

Biosynthesis of γ -Polyglutamic Acid by *Bacillus licheniformis* Through Submerged Fermentation (SmF) and Solid-state Fermentation (SSF)



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Gamma-Polyglutamic acid (γ -PGA) was produced from fermentation processes by inoculating the bacterium *Bacillus licheniformis* on different substrates. In submerged fermentation (SmF), glycerol and glucose were used as the main carbon substrates while ammonium sulfate served to provide nitrogen. In solid-state fermentation (SSF), soybean was used as the main substrate. Tests carried out in an airlift bioreactor with *B. licheniformis* showed a maximum productivity of 0.789 g L⁻¹ h⁻¹ and a yield of 0.4 g g⁻¹. Different soybean cultivars from the 2015 and 2016 crops were used in the SSF. The BRS 1001IPRO cultivar, from the 2016 crop, showed the highest production, i.e., 1.2 g L⁻¹ of γ -PGA. This cultivar was subjected to different levels of milling (fine, intermediate and coarse). Coarsely milled grains increased γ -PGA production to 2.7 g L⁻¹. Moreover, coarse milling made the internal nutrients of the grains available for fermentation and this particle size did not compromise aeration during fermentation.

Keywords:

γ -polyglutamic acid, biosynthesis, *B. licheniformis*, SmF, SSF

Introduction

The industrial importance of bioprocessing and bioprospecting of microorganisms cannot be overstated. In recent decades, in addition to basic nutrition functions, food has been found to possess other functional properties that benefit the chemical and pharmaceutical industry, and more recently, that have been applied to the treatment of waste and effluents. The use of compounds made from biodegradable polymers of vegetable or microbial origin is extremely important from the standpoint of saving resources. Since such biodegradable polymers are of natural origin, they can be incorporated into the natural carbon cycle, which involves natural recycling.

The use of renewable sources in the production of consumer goods offers substantial socioeconomic and environmental benefits. The final destination of soybeans must go beyond oil and bran processing, extending its use to different sectors of the economy. The development of a bioprocess to produce bioactive compounds is of interest to environmental and human health, reducing the generation of sludge from water treatment plants, and hence, of

aluminum concentrations that contribute to Alzheimer's disease.

Brazil is the world's biggest soybean producer; hence, the production of γ -PGA in fermented soy substrate shows a very promising potential to add economic and technological value to commodities, as well as future research. γ -PGA is naturally present in products of fermented soybean mucilage consumed in Asian countries, and soy and its derivatives are therefore potential substrates for the production of biopolymer. Thus, glycerol considered a by-product in the production of biodiesel from soy, can be an inexpensive source of nutrients to produce γ -PGA.

Soybean bagasse, which is also a by-product of the biodiesel extraction process, still contains quantities of nutrients that serve as food sources for many microorganisms, thereby producing new compounds. γ -PGA is produced from carbon and nitrogen-based nutrients that are present in soybeans. Therefore, it is important to know the variations in cultivars in order to select the best properties for conversion in the fermentation process.

Since γ -PGA has various biochemical properties, this polymer can perform different functions, depending on the organism and the environment^{1,2}.

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The limitation of γ -PGA production due to the increase in the viscosity of liquid culture media and the search for cheaper substrates have led to the development of solid-state fermentation (SSF) processes. However, reports on the production of γ -PGA in SSF are much less frequent than those in submerged fermentation, e.g., the works of Oh³, Xu⁴, and Chen^{5,6}. Worldwide interest in these polymers has grown considerably in recent times⁷. Although their application is advantageous for environmental conservation, their high production cost compared to that of conventional products for treating water and effluents is still a major disadvantage. This can be exemplified by comparing the cost of aluminum sulfate – US\$ 1.10/kg, and aluminum polychloride – US\$ 7.50/kg, against that of 30 % γ -PGA – US\$ 32.00/kg and pure γ -PGA – US\$ 620.00/kg.

Biopolymers appear as a substitute for conventional products traditionally used in the market^{8,9}. 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 PG α 21Ca for water treatment¹⁰. γ -PGA is considered a superior bioflocculant because of its ability to flocculate a wide range of organic and inorganic compounds¹¹.

γ -PGA can be extracted from natto, a fermented soybean food produced and consumed mainly in Japan. Natto, which has an odor characteristic of short-chain fatty acids and ammonia, is composed of a highly viscous polymer. 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 citric acid. Through glycolysis and the Krebs cycle, these carbons will synthesize α -ketoglutaric acid, which is a key intermediate for the synthesis of γ -PGA, which is a direct precursor of glutamic acid¹². Another route to produce glutamic acid is from glutamine, which involves synthesizing glutamic acid by means of the enzyme glutaminase^{13,14}. γ -PGA is produced predominantly by bacteria belonging to *Bacillus* spp., such as *B. licheniformis*, *B. subtilis*, *B. megaterium*, *B. pumilus*, *B. mojavensis*, and *B. amyloliquefaciens*. *B. licheniformis* and *B. subtilis* have been used mostly for the fermentative production of γ -PGA¹¹.

Bacillus licheniformis, which is the object of this study, is used industrially because of its ability to secrete large amounts of enzymes in the culture medium, and because it is a producer of important

compounds, such as γ -PGA^{11,15,16}. *B. licheniformis* can also be used as an expression platform for enzymes, amino acids, antibiotics, biofuels, and other secondary metabolites^{17,18}. *B. licheniformis* has been qualified as a producer of antimicrobial peptides, with activity against several pathogenic or deteriorating microorganisms.

The main purpose of this work was to study the process of γ -PGA production from *B. licheniformis* by means of SmF and SSF. In parallel, the 2015 and 2016 soybean crops were tested to measure their protein content, and hence, their greater production of γ -PGA. This study highlights the relevance and appropriate use of biomass for the development of bioproducts.

Materials and methods

The *Bacillus licheniformis* strain NCTC 10341 used in this research was donated by the Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, Brazil.

Three soybean cultivars developed by the Brazilian Agricultural Research Corporation (EMBRAPA), namely, BRS 232, BRS 360 RR, and BRS 1001 IPRO, were used to produce γ -PGA via SSF. The harvested soybeans were subjected to no chemical or biological treatments. In order to ensure reproducible results, cultivars were used from two different crops, 2015 and 2016. The soybeans were processed in a Willey knife mill and sifted through 16-mesh (fine fraction), 9-mesh (medium fraction), and 4-mesh (coarse fraction) sieves.

The glycerol used in this study, a by-product of the soybean biodiesel extraction process, resulting from the transesterification of biodiesel, was provided by IGTPAN – Granado Polyacrylonitrile Technology Institute.

The standard used here was γ -PGA in sodium salt form, a product of Merck. The solutions used in all the experiments were prepared using analytical grade reagents and ultrapure water from a Millipore Milli-Q water purification system.

The materials were characterized using methods such as elemental analysis and infrared spectroscopy. 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 analyzer.

The starting and modified samples were mounted on metal stub holders and coated with gold. The soybean cultivar BRS 1001 IPRO was then subjected to electron detection in a LEO 440i SEM coupled to an Oxford Si(Li) X-ray Energy Dispersive Spectrometer.

The presence of γ -PGA functional groups was investigated by means of infrared spectroscopy. The infrared spectra from the compounds embedded in potassium bromide for IR spectroscopy were obtained in the range of 4000 – 400 cm^{-1} , using a Perkin Elmer 16 PC FTIR spectrophotometer and Agilent Cary 660.

The glucose concentration was determined by the GOD-Trinder enzymatic oxidase method¹⁹. The glycerol kinase colorimetric assay was performed following the method described by Huang²⁰.

The amount of γ -PGA was determined by the spectrophotometric method based on the complex reaction of γ -PGA with cetyltrimethylammonium bromide (CTAB), as described by Kanno and Takamatsu²¹. The aqueous solution of γ -PGA (300 μL) was mixed with phosphate buffer and CTAB (0.1 M/NaCl 1 M), and held in ultrasonic bath at a temperature of 30 °C for 25 min. The γ -PGA concentration was determined by measuring the turbidity of the mixture with the spectrophotometer at 400 nm, and comparing this with a standard curve. The calibration curve was constructed from complexation with CTAB. Turbidity based calibration curve ($y = 0.0079x + 0.0001$, where x and y represent the concentration of γ -PGA and the turbidity at 400 nm of the mixtures) gave a good linearity over a range of at least 4.0–40 mg L^{-1} γ -PGA. Moisture and volatiles were quantified using the American Oil Chemists Society (AOCS) method Bc 2–49²². Ash content was determined by AOCS method 945.38C, while total lipids were determined using the AOCS method Ac 3–44²². The protein content was determined using Method 037 of the Adolfo Lutz Institute²³.

Experimental

Microorganism

Bacillus licheniformis strain NCTC 10341, donated by Fiocruz, was used for the production of γ -PGA. The ideal medium to grow this strain is in nutrient broth at a temperature of 33 °C and pH between 6.8 and 7.2. The recommended incubation time for aerobic culture was 24 h.

Strips containing *B. licheniformis* spores were placed in a sterile medium, and incubated at 33 °C for 48 hours under orbital shaking at 150 rpm. Aliquots were centrifuged, supplemented with sterile glycerol (20 % v/v), and stored at 25 °C.

The culture media were sterilized in an autoclave at 121 °C for 15 minutes. The sugars were sterilized separately and added before inoculation to prevent the precipitation of ions such as iron, manganese, and zinc. Sterilization can affect the availability of carbohydrates, which may react with am-

monium ions and amino acids that are present in solutions for the production of γ -PGA.

The medium for the production of γ -PGA via SmF in an airlift bioreactor was composed of (g L^{-1}): glucose 30; glycerol 30; L-glutamic acid 50; citric acid 10; NH_4NO_3 8; NH_4SO_4 8; K_2HPO_4 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1; 0.03 MnSO_4 and 0.3 % n-heptane.

Submerged fermentation (SmF) in an airlift bioreactor

In this work, we used an external-loop airlift bioreactor, which allowed for greater customization of the connections, the ascending gas-liquid flow in the riser, and the descending flow in the downcomer. Airlift bioreactors are pneumatically agitated reactors equipped with simple, compact, low-cost, and easy-to-operate devices. In addition to presenting high rates of mass transfer and low energy consumption, another advantage of airlift bioreactors is that they can be used for culturing microorganisms with minimal mechanical impact. According to Rossi²⁴, the use of an airlift bioreactor with forced air injection enables recirculation of the liquid medium. This recirculation also generates turbulence and mixing, contributing significantly to mass transfer in the system. The greater distance covered by the gas bubbles while passing through the system without the inconvenient agitation of turbines favors good transfer of oxygen, ensuring the integrity of the cells due to low shear stress.

The 2.3-L capacity airlift bioreactor used here had external circulation. The bioreactor was sterilized internally with a 3 % peracetic acid solution. After removing the sterilization solution, the bioreactor was sealed until the inoculated culture medium was added, whereupon the fermentation process began. The pH level was adjusted to 7.0 and kept constant throughout fermentation. Oxygen content was monitored with a polarographic probe, and air flow was regulated manually in order to keep the dissolved oxygen (DO) above a critical concentration of 10 % saturation. The second step consisted of feeding nutrients, i.e., glycerol, glucose, calcium chloride, and zinc sulfate, into the bioreactor. Tests in the bioreactor were conducted as described by Kumar and Pal²⁵ and Yoon *et al.*²⁶, by inoculating 46 mL of *B. licheniformis* (2 % v/v) in 2.3 L of sterilized culture medium in an autoclave. The pH level was kept stable between 6.8 and 7.2 in a controller containing solutions for automatic adjustment, with the addition of 0.2 M NaOH and 0.1 M H_2SO_4 solutions.

The temperature in the bioreactor was controlled by circulating water in a heat exchanger in the downcomer, using a thermostatic bath. The inlet air was produced by compressed air, purified with

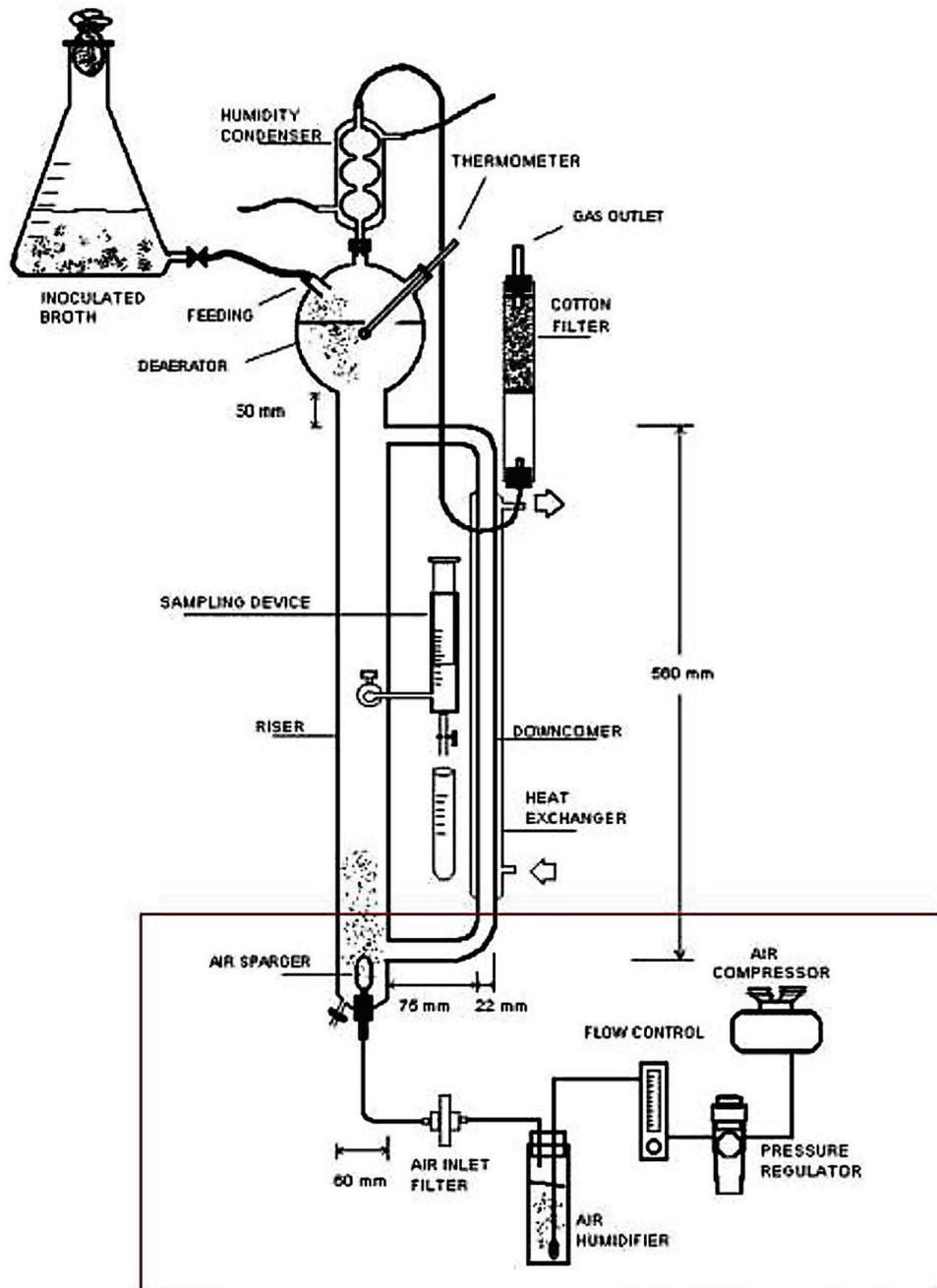


Fig. 1 – Airlift bioreactor system and inlet air flow control

the aid of PTFE (polytetrafluoroethylene) hydrophobic filter membranes with 0.22 mm diameter pores. Airflow and pressure were controlled manually, as shown in the schematic diagram in Fig. 1²⁷. The bioreactor was also equipped with a diffuser to reduce the size of bubbles, a porous ceramic sparger at the base of the riser, from which 10 mL aliquots were removed to analyze γ -PGA, without the need to stop the process and with less risk of contamination. The glucose and glycerol solutions used in the medium for the inoculation and production of γ -PGA were sterilized separately and incorporated into the medium prior to inoculation.

Solid-state fermentation (SSF) using soy as substrate

γ -PGA was produced in triplicate with *B. licheniformis* in three cultivars, and the values were expressed as means \pm standard deviation. The tests were carried out in a 250-mL Erlenmeyer flask, with a hydrophobic cotton plug and gauze. The soybeans were milled in a Wiley mill, and sifted through 16 mesh (fine fraction), 9 mesh (medium fraction), and 4 mesh (coarse fraction) Tyler sieves.

Ten grams of soybeans (dry weight) were selected, immersed in water (pH 7.0) to obtain an ini-

Table 1 – Coded and original values used in the CCRD to evaluate the effect of the initial moisture content, inoculum volume, amount of substrate and temperature in the production of γ -PGA by SSF

Variable	-2	-1	0	+1	+2
Initial moisture content (%)	40	45	50	55	60
Inoculum volume (%)	10	15	20	25	30
Amount of substrate (g)	5	10	15	20	25
Temperature (°C)	31	33	35	37	39

tial moisture content of the substrate of 60 %, and then stored at 10 °C for 18 h, using a modified version of the procedure proposed by Kiuchi and Watanabe²⁸. The grains were then autoclaved for 1 hour at 130 °C, and cooled to 50 °C prior to inoculation (patent CN1718735B). The inoculum, at a concentration of 10 % (m/v), was spread over the surface of the substrate under aseptic conditions, and then subjected to static incubation at 33 °C for 48 h, using a modified procedure described by Chen⁵. To interrupt the fermentation process, the mixture was refrigerated for eight hours at a temperature of 3 to 10 °C, with low moisture content²⁸.

Upon completion of the fermentation, 30 mL of deionized water was added to 10 g of fermented substrates in the Erlenmeyer flask. After agitating at 200 rpm and 28 °C for 1 h on a rotary shaker²⁸, the mixture was filtered through muslin cloth and centrifuged at 14800 rpm for 20 min. Then, 300 μ L of the supernatant was poured into four volumes of cold methanol to precipitate the γ -PGA. The sediment was collected by centrifugation at 14800 rpm for 20 min, and redissolved with 300 μ L of deionized water. To obtain a clarified aqueous solution of γ -PGA, any insoluble contaminants were removed by centrifugation at 14800 rpm for 20 min^{30,31}.

To maximize the production of γ -PGA in SSF, factors such as initial moisture content, inoculum volume, substrate quantity, and temperature were evaluated. Tests for the four factors were performed according to the Central Composite Rotatable Design (CCRD), totaling 31 tests with seven repetitions at the central point. The values were determined using Minitab software. Table 1 shows the coded and original values of the factors. The pH level of the substrates was adjusted to 7.0, and all the substrates were incubated for 48 hours in static mode.

Results and discussion

Production of γ -PGA in an airlift bioreactor

According to Hsueh³², γ -PGA is biosynthesized only at the beginning of the stationary phase, which is explained by the limited availability or even lack

of nutrients needed for the growth of *Bacillus* cells. The resulting biopolymer is degraded at the end of the stationary phase, especially when basic carbon and nitrogen substrates are scanty. The concentration of carbon source and the ideal concentration of nitrogen are essential for bacterial growth, considering the formation of product with amine groups, where an adequate C/N ratio in the environment is crucial to ensure a good yield, preventing the formation of undesirable by-products.

In the airlift system, glycerol was used as the main carbon source because of its favorable organic composition and the fact that it is part of the Krebs cycle, where part of the reactions of biopolymer formation occur. In the process by inoculation of *B. licheniformis* conducted in a SmF according to the methodology described by Yoon²⁵, 35.0 g L⁻¹ was obtained, while the production with *B. subtilis* was 31.7 g L⁻¹ following the same methodology also described by Wu³³ with glycerol as substrate in submerge medium. According to Wu³³, the addition of glycerol contributed to increase productivity and reduce viscosity, mainly when the process was carried out with *B. licheniformis*. The authors emphasized the importance of adding fractionated glycerol to enable the microorganism to make the best possible of the substrate, thereby increasing its molecular weight.

In the first experiment, the pH and DO were controlled (Fig. 2), and 15.21 g L⁻¹ of biopolymer was produced in only 19 hours of fermentation. The production yield reached 21.31 g L⁻¹ 27 hours after the beginning of the process, when the glycerol and glycoside substrates were depleted and the minimum saturation of DO occurred, with a productivity of 0.789 g L⁻¹ h⁻¹. The temperature was kept at 35 °C, the pH level was adjusted to 7.0, and automatically corrected using 0.2 M of NaOH or 0.1 M of H₂SO₄ to remain between 6.8 and 7.2.

After 30 hours, a new test was carried out; this time to investigate γ -PGA productivity through the addition of carbon and enzyme inducers. In this process, 20 % of the medium of the previous test was maintained, keeping the (active and inactive) microorganisms present in the medium. The new culture medium was prepared in the same conditions as those used previously, but with lower concentrations of carbon substrates. The medium was composed of (g L⁻¹): glucose 15; glycerol 15; L-glutamic acid 25; citric acid 5; NH₄NO₃ 4; NH₄SO₄ 4; K₂HPO₄ 1; MgSO₄·7H₂O 0.05; 0.015 MnSO₄ and 0.3 % n-heptane.

Fig. 3 shows the production of 20.30 g L⁻¹ after 43 h of fermentation and the addition of substrates (7.5 g L⁻¹ of glycerol and 6.0 g L⁻¹ of glucose). In this reaction, there was a decrease in DO saturation (Fig. 3). The residual glucose in this range was

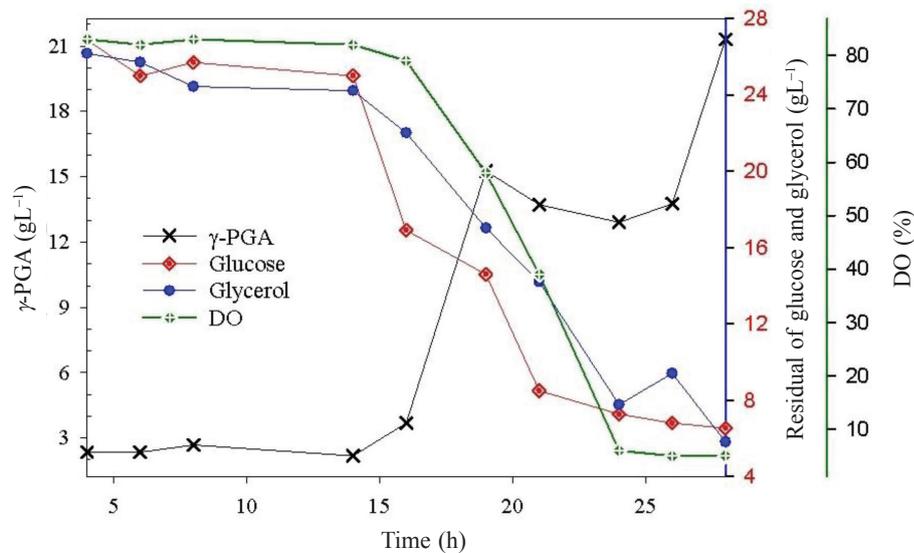


Fig. 2 – γ -PGA production and carbon source consumption, and oxygen consumption in an airlift bioreactor

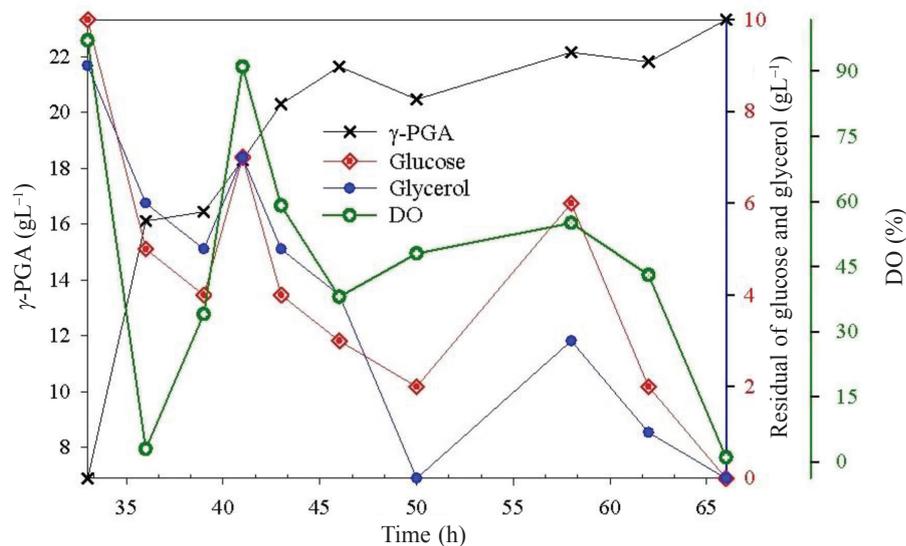


Fig. 3 – γ -PGA production and carbon source consumption, and oxygen consumption

4 g L⁻¹ and glycerol 5 g L⁻¹. With the addition of 50 mL of 1 g L⁻¹ of the inducers calcium chloride and zinc sulfate, maximum production (23.32 g L⁻¹) was achieved in 66 hours, yielding 0.4 g g⁻¹ γ -PGA of carbon substrate. γ -PGA production has been widely studied in different bioreactors, mainly in liquid media. However, what stands out in the current study is the combined use of nutrient sources, particularly of carbon and inducers, as well as cofactors inherent to the airlift bioreactor system. This study can be further expanded.

Table 2 presents the production, productivity, and yield obtained in the tests with different nutrient concentrations. Note the productivity in the airlift bioreactor, where oxygen is more available to microorganisms. Also note the γ -PGA production yield considering the limitation of nutrients and

their addition according to the consumption by *B. licheniformis*. This prevents an overload of carbon sources and the production of undesirable intermediates. Moreover, agitation is produced by the flow of filtered air, reducing the risk of contamination of the medium. An important point about this system is the need to control the parameters of the process, particularly DO, which is essential in aerobic cultures, and a limiting factor for the growth of *Bacillus* spp.

Production of γ -PGA in solid-state fermentation

Using the parameter of rainfall as a determinant revealed that the 2015 and 2016 soybean crops varied in terms of their proximate composition. The cultivars BRS 232, BRS 1001 IPRO, and BRS 360 RR

Table 2 – Production, productivity and yield obtained in tests with different concentrations of nutrients in this study compared to the literature

Strain	Nutrient	Time (h)	Production γ -PGA (g L ⁻¹)	Productivity γ -PGA (g L ⁻¹ h ⁻¹)	Yield (g g ⁻¹)	Ref.
<i>B. subtilis</i> NX-2	Glycerol	48 h	31.7	0.66	0.264	[33]
<i>B. licheniformis</i> ATCC 9445A	Glutamic acid and citric acid	42 h	35	0.83	0.188	[26]
<i>B. licheniformis</i> NCTC 10341	Glutamic acid glucose and glycerol	27 h	21.31	0.79	0.178	This study (SmF)
<i>B. licheniformis</i> NCTC 10341	Glucose and glycerol	66 h	23.32	0.36	0.462	This study (SmF)

Table 3 – Proximate composition of three different soybean cultivars (g 100 g⁻¹) from the 2015 and 2016 crop

Cultivar	Moisture and volatiles	Protein (Nx6.5) ¹	Total lipids	Carbohydrate _(t)	Ash	
BRS 232	2015	12.82 (0.15) ^a	33.66 (0.04) ^a	20.64 (0.19) ^a	28.16	4.72 (0.01) ^a
	2016	9.05 (0.15) ^a	36.90 (0.13) ^a	21.33 (0.08) ^a	28.14	4.58 (0.04) ^a
BRS 360	2015	13.42 (0.26) ^a	30.60 (0.03) ^a	21.31 (0.08) ^a	29.33	5.34 (0.04) ^a
	2016	8.95 (0.03) ^a	38.49 (0.06) ^a	20.86 (0.18) ^a	26.92	4.78 (0.04) ^a
BRS 1001	2015	13.34 (0.28) ^a	34.07 (0.09) ^a	21.73 (0.18) ^a	26.39	4.47 (0.03) ^a
	2016	9.92 (0.18) ^a	35.98 (0.43) ^a	21.32 (0.10) ^a	28.24	4.54 (0.02) ^a

¹The protein content of the sample is calculated by multiplying the total nitrogen content by a specific factor for converting nitrogen to protein. ^aMean and estimated standard deviation.

presented a total rainfall of 762, 749, and 461 mm, respectively, in the 2015 crop, and of 1452, 1023, and 1023 mm, respectively, in the 2016 crop. The increase in precipitation from one crop to another affected the amount of protein in all the cultivars, the main source of chemical compounds for the production of γ -PGA.

Water stress caused by scarcity can reduce the protein content of soybeans by 16 % and lipids by 25 %³⁴. A comparison of soybean cultivars from the 2015 and 2016 crops revealed differences in some parameters (Table 3). The SSF experiments were carried out with three replicates. The data were represented as means \pm standard deviation. The level of significance of the differences was evaluated by means of the *t* test. An analysis of variance was performed using ANOVA procedures. The level of significance was set at $p < 0.05$.

From 2015 to 2016, the protein content increased by 25.8 % in the BRS 360 RR cultivar, by 9.6 % in the BRS 232 cultivar, and by 5.6 % in the BRS 1001 IPRO cultivar. Although the soybean protein content is genetically determined, environmental factors can cause changes in nitrogen availability during grain formation³⁵. Specht³⁶ reported that soil irrigation has a positive effect on soybean protein content, and that severe droughts can cause the protein content to decrease. Soil water stress causes the proteins in soybean seeds to decrease during the stage of reproductive growth³⁷. Pipolo³⁸ stated that rainfall during the grain development

stage, more than changes in temperature, explains differences in protein content. Therefore, changes in this parameter may be linked to water stress in this plant species. On the other hand, the proximate composition depends basically on genetic characteristics (type of cultivar), climate and regional conditions, varying according to the crop^{39–41}.

Solid-state fermentation with *B. licheniformis* was performed using the three soybean cultivars of the 2015 and 2016 crops (Fig. 4). From the cultivars of the 2015 crop – BRS 232, BRS 360 RR, and BRS 1001 IPRO – 0.731 g L⁻¹, 0.643 g L⁻¹, and 0.898 g L⁻¹ of γ -PGA, respectively, were produced. From the 2016 crop, 1120.2 mg L⁻¹ of γ -PGA was produced from the cultivar BRS 232, while 1.112 g L⁻¹ and 1.177 g L⁻¹, respectively, were produced from the cultivars BRS 360 RR and BRS 1001 IPRO.

The cultivars BRS 232 and 360 RR showed a statistically significant difference in means ($p < 0.05$) between crops and cultivars. The means between the cultivars of crop 2016 were statistically equal, but the BRS 1001 IPRO cultivar was the one that presented the highest average production of γ -PGA. Crop 2016 cultivars showed greater potential as a substrate for solid-state fermentation (SSF), as they had a higher protein content, which is desirable in γ -PGA production. Although the microorganisms use glutamic acid as monomers to polymerize γ -PGA, the higher amount of total protein found in this study also indicated the greater pres-

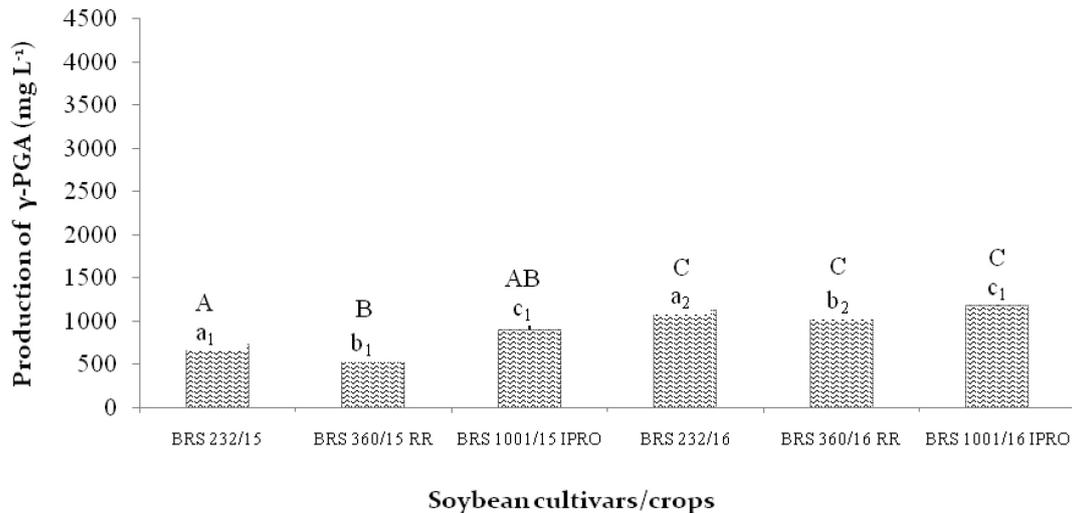


Fig. 4 – Production of γ -PGA by *Bacillus licheniformis* on different soybean cultivars from the 2015 and 2016 crops. All the data are presented as means \pm standard deviation. Lower case letters indicate a relationship between the means of the cultivars in the two crops, while the different numbers indicate statistically significant differences ($p < 0.05$). Different capital letters indicate statistically significant differences ($p < 0.05$) between the means of the different cultivars in the same crop.

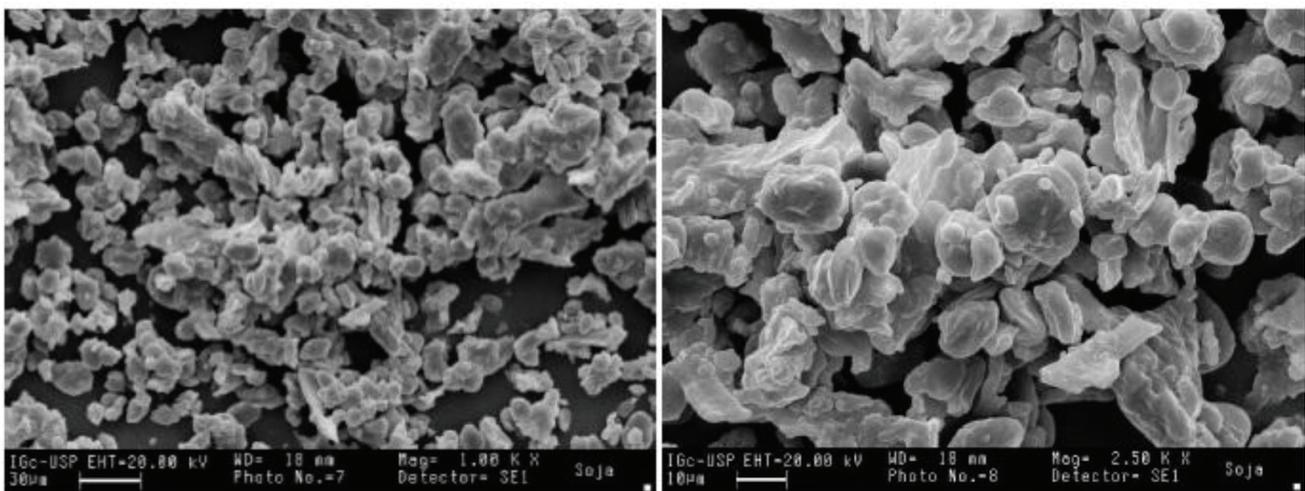


Fig. 5 – SEM images of soybean BRS 1001 IPRO (1000 and 2500X magnification)

ence of this amino acid and others that indirectly influence the production of γ -PGA, such as the amino acid alanine⁴².

To determine whether the soybean's physical structure affects the production of biopolymer, the BRS 1001 IPRO cultivar (Fig. 5) was milled to change the grain structure. The processed grains, i.e., fine, medium, and coarse fractions, produced 1.939 g L⁻¹, 2.253 g L⁻¹ and 2.722 g L⁻¹ of biopolymer, respectively (Fig. 6). These biopolymer yields show a statistically significant difference in means ($p < 0.05$) when compared to those obtained with the whole grains of the same soybeans and soybean crop.

Milling the soybeans increased the γ -PGA production in all the particle sizes when compared to the whole grains. The external protective layer of the beans was completely broken by milling, expos-

ing their internal content. With the nutrients made widely available to microorganisms, biopolymer production had intensified. However, the particle size interfered with fermentation. The production of γ -PGA was lowest with the fine fraction, followed by the medium and coarse fractions. Although smaller particles have larger surface area, they cause the material to become compacted during fermentation, reducing the aeration of the entire content and the production of biopolymer. Larger particle sizes also provide greater aeration during fermentation, as observed in the medium and coarse fractions. The fine and medium fractions showed a statistically significant difference in means ($p < 0.05$) when compared to the coarse fraction.

Notwithstanding the difference in results in the two fermentation processes, SmF and SSF, both

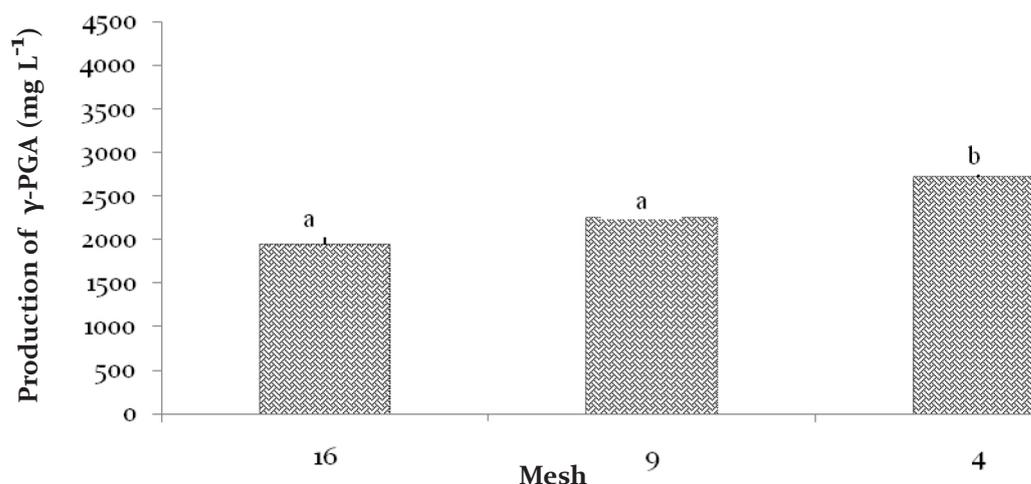


Fig. 6 – Production of γ -PGA by *Bacillus licheniformis* on substrate (BRS 1001 IPRO, 2016 crop) with different particle sizes. All the data are presented as means \pm standard deviation and the different letters indicate statistically significant differences ($p < 0.05$).

Table 4 – CHN content of soybean grain, standard γ -PGA and γ -polyglutamic acid after extraction in SSF

Sample	C/%	H/%	N/%	C/N
soybean	47.06	7.17	7.18	6.55
γ -PGA (SSF)	48.03	7.92	6.85	7.01
standard γ -PGA	44.04	6.93	6.92	6.36

processes required constant aeration. High oxygen ratios resulted in greater production of γ -PGA, and the same applies to the ideal amount of available nutrients in both processes.

Characterization by elemental analysis and FTIR

Table 4 presents the results of the elemental analysis of carbon, hydrogen, and nitrogen (CHN), the materials of this study. The CHN results indicate very similar nitrogen/carbon values for standard γ -PGA and soybean grains. As can be seen, the carbon/nitrogen ratio for the reference γ -PGA and for the γ -PGA extracted from soybean grains is 7.96, which is consistent with the composition of the γ -PGA molecule. Considering that the formula of γ -PGA is $(C_5H_6NO_3)_nNa$ and based only on the monomer $C_5H_6NO_3$, the γ -PGA extracted from SSF

contained 50.33 % of C, 7.27 % of H, 6.32 % of N, and 36.08 % of O (Table 5). In a typical elemental analysis of a γ -(D,L)-polyglutamic acid sample, Ho⁴³ found that purified γ -PGA contained 46.51 % of C, 5.43 % of H, and 10.85 % of N.

Table 5 lists the results of the FTIR analysis of soybean grain, standard γ -PGA, SSF and SmF. The peaks of the standard γ -PGA and γ -PGA produced by *B. licheniformis* spectra are similar, given that the samples have the same composition because natto is produced from fermented soybeans, i.e., it is composed of soybeans, γ -PGA, and levan polysaccharide. The diagnostic region of the soybean and other solutions spectra shows peaks at 1640 cm^{-1} . These peaks correspond to C–H stretching with sp^3 hybridization, and the peaks originate from symmetric and asymmetric stretching of C–H. The spectra of soybeans and other products also show peaks at around 1450 cm^{-1} , which represent the C=O functional group. The soybean, SSF and γ -PGA spectra show strong peaks at around 1640 cm^{-1} , which is characteristic of the amide I group, N–H bending band. These spectra also show a broad absorption band near 3400 cm^{-1} , which corresponds to the O–H bond of an alcohol functional group. Miller⁴⁴ used FTIR to analyze soybean grains, and based on the characteristic peaks of each component, de-

Table 5 – FTIR absorption peaks (cm^{-1}) for standard γ -PGA used as reference material, and after the process of γ -PGA extraction from SSF and SmF

Sample	Amide I	Amide II	C=O	C–N	N–H	O–H
Soybeans	1643.32	1547.43	1401.74	1143.1	699.17	3419.56
Standard γ -PGA	1643.28	1556.75	1405.23	1163.09	704.38	3436.71
γ -PGA (SSF)	1643.04	1554.47	1401.89	1159.91	699.81	3428.01
γ -PGA (SmF)	1643.73	1550.02	1417.46	1211.02	697.26	3446.21

terminated the presence of the groups: amide I at 1650 cm^{-1} associated with protein, lipid ester at 1545 cm^{-1} , and carbohydrates in the region of 1200 to 900 cm^{-1} . These authors stated that the amount and configuration of proteins varied along the cotyledon, as well as the amount of lipids and carbohydrates^{44,45}. An FTIR analysis of the soybeans revealed the presence of the amide I group at 1643.32 cm^{-1} associated with protein, and lipid ester at 1547.43 cm^{-1} .

The FTIR absorption bands for standard γ -PGA in KBr pellets are: amide I, N–H bending band at 1643 cm^{-1} ; amide II, stretching band at 1456 cm^{-1} ; C=O symmetric stretching band at 1445 cm^{-1} ; C–N stretching band at 1263 cm^{-1} ; N–H out of plane bending band at 667 cm^{-1} ; and O–H stretching band at 3436 cm^{-1} .

Conclusions

One of the goals of this work was to test the performance of the airlift bioreactor, which is easy to use and inexpensive, in the production of γ -PGA from *B. licheniformis* in a medium containing a low down combination of carbon and nitrogen nutrients. The airlift bioreactor tests showed promising results, since the system enabled the constant movement of the medium without the risk of the cells undergoing shear stress. Moreover, viscosity in this system did not increase, which can be explained by the addition of glycerol with *B. licheniformis*. The tests carried out in the airlift bioreactor showed a maximum productivity of 0.789 $\text{g L}^{-1} \text{h}^{-1}$ with *B. licheniformis* and yield of 0.4 g g^{-1} .

The genetic variation of soybean cultivars did not influence the protein concentration. However, the increase in rainfall during the growing period (2016) resulted in a higher protein content in the soybeans. In this study, the cultivars BRS 232, BRS 360 RR, and BRS 1001 IPRO from the 2016 crop showed a greater potential as a substrate in SSF because of their high total protein content, which resulted in a higher yield of γ -PGA. The production of biopolymer was influenced by the physical structure of soybeans. The use of milled soybeans increased the γ -PGA production yield, which was highest with the coarse fraction. Milling the soybeans increased the availability of nutrients to microorganisms, and the coarse fraction reduced the impact of compaction, enabling aeration of the substrate. The initial moisture content and volume of inoculum were considered significant factors for the production of γ -PGA. The optimization of significant factors increased the γ -PGA production yield to 4.4 g L^{-1} .

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