Inhibition of Toxigenic *Aspergillus niger* by Microbial Metabolites

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The aim of this study was to assess the potential of microbial antagonists to control the growth and mycotoxin production by toxigenic fungal strain Aspergillus niger. Mycotoxins are secondary metabolites that produce acute and chronic effects on human and animal health. Among the mycotoxins of great interest produced by Aspergillus are ochratoxins and aflatoxins. Ochratoxin is considered to be nephrotoxic, teratogenic, immunotoxic, and probable human carcinogen. From this group, ochratoxin A is found in wheat, corn, grapes, raisins, wines and wine vinegars, cheese and meat products of animals fed with contaminated grains. Six microbial strains, two Bacillus, one Trichoderma and three Pseudomonas were evaluated for their potential as biocontrol agents. The antifungal activity of extracellular compounds released by bacterial strains was investigated by dry matter determination, microscopic observations, and enzyme linked immunosorbent assay (ELISA). Out of tested strains, B. amyloliquefaciens 1014 cultures produces metabolites with higher antifungal activity against A.niger. The active extracellular metabolites were partial characterized as referring to thermal stability and their production was optimized by varying the composition of the culture medium. Our results revealed that the metabolites from B. amyloliquefacines 1014 are thermostable and they are able to conserve the antifungal activity after sterilization.

Keywords : Aspergillus niger, Bacillus sp., biocontrol, metabolites, antifungal activity

Fungal phytopathogens cause serious problems in the cultivation of economically important plants [1-4]. The use of chemical compounds has failed to control plant diseases due to resistance, environment pollution, and damage to human health. The application of microorganisms for pathogen control is considered a viable disease control technology that can overcome these disadvantages. Biocontrol method has great relevance for the modern and echo-compatible agriculture. There are many microorganisms such as *Pseudomonas* [5-7], *Trichoderma* [8-10], *Rhodotorula* [11-13] and *Bacillus* [14-17] with antagonistic activity against phytopathogenic fungi. *Aspergillus* genera considered to be the most important toxigenic fungi are characterized by a fast growth, *p*H tolerance, and high abundance in many environments. Some *Aspergilli* are capable to secrete mycotoxins, toxic secondary metabolites that produce acute and chronic effects on human and animal health [18]. Among the mycotoxins of great interest produced by *Aspergillus* are aflatoxins and ochratoxins.

The aim of this study was to find microbial strains producing extracellular metabolites able to inhibit the fungal growth and ochratoxin A (OA) production by an *Aspergillus niger* strain. The culture conditions such as time of cultivation and composition of culture medium were also studied to obtain bacterial metabolites with higher antifungal activity. A special emphasis was put on the thermal stability of metabolites, important feature for possible conditioning processes.

Experimental part

Materials and methods

Fungal pathogen strains and culture conditions

The target strain as pathogen agent was *Aspergillus niger* 105 from Microbial Collection of ICECHIM. The strain was grown on potato dextrose agar (PDA) incubated at 28° C for 7 days.

Microbial strains as biocontrol agents

The strains selected as potential biocontrol agents were: *Bacillus subtilis 1016, B. amyloliquefaciens 1014* and *Trichoderma sp. 36* from Microbial Collection of INCDCP-ICECHIM); *B. licheniformis W7, Pseudomonas aeruginosa 342, P. aeruginosa ST1, P. fluorescens DP1* from Microbial Collection of Institute of Biology. The strains were maintained on PDA slants and stored at 4° C. The inoculum of *Trichoderma sp*. was prepared by growing on PDA slants, at 28° C for 7 days. Bacteria were growing in Agar Nutrients Slants tubes at 28^0 C for 24 h.

Culture medium and growth conditions for the production of antifungal metabolites

The microorganisms as biocontrol agents were grown on Sabouraud liquid medium [14], at 200 rpm, 28^{0}C for 24 hours. To study the growth and production of antifungal metabolites, 10 mL of bacterial suspension was inoculated in Erlenmayer flasks with 90 mL Sabouraud medium and incubated for 6 days at 28° C and 200 rpm. At regular time intervals, an aliquot of the bacterial culture was taken to

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Microbial strain	OD_{560nm}	Inhibition zone (mm)*
Pseudomonas aeruginosa 342	1.386	16.5
Pseudomonas aeruginosa ST1	2.105	18.0
Bacillus licheniformis W7	0.802	17.5
Bacillus subtilis 1016	1.538	18.5
Pseudomonas fluorescens DP1	1.191	17.0
Trichoderma sp. 36	0.654	19.5
B. amyloliquefaciens 1014	2.175	20.5

*results of three experiments

Table 1
GROWTH INHIBITION OF *ASPERGILLUS NIGER* BY METABOLITES OF ANTAGONISTIC MICROORGANISMS

determine the optical density ($OD_{560\ nm}$) on a spectrophotometer BioMate (Thermo Electron Scientific Instruments Corp., USA). The antifungal capacity of the supernatants containing metabolites were tested *in vitro* assay against *A. niger* grown on Sabouraud solid media. The antifungal activity of the metabolites from bacterial strains cultivated on four liquid media (i) Luria Bertani (LB) [19], (ii) OM medium [20], (iii) peptone-potato-dextrose (PPD) [21] and (iv) Sabouraud medium was also evaluated. After cultivation, the liquid cultures were centrifuged (4000 rpm, 4°C) and tested for the antifungal activity using *in vitro* assay on solid and liquid media.

Antifungal activity assay

The ability of the strains to inhibit the growth of fungal pathogen was tested in Petri plates by dual culture technique. The Sabouraud solid medium in Petri plates was inoculated with fungal culture of *A. niger*. The composition of solid Sabouraud medium was the same, including agar (15 g/L). Then, a paper disc (50 mm diameter) was placed into the center of the plate and was incubated with 1 mL ($1x10^6$ spores/mL) of supernatant from each biocontrol agent tested. The plates were incubated at 28°C and the colony diameter was measured daily. The tests were performed in triplicates. The inhibition halos measured were considered as antimicrobial activity (mm). The bacterial culture broth of different ages from *B. amyloliquefaciens* was centrifuged and used for testing the antifungal properties in Petri plates, incubated at 28°C, for 96 h. The antifungal activity was determined also in Sabouraud liquid medium by incubating mixed cultures of *A. niger 105* and *B. amyloliquefacines*, for 72 h, at 150 rpm and 28°C. In broth samples, dry weight determinations were performed and the results expressed as percentage of inhibition growth. The weight of mycelium was expressed in g/100 mL. The supernatants were assayed for mycotoxins content.

Heat stability

Metabolite supernatants, non-sterilized or sterilized at 110° C, for 30 min. were inoculated with fungal suspension (150 µL) of *Aspergillus niger* 105. The antifungal activity was evaluated by dry weight of fungal biomass and ochratoxin content.

Microscopic observations

The effect of biocontrol action against fungal growth was observed using optic microscope Olympus BX 51 (40 X photos) (Olympus, Japan).

Mycotoxins determination

The ochratoxin A content in the supernatants obtained from bacterial culture was determined by competitive enzyme linked immunosorbent assay (ELISA) [22] using RIDA SCREEN kit (R-Biopharm, Darmstadt, Germany). The mycotoxin assay respects the kit instructions.

Results and discussions

Antagonistic strains produce various secondary metabolites which can play a role in the mechanism of their biological activity. The antifungal activity of extracellular metabolites against fungal pathogen was evaluated by several methods, including measurement of dry matter, visual and microscopic observations, and mycotoxins (ochratoxin) content determination. The thermal stability of the extracellular metabolites was also evaluated.

Ochratoxin A (OA) has been shown to have nephrotoxic, immunotoxic, genotoxic and teratogenic properties and it is a possible human carcinogen. The mycotoxin contaminates cereals and also it has been detected in a large variety of foods such as coffee, beer and wine. Different species of *Aspergillus* are known to be producers of such secondary metabolites. Since *Aspergillus niger* is used in food industry, a special attention was focused on the inhibition of mycotoxins production [23-24].

In a previous paper, several strains were tested for the antimicrobial activity using agar diffusion technique [25]. As a result of the screening, Bacillus subtilis 1016, *B. amyloliquefaciens 1014, B. licheniformis W7, Pseudomonas aeruginosa 342, P. aeruginosa ST1, P. fluorescens DP1 and Trichoderma sp. 36* were selected as microorganisms which produce extracellular metabolites with antifungal activity. The microbial strains were cultivated on Sabouraud medium which contain the adequate nutrients to obtain rich microbial cultures. The bacterial cell growth ($OD_{560\ nm}$) and antifungal activity of each microbial culture (size of the inhibition zone) are presented in table 1.

The larger diameter of inhibition zone reached 20.5 mm for metabolites released by *B. amyloliquefacines 1014,* a strain found to produce metabolites with higher antifungal activity. The diameter decreases in following order: *Trichoderma sp. 36, Bacillus subtilis 1016, Pseudomonas aeruginosa ST1, Bacillus licheniformis W7, Pseudomonas fluorescens DP1* and *Pseudomonas aeruginosa 342.* Based on these results, *B. amyloliquefacines 1014* was selected for further experiments performed in order to obtain extracellular metabolites with improved antifungal activity against *A. niger*.

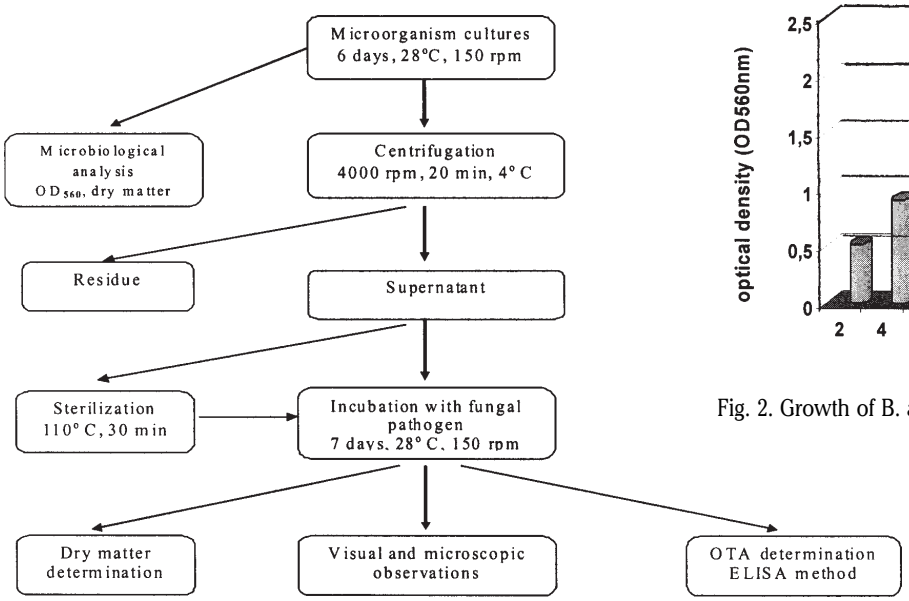


Fig. 1. Experimental protocol used to obtain and characterize bacterial metabolites

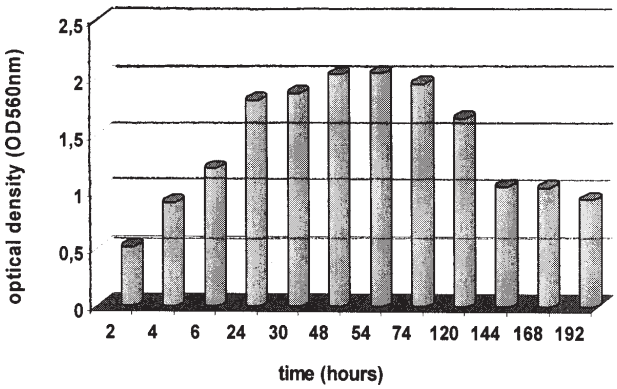


Fig. 2. Growth of B. amyloliquefaciens 1014 on Sabouraud liquid medium

The flow chart of the experiment performed to obtain and characterize the extracellular metabolites from *B. amyloliquefacines* 1014 is shown in figure 1.

B. amyloliquefacines 1014 strain was investigated to establish the optimum conditions for producing antifungal metabolites. *Bacillus* culture reached its maximum growth, measured in terms of optical density, after 48-54 h of incubation on Sabouraud medium (fig. 2).

In each culture sample collected after 2 to 192 h of incubation, the antifungal activity was determined using dual culture technique (fig. 3.). After 5 days of incubation, the fungal hyphae reached the bacterial culture and the inhibition zone was established. As shown in figure 3, the most active metabolites against fungitoxic strain are produced within 96 to 120 h of *B. amyloliquefacines* cultivation.

The visual observations of the dual cultures, grown on solid medium in Petri plates revealed the antagonism between *B. amyloliquefaciens – A. niger* (fig.4). The inhibitory effect induced by bacterial metabolites against the growth of *A.niger* was maxim within 96 to 120 h of incubation. Thus, after reaching the maxim inhibition diameter of 19.5 mm, the antifungal effect decreased with the extension of incubation period.

In the next step, to improve the antifungal activity of the bacterial strain different composition of the bacterial culture medium was used. The chosen media have different nutrients content, from Sabouraud medium with relative simple composition to OM medium supplemented with vitamins and growth factors.

Efficiency of antifungal capacity of extracellular metabolites released by bacterial strain grown on the four media was evaluated after incubation with *A. niger* in liquid and solid medium. As revealed the results depicted in figure 6, the highest inhibition effect was obtained with the metabolites obtained from *B. amyloliquefacines* 1014 cultured for 120 h on a rich culture medium.

The finding that extracellular metabolites from culture filtrates of *Bacillus amyloliquefaciens* 1014 inhibited the growth of pathogenic fungi led to the investigation of the effects caused on the morphology of *A. niger* hyphae. As observed in figure 7, the morphology of *A. niger* hyphae from the contact zone between biocontrol agent and toxigenic strain indicated several modifications. Thus,

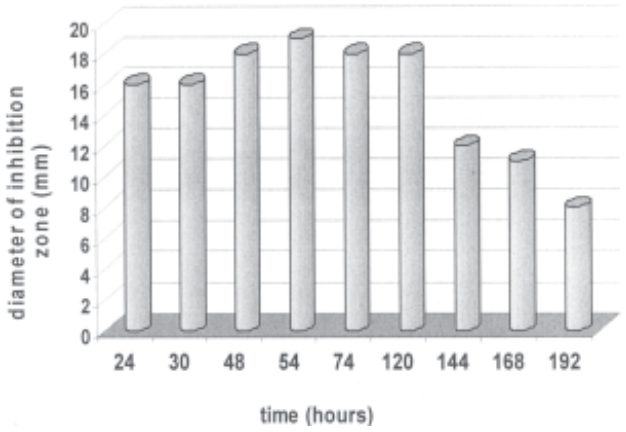


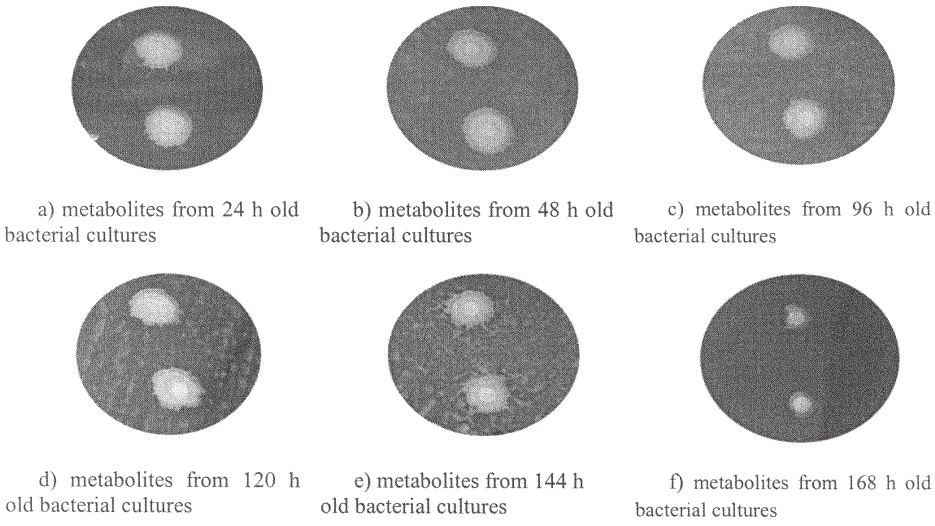
Fig. 3. Effect of B. amyloliquefaciens 1014 culture age on the growth of A. niger

hyphal tips of the fungus became deformed and hyphae thickened and vacuolar compared with hyphae from the outside area of inhibition. Many swellings occurred in the hyphae or at the tips of hyphal strands, whereas normal hyphal walls were smooth with no swellings or vacuolation.

The microscopic observations are similar to those reported by other studies indicating modifications of fungal hyphal morphology induced by the crude extracellular metabolites. Hyphal swelling and the inhibition of spore germination have been shown at the contact between antifungal metabolites of *Streptomyces violaceusniger* and *Fusarium oxysporum* [26]. The culture filtrate of *Pseudomonas aeruginosa* K-187 was found to cause growth aberration, hyphal swelling, and lysis of many fungi due to its high content of chitinase enzyme [27]. The lysis and dissolution of fungal mycelium of *Curvularia lunata* by metabolites from *Bacillus* sp. strain BC121 were also reported [28].

The relation between thermal stability of antifungal metabolites and fungal growth and ochratoxin inhibition was next investigated. The results are presented in table 2.

The percentage of inhibition of *A. niger* grown in presence of non diluted metabolites released from *B. amyloliquefacines 1014* cultured on OM medium was not significant affected by thermal stress. The same behaviour was recorded for ochratoxin inhibition. In the case of



a) metabolites from 24 h old bacterial cultures

b) metabolites from 48 h old bacterial cultures

c) metabolites from 96 h old bacterial cultures

d) metabolites from 120 h old bacterial cultures

e) metabolites from 144 h old bacterial cultures

f) metabolites from 168 h old bacterial cultures

Fig. 4. Antagonism between *B. amyloliquefaciens* 1014 and *A. niger*

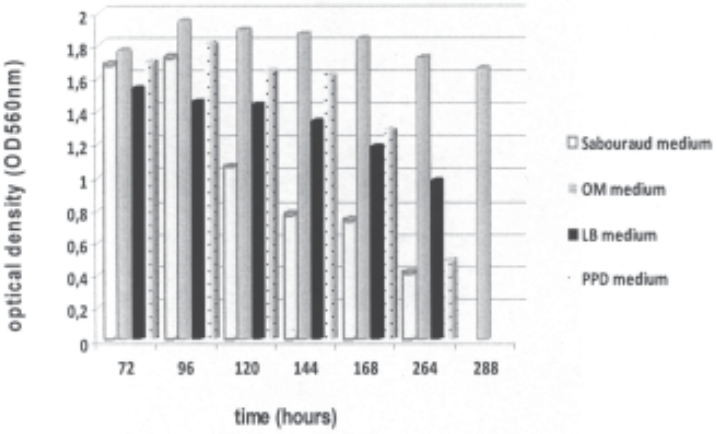


Fig. 5. Growth of B. amyloliquefaciens 1014 on different nutrient media

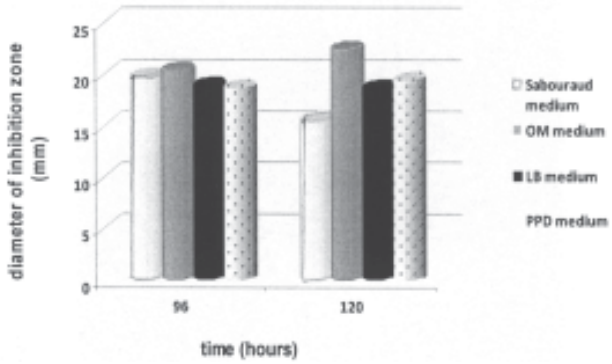


Fig. 6. Effect of medium composition on antifungal activity of bacterial metabolites

metabolites 50% diluted, the heat treatment causes the decrease of fungal biomass amount from 89.0 to 85.1 %, while the inhibition of mycotoxin secretion is decreasing form 88 to 83.0 %.

All together, our data demonstrate that the metabolites produced by *B. amyloliquefacines* are thermostable and able to conserve the antifungal activity after sterilization. These results are in agreement with other studies performed with microbial metabolites secreted by a *Bacillus polymyxa* strain [29], and *Bacillus* strain isolated from the soil of a lemon plantation [30]. Finally, *B. amyloliquefaciens* 1014 constitutes a promising biocontrol agent which produces persistent antifungal extracellular metabolites against toxigenic *A. niger*.

Our future vision will be the use of the *B. amyloliquefaciens* 1014 strain under greenhouse and field conditions to study its effect in reducing the effect of others pathogens. Also, it will be of interest the entrapment of bioactive metabolites to offer protection from environmental exposure and resultant chemical and biological degradation [31].

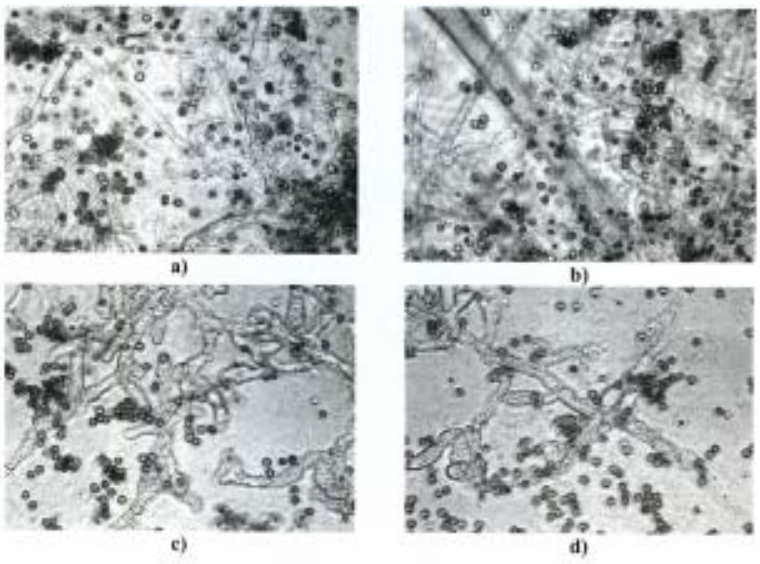


Fig. 7. Hyphal morphology of *Aspergillus niger* in the presence of metabolites from *B. amyloliquefaciens 1014*. a) and b) normal fungal hyphae from outside region of interaction; c) and d) occurrence of vacuoles, bubbles and swelling in hyphae from region of interaction between biocontrol agent and phytopathogen

Supernatant processing	Supernatant concentration (%)	Fungal biomass decrease (%; w/w)	Ochratoxin inhibition (%)
Non treated	100	91.8	93,3
Heat treated	100	89.5	92.5
Non treated	50	89.0	88.0
Heat treated	50	85.1	83.0

Table 2
INFLUENCE OF HEAT TREATMENT UPON FUNGAL PATHOGEN GROWTH AND MYCOTOXIN PRODUCTION DETERMINED BY THE METABOLITES FROM *B. AMYLOLIQUEFACIENS 1014*

All data are the mean of at least three independent experiments showing consistent results.

Conclusions

The results showed that *B. amyloliquefaciens* 1014 produced an inhibitory effect on growth and mycotoxin production by toxigenic *A. niger*. The heat treatment does not affect the antifungal activity of the bacterial metabolites exhibited through inhibition of fungal growth and mycotoxin production. These characteristics make *B. amyloliquefaciens* 1014 strain a good candidate to be exploited for its future use as a biocontrol agent against plant diseases. It is expected that the replacement of traditional chemical to control plant disease by the use of microorganisms and their active metabolites could reduce the environmental impact of agricultural productions.

Acknowledgments: A part of these scientific works were financial supported through projects PN.09.09.01.09 and COST Action FA 1105. The authors are gratefully to dr. Anca Voicu from Institute of Biology for the scientific assistance.

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Manuscript received: 11.12.2013