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ORIGINAL ARTICLE

Active metabolites produced by *Penicillium chrysogenum* IFL1 growing on agro-industrial residues

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Abstract Microbial extracts continue to be a productive source of new molecules with biotechnological importance. Fungi of the genus *Penicillium* are known to produce biologically active secondary metabolites. The goal of this work is verify the production of antimicrobial metabolites by *Penicillium chrysogenum* IFL1 using agro-industrial residues. *P. chrysogenum* IFL1 produced active metabolites growing on the agro-industrial residues, grape waste and cheese whey. The 7-day cultures showed antimicrobial activities against bacteria, fungi and amoebae. The filtrate of the cheese whey culture inhibited the growth of the bacteria *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa*, the fungus *Fusarium oxysporum* and the amoeba *Acanthamoeba polyphaga*. Due to the greater antimicrobial activity of the cheese whey culture, a footprinting profile was carried out using the ESI-MS and ESI-MS/MS techniques. The presence of penicillin G and other metabolites that have antimicrobial activity such as penicillin V and rugulosin can be suggested. *P. chrysogenum* IFL1 was able to produce a wide variety of antimicrobial compounds on agro-industrial residues, which makes the process ecologically friendly.

Keywords *Penicillium chrysogenum* · Agro-industrial residues · Secondary metabolites · Antimicrobial activity · ESI-MS

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Introduction

Infectious diseases caused by bacteria, viruses, parasites and fungi are among the leading causes of death (WHO 2012). Resistance to antibiotics has led to the emergence of new and the reemergence of old infectious diseases (Song 2003; Hemaiswarya et al. 2008). Food-borne illnesses, resulting from the consumption of contaminated foods, have been of great concern for public health, although there is an increased consumer concern and a demand for natural and minimally processed food (Serra et al. 2008; Cleveland et al. 2011). In addition, infectious diseases have led to important economic losses worldwide in agriculture. The intensive use of chemicals has led to the emergence of frequent problem of resistant microbial pathogens and has also caused serious problems affecting not only human health but also the quality of the environment (Pérez-Garcia et al. 2011).

Food, agrochemical and pharmaceutical industries exploit the biosynthetic capabilities of filamentous fungi to produce secondary metabolites, mainly antimicrobial metabolites. The success of filamentous fungi for the industrial production of biotechnological products is largely due to the metabolic versatility of these microorganisms (Goodacre et al. 1995; El-Enshasy 2007; Pimenta et al. 2010). A large number of

fungal extracts and/or extracellular products have antimicrobial activity, mainly related to *Aspergillus* and *Penicillium* species. Since the discovery of penicillin, the micromycetes have been famous as producers of secondary metabolites with biological activity, including antibacterial, antifungal, immunosuppressant, cholesterol-lowering agents and mycotoxins (Rancic et al. 2006; Petit et al. 2009).

Agro-industrial residues can be used as a natural bioresource for the production of bioactive compounds, because they are inexpensive and abundant and their use may result in environmental and economic benefits (Nigam 2009; Nigam et al. 2009). In this context, the objective of this work was to verify the production of antimicrobial metabolites against bacteria, fungi and amoebae by the fungi *Penicillium chrysogenum* IFL1 using agro-industrial residues as the sole source of nutrients and to analyze the secondary metabolites produced using mass spectrometry (MS).

Materials and methods

Microorganism

The fungus *P. chrysogenum* IFL1 belongs to the collection of the Laboratory of Biochemistry and Applied Microbiology (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil). This isolate was identified by sequencing the ITS region rDNA (GenBank JQ614063) and part of the β-tubulin gene (GenBank JQ614055). The fungus was maintained on Potato Dextrose Agar (PDA) slants covered with mineral oil at 4°C and periodically subcultured.

Culture media and growth conditions

The dried cheese whey powder was obtained from Parmalat (Porto Alegre, Brazil) and the grape waste was from a wine producer of southern Brazil (Vinícola Valmarino, Bento Gonçalves, Brazil). The conidia suspension was performed according to Lopes et al. (2011).

The fungus was cultivated in Erlenmeyer flasks (250 mL) containing 50 mL of medium (10 g L^{-1} of either cheese whey or grape waste). The growth conditions were 7 days at 30 °C and 120 rpm. The samples were filtered through Whatman no. 1 filter paper. The filtrates were lyophilized and then resuspended in distilled water to obtain a 100 mg mL^{-1} suspension. Samples were sterilized through 0.22-μm filters prior to the analysis of biological activities.

Antibacterial activity

The antibacterial activity was determined using the agar diffusion method according to Motta and Brandelli (2002) with minor modifications. The bacteria tested were *Salmonella typhimurium* ATCC 14078, *Listeria monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 9801 and *Pseudomonas aeruginosa* (environmental isolate).

The minimum inhibitory concentration (MIC) was determined using the concentrations of 50, 25, 12.5, 6.25, 3.13 and 1.56 mg mL^{-1} of the filtrates, and aliquots of each dilution were applied on the plates. Both culture media were used as controls, and experiments were performed in triplicate.

Antifungal activity

The filamentous fungi *Fusarium oxysporum* f. sp. *lycopersici* (environmental isolate), *Aspergillus fumigatus* (environmental isolate), *Aspergillus flavus* (food isolate), *Penicillium expansum* (food isolate) and the yeast *Candida tropicalis* (clinical isolate) were used. The fungi were cultivated on PDA dishes for 5 days at 30 °C. After the conidia suspension was prepared, as previously mentioned for *P. chrysogenum*, it was added to the PDA at 50°C in an enough volume to provide a final concentration of 10^6 conidia mL^{-1}. The medium was poured on plates, and after the solidification, 15 μL of the filtrates were added. The plates were incubated at 30 °C for 48 h and subsequently observed for inhibitory activity against the fungal indicator (adapted from Rouse et al. 2008). Activity against *C. tropicalis* and the MIC were developed by similar protocols described for the antibacterial activity. The filtrates of the culture medium were also used as negative control and the experiments were performed in triplicate.

The effect of the filtrates on the spore germination according to Becker-Ritt et al. (2007) was determined. Approximately 10^6 spores were suspended in 15 μL of the filtrates, incubated for 2 h at 28 °C, and then inoculated onto the PDA-containing Petri dishes. The plates were incubated for 48 h at 30 °C. The effect of the filtrates was assessed by visual comparison of the mycelial development with the control group.

Amoebicidal activity

The amoebicidal activity was determined according to Ródio et al. (2008). Concentrations of 50, 25, 10, 5, 2.5 and 1 mg mL^{-1} of the filtrates were tested against the strain *Acanthamoeba polyphaga* ATCC 30461. For the assessment of amoebicidal activity, 100 μL of *A. polyphaga* (8×10^3 trophozoites mL^{-1}) cultures and 100 μL of each test solution were inoculated into a 96-well plate. The plate was sealed and incubated at 30°C, monitored by means of an inverted microscope, and counted in a Fuchs-Rosenthal counting chamber after 24, 48 and 72 h. The controls used were sterile water and culture media. The experiments were performed in triplicate.

Extraction of metabolites

The extraction was performed according to Senyuva et al. (2008) with modifications. The lyophilized filtrates were extracted twice in 3 mL acetonitrile and 2 mL 1% formic acid in ethyl acetate and ultrasonicated for 10 min. The solvents were evaporated, and the residue was suspended in 1% (v/v) formic acid aqueous solution and passed through a 0.2-μm disposable filter before further analysis.

TOF-MS analysis

A Waters Q-TOF micro was used to determine the mass profile of the cheese whey extract. The instrument is equipped with a nano-electrospray ionization (nano-ESI) source. Data were analyzed with the Waters MassLynx software. MS spectra were processed using background subtract followed by smoothing the spectrum with Savitzky-Golay smoothing, and measuring the peak top with a centroid top of 80%. The direct injection with 1 μL min^{-1} flow was performed. The conditions of ESI MS and ESI MS/MS were according to Senyuva et al. (2008). The standard Penicillin G (PenG) with purity ≥98.0% (Sigma-Aldrich, St. Louis, MO, USA) was used to confirm the presence of this metabolite in the extract.

Aqueous biphasic separation and analysis of Penicillin G

Aliquots of 2 mL culture filtrates were mixed with 4 mL of 50% (w/v) ammonium sulfate, and then 4 mL of 50% (w/v) polyethylene glycol (PEG) solution was cautiously added. The mixture was homogenized in vortex by 15 s and then centrifuged at 3,000 *g* at 4 °C for 10 min. The PEG upper phase containing PenG was collected (Bi et al. 2009) and analyzed by high-performance liquid chromatography (HPLC). The analyses were carried out in a HPLC unit (Agilent, Waldbronn, Germany), equipped with a degasser, quaternary solvent pump, UV/vis detector and ChemStation database acquisition system. Samples (10 μL) were applied to a 250 mm×4.6 mm i.d., 5 μm, C18 polymeric column (Vydac, Southborough, MA, USA), eluted with 50 mM phosphoric acid: methanol (40:60, v/v) at a flow rate of 1 mL min^{-1} and UV detection at 215 nm. A standard curve (R^2=0.997) was prepared with Penicillin G (Sigma) in a concentration range from 1 to 0.001 mg mL^{-1}.

Results

Determination of biological activities

The results of the antimicrobial activity of culture filtrates of *P. chrysogenum* IFL1 are summarized in Table 1. The lyophilized samples were assayed against Gram-positive and Gram-negative bacteria and the inhibition occurred predominantly on Gram-positive bacteria, *S. aureus* and *B. cereus*. The phytopathogenic fungus *Fusarium oxysporum* was inhibited by the filtrate of cheese whey culture. However, fungal sporulation was not inhibited when the spores were pre-incubated with filtrates of cheese whey or grape waste cultures.

Table 1 Growth inhibition caused by *Penicillium chrysogenum* filtrates produced on agroindustrial residues

Microorganism	Filtrate (100 mg/mL)	
	Grape waste	Cheese whey
Gram positive bacteria		
Bacillus cereus	8±1.2 (25)	11±1.0 (12.5)
Staphylococcus aureus	16 ±3.6 (25)	10 (50)
Listeria monocytogenes	–	–
Gram negative bacteria		
Escherichia coli	–	–
Pseudomonas aeruginosa	–	18.7±0.6 (50)
Salmonella enteritidis	–	–
Yeast		
Candida tropicalis	–	–
Filamentous fungi		
Fusarium oxysporum	–	5±1.0 (50)
Aspergillus flavus	–	–

The values in parentheses are the minimum inhibitory concentration (MIC) of each filtrate

– No inhibition

The culture filtrates were also tested against a strain of *Acanthamoeba polyphaga* (Fig. 1). Both filtrates showed amoebicidal activity, being more efficient in 72 h of treatment and at concentrations of 50, 25 and 10 mg mL^{-1}, when the cyst formation occurred. All concentrations of the cheese whey filtrate caused a decrease of trophozoites in 72 h. In the same period, the grape waste filtrate showed absence of trophozoites in the concentration of 50 mg mL^{-1}, while in the same concentration the cheese whey showed a reduction to 2% of trophozoites in relation to the control group. Trophozoites encyst in double cellulose walls under adverse environmental conditions such as starvation, desiccation, extreme temperatures, and exposure to antimicrobial agents (Boonman et al. 2008).

Footprinting of metabolites and Penicillin G identification

A mass profile of the cheese whey culture filtrate was generated to investigate the metabolites produced by *P. chrysogenum*, since this filtrate showed a broader spectrum of biological activities. The footprinting of the most abundant metabolites is shown in Fig. 2. Initially, a screening for

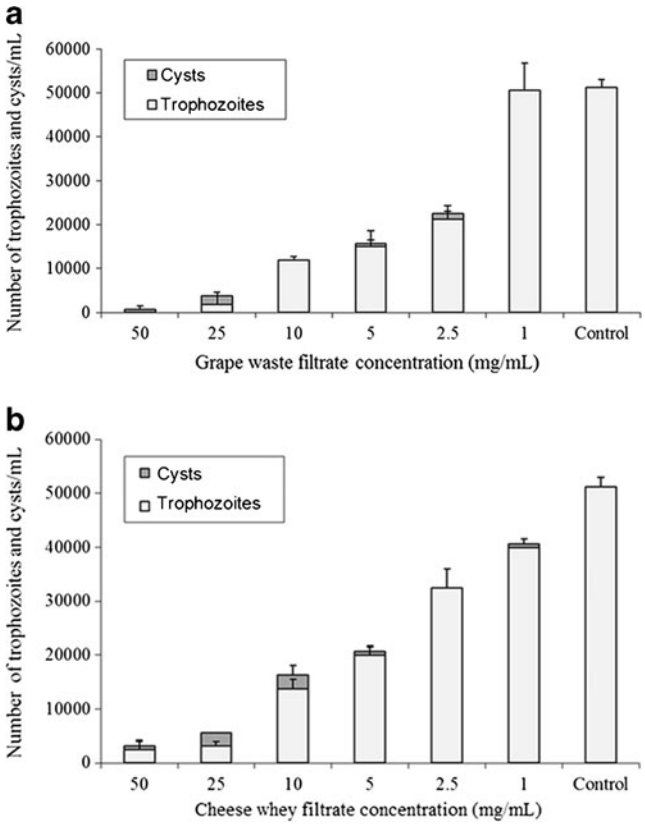

Fig. 1 Susceptibility of *Acanthamoeba polyphaga* to **a** grape waste filtrates and **b** cheese whey filtrates. These graphs show 72 h of treatment and the control. The cysts are shown as *dark bars* and the trophozoites as *grey bars*

metabolites commonly produced by this fungus was performed including roquefortine C, chrysogine (Smedsgaard et al. 2004), penicillins, PR toxin, secalonic acids (Pitt and Hocking 2009), cyclopiazonic acid (Rundberget et al. 2004), meleagrin, xanthocillins, sorbicillins, questiomycin, chrysogenin, negapillin, notatin, PAF and fungisporin (Frisvad et al. 2004). There are reports on the production of ochratoxin A, penicillic acid and patulin by some strains of *P. chrysogenum* (Barkai-Golan 2008). Then,a search for other metabolites produced was also performed using mass databases as found in Nielsen and Smedsgaard (2003) and Vishwanath et al. (2009).

Molecular masses of some mycotoxins as roquefortine C, secalonic acid, ochratoxin A, citrinin, PR toxin and patulin were not found in this extract. The presence of the mycotoxin ciclopiazonic acid was evidenced in the extract. Some metabolites like Penicillin G (PenG) and V, Rugulosin and Formyl-xanthocilin X were found in the filtrate of cheese whey culture.

The presence of PenG on cheese whey extract was also evidenced in the analysis of ESI-MS and ESI-MS/MS (Fig. 3). The presence of the mass of 335.1179 in the crude extract (Fig. 3c) and the same pattern of fragmentation of the penicillin standard (Fig. 3d) confirm the identity of this metabolite.

The occurrence of PenG in the filtrates of grape waste and cheese whey cultures was demonstrated by HPLC. The culture filtrates were initially submitted to aqueous two-phase extraction using PEG and ammonium sulfate solutions, and HPLC analysis was performed with the PEG phase. The amounts of PenG on grape waste and cheese whey filtrates were 32 and 10 μg mL^{-1}, respectively.

Discussion

Penicillium chrysogenum IFL1 was capable of producing bioactive metabolites during growth on important agro-industrial byproducts, such as cheese whey and grape waste. Among the observed bioactivities, amoebicidal activity has not previously been associated with metabolites from *Penicillium* spp. According to Boonman et al. (2008), the first evidence that a fungal metabolite can effectively kill *Acanthamoeba* trophozoites in vitro was obtained from *Fusarium* sp. Tlau3, an endophytic isolate from *Thunbergia laurifolia* Lindl. Some cultures of *Acanthamoeba castellanii*

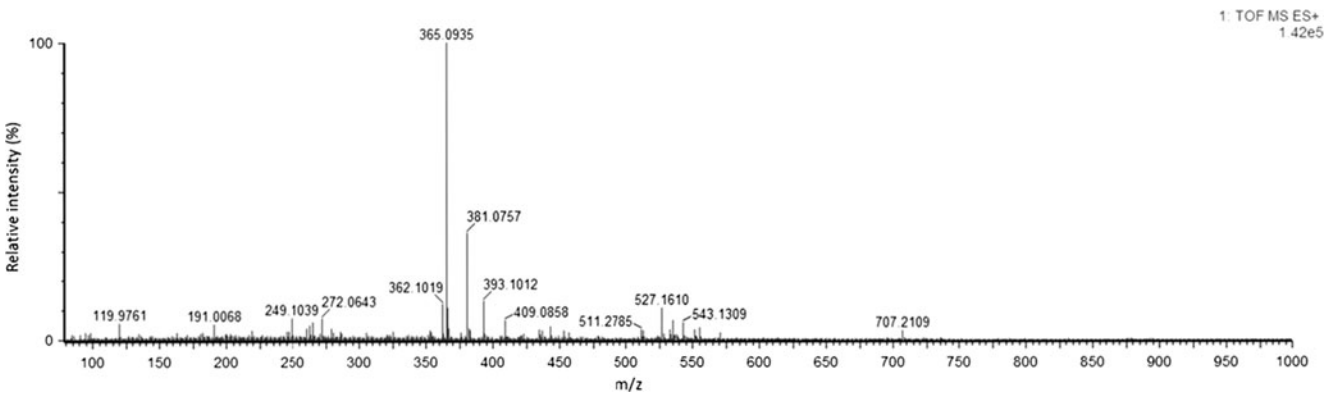

Fig. 2 ESI Q-TOF MS fingerprinting of produced metabolites by *Penicillium chrysogenum* on cheese whey. The spectrum shows the more abundant metabolites using 6 V collision energy

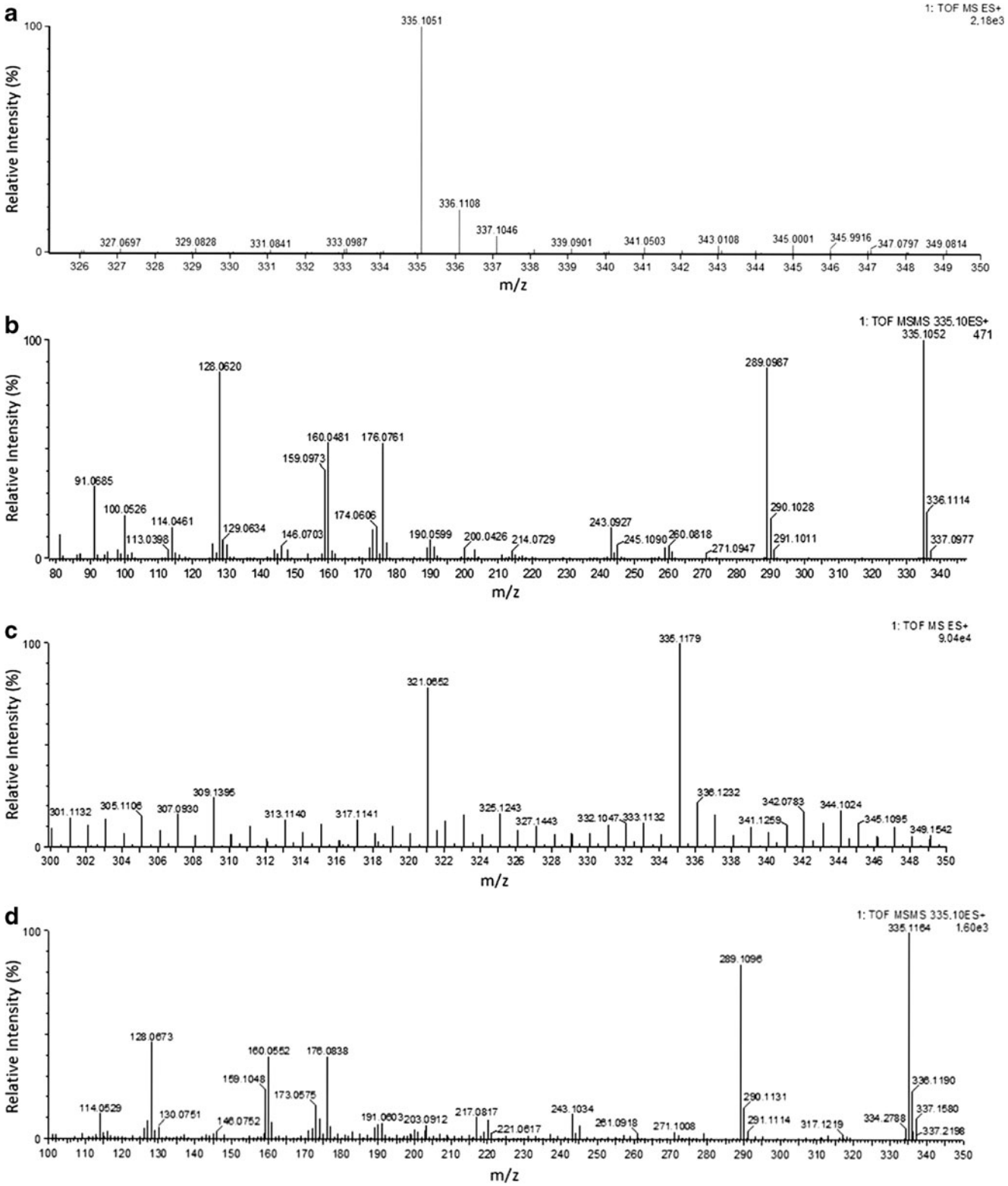

Fig. 3 Spectra in: **a** TOF MS of penicillin standard with the mass 335.1051; **b** TOF MS/MS of penicillin standard; **c** TOF MS *Penicillium chrysogenum* on cheese whey extract showing the presence of penicillin with the mass 335.1179 and **d** pattern of fragmentation using TOF MS/MS of the penicillin found in the sample

superficially infected with *Penicillium* sp. showed decreased numbers of amoebae after 24 and 72 h (Chomicz et al. 2010). However, those authors could not explain the influence of the fungus on the amoeba growth. Most literature reports the

amoebicidal activity of plant extracts from *Salvia* spp. (Goze et al. 2009), *Allium* spp. (Polat et al. 2008) and some medicinal herbs like *Solidago virgaurea, Solidago graminifolia, Rubus chamaemorus* and *Pueraria lobata* (Derda et al. 2009). *Acanthamoeba* causes granulomatous amebic encephalitis, a chronic fatal brain infection that occurs mostly in immunodeficient individuals, amebic keratitis, a sight-threatening corneal infection related to contact lens misuse, and cutaneous, nasopharyngeal and systemic infections (Benitez et al. 2010). Thus, the discovery of novel fungal metabolites to combat *Acanthamoeba* infections is a topic of utmost relevance.

Considering that *P. chrysogenum* is able to produce β-lactam antibiotics, which are active mainly against Gram-positive bacteria, the antibacterial activity could possibly be caused by the presence of penicillins in the filtrates. However, it is important to highlight the inhibition of the Gram-negative bacteria *P. aeruginosa*. As Gram-negative bacteria are naturally resistant to natural penicillins (Poole 2001), the presence of another metabolite with antibacterial activity can be suggested. *S. aureus* and *P. aeruginosa* are versatile organisms with several virulent characteristics and resistance mechanisms. These pathogenic bacteria are associated with a broad spectrum of human infections (Kanafani and Fowler 2006; Kerr and Snelling 2009). The antimicrobial activity observed against *B. cereus* is also relevant. Several *B. cereus* strains have been identified as the causative agent of two different types of food poisoning: the emetic type and the diarrheal type, and both can occasionally be fatal (Martínez-Blanch et al. 2009).

PenG was quantified in the culture filtrates and was found at 32 and 10 μg mL^{-1} in grape waste and cheese whey filtrates, respectively. In spite of the titer currently obtained in industrial penicillin production not being available in the open literature, the estimated titer is around 145 mM of penicillin (corresponding to 50 g L^{-1}) after 200 h of cultivation, according to Nielsen (1997). In this work, the titers were lower comparing to the industrial strains as *P. chrysogenum* NRRL 1951; however, it is possible to increase the titers of PenG, using optimization methods. Factorial design, response surface methodology, and design of experiments provide powerful and efficient ways to optimize cultivations (Mandenius and Brundin 2008). In addition, the penicillin production by solid state fermentation (Barrios-Gonzales et al. 1988) could be another approach, mainly because of the utilization of agro-industrial residues in this work.

Some strains of *Fusarium* are responsible for severe vascular wilt or root rot diseases in many plants (Edel et al. 2001; Mandal et al. 2009). Synthetic chemical fungicides have long been used to reduce the incidence of such plant diseases, but the resulting environmental pollution and induction of resistance indicate the need for alternative control methods (Wang et al. 2002). Novel natural antifungal compounds need to be discovered, with the filamentous fungi being valuable sources of small-size antifungal peptides, like the peptide PAF produced by *P. chrysogenum* (Skouri-Gargouri et al. 2009). Metabolites from *P. chrysogenum* IFL1 may be valuable in combatting phytopathogenic fungi such as *Fusarium* spp.

The footprinting performed with cheese whey culture filtrate suggests the production of active metabolites like penicillins G and V, rugulosin, formyl-xanthocilin X and the mycotoxin cyclopiazonic acid. Cyclopiazonic acid is a mycotoxin that has been isolated from numerous species of *Aspergillus* and *Penicillium* (Monaci et al. 2002). This toxin has been found as a natural contaminant of corn and peanuts as well as of cheese colonized with *Penicillium camemberti* or other *Penicillium* species. This fact could explain the production of this mycotoxin on cheese whey. Despite the production of this mycotoxin, Pitt and Hocking (2009) reported that *P. chrysogenum* does not appear to be a serious source of mycotoxins. In addition, the production of mycotoxins can be controlled through appropriate cultivation parameters (Blumenthal 2004). Rugulosin was first described in *Penicillium rugulosum* and has been reported as a toxic substrate to *Drosophila melanogaster* and to ovarian cells of the fall armyworm *Spodoptera frugiperda*. It has been also reported as an antibiotic to both Gram-positive and Gram-negative bacteria, showing moderately antifungal activity (Sumarah et al. 2008). The production of rugulosin by *P. chrysogenum* IWW 1053 has been described and no cytotoxicity was found in extracts containing rugulosin (Brunati et al. 2009). The presence of formyl-xanthocilin X has also been observed, while studies about xanthocillins are relatively scarce. Xanthocillin X dimethylether and mono-methylether, which showed antiviral activity against Newcastle disease virus, vaccinia and herpes simplex viruses, are produced by *Aspergillus* sp. and *Penicillium notatum* (synonymized as *Penicillium chrysogenum*). The antibacterial activity of these compounds has also been reported (Tatsuta and Yamaguchi 2005).

The strain *P. chrysogenum* IFL1 produced active metabolites with antibacterial, antifungal and amoebicidal activities on inexpensive media such as grape waste and cheese whey. Some metabolites previously shown to display insecticidal, antifungal and antibacterial activities were also detected, showing that, despite the long period of studies on *P. chrysogenum*, there are potential new metabolites to be used as drugs or prototypes. It was not possible to identify the amoebicidal metabolite, further studies being necessary to isolate active compounds and to study each one individually.

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