the cell mass (Ryan et al., 2013;. Torrego-Solana et al., 2012), Isolated UEA-20.171 was able to accumulate PHA as can be seen in Figure 2. Among the microorganisms producers of PHA, *Bacillus* are commonly found in the environment, however, despite the variety of species, they are largely unexplored. The identification showed that the isolated UEA-20.171 belongs to the genus *Lysinibacillus* as shown in the phylogeny (Figure 3).

*Bacillus* exhibit desirable characteristics for the production of PHAs, such as short generation time, easily reach a high cell density, use of low cost carbon and nitrogen sources, they are able to secrete large amount of enzymes, and are good "hosts" for expression of heterologous genes (Law et al., 2003). Members of the genus *Bacillus*, as well as several other microorganisms that produce PHA, also produce copolymers containing 3HB and 3HV units when grown on different substrates such as glucose, molasses, propionic acid, among others (Gomes, 2013; Krueger, 2009). Furthermore, *Bacillus* can produce a variety of extracellular enzymes, including α-amylases and proteases. Due to these enzymes, *Bacillus* strains are able to utilize polysaccharides and polypeptides for cell growth and direct accumulation of PHA (Halami, 2008). The *Lysinibacillus* is a bacteria of rapid growth, and low nutritional exigency, and might become an important producer of PHA. The synthesis of PHB by *Bacillus cereus* strain T, starts after the end of

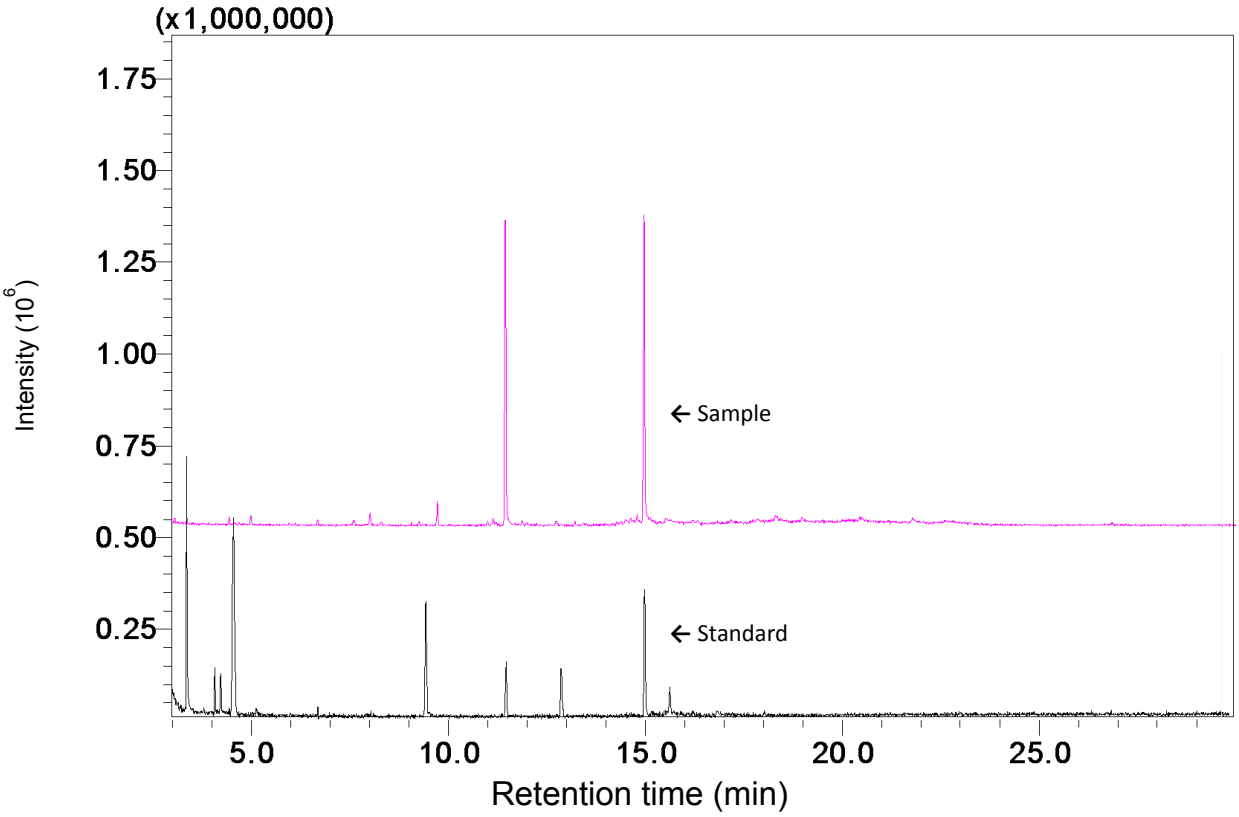



**Figure 4.** Chromatogram of the PHA produced from glucose by isolated UEA-20.171 and the pattern.

the logarithmic phase, reaching a maximum accumulation until the formation of spore, and degradation occurs during the process of sporulation (Valappil et al., 2007b). Already, in *B. megaterium*, during stationary phase, the polymer beads decay in size and number per cell (McCool et al., 1996). For *Lysinibacillus,* cell growth was observed from 12 to 24 h and the PHA production from 36 to 48 h as shown in Figure 5. Some unique properties of *Bacillus subtilis* as lacking lipopolysaccharide layer, the expression of genes of autolysis on completion of the PHA biosynthesis process, and the use of industrial biowaste enable it to compete as a potential candidate for the commercial production of PHAs (Singh et al., 2009). Gram-negative bacteria contain lipo-polysaccharides (LPS), which are pyrogenic endotoxins, in the outer membrane. In contrast, most Gram-positive bacteria do not contain LPS (Singh et al., 2009). The lack of LPS is an advantage of using a Gram-positive PHA producer to produce endotoxin-free PHA for medical applications. Additionally, *B. subtilis* is generally regarded as a safe (GRAS) organism by the Food and Drug Administration (Singh et al., 2009). PHA produced by *Lysinibacillus* was similar to that produced by *Bacillus (*Figure 4). For PHA recovery, solvent, or both are usually used for the extraction of PHA, for the digestion of non-PHA cellular materials, or for both processes. Gram-positive bacteria, including *Bacillus*, have relatively thick cell walls; thus, more energy and chemicals are required to disrupt the cells for recovery of the intracellular PHA (Tsuge et al., 2015).

However in the study of Costa (2012), 217 bacteria were isolated from sponges collected from the cities of Parintins and Manaus - Amazonas. Of this total, they identified 30 strains of bacteria in the city of Parintins, noting the presence of *Lysinibacillus* genus, at a frequency of 13.6%, associated with freshwater sponges, with symbiotic functions. The *Lysinibacillus* genus has not been described until now for the production of PHA. Other studies have also shown the *Bacillus* ability to produce poly-3-hydroxibutirato - PHB (Lee et al., 2004; Takaku et al., 2006) being the most promising of biopolymers existing in the form of amorphous semi-crystalline granules inside the bacteria (Jendrossek and Handrick, 2002 ).

Valappil et al. (2007a) have reported some *Bacillus* species studied for their ability to accumulate PHA. Among these, the *B. thuringiensis* strain DSM2046 stands out in the presence of acetate as a carbon

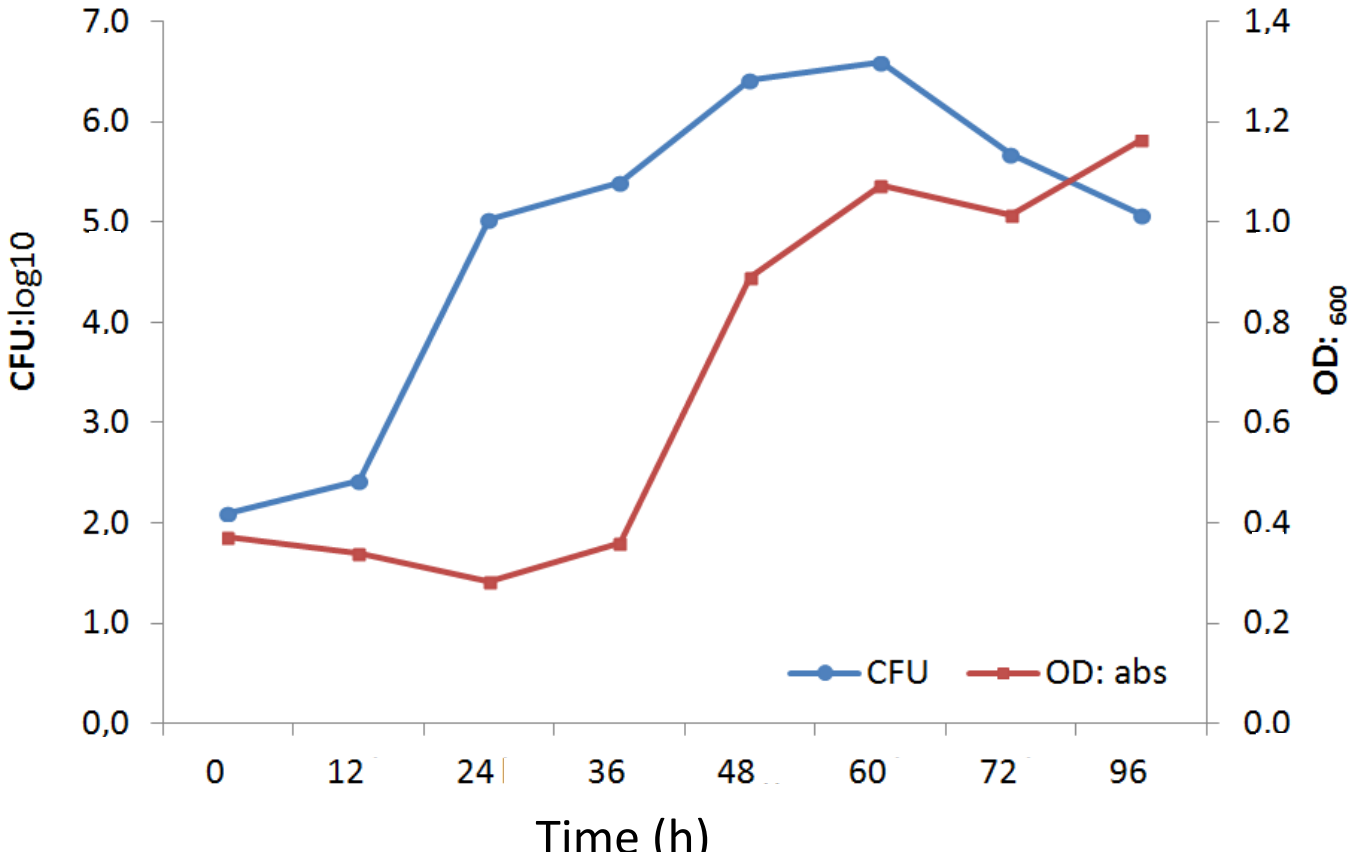



**Figure 5.** Cell division and biopolymers formation in the bioreactor using the isolated UEA-20.171 of *Lysinibacillus* sp.

source, producing about 47.6% by weight of PHB.

It is known that one of the factors limiting the commercialization of PHAs is the cost of the substrate for its production, so many researches with the use of low cost substrates, such as sugar cane molasses used for the production of P (3HB) by *B. megaterium*, with a yield of 46.2% P(3HB) on a dry weight biomass (Gouda et al., 2001). Omar et al. (2001) cultivated *B. megaterium*, using date syrup and sugar beet molasses as carbon source, and obtained an accumulation of 52 and 50%, respectively. In addition, they found that the accumulation of P(3HB) is associated with cell growth. Already *Bacillus* sp. JMa5 grown on molasses, accumulated 35% of P(3HB) on dry weight (Wu et al., 2001). Borah and colleagues (2002) studied the influence of nutritional and environmental conditions for the accumulation of P(3HB) in *B. mycoides*, indicating that sucrose, meat extract and ammonium sulfate are important elements for growth and polymer accumulation, obtaining a 69.4% yield of P (3HB) of the dry weight of the biomass. *B. cereus* accumulated polymer 40.3% (75% 3HB and 25% 4HB) when grown on medium containing fructose (Valappil et al., 2007c). Values higher than 60% of cell dry weight P3HB were obtained when given glucose or xylose as carbon sources, making it a candidate strain for the production of PHA using lignocellulosic residues as substrates (Lopes et al., 2009). Further testing should be made with UEA -20.171, using industrial waste as a carbon source, to evaluate the cost of PHA production by this bacterium.

## Conclusions

*Bacillus* spp. are the most versatile PHA producers, their abilities to produce PHAs range from homopolymers to copolymers from simple sugars to complex industrial wastes. Isolated UEA -20.171 of *Lysinibacillus* sp. was able to grow and accumulate large amounts of PHA in a short period of time. P3HB production by *Lysinibacillus* has some advantage, and can use various carbon sources; secretion of a variety of extracellular enzymes, endotoxin-free PHA production. These benefits will contribute to a low-cost production of high-performance PHA, since the major obstacles of PHA commercialization are high production costs and poor material properties.

## Conflict of interests

The authors have not declared any conflicts of interests.

## ACKNOWLEDGEMENTS

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