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Optimization of fermentation temperature and time for production of an antifungal extract from *Bacillus amyloliquefaciens* B17

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ABSTRACT

Background/Objective. Species of *Bacillus* are currently gaining interest because its ability to produce secondary metabolites with antifungal properties against various plant pathogenic fungi. The objective of this study was to optimize fermentation temperature and time for antifungal extract production by *Bacillus amyloliquefaciens* B17 and to verify its activity against plant pathogenic fungi *Gilbertella persicaria, Choanephora cucurbitarum, Colletotrichum asianum, and Botrytis cinerea.*

Materials and Methods. A central composite design (CCD) with two factors and five levels (fermentation temperature: 23.7, 25, 28, 31, and 32.2 °C and fermentation time: 25, 46, 95, 144, and 164. 3 h) was used. Thirteen combinations of temperature and fermentation time were randomly performed. The thirteen crude extracts of *B. amyloliquefaciens* B17 were obtained from the cell-free fermentation broth by acid precipitation followed by alkaline solubilization. The response variable was the diameter of the inhibition halos generated by placing drops of the different crude extracts onto the medium already inoculated with a suspension of *Gilbertella persicaria* spores.

Results. The optimal conditions for the production of the extract with the greatest antifungal activity in *B. amyloliquefaciens* B17 were 26.8 °C and 158.6 h.

Conclusion. The optimized crude extract from *B. amyloliquefaciens* B17 exhibited a strong ability to inhibit mycelial growth and spore germination of *Gilbertella*

persicaria, Choanephora cucurbitarum, Colletotrichum asianum, and *Botrytis cinerea.*

Keywords: *Bacillus,* Biocontrol, Antifungal extract, Fungi.

Introduction

Plant pathogenic fungi cause significant losses in agricultural crops throughout the world, and the annual economic losses in the agricultural sector due to fungal diseases reach 200 billion dollars worldwide (Horbach *et al.*, 2011; Wang *et al.,* 2022). Within the wide diversity of plant pathogenic fungi, the Ascomycota division includes around 75% of the fungal species that cause significant damage to cereals, fruits and vegetables, being the most important genera: *Alternaria, Fusarium, Diplodia, Monilinia, Penicillium, Botrytis, and Colletotrichum* (Arroyave-Toro *et al.,* 2017; García-Rico and Fierro, 2017; Hoffmann *et al*., 2013). In addition, the division Mucoromycota also contains important and fast-growing plant pathogenic species such as *Rhizopus* spp., *Mucor* spp., *Choanephora cucurbitarum,* and *Gilbertella persicaria* (Benny, 1991; Hoffmann *et al*., 2013).

Although chemical control is essential for limiting plant diseases caused by plant pathogenic fungi, the indiscriminate use of chemical fungicides has contributed to antifungal resistance, as well as environmental pollution and human health deterioration. Therefore, it is necessary to develop alternative control methods that have minimal impacts on the environment and human health (Ginting *et al.*, 1996; Ragazzo-Sánchez *et al.,* 2011). Biological control, including the use of microorganisms or certain microbial metabolites, has turned out to be a viable alternative for control of pre- and postharvest fungal diseases (Juárez-Becerra *et al.,* 2010; Pérez-García *et al.,* 2011; Ragazzo-Sánchez *et al*., 2011).

Bacteria of the genus *Bacillus* are currently of great interest because they have multiple mechanisms of action for the biological control of plant pathogenic microorganisms. The biocontrol mechanisms of *Bacillus* species include competition with plant pathogen for substrate, stimulation of plant defense, and production of volatile organic and lytic enzymes, and antimicrobial compounds. Among the antimicrobial compounds produce by *Bacillus* spp., lipopeptides are of particular importance, since these molecules are bioactive against a wide range of plant pathogenic agents (bacteria, fungi and oomycetes). Lipopeptides consist of a cyclic peptide attached to a fatty acid chain, which, based on its structure, are mainly classified into three groups or families: surfactin, iturin, and fengycin (Bonmatin *et al.,* 2003; Cawoy *et al*., 2014; Fira *et al.,* 2018; Pedraza-Herrera *et al.,* 2020).

Lipopeptide production is influenced by several factors, such as strain type, media composition, and fermentation conditions. Fermentation time can influence the type and concentration of lipopeptide produced (Inès and Dhouha, 2015). Lipopeptides with antimicrobial activity, such as surfactins, are produced during the exponential phase of bacterial growth, while lipopeptides with antifungal activity, such as iturins and fengycins, are produced during the stationary phase (Abushady *et al.,* 2005; Wang *et al*., 2008; Xu *et al*., 2020). Although lipopeptide biosynthesis can occur at temperatures between 25 and 45 °C, the fermentation temperature also affects the type of lipopeptide produced. In some types of *Bacillus* strains, it has been reported that a temperature of 37 °C profits surfactin production, while a temperature of 25 °C benefits iturin production (Abushady *et al*., 2005; Inès and Dhouha, 2015; Ohno *et al.,* 1995).

In previous studies, the significant antimicrobial activity of *Bacillus amyloliquefaciens* B17 has been reported. This strain, isolated from the rhizosphere of tomato plants, has demonstrated significant oomyceticidal activity; however, its antifungal activity has not yet been reported (Ley-López *et al*., 2022; Ley-López *et al*., 2018). The objective of this research was to optimize the fermentation temperature and time for the production of antifungal extract from *Bacillus amyloliquefaciens* and to determine the effect of extract on the mycelial growth and spore germination of the plant pathogenic fungi *Gilbertella persicaria, Choanephora cucurbitarum, Colletotrichum asianum,* and *Botrytis cinerea.*

Materials and Methods

Microorganisms and culture conditions. The microorganisms used in this study were provided by the Phytopathology Laboratory of the Food and Development Research Center (Culiacán Regional Coordination). *Bacillus amyloliquefaciens* B17 (GenBank accession number: KX953161), isolated from the rhizosphere of tomato crops at different geographical locations in Sinaloa, was maintained on nutrient agar (NA, BD Bioxon) at 27 °C. *Gilbertella persicaria* HP15 (accession numbers: KR076758 and KR076761), *Choanephora cucurbitarum* CCCFMX01 (accession numbers: OQ269823 and OQ269827) and *Colletotrichum asianum* UACH299 (accession number: MK016315) were grown in potato dextrose agar (PDA, BD Bioxon) to 27 °C. *Botrytis cinerea* HF02 (accession numbers: OQ191231, OQ286120, and OQ286119) was maintained on PDA plates at 22 °C.

Inoculum preparation. *Bacillus amyloliquefaciens* grown in LB medium was used as inoculum. Bacterial cells were scraped from the NA plate and added into a 250 mL flask containing 120 mL of LB broth. After incubating at 27 °C and

shaking 150 rpm for 24 h, the concentration of bacterial cells was 1.2 x 10^9 CFU/mL (according to the McFarland scale) (Ley-López *et al.,*2022).

Obtaining crude extracts. Eight milliliters of inoculum were placed in a 1 L flask containing 200 mL of Landy medium (glucose, 20 g/L; L-glutamic acid, 5g/L; yeast extract, 1g/L; K_2HPO_4, 1g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; KCl, 0.5 g/L; $CuSO_4 \cdot 5H_2O$, 1.6 mg/L; $Fe_2(SO_4)_3 \cdot 7H_2O$, 0.4 mg/L; $MnSO_4 \cdot H_2O$, 1.2 mg/L) previously adjusted to an initial pH of 7. Landy medium, a usual culture medium used for lipopeptide production by *Bacillus* spp., was used to carry out fermentation in shaken flasks (230 rpm). After the fermentation time elapsed, the bacterial cells in the Landy medium were removed by centrifugation at 10,000 rpm for 12 min at 4 °C, and the cell-free supernatant was collected. The supernatant was acidified with 6N HCl to pH of 2 and stored overnight at 4 °C to precipitate peptide compounds. The precipitate was recovered by centrifugation (10,000 rpm, 20 min at 4 °C) and was dissolved in 10 ml of distilled water. The pH of the solution was adjusted to 8 with 0.5 M NaOH. Afterwards, this solution was filtered with 0.45 mm nylon membranes to obtain the *Bacillus c*rude extract. Since a single method is insufficient for purifying peptide compounds, it is common to include multiple steps in the purification of these compounds. Therefore, for this study, a combination of acid precipitation, alkaline solubilization and filtration was used for extraction. For acid precipitation, the pH of the fermentation medium was lowered (~2) using concentrated hydrochloric acid. Thus, the negative charges on the peptide molecules were neutralized, which decreases their solubility in the aqueous phase and facilitates their separation by precipitation. Subsequently, the compounds that precipitated were again solubilized in the aqueous phase by making the medium alkaline (pH=8), and the peptide compounds that were not solubilized were eliminated by filtration (Ley-Lopez *et al.,* 2022; Motta Dos Santos *et al*., 2016; Torres *et al.,* 2016).

Optimization of temperature and fermentation time by RSM. To determine the optimal fermentation temperature and time for production of a crude extract from *Bacillus amyloliquefaciens* B17 with the greatest antifungal activity, a central composite design (CCD) with two factors and five levels (fermentation temperature: 23.7, 25, 28, 31, and 32.2 °C and fermentation time: 25, 46, 95, 144, and 164.3 h) was used. Thirteen combinations (including 5 replicates of the central point) of fermentation temperature and time were randomly performed (Table 1). The response variable was the diameter of the inhibition halos generated by placing aliquots (5 µL) of the different crude extracts, applied directly as drops onto the medium (PDA) already inoculated with 100 µL of *G. persicaria* spore suspension (1 x 10^6 sporangiospores/mL).

Table 1. Experimental results of the inhibition halos generated by the crude extracts of *B. amyloliquefaciens* obtained under different fermentation conditions.

Experimental runs	Process variables		Response variable
	Fermentation temperature (°C)	Fermentation time (h)	Diameter of inhibition halo (mm)
1	28	95	8.9
2	31	46	0
3	25	144	11.8
4	25	46	0
5	28	25.7	0
6	32.2	95	0.9
7	28	164.3	13.0
8	28	95	7.7
9	28	95	10.0
10	28	95	11.1
11	23.7	95	9.1
12	31	144	7.6
13	28	95	10.8

Yield of optimized crude extract. After the optimal fermentation temperature and time conditions were validated, the optimized crude extract was freeze-dried (FreeZone Triad Benchtop Freeze Dryer, LABCONCO) and weighed to determine the yield of the fermentation process. The optimized and lyophilized extract was stored at 4 °C for further analysis.

Determination of the mean effective concentration (EC_{50}). To determine the concentration of the optimized crude extract that inhibits 50% of the mycelial growth (EC_{50}), different concentrations of freeze dried extract (3.75, 7.5, 15, 30, 45, 60 µg/mL) were added to sterile PDA medium (45 °C) before being poured into Petri dishes. PDA medium without optimized extract was used as a control. The plates were allowed to gel at room temperature for 24 h, after which mycelial discs (6 mm in diameter) of *G. persicaria, C. cucurbitarum, C. asianum, and B. cinerea* (after 7 days of growth) were placed in the center of the plates. The plates with *G. persicaria, C. cucurbitarum,* and *C. asianum* were incubated at 27 °C, meanwhile, the plates with *B. cinerea* were incubated at 22°C, until mycelial growth covered each of the respective control plates. The percentage of growth inhibition was determined using the following formula (Ramírez-Benítez *et al*., 2019).

$$\% \text{ Inhibition} = \frac{(\text{mycelial growth diameter in control}) - (\text{mycelial growth diameter in treated Petri plates})}{(\text{mycelial growth diameter in control})} \; x\, 100$$

Using the results of the percentage of growth inhibition, a dose-response curve was generated and the EC_{50} was determined.

Determination of the minimum inhibitory concentration (MIC). The MIC was determined according to the methodology of Toral *et al.,* (2018) with some modifications. One hundred microliters of spore suspension (1 x 10^6 spores/mL) of each of the fungi (*G. persicaria, C. cucurbitarum, C. asianum,* and *B. cinerea*) was added in each well of the microplates containing 900 µL of potato dextrose broth (PDB, Difco) and different extract concentrations (0, 0.75, 1.5, 2.25, 3, 3.75, 4.5, 5.25, 6, 6.75, 7.5, 9, 10.5, 12, 13.5, and 15 mg/mL). PDB without crude extract was used as a control. The microplates with *G. persicaria and C. cucurbitarum* were incubated at 27 °C for 48 h, while *C. asianum* were incubated at 27 °C for 96 h and the microplates with *B. cinerea* were incubated 22 °C for 96 h. The experiment was performed in triplicate and the MIC was determined visually as the lowest concentration of the optimized extract that completely inhibited fungal growth.

Determination of the minimum fungicidal concentration (MFC). The MFC was determined after evaluating the corresponding MIC. Aliquots of 5 µL from each well of the microplates that did not show growth of the fungi were transferred in triplicate to PDA plates. The inoculated plates were incubated at 27 °C for 96 h for *G. persicaria* and *C. cucurbitarum*; 27 °C for 144 h for *C. asianum* and 22 °C for 144 h for *B. cinerea.* The MFC was determined as the lowest concentration of extract that kills each of the fungi (Cortés *et al.,* 2020).

Effect of optimized crude extract. After evaluating the MIC, aliquots of 10 µL from each well of the microplates containing PDB, fungal spores, and different extract concentrations were placed on slides and the mycelial growth and spores germination were observed by triplicate using an Imager A2 microscope (Zeiss, Germany).

Data analysis. The diameters of the inhibition halos were analyzed, by multiple regression, to determine the mathematical model that adequately explains the variation in the response variable. An analysis of variance (ANOVA) was performed to examine the statistical significance of the terms of the model obtained by regression. Once the adjusted prediction model for the studied response variable (diameter of the inhibition halos) was obtained, the numerical desirability method (D) was used to determine the fermentation temperature and time that maximized the diameters of the inhibition halos. The aim of the optimization was to find the highest possible values of D that were associated with the optimal temperature and fermentation time conditions. Minitab Statistical Software (version 18) was used for experimental design and regression analysis of the experimental data.

Results

RSM optimization. The experimental design (CCD) and the results of the response variable (diameter of the inhibition halos) generated by the 13 crude extracts are shown in Table 1. The diameter of the inhibition halos ranged from 0 to 13 mm. The response variables obtained from the 13 crude extracts were analyzed by multiple regression, and mathematical model that adequately explained the variation in the response variables was constructed.

The results of the ANOVA and the regression analysis for the quadratic model of the inhibition halo are presented in Table 2. The regression model explained 94.23% of the total variability in the diameters of the inhibition halos, with a significance level of 0.05. ANOVA revealed that the *p* value of the model was 0.000, which implies that the model was significant ($P < 0.05$).

Table 2. ANOVA and regression analysis for the response variable (diameter of the inhibition halo) generated by the crude extracts of *B. amyloliquefaciens* obtained under different fermentation conditions.

Source	Coeficient model	Sum of squares	df	Mean square	F value	*p*-value
Model		275.94	5	55.19	22.88	0.000*
Intercept	9.702					
X_1:Temperature	-1.983	31.47	1	31.47	13.05	0.009*
X_2:Time	4.720	178.25	1	178.25	73.91	0.000*
X_1X_2	-1.05	4.41	1	4.41	1.83	0.218
X_1^2	-2.571	38.49	1	45.98	19.07	0.003*
X_2^2	-1.831	23.32	1	23.32	9.67	0.017*
Residual		16.88	7	2.41		
Lack of Fit		8.70	3	2.9	1.42	0.330
Pure Error		8.18	4	2.05		

*Significant (for a *p-value*<0.05); df = degrees of freedom; $R^2 = 0.9423$; Adj $R^2 = 0.9012$

The regression model with coded variables obtained to predict the diameter of the inhibition halos was as follows:

$$Y = 9.702 - 1.983X_1 + 4.72X_2 - 1.050X_1X_2 - 2.571X_1^2 - 1.831X_2^2$$

where Y corresponds to the diameter of the estimated inhibition halo and X_1 and X_2 are the coded values of temperature and fermentation time, respectively. The terms that had significant effects ($p < 0.05$) on the diameter of the inhibition halos were the linear (X_1, X_2) and quadratic (X_1^2, X_2^2) terms of temperature and fermentation time. The lack of fit of the data was not significant because the *p* value was greater than 0.05 for the test of lack of fit.

Considering only the significant terms, the model for the diameter of the inhibition halos (Y) can be rewritten as:

$$Y = 9.702 - 1.983X_1 + 4.72X_2 - 2.571X_1^2 - 1.831X_2^2$$

Figure 1 shows the response surface graph obtained with the data estimated by the mathematical prediction model. As the desired values for the diameter of the inhibition halo were the highest, these values were found in the area where there were intermediate values of temperature (T) and high values of fermentation time (t).

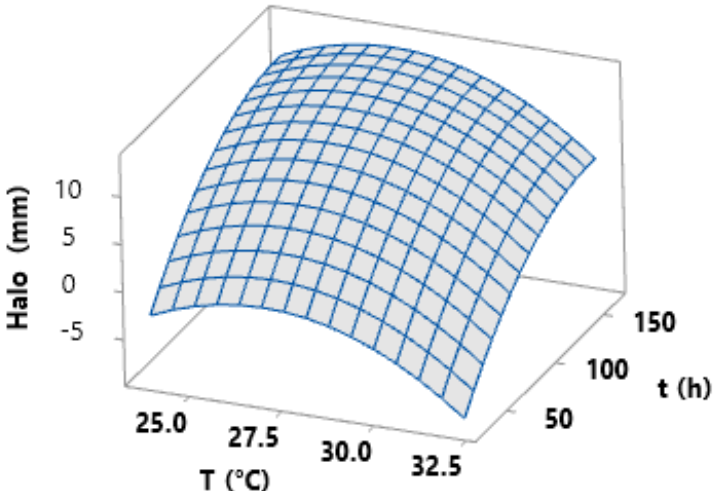

Figure 1. Response surface plot for the inhibition halo diameter as a function of fermentation time and temperature.

Optimization using the numerical desirability (D) method was carried out to find the highest possible values of D (values close to 1), which are associated with the best fermentation conditions. The optimal temperature and fermentation time were 26.8 °C and 158.6 h, respectively. These optimal conditions corresponded to a value of D = 1 (Figure 2).

Determination of EC_{50}, MIC and MFC. To estimate the EC_{50} of the extract on *G. persicaria*, *C. cucurbitarum, C. asianum,* and *B. cinerea,* the percentages of radial fungal growth inhibition in solid media were determined at different concentrations of the crude extract. These results revealed a reduction in the radial growth in all the fungi studied, in all the concentrations evaluated (Figure 3). The concentrations of

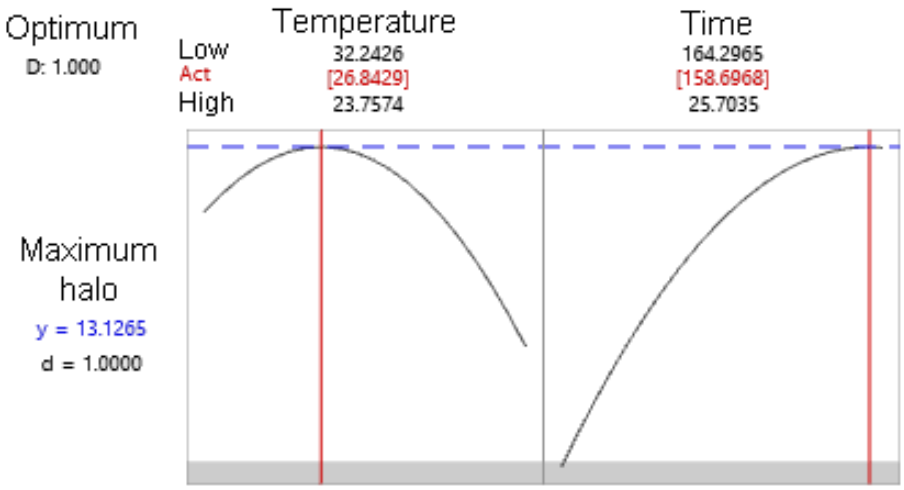

Figure 2. Optimization plot showing the effect of each factor (temperature and time) on the response (inhibition halo). The vertical lines and red numbers represent the optimal configuration of the factors. The horizontal line and blue number represent the response for the optimal level of the factors.

optimized crude extract that inhibited 50% of the radial growth (EC_{50}) of the fungi *G. persicaria, C. cucurbitarum, C. asianum,* and *B. cinerea* were 3.7, 22.6, 34.5, and 36.3 µg/mL, respectively. The MIC and MFC of the optimized crude extract were estimated to be 1.2 and 1.5 µg/mL for *G. persicaria*; 5.25 and 6 µg/mL for *C. cucurbitarum*; 3 and 4.5 for *C. asianum* and for *B. cinerea*, the MIC and MFC were estimated to have the same value of 3 mg/mL (Table 3).

Effect of optimized crude extract. The optimized crude extract of *B. amyloliquefaciens* B17 had adverse effects on spore germination of the four fungi (*G. persicaria, C. cucurbitarum, C. asianum,* and *B. cinerea*). At a low extract concentration (1.5 mg/mL) in the culture medium, a decrease in the speed of spore germination was observed (Figures 4C, 5C, 6C, and 7C), in addition to the fact that the germinated spores present multiples germ tubes (Figure 4C and Figure 5B) or the generated hyphae present abnormal bulges (Figures 4C, 6B, and 7B). The presence of giant cells was observed in *G. persicaria* and *C. cucurbitarum* (Figures 4D and 5C), the giant cells correspond to spores that only present spherical growth without the development of the germ tube. On the other hand, the spores that germinated in PDB without the addition of extract (controls) showed normal germination, since the spores germinated and the germ tubes elongated to form mycelia without bulging or deformation (Figure 4A, 5A, 6A and 7A).

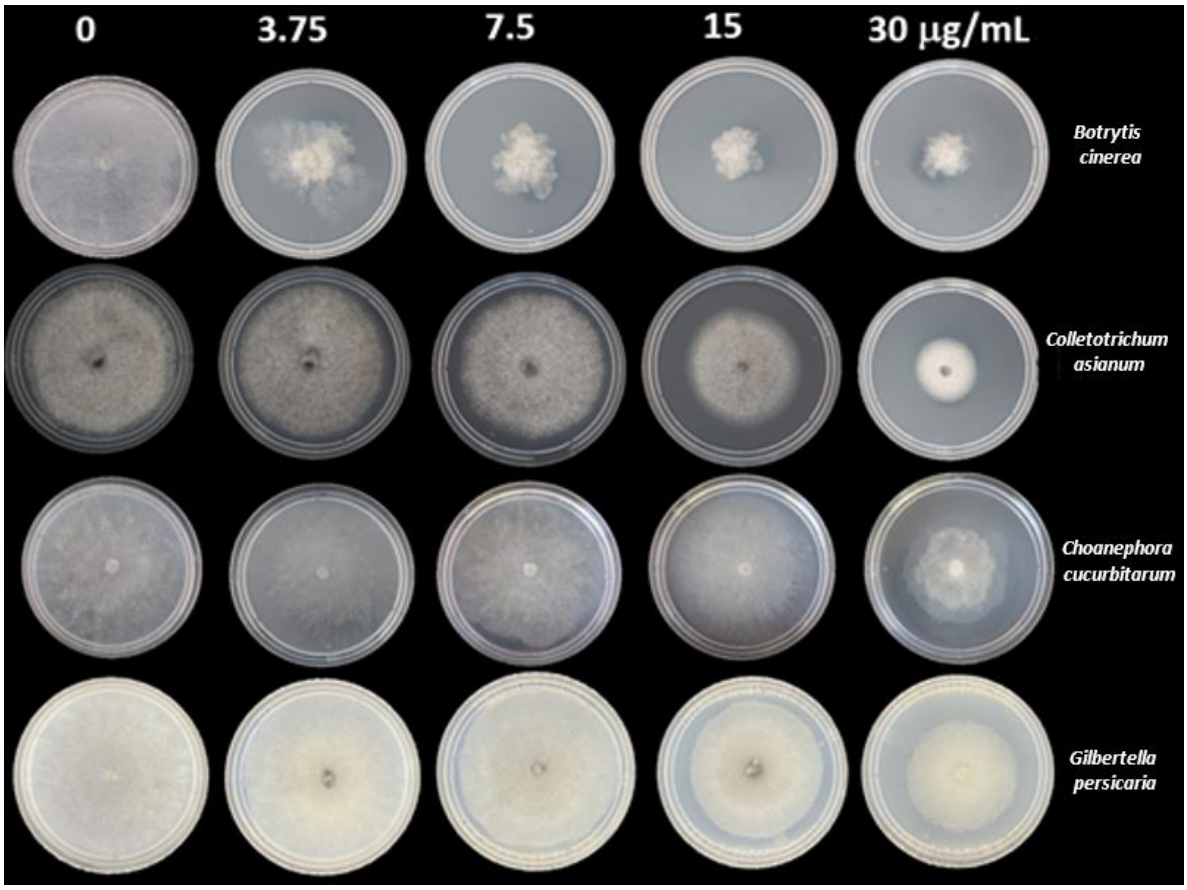

Figure 3. Effect of *Bacillus amyloliquefaciens* B17 crude extract on mycelial growth. The radial fungal growth inhibition in solid media (PDA) were determined at different concentrations of the crude extract (0, 3.75, 7.5, 15, and 30 µg/mL). These series of images reveal a reduction in the radial growth of all the fungi evaluated: *Botrytis cinerea* (22 °C, 96 h), *Colletotrichum asianum* (27 °C, 168 h), *Choanephora cucurbitarum* (27 °C, 48 h), and *Gilbertella persicaria* (27 °C, 48 h). These observations correspond to three repetitions of each series.

Table 3. MIC and MFC of optimized extract against *Gilbertella persicaria, Choanephora cucurbitarum, Colletotrichum asianum* and *Botrytis cinerea.*

Optimized crude extract (mg/mL)	*G. persicaria*		*C. cucurbitarum*		*C. asianum*		*B. cinerea*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
0.75	+	+	+	+	+	+	+	+
1.5	+	+	+	+	+	+	+	+
2.25	+	+	+	+	+	+	+	+
3	+	+	+	+	-	+	-	-
3.75	+	+	+	+	-	+	-	-
4.5	+	+	+	+	-	-	-	-
5.25	+	+	-	+	-	-	-	-
6	+	+	-	-	-	-	-	-
6.75	+	+	-	-	-	-	-	-
7.5	+	+	-	-	-	-	-	-
9	+	+	-	-	-	-	-	-
10.5	+	+	-	-	-	-	-	-
12	-	+	-	-	-	-	-	-
13.5	-	+	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-

"+" = Presence of mycelial growth; "-" = Absence of mycelial growth.

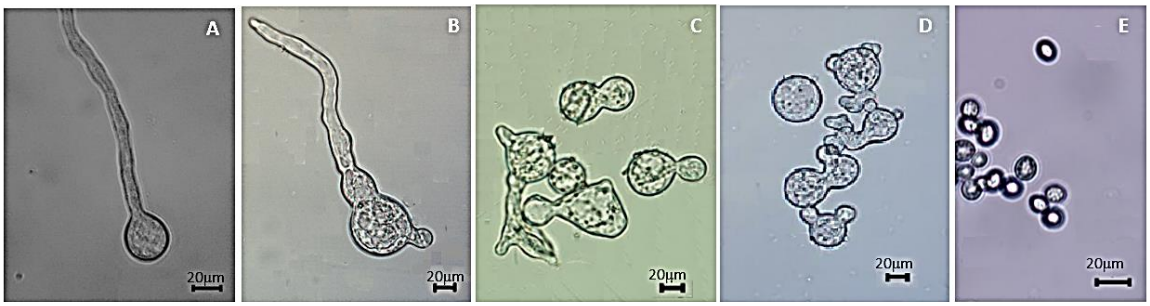

Figure 4. Effect of *Bacillus amyloliquefaciens* B17 crude extract on sporangiospores germination (27 °C, 48 h) of *Gilbertella persicaria*. (A) Control: *Gilbertella* sporangiospores in PDB medium without extract; (B) *Gilbertella* sporangiospores exposed to 0.75 mg/mL extract dose; (C) *Gilbertella* sporangiospores exposed to 1.5 mg/mL extract dose; (D) *Gilbertella* sporangiospores exposed to 6 mg/mL extract dose; (E) *Gilbertella* sporangiospores exposed to 15 mg/mL extract dose. Each image represents a consensus of 3 observations.

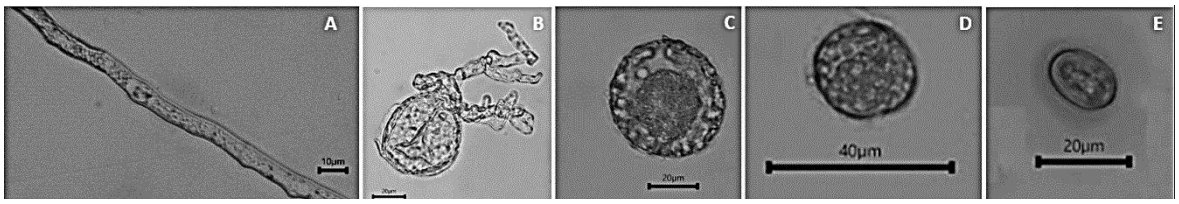

Figure 5. Effect of *Bacillus amyloliquefaciens* B17 crude extract on sporangiospores germination (27 °C, 48 h) of *Choanephora cucurbitarum*. (A) Control: *Choanephora* sporangiospores in PDB medium without extract; (B) *Choanephora* sporangiospores exposed to 0.75 mg/mL extract dose; (C) *Choanephora* sporangiospores exposed to 1.5 mg/mL extract dose; (D) *Choanephora* sporangiospores exposed to 2.25 mg/mL extract dose; (E) *Choanephora* sporangiospores exposed to 4.5 mg/mL extract dose. Each image represents a consensus of 3 observations.

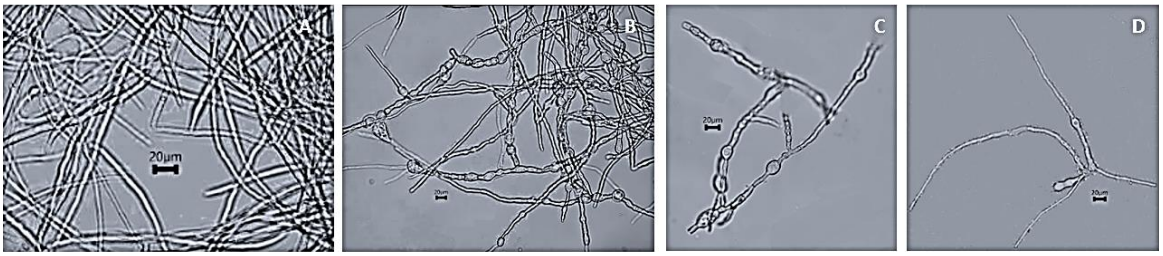

Figure 6. Effect of *Bacillus amyloliquefaciens* B17 crude extract on conidia germination (27 °C, 96 h) of *Colletotrichum asianum.* (A) Control: *Colletotrichum* conidia in PDB medium without extract; (B) *Colletotrichum* conidia exposed to 0.75 mg/mL extract dose; (C) *Colletotrichum* conidia exposed to 1.5 mg/mL extract dose; (D) *Colletotrichum* conidia exposed to 2.25 mg/mL extract dose. Each image represents a consensus of 3 observations.

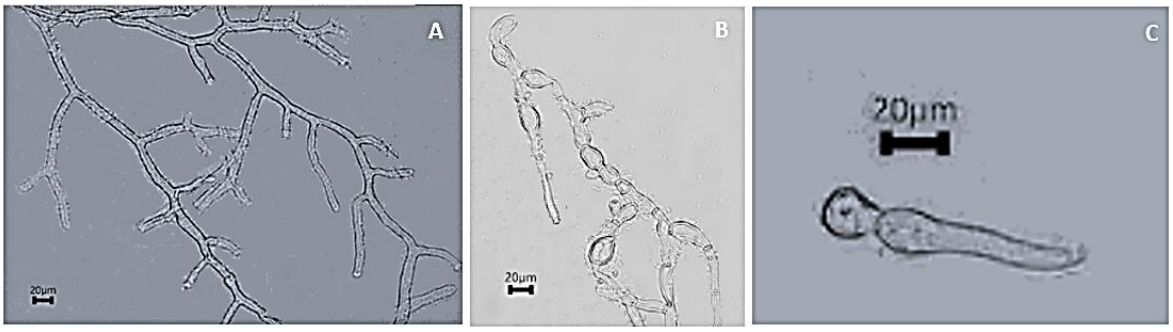

Figure 7. Effect of *Bacillus amyloliquefaciens* B17 crude extract on conidia germination (27 °C, 48 h) of *Botrytis cinerea.* (A) Control: *Botrytis* conidia in PDB medium without extract; (B) *Botrytis* conidia exposed to 0.75 mg/mL extract dose; (C) *Botrytis* conidia exposed to 1.5 mg/mL extract dose. Each image represents a consensus of 3 observations.

Discussion

In the present study, the importance of temperature and fermentation time for *Bacillus amyloliquefaciens* to produce secondary metabolites, which have potential for controlling plant pathogenic fungi, was highlighted. Lipopeptides are the most widely studied secondary metabolites in numerous *Bacillus* species (*B. subtilis, B. amyloliquefaciens, B. licheniformis, B. megaterium, B. mycoides and B. thuringiensis*) (Bhusal and Mmbaga, 2020; Ley-López *et al.,* 2022; Li *et al.,* 2020; Meena *et al.*, 2020). In *B. amyloliquefaciens* B17, the optimal conditions for the production of compounds with antifungal activity were 26.8 °C and 158.6 h.

Under this production and extraction scheme, the optimized extract yield of *B. amyloliquefaciens* B17 was 756 mg of crude precipitate per 1L from Landy medium, fermented at 26.8 °C for 158.6 h. This yield was higher than that reported for the *B. subtilis* strain KLP2015, which produced a yield of peptide precipitate (specifically identified as lipopeptides) of 545 mg/L (from Luria Bertani broth, fermented at 30 °C for 72 h) (Meena *et al.,* 2020). The differences in yield can be explained by the type of strain used, in addition to the time and composition of the fermentation medium used (Cozzolino *et al.,* 2020), since it has been reported that fermentation media rich in salts of divalent cations (such as Landy medium) promote greater production of secondary metabolites such as lipopeptides since many of these cations ($Mg^{2+,}$ $Mn^{2+,}$ Cu^{2+}, Fe^{2+}) act as cofactors of bacterial enzymes (Abushady *et al.*, 2005; Wang *et al.,* 2008; Xu *et al.*, 2020). Regarding the antifungal activity, several studies have reported the antifungal activity of lipopeptides produced by *Bacillus* spp. against various plant pathogenic fungi (*Rhizopus stolonifer, Botrytis cinerea, Colletotrichum gloeosporioides, Rhizoctonia solani, Fusarium oxysporum, Aspergillus flavus, Aspergillus niger* and *Mucor* sp.) (Meena *et al.,* 2020; Moyne *et al.,* 2001; Tao *et al.,* 2011; Toral *et al.,* 2018; Villegas-Escobar *et al.,* 2018; Yan *et al.,* 2020; Yu *et al.,* 2002; Zhu *et al.*, 2020).

In a previous study using the same *B. amyloliquefaciens* strain (B17), but under different fermentation conditions (31 °C and 144 h), only the production of fengycin and surfactin was reported, these compounds being responsible for the oomyceticidal activity against *Phytophthora capsici,* in our research it is necessary to identify previously if lipopeptides are present in the crude extract and if these compounds are responsible for the antifungal activity of the extract (Ley-López *et al*., 2022).

The optimized crude extract of *B. amyloliquefaciens* was effective against the four plant pathogenic fungi. Being *B. cinerea* and *C. asianum* the most sensitive fungi (EC_{50}=3.7 and 22.6 µg mL^{-1} respectively) and *G. persicaria* and *C. cucurbitarum* the least sensitive fungus (EC_{50}= 36.3 and 34.5 µg mL^{-1} respectively).

In this study, the antifungal activity of the crude extract of *B. amyloliquefaciens* B17 against *G. persicaria*, *C. cucurbitarum, C. asianum* and *B. cinerea* was demonstrated, given that the extract produced malformations in the germ tubes, as well as abnormal bulges in the hyphae of the fungi. Deformations in the hyphae or swollen hyphal segments similar to those obtained in this study have been reported in *B. cinerea, C. gloeosporioides, F. oxysporum,* and *Mucor* sp. due to the effect of lipopeptides, mainly fengycin and iturin A (Meena *et al.*, 2020; Toral *et al.*, 2018; Yan *et al.*, 2020; Zhu *et al.,* 2020).

Conclusions

The fermentation temperature and time were optimized for the production of antifungal extract by *B. amyloliquefaciens* B17. The antifungal activity of the optimized extract produced by *B. amyloliquefaciens* B17 was evaluated against *Gilbertella persicaria*, *Choanephora cucurbitarum, Colletotrichum asianum* and *Botrytis cinerea* and it was observe that the extract produced under optimal fermentation conditions (26.8 °C and 158.6 h) had adverse effects not only on mycelial growth but also on the spore germination of these four plant pathogenic fungi.

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