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Study of *Bacillus* spp. strains used in the production of γ-polyglutamic acid in submerged culture

[1*]Campos, V., [2]Pimentel Filho, N. J. and [1]Medeiros, T. A. M.

[1]Science and Technology Institute of Sorocaba (ICTS), São Paulo State University, Brazil
[2]Center of Nature Science, Universidade Federal de São Carlos, Buri, São Paulo, Brazil

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Abstract

Gamma-polyglutamic (γ-polyglutamic) acid has received considerable attention for environmental applications. γ-polyglutamic acid can be produced by fermenting soybeans and by submerged culture using the bacterium *Bacillus subtilis*. Although it is practically impossible to chemically synthesise γ-polyglutamic acid, this biopolymer can be obtained through microbial culture. The microorganism most commonly used in the production of γ-polyglutamic acid, among other strains, is *B. subtilis*, which has been investigated for the production of biopolymers. The purpose of the present work was to evaluate the production of γ-polyglutamic acid from submerged culture of different *Bacillus* strains. The evaluation of the efficiency of *Bacillus* strains in producing γ-polyglutamic acid indicated that *B. licheniformis* produced a higher volume of the biopolymer than *B. subtilis*. The highest γ-polyglutamic acid production by *B. licheniformis* was 398.1 mg L^{-1}. *B. licheniformis* was found to be an L-glutamic acid-independent bacterium for the production of γ-polyglutamic acid. *B. licheniformis* and *B. subtilis* growth and γ-polyglutamic acid production rates were also influenced by the culture media.

Introduction

Gamma-polyglutamic acid (γ-PGA) is a water-soluble, biodegradable, biocompatible, and non-toxic anionic biopolymer obtained through fermentation by bacteria of the genus *Bacillus*. Biopolymers are of interest mainly because they offer an alternative for the substitution of synthetic polymers derived from petroleum, which pollute the environment since they do not undergo biological degradation (non-biodegradable), and that chemical degradation occurs in decades. Another important factor for the use of biopolymers is that they originate from renewable sources, such as soy, which makes them promising for industrial applications. The use of soy in the extraction of γ-PGA can add economic value to a soft commodity, since the volume of agricultural derivatives traded in the international market represents about 5% of the total volume of financial derivatives (Campos *et al.,* 2016).

γ-PGA, which is produced exclusively by microbial metabolism, is obtained by enzymatic synthesis, mainly by *B. subtilis*. To date, there is no methodology for the chemical synthesis of γ-PGA (Shih and Van, 2001; Nagai and Tamang, 2010). Growing interest has focused on the development of solid-state culture (SSC) processes, given the limitations of γ-PGA production due to the increase in viscosity of liquid culture media, and the search for low-cost substrates. However, there are only few reports about solid-state production of γ-PGA (Xu *et al.,* 2005; Chen *et al.,* 2005; Oh *et al.,* 2007).

γ-PGA has been produced on an industrial scale via extracellular fermentation of bacteria on renewable sources such as soybean substrate (Shih and Van, 2001). Some companies produce γ-PGA commercially for different sectors of the economy. Shandong Freda Biotechnology Co. Ltd., a biotechnology company, produces, extracts, and sells γ-PGA products for the food industry, cosmetics, health care, water treatment, hygiene products, medical applications, *etc.* Nippon PolyGlu Co. Ltd. produces PGα 21Ca for water treatment. Campos *et al.* (2016) performed coagulation-flocculation tests and solid-liquid phase separation by sedimentation with PGα21Ca, reporting significant results in the removal of apparent colour and turbidity in a water treatment plant, in both the dry and rainy seasons, especially with the coagulants polyaluminium chloride and aluminium sulphate. According to Oppermann-Sanio and Steinbüchel (2002), γ-PGA hydrogel with flocculating activity can be synthesised. Modifying the γ-PGA molecule can increase the polymer's coagulant activity (Kunioka, 1997; Taniguchi *et al.,* 2005; Campos *et al.,* 2017).

*Corresponding author.
Email: v.campos@unesp.br

γ-PGA can also be extracted from natto, a fermented soybean food product mainly consumed in Japan. Figure 1 shows a schematic diagram of the polymer production chain. In bacterial biosynthesis, glutamic acid, the monomer that constitutes γ-PGA, may be exogenous or endogenous, *i.e.*, it can be supplied to the microorganisms in culture media or through the Krebs cycle. Various carbon sources can be used for the synthesis of γ-PGA, such as glucose and other carbohydrates. Through glycolysis and the Krebs cycle, these carbons will synthesize α-ketoglutaric acid, which is a key intermediate for the synthesis of γ-PGA, a direct precursor of glutamic acid. Another route to produce glutamic acid is from glutamine, which involves synthesizing glutamic acid by means of the enzyme glutaminase (Ikeda and Tsuno, 1984; Wu *et al.,* 2008).

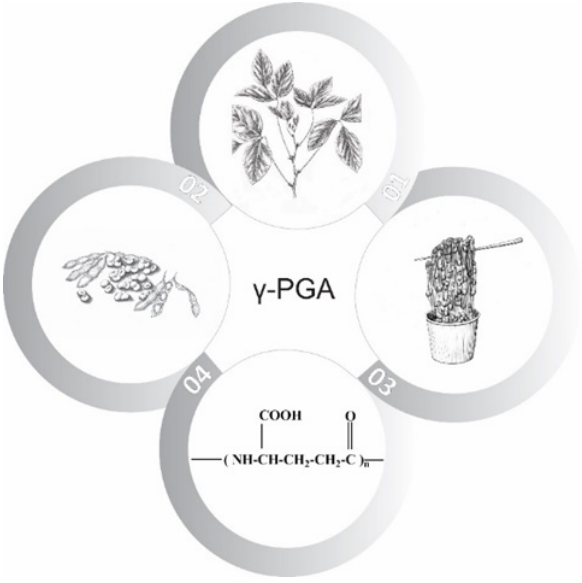

Figure 1. Illustrative representation of the sequence for obtaining γ-PGA: 1 = soy [*Glycine max* (L.) Merrill]; 2 = soy beans; 3 = natto, with apparent viscosity; and 4 = chemical formula of γ-polyglutamic acid.

Although it is practically impossible to chemically synthesise γ-PGA, this biopolymer can be obtained through microbial culture (Shih and Van, 2001). The microorganism most commonly used in the production of γ-PGA, among other strains, is *Bacillus subtilis*, which has been investigated for the production of biopolymers (Meerak *et al.,* 2007; Bajaj and Singhal, 2009; Yong *et al.,* 2011; Kongklom *et al.,* 2017). Strains of the genus *Bacillus* are promising for the production of γ-PGA because of their high growth rates, short fermentation cycles, secretion of proteins in the extracellular medium, and their GRAS (*generally recognized as safe*) status (Schallmey *et al.,* 2004). Although many studies have reported the submerged culture of γ-PGA fermentation (Soliman *et al.,* 2005; Jiang *et al.,* 2006; Wu *et al.,* 2008), the present work instead reported the effects of nutrient control in submerged culture of γ-PGA production. Specific objectives were to understand the production mechanisms using different *Bacillus* strains. The present work thus highlights the relevance of appropriate use of microbial strains for the development of bioproducts.

Materials and methods

The standard used was γ-PGA in sodium salt form, a product of Shandong Freda Biotechnology (90.18% purity). The solutions used in all the experiments were prepared using analytical-grade reagents and ultrapure water. Ultrapure water (resistivity of 18.2 MΩ cm^{-1} at 25°C) was obtained from the Gehaka Master System MS 2000 water purification system.

Apparent viscosity was determined using an A&D SV10 viscometer. Elements commonly found in organic compounds, such as carbon, hydrogen, and nitrogen, were identified by means of elemental analysis, using a Perkin Elmer 2400 CHNS/O Elemental Analyser.

The presence of γ-PGA functional groups was investigated by means of infrared (IR) spectroscopy. The infrared spectra from the compounds embedded in potassium bromide for IR spectroscopy were obtained in the range of 4,000 - 400 cm^{-1}, using a Perkin Elmer 16 PC FTIR spectrophotometer.

The amount of γ-PGA was determined with the spectrophotometric method based on the complexation reaction of γ-PGA with cetyltrimethylammonium bromide (CTAB), as described by Kanno and Takamatsu (1995). The γ-PGA calibration curve was constructed at 400 nm using a spectrophotometer.

The γ-PGA was produced using two *Bacillus* strains not yet studied for the production of γ-PGA, namely, *B. subtilis* NCTC 3610 and *B. licheniformis* NCTC 10341. All the strains were grown at an ideal temperature of 33°C and pH of 7.0 (± 0.2). The inoculum was incubated in nutrient broth and E broth for 24 h in an orbital shaking incubator. The inoculum was standardised at absorbance 1.0 (± 0.1) for fermentation in E broth. Bacterial growth in nutrient broth and E broth was studied and γ-PGA production mechanisms were evaluated based on the cultivation of microbial consortia and in the absence of L-glutamic acid. The experiments were carried out in triplicates and values were expressed as mean ± standard deviation. The *t*-test was conducted to determine the significance. A probability level of $p < 0.05$ was considered to be statistically significant.

The experimental setup consisted of a Biogreen model 40 vertical laminar flow hood, a Class A3 HEPA filter. In the fermentation process, the temperature was controlled and the flask shaken using a orbital shaking incubator Lucadema. The Thermo Fisher Scientific Legend Micro 21R refrigerated microcentrifuge operating at a maximum rotational speed of 21,100 *g*. The bacterial growth was analysed and γ-PGA was quantified by the spectrophotometric method, using a Nova Instruments NI-1600 UV spectrophotometer operating at wavelengths of 600 and 400 nm.

Results and discussion

γ-PGA quantification curve

Shandong Freda Biotechnology Co. Ltd. produces the sodium form of γ-PGA, so the sodium concentration was analysed here by ICP-OES and quantified as 9.82 wt%. The theoretical yield of sodium in $C_5H_8NO_4Na$ γ-PGA was 10.71%. Hence, based on stoichiometric calculations, the theoretical yield of γ-PGA in sodium salt was 82.03%. Therefore, 1.1895 g of γ-PGA from Shandong Co. Ltd, weighed to an accuracy of ± 0.1 mg, was placed in a platinum crucible and calcined at 700°C for 6 h. After cooling in a desiccator, the white residue, which consisted of sodium carbonate, yielded a weight of 0.3162 g.

Based on the elemental analysis of the materials used in the present work (Table 1), the CHN results indicated very similar nitrogen/carbon values for pure γ-PGA and *B. licheniformis* γ-PGA. The CHN content of pure γ-PGA was 29.72% C, 6.06% H, and 6.93% N, and *B. licheniformis* γ-PGA was 48.79% C, 7.61% H, and 6.22% N. As can be seen, the C:N ratio for pure γ-PGA and *B. licheniformis* γ-PGA was 4.28, which is consistent with the composition of the γ-PGA molecule. Considering that the formula of γ-PGA is $(C_5H_7NO_3)n$ and based only on the monomer $C_5H_7NO_3$, the γ-PGA produced from *B. licheniformis* contained 48.79% of C, 7.61% of H, 6.22% of N, and 37.38% of O. In a typical elemental analysis of a γ-(D, L)-polyglutamic acid sample, Ho *et al.* (2006) found that purified γ-PGA contained 46.51% of C, 5.43% of H, and 10.85% of N.

As it binds to γ-PGA, CTAB forms a complex of highly dispersed, water-insoluble type micelles, thereby increasing turbidity (Ashiuchi, 2011). This increase in turbidity, indicated by absorbance values read at a 400 nm wavelength, is directly proportional to the concentration of biopolymer in the solution. In the present work, the γ-PGA quantification curve showed an R^2 value of 0.9964 (Figure 2), and equation of $y = 0.0079x + 0.0001$.

The apparent viscosity of the pure γ-PGA was 23.8 mPa.s^{-1} at 23.8°C when dissolved in water at a concentration of 1%. The broth containing the *B. licheniformis* γ-PGA had an apparent viscosity of 21.9 mPa.s^{-1} at 25.2°C.

The γ-PGA extracted from the submerged culture was quantified by complexation with CTAB. The CTAB solution employed to quantify γ-PGA should have a concentration of 0.1 M. This solution was homogenised with sodium chloride (NaCl) solution at a concentration of 0.1 M in the same volume. An aqueous solution of 300 µL of crude γ-PGA was mixed in a 2 mL microtube with 1.2 mL of phosphate buffer, pH 7.0, and 300 µL of CTAB 0.1 M/NaCl 1M (1:4:1 v/v). The resulting complex yielded turbidity proportional to the concentration of γ-PGA, and this compound was spectrophotometrically quantified at 400 nm, using an analytical standard (Kanno and Takamatsu, 1995).

Production of γ-PGA in submerged culture

Nutrient broth was chosen as the inoculum culture medium for γ-PGA production in E broth, as described in the literature. The growth of microorganisms in nutrient broth and E broth was examined, aiming to gain insight into the relationship between microbial growth and γ-PGA production. *B. subtilis* produced the highest yield of γ-PGA (290.5 mg L^{-1}) in 144 h of culture (Figure 3). Richard and Margaritis (2003) were able to produce 23 g L^{-1} of γ-PGA using a medium similar to E broth, obtaining 20 g L^{-1} of glycerol in a culture with *B. subtilis* IFO 3336. According to Wu *et al.* (2008), *B. subtilis* CGMCC 0833 isolated from soil reached a yield of 34.4 g L^{-1}

Table 1. FTIR absorption peaks (cm^{-1}) for γ-PGA sodium salt from Shandong Freda Biotechnology (SFB), used as reference material, and after the process of γ-polyglutamic acid production in submerged culture by the bacterium *B. licheniformis*.

Sample	Amide I	Amide II	C=O	C-N	N-H	O-H
γ-PGA (SFB)	1,637.78	1,448.00	1,399.80	1,127.01	779.24	3,435.07
γ-PGA (*B. licheniformis*)	1,643.04	1,548.25	1,401.89	1,159.91	702.60	3,428.01

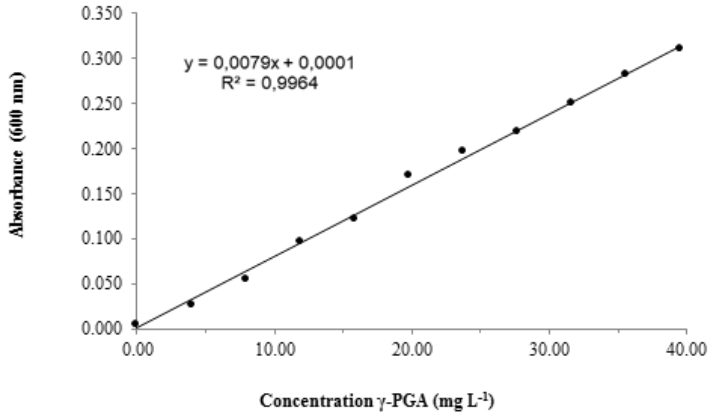

Figure 2. Calibration curve for the quantification of γ-polyglutamic acid.

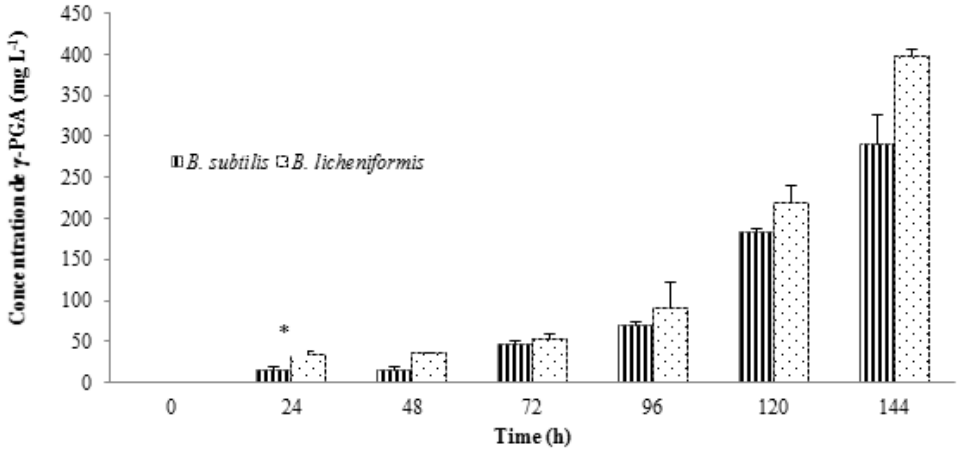

Figure 3. γ-PGA production in E broth. *statistically significant difference between the means ($p < 0.05$).

of the biopolymer. *B. licheniformis* presented the highest γ-PGA production in all the culturing times, with statistically significant difference in the mean of γ-PGA production at times 24, 48, and 144 h. The highest γ-PGA production yield was 398.1 mg L^{-1} in 144 h of culture, and in the first 24 h of culture, the bacterium had already produced a relatively large amount of the biopolymer, *i.e.*, about 32.9 mg L^{-1} (Figure 3). Soliman *et al.* (2005) fermented *B. licheniformis* in a medium similar to E broth, without L-glutamic acid and with casein, $(NH_4)_2SO_4$, and NH_4Cl, and obtained a maximum yield of 33.5 g L^{-1} of γ-PGA. *B. licheniformis* CCRC 12826 produced a yield of 19.8 g L^{-1} in the fermentation in modified E broth (Shih *et al.,* 2002).

Even though *B. licheniformis* strain used in the present work yielded the highest capacity to produce the biopolymer, production was low as compared to that achieved with other strains of the same species. This strain thus did not show a potential to produce high γ-PGA yields. γ-PGA production was stable during the stationary growth phase. From an economic standpoint, this is crucial since production does not only occur during the exponential growth phase (Figure 4).

In another experiment, γ-PGA production was carried out using a microbial consortium in order to determine if a higher yield could be obtained in this condition. The γ-PGA production yield was found to be lower when the two microorganisms were grown together. The consortium presented a decrease in production yield, with a maximum of 185.2 mg L^{-1} following 120 h of cultivation (Figure 5). Even the considerable production yield of the biopolymer grown in microbial consortia did not exceed the γ-PGA production yield achieved in the culture using only *B. licheniformis* or *B. subtilis*. Consortial production was probably a limiting factor in the production of γ-PGA because these bacteria compete for nutritional resources.

B. licheniformis showed the lowest γ-PGA production efficiency in the absence of L-glutamic acid and in the presence of glycerol (75.7 mg L^{-1}), sucrose (71.1 mg L^{-1}), and glucose (46.2 mg L^{-1}).

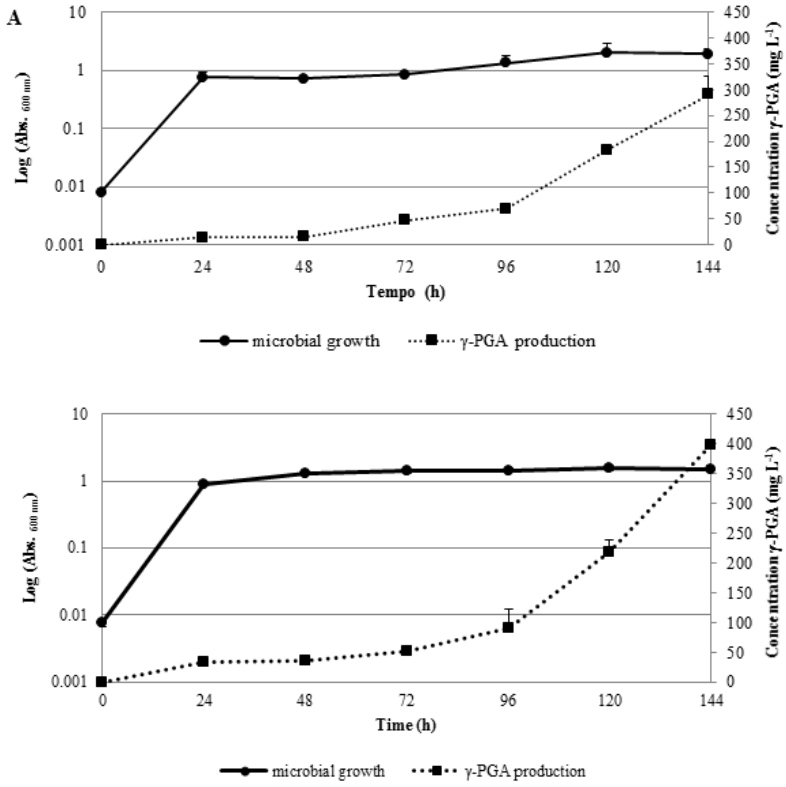

Figure 4. Microbial growth curve and γ-PGA production in *B. subtilis* (A) and *B. licheniformis* (B) in E broth.

With glycerol as the carbon source, the production yield was 75.7 mg L^{-1} (96 h). The use of glycerol to produce γ-PGA in *B. licheniformis* has been previously reported (Cromwick *et al.,* 1996; Shih *et al.,* 2002).

It can be stated that *B. licheniformis* is a bacterium independent of L-glutamic acid, which is a favourable characteristic in the biopolymer production process, since the bacterium can use different routes to produce γ-PGA, thereby intensifying its production. *B. licheniformis* potentiated the production of γ-PGA, as it used a larger spectrum of carbon sources, as well as glutamic acid, for the production of the biopolymer. On the other hand, *B. subtilis* was able to produce only in the presence of glutamic acid and glucose. The use of carbohydrate sources in γ-PGA production can reduce production costs, since L-glutamic acid represents 50% of the money spent on the preparation of E broth. The suitable carbon sources for the biosynthesis of γ-PGA strongly depend on the bacterial strains. Glucose, glycerol, maltose, citric acid, fructose, and sucrose are the carbon sources normally employed in γ-PGA production, in the form of single or combined components of the production medium. Cheng *et al.* (1989) found that the ideal carbon source for the production of γ-PGA by *B. licheniformis* A35 was glucose, at a concentration of 8 g L^{-1}. On the other

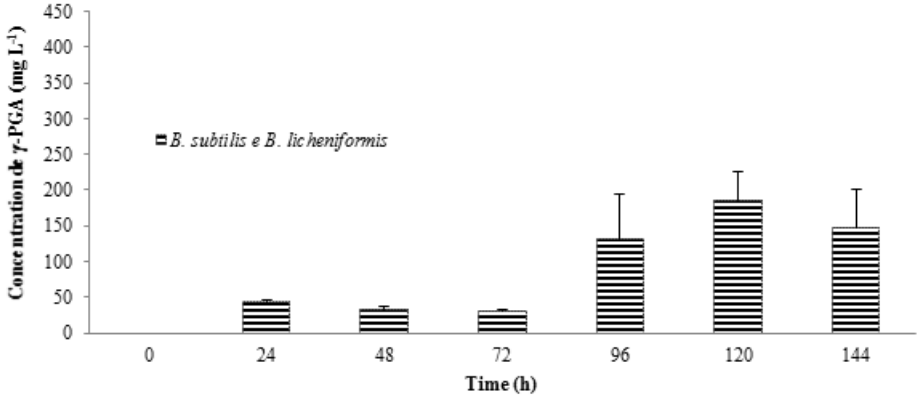

Figure 5. γ-PGA production in a microbial consortium containing the *Bacillus* strains.

hand, sucrose was the ideal carbon source for *B. subtilis* ZJU-7. In the present work, *B. licheniformis* presented higher γ-PGA production in the presence of L-glutamic acid in the medium. This is desirable since the use of other carbon sources could reduce the production cost.

Characterisation by FTIR

Table 1 depicts the FTIR analysis results of pure γ-PGA and *B. licheniformis* which show similar peaks given that the samples had the same composition since γ-PGA is also produced from fermented soybeans.

According to Ho *et al.* (2006), the FTIR absorption bands for sodium γ-PGA in KBr pellets are: amide I, N-H bending band at 1,638 cm^{-1}; amide II, stretching band at 1,585 cm^{-1}; C=O symmetric stretching band at 1,402 cm^{-1}; C-N stretching band at 1,131 cm^{-1}; N-H out of plane bending band at 707 cm^{-1}; and O-H stretching band at 3436 cm^{-1}.

The γ-PGA solution extracted from the submerged culture of *B. licheniformis* showed bands such as amide I at 1,643 cm^{-1} and C=O stretching vibrations of carbonyl groups at 1,401 cm^{-1}. Contributions on a smaller scale come from C-N stretching vibrations at 1,159 cm^{-1} and N-H folding vibrations at 702 cm^{-1}, in addition to the O-H stretching band at 3,428 cm^{-1}. The absorption peak at 3,400 – 3,450 cm^{-1} is characteristic of OH stretching of adsorbed water molecules (Table 1). In biopolymers such as γ-PGA, glutamate is synthesised independently of the ribosomes and is polymerised through amide bonds, which differentiates it structurally from proteins, in addition to distinguishing it in terms of its functionality (Shih and Van, 2001).

The absorptions in the average wavelength of the infrared spectrum for amide allow one to identify secondary structures. γ-PGA can appear in five different conformations, depending on the conditions of the medium in which the biopolymer is located (Rajan, 2014). Based on the identification of secondary structures in amides, absorption bands in the range of 1,648 – 1,660 cm^{-1} are characteristic of the structural conformation of the α-helix motif. The ionisation of lateral groups of γ-PGA is identifiable through infrared analysis. In the γ-PGA, after extraction from the submerged culture, it was possible to identify the band characteristic of lateral ionization of the COO^- carboxyl groups from the spectrum in the band at 1,548 cm^{-1}. Ionization of the lateral groups is responsible for influencing the structural conformation of γ-PGA, and directly linked to the pH levels of the medium and to the temperature. An increase in the absorption values in this range is indicative of the moment when the structural conformation of the γ-PGA molecule shifts from the α-helical structure to a random chain structure (Ho *et al.,* 2006; Carbonaro and Nucara, 2010).

Conclusion

The microorganisms yielded low γ-PGA production when compared with the data obtained from available literature. The highest γ-PGA production potential was shown by the bacterium *B. licheniformis*. The cultivation of bacterial consortia did not positively affect γ-PGA production, since the bacteria presented superior results in separate cultures.

B. licheniformis can be considered an L-glutamic acid-independent bacterium since it is able to produce γ-PGA in the absence of L-glutamic acid. The γ-PGA production yield was highest in the culture using solely *B. licheniformis*, since this bacterium did not compete for resources and used more than one route to produce the biopolymer.

Considering the base of the $C_5H_7NO_3$ monomer, the γ-PGA produced from *B. licheniformis* showed C: 48.79%, H: 7.61%, N: 6.22%, and O: 37.38%. The spectra of the γ-PGA produced by *B. licheniformis* were consistent with the presence of carboxyl, hydroxyl, carbonyl, and amide groups, which are expected in the structure of this biopolymer.

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