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ORIGINAL RESEARCH ARTICLE

Production of a Mixture of Fengycins with Surfactant and Antifungal Activities by *Bacillus* sp. MA04, a Versatile PGPR

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Abstract *Bacillus* sp. strain MA04 a plant growth-promoting rhizobacteria (PGPR) showed hemolytic activity on blood agar plates, and the supernatant from liquid culture in nutrient broth at 24 h exhibited emulsification activity, suggesting the production of biosurfactants. In antagonist assays, the supernatant showed antifungal activity against phytopathogenic fungi such as *Penicillium expansum*, *Fusarium stilboides*, *Sclerotium rolfsii* y *Rhizoctonia solani*, finding a reduction of mycelial growth of all fungi tested, ranging from 35 to 69%, this activity was increased with time of culture, accomplishing percentages of inhibition up to 85% with supernatants obtained at 72 h. Then, the crude biorsurfactant (CB) was isolated from the supernatant in order to assay its antagonistic effect on the phytopathogens previously tested, finding an increase in the inhibition up to 97% at 500 mg/L of CB. The composition of CB was determined by infrared spectroscopy, identifying various functional groups related to lipopeptides, which were purified by high-performance liquid chromatography and analyzed by MALDI-TOF/TOF–MS, revealing a mixture of fengycins A and B whose high antifungal activity is been widely recognized. These results show that PGPR *Bacillus* sp. MA04 could also contribute to plant health status through the production of metabolites with antimicrobial activity.

Keywords Hemolytic activity · Emulsification index · Lipopeptides · Adduct · Biological control

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Introduction

Plant growth-promoting bacteria (PGPB) present at rhizosphere level, encourage the growth and development of the host plant through bacterial physiological processes as the biological nitrogen fixation and the solubilization of phosphates and micronutrients, that improve the nutritional status, and by the production of bacterial phytohormones such as auxins, cytokinins and gibberellins, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity that regulate plant physiological processes involved in seed germination, vegetative development, flowering and stress caused by environmental factors. So that, plants of economic interest inoculated artificially with PGPB can better express their genetic potential, reflected in their growth, yield and quality [1].

Moreover, the incidence of pest and diseases at any stage of the crop cycle can compromise the production yield, giving rise to significant economic losses [2]. Plants associated with PGPB may suffer less damage when they are affected by bacteria, fungi, virus and nematodes, not only for having a better nutrition or a lower level of stress, but also because some PGPB can produce enzymes and metabolites such as chitinases and antibiotics with antagonistic activity against any of these phytopathogens [3, 4].

In recent years, some *Bacillus* species have been recognized as biocontrol agents of soil-borne phytopathogens,

by their ability to produce biorsurfactant lipopeptides with antimicrobial activity, these compounds that comprise four structural families: surfactins, iturins, mycosubtilins and fengicins are synthesized by non-ribosomal peptide synthetases [4]. However, the production of the lipopeptides depends on the presence of genes encoding for these synthetases and of the composition of culture media and culture conditions [5].

Bacillus sp. strain MA04 is a strain isolated from tomato rhizosphere soil [6], that exhibits in vitro activities such as ACC deaminase, phosphate solubilizing and indole production, probably related to their ability to improve the seedling vigor of tomato and pepper, but it is unknown if it can also contribute to plant health by suppression of phytopathogens. Therefore, the aim of this study was to characterize MA04 in its ability to inhibit fungal phytophatogens in vitro, identifying the metabolites involved in the antagonism.

Materials and Methods

Microbial Strains and Culture Media

The fungi used in this study were chosen for their economic importance as phytopathogens of ornamentals, seed, fruits and vegetables. *Rhizoctonia solani*, *Sclerotium rolfsii* and *Alternaria* sp. are considered soil-borne fungal pathogens; particularly *R. solani* cause damping off on tomato seeds, these strains has been used in antagonism essays using *Trichoderma virens* [7]. *Penicillium expansum* Link CFNL-2016, isolated from stored apple [8] and *Fusarium stilboide*s CFNL-2768, isolated from pepper [9], these postharvest pathogens cause the disease of blue mold and the peduncle rot in fruits, respectively. *Aspergillus niger*, *Colletotrichum* sp., *Diplocarpon rosae* and *Phytophtora capsici* belong to our laboratory collection, and were isolated from soil, mango fruits with anthracnose, roses with black spot and pepper plants wilting, respectively.

The PGPR *Bacillus subtilis* strain MA04 was cultivated in nutrient broth (NB) for antifungal characterization of supernatants, and the fungal phytopathogens were grown on NB-potato dextrose agar (PDA) to obtain active mycelia for the antagonism tests. All strains were conserved under refrigeration at − 21 °C in glycerol stocks.

Hemolytic Activity Determination

The hemolytic activity in *Bacillus* sp. MA04 was determined by streaking onto a sheep's blood agar plates, and incubating them for 72 h at 30 °C. Haemolysis was identified by the presence of a clear halo formation around the colonies [10].

Characterization of Supernatants

The bacterial production of biosurfactants in the culture medium was determined through the emulsification of kerosene. *Bacillus* MA04 was grown in nutrient broth (NB) medium at 30 °C at 180 rpm for 24, 48 and 72 h. After each time, cell-free culture supernatants (CFS) were obtained by centrifugation at 2500×*g* for 30 min, and the emulsification capacity of CFS was estimated by determining the emulsification index (EI_{24}) and the emulsion stability (ES_t) every 24 h for 5 days, according with the method of Zhu et al. [11].

The antifungal activity of CFS against plant pathogens was performed in vitro. For this, 39 g of potato dextrose agar (PDA) were supplemented with 1 L of CFS and sterilized by autoclaving at 121 °C for 15 min, and then the media was poured into Petri dishes. For this preliminary test, a mycelial disc of 5 mm from actively growing culture of *R. solani*, *S. rolfsii*, *P. expansum* or *F. stilboides* was placed in the center of the plate prepared with the CFS. Controls were performed in the same manner, using medium without CFS. Plates were incubated at 30 °C and the mycelial diameter was daily measured, the antagonistic activity was expressed as the percent of reduction (inhibition) of mycelia growth with respect to control [12].

Isolation and Activity of Crude Biosurfactant

Bacillus sp. MA04 was cultivated in 2 L Erlenmeyer flasks containing 800 mL of nutrient broth at 30 °C and 180 rpm for 72 h, the cells were then harvested by centrifugation at 2500×*g* for 30 min to recover the supernatant, which were adjusted to pH 2 with HCl 6 N and stored overnight at 4 °C, the insoluble precipitate was recovered by centrifugation at 2500×*g* for 30 min and washed twice with acidified water to pH 2. Finally, the precipitate was subjected to fractionated extraction with methanol, and the extract was evaporated to dryness in a rotary evaporator (Buchi RII) under reduced pressure at 50 °C [5]. The resultant residue considered as the crude biosurfactant (CB) was used to measure the antifungal and emulsifying activities.

For the assays, CB (62.5 mg) was weighed and redissolved in 25 mL of phosphate buffered saline (PBS) 0.01 M at pH 7.4 [6], to obtain a stock solution of 2500 mg/L. Aliquots of this were rediluted to concentrations of 50, 100, 200, 500 and 1000 mg/L to determine the EI_{24} and ES_t [11]. In the same way, plates containing NB and PDA with CB were prepared at concentrations of 100, 200 and 500 mg/L to evaluate the antifungal activity, as was described previously to the characterization of supernatants, now including the phytopathogens *Alternaria* sp., *A. niger*, *Colletotrichum* sp., *D. rosae* and *P. capsici*.

Analysis by FT-IR Spectroscopy

The chemical nature of CB was identified by Fourier Transform Infrared Spectroscopy (FT-IR) analysis, using a Perkin-Elmer Spectrum 100 FT-IR spectrometer. The CB was reduced to powder and directly recorded on the spectrophotometer in a dry atmosphere over the range of 650-4000 cm^{-1} with a resolution of 4 cm^{-1}.

Identification of Antifungal Compounds by MALDI TOF/TOF MS

To identify the compounds in the CB related to antifungal activity. First it was fractionated by HPLC on Zorbax 300SB-C8 column (150 × 4.6 mm, 5 µm particle size, 300 Å pore size; Agilent technologies), and eluted by using a gradient of 80% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) over 60 min, at a flow rate of 1.0 mL/min. The detection of compounds was carried out using a diode-array detector (DAD) set at 215 nm, and the fractions collected were tested for fungal antagonism in vitro, impregnating paper discs (6 mm in diameter) with 10 µL of each fraction and placing them around of active mycelia of *Alternaria* sp. growing on NB-PDA plates. The active fractions that inhibited the mycelia growth were dried and resuspended them in 50% acetonitrile/0.1% TFA, and 1 µL of this was mixed with a equal volume of α-cyano-hydroxy-cinnamic acid (10 mg/mL-1 in solution of 50% acetonitrile/0.1% TFA) [13]. Later, 1 µL of this later mixture was analyzed by MALDI-TOF/TOF MS using an AutoFlex III mass spectrometer (Bruker Daltonics). These analyses were performed in the chromatography and proteomic laboratories of the Center for Research and Advanced Studies of the National Polytechnic (CINVESTAV) Irapuato unit.

Statistical Analyses

Emulsification and antagonist assays were performed by triplicate and data obtained were analyzed by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and the differences among means were reported as statistically significant when $p < 0.05$.

Results and Discussion

Bacterial Biosurfactant Production

The growth of *Bacillus* MA04 on blood agar plates exhibited clear halos around of the colonies, which are due to the production of metabolites inducing erythrocytes lysis. Furthermore, the supernatant from growth culture gave a positive result to the emulsification test; obtaining an IE_{24} of 55.9% at 24 h (Table 1), a value similar to that obtained from other *Bacillus* producers of biosurfactants isolated from soils and sediments [14, 15]. Both tests have been proposed as preliminary criteria for identifying environmental isolates with potential of production of biosurfactants, compounds also are recognized for its antifungal capacity [16].

Antifungal and Emulsifying Properties of Supernatant

The Table 2 shows that supernatant from MA04 at 24 h of culture inhibited the mycelium growth of four phytopathogenic fungi in percentages ranging from 35.7 (*F. stilboides*) to 69.5% (*S. rolfsii*), and this inhibition increased with the time of culture probably due to the biosurfactant accumulation, supernatants obtained at 48 and 72 h accomplishing average inhibitions of 84.8% (*P. expansum*) and 85.3% (*S. rolfsii*). But not all pathogens showed the same response, *F. stilboides* and *R. solani* exhibited similar inhibition rates with supernatants from cultures at 24 and 72 h.

During the growth culture of MA04, the values of some parameters such as cellular density and EI_{24} were constants within 72 h, while the optical density ($O.D._{600}$ nm) and biomass were reduced over the time (Table 1), which is probably due to the increase in the biosurfactant concentration that may cause lysis and degradation of bacterial cell membranes [11]. El-Sheshtawy et al. [17] reported that the production of biosurfactant by *Bacillus licheniformis* in a mineral salt medium may be monitored through the measurement of EI_{24} and surface tension. In our case, related to *Bacillus* sp. MA04, none of growth culture parameters tested could be used to measure the increase in biosurfactant production on supernatant, however other surface properties such as the interfacial tension and the critical micelle concentration (CMC), among others could be considered [18].

Characterization of Biosurfactant

The recovery of crude biosurfactant (CB) from supernatants by precipitation and extraction indicated that MA04 yielded the highest amount of biosurfactant after 72 h of culture (145.5 mg/L) (Table 1). The antagonism assays using the CB on plates showed that the concentration at 100 mg/L was able to inhibit the mycelial growth of seven from nine pathogens tested in more than 62%, and at 500 mg/L the inhibitions were greater than 91% in *A. niger*, *D. rosae* and *P. expansum*. Conversely, a reduced effect (6.2%) was recorded on *P. capsici*, even at the highest concentrations tested (6.2%) (Table 3).

Table 1 Cultural characteristics of *Bacillus* sp. MA04 growing on nutrient broth

Variable culture	Time (h)		
	24	48	72
pH	6.83^{c}	7.20^{b}	7.65^{a}
$O.D._{600}$ nm	0.53^{ab}	0.56^{a}	0.45^{b}
Celular density (cells/mL)	8.4×10^{7a}	7.1×10^{7a}	8.1×10^{7a}
Biomass (g/L)	0.42^{b}	0.58^{a}	0.36^{c}
EI_{24} (%)	55.9^{a}	55.0^{a}	52.2^{a}
Crude biosurfactant production (mg/L)	43.71^{b}	51.44^{b}	145.58^{a}

The values are the average of three replicates; means with the same letter by rows are not significantly different by Tukey's test ($p < 0.05$)

Table 2 Inhibition *in vitro* of phytopathogenic fungi using supernatants of *Bacillus* sp. MA04 obtained at different times of culture

Fungal phytopathogen	Percentage inhibition (%)		
	24 h	48 h	72 h
P. expansum	46.8^{c}	78.6^{b}	84.8^{a}
F. stilboides	35.7^{b}	45.7^{a}	44.8^{a}
S. rolfsii	69.5^{b}	78.8^{ab}	85.3^{a}
R. solani	67.2^{a}	60.8^{a}	64.1^{a}

The values are the average of three replicates; means with the same letter by rows are not significantly different by Tukey's test ($p < 0.05$)

Table 3 Antifungal activity of bacterial crude biosurfactant at different concentrations

Fungal phytopathogen	Percentage inhibition (%)		
	Crude biosurfactant (mg/L)		
	100	200	500
Alternaria sp.	21.7^{c}	33.8^{b}	57.4^{a}
A. niger	81.8^{a}	85.0^{b}	91.1^{c}
Colletotrichum sp.	67.1^{c}	70.2^{b}	83.0^{a}
D. rosae	85.1^{c}	88.3^{b}	92.7^{a}
F. stilboides	69.1^{c}	80.8^{b}	83.7^{a}
P. capsici	2.8^{a}	4.7^{a}	6.2^{a}
P. expansum	62.4^{c}	79.1^{b}	97.1^{a}
R. solani	72.5^{b}	77.4^{a}	81.0^{a}
S. rolfsii	74.0_{b}	77.7^{b}	84.6^{a}

The values are the average of three replicates; means with the same letter by rows are not significantly different by Tukey's test ($p < 0.05$)

Table 4 Emulsifying properties of crude biosurfactant of *Bacillus* sp. MA04 at different concentrations

Conc. (mg/L)	IE_{24} (%)	ES_t (%)				
		24 h	48 h	72 h	96 h	120 h
100	33.9b[a]	72.4^{a}	70.0^{a}	66.6^{a}	66.4a	65.2a
200	36.2^{b}	76.9^{a}	76.5^{a}	69.2^{a}	67.7^{a}	67.2^{a}
500	51.4^{a}	77.5^{a}	77.0^{a}	75.8^{a}	75.7^{a}	74.3^{a}

The values are the average of three replicates; means with the same letter are not significantly different by Tukey's test ($p < 0.05$)

IE_{24} emulsifying activity, *ES* emulsion stability

Concerning physicochemical properties, CB presented a good ability to emulsify kerosene (aliphatic hydrocarbon) reaching EI_{24} of 51.4% at 500 mg/L, producing stable emulsions (ES_t) during 120 h (Table 4). Values of EI24 greater than 50% imply that entire hydrocarbon phase is emulsified [19]. Zhu et al. [11] performed similar assays using the CB from *Bacillus amyloliquefaciens* XZ-173, finding only at 500 mg/L a greater value of EI_{24} (63.4%) than that obtained by MA04 (51.4%), but all emulsions formed had a lower stability when compared with results of this study.

Chemical Nature of Biosurfactant

Spectroscopy analysis of CB by FT-IR spectrum (Fig. 1) showed the absorption bands related to the chemical bonds and functional groups. The bands at 3288 and 1639 cm^{-1} correspond to the stretching modes of N–H and CO–N bonds, respectively, which are characteristics of lipopeptides. Besides, the absorbance bands at 2924 and 2854 cm^{-1} reflect the presence of aliphatic chains ($–CH_2$, $–CH_3$) by C–H stretching. Finally, band at 1277 cm^{-1} corresponds to C–O stretching vibrations related to ester carbonyl absorption. All the above functional groups in the analysis suggest the presence of lipopeptides on the CB [11, 20].

The fractionation of CB by HPLC produced twelve fractions; the six the most abundant were then tested for antifungal activity against *Alternaria* sp., showing inhibition of mycelia growth those fractions identified as 3, 4 and

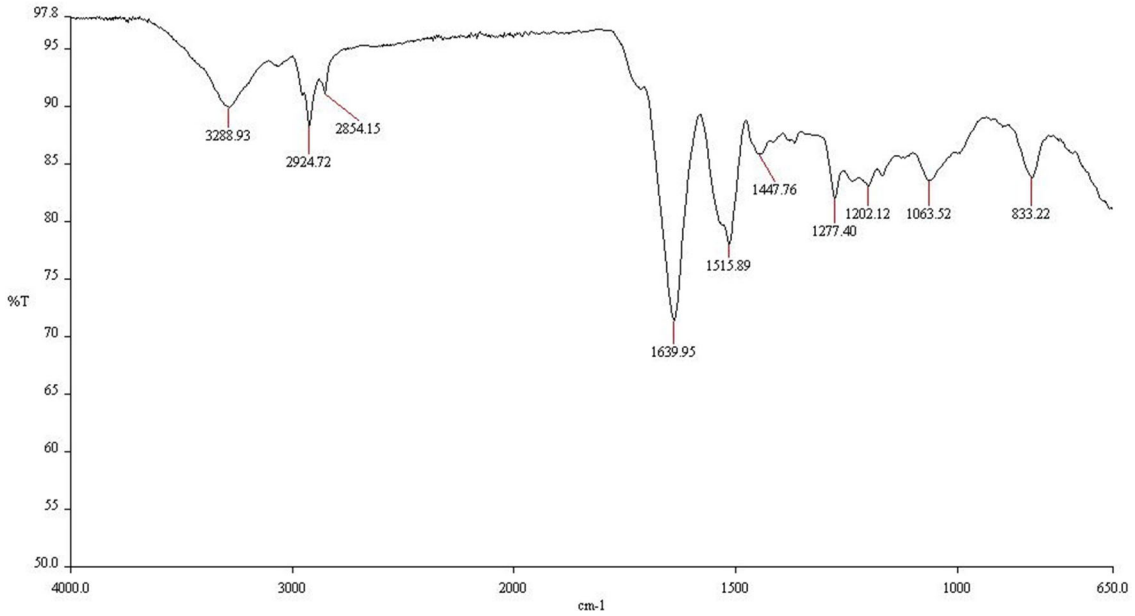

Fig. 1 FT-IR spectra of crude biosurfactant produced by *Bacillus* MA04

5 (Figure not shown); being further analyzed by MALDITOF/TOF MS. The resultants chromatograms showed molecular ion peaks from m/z 1397 to 1584 with different relative abundances (Table 5). The peaks at m/z 1449, 1463, 1477, 1491 and 1505 $[M + H]^+$ could correspondent to variants of protonated forms of fengycin type A or B (a class of lipopeptides), with mass differences of 14 Da (-CH2-), indicating the occurrence of fatty acids of chain lengths of C15, C16 and C17, respectively. The similarity of structure between the compounds of this family makes difficult their separation them by traditional HPLC methods, so that the fractions that exhibited antagonism could contain fengycin homologues [5, 21]. An alternative for this approach is the strategy developed by Yang et al. [22] using a three-stage linear gradient on HPLC phase reverse system.

A series of peaks at m/z 1471, 1485, 1499, 1513, 1515, 1527 and 1543, that differ in mass from the protonated forms of fengycins by 22 Da (Na^+) or 38 Da (K^+), suggest the formation of adducts of sodium o potassium, respectively, which is a common characteristic observed in the analysis of peptides by MALDI TOF as reported by Pathak and Keharia [23].

With respect to other families of lipopeptides, members of fengycin family particularly, have been reported for possessing the highest antifungal activity [4]. Bin et al. [24] described the production of fengicyns by *Bacillus subtilis* B-FS01, similar to found in our study, which, in in vitro test, showed high antifungal activity against *P. capsici*, *Fusarium moniliforme*, *Fusarium graminearum*, *Sclerotinia sclerotium* and *Colletotrichum musae*, pathogens of vegetables, ornamentals, cereals and fruits. In our study, this could explain the high antagonistic activity of *Bacillus* sp. MA04 found against almost all soil-borne and postharvest fungal pathogens tested.

In general, LP show a huge potential for agroindustry, not only for their antimicrobial properties, but also for their physicochemical property to produce stable emulsions, such as those found in the present study, which could be used to formulate organic and chemical molecules as fertilizers and pesticides. In soil bioremediation, LP can be also used to remove metals such as iron, lead, nickel, cadmium, copper, cobalt and zinc, and hydrocarbons through soil washing [25, 26]. In conclusion, the present study shows the ability of *Bacillus* sp. MA04 to produce fengycin-type lipopeptides that could contribute to enhance

Table 5 Main mass peaks of the fengycin obtained by MALDI TOF/TOF mass spectrometry of biosurfactant produced by *Bacillus* sp. MA04

Main mass peak (m/z)	Putative fengycin	Fraction
1449.7	C15$[M + H]^+$Ala C15 Fengycin A	3, 4
1463.7	C16$[M + H]^+$Ala C16 Fengycin A	3, 4, 5
1477.7	C17$[M + H]^+$Ala C17 Fengycin A	3, 4, 5
1491.9	C16$[M + H]^+$Val C16 Fengycin B	4, 5
1505.8	C17$[M + H]^+$Val C17 Fengycin B	5

its activity as plant growth promoter through the control of fungal phytopathogens.

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