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Evaluation of a Fermented Millet Product of *Bacillus subtilis* ATCC 55033 as Potential in Biocontrol of Bacteriosis (*Xanthomonas axonopodis*) in Gulupa (*Passiflora edulis* Sims)

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Abstract: The "oil stain disease" has become a phytosanitary status that involves economic losses for farmers who produce gulupa (*Passiflora edulis* Sims). For this reason, this research aimed to evaluate the biocontrol activity of *Bacillus subtilis* ATCC 55033 in a culture medium based on fermented millet as a biocontrol product of the disease. Initially, metabolite production of seven strains of *B. subtilis* and a reference strain *B.s* (ATCC 55033) was evaluated; in addition, the *in vitro* antagonistic ability was evaluated against *Xanthomonas axonopodis*. Two experiments were performed under *in vitro* conditions: (1) study of the presence of halos of hemolysis due to metabolite production; (2) analysis of the antagonistic ability by using double layer. It was observed that concerning the average, a number five times bigger of halos of hemolysis and 10 times bigger halos of inhibition *in vitro* on the growth of *X. axonopodis* in the strains *B.s* (ATCC 55033) and *B.s* TB2 over other strains. Subsequently, the evaluation was performed under greenhouse conditions, based on a randomized block design, in which two treatments were applied over the soil: (1) application of the fermented medium based on millet with *B.s* ATCC 55033, 24 h before *X.a* inoculation; (2) application of 5 d post-inoculation. It was observed that the treatment, previous infective process, obtained an average percentage of severity equal to 42.044%. In addition, after the evaluation month, a foliage restoration was presented. In conclusion, it is suggested that these products could act as inducers in the plant systemic resistance to trigger defense responses to infection of *X.a*.

Key words: *Xanthomonas*, *Bacillus*, biocontrol, bacterioses.

1. Introduction

Agricultural activities are directly related to population growth. In the last 40 years, the world population has increased by 90%, and crop production has increased by 25% worldwide [1]. In 2011, the world's population reached six million people who lived and fed on products mostly originated from farming. If a population of 1.5 million people is projected for 2020, grain production should increase by 39% [2].

Colombia is one of the Latin American countries with the most significant agricultural activity due to its large tracts of land and cattle ranching. For 2016, in 26 departments of Colombia, there is an area of 43 million hectares for agricultural use that corresponds to 38.6% of the dispersed rural area registered by the Departamento Administrativo Nacional de Estadística (DANE), 80% corresponds to pasture and stubble and 19.7% to agricultural use corresponding to 8.5 million hectares [3]. Gulupa is a fruit plant of the genus

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Pasifloras (passion fruit) with an area of 15,000 ha and a production of approximately 160,000 t with a yield of 15 t/ha according to the data of Asohofrucol of 2017. The operating costs of production of the gulupa in 2016 corresponded to $96,642,000 [4].

The applications of pesticides of synthetic origin inputs were the only options for crop management since the 20th century. In addition, excessive and erroneous use of the chemicals has impacted the polluted environment, and they are a major cause of resistance of pathogens [1]. For that reason, they are currently looking for other alternatives to mitigate phytosanitary problems and achieve green markets and clean production [5].

The use of chemical compounds to fumigate crops to control pests of bacterial, fungal, or insect origin has caused alterations in the soils, and their toxicity generates chronic diseases in human beings. Therefore, the use of natural origin substances is a promising measure for the biological control of pests, which are environmentally friendly [5].

In this sense, biopesticides originate from plant extracts and/or secondary metabolites of some microbial species. *Bacillus subtilis* has been associated with rhizosphere of some plant species such as *Ornithogalum umbellatum*. This ornamental plant contains a number of secondary metabolites of alkaloid nature and others that may help enhance the bio-controlling effect of some microbial species in their rhizosphere [6]. In fact, other plant extracts may have the property of stimulating the production of *Bacillus* lipopeptide secondary metabolites such as surfactin. Millet, banana peels and corn extracts may increase the production of this antimicrobial molecule [7].

Some of the genus *Bacillus* sp. and the strain *B. subtillis* ATCC 55033 can produce surfactin and other metabolites of tensoactive metabolites antimicrobial agents in a standard medium based on millet, the latter being the nutritional source for the production of secondary metabolites [8]. Therefore, the objective of the project was to evaluate the antagonistic effect *in vitro* and *in vivo* of the fermentation products based on millet of both *Bacillus* sp. and *B.s* ATCC 55033 strains in *Xanthomonas* sp. (*Passifloras edulis* Sims), the latter, being a disease with almost 80% in the gulupa production areas of the Sumapaz [9] and the management of this disease has been limited by resistance to conventional bactericidal agrochemicals and antibiotics [10].

2. Materials and Methods

2.1 Selection of Bacterial Strains Bacillus and Xanthomonas

2.1.1 Isolation and Identification of Phytobacteria *X. axonopodis*

The analyzed microorganisms (strains of *X. axonopodis* pv. *passiflorae*) were extracted from plant material collected in a gulupa crop located in the municipality of Pasca, Colombia. The bacteria were recovered employing the maceration method [11]. As it allows the removal of the epiphyte bacteria with greater ease, the recovered strains were identified with the following nomenclature: UCMC102-2, UCMC106-1, UCMC107-1, UCMC108-1, and they were stored in glycerol at -70 °C until required for *in vitro* and *in vivo* assays.

2.1.2 Obtaining and Preserving *B. subtilis* ATCC 55033 and Other Native Strains of *Bacillus* sp.

The *B. subtilis* strain ATCC 55033 was cryopreserved in brain heart infusion (BHI) broth with 10% *v*/*v* glycerol and stored at -70 °C. Prior to each test, the microorganism was grown on BHI broth for 24 h at 37 °C under aerobic conditions. A purity test was performed before each assay to confirm cell morphology. Nutrient agar glass tubes to evaluate the strains under study's viability and establish macroscopic characteristics (i.e., large, flat, whitish and irregularly shaped colonies) grew it. Qualitative observations were made of the colonies' pigmentation and size in the medium HiCrome® *Bacillus* Agar to confirm its authenticity.

2.2 Design of a Millet-Based Culture Medium with B. subtilis ATCC 55033

The *B. subtilis* ATCC 55033 strain was developed as an experimental subject in a sweet sorghum stalk biomass culture medium (SSB) that was designed based on the formula described by Monteiro [12]. This SSB culture medium was supplemented with millet maize, because of its high nutritional content and important iron source [13], and with sucrose that stimulates the growth and production of metabolites [14]. For its formulation, the response surface methodology strategy was followed to find optimum laboratory-scale growth. Additionally, the response variables were the hemolysis halo and biomass production, and the design variables were the different concentrations of millet and sucrose. The analyses were performed using Statgraphics Centurion XVII statistical software and the relationship of the design variables on the production of biomass and fermentation products surfactants was established.

2.2.1 Growth Curve of Strain *B. subtilis* ATCC 55033 and Semi-quantitative Evaluation of Secondary Metabolites *in Vitro* (Haemolysis Halo)

Evaluation of the growth kinetics of strain *B. subtilis* ATCC 55033 was carried out over a period of 20 h. One hundred microliters (100 µL) of the initial suspension was added in 5 mL of BHI broth medium, and then the tubes were incubated at 37 °C in an aerobic condition. To determine the growth curve, an hourly plate count was taken from each 100 µL BHI broth to make serial dilutions in 0.1% peptone water; from each dilution, 10 µL was taken and plated on nutrient agar. These plates were incubated at 37 °C for 24 h in an aerobic condition. After this time, a colony count was performed.

2.2.2 Growth Curve and Simultaneous Production of Surfactant Metabolites

The hemolysis halo in blood agar was semi-quantified at different concentrations of microorganisms since in this test the degree of hemolysis corresponds to the production of metabolites surfactants. Therefore, this test was carried out in order to determine if their formation also depends on the cell concentration. Simultaneously, the microorganism was grown on BHI broth culture, and according to the data obtained in the growth curve, the counting of microorganisms was carried out through the plaque counting technique (UFC).

2.2.3 Optimization of the Concentration of Millet and Sucrose to Obtain an Adequate Yield of Secondary Metabolites and Biomass of *B. subtilis* ATCC 55033

In order to evaluate the nutritional requirements necessary for the production of surfactin by *B. subtilis* ATCC 55033, a preliminary culture medium was designed to a final volume of 10 mL with the following composition: a nitrogen source (5 mg of yeast extract) and a culture medium buffer solution (0.105 mg $NaHSO_4{\cdot}H_2O$ and 5 mg of $MgSO_4{\cdot}7H_2O$) [12]. These components were kept stable, unlike the concentrations of millet corn, sucrose and iron source that were varied.

A batch fermentation process was performed. Prior to each test, the millet was weighed and then germinated for 12 h. After that time, it was macerated and the sucrose concentration indicated by the experimental design was added. The medium was sterilized at 121 °C for 15 min. The salts and the yeast extract were separately prepared, sterilized, and added to the medium prior to each fermentation.

Factorial designs 32 and 42 were completely randomized, considering that factor 1: the concentration of millet in mg/10 mL and the factor 2: the concentration of sucrose in mg/10 mL. Each factor had three levels representing a low, medium and high concentration. The experiment consisted of nine runs with their respective replica plus one control in BHI broth for 19 experimental runs. The response variable was hemolysis halo and Log_{10} CFU/mL.

The inoculum of 24 h growth at 37 °C and adjusted to 0.5 MacFarland was added to the medium. It was incubated for 17 h at 37 °C. After the incubation time,

15 serial dilutions were performed on each experimental medium. The last eight dilutions were then grown with an inoculum of 10 µL in nutrient agar and 20 µL of the initial agar in blood agar in duplicate. They were incubated for 24 h at 37 °C. The tests were carried out without agitation and with agitation and the models were selected to include this variable. With this, its effect was observed on the response variables. The containers used were flasks with dimensions 7 cm high and 18.5 cm in diameter, the ratio of the medium and the container were 1:5.

2.2.4 Semi-quantitative Evaluation of the Presence of Fermentation Products as Surfactants in *Bacillus*

To observe the production of these metabolites, the well diffusion technique was performed where 1 mL of the *B. subtilis* strain ATCC 55033 was inoculated in 9 mL of BHI broth. It was incubated at 37 °C for 24 h in an aerobic environment. A 3 mm well was made to the blood agar, 20 µL of the *B. subtilis* suspension was dispensed into the well after completion of the incubation time in duplicate. Samples on blood agar were incubated at 37 °C for 24 h in an aerobic environment. After the incubation, the ImageJ program's hemolysis halo (version 1.45 K) was evaluated.

2.2.5 Qualitative Analysis by Thin Layer Chromatography (TLC)

This technique was used to observe the presence of the metabolite in a purification process of sulfactinand/or other secondary metabolites such as iturin and fengycin. This methodology was developed by Romero *et al*. [15]. In the run of the samples, n-butanol was used as solvent. Measurement of the flow rate value was performed on the analyzed strains *B.s* ATCC 55033 and *B.s* TB2.

2.3 Antimicrobial and Antagonistic Evaluation in Vitro of Native Bacillus Strains and B. subtilis ATCC 55033 against X. axonopodis (UCMC108-1)

The antagonism tests were performed using the double-layer technique, which was described by Ramirez [16] by modifying the inoculation of the phytobacteria (*X. axonopodis*). Twenty milliliters (20 mL) of nutrient agar per box was prepared in which the strain of *X. axonopodis* pv. *passiflorae* (UCMC108-1) was grown and incubated for 24 h at 27 °C there. After that time, a well of 6.58 mm of diameter was made inside the laminar flow cabinet in the center of the plate culture, where 50 µL of *B. subtilis* was inoculated, then incubated for 24 h at 27 °C. Once the time was over, the confrontations were read. This procedure was applied to evaluate the eight strains of *Bacillus* sp. For the tests with the fermentation products, the same procedure was performed using 100 µL of the product.

2.4 Evaluation of Efficiency of Fermentation Products from a Millet Medium with B. subtilis ATCC 55033 against X. axonopodis under Greenhouse Conditions

2.4.1 Plants of Passion Fruit

The plant material used for the tests on detached leaves and on seedlings cultivated under greenhouse conditions was supplied by the companies OCATI and Agrícola Olimpia S.A.S. These plants were acquired from three months of age and transplanted to bags for nursery plants (27 cm × 27 cm).

2.4.2 Efficiency Tests of Fermentation Products in Seedlings under Greenhouse Conditions

The plants conserved in the Universidad Nacional of Colombia (Bogotá) were treated to determine the efficiency of the fermentation product previously prepared. Four treatments were described for this process (Table 1). The treatments were in triplicate. The monitoring of the experimental units was done by means of periodic readings of the symptomatology every 3 d to determine the severity indexes.

2.5 Statistical Analysis

For the design of the millet culture medium, a response surface design was performed to determine the optimal concentrations of the independent variables that would stimulate surfactin synthesis and

Table 1 Treatments used in the experimental design of blocks completely random.

Description of the treatments	
Treatment 1	Negative control (inoculation of distilled water)
Treatment 2	Positive control (inoculation of *Xanthomonas axonopodis* pv. *passiflorae* UCMC108-1)
Treatment 3	First inoculation of *X. axonopodis* pv. *passiflorae* UCMC108-1, 5 d later were applied from the fermentation products with strain *Bacillus subtilis* ATCC 55033
Treatment 4	First application of the fermentation products with strain *B. subtilis* ATCC 55033, after 5 d the inoculation of *X. axonopodis* pv. *passiflorae* UCMC108-1

cell growth. Statistical analysis was performed using one-way analysis of variance (ANOVA) to determine if there were statistically significant relationships between the variables for $p > 0.05$. The statistical software Statgraphics Centurión XVII was used for this purpose. Duplicate trials were performed in two independent trials ($n = 4$).

On the other hand, *in vitro* antagonism tests and blood agar hemolysis assays were analyzed using the Image software (version 1.48) for halo measurements. Treatment-based disease dynamics was analyzed by single-way ANOVA, using the SAS 9.0 program to determine if there were statistically significant relationships between the variables for $p > 0.05$ and a test of means test for the Tukey treatments.

3. Results

3.1 Development of Millet-Based Culture Medium with B. subtilis ATCC 55033

3.1.1 Production of Secondary Metabolites Surfactants vs. Bacterial Growth of *B. subtilis* ATCC 55033

Surfactin stimulation, represented in haemolysis halos, was evidenced from a concentration of 6.27 Log_{10} UFC/mL, corresponding to the same cell concentration at hour one. It is observed that the production of surfactin is independent of cellular concentration.

3.1.2 The Concentration of Essential Millet and Sucrose in the Optimal Production of Secondary Metabolites Surfactants

The results were analyzed by surface response methodology for variability of hemolysis halos and Log_{10} CFU/mL. Five designs were made to find the best means to maximize the hemolysis halo and Log_{10} CFU/mL (Figs. 1 and 2).

The optimal conditions of the independent variables were: millet (30-60 mg/10 mL) and sucrose (3-15 mg/10 mL), and these ranges were applied for the production of surfactin and the increase in the concentration of cells of *B.s* ATCC 55033.

3.1.3 The Presence of Fermentation Products as Surfactants in *Bacillus* sp. by the Greater Halos of Hemolysis in Blood Agar

When evaluations of the resulting fermentation products were performed with the optimum millet medium described above, it was obtained that with respect to the *B.s* ATCC 55033 control, the most extensive hemolysis halos were obtained from strains *B.s* TB2, B1015 and B109 (41.17, 39.62 and 39.66 mm, respectively). These strains are potential producers of secondary metabolites surfactants, which may be efficient in their antimicrobial and antagonistic activity both *in vitro* and *in vivo* (Fig. 2).

3.1.4 Milk-Based Fermentation Products Would Indicate Iturin in the Implication of Its Activity

Within the butanol run with the two strains *B.s* ATCC 55033 and *B.s* TB2, flow rate (mL/min) values were determined. *B.s* ATCC 55033 obtained a flow rate value of 0.7, corresponding to its nature within the surfactin range. Moreover, *B.s* TB2 obtained a flow rate value of 0.5, within the range of iturin. The strain *B.s* TB2 also showed the highest level of hemolysis halos production.

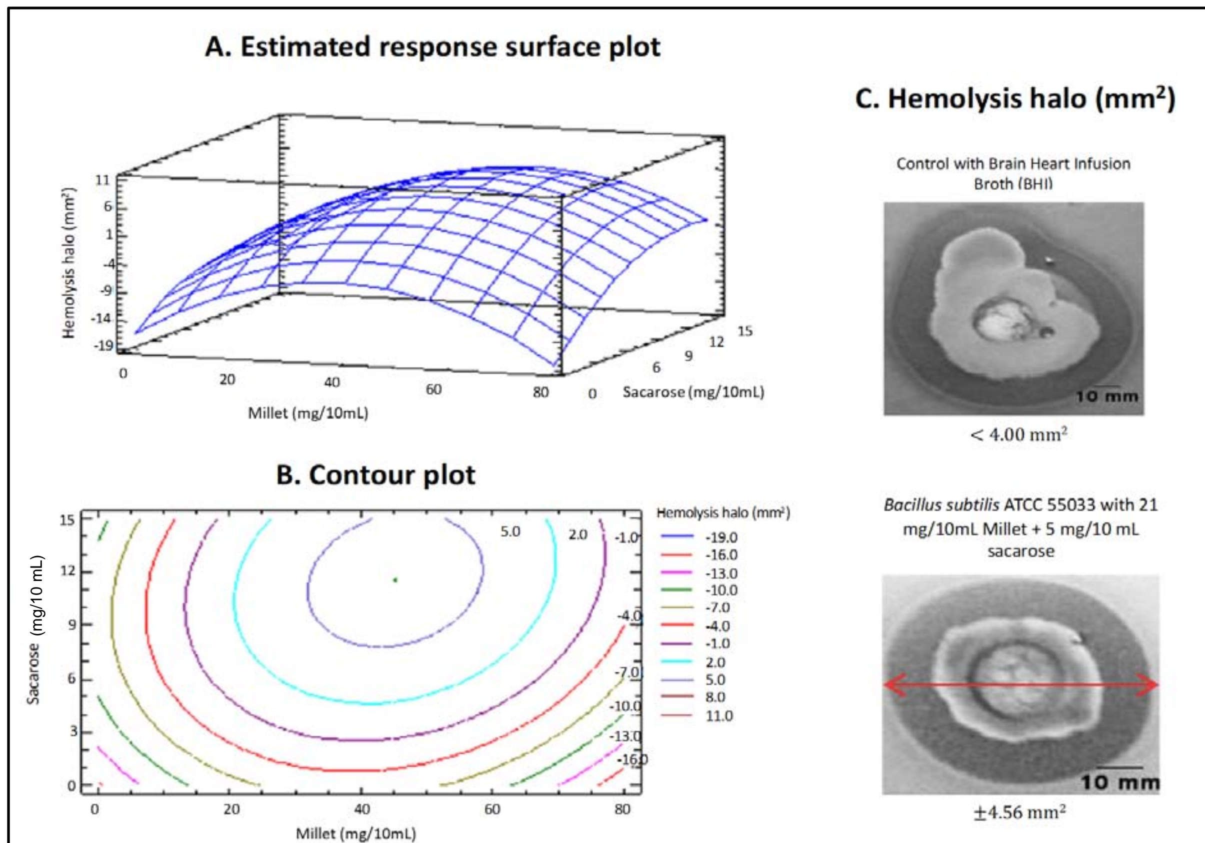

Fig. 1 Response surface for the interaction between millet and sucrose concentration during the halo variable hemolysis. Coefficient of statistical determination indicates that the model explains 61.2057% of the variability in halo hemolysis (mm^2) for a value of $p < 0.05$ with a 95% confidence level since the millet's variable concentration has a more significant effect on the design, with a value of $p = 0.0094$.

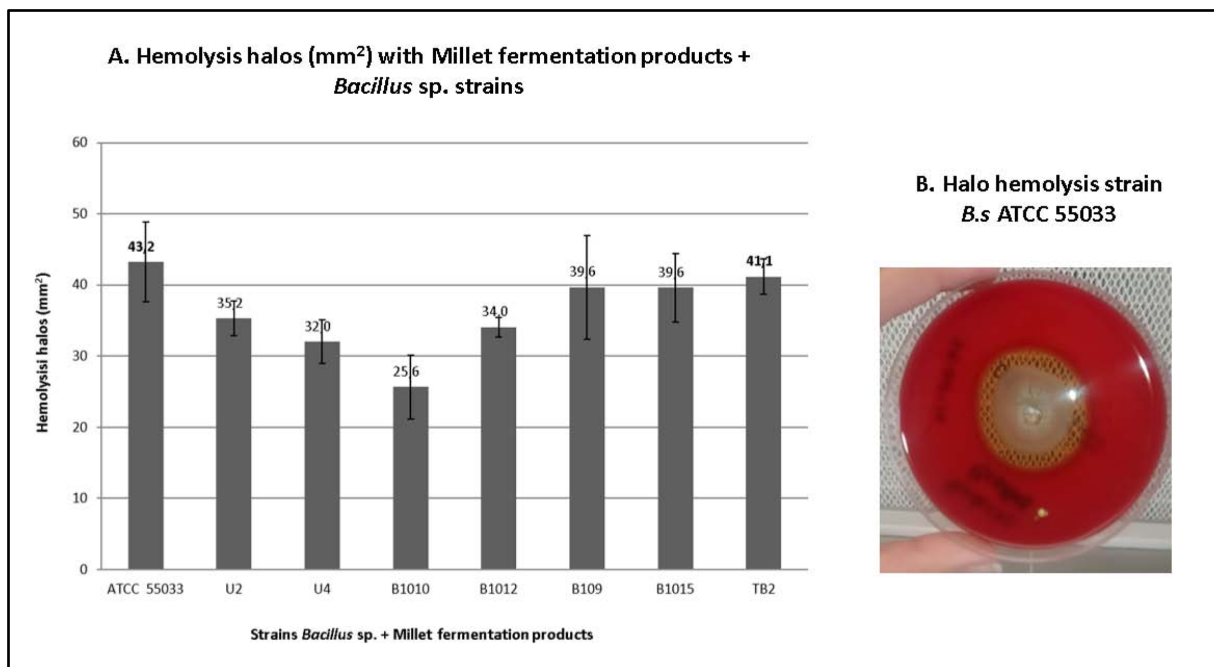

Fig. 2 Evaluation of fermentation products with native strains of *Bacillus* sp. inhibition halos of the tests 1 and 2. *B.s* ATCC 55033 and *B.s* TB2 had the best zones of inhibition on the growth of *X.a* in both tests.

3.2 The Native Strains of Bacillus UCMC and B. subtilis ATCC 55033 together with Their in Vitro Millet Fermentation Products Have an Antimicrobial Effect on X. axonopodis

The antimicrobial effect on *X. axonopodis* was analyzed according to the comparison between two broth cultures: test 1, the use of *Bacillus* sp. with media millet, and test 2 fermentation products without *Bacillus* sp. It was observed that fermentation products with these microorganisms have larger diameters in inhibition halos (Fig. 3) suggesting a higher production of secondary metabolites of interest for biological control (iturins, fengicines and surfactins).

3.3 Efficacy of Fermentation Products of B. subtilis ATCC 55033 in Gulupa Plants Applied in Pre-inoculation with X. axonopodis under Greenhouse Conditions

The severity of the infection process was lower in the treatment when the fermentation products were applied before inoculation with the phytopathogenic bacterium *X. axonopodis*. That is to say, on the range 39-54 days post-inoculation (dpi), a percentage of the severity of 87.5% was observed in the infected plants.

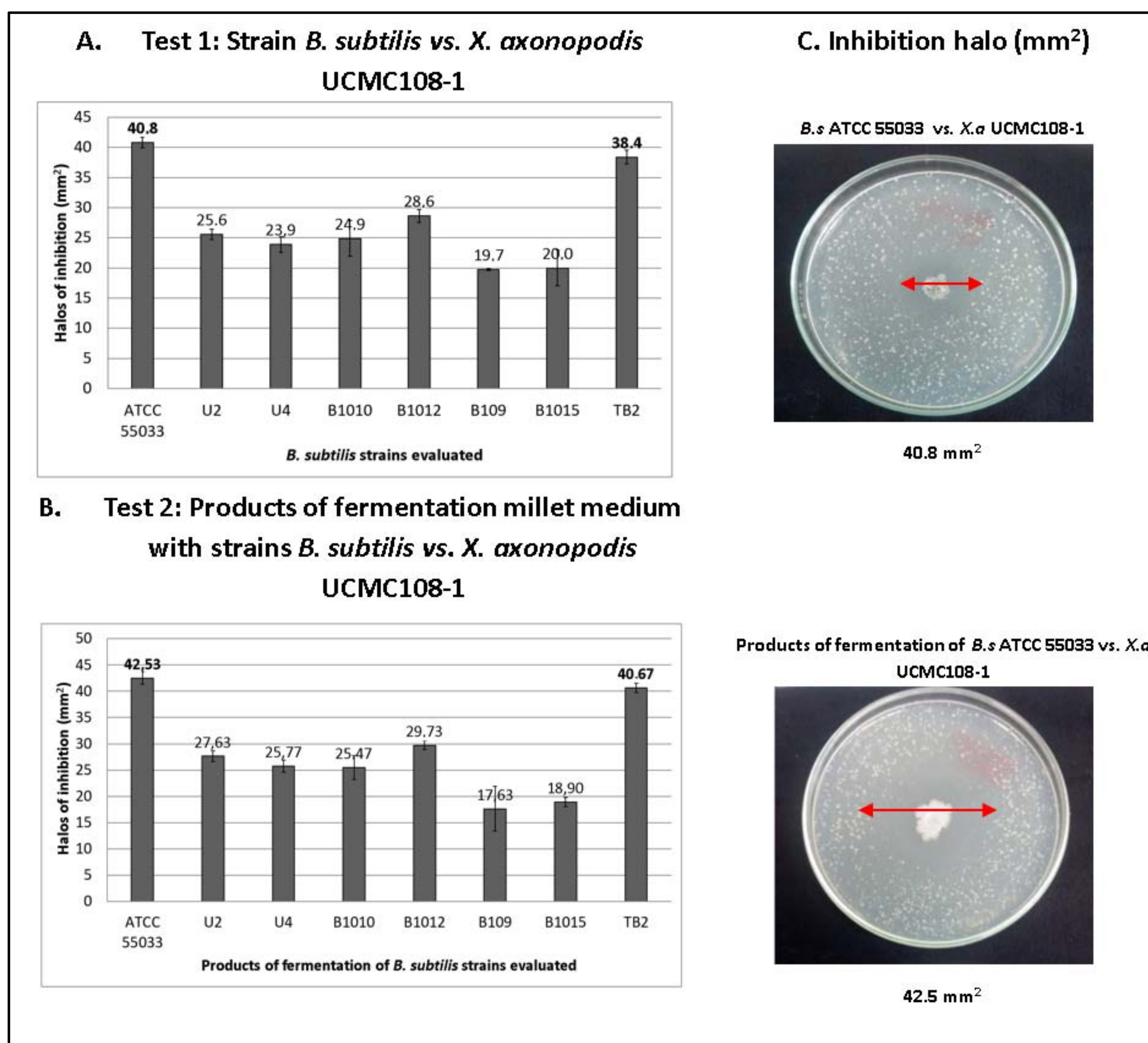

Fig. 3 Inhibition halos from tests 1 and 2.
B.s ATCC 55033 and *B.s* TB2 had the best inhibition halos in *X.a* growth in both tests.

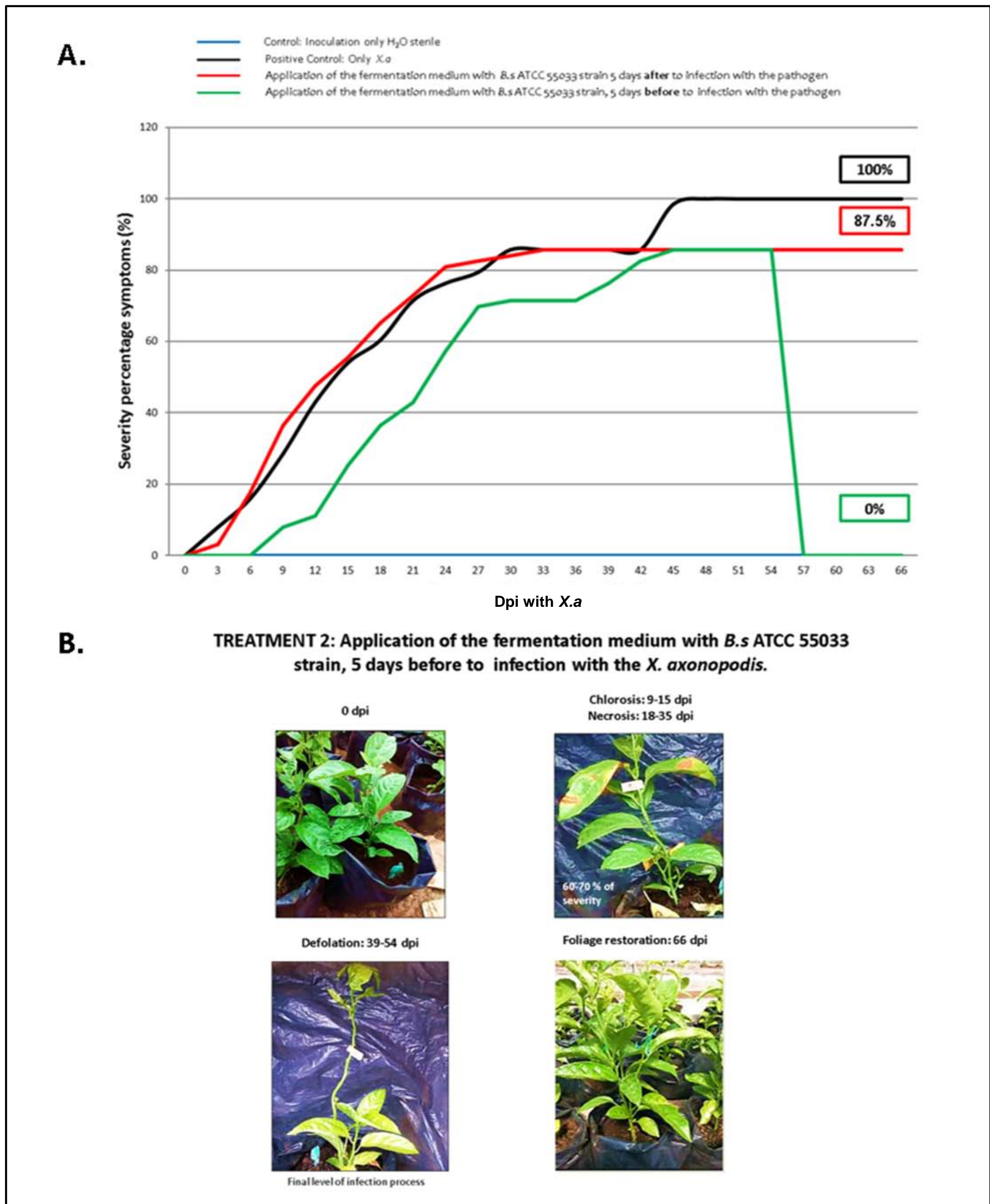

Fig. 4 Efficacy of fermentation products of *B. subtilis* ATCC 55033 in gulupa plants applied in pre-inoculation with *X. axonopodis* under greenhouse conditions: (A) percentage of severity in gulupa seedlings with four treatments during their infective process and the interaction with the application of the fermentation product ($p < 0.001$); (B) infection progress with application of treatment 2. In 66 days post-inoculation (dpi), it observed a foliage restoration.

Table 2 Efficiency of fermentation products in treatments.

Treatments	Percentage final severity	Efficiency (percentage of health)
1. Negative control[c]	0	100
2. Positive control[a]	72.37	23.63
3. Inoculation of the pathogen before application of the product[a]	68.43	31.57
4. Application of the product before inoculation of the pathogen[b]	42.044	57.95*

* 57.9% efficacy percentage of the fermentation medium was observed with *B.s* ATCC 55033 in gulupa seedlings prior to inoculation of *X. axonopodis* under greenhouse conditions.
Tukey test with $\alpha = 0.05$. Stockings with the same letter are not significantly different.

After about 3 d, there was defoliation, and therefore, from 57 dpi, new leaf buds began to be found in the plants that were infected by *X.a*. This result could be correlated with an induction effect of resistance in the host plant (Fig. 4A).

The values obtained by the observations with the severity scale were grouped by means of the Tukey test (Table 2). It was observed that the treatments 2 (positive control) and 3 (infected seedlings prior to the application of the product) were performed similarly, that is to say, in both treatments of disease dynamics, there were significant differences, being a relevant finding that the treatment 4 with previous application of the fermentation products obtained only a severity percentage of 42% with 57.95% of efficacy again infection of *X. axonopodis*.

Statistical analyses performed with the SAS 9.0 program (Table 2) suggest a very similar disease dynamic between treatments 2 and 3. There were correlations with the data observed in the follow-up of the disease process. Both treatments developed the symptoms over time, similar to those observed in the positive control (treatment 2). On the other hand, treatments 4 and 1 were grouped into different categories, indicating that these treatments are significantly different than the other results reported in the trial (Fig. 4B).

4. Discussion

Biological control is one of the alternatives for the treatment of phytopathogens that greatly affect crop production. In addition, biological control offers a great advantage as a control option as they represent an ecological, environmentally-friendly, non-polluting alternative, and significantly reduces the risk of resistance of pathogens [2, 5, 17]. In addition, as it is selective in its mode of action, biological control unlikely harms other beneficial organisms since in many cases it favors the ecosystem and stimulates plant growth. In like manner, the effects on human health are minimal or null [2, 5, 18].

Currently, rhizospheric microorganisms such as fungi and bacteria have become important in the promotion of plant growth and the biological control of phytopathological agents [19]. Within this group, there are growth promoting rhizobacteria (acronym: PGPR), which have shown their excellent performance in the management of abiotic and biotic stress. Undoubtedly, the use of this type of bacteria is within of sustainable agricultural practices. However, it is still necessary to know the mechanisms in the production of their secondary metabolites, the appropriate applications in the field, and evaluate the possible factors that influence their efficacy [20].

Studies conducted in an attempt to elucidate the mechanisms involved in biological control have shown antagonistic activities of *Bacillus* sp. [21] often associated with the production of secondary metabolites with antibiotic and resistance-inducing properties, considered this genus as PGPR rhizobacteria [22]. These metabolites are essential in the formation of root biofilm, cell-to-cell communication (quorum sensing), and the interaction with plants for the mediation of diverse physiological activities within them, especially in the induction of systemic resistance (ISR) [23].

Bacillus produces a large group of surfactants, such as surfactin and consists of a cyclic heptapeptide linked to a β-hydroxy fatty acid chain. The isomerism and the length of the fatty acid chain are responsible for the activities of the surface [24] and, therefore, there are four isoforms: surfactin (A-D) [25]. Surfactin is present from the beginning of the latency phase because the microorganism needs it for its development. External factors, such as increased cell frequency and substrate availability, are useful for the stimulation of *Bacillus*. Biosynthesis of surfactin is regulated by a quorum detection system, which in turn coordinates competition and sporulation [26]. Extrinsic factors increase the expression of the surfactin synthase gene, activated by the phosphorylation of a second messenger (Com A). Transmembrane proteins (Com P) which trigger the synthesis of compounds and finally, the secretion of surfactin to the medium count this messenger [27].

The description of the experimental results using mathematical models is adequate to explain the behavior of the independent variables on the response variables. It is also taken into account that when using gradient factors of the variables, an improvement in the process occurs or, on the contrary, a negative effect on the process [28].

In the methodology implemented, a means of fermentation was carried out based on the literary description to stimulate surfactin production. It was expected to generate significant changes in the maximum yield in surfactin, expressed as halos of hemolysis, which was obtained from the optimal concentrations of the millet and sucrose. Several sources of carbon are essential in the production of surfactin. In the strain *B. subtilis* BBG208 carbon sources such as yeast extract and mannitol, higher yields were obtained than other substrates used in fermentation. In addition, substrates as a nitrogen source such as urea and mixed with NH_4HCO_3 can also increase their production, are low cost offering an economic scale in the production yield [29].

Millet, the primary source of this culture medium, was germinated to increase the availability of the nutrients it possesses, considering that a germination time of more than 12 h produces grain roasting [30]. In addition, this is cheap and provides the microorganism with energy requirements, carbohydrates, iron, vitamins and appropriate sucrose concentrations [13]. Therefore, agricultural residues of vegetable origin such as fruit extracts, beets [31], and the corncob hydrolyzate have high oligosaccharides [32]. Xylose was essential in obtaining surfactin from the strain *B. subtilis* BS-37 [32]. Research by Zhou *et al.* [33] also support this, who obtained high yields of surfactin with appropriate glucose and cellulose from *B. subtilis* HH2 strain isolated from intestinal substances of herbivores such as giant panda. So, the use of plant remains is a vital source of fermentable carbon indispensable in obtaining this type of surfactants.

The results obtained in the agitation tests at 150 rpm showed a correlation between the data, which indicates that the model equation approaches the values obtained in the experimental tests. Therefore, the results allow these models to be replicated. This could occur due to the fact that the constant agitation allowed a homogeneous medium and, therefore, a more homogenous and representative sample for the development of semi-quantitative evaluation tests of the presence of surfactin and cell count by plaque count. The agitation favored the increase in cellular concentration. An increase in test 1 of a logarithmic unit (13.04 Log_{10} CFU/mL) was obtained. In test 6, the concentration was maintained in the two experiments, and test 7 presented 16 Log_{10} CFU/mL, 4 Log_{10} CFU/mL more than the fermentation medium static.

The agitation variable had already been reported as an inducer of cell growth in trials of scaling of the *B. subtilis* strain performed by Pabón [34] where it was concluded that the agitation treatment positively influences cell growth. The reported UFCs were 1.2 ×

10^9 cells/mL [34]. Ramírez (2005) [16] evaluated the production of *B. subtilis* against *Streptomyces scabies* using these growing conditions: to 200 rpm agitation at a temperature of 30 °C. They obtained a cell concentration of 2.9×10^8 cells/mL. This increase in cell concentration is attributed to the advantage generated by the agitation of putting the microorganism in contact with all the nutrients of the medium, thus, the bacterium has each one of the components of the medium that allows its multiplication at its disposal [35].

The interest in being able to achieve the optimal conditions of production of this type of surfactant is to be used in the field. Several studies have shown that among several species of *Bacillus*, specifically *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. cereus* and *B. thuringiensis*, almost 95% of this group can produce surfactin and have an antibiotic effect against phytopathogenic bacteria such as *Ralstonia solanacearum*, *R. radiobacter*, *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae* and *X. axonopodis* pv. *vesicatoria*, which was related to the expression of the *srfAA* and *fenD* genes [36].

Studies performed by Zeriouh *et al*. [37] allow corroborating that the metabolites iturin, fengicin and surfactin, tested against bacterial agents *X. campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum*, present a generalized inhibitory effect against these bacterial pathogens. The visualization of the cellular damage caused by the metabolites in *X. campestris* pv. *cucurbitae* at the ultrastructural level was followed by negative staining and transmission electron microscopy. After 1 h of exposure, the first cytotoxic effects of the metabolites can be clearly seen [37].

Initially, there was a general disorganization of the cytoplasm, which became less electrodensing. After 8 h, the disintegration of the plasma membrane became evident, resulting in the formation of small vesicles in the membrane, and finally, after 24 h of exposure to the metabolite, lysis of the bacterial cell and the exit of the cytoplasmic content. This study makes it possible to suggest these metabolites are attractive candidates for compounds in the next generation of phytosanitary products [37].

When compared to Etchegaray *et al.* analysis [38], these results used *B. subtilis* (strain OG) against *X. axonopodis* and *X. campestris* pv. *campestris*, show that aggregates of surfactin and iturin interact with these phytopathogens by inducing dramatic cell morphology changes [38, 39]. The organized structures of these aggregates that form the metabolites succeed in removing cations such as calcium and/or magnesium that may be bound to the pathogen's negative charge cell wall. This removal of cations can induce a collapse of the cell wall, allowing the absorption of these peptides to the membrane and forming pores [40, 41].

Another example, Elshakh *et al*. [42] found three strains: *B. subtilis* A15, *B. amyloliquefaciens* D29 and *B. methylotrophicus* H8, reduced growth and biofilm formation significantly from *X. oryzae* pv. *oryzae* causal agent of bacterial leaf blight (BLB). Besides, by an analysis of transmission electron microscopy, they observed changes in bacterial cell morphology, such as cell elongation and severe alterations of the cell wall. And finally, they demonstrated by molecular analysis as PCR that the three strains amplified the genes associated with the biosynthesis of bacillomycin, fengycin, iturin and surfactin [42].

The tests carried out with different native strains of *Bacillus*, with their respective secondary metabolites, which were tested against bacterial pathogens such as *X. axonopodis* allowed observing in this study percentages of antagonism that suggest their inhibitory capacity against the phytopathogen. Also, these metabolites can be inducers of systemic resistance in the host of the plant [43]. Notably, the host plant can gain defense pathways dependent on salicylic acid (SA) and jasmonic acid (JA) due to these microbial compounds [44, 45]. Almost, bacterial phytopathogenic diseases when treated with *Bacillus*

bacteria, trigger in the plant the increase in the enzymatic activity of polyphenol oxidase (PPO), peroxidase (POD), phenyl ammonium lyase (PAL), superoxide dismutase (SOD) and catalase (CAT) associated with these defense pathways [42, 46].

Some beneficial bacteria can indirectly protect plants through the stimulation of inducible defense mechanisms that make the host plant more resistant to the phytopathogen's penetration and colonization [21]. In fact, *Bacillus* also gives the plant tolerance to adverse environmental conditions such as high salinity, drought and heavy metals in soils [47]. The secondary metabolites can be multifunctional in helping the plant in both abiotic and abiotic stresses. In this study, related to biotic stress such as bacterial diseases, *B. subtilis* strengthens the defensive potential of the plant when it stimulates the ISR through the production of these secondary metabolites [44, 48].

The reduced symptoms of the disease caused by the metabolites of *B. subtilis* were associated with metabolic changes related to a priming event [23], after the first defense response triggered by the phytobacteria and potentiated with the application of the fermentation product of the prepared culture medium. Beneficial rhizobacteria such as *Bacillus* induce priming by these secondary metabolites, which are not only the surfactants but also the volatile organic compounds [49]. At the beginning of this mechanism, there is a transient inhibition of the local immune responses of the plant so that they can colonize *Bacillus* [50]. Then, internally, the expression of associated transcription factors is triggered, such as MYb72, which is related to expanding the defense response systemically [51].

Concerning these molecular mechanisms associated with ISR and priming, it was found that in *B. cereus* AR156 against *P. syringae* pv. *Tomato* DC3000 in *Arabidopsis* two transcription factors (WRKY11 and WRKY70) were identified. These are essential regulators involved in ISR, activating cellular defense responses such as the generation of hypersensitivity response (HR) and the level of transcription of the plant's defense response gene. In addition, they observed that each one corresponded to a defense pathway: WRKY11 associated with the SA pathway and WRKY70 are dependent on JA/ET [52].

This reduction of symptoms is associated with the cellular defense responses associated with the RH triggering. Ion fluxes through the plasma membrane and the oxidative burst generated by reactive oxygen species (ROS), later expressed as a defense detection mechanism, further limit and inhibit the penetration of pathogens into the tissues of the plant and therefore, decrease the symptoms of the disease [36, 45, 53, 54]. In addition, the activity of other plant defense enzymes such as β-1,3-glucanase and phenylalanine ammonia-lyase (PAL) important in this cellular defense activity can be observed. These were significantly higher than in tomato control plants at 72 h after inoculation with *X. campestris* pv. *vesicatoria* when these plants were treated from seed with *B. subtilis* CBR05 [55].

Not only is this type of PGPR bacteria capable of triggering ISR. In these experiments, it was observed that in plants previously treated with *B. subtilis* after being infected 57 d later, the regeneration of new leaf shoots was observed in the places where *X. axonopodis* had been inoculated. This interesting result is related to the results of Shahzad *et al*. [56] where they observed a significant plant growth due to a high production of gibberellins due to the application of the endophytic strain *B. amyloliquefaciens* RWL-1 [56, 57]. Both *Bacillus* and rhizosphere bacteria have a high potential in sustainable agricultural practices due to their ability to trigger various defense mechanisms in the plant and help tolerance with the restoration of plant growth in the face of pathogen attack and even insect-plague.

5. Conclusions

- The optimum conditions of the independent

variables were established in the millet culture medium (between 30 mg/10 mL and 60 mg/10 mL) and sucrose (between 3 mg/10 mL and 15 mg/10 mL), noting that these ranges were applied for the production of secondary effects metabolites and the production of cellular concentration.

- The hemolytic activity allowed knowing in a semi-quantitative way the capacity of *B. subtilis* as the producer of secondary metabolites, especially surfactin and iturin, being *B. subtilis* ATCC 55033 with 43.24% the largest producer and followed by *B. subtilis* TB2 with 41.17%. Potential bio-controllers of phytopathogens are considered for their antimicrobial activity, given the action conferred by such metabolites.
- *In vitro* tests demonstrate the antagonistic capacity of the fermentation medium and strains of *Bacillus* sp. The following inhibition halos were obtained: *B. subtilis* ATCC 55033 (40.8 mm^2) and *B. subtilis* TB2 (38.4 mm^2). However, in the tests with the microorganism without culture medium an increase in the diameter of the halo was observed, there were no significant differences between *B.s* ATTC 55033 (42.53 mm^2) and *B.s* TB2 (40.67 mm^2).

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