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Isolation and Partial Characterization of Antifungal Metabolites Produced by *Bacillus* sp. IBA 33

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Abstract Antifungal proteins produced by *Bacillus* sp. IBA 33 were purified by ammonium sulfate precipitation and DEAE-Sephacel column chromatography. The two purified proteins inhibited the growth of *Geotrichum candidum*, the sour rot disease agent in lemon. The proteins were stable at 20 (3 months), 40, 60 and 100°C (30 min) and remained active after sterilization at 121°C for 15 min. Their hydrophobic nature was proved and when were developed with ninhydrin they did not show any free amino groups. The infrared spectrum showed vibrational modes corresponding to peptide, ester or ketone links and saturated CH links corresponding to long chain fatty acids. UV scan spectroscopy showed tyrosine and or tryptophan amino acids in their composition. The remarkable thermoresistance of proteins may be a good feature to be used in the development of a new biocontrol method of *Geotrichum candidum.*

Introduction

Postharvest disease biological control is advisable when lemons are in cold-storage chambers because the fruit is under controlled conditions of relative humidity, temperature, and gas concentration.

It has been suggested that the use of antimicrobially active species and strains of the genus *Bacillus*, or use of their metabolites, may be an alternative or supplementary method to chemical plant protection [9, 21]. Successful use of existing microorganisms to control plant disease will be enhanced by understanding molecular and biochemical biological control mechanisms.

Fungi are the chief causal agents of spoilage in fruit stored in cold chambers. Citrus fruits are affected by: *Penicillium digitatum*, *Penicillium italicum*, *Alternaria citri*, *Botrytis cinerea*, *Colletrotrichum gloeosporoides*, *Geotrichum candidum*, *Rhizopus stolonifer*, *Cladosporium herbarum*, *Alternaria alternata*, *Phitophtora citrophtora*, *Trichoderma viridae*, *Trichotecium roseum*, *Fusarium oxysporum*, *Fusarium solani*, *Sclerotinia sclerotium*, *Diplodia mutila*, *Phomopsis citri* [22]. The ones that cause the largest losses in the lemon industry are *P. digitatum* and *G. candidum*, green mold and sour rot agents, respectively.

Although several bacteria species are known for their antifungal activity, *Bacillus* sp., as an endospore producer, has an extreme tolerance to heat and desiccation [7, 12].

Production and involvement of antifungal protein by *Bacillus* sp. in the suppression of various diseases is not an uncommon phenomenon. These antifungal metabolites (AFM) are usually polypeptides composed entirely of aminoacids, but some may contain other residues. For example, fungicin M-4 produced by *B. licheniformis* M-4 is composed of 34 aminoacid residues of seven types [15], whereas rhizocticin A produced by *B. subtilis* ATCC 6633 is a phosphono-oligopeptide [14]. These antifungal peptides are either linear or cyclic [4, 14].

Bacillomycin D is a lipopeptide belonging to the iturin group of peptide antibiotics isolated from *B. subtilis* [3].

Bacillus polymixa, purified and characterized by Kavitha et al. [13], produces an antifungal protein against *Pyricularia grisea* and *Rhizoctonia solani*, which are rice pathogens.

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The present study reports on the purification and partial characterization of two metabolites produced by *Bacillus* sp. strain IBA 33 isolated from the soil of lemon plantations in Tucumán, Argentina.

Materials and Methods

Microorganisms

Bacillus sp. IBA 33 strain, producer of AFM, was isolated from the soil of lemon plantations [11], maintained in LB medium, transferred every 30 days and kept at 4°C.

Geotrichum candidum was the pathogen used. It was isolated from diseased lemons, maintained in PDA, transferred every 30 days and kept at 4°C.

Production of Metabolites from *Bacillus* IBA 33

100 ml of Landy medium (LM), composed of glutamic acid 5 g/l; glucose 20 g/l; $Fe_2(SO_4)_3{\cdot}H_2O$ 5 mg/l; $CuSO_4{\cdot}H_2O$ 0.16 mg/l; K_2HPO_4 0.5 g/l; KCl 0.25 g/l; $MgSO_4$ 0.25 g/l, pH 6 [6] were inoculated with 10 ml of the suspension of 10^{10} UFC ml^{-1} incubated at 28°C for 72 h with constant shaking in a rotary shaker at 250 rpm.

Isolation of Antifungal Metabolites

Metabolites were centrifuged from the culture at 6708*g* for 15 min. The supernatant was concentrated at 57% using 100 A° dialysis membranes and carboxymethylcellulose (crude extract).

The crude extract was sterilized at 121°C for 15 min.

Metabolite Purification

50 ml of the crude extract were precipitated with 40% $(NH_4)_2SO_4$ by slowly stirring at 5°C for 1 h. The precipitate was dissolved in distilled water and dialyzed using a cellulose membrane against distilled water. The insoluble residues were then removed by centrifugation at 9660*g* for 15 min.

The resulting soluble portion (with antifungal activity) was stored at 4°C for further purification. 5 ml were loaded onto a DEAE-Sephacel (1.5 cm × 15 cm) column previously equilibrated with 50 mM Tris–HCl buffer pH 7.5 [2] and protein monitored by measuring the absorbance at 280 nm using a Beckman DU 650 Spectrophotometer. The column was then eluted (2 ml fractions at a 0.5 ml/min flow rate) with a NaCl 0–0.4 M gradient in Tris–HCl buffer pH 7.5.

Inhibition tests were carried out at the end of each purification stage by the microdilution method [20].

Liquid–Liquid Extraction

In a 50-ml centrifuge tube, 5 ml of antifungal extract obtained in the previous step and 5 ml of isobutanol were shaken vigorously and centrifuged at 6708*g* for 15 min. The aqueous phase was discarded while the organic one was evaporated with gaseous nitrogen [16].

Inhibition tests were carried out by bioautographical methods [10].

Characterization of Antifungal Metabolites

Stability at Different Temperatures

Metabolites were stored either for 3 month at 20°C, or treated 30 min at 40, 60 and 100°C, or subjected to autoclaving for 15 min at 121°C. After allowing the heated samples to cool to 20°C, all metabolites samples were subjected to a bio-assay.

For control, the metabolites sample was filtrate using a cellulose nitrate filter pore 0.22 μm. The experiment was done in triplicate.

Thin Layer Chromatography (TLC)

Silica gel plates 60 F254 (Merck, 2 mm) were spotted and the run was carried out with a chloroform–methanol–acetic acid (40:4:1) mixture [1]. Plates were developed under UV light at 254 and 365 nm and later sprayed with ninhydrin and heated at 140°C to determine the presence of free amino groups.

Agar Layer Bioautography

50 ml of liquid PDA medium were inoculated with a 2.5 ml *G. candidum* (10^6 spores ml^{-1}) spore suspension. The mixture was spread on a silica gel plate previously spotted with 20 μl metabolites. Once it was solidified into a thin gel it was incubated at 28°C for 24 h. Plates were developed by spraying with 2,3,5 triphenyltetrazolium and incubated for 24 h at 28°C [10].

FT-IR Studies

The FT-IR spectrum of the AFM in D_2O, at a concentration of 1 mg/ml was recorded on a NICOLET 5700 Spectrometer at room temperature. Samples were placed in a liquid cell assembled with CaF_2 windows and 0.056 mm lead spacers. The spectrum was taken with a resolution of 2 cm^{-1}.

UV Spectroscopy

The samples were resuspended in Hepes buffer 10 mM NaCl 100 mM pH 7.1 to carry out absorption spectra between 250 and 280 nm wavelengths. A Beckman DU 7500 spectrophotometer was used.

Antifungal Activity

45 ml of potato dextrose broth pH 5; 2.5 ml spore suspension of *G. candidum* (10^6 spores ml^{-1}) and 2.5 ml of the crude extract were placed in 250-ml Erlenmeyer flasks. Flasks were incubated at 28°C and 250 rpm for 5 days. Dry weight determinations were performed and the results expressed as percentage of inhibition growth. Control assays were also made in the same way, but did not contain the crude extract.

Minimum Inhibitory Concentration (MIC) Determination

Metabolite dilutions M1 155 µg prot/ml (tubes 4–15 from DEAE-Sephacel), M2 390 µg prot/ml (tubes 29–43 from DEAE Sepahacel) were made with sterile distilled water (1/2; 1/4; 1/8; 1/16; 1/32; 1/64; 1/128 and 1/256). On a sterile microplate, 50 µl of potato broth with glucose (10 g/l), 50 µl *G. candidum* (10^6 spores ml^{-1}) and 100 µl of the respective dilutions were incubated for 12 h at 28°C. 100 µl were drawn out of each well, disseminated in Petri dishes with APG and incubated for 24 h at 28°C [20].

Results and Discussion

The *Bacillus* strain IBA 33 used in this study showed antagonistic activity against different lemon pathogenic fungi and saprophytes. *G. candidum* was chosen for the inhibition assays because it is one the most harmful post-harvest pathogens [11].

Bacillus metabolites proved to be stable at different temperatures. No activity loss was observed in the samples stored for 3 month at 20°C, or incubated for 30 min at 40, 60 or 100°C. Activity of the metabolites sample was not affected for autoclaving 15 min at 121°C.

After purification, the chromatogram showed two inhibition peaks in a DEAE-Sephacel column (Fig. 1). The first one, between tubes 4 and 15 (M1), while the second peak, between tubes 29 and 43 (M2), exhibited 77 and 64% inhibition against *G. candidum*, respectively.

Our results are somewhat different from those obtained by Bechard et al. [2] because they got only one antifungal activity peak. Phister et al. [19] reported a *Bacillus* sp.

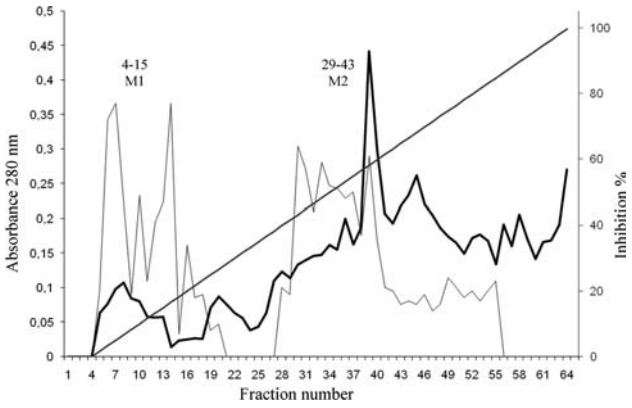

Fig. 1 *Bacillus* sp. IBA 33 AFM purification step by DEAE-Sephacel chromatography. Experimental conditions are described under "Materials and Methods". *Dark line*: protein (absorbance at 280 nm), *light line*: AFM (% inhibition)

CS93 strain basilysin, iturin A and chlorotetaine producer (lipopeptides with antifungal activity).

When the liquid–liquid extraction was carried out, antifungal activity was detected in the organic phase; hence its hydrophobic nature was assumed. Similar results were obtained by Cho et al. [5]. TLC plates were developed by bioautography (Fig. 2). The growth inhibition halo of *G. candidum* observed in contact with M1 was 1 cm, whereas M2 was 3 mm.

After performing the run of samples spotted in TLC in the chloroform, methanol and acetic acid mixture and when they were exposed to UV light, only one spot of Rf 0.67 for M1 and M2 was detected. Hence, it may be assumed that both samples have the same polarity. The inhibitory

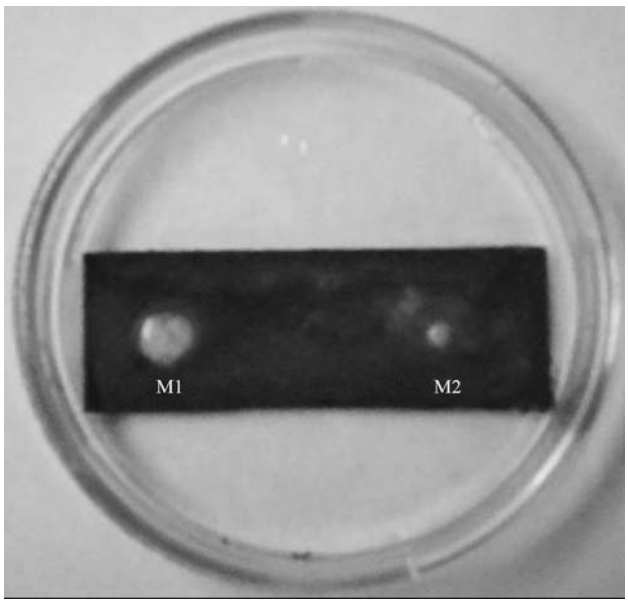

Fig. 2 Inhibition test carried out by bioautographical method. Left: M1; Rigth: M2

activity of the spots was confirmed after TLC by bioautographic assay. Besides the TLC plates were developed with ninhydrin and no spots were observed. Thus, a peptide without free amino groups (cyclic structure) may be presumed.

The AFM infrared spectra are shown in Fig. 3 and three regions were observed. The one at 1650 cm^{-1} assigned to the vibrational amida I mode which shows the peptide link; another at 1710–1740 cm^{-1} characteristic of carbonyl groups in esther or ketone groups (Fig. 3a) and another at 2850–2950 cm^{-1} bands corresponding to saturated CH links assigned to long chain fatty acids (Fig. 3b).

Besson et al. [3], Eschita et al. [8], and Peypoux et al. [18] reported that *Bacillus subtilis* is the producer of cyclic lipopeptides, i.e., molecules of seven amino acids with a β-fatty acid [23]. We obtained the same absorption bands for both samples. Hence, our strain might be a producer of cyclic lipopeptides with antifungal activity belonging to the iturin family. When the wavelength scanning was performed between 250 and 380 nm, we found absorbance maximums at 280 nm for both samples (Fig. 4a, b). Thus, we might infer that the AFM produced by *Bacillus* sp. IBA 33 have tyrosine or tryptophan or both in their composition [17]. Bechard et al. [2] reported an absorbance maximum between 210 and 230 nm which they thought was due to the presence of tyrosine. However, they later discovered that it was a residue of α-aminoacid 4 hydroxyphenilacetic which is structurally similar to tyrosine.

The CIM for M1 (155 μg prot/ml) corresponded to a 1/64 dilution, while for M2 (390 μg prot/ml) the dilution was 1/32. These results coincide with those obtained in the whole study where M1 always has higher inhibition values than M2.

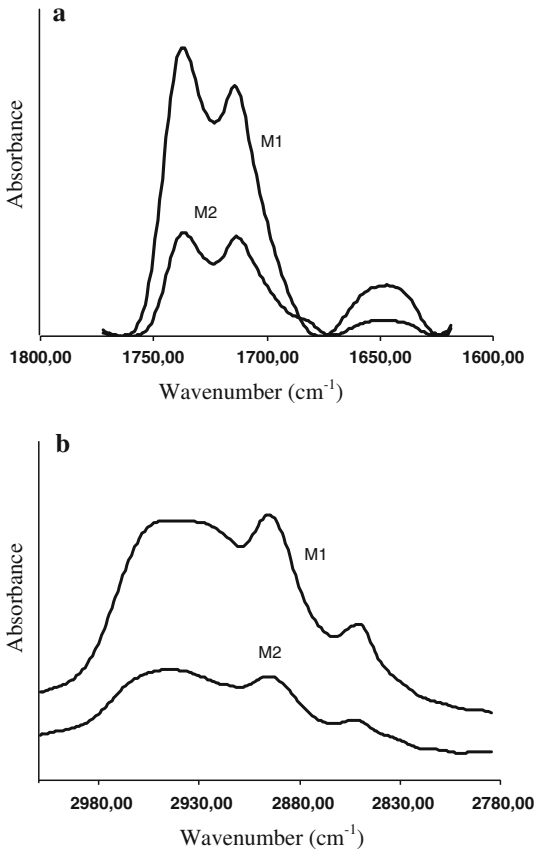

Fig. 3 **a** Amida I region of FT-IR spectrum (1650 cm^{-1}) of the AFM at a concentration of 1 mg/ml in D_2O. Carbonyl groups in ester or ketone groups region (1710–1740 cm^{-1}). **b** Saturated CH links region of FT-IR spectrum (2850–2950 cm^{-1})

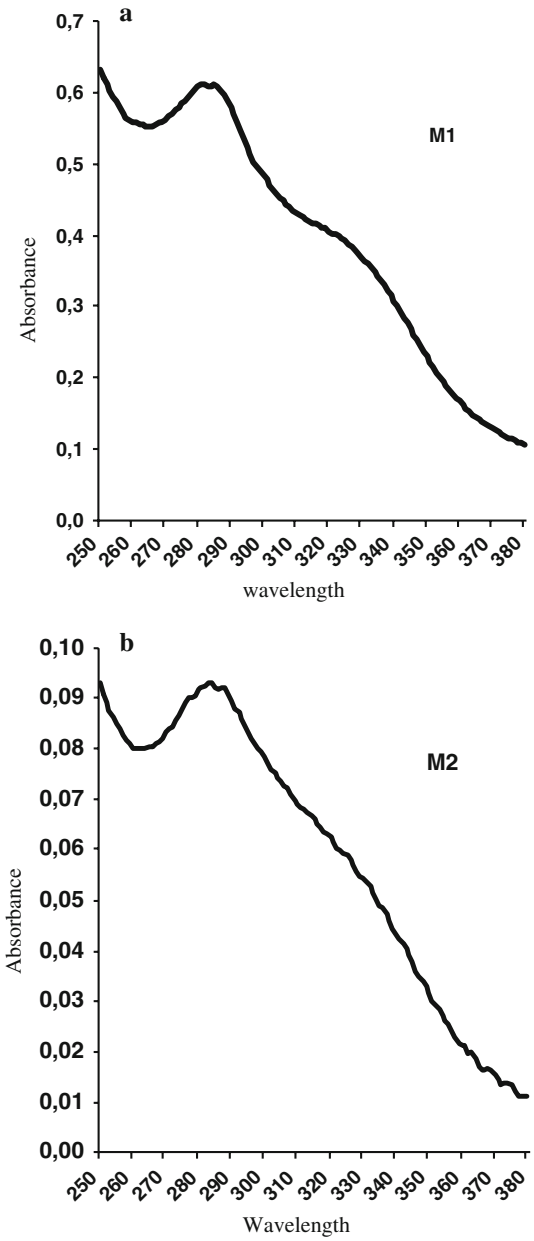

Fig. 4 **a** UV absortion spectra between 250 and 280 nm wavelengths (M1). **b** UV absorption spectra between 250 and 280 nm wavelengths (M2)

We showed evidences of potential AFM in the *Bacillus* sp. strain IBA 33 studied, which is active against sour rot, as was demonstrated in our previous work [11]; and in this study we described the isolation and partial characterization of them which is necessary to further characterize these new fungicides.

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