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# Soy Molasses as Culture Medium for *Bacillus* Species Aiming at Plant Growth Promotion

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**Abstract:** Soy molasses, a by-product from the processing of soy protein concentrate, is a low-cost feedstock for fermentation processes due to its high content of fermentable sugars. This work investigates the use of soy molasses for growing *Bacillus* species, aiming at their potential application as plant growth promoters. Firstly, six *Bacillus* strains were screened for their ability to grow in increasing concentrations of soy molasses in a microplate assay. Following this, shaken-flask assays for growth and γ-polyglutamic acid (γ-PGA) production by three *Bacillus* strains in medium E and soy molasses media with 28 and 56 g $L^{-1}$ of total reducing sugars (TRS) were carried out. An in vivo experiment evaluated the effect of the bacterial fermented broths on the germination and initial development of maize. Soy molasses supported the growth of *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Bacillus licheniformis* in concentrations of 28 and 56 g $L^{-1}$ TRS, but it was inhibitory at 112 and 224 g $L^{-1}$ TRS. In soy molasses media, growth was not always associated with γ-PGA production, which was a maximum of 56 g $L^{-1}$ TRS for *B. amyloliquefaciens* and *B. licheniformis*. Fermented broths with *B. subtilis* and *B. licheniformis* in soy molasses media (56 and 28 g $L^{-1}$ TRS, respectively) applied to maize seeds resulted in the highest Vigor Indexes of the seedlings, which correlated negatively with the broth pH and were not impacted by the γ-PGA and indole acetic acid produced by the bacteria. The low-cost and easily available feedstock soy molasses constitutes a potential culture medium for the growth of plant growth-promoting bacteria.

**Keywords:** soybean; beneficial bacteria; growth promotion; bacterial growth; bacterial polymer



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## 1. Introduction

Brazil is one of the largest producers and exporters of soybean and corn, estimating a production of 146.52 million tons of soybean and 110.96 million tons of corn in the 2023/24 harvest [1]. At the same time, the requirement for fertilizers is high, approximately 16 million tons per harvest, and around 70% are imported [2].

Considering the need to adapt production systems to the sustainable development objectives established by the United Nations (UN), "Zero Hunger" and sustainable agriculture and actions regarding global climate change [3], it is vital and urgent to develop technologies that can contribute to food production, reducing greenhouse gas emissions ($CO_2$, $N_2O$, and $CH_4$) and energy consumption by optimizing the use of fertilizers by

plants. Among the most efficient strategies to increase the sustainability of agroecosystems, maintain soil quality, and with the potential to reduce the consumption of mineral fertilizers, the beneficial microorganisms and the compounds they secrete stand up [4].

Bacteria of the genus *Bacillus* are important plant growth promoters [5–7]. Due to their ability to form spores, these can germinate when applied to plants, and through chemotaxis, they end up starting the root colonization process. Growth-promoting effects can be direct, such as the siderophore production, nitrogen fixation, phytohormone production, and nutrient solubilization, or indirect such, as the production of exopolysaccharides, hydrogen cyanide and lytic enzymes, and biofilm formation [7,8]. The combined production of gibberellins, abscisic acid, and phosphate solubilization presented by a strain of *Bacillus amyloliquefaciens* (H-2-5) resulted in plant growth promotion, causing physiological changes in soybean in saline soil [9].

Indole acetic acid (IAA) is one of the phytohormones produced by the *Bacillus* species, which acts as a potent signaling molecule involved in plant–microbe interactions [10]. Exogenous IAA is able to stimulate the elongation of the primary root when in low concentration. However, with high IAA levels, the formation of lateral roots is stimulated. With increased root surface and length, the access to soil nutrients is enhanced in the presence of IAA produced by bacteria in the rhizosphere [11].

Gamma-polyglutamic acid (γ-PGA) is a non-toxic and biodegradable biopolymer produced by several species of *Bacillus*, including *B. licheniformis* and *B. subtilis* [12,13]. Consisting of repeated units of L- or D-glutamic acid or a combination of both, γ-PGA has multiple functions, such as the sequestration of toxic metal ions, improving bacterial survival during stressful conditions, and also playing a role in virulence, for example, in *Bacillus anthracis*, in which the polymer is secreted and remains attached to the cell as a capsule [14,15]. In *B. subtilis*, the production of γ-PGA was enhanced when zinc and copper were in excess, consisting of a protective mechanism of metal resistance in bacteria [15]. Thapa et al. [16] tested 22 *Bacillus* isolates under optimal conditions of temperature (30 to 37 °C), pH (6.5–7.0), incubation time (3 days), and sodium chloride concentration (3%), and identified the species *B. subtilis*, *B. cereus*, *B. paranthracis*, and *B. licheniformis* as potential producers of γ-PGA, with emphasis on the last species.

With high potential to be used in agriculture, γ-PGA increased the biomass, root activity, and absorption of N, P, and K of *Brassica rapa* subsp. *chinensis* [17], increased the absorption of N by *Brassica rapa* subsp. *pekinensis* [18], and stimulated rice growth [4]. Due to its high solubility, γ-PGA can sequester water molecules [19], helping to mitigate the effects of water stress on seeds and plants.

Studies have shown that the growth of bacteria in agro-industrial wastes can positively affect the plant growth. Wang et al. [20] concluded that soybean curd and sweet potato residues cultivated with *B. subtilis* applied to cucumber seeds resulted in effective suppression of the disease caused by *Fusarium* and also increased the dry weight of roots and shoots of cucumber seedlings. The authors attributed the results to the co-production of γ-PGA production and lipopeptides (the last one acting as antifungal) in these residues, which was not possible to obtain concomitantly by using optimized media for each compound.

One of the by-products from the processing of soy protein concentrate is the soy molasses, which is a low-cost feedstock for fermentation processes due to its high content of fermentable sugars. Soy molasses is a viscous and brown syrup typically containing 50% of soluble solids consisting mainly of 60% carbohydrates (stachyose, raffinose, and sucrose as main sugars), 10% nitrogenous compounds, 20% lipids, and 10% minerals [21]. In terms of monosaccharides and oligosaccharides, crude soy molasses contains 13.26 g $L^{-1}$ glucose, 9.53 g $L^{-1}$ galactose, 1.99 g $L^{-1}$ fructose, 74.51 g $L^{-1}$ sucrose, 96.58 g $L^{-1}$ raffinose, and 114.02 g $L^{-1}$ stachyose, for a total carbohydrate content of 59.80 g 100 $g^{-1}$ [22]. As fermentation medium, soy molasses has been used to obtain bioethanol [21,23], butanol [24], vinegar [25], exopolysaccharides [26,27], β-glucan [22], biosurfactant [28], enzymes [29,30], and single-cell proteins [31].

As far as we know, the utilization of soy molasses for cultivating *Bacillus* species aiming at their potential application as plant growth promoters has not been reported yet. This investigation first evaluated the soy molasses, in various sugar concentrations, as a culture medium for the growth of the *Bacillus* species (*amyloliquefaciens*, *subtilis*, and *licheniformis*). Subsequently, the production of γ-PGA and IAA was analyzed along with the bacterial growth in soy molasses medium compared to a well-known medium for γ-PGA production (medium E). An in vivo experiment was carried out to evaluate the effects of the bacterial fermented broths on the germination and initial development of maize. Our hypothesis is that soy molasses can support *Bacillus* growth and stimulate the production of important metabolites that could result in plant growth promotion.

## 2. Material and Methods

### *2.1. Physico-Chemical Characteristics of Soy Molasses*

Soy molasses was kindly provided by CJ Selecta (Araguari, Minas Gerais State, Brazil) and stored under freezing before use. The physico-chemical characteristics of soy molasses are displayed in Table 1. The pH value was determined using a digital pH meter. The density was estimated using the mass/volume ratio. The determination of phosphorus, potassium/sodium, and sulfur was carried out by acid digestion followed by a colorimetric assay, the flame photometric method, and the barium sulfate gravimetric method, respectively. For the analysis of copper, zinc, iron, calcium, manganese, and magnesium, acid digestion and atomic absorption determination were utilized [32]. Nitrogen was estimated using sulfuric digestion with Kjeldahl's method [33]. Mineral residue (insoluble and total) and organic matter were determined using ignition loss, while organic carbon was determined using dichromate extraction and a titrimetric assay [34]. For the determination of total reducing sugar, 3,5-dinitrosalycilic acid [35] after acid hydrolysis was utilized.

**Table 1.** Physico-chemical characteristics of the soy molasses.

| Analysis | Value |
|---|---|
| pH | 6.00 |
| Density (g $L^{-1}$) | 1.31 |
| Total reducing sugars—TRS (g $L^{-1}$) | 432.00 |
| Organic matter (g $L^{-1}$) | 370.58 |
| Organic carbon (g $L^{-1}$) | 205.88 |
| Mineral residue + organic (g $L^{-1}$) | 528.62 |
| Total mineral residue (g $L^{-1}$) | 158.04 |
| Insoluble mineral residue (g $L^{-1}$) | 0.04 |
| Soluble mineral residue (g $L^{-1}$) | 158.00 |
| Nitrogen (g $L^{-1}$) | 7.98 |
| Phosphorus (g $L^{-1}$) | 6.07 |
| Potassium (g $L^{-1}$) | 20.50 |
| Sodium (mg $L^{-1}$) | 110.00 |
| Calcium (g $L^{-1}$) | 0.18 |
| Magnesium (g $L^{-1}$) | 1.51 |
| Sulfur (g $L^{-1}$) | 1.44 |
| Copper (mg $L^{-1}$) | 25.50 |
| Iron (mg $L^{-1}$) | 210.00 |
| Manganese (mg $L^{-1}$) | 0.50 |
| Zinc (mg $L^{-1}$) | 24.00 |

### *2.2. Microorganisms*

Two strains of *B. subtilis* from culture collection (CCT2471 and CCT7719) and one strain isolated from sugarcane (ENDO26, [36], named M-2 here), one strain of *B. amyloliquefaciens* from culture collection (CCT7690) and one strain isolated from a biofertilizer commercial product (S-1), and one strain of *B. licheniformis* isolated from sugarcane (RIZ-4, [36], named M-1 here) were utilized in the experiments. All of the strains were maintained in slants with

Nutrient Agar medium (1.5 g $L^{-1}$ beef extract; 1.5 g $L^{-1}$ yeast extract; 5 g $L^{-1}$ bacteriological peptone; 5 g $L^{-1}$ sodium chloride; and 20 g $L^{-1}$ agar; distilled water) under mineral oil at 4 °C. The bacteria were transferred to a fresh medium before use.

### *2.3. Culture Media*

The following culture media were utilized: (1) Nutrient Broth—1.5 g $L^{-1}$ beef extract, 1.5 g $L^{-1}$ yeast extract, 5 g $L^{-1}$ bacteriological peptone, 5 g $L^{-1}$ sodium chloride, distilled water; (2) Soy molasses 28 g $L^{-1}$ TRS—65 mL (85 g) of soy molasses to 1000 mL distilled water; (3) Soy molasses 56 g $L^{-1}$ TRS—130 mL (170 g) of soy molasses to 1000 mL distilled water; (4) Soy molasses 112 g $L^{-1}$ TRS—260 mL (340 g) of soy molasses to 1000 mL distilled water; (5) Soy molasses 224 g $L^{-1}$ TRS—520 mL (680 g) of soy molasses to 1000 mL distilled water; (6) Medium E—20 g $L^{-1}$ L-glutamic acid, 12 g $L^{-1}$ citric acid, 80 g $L^{-1}$ glycerol, 7 g $L^{-1}$ ammonium chloride, 0.5 g $L^{-1}$ dipotassium phosphate, 0.5 g $L^{-1}$ magnesium sulfate, 0.04 g $L^{-1}$ iron trichloride, 0.15 g $L^{-1}$ calcium chloride, 0.42 g $L^{-1}$ manganese sulfate, distilled water [37].

The pH of the culture media was adjusted to 7.0 with NaOH 1 M. The media were autoclaved at 120 °C for 15 min at 1 atm. The concentration of TRS in the soy molasses was determined using the 3,5-dinitrosalycilic acid according to Miller [35] after acid hydrolysis.

### *2.4. Preliminary Evaluation of Bacillus Species Growth in Soy Molasses with Increasing Concentrations of Total Reducing Sugars*

#### 2.4.1. Inoculum Preparation

Initially, three loops of bacterial cells grown in Nutrient Agar previously were transferred to Falcon tubes containing 10 mL of sterile Nutrient Broth and incubated at 35 °C, 150 rpm, for 24 h. The optical density (OD) of the bacterial suspensions was determined at 600 nm and diluted in Nutrient Broth to approximately 0.1. Five aliquots of 1.5 mL were then transferred to sterile 2 mL tubes and centrifuged at 8000 rpm for 8 min at 4 °C, discarding the supernatants and suspending the bacterial cell mass in the culture media 1 to 5 according to Section 2.3. These cell suspensions constituted the bacterial inocula.

#### 2.4.2. Bacterial Growth in Microplates

The growth assays were performed in Corning® Costar 96-well sterile polystyrene microplates with transparent flat bottoms and lids. A volume of 180 µL of each medium (1 to 5) was added aseptically, in triplicates, followed by 20 µL of each bacterial inoculum. The microplates were incubated at 35 °C at 160 rpm (approximately 5 mm of orbital amplitude) for 24 h. The growth was monitored by measuring the OD at 600 nm every 15 min using a microplate reader (Tecan Infinite M200, Mannedorf, Switzerland).

The results obtained for each medium and bacterial strain were analyzed, initially discounting the OD values obtained after each time measurement from the values obtained in the control treatments (culture medium without bacterial inoculation). Following this, the OD variation (final OD–initial OD) was calculated for each culture medium and bacterial strain at the end of the cultivation time.

The results were evaluated using the Analysis of Variance test, and the means were compared using Tukey's test ($p < 0.05$) for each bacterial strain among the culture media.

### *2.5. Shaken-Flask Assays for Growing Bacillus Strains from Soy Molasses*

#### 2.5.1. Inoculum and Assay Preparation

The strains *B. subtilis* CCT2417, *B. amyloliquefaciens* CCT7690, and *B. licheniformis* M-1 were grown in Nutrient Broth by transferring three loops of bacterial cells to Falcon tubes containing 20 mL of the culture medium, and were incubated at 35 °C, 150 rpm, for 24 h. The optical density (OD) of the bacterial suspensions was determined at 600 nm, and was diluted in Nutrient Broth to approximately 1.0. The tubes were centrifuged at 8000 rpm for 8 min at 4 °C, discarding the supernatants and suspending the bacterial cell mass in the culture media soy molasses with 28 g $L^{-1}$ and 56 g $L^{-1}$ TRS and in Medium E (media

2, 3, and 6, respectively, according to Section 2.3). These cell suspensions constituted the bacterial inocula.

A volume of 2 mL of the bacterial suspension was inoculated into 500 mL Erlenmeyer flasks to a final volume of 200 mL of culture medium in triplicates. The flasks were incubated at 35 °C, 150 rpm, for 8 days. Samples (10 mL) were taken daily for the analysis.

#### 2.5.2. Analysis

A sample volume of 1.5 mL was transferred to a 2 mL Eppendorf tube and centrifuged at 13,000 rpm for 20 min. The supernatant was discarded, and the cell pellet was suspended in distilled water. After homogenization, the optical density of the cell suspensions was determined at 600 nm. The same procedure was utilized for the culture media without bacterial inoculation to constitute the blanks for the respective media. When necessary, the samples were diluted with distilled water.

The remaining sample was centrifuged at 13,000 rpm for 20 min, and the pH value of the supernatant was analyzed using a digital pH meter. The TRS concentration was determined using the 3,5-dinitrosalycilic acid method [35] after acid hydrolysis.

The extraction of γ-PGA from the supernatants followed the methodologies of Goto and Kunioka [38] and Zeng et al. [39], with modifications. Aliquots of 1 mL were transferred to 2 mL Eppendorf tubes and centrifuged at 13,000 rpm for 20 min to remove the bacterial cells. A volume of 300 µL was collected, and 1.2 mL of cold methanol was added. The tubes were manually agitated and left at rest for 10 min at 4 °C. The microtubes were centrifuged again under the same conditions, the supernatant was discarded, and they were left inverted at 30 °C for 20 min for the methanol evaporation. Distilled water (300 µL) was added, the tubes were agitated, and the aqueous solution was prepared for γ-PGA quantification. The polymer concentration was determined using the UV-VIS spectrophotometric method of complexation with cetyltrimethylammonium bromide (CTAB), according to Kanno and Takamatsu [40]. A volume of 300 µL of the aqueous solution (after the extraction procedure) was mixed with 1.2 mL of phosphate buffer (pH 7.0) and 300 µL of the solution CTAB 0.1 M/NaCl 1 M, and was maintained in a water bath at 30 °C for 25 min. The readings were taken at 400 nm in a spectrophotometer utilizing distilled water as the reaction blank. A standard curve with known concentrations of γ-PGA was made for the quantification of γ-PGA production.

For IAA analysis, 0.5 mL of the supernatant was added to a microtube with 0.5 mL of Salkowski reagent [41], and was incubated for 30 min at room temperature in a dark environment. Following this, the readings were taken at 530 nm in a spectrophotometer. A standard curve with known concentrations of IAA was made for the quantification of IAA production. All analyses were carried out in every time, except for the IAA concentration, in which only the samples on the 8th day of cultivation were analyzed.

The fermented broths left after the 8th day of bacterial growth were mixed (in equal parts of each replicate for each bacterium and medium) and maintained in freezing conditions until the assay of the in vivo experiment with maize.

### *2.6. Structural Characterization of γ-PGA Using FTIR Analysis*

After the extraction procedure, the samples from the assay with *B. amyloliquefaciens* in Medium E (4 days) and soy molasses with 56 g $L^{-1}$ TRS in three situations (without bacterial inoculation, with the bacterium inoculated at the initial time and after 5 days) were dried at 35 °C in Eppendorf tubes. The Fourier Transform Infrared (FTIR) spectra were obtained with the dried samples and recorded using a Tensor II (Bruker®, Karlsruhe, Germany) spectrometer operating with the Attenuated Total Reflectance accessory and with KBr disks over the range of 4000 $cm^{-1}$ to 500 $cm^{-1}$, obtained using the accumulation of 128 scans with 4 $cm^{-1}$ resolution. The sample spectra were compared with the spectra obtained for the pure γ-PGA (Sigma®, New York, NY, USA).

### *2.7. Effect of the Bacterial Fermented Broth on the Germination and Initial Development of Maize*

The broths obtained after bacterial growth for 8 days were utilized in an in vivo experiment performed with maize seeds (cv. IAC Airan). The seeds were disinfected by immersion in a 70% hydroalcoholic solution for 2 min followed by a 1% sodium hypochlorite solution for 15 min. The seeds were then washed with sterile distilled water several times [42]. An amount of 100 g of maize seeds was placed together with 5 mL of the bacterial broths into transparent plastic bags, which were inflated with air and agitated for 1 min [43]. The control treatments consisted of the soy molasses media without bacterial inoculation and sterile distilled water. The seeds were sown over two sheets of Germitest paper that were covered for another paper sheet (all moistened with sterile distilled water in the proportion of 2.5 times the dry weight) and curled. Four replicates per treatment were utilized, each replicate consisting of a roll of Germitest paper with 50 seeds each and placed in a germination cabinet at 25 °C with a 12:12 h light-dark cycle (15,000 lux) for 7 days [44]. The percent of germination and the length (cm) of root and shoot for each seedling were determined.

The seedling Vigor index was calculated according to the following formula [45]:

$$\text{Vigor index} = \text{Germination \%} \times (\text{Shoot length} + \text{Root length})/100$$

The Principal Component Analysis (PCA) was applied to the results. A binary data matrix was considered, in which the lines contained the strains, and the columns presented the means of variables such as growth ($OD_{600}$), pH, TRS, γ-PGA, IAA, and Vigor Index. The input of data and the biplot were obtained with the SRplot tool [46].

## 3. Results and Discussion

### *3.1. Preliminary Evaluation of Bacillus Species Growth in Soy Molasses with Increasing Concentrations of Total Reducing Sugars*

The growth profiles of *Bacillus* strains in soybean molasses with different TRS concentrations in comparison with the Nutrient Broth medium indicated that in molasses media with 28 and 56 g $L^{-1}$ TRS, there was significantly greater bacterial growth, that is, greater variation in optical density at the end of 24 h of cultivation, except for the strains *B. subtilis* 2471 and *B. licheniformis*. With 112 g $L^{-1}$ TRS, there was a significant decrease in growth compared to the concentration of 56 g $L^{-1}$, except for *B. licheniformis*. The concentration of 224 g $L^{-1}$ TRS in soy molasses was inhibitory for the growth of all strains (Figure 1).

Based on the results above, the strains *B. subtilis* 7719, *B. amyloliquefaciens* 7690, and *B. licheniformis*, and soy molasses media with 28 and 56 g $L^{-1}$ TRS, were selected for the next experimental steps. The choice for the *B. subtilis* strain was because it was the one with the best growth profile in the molasses medium, including the concentration of 112 g $L^{-1}$ TRS. Regarding *B. amyloliquefaciens*, the strain 7690 was chosen because it presents more distinctive growth in relation to the Nutrient Broth. As for *B. licheniformis*, although the growth in soy molasses media was lower than in Nutrient Broth medium, it is one of the most common *Bacillus* species used in the production of γ-PGA [12,16,47], besides the fact that only one strain of this species was tested here. As for soy molasses media with 28 and 56 g $L^{-1}$ TRS, the choice was determined by the best growth results of the aforementioned strains in 24 h of cultivation.

Oliveira et al. [26] utilized soy molasses (10 g $L^{-1}$) as a carbon source, replacing mannitol for the growth and production of extracellular polymeric substances (EPS) from diazotrophic bacteria (*Rhizobium hautlense*, *Mesorhizobium* sp., and *Ensifer meliloti*). Bacterial growth was greater with soy molasses or similar to mannitol for two bacteria, while EPS production was greater with soy molasses for all three bacteria. In the present work, the concentration of soy molasses was 8.5 g $L^{-1}$ in the medium 28 g $L^{-1}$ TRS, but without any supplementation.

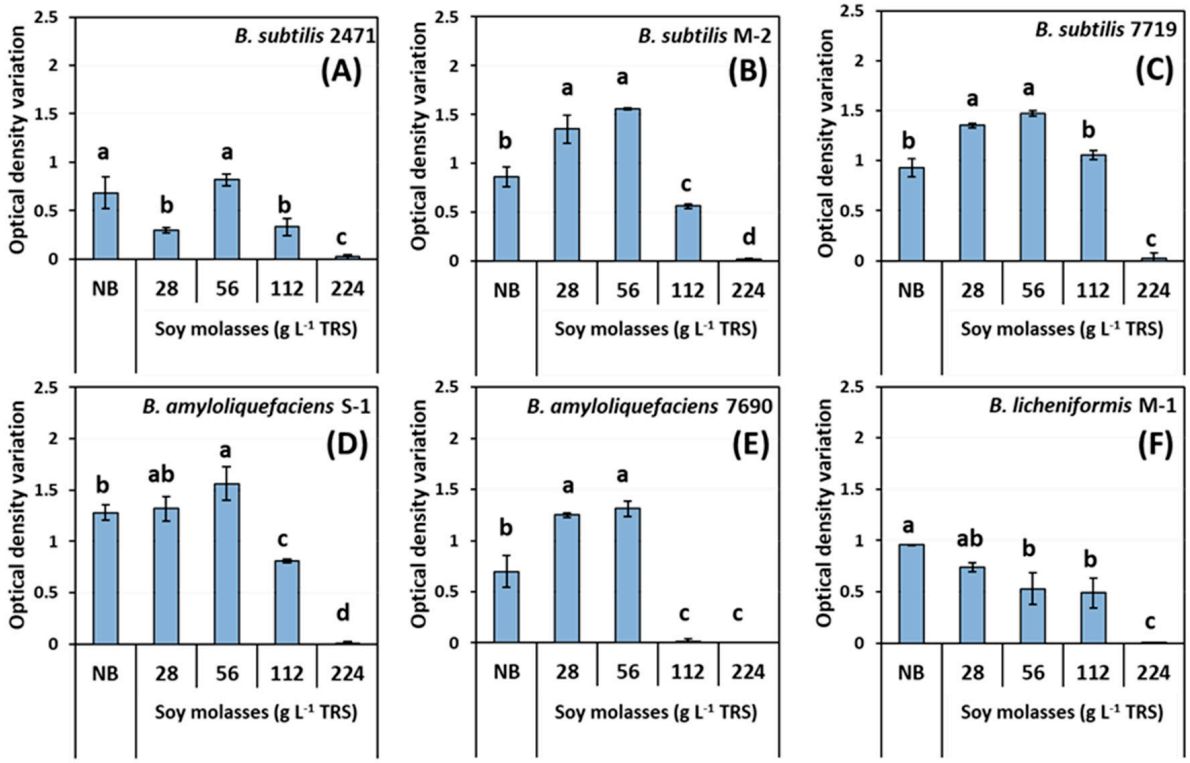


**Figure 1.** Optical density variation of *Bacillus* (*subtilis*, (**A**–**C**); *amyloliquefaciens*, (**D**,**E**); *licheniformis*, (**F**)) strains in Nutrient Broth (NB) and soy molasses medium with total reducing sugar concentration (TRS) varying from 28 to 224 g $L^{-1}$ at 35 °C, 150 rpm, for 24 h. Different letters over the bars indicate significant differences found using Tukey´s test ($p < 0.05$).

### *3.2. Shaken-Flask Assays for Growing Bacillus Strains from Soy Molasses*

The bacterium *B. amyloliquefaciens* showed significant growth in medium E, reaching 6.0 OD after 3 days. Although it showed a tendency to stabilize growth up to 6 days, there was an increase in OD until reaching 8 days, but the growth speed was much lower than that in the 6-day period. The production of γ-PGA increased significantly from 2 to 3 days, coinciding with the decrease in growth rate. From 3 to 8 days, there was a fluctuation in values, with a tendency to decrease, but reduced from approximately 800 to 700 μg $mL^{-1}$. This decrease coincided with the increase in OD after 3 days of growth. There was no significant change in pH throughout the cultivation time (Figure 2A).

Medium E is a well-known medium used for γ-PGA production, with glycerol (80 g $L^{-1}$) and citric acid (12 g $L^{-1}$) as carbon sources, in addition to glutamic acid and salts [37], but no sugar is present. Song et al. [48] concluded that citric acid, glutamic acid, and yeast extract substantially increased the production of γ-PGA by *Bacillus* sp. In soybean molasses, the predominant carbon sources are stachyose, raffinose, and sucrose [31], but sucrose, galactose, glucose, and fructose are also found [49,50]. Glucose, glycerol, maltose, citric acid, fructose, and sucrose are the carbon sources reported for γ-PGA production [12,51,52]; however depending on the *Bacillus* species, with sucrose (*B. subtilis*, [48]) or glycerol (*B. amyloliquefaciens*, [53]), greater production of γ-PGA was observed.

When glycerol was used as a carbon source (50 g $L^{-1}$) in a medium with ethanol and meat extract, the growth of *B. amyloliquefaciens* was 1.6 times greater than that with glucose and 3.2 times greater than that with sucrose [54]. Glycerol is most efficiently metabolized in the central metabolic pathways of *B. amyloliquefaciens.* Through metabolic engineering, it was possible to increase the production of γ–PGA 3.72 times from crude glycerol as a substrate, with growth reaching OD around 6.0 after 3 days of cultivation [53], similar to that observed in the present work (Figure 2A). In both studies, growth was associated with

γ-PGA production. However, the γ-PGA concentration in medium E was much lower here than that found in the aforementioned work.

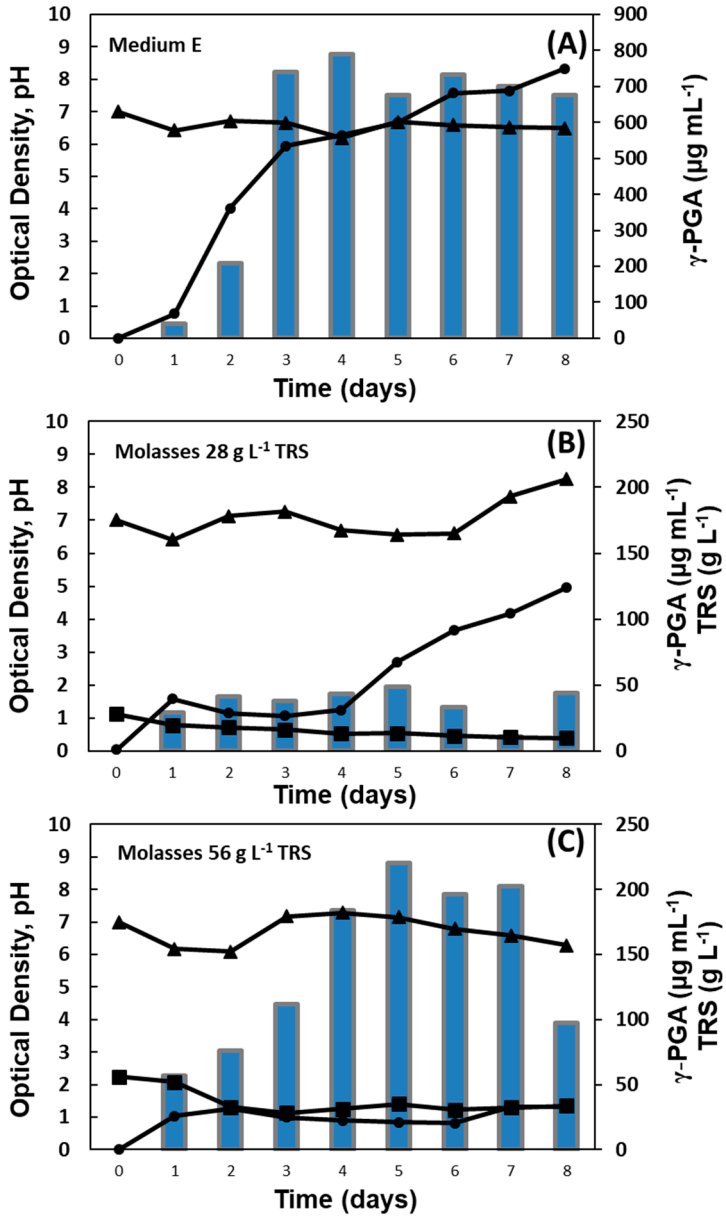


**Figure 2.** Optical density (●), pH (▲), total reducing sugars (TRS, ■), and γ-PGA production (blue bar) by *B. amyloliquefaciens* 7690 in medium E (**A**) and soy molasses medium with 28 (**B**) and 56 g $L^{-1}$ (**C**) TRS at 35 °C, 150 rpm, for 8 days.

In soy molasses media, the association of growth and γ-PGA production was not observed. As with 28 g $L^{-1}$ TRS, approximately 50 µg $mL^{-1}$ of γ-PGA was produced from 2 days onwards, with fluctuations around this value for up to 8 days. Regarding growth, there was an increase in OD after 1 day, and a stabilization and variation of 4 OD from 4 days to 8 days. The pH of the culture medium varied in the range of 6.5–7.5 within 6 days of cultivation, increasing to around 8.2 at the end of the 8th day. The highest consumption of TRS occurred after 1 day of growth, slowing afterwards. The growth observed after 4 days may be related to the consumption of γ-PGA, since there was a decrease in values within this time, increasing only after 8 days of growth (Figure 2B). Bacteria of the genus *Bacillus* can degrade and use γ-PGA as substrates, since this compound is a secondary metabolite [55].

The same behavior was observed with 56 g $L^{-1}$ TRS, but the OD remained at 1 until the end of the 8th day. However, the γ-PGA concentration reached approximately 250 μg $mL^{-1}$ after 5 days, decreasing to approximately 100 μg $mL^{-1}$ after 8 days. The pH dropped within 2 days, and after this remained at around 6–7. Sugar consumption increased from 1 to 2 days, and then stabilized (Figura 2C).

Summing up, for *B. amyloliquefaciens* the highest production of γ-PGA and concomitant growth were obtained in broth E; however, in soy molasses media, higher growth was shown with 28 g $L^{-1}$, but greater production of γ-PGA was shown with 56 g $L^{-1}$ TRS.

For *B. licheniformis*, the growth curves obtained in medium E and soy molasses medium with 28 g $L^{-1}$ TRS were similar, reaching OD 4 at the end of the 8th day. However, the pH of the culture medium was very different in the two conditions: in medium E, there was an increase from 7 to 9, while in the soy molasses medium with 28 g $L^{-1}$ TRS, the pH value dropped from 7 to approximately 5.5. TRS consumption was low in the soy molasses medium. γ-PGA production was below 10 μg $mL^{-1}$ in 28 g $L^{-1}$ TRS molasses medium, but reached 40 μg $mL^{-1}$ at the 8th day in medium E (Figure 3A,B). With 56 g $L^{-1}$ TRS, the OD did not exceed 2, and γ-PGA production reached 40 μg $mL^{-1}$ after 7 days. The pH fluctuated from 7 to approximately 6.0, and almost 50% of the sugar in the medium was consumed within 5 days, stabilizing afterward (Figure 3C). The same behavior—growth not associated with γ-PGA production—was observed with *B. licheniformis* in soy molasses media.

Glucose was a better carbon source than glycerol for the growth of *B. licheniformis* [56]. As for the production of γ-PGA, the same authors found that the mixture of glucose and glycerol increased the production of the biopolymer. Even considering the fluctuations in γ-PGA values, the polymer production was similar in medium E (without sugars, but with glycerol) and in the molasses medium with 56 g $L^{-1}$ TRS (with sugars, no glycerol), regardless of the carbon source of the medium. It is noteworthy that stachyose and raffinose account for approximately 25% of the total carbohydrates in soy molasses [28], and may not be assimilated by the bacteria. As for fermentable sugars such as sucrose, glucose, and fructose, they represent about 30% of the total carbohydrates in soy molasses [57], and they must be the preferable carbon sources.

The strain of *B. subtilis* showed a similar growth profile in medium E and molasses media with 28 g $L^{-1}$ TRS over 8 days of cultivation, reaching a maximum DO 4. The pH dropped to approximately 6.0 in the medium E and remained at this value until the end of the cultivation, while in the molasses medium with 28 g $L^{-1}$ TRS, the pH fluctuated and maintained at 7.0 until the 8th day (Figure 4A,B). However, the medium with 56 g $L^{-1}$ TRS provided greater growth of *B. subtilis*, reaching OD 7 after 8 days. In this medium, there was acidification, with the pH remaining around 5.5 at the end of the cultivation. TRS consumption was highest on the first day of cultivation (approximately 50%), slowing afterwards (Figure 4C).

The rapid and high consumption of TRS within 1 day of cultivation resulted in the pH dropping from 7 to approximately 5.5, which may have impacted γ-PGA production. Among the bacteria and culture media studied, the highest sugar consumption in the shortest cultivation time and the lowest pH value was observed with *B. subtilis*. Wang et al. [13] concluded that γ-PGA production by *B. subtilis* was repressed by pH values below 5.5. The optimal pH for glutamate utilization was 6.5, in which specific glutamate utilization and γ-PGA formation rates reached the maximum values with *B. subtilis* [58].

Although the growth in medium E and molasses media 28 g $L^{-1}$ TRS was similar, γ-PGA production was greater in the molasses medium (between 50 and 60 μg $mL^{-1}$) than in medium E (maximum 10 μg $mL^{-1}$). There was no production of γ-PGA in the molasses medium with 56 g $L^{-1}$ TRS, despite the expressive bacterial growth (Figure 4C). For *B. subtilis*, in soy molasses media, the greatest growth was also not associated with the greatest production of γ-PGA.

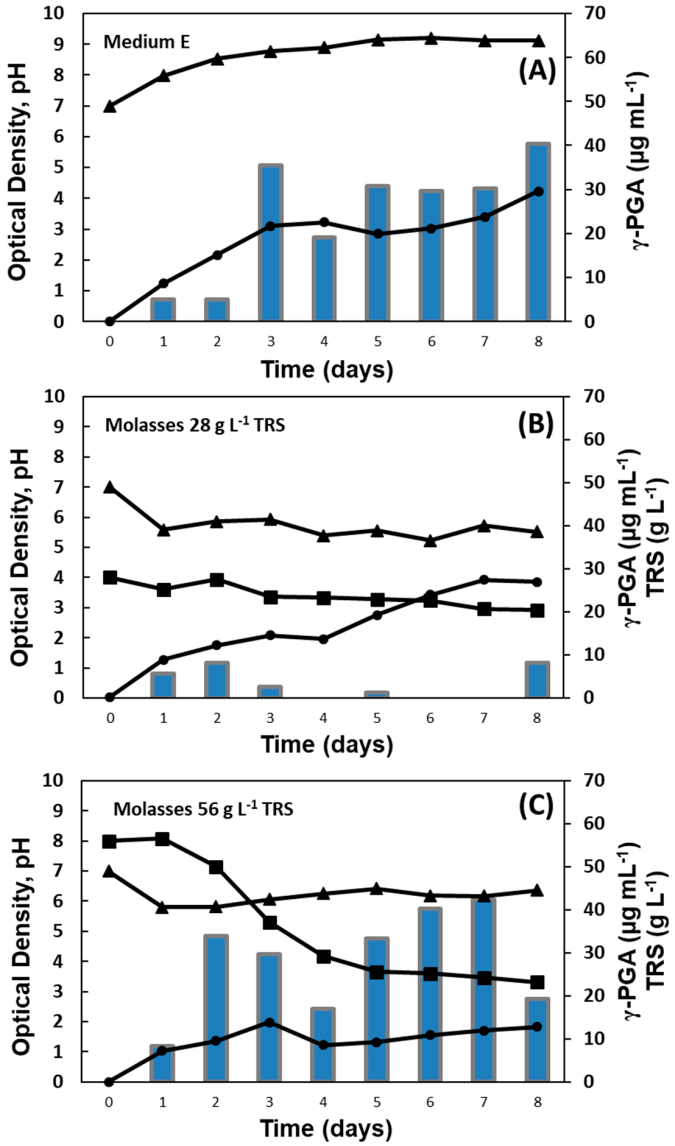


**Figure 3.** Optical density (●), pH (▲), total reducing sugars (TRS, ■), and γ-PGA production (blue bar) by *B. licheniformis* M-1 in medium E (**A**) and soy molasses medium with 28 (**B**) and 56 g $L^{-1}$ (**C**) TRS at 35 °C, 150 rpm, for 8 days.

γ-PGA-producing bacteria are classified as glutamic acid-dependent and -independent. In the first case, exogenous glutamic acid for polymerization and production of γ-PGA chains is required. In the second case, the glutamic acid necessary to produce the polymer is generated with the tricarboxylic acid cycle, glycolysis, pentose phosphate pathway, amino acid metabolism, and glutamate synthesis [47]. In the case of soy molasses, glutamic acid is the amino acid present in the greatest amount; however, it may not be enough for the synthesis of high amounts of this polymer. In this case, for example, sugars are metabolized in the glycolytic pathway, following the tricarboxylic acid cycle, where ketoglutaric acid can be diverted to glutamic acid synthesis [55]. It is expected that there will be decreased growth but enhanced γ-PGA production or vice versa, as it was observed in soy molasses media for the three bacteria studied here. The medium with the highest TRS concentration (56 g $L^{-1}$) provided greater production of γ-PGA for two bacteria (*B. amyloliquefaciens* and *B. licheniformis*); however, for *B. subtilis*, there was no production of γ-PGA at this concentration, which may be related to the metabolic characteristics of this strain specifically, since in medium E, which is the recommended medium for γ-PGA production, the concentration obtained was very low.

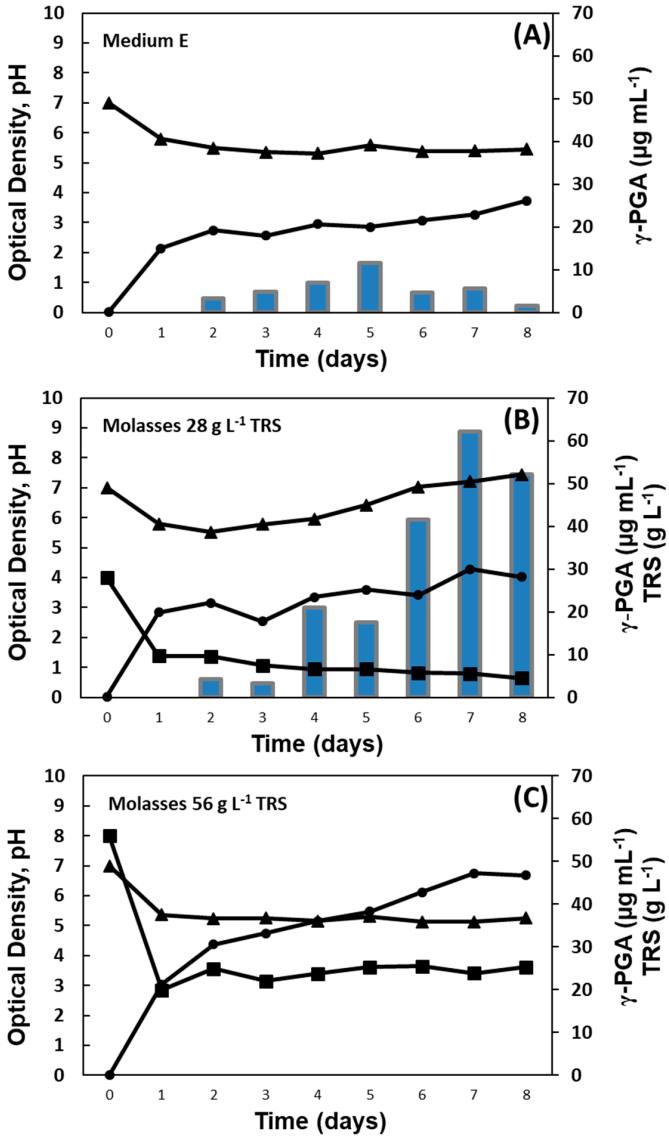


**Figure 4.** Optical density (●), pH (▲), total reducing sugars (TRS, ■), and γ-PGA production (blue bar) by *B. subtilis* 7719 in medium E (**A**) and soy molasses medium with 28 (**B**) and 56 g $L^{-1}$ (**C**) TRS at 35 °C, 150 rpm, for 8 days.

The production of γ-PGA by all three bacteria was low when compared to those reported in the literature [55,59], but it is noteworthy that the aim here was not to produce the polymer per se, but to evaluate whether it is synthesized in soy molasses medium and how its presence could contribute to the promotion of plant growth in subsequent experiments.

### *3.3. Structural Characterization of γ-PGA Using FTIR Analysis*

The presence of y-PGA in the fermented broths was confirmed using FTIR spectra compared with the spectrum of y-PGA Sigma®. For this, samples of medium E fermented with *B. amyloliquefaciens* (where the highest production of y-PGA was observed after 4 days of growth) and samples of molasses medium with 56 g $L^{-1}$ TRS without bacterial inoculation, inoculated with the bacteria at time zero (initial), and after 5 days of growth were evaluated. This analysis was carried out to reject the hypothesis that any compound present in the molasses could be extracted using the method adopted and had absorption at the wavelength used to determine y-PGA, leading to misleading results, according to the methodology used. This hypothesis was raised due to the turbidity of the sample after the y-PGA extraction procedure in molasses samples where no bacterium was inoculated.

FTIR spectroscopy was used to identify γ-PGA (Figure 5A–C). All samples presented the following characteristic bands of the polymer: 3424 $cm^{-1}$ relative to the O–H and N–H groups; 2924 $cm^{-1}$ relative to the C–H groups; 1632 $cm^{-1}$ relative to C=O stretching of amide I; a shoulder around 1550 $cm^{-1}$ relative to the N-H groups of amide II; 1403 $cm^{-1}$ relative to the O–H bending of COOH groups; at approximately 1100 $cm^{-1}$ due to the C–N and C–O stretching; and 680 $cm^{-1}$ due to the N–H groups of the polymer [60–62].

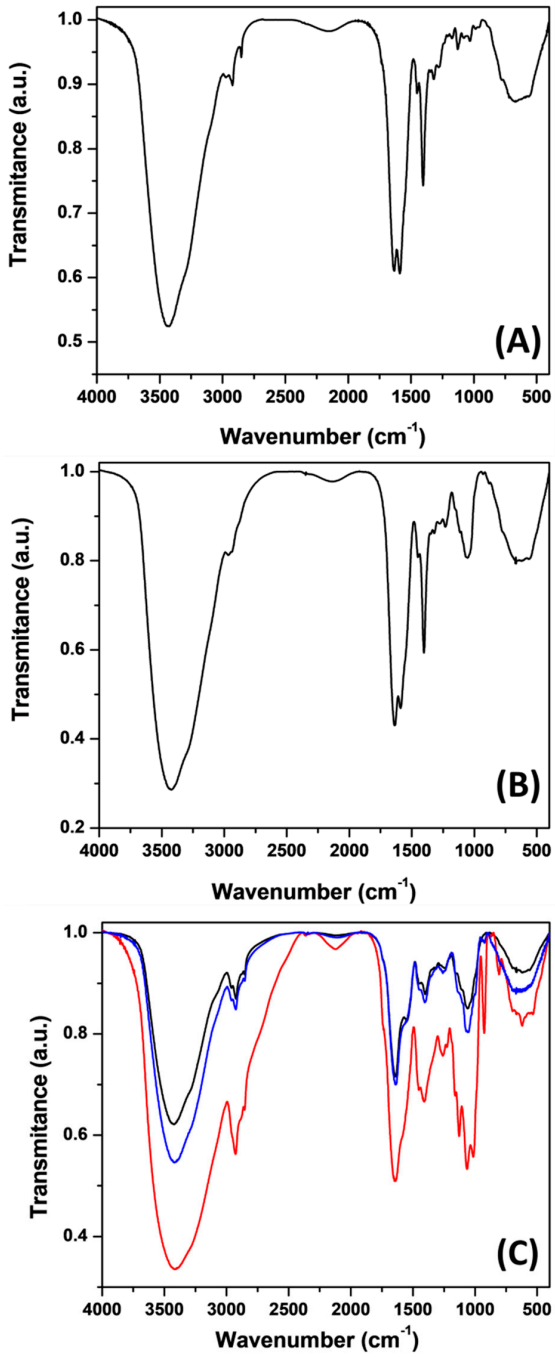


**Figure 5.** FTIR spectra of γ-PGA Sigma® (**A**), sample of γ-PGA extracted from the medium E after 4 days of *B. amyloliquefaciens* growth (**B**), and samples of γ-PGA extracted from the soy molasses medium 56 g $L^{-1}$ TRS without bacterial inoculation (blue line), from the soy molasses medium 56 g $L^{-1}$ TRS right after bacterial inoculation (black line), and from the soy molasses medium 56 g $L^{-1}$ TRS after 5 days of bacterial inoculation (red line) (**C**).

Comparing the spectra of samples in soy molasses medium with 56 g $L^{-1}$ TRS at time zero and after five days of growth with *B. amyloliquefaciens* indicate that the bands in the regions of 1630 and 1400 $cm^{-1}$ are more intense for the sample taken at five days (Figure 5C), suggesting that the y-PGA is more present and purer at this cultivation time than in the sample at time zero.

In summary, the turbidity of the sample observed after extraction of γ-PGA from the molasses medium without bacterial inoculation indicates that there are some substances present in the soy molasses that are extracted with methanol and absorbed in the wavelength range of 400 nm, also corroborated with the infrared spectrum, which showed characteristic bands of the y-PGA polymer in soy molasses. However, it is noteworthy that *B. amyloliquefaciens* produced γ-PGA in the soy molasses medium with 56 g $L^{-1}$ TRS after 5 days of growth, as the characteristic bands of the polymer are more intense in this sample cultivated with the bacterium, agreeing with the spectrophotometry results. The growth of the bacteria in the soy molasses medium may have led to the consumption of substances that presented bands in the FTIR spectra close to the γ-PGA bands. Therefore, the characteristic and intense bands of γ-PGA in the bacteria culture sample after 5 days indicate that the polymer is purer.

### *3.4. Effect of the Bacterial Fermented Broth on the Germination and Initial Development of Maize*

The bacterial fermented broths in each culture medium after 8 days of cultivation were evaluated for their effect on the germination of maize seeds, as well as on the length of the main root and shoot of the seedlings obtained after 7 days of germination. The characteristics of each broth are described in Table 2, as well as the controls used. The results of the germination and initial development of corn seedlings (root and shoot length) are presented in Table S1 (Supplementary Materials).

**Table 2.** Characteristics of the fermented broths of *B. amyloliquefaciens* (BA), *B. licheniformis* (BL), and B. *subtilis* (BS) after 8 days, and of the controls used in the germination and initial development experiment of corn seedlings.

| Bacteria | Medium [1] | $OD_{600}$ [2] | γ-PGA (μg $mL^{-1}$) | pH | TRS [3] (g $L^{-1}$) | IAA [4] (μg $mL^{-1}$) |
|---|---|---|---|---|---|---|
| BA | E | 8.33 | 675.71 | 6.48 | 0 | 36.73 |
| | 28 | 4.96 | 43.97 | 8.24 | 9.68 | 1.31 |
| | 56 | 1.31 | 97.48 | 6.29 | 33.95 | 1.05 |
| BL | E | 4.23 | 40.38 | 9.12 | 0 | 40.42 |
| | 28 | 3.85 | 8.10 | 5.51 | 20.45 | 0 |
| | 56 | 1.83 | 19.24 | 6.36 | 23.16 | 0 |
| BS | E | 3.73 | 1.49 | 5.45 | 0 | 11.47 |
| | 28 | 4.02 | 52.06 | 7.45 | 4.47 | 14.97 |
| | 56 | 6.68 | 0 | 5.26 | 25.30 | 0 |
| Control | E | - | - | 7.0 | 0 | - |
| | 28 | - | - | 7.0 | 28 | - |
| | 56 | - | - | 7.0 | 56 | - |
| | Water | - | - | 7.5 | 0 | - |

[1] E = Medium E; 28 = soy molasses 28 g $L^{-1}$ TRS; 56 = 56 soy molasses 28 g $L^{-1}$ TRS. [2] Optical density at 600 nm. [3] Total reducing sugars. [4] Indole acetic acid.

To verify the contribution of the fermented broths to the germination and initial development of maize, expressed as the Vigor Index, and which characteristics of the broths most influenced this parameter, the Principal Component Analysis (PCA) was applied, as presented in Figure 6.

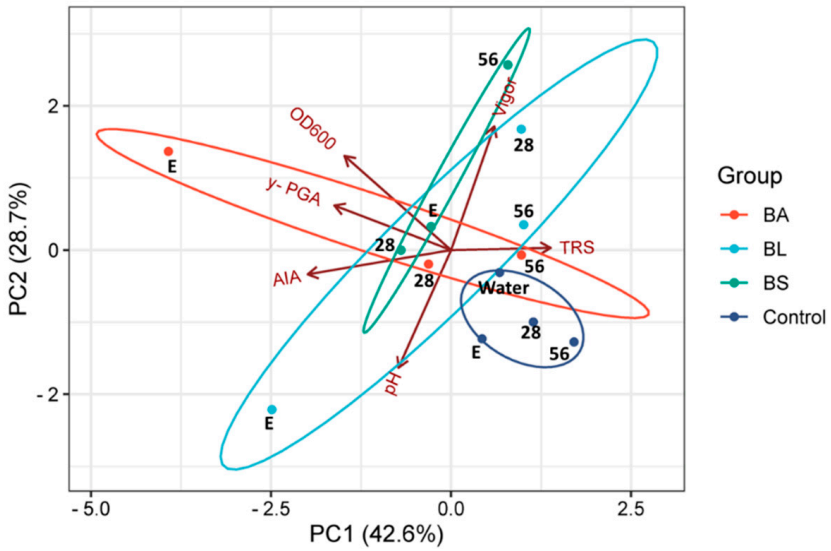


**Figure 6.** Principal Component Analysis (F1 and F2) of the *Bacillus* species (BA, *B. amyloliquefaciens*; BL, *B. licheniformis*; BS, *B. subtilis*) cultivated in Medium E (E) and soy molasses medium with 28 g $L^{-1}$ (28) and 56 g $L^{-1}$ (56) total reducing sugars (TRS) regarding their results for growth (OD600), medium pH (pH), TRS, γ-PGA, and IAA concentrations after 8 days of cultivation. The Vigor Index refers to the results obtained for the maize seeds treated with the fermented broth of each bacterium in each culture medium. The control group includes all the media tested without bacterial inoculation besides water.

The F1 and F2 components explained 71.3% of the total variability of the data. The Vigor Index correlated negatively with the broth pH, so higher pH values harmed the germination and initial development of maize seedlings. The Seed Analysis Rules [44] and the International Seed Testing Association [63] establish that the substrate in the germination test must have a pH between 6.0 and 7.5. It is known that soil alkalinity, characterized by higher pH (8.5–11), disrupts cellular stability and destroys the membrane stability and root activity of plants [64]. The harmful effects on maize development, including a reduction in height, and fresh and dry mass of shoots and roots are already noticeable at an intermediate level of soil alkalinity [65]. According to the PCA (Figure 6), the soy molasses media with 56 g $L^{-1}$ TRS fermented with *B. subtilis* and the 28 g $L^{-1}$ TRS fermented with *B. licheniformis*, related to the highest Vigor Index values, presented pH 5.26 and 5.51, respectively. Medium E fermented with *B. licheniformis*, which was the furthest from the Vigor Index, presented a pH of 9.12 (Table 2).

The Vigor Index also correlated with the TRS concentration of the fermented broths, since concentrations of around 20 g $L^{-1}$ were found in broths fermented with *B. subtilis* and *B. licheniformis*, with 56 and 28 g $L^{-1}$ TRS, respectively (Table 1), resulting in the highest Vigor Indexes (Figure 6). The growth of the bacteria in soy molasses media contributed significantly to the increased Vigor Index compared to the results obtained in the control media without bacterial inoculation. Furthermore, the presence of bacteria in the fermented broths (around $10^9$ CFU $mL^{-1}$) should be highlighted. The adherence and survival of growth-promoting bacteria in the seeds during the germination process are important for infection to occur. Sugar solutions with concentrations ranging from 10% to 25% are used to increase the distribution and survival of rhizobia in soybean (*Glycine max* L.) and bean (*Phaseolus vulgaris* L.) seeds, resulting in an improved symbiotic effect on plants [66,67]. In addition, the TRS content in molasses fermented broth (approximately 20 g $L^{-1}$) may have contributed to the bacterial cells remaining viable for longer and secreting metabolites with effects on the initial development of maize.

γ-PGA, IAA, and the optical density values of the broths displayed no correlation with the Vigor Index, which means that the highest Vigor Indexes of the maize seedlings were

not obtained with bacterial broths that presented higher concentrations of γ-PGA and IAA or higher optical density (Figure 6). Ngearnpat et al. [4] reported that the effect of γ-PGA seems more effective in the lateral root development, to the detriment of the primary root (value used here to compose the Vigor Index). According to Pereira et al. [68], nano γ-PGA used as a transport system for the plant growth regulator gibberellic acid resulted in a superior increase in bean lateral roots compared to the application of gibberellic acid alone.

In the work of Ngearnpat et al. [4], the γ-PGA treatment that most contributed to the highest Vigor Index of rice seedlings was 500 mg $kg^{-1}$ of soil. However, the (pure) polymer was applied directly to the soil. With the results obtained here, less than 1 L (approximately 700 mL) of broth fermented with *B. amyloliquefaciens* in medium E (where there was the greatest production of γ-PGA) would be needed for each kilo of soil to obtain the same concentration (500 mg $kg^{-1}$) used by the authors above. However, this bacterium growing in medium E did not present satisfactory results regarding the Vigor Index, probably due to the absence of sugar in the fermented broth and the presence of other substances produced that may have impacted the development of maize seedlings when applied to the seeds. In fact, the two treatments that resulted in the highest Vigor Index presented a low concentration of γ-PGA (0 and 8 μg $mL^{-1}$) and absence of IAA, indicating that other characteristics of the growth medium—soybean molasses—after fermentation had a more effective contribution to the Vigor Index of maize seedlings.

Wang et al. [20] found that the addition of crop residues (soybean curd and sweet potato residues) inoculated with *B. subtilis* obtained after solid state fermentation, containing 3.63% (*w*/*w*) γ-PGA, significantly increased the dry weight of roots and aerial parts, as well as the ratio between the size of the root and the aerial parts of cucumber. The number of bacteria was approximately $10^{10}$ CFU $g^{-1}$ and had a pH of 8.8 in the fermented residues. The composition of the culture medium, the number of bacteria, and the pH after fermentation are factors that must be considered when applying bacterial suspensions to promote plant growth, as they affect the colonization of the plant by bacteria and their survival. For this same reason, agro-industrial residues can be important substrates for the cultivation of growth-promoting bacteria, as they can add substances that maximize the effect of inoculating the bacteria to the plant. In this context, soy molasses constitutes a potential culture medium for the cultivation of growth-promoting bacteria, as in addition to providing the production of potentially important substances for promoting plant growth, such as γ-PGA and IAA, it is low cost and has high availability in Brazil.

## 4. Conclusions

Soy molasses constitutes a potential culture medium for the growth of *Bacillus* species (*amyloliquefaciens*, *subtilis*, and *licheniformis*) in concentrations of up to 56 g $L^{-1}$ total reducing sugars (TRS) without supplementation. Growth was not always associated with γ-PGA production, which was a maximum of 56 g $L^{-1}$ TRS for *B. amyloliquefaciens* and *B. licheniformis*. Fermented broths with *B. subtilis* and *B. licheniformis* in soy molasses media (56 and 28 g $L^{-1}$ TRS, respectively) applied to maize seeds resulted in the highest Vigor Indexes of the seedlings, which correlated negatively with the broth pH, and were not impacted by the γ-PGA and indole acetic acid produced by the bacteria. The low-cost and easily available feedstock soy molasses constitutes a potential culture medium for the growth of plant growth-promoting bacteria.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10080403/s1, Table S1: Results of the maize seed germination and initial development after application of bacterial fermented broths, in soy molasses and medium E.

**Author Contributions:** Conceptualization, A.P.F.C.d.S., A.F. and S.R.C.-A.; methodology, A.P.F.C.d.S., A.F., M.M.R.-M. and S.R.C.-A.; investigation, A.P.F.C.d.S., B.S.R.D., J.M.d.S.-N., K.R.F., A.F. and S.R.C.-A.; writing—original draft preparation, S.R.C.-A., A.F. and K.R.F.; writing—review and editing, S.R.C.-A., A.F., K.R.F., F.R., M.M.R.-M., A.P.F.C.d.S., J.M.d.S.-N. and B.S.R.D.; funding acquisition, S.R.C.-A. and A.F. All authors have read and agreed to the published version of the manuscript.

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