Antibacterial activity against plant pathogens by cruded extracts and compounds from *Idriella* sp.

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ORIGINAL

Actividad antibacteriana de compuestos y extractos crudos de *Idriella* sp. contra patógenos de plantas

Resumen. Un nuevo aislamiento, *Idriella* sp. GH5608 fue colectado en la región de Xalapa, Veracruz, México. Para explorar la existencia de actividad antibacteriana intracelular o extracelular de dicha cepa, los caldos de cultivo y el extracto metanólico de la biomasa fueron evaluados por el método de microdilución en caldo en contra de especies patógenas tanto para humanos como para plantas. De la purificación de los extractos de este hongo se lograron identificar el 5-hidroximetil-2-furaldehido y el 1-n-Butil-β-D-fructopiránosido. Estas estructuras fueron determinadas por métodos espectroscópicos. Ambos compuestos mostraron actividades antibacterianas en contra de *Xanthomonas axonopodis, Pectobacterium carotovorum, P. crhysanthemi* y *Erwinia amylovora* (CMI = 0. 625 mg/ml).
Palabras clave: 5-hidroximetil-2-furaldehido, 1-n-Butil-β-D-fructopiránosido.

Abstract. A new isolate *Idriella* sp. GH5608 was collected in a region of Xalapa, Veracruz, Mexico. In order to explore the existence of intracellular or extracellular antibacterial activity from this strain, culture filtrates and biomass methanolic extracts were screened by microdilution method against pathogenic species to either humans or plants. Biossays-guided fractionation of the extracellular extracts from *Idriella* sp. GH5608 led to the identification of 5-hydroxymethyl-2-furaldehyde and 1-n-Butyl-β-D-fructopyranoside. These structures were determined by spectroscopic methods. Both compounds showed inhibitory antibacterial activities against *Xanthomonas axonopodis, Pectobacterium carotovorum, P. crhysanthemi* and *Erwinia amylovora* (MIC = 0. 625 mg/ml).
Key words: 5-hydroxymethyl-2-furaldehyde, 1-n-Butyl-β-D-fructopyranoside.

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Introduction

The use of microorganisms for biological control purposes has become an effective alternative to control plant pathogens. There are many examples of formulations using either bacterial or fungal strains with biocontrol applications (Bernal *et al*., 2002; Fravel and Roberts, 1991; Penyalver *et al*., 2000; Wilson and Backman, 1999). Different authors have discussed the potential of tropical fungal species as producers of active metabolites (Rossman, 1997; Wildman, 1997). In nature, plant debris support the growth of a wide variety of filamentous fungi. Among the microfungi colonizing leaf litter, species of the genus *Idriella* are found frequently. The genus *Idriella* was established in 1956 (Nelson and Stephen, 1956), around 36 species have been described so far (Kirk, 2004). In addition to plant debris,

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species of *Idriella* prosper in soil and living plants, hence there are parasites, saprotrophs and endophytes species (Domsch *et al*., 1980; Rodrigues and Samuels, 1992). *Idriella bolleyi* was identified as a potential biocontrol agent for wheat protection against the "take all" infection caused by *Gaeumannomyces graminis* (Kirk and Deacon, 1987; Lascaris and Deacon, 1991), which is responsible for root and stem infection in grain crops throughout the world (Jadubansa *et al*., 1994). Recently Espinoza *et al*. (2008) showed that culture filtrates from *Idriella* sp. exhibited fungicide activity against of *Alternaria citri*, *A. tenuis, Colletotrichum gloeosporoides, Curvularia lunata, Fusarium moniliforme*, *Geotrichum* spp., *Helminthosporium turcicum*, *Pestalotia* spp. and *Ulocladium* spp. Despite the wide occurrence of *Idriella* species in nature, there are only few studies exploring its potential as biocontrol agent (Kirk and Deacon, 1987; Lascaris and Deacon, 1991; Espinoza *et al.,* 2008). Thus, it could be interesting to analyze antibacterial activity by this fungus against other microorganisms such phytopathogenic bacteria affecting agricultural plantations or even strains of clinical relevance. Here we report the production of extracts showing antibiotic properties against bacterial pathogens of economical importance for either agricultural or clinical fields; additionally, structures of some compounds were elucidated from samples showing antibacterial activities in culture filtrates.

Materials and methods

Strain isolation

Fungal strain *Idriella* sp. GH5608, was isolated from a sample of leaf litter collected in a patch of a cloud forest near Xalapa, Veracruz, México. The taxonomic identification of the strain was based on morphological characteristics of the fungi growing on a natural substrate (Heredia-Abarca *et al*., 2004). The isolate was cultured and maintained in potato dextrose agar plates (250 g of potato, 10 g of anhydrous glucose, 10 g of meat peptone, 15 g of agar and 1000 ml of water) at a constant temperature of 27ºC (Lascaris and Deacon, 1994). Cultures are deposited in the Instituto de Ecología A.C. culture collection and in the Laboratorio de Alta Tecnología de Xalapa (LATEX).

Culturing and growth conditions

A culture of *Idriella* sp. grown for 7 days on potato dextrose agar plates was scraped off with Tween 0.1% (v:v) to obtain a vegetative cell suspension. Two milliliters of the suspension were inoculated into a 500 ml Erlenmeyer flash containing 100 ml of a seed medium containing 4% sucrose, 2.5% corn flour, 0.05% yeast extract. An initial value of pH 6.6 was obtained with no adjustment. A total of 100 flasks (100 mL of medium in every 500 mL flask) were inoculated to reach a total volume of 10 L in the same way and maintained in continuous agitation (150 rpm) for 7 days at 27ºC.

Bioassay and test organisms

Antibacterial activity *in vitro* was assayed using culture filtrates against pathogenic bacteria listed in Table 1. Samples were assayed by duplicate using the microdilution method with a positive control (bacterial suspensions and broth medium), a negative control (broth medium), and also a dissolvent control (methanol) as suggested by Trigos *et al*. (2006). Methanolic extracts from biomass were used for determination of Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs). *Escherichia coli* (Migula) Castellani and Chalmers (BM-001) and *Sthaphylococcus aureus* Rosenbanch (BM-002) were used as examples of strains of clinical interest. Additionally some plant pathogenic bacteria were analyzed; these included *Xanthomonas axonopodis* Starr & Gárces (BF-006), *Pectobacterium carotovorum* subsp. *atrosepticum* (Jones) Hauben *et al.* emend Gardan *et al.* (BF-001), *P. chrysanthemi* burkholder, Mc Fadden & Dimock (BF-003), *Erwinia amylovora* Burrill (BF-004) and *Herbaspirillum rubrisubalbicans* Christopher & Edgerton (BF-009). Medium for bioassay and maintenance of the test organism was Müeller–Hinton (Trigos *et al*., 2005). All strains used for this study are deposited at the LATEX culture collection.

Table 1. Minimal Inhibitory Concentration (MIC, µl/ml) of culture filtrate and crude methanolic extract from *Idriella* sp. biomass showing bacteriostatic activity against human pathogenic and phytopathogenic bacteria

Test organism	MIC (µl/ml)		Inhibition (%)	
	Culture filtrates	Crude methanolic extract of biomass	Culture filtrates	Crude methanolic extract of biomass
Pathogenic bacteria				
Escherichia coli	500	62.5	13	86
Plant pathogenic bacteria				
Pectobacterium carotovorum subs. *atrosepticum*	250	500	78	20

Note: Dilutions of culture filtrates and crude extract 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 were equivalent to 500, 250, 125, 62.5, 31.25 and 15.62 µl/ml, respectively.

Culture filtrate assays

Culture broth samples of 100 ml were collected for the measurements of cell growth wich was determined by dry weight after 24 h at 60 °C; high performance liquid chromatography (HPLC) was performed on chromatography station for windows Empyre of Waters, at wavelength of 284 and 334 nm on UV-Visible detector, a ODS column (C-18, 300 x 4.5 x 5 µm) of Waters, mobile phase was Hx:AcOEt 6:4 and MeOH: AcOEt 1:1 at a flow rate of 1ml/min were used for kinetics of metabolites formation and pH values were also recorded at different fermentation times. For the two compounds described below, showing antibacterial activity, retention times in HPLC were 7.4 and 4.1 min for compound **1** and **2,** respectively.

Isolation and purification of fungal metabolites

The harvested broth (10 L) was separated by filtration with filter papers (Whatman No. 42). Mycelia-free supernatant (8 L) was extracted with n-buthanol. The extract was concentrated to dryness under reduced pressure to give crude buthanolic extract. This residue was purified via column chromatography, using silica gel (Merck 0.040-0.063 mm). The column was eluted with a hexane-ethyl acetate gradient. Thin Layer Chromatography (TLC) plates of silica gel (Merck 60 GF254 of 0.2 mm of thickness) were used for separation of compounds. Melting points were determined in a Fisher-Johns device and were not corrected. NMR spectra of the pure compounds was furnished by 1D and 2D measurements spectra were recorded on a Bruker DMX500 in $CDCl_3$ and CD_3OD, operating at 500 MHz for ^1H and 100 MHz for ^{13}C; and tetramethylsilane (TMS) was used as internal standard. The chemical shifts were recorded in ppm (δ) and coupling constants (J) in Hz. The GC-MS spectrum was recorded on Hewlett Packard 890-2 at 70 eV using the DI method. IR spectrum was recorded on Perkin Elmer Spectrum 2000 Explorer and UV spectra were recorded on Perkin Elmer Lambda 40 spectrometer.

Isolation of 5-hydroxymethyl-2-furaldehyde (1). From the fractions eluted with hexane: ethyl acetate 6:4, 8 mg of a oily brownish compound was obtained. TLC (Hx:/AcOEt 6:4, silica gel Merck) Rf 0.44 revealing with UV light at 254 nm and iodine steam. IR ($CHCl_3$) ν_{max}, (cm^{-1}): 3437, 1732, and 1681. MS m/z= 126 ($C_6H_6O_3$); 109 $[M-17]^+$; 97 $[M-29]^+$; . ^1H

NMR ($CDCl_3$) δ, ppm: 9.57 (1H, s, H-1); 7.22 (1H, d, J=3.6 Hz, H-3); 6.52 (1H, dt, J=3.6 y 1.0 Hz, H-4); 4.71 (2H, s, 2H-6). ^{13}C NMR ($CDCl_3$) δ, ppm: 177.7 (C-1); 160.8 (C-5); 152.3 (C-2); 122.9 (C-3); 109.9 (C-4); 57.5 (C-6).

Isolation of 1-n-Butyl-β-D-fructopyranoside (2). From the fractions eluted with ethyl acetate, 620 mg of white crystals with melting point 149-150°C was obtained. TLC (AcOEt:MeOH 1:1 silica gel Merck) Rf 0.65 revealing with fosfomolibdic acid. IR (MeOH) ν_{max}, (cm^{-1}): 3377, 2954, 1370, 1120, 1050, 911, 890, 863, 779, 658; ^{1}H NMR (CD_3OD) δ, ppm: 3.90 (1H, d, J=9.89 Hz, H-3'); 3.83 (1H, c, H-5'); 3.77 (1H, dd, J=3.45 y 3.48 Hz, H-4'); 3.75 (1H, dd, J=2.1 y 1.42 Hz, H-6'a); 3.72 (2H, dd, J=11.33 y 11.32 Hz, H-1'); 3.65 (1H, dd, J=1.88 y 1.88 Hz, H-6'b); 3.50 (2H, c, H-1); 1.56 (2H, c, H-2); 1.40 (2H, c, H-3); 0.93 (3H, t, J=7.4 Hz, H-4). ^{13}C NMR (DMSO) δ, ppm: 99.8 (C- 2/); 69.4 (C-4/); 69.2 (C-5/); 69.0 (C-3/); 63.9 (C-6/); 62.2 (C-1/); 59.6 (C-1); 32.4 (C-2); 19.7 (C-3); 14.5 (C-4); ^{13}C NMR (CD_3OD) δ, ppm: 101.6 (C- 2'); 71.6 (C-4'); 71.1 (C-5'); 70.6 (C-3'); 65.2 (C-6'); 63.5 (C-1'); 61.6 (C-1); 33.3 (C-2); 20.5 (C-3); 14.3 (C-4).

Results

Antimicrobial activity screening

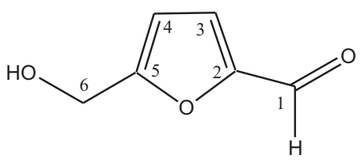
In order to determined if antibacterial activity were found in culture broth (extracellular) or bound to biomass (intracellular), a preliminary test was carried out using both culture filtrates and biomass methanolic extracts. These fractions were tested against a human pathogenic strain (*E. coli* ATCC 25922) and also against a phytopathogenic strain (*P. carotovorum* subsp. *atrosepticum* BF001). Table 1 shows that culture filtrates had antibacterial activity against phytopathogenic strain; whereas methanolic extracts from biomass showed inhibition against *E. coli* (ATCC 25922).

Purification and structural elucidation

As a sequential purification strategy, buthanol extractions of culture filtrates from *Idriella* sp. were followed by chromatography of the organic residue, allowing the isolation of two compounds, which were shown to be 5-hydroxymethyl-2-furaldehyde and 1-n-Butyl-β-D-fructopyranoside (Figure 1) by comparison with authentic samples and spectral data (Ayer and Racok, 1990; Zhu *et al*., 1991; Zhang *et al*., 1996).

Kinetics Profiles

A typical time course of the fermentation is shown in Figure 2. Maximal biomass was reached after of 7 days. A rapid pH reduction was observed during the first three fermentation days. Subsequently, pH value was stabilized around 4.6 (Figure 2). Production of compound **1** was observed only after 5 day and reached a maximum at day 7; whereas compound **2** was detected at day 3 and reached a maximum at day 8 (Figure 2). Formation of compound **1** is partially associated to fungal growth, in comparison to compound **2**.



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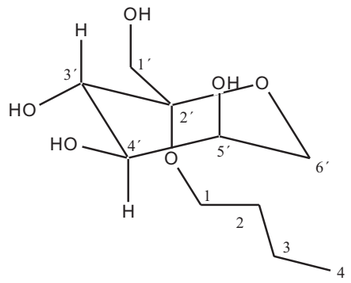

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Figure 1. Chemical structures of compounds **1** (5-hydroxymethyl-2-furaldehyde) and **2** (1-n-Butyl-β-D-fructopyranoside).

Biological Activity

Antibacterial activities *in vitro* for both compounds **1** and **2** against several plant pathogenic bacteria are summarized in Table 2. Both compounds displayed antimicrobial activity against plant pathogenic bacteria such *X. axonopodis, P. carotovorum* subs. *atrosepticum, P. crhysanthemi* and *E. amylovora* (MIC = 0.625 mg/mL). There was not detected effect against clinically important strains such *E. coli* and *S. aureus.*

Discussion

In the course of screening for new antibacterial compounds from microfungi colonizing and leaf litter, a fungal strain of *Idriella* sp. displayed antibacterial activity (Trigos, *et al*., 2005). The present study showed that both culture filtrates

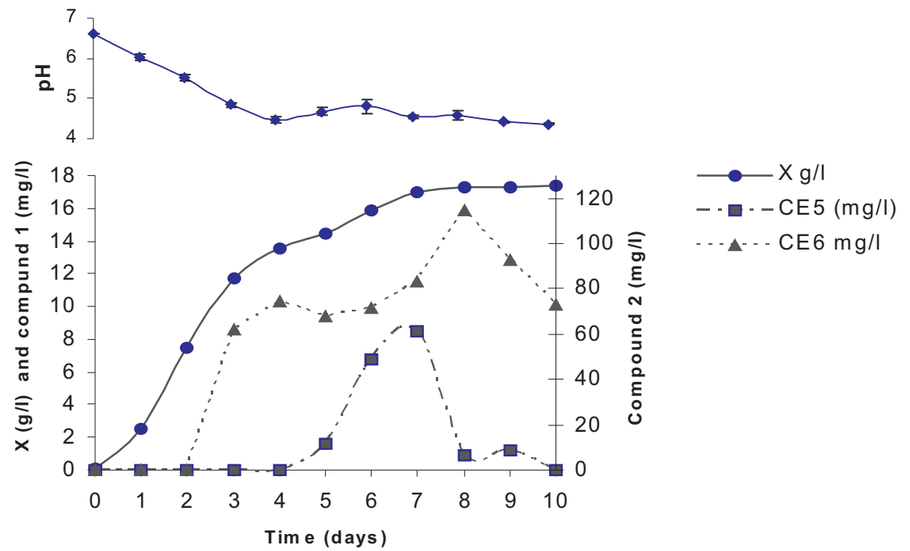

Figure 2. A typical time course of **1** and **2** production by *Idriella* sp. GH5608■, compound **1**;▲, compound **2**;●, biomass (X);♦, pH.

Table 2. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) for compounds 1and 2 against plant pathogenic bacteria

Test organism	**1** MIC (mg/ml)	**1** MBC (mg/ml)	**2** MIC (mg/ml)	**2** MBC (mg/ml)
Plant pathogenic bacteria				
Xanthomonas axonopodis	0.625	2.5	0.625	2.5
Pectobacterium carotovorum subsp. *atrosepticum*	0.625	1.25	0.625	1.25
Pectobacterium crhysanthemi	0.625	ND	0.625	ND
Erwinia amylovora	0.625	ND	0.625	ND
Herbaspirillum rubrisubalbicans	1.25	ND	1.25	ND

ND: not detected.

and biomass extracts from *Idriella* sp. exhibited growth inhibition against several plant pathogenic and human pathogenic bacteria. All these sensitive bacteria are Gram (-), which should be considered for the potential applications of these extracts. Thus, the topical use of crude extracts from *Idriella* sp. could be considered as a control to bacterial plant pathogens, without massive aspersion all over crop areas. Compound 5-hydroxymethyl-2-furaldehyde (1) is a major organic breakdown product derived from the bacterial foodstuffs and is also found in spoilt preparations of solutions used in parenteral nutrition (Hryncewicz *et al*., 1996). In addition this compound has showed significant cytotoxic activities in a brine shrimp bioassay (Khondar *et al*., 2005). Also, it has been found to be an insecticidal compound by bioassay-guided fractionation (Miyazawa *et al*., 2003). On the other hand, 1-n-Butyl-β-D-fructopyranoside (2) was isolated previously as component of plants (Zhu *et al*., 1991) with the ability to display antistress activity in mice (Wakunaga Pharmaceutical Co., Ltd., 1984). This is the first report describing the isolation of these two compounds from a fungal strain. Interestingly, these two compounds showed antimicrobial activities against Gram (-) plant pathogenic bacteria.

Thus, *Idriella* sp. can be considered as a novel source for obtaining new molecules showing antibiotic activities against other microorganisms. It is worth noticing that Compound 2 was produced by *Idriella* sp. during most of the time course of the fungal growth. On the other hand, Compound 1 is produced only at the end of the fungal growth. This indicates that each compound is produced by different processes related to the fungal culture development. For example, Compound 2 may be more related to biosynthesis, as compared to Compound 1, as more related to secondary metabolism. This observation seems to indicate that it would be possible to manipulate the physiology of *Idriella* sp. in order to be more specific for the production of either compound.

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