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Chitinase, chitosanase, and antifungal activities from thermophilic streptomycetes isolated from compost

Actividades quitinolíticas, quitosanolíticas y antifúngicas de estreptomicetos termofílicos aislados de composta

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Abstract. The *Streptomyces* genus comprises a large and diverse group of bacteria, many of which are commercially exploited for the production of antibiotics and lytic enzymes. The thermophilic species are less studied than the predominant mesophilic species. However, the first ones are a potential source of thermostable bioactive products and enzymes with novel properties. In this study, two selected thermophilic streptomycetes were identified and their chitinolytic activities were evaluated. The identification of these two isolates was performed by microscopic morphology, partial 16S rDNA sequences, and its phylogenetic analysis. To study the chitinolytic activities of these isolates, the effects of colloidal chitin (CC) and fungal cell walls (FCW) on the chitinase activities and chitinase and chitosanase isoform patterns were determined. Additionally, *in vitro* confrontations against chitinolytic phytopathogenic fungi were performed at 45 ˚C and 65 ˚C. Both isolates (AC4 y AC7) were identified as members of the streptomycete thermophilic clade. The highest chitinolytic activities were observed in the combinations 0.1% FCW/0.1% CC and 0.1% FCW/0.3% CC with maximum values of 0.7 U/μg and 0.45 U/μg, respectively for the AC4 strain, and with values of 0.48 U/μg in both treatments for the AC7 strain. The electrophoretic profiles of chitinase and chitosanase activity showed not only differences in bands intensity, but also few new bands were observed. Both isolates inhibited the growth of *Rhizoctonia solani* and *Fusarium oxysporum*. The present study shows that thermophilic streptomycetes have potential bioactivities that might be exploited in horticulture.

Keywords: Hidrolytic enzymes; *Fusarium oxysporum*; *Rhizoctoria solani*; Phylogenetic analysis; Actinomycetes.

Resumen. El género *Streptomyces* constituye un grupo muy amplio y diverso de bacterias, y muchas de ellas son explotadas comercialmente para la producción de antibióticos y enzimas líticas. Las especies termofílicas son menos estudiadas que las mesofílicas; sin embargo, las primeras son una fuente potencial de productos bioactivos y de enzimas termoestables con propiedades novedosas. En este estudio, dos estreptomicetos termofílicos selectos se identificaron y sus actividades quitinolíticas fueron evaluadas. La identificación de los aislados se realizó por morfología microscópica, secuenciación del gen ribosomal 16s y su análisis filogenético. Para estudiar las actividades quitinolíticas de estos aislados, se determinó la influencia de la quitina coloidal (CC) y la pared celular fúngica (FCW) sobre la actividad de quitinasas y los patrones electroforéticos de las isoformas de quitinasas y quitosanasas. Adicionalmente, se realizaron confrontaciones *in vitro* contra hongos fitopatógenos quitinolíticos a 45 ˚C y 65 ˚C. Ambos aislados (AC4 y AC7) se identificaron como miembros del grupo de estreptomicetos termofílicos. Los tratamientos con las mayores actividades quitinolíticas fueron las combinaciones 0,1% FCW/0,1% CC y 0,1% FCW/0,3% CC con valores máximos de 0,7 U/μg y 0,45 U/μg, respectivamente en la cepa AC4 y con valores de 0,48 U/ug en ambos tratamientos con la cepa AC7. Los perfiles electroforéticos de actividades de quitinasas y quitosanasas mostraron diferencias en la intensidad de bandas, pero también se observaron nuevas bandas en menor grado. Los dos estreptomicetos inhibieron el crecimiento de *Rhizoctonia solani* y *Fusarium oxysporum*. El presente estudio muestra que los estreptomicetos termofílicos tienen actividades biológicas que podrían ser explotadas en horticultura.

Palabras clave: Hidrolytic enzymes; *Fusarium oxysporum*; *Rhizoctoria solani*; Análisis filogenético; Actinomicetos.

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INTRODUCTION

Soil actinomycetes, especially those that belong to the genus *Streptomyces*, have been the focus of intensive research (Hopwood, 1999; Challis & Hopwood, 2003). Early interest in *Streptomyces* arose from the finding that this group of bacteria has the ability to produce a large variety of bioactive compounds and extracellular hydrolases. Their capacity to produce a wide variety of antibiotics and extracellular enzymes gives them an important role in the decomposition of organic matter in the soil and in the control of phytopathogens. In the latter case, actinomycete-fungus antagonism has been demonstrated for a wide variety of plant pathogens, such as *Alternaria* (el-Abyad et al., 1996; Chattopadhyay & Nandi, 1982), *Rhizoctonia* (Rothrock & Gottlieb, 1981; Hwang et al., 2001), *Verticillium* and *Pythium* (Yuan & Crawford, 1995; Chamberlain & Crawford, 1999), *Aspergillus* (Gomes et al., 2000), *Phytophthora* (You et al., 1996; Trejo-Estrada et al., 1998), and *Fusarium* (Getha & Vikineswary, 2002; Smith et al., 2002; González-Franco et al., 2003).

Mesophilic species have been extensively isolated and characterized, and the search for novel bioactive metabolites such as antibiotics, and highly active lytic enzymes has switched in focus to more rare genera of actinomycetes or to well-characterized ones that are found in unusual environments. Thermophilic actinomycetes have not been widely explored, though they have potential as a source of novel bioactive compounds and enzymes with novel properties. For example, a highly thermostable chitinase with dual active sites and triple substrate binding sites was isolated from a thermophilic microorganism (Tanaka et al., 1999). Highly thermostable amyloglucosidase and pullulanase enzymes with activities at acid pHs were isolated from two thermophilic actinomycetes (Sanglier et al., 1993).Compost, a source for thermophilic actinomycetes, has been used as a soil amendment or as a container medium but may also protect plants from diseases caused by soilborne root pathogens (Ellis et al., 1986; Chen et al., 1987; Pane et al., 2011; Larkin & Tavantzis, 2013). Several organisms antagonistic to soilborne root pathogens have been isolated from fungi suppressive composts (Kuter et al., 1983; McKellar & Nelson, 2003; Suárez-Estrella et al., 2013), including actinomycetes which contribute significantly to biological control. These findings suggest that suppressive organisms may be at least partly responsible for the decreased disease incidence observed for plants grown in composted soils.

A wide variety of lytic enzymes have been studied in thermophilic actinomycetes, especially those that degrade plant derived materials. For example, cellulases, xylanases, α-amylases, and β-xylosidases (Crawford & McCoy, 1972; Hoitink & Fahy, 1986; Warren, 1996; Petrosyan et al., 2003). Previously, a thermostable chitinase produced by *Streptomyces thermoviolaceus* OPC-520 was purified and characterized (Tsujibo et al., 1993).

It is known that the innate thermal stability of proteins of thermophilic microorganisms is greater than that of their counterparts from mesophilic organisms (Farrell & Rose, 1967; Vieille et al., 1996; Niehaus et al., 1999). Thus, metabolites and enzymes excreted by thermophilic actinomycetes can be of great interest not only for their bioactivities, but also for their thermostability and resistance to denaturation (Sanglier et al., 1993; Goh et al., 2003).

There are relatively few reports concerning antibiotic production by thermophilic actinomycetes. Some of the antibacterial metabolites produced by these organisms include thermomycin, thermorubin, thermoviridin, thermothiocin and granaticin (Edwards, 1993).

Although many studies have been conducted to determine the effects of mesophilic chitinolytic microorganisms on the growth and development of fungal pathogens (Chet et al., 1990; Inbar & Chet, 1991; Lorito et al., 1994; Chernin et al., 1995; Trejo-Estrada et al., 1998; Gomes et al., 2000; Melent'ev et al., 2001), few studies have directly studied the chitinolytic enzymes of antifungal thermophilic actinomycetes, which could be used to generate products for agricultural applications (e.g., suppressive compost, biofertilizers). Chitinases from thermophilic actinomycetes could have important biotechnological applications. For instance, they could be used for the bioconversion of chitin, an abundant carbohydrate, as a renewable resource and for the production of oligosaccharides. Furthermore, some chitooligosaccharides can be used in phagocyte activation, or as growth inhibitors of certain tumors (Muzzarelli, 1983; Felse & Panda, 1999), and therefore have importance in human medicine.

The objectives of the present study were to identify selected thermophilic streptomycetes with chitinolytic activity isolated from a compost from horse litter, to determine their antifungal activities, and to evaluate the effects of different substrates on the activities of their chitinases and the electroforetic profile of chitinase and chitosanase isoforms.

MATERIALS AND METHODS

Isolation and selection of actinomycetes and culture conditions. Thermophilic actinomycetes were isolated by spreading dilutions of a horse manure compost, obtained from a horse farm near Moscow, Idaho, on International Streptomyces Project medium 2 (ISP2) plates. This compost contained horse manure mixed with wood shavings, hay and other materials typically taken from horse stables. The plates were incubated at 50 °C, to enrich for thermophilic actinomycetes, in a humid atmosphere for 7 to 10 days. Characteristic actinomycete colonies were picked onto tryptone yeast extract (TYE) plates and tentatively classified on the basis of their morphological characteristics under light microscopy. Stock cultures were maintained on TYE plates, and as either mycelial or spore suspensions in 10% glycerol at -20 °C. Composition

of the ISP2 and TYE media are published elsewhere (Shirling & Gottlieb, 1966; Atlas, 1997).

Eight isolates were pre-screened for chitinolytic activity by inoculation onto Chitin Medium (CM) agar, containing 0.3% (w/v) colloidal chitin (CC) (González-Franco et al., 2003). Cultures were incubated at 45 °C until zones of chitin clearing were observed around the colonies. The highly active chitinase producing isolates, as judged from the size of chitin-clearance zones (measured from the edge of the bacterial growth to the limit of the clear zone) were subsequently screened in CM broth supplemented with 0.3% (w/v) of colloidal chitin. Cultures were incubated at 45 °C for 6 days at 250 rpm in an incubator shaker. Based upon their potent chitinase activity, two actinomycetes were chosen for further study.

DNA template preparation and PCR amplification of partial 16S rRNA gene sequences

DNA extraction. Total chromosomal DNA was extracted from the isolates after protoplast formation. First, the isolates were grown for three days in super yeast extract-malt extract broth (Hopwood et al., 1985). Then, the cells were harvested by centrifugation and washed three times with five volumes of 10.3 % (w/v) chilled sucrose. Protoplasts were prepared according to Hopwood et al. (Hopwood et al., 1985). Lysozyme treatment (3 mg/ml) was for approximately 90 minutes, but depended on the isolate. Protoplast formation was monitored by phase-contrast microscopy. Protoplasts were separated from the undigested mycelium by filtration through cotton wool. The supernatant was removed from the protoplasted cells by centrifugation, and chromosomal DNA was extracted from the pellet by adding sterile distilled water and triturating. The lysates were then centrifuged at 13000 rpm for 4 min, and 4 µL of the supernatant was used as template for PCR.

PCR. The 16S rDNA partial sequences of the thermophilic isolates were amplified by using oligonucleotide primers designed to anneal to conserved positions in the 3' and 5' regions of bacterial 16s rRNA genes. The forward primer was 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (Lane et al., 1985) and the reverse primer was 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (Thomsen et al., 2001). Each PCR mixture contained 20 µM of each primer, 0.2 mM of each dNTPs, 25 mM of MgCl2, 5 µL of 10x PCR universal buffer (Invitrogen TECH-LINE™, USA), 4 µL of the DNA template, and 1.25 U of Taq DNA polymerase (Invitrogen TECH-LINE℠, USA) to a final volume of 50 µL. The reaction conditions were as follows: 95°C for 5 min for one cycle followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, and finally one cycle at 72 °C for 7 min in a Gene Amp PCR System 2400 thermocycler (Applied Biosystems). Positive and negative controls were included for each PCR experiment. The positive control consisted of reaction mixtures containing 8 ng of chromosomal DNA of *Streptomyces lydicus* WYEC108. The negative control lacked the DNA template but contained all other reactants. The PCR products were checked on a 1% agarose gel stained with ethidium bromide. A 1 Kb plus ladder (Gibco BRL Life Technologies, Gaithersburg, Md.) was used as a DNA size marker. The resultant PCR product was purified using the UltraClean™ PCR Clean-up™ kit (Mo Bio Laboratories, Inc. Solana Beach, CA) according to the manufacturer's instructions. The purified PCR amplicons were sequenced by the Laboratory of Biotechnology and Bioanalysis at Washington State University (Pullman, WA) using 27F and 907R as sequencing primers. Sequences were compared against known sequences using the NCBI BLAST database (Altschul et al., 1997).

Phylogenetic analysis. The 16S rDNA sequences of strains AC4 and AC7 were aligned with 16S rDNA sequences of other *Streptomyces* retrieved from the EMBL/GenBank database. The BioEdit program (Hall, 1999) was use as an editing tool. Multiple alignments were obtained with the Clustal W program (Thompson et al., 1994). Phylogenetic trees were inferred by three algorithms, maximum-parsimony (Kluge & Farris, 1969), neighbor-joining (Saitou & Nei, 1987), and maximum-likelihood using the PAUP* package (Swofford, 2002). Bootstrap analyses for the neighbor-joining and the maximum-likelihood results were generated based on 200 resamplings.

Evolutionary distance matrices for the neighbor-joining method were generated as described by Lockhart et al. (1994). The TreeView (WIN 32) program (Roderic, 2001) was used for viewing the trees generated by the three algorithms.

Cellulase activity. Isolates were tested for cellulase activity by inoculating the isolates in a CM agar supplemented with 0.3% (w/v) of carboxymethylcellulose (CMC) instead of CC and incubating for 3 days at 45 °C. Activity was developed as described earlier (Teather & Wood, 1982).

Growth temperature. Growth of selected isolates were tested for up to ten days at different temperatures (30 °C, 37 °C, 45 °C, 55 °C, 65 °C, and 75 °C) on TYE plates. Morphological characteristics were also recorded.

Fungal antagonist bioassays. Selected isolates were tested for antifungal antagonism directly against the phytopathogens *Rhizoctonia solani* [Kindly provided by Dr. W. C. Chun (Department of Plant Soil and Entomological Sciences, University of Idaho)] and *Fusarium oxysporum* ATCC 070233 from the American Type Culture Collection. *In vitro* plate bioassays were used to assess antagonism by selected isolates. The actinomycete isolates were streak-inoculated to one side of TYE plates and incubated at 45 °C, a common temperature to assay thermophilic actinomycetes, or 65 °C, the highest temperature at which the selected thermophilic actinomycetes grow, for 3 days to allow for growth and the production and diffusion of

metabolites. Two temperatures were used in order to determine variability of the antagonistic activity. An agar plug containing actively growing fungus was then placed onto the opposite side of the inoculated plates, and the plates were incubated at 30 °C to allow for fungal growth. Fungal mycelial plugs were placed on non-inoculated plates as controls and were also incubated at 30 °C. Growth inhibition was recorded at different time intervals, depending on the growth rate of the test fungus.

Enzyme production with different substrates and electrophoretic profiles

Crude enzyme production. Selected thermophilic isolates were grown in CM broth containing one of the following substrates (% w/v): 0.3% colloidal chitin (CC), 0.1% fungal cell wall (FCW), 0.1% CC/0.1% FCW, 0.1% CC/0.3% FCW or 0.3% CC/0.1% FCW. Culture conditions were as stated above. The FCW were prepared from *Fusarium sambucinum* as described previously (González-Franco et al., 2003). Chitinase activity was determined at 24 h, 48 h, 72 h, and 96 h.

Chitinase and protein determination. Chitinase-like activity was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-N, N'-diacetylchitobiose [pNP-$(GlcNAc)_2$], as described by Frandberg and Schnurer (1994). The *p*-nitrophenyl-N, N'-diacetylchitobiose, was dissolved in 50 mmol/L potassium phosphate buffer at pH 6.1 to a final concentration of 0.05 M. Enzyme assays were performed in a microtitre plate at 37 °C with 10 µL crude enzyme and 90 µL of the pNP- $(GlcNAc)_2$. The reaction was terminated by adding 10 µL of 1 mol/L NaOH. Absorption was measured at 405 nm in an Automated Microplate Reader (Model Elx800, Bio-Tek Instruments, Inc, Winooski, Vermont, USA). Chitinase-like activity was estimated from a standard curve of *p*-nitrophenol. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 µmol of p-nitrophenol/min. Total protein of microbial culture supernatant was determined by the method of Bradford (Bradford, 1976).

Polyacrylamide gel electrophoresis (PAGE) under native conditions. Native PAGE was performed at pH 8.9 according to the procedure described by Davis (Davis, 1964) using 8 and 12% (w/v) polyacrylamide resolving gels, containing 0.01% (w/v) glycol chitin (for the detection of chitinase activity) or glycol chitosan (for the detection of chitosanase activity) and 5% (w/v) polyacrylamide stacking gels. Samples contained 62.5 mM Tris-Cl buffer (pH 6.8), 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue. Chitinase (EC 3.2.2.14) from *Streptomyces griseus* and a-Lactoalbumin Type III from Bovine Milk were used as protein standards. Electrophoresis was performed in a Mini-Protean II Electrophoresis system (Bio Rad, Hercules CA) at room temperature. Samples (20 µg ± 2 per well) were electrophoresed through the stacking gel at 95V and through the resolving gel at 118V.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed by the method of Laemmli (Laemmli, 1970). The samples were applied to a 11.25 % (w/v) polyacrylamide resolving gel containing 0.01% (w/v) glycol chitin (for chitinase activity) or glycol chitosan (for chitosanase activity), and 0.1% (w/v) SDS. Protein samples were previously treated with pefabloc SC [4-(2-Aminoethyl)-benzenesulfonyl-flouride, hydrochloride] (Roche Diagnostics Co., Indianapolis, IN) (2 mM) to inhibit the hydrolytic action of proteases. The samples were boiled for 5 minutes in 12 mM Tris-HCl buffer (pH 6.8) containing 5% (v/v) glycerol, 0.4% (w/v) SDS with or without 1.1% (v/v) β-mercaptoethanol and bromophenol blue (0.01%) (w/v). Gels were run as described for native gels. The protein loaded per well for all strain samples was 20 µg ±2.

Detection of chitinase and chitosanase activity in native gels. Chitinase activity was assayed in crude extracts by a modified method of Trudel and Asselin (Trudel & Asselin, 1989). Briefly, after electrophoresis, the gels were incubated in 100 mM sodium acetate buffer (pH 5) for 7 minutes at room temperature with reciprocal shaking. The gels were removed, and then after a further incubation at 37 °C for 2 hours, under moist conditions, the gels were stained with 0.01% (w/v) Calcofluor white M2R in 500 mM Tris-HCl (pH 8.9). After 5 min, the fluorescent solution was removed and the gels were incubated for an hour in distilled water at room temperature. Lytic zones were visualized on a Gel Doc 1000 fluorescent gel documentation system with uv illumination. Gels were also stained with Coomasie Brillant Blue R-250 to visualize protein.

Detection of chitinase and chitosanase activity after SDS-PAGE. Gels were subjected to renaturation for 30 min at room temperature in 150 mM sodium acetate buffer (pH 5) supplemented with 1% (v/v) Triton X-100. Fresh solution was replaced in gels, following a four-hour incubation at 37 °C. Gels were then stained and lytic zones were visualized under uv illumination as for native gels.

Statistical analysis. The data were statistically analyzed (ANOVA) and the treatment means were compared by Tukey's Studentized Range (HSD). All results reported are the average of three determinations. The package used for analysis was SAS 8.2 Copyright (c) 1999-2001 by SAS Institute Inc., Cary, NC, USA.

RESULTS

Screening of isolates. Two thermophilic actinomycete strains AC4 and AC7, from the horse manure compost were selected for their ability to degrade CC in solid fermentation and to produce chitinase-like activity in liquid media. These isolates grew slowly (7 days) on CM agar supplemented with CC, but the colonies were surrounded by a clear hydrolysis zone. In

the cellulase assay on CM agar supplemented with CMC the strains grew fast (3 days) and similar clear zones were observed, though a little bigger with strain AC7 (Data not shown).

The microscopic morphology of the two isolates was observed after growth on ISP2 agar. Both strains, AC4 and AC7 were Gram-positive filamentous cells, with branched aerial mycelium. The initial growth stage of AC4 and AC7 was observed in figure 4a and 4e respectively; it was characterized by unfragmented vegetative mycelium, which starts differentiating into aerial mycelium. Sporophores were observed which contained chains of more than 10 spores in an arrangement of open loops and hooks (retinaculum-apertum), though short spiral spore chains were also observed in the mature stage (Fig. 1).

Molecular identification of selected isolates. Comparison of partial 16S rRNA gene sequences to those of previously described *Streptomyces*, showed that sequences of strains AC4 and AC7 were similar to some members of the genus *Streptomyces*. The partial 16S rDNA sequence (689 bp) of strain AC7 had 100% identity with *Streptomyces thermovulgaris* (accession No. Z68094); while sequence of strain AC4 (689 bp) had 99% identity with *Streptomyces thermovulgaris* (accession No. Z68094) and *Streptomyces thermonitrificans* (accession No. Z68098). To determine the relatedness of both Streptomycete isolates with other Streptomyces species, including mesophilic and thermophilic strains, phylogenetic analysis was performed with selected streptomycete type strains (Fig. 2). It was evident from the phylogenetic tree that both strains, AC4 and AC7, were located in the major cluster of thermophilic streptomycetes in closed proximity to the thermophilic *Streptomyces thermovulgaris* and *Streptomyces thermoviolaceus*.

Growth temperature assay and macroscopic culture characteristics. The isolates AC4 and AC7 grew up aerobically at different temperatures ranging from 30 ˚C to 65 ˚C on TYE plates, but none of the isolates grew at 75 ˚C. Poor growth and sporulation was observed at 30 ˚C (10 days), while good sporulation and robust growth were observed at 37 ˚C (48 h) and 55 ˚C (48 h). At 45 ˚C both strains showed good growth by 96 h and produced gray spores. Growth at 65 ˚C was observed after 24-30 h for both strains, with dark gray spores for strain AC4 and white aerial growth (no spore chains were generated from the aerial mycelium) for strain AC7 (longer incubation times did not change these results). The aerial mycelium of strain AC4 characteristically had dark brown to black areas, with a hygroscopic appearance, especially when grown at 37 ˚C and 45 ˚C. The color of the substrate mycelium of strain AC4 was yellow, with a brown tint in some growth edges. For isolate AC7, it was light yellow to yellow-brownish depending on growth temperature. Diffusible pigments were not produced by either strain. These isolates also showed antifungal activity in the antagonist assay along with the production of chitinase-like activity in solid and liquid media.

Fungal inhibition bioassays. Antifungal bioactivities of isolates AC4 and AC7 were tested against the filamentous fungi at two different temperatures. Both strains exhibited antifungal activity. Incubation of both strains at 45 ˚C or 65 ˚C showed no difference in antagonistic activities against *R. solani* (100% radial growth inhibition). However, isolate AC4 showed higher anti-*Fusarium* activity at 45 ˚C (60% radial growth reduction) than at 65 ˚C (53% radial growth reduction). Isolate AC7 showed higher anti-*Fusarium* activity at 65 ˚C (53% radial growth reduction) than at 45 ˚C (44% radial growth reduction).

Crude enzyme production. Highly significant differences were observed in chitinase production by Streptomyces sp. strains AC4 and AC7 depending on the substrate. The substrate combination of colloidal chitin (CC) with fungal cell wall (FCW), especially the 0.1% CC/0.3% FCW and 0.3% CC/0.1% FCW media, yielded the highest production, followed by 0.1% CC/0.1% FCW, 0.3% FCW, and 0.3% CC treatment, respectively (Table 1). Maximum chitinase production peaked between 24 to 48 hours, depending on the substrate and strain (Fig. 3).

Chitinase and chitosanase activity on native gels. Chitinase active proteins were readily detected on PAGE gels containing 0.01% of glycol-chitin, a substrate used by most chitinases. The Davis system was run at alkaline pH (8.9) and was used to separate neutral or acidic proteins. Both strains AC4 and AC7 produced proteins that were able to hydrolyze the glycol-chitin under native conditions. Figure 4a shows a PAGE gel of AC4, which yielded one chitinolytic band with moderate electrophoretic mobility in all substrate treatments (band b), and another band that migrated a bit further (band c) was detected in all substrate treatments except 0.3% CC. In this medium, we observed only a chitinolytic active band with low electrophoretic mobility (band a). A wide chitinolytic area was also detected on the top of the resolving gel; however, under the Davis native PAGE system, it was not possible to separate individual chitinolytic bands from this area, since poor electrophoretic mobility was observed. Reduction of the polyacrylamide in the gel did not enhance band separation, nor mobility of the chitinolytic active zone, suggesting the presence of basic chitinases (Trudel & Asselin, 1989). The AC7 PAGE gel showed only an active chitinase zone on top of the resolving gel with no mobility with all the substrate treatments (Fig. 4b) probably due to the reasons discussed above. Chitosanase activity was also detected on PAGE gels containing 0.01% of glycol-chitosan. Chitosanase activity was detected in two bands with moderate electrophoretic mobility in both strains, with all substrate treatments of the culture media (Fig. 4, c and d).

Chitinase activity after SDS-PAGE. To estimate the molecular weights of the chitinase-like enzymes, the cul-

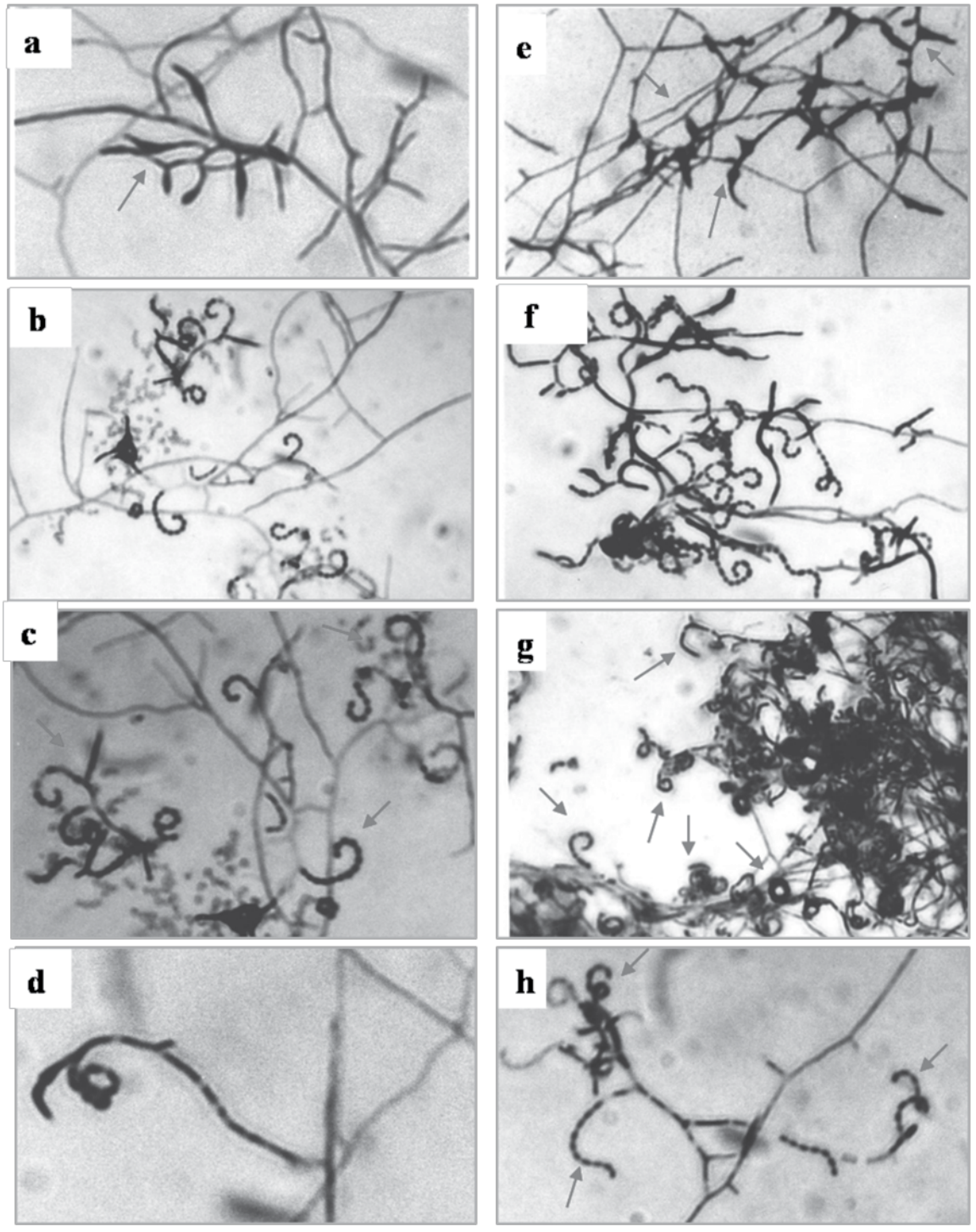

Fig. 1. Microscopic morphological structures of *Streptomyces* sp. AC4 (a-d) and AC7 (e-h).
Fig. 1. Morfología microscópica de *Streptomyces* sp. AC4 (a-d) y AC7 (e-h).

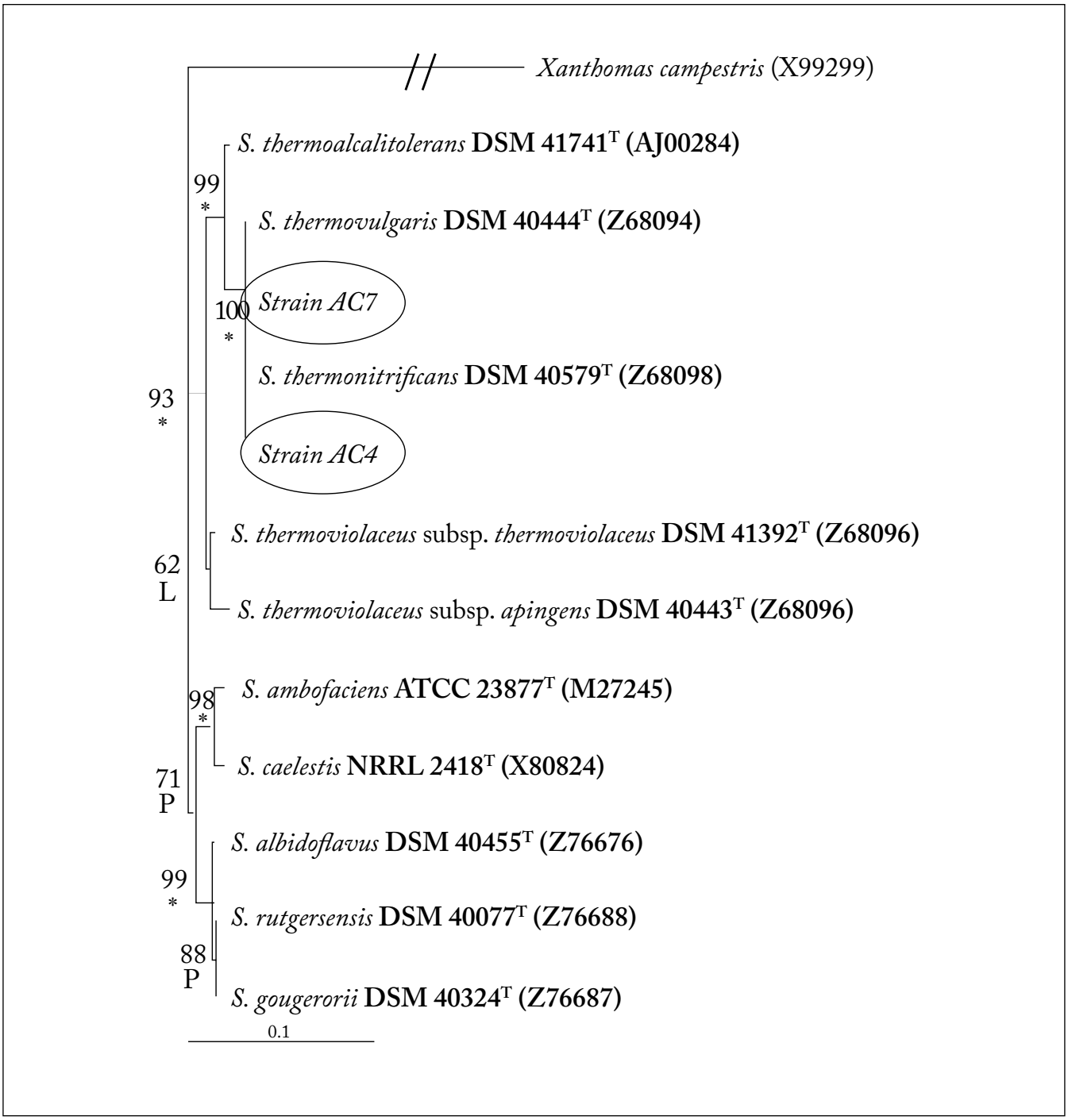

Fig. 2. Relationship of *Streptomyces* sp. AC4 and AC7 with their nearest phylogenetic relatives in a neighbor-joining three based on partial 16S rDNA sequences. L and P indicate branches that were also found by the maximum-likelihood and maximum-parsimony methods, respectively; the asterisks indicate branches recovered with all three methods. The numbers at the nodes indicate the level of bootstrap support based on a neighbor-joining analysis; only values that were >56% are given. The scale bar indicates 0.1 substitutions per nucleotide position.

Fig. 2. Relación filogenética de *Streptomyces* sp. AC4 and AC7 con sus similares empleando el algoritmo de neighbor-joining basado en las secuencias parciales del gen ribosomal 16S. L y P indican las ramas que también fueron encontradas con los métodos de máxima verosimilitud y máxima parsimonia, respectivamente; los asteriscos indican las ramas que se recobraron con los 3 métodos. El número en cada nodo indica el nivel de soporte estadístico basado en un análisis de nj; sólo valores superiores a 56% son proporcionados. La escala de la barra indican 0,1 sustituciones por posición de nucleótido.

Table 1. Effect of different combinations of colloidal chitin and fungal cell wall on the chitinase activity of two thermophilic streptomycetes.
Tabla 1. Efecto de diferentes combinaciones de quitina coloidal y pared celular fúngica en la actividad quitinolítica de dos estreptomicetos termofílicos.

Substrates [b]	Strains [a]	
	AC4	AC7
	Chitinase-like activity (U/μg protein)	Chitinase-like activity (U/μg protein)
0.3% CC	0.1618 d	0.1388 a
0.3% FCW	0.2420 cd	0.2523 ab
0.1% CC/0.1% FCW	0.6263 a	0.3291 a
0.1% CC/0.3% FCW	0.3146 bc	0.2635 ab
0.3% CC/0.1% FCW	0.4060 b	0.3671 a

Means followed by the same letter within a column are not significantly different (P=0.05) by Tukey's Studentized Range (HSD) Test. Values are the mean of 3 independent experiments.
CC: colloidal chitin; FCW: cell wall of *Fusarium sambucinum*.

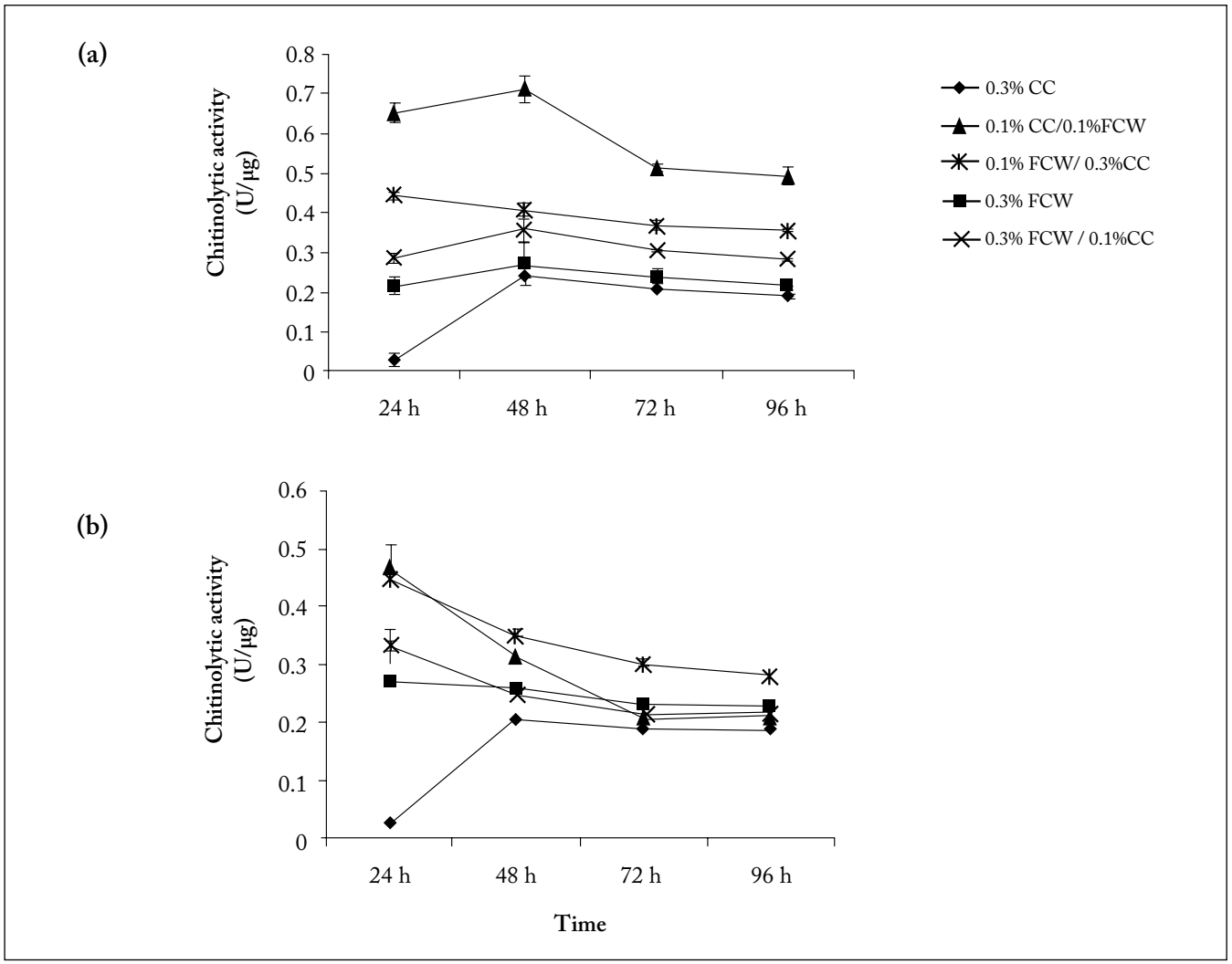

Fig. 3. Time course of chitinase-like production with five different substrates during the culture of the thermophilic strains AC4 (a) and AC7 (b).
Fig. 3. Dinámica en la producción de quitinasas con cinco diferentes substratos durante la cultivo de las cepas termofílicas AC4 (a) y AC7 (b).

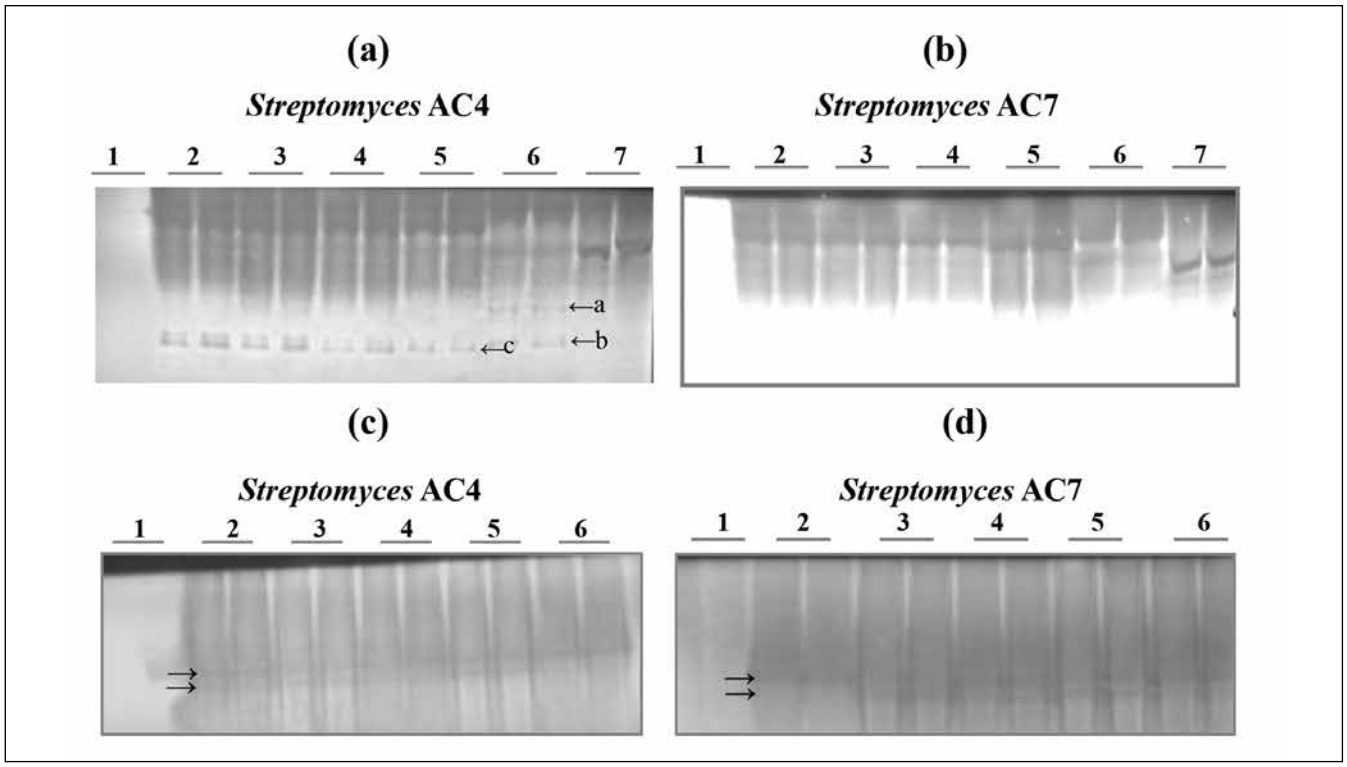

Fig. 4. Detection of chitinolytic (a and b) and chitosanolytic (c and d) activities of extracellular proteins of isolates AC4 and AC7 after native-PAGE. Strains were grown on five different combinations of coloidal chitin (CC) and fungal cell wall (FCW): 0.3% CC/0.1% FCW (lane 2), 0.1% CC/0.3% FCW (lane 3), 0.1% CC/0.1% FCW (lane 4), 0.1% FCW (lane 5), 0.3% CC (lane 6). Bovine milk α-lactalbumin (38 μg) (lane 1 of upper gels) was included as negative control. Chitinases of *S. griseus* (0.28U) (lane 7) were used as a positive control for chitinase activity, and was included in gels c and d (lane 1) as negative control for chitosanase activity.

Fig. 4. Detección de actividades quitinolíticas (a y b) y quitosanolíticas (c y d) de proteínas extracelulares de cultivos de AC4 y AC7 despues de PAGE nativo. Las cepas fueron crecidas en cinco diferentes combinaciones de quitina coloidal (CC) y pared celular fúngica (FCW): 0,3% CC/0,1% FCW (carril 2); 0,1% CC/0,3% FCW (carril 3); 0,1% CC/0,1% FCW (carril 4); 0,1% FCW (carril 5); 0,3% CC (carril 6). α-Lactalbumina de leche bovina (38 μg) (carril 1 del gel superior) fue incluida como control negativo. Chitinasas de *S. griseus* (0,28U) (carril 7) fueron usadas como control positivo para la actividad quitinolítica en los geles a y b (carril 1) y como control negativo para la actividad de quitosanasas en los geles c y d (carril 1).

ture supernatants were also examined under non-native PAGE conditions. Denaturation of the proteins was carried out with SDS and SDS plus β-mercaptoethanol (β-ME) (González-Franco et al., 2003). After renaturation, active chitinase isoforms were detected in the glycol-chitin containing gels. The effect of denaturation with SDS or SDS plus β-ME on renaturation of chitinases produced by the two thermophilic isolates was observed. Under both denaturation conditions, two chitinolytic bands of 32 and 34 kDa were present after renaturation, though less intense activity was detected when SDS plus β-ME was used as the denaturant (Fig. 5). The combination of β-ME and SDS further reduced the renaturation efficiency of the enzymes, suggesting the presence of susceptible disulfide bonds in the isoforms.

DISCUSSION

Two thermophilic actinomycetes were selected for study based upon their chitinolytic activity produced in CM medium supplemented with 0.3% of CC as a carbon source. Identification of the selected isolates, AC4 and AC7, was performed by morphological characteristics, 16S partial sequences and phylogenetic analysis.

The nucleotide sequence of the partial 16S rDNA gene of strains AC4 and AC7, and their comparison with the 16S rDNA sequences of previously studied *Streptomyces* confirmed the assignment of strains AC4 and AC7 into species of thermophilic *Streptomyces*.

Morphological characteristics of strains AC4 and AC7 fit the descriptions of those matches, which are very similar

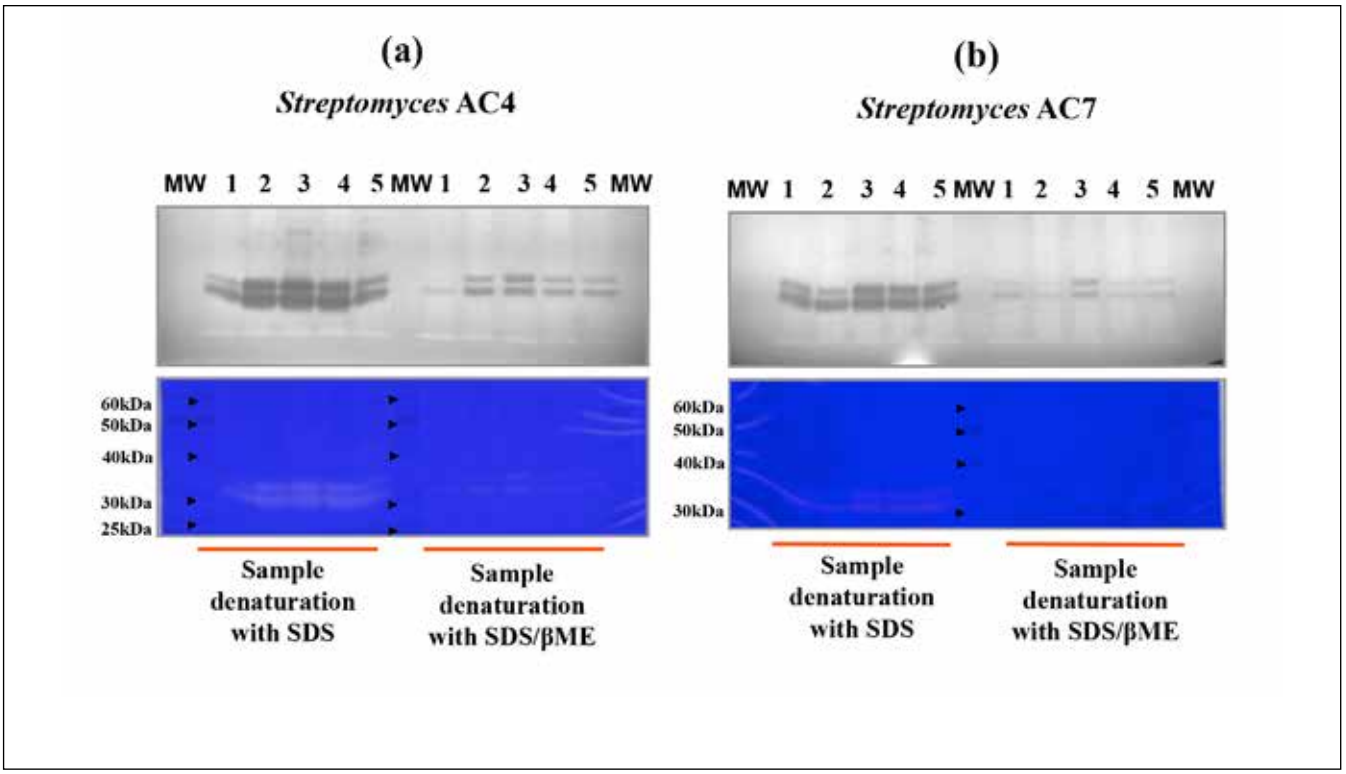

Fig. 5. Chitinolytic activity of extracellular proteins after SDS-PAGE in gels containing glycol chitin. The samples were obtained from cultures of the thermophilic isolate (a) AC4 and (b) AC7 grown in CM medium containing one of the following substrates: 0.3% (w/v) CC (1), 0.1% (w/v) FCW (2), 0.1% (w/v) CC/0.1% (w/v) FCW (3), 0.1% (w/v) CC/0.3% (w/v) FCW (4) and 0.3% (w/v) CC/0.1% (w/v) FCW (5). Samples were denatured with SDS or SDS plus β-mercaptoethanol as indicated in each gel. The upper gel shows bands with chitinase activity which appear as dark zones under uv illumination after staining with the fluorescent Calcofluor white M2R while the bottom gels are photographs of the Coomassie blue stain of the same gel to estimate the molecular weights of the isozymes.

Fig. 5. Actividad quitinolítica de proteínas extracelulares después de SDS-PAGE en geles que contienen chitina glicol. Las muestras fueron tomadas del cultivo de los aislados termofílicos AC4 (a) y AC7 (b) crecidos en medio CM conteniendo como sustrato diferentes combinaciones de quitina coloidal (CC) y pared celular fúngica (FCW): 0,3% (w/v) CC (1); 0,1% (w/v) FCW (2); 0,1% (w/v) CC/0,1% (w/v) FCW (3); 0,1% (w/v) CC/0,3% (w/v) FCW (4), y 0,3% (w/v) CC/0,1% (w/v) FCW (5). Las muestras fueron desnaturalizadas con SDS o SDS más β-mercaptoetanol como se indica en cada gel. El gel superior muestra las bandas con actividad quitinolítica mientras que los geles inferiores fueron teñidos con azul de Coomassie para estimar el peso molecular de las isozimas.

(Goodfellow et al., 1987; Williams et al., 1989). *Streptomyces* sp. AC7 was identified as member of the *Streptomyces thermovulgaris* species and located in the *S. thermovulgaris* clade (Goodfellow et al., 1987). On the other hand, *Streptomyces* sp. AC4 could not be assigned to a specific species since it shared 99% identity with both *S. thermovulgaris* and *S. thermonitrificans*, and in the phylogenetic analysis, the type strains of those best matches clustered together showing high similarity in their 16S rDNA sequences. Moreover, Kim *et al.* (1999) proposed that *S. thermonitrificans* Desai and Dhala 1967 (DSM 40579^T) be recognized as a subjective synonym of *S. thermovulgaris* Henssen 1957 (DSM 40444^T) based on DNA-DNA homology studies (Kim et al., 1999). Consequently, both strains (AC4 and AC7) could be located in the *S. thermovulgaris* clade, as members of the same species, but some differences were detected between the two strains, such as temperature effect in growth and sporulation. The thermophilic actinomycetes have growth temperatures between 28 °C and 60 °C, although there have been some reports of thermophilic *Streptomyces* growing at 65 °C (Waksman et al., 1939). Most of the studies of thermophilic streptomycetes, including those focused on optimization for production of metabolites and enzymes, were typically performed at 45 °C or 55 °C (James & Edwards, 1989; Edwards, 1993; Kim et al., 1999). In the present study, isolates AC4 and AC7 were tested for growth at different temperatures, and they were able to grow between 30 °C and 65 °C; though some differences between isolates were detected. Strain AC4 grew at 65 °C more robustly and with greater gray sporulation than at 45 °C, whereas the opposite was true for strain AC7. Growth at 65 °C is not common in the thermophilic streptomycetes, but these two isolates, especially AC4 are able to do so.

Although slight differences were detected in the native PAGE gels for the chitinolytic activities detected in AC4 cul-

ture fluids produced with different carbon sources, under denaturation conditions only two chitinolytic bands in all the substrate treatments were observed. The same isoform profiles were observed with isolate AC7. Similar tendencies of low profile diversity were observed for different fungi (primarily representing mycoparasitic and biocontrol fungi) by Inglis and Kawchuk (2002), who used different FCW as their carbon source (Inglis & Kawchuk, 2002). Reduction of band intensities in the chitinolytic isoforms after β-ME treatment, in samples of both actinomycetes, suggests the reduction of disulfide bonds compromises the activity of the chitinases (Trudel & Asselin, 1989).

Detection of chitosanase activity in all the substrate combinations was interesting given that chitosan is a deacetylated analog of chitin that is a characteristic component of the cell walls of zygomycetous fungi (Ruiz-Herrera, 1992). Induction of chitosanase activity by CC is explained because chitosan, a deacetylated analog of chitin, can be formed during the synthesis of CC, due to a strong acid hydrolysis of the crab shell chitin followed by neutralization with a strong alkali step that is required in the process (Hsu & Lockwood, 1975). However, chitosanase activity was also detected when only cell walls of *Fusarium sambucinum* (an ascomycetous fungus) were used as the carbon source. The CW of ascomycetous fungi does not contain chitosan (Ruiz-Herrera, 1992).

Chitinases are the most important enzyme class conferring antagonism against phytopathogens, considering that chitin is the most abundant component of the cell wall of many fungi (Bartnicki-Garcia, 1968). *Rhizoctonia solani* and *Fusarium oxysporum* are good examples of chitinous fungal phytopathogens. Actinomycete strains AC4 and AC7, which produce both chitinase and β1-4 glucanase activities, were assayed for antagonistic activity against *R. solani*, *F. oxysporum*. No effect of incubation temperature for either of the isolates was observed in the antagonistic activities against *R. solani*, which were very efficient (100% growth inhibition) with both isolates. However, slight differences in antagonistic activities against *F. oxysporum* were observed between the strains at the temperatures tested. AC4 antagonistic activity against *F. oxysporum* was slightly higher at 45 °C (60%) than at 65 °C (53%), while for AC7 antagonistic activity was higher at 65 °C (53%) than at 45 °C (44%). Effects of temperature on antimicrobial activities of microorganisms have been reported by others (Farrell & Rose, 1967). Differences in antagonistic activity of the culture fluids at different temperatures may be due to differences in rates of production of the metabolites by the strains, or due to intrinsic stabilities of the bioactive compounds. Although chitin is the major component of fungal phytopathogens, different degrees of antagonism have been observed. Sivan and Chet (1989) found that cell walls of *F. oxysporum* were more resistant to cell-wall degrading enzymes compared with those of *R. solani* (Sivan & Chet, 1989), while opposite results were obtained by Potgieter and Alexander (1966) (Potgieter & Alexander, 1966). Degradation of fungal cell walls is complex and most of the time synergistic enzymes of various classes are required to disrupt it, depending on the fungi (Driskill et al., 1999; Inglis & Kawchuk, 2002).

In an attempt to determine if the antifungal activity was mainly located in high molecular mass (protein phase) or in low molecular weight compounds (antibiotics), ultrafiltration of culture fluids of AC4 and AC7 was performed in a micro concentrator with a 10,000 Da molecular weight cut off membrane (Millipore, Marlborough, MA).

The culture fluids of both actinomycetes were obtained from growth in CM broth supplemented with 0.1% FCW/0.3% CC (substrate selected for its induction effect on chitinolytic activity). The 10-fold concentrated protein-containing phase and the filtrates (freeze-dried and resuspended at a 10-fold concentration in 50 mM phosphate buffer at pH 7), were tested by using disc diffusion assays against the same fungi used previously for antagonism assays. However, no inhibitory activity was detected in either fraction. One possible explanation is that the antagonism bioassays were performed on TYE plates under the effect of an agar matrix saturated with metabolites generated from the streptomycete growth, while the cultures used for the separation of enzymes and antibiotic phases were grown in CM broth medium (mainly chitinase inducer medium), and although the supernatant fractions (enzymes and antibiotics) were concentrated, the supplied metabolites (300 µL imbibed paper disc) were of insufficient concentration. Another explanation may be that the bacteria may not have been grown under appropriate conditions and that the chitinolytic proteins may not have a fungicidal effect by themselves, requiring the presence of other hydrolytic enzymes such as glucanases and secondary metabolites with antibiotic activity (that may not be produced under the colloidal chitin medium) to synergistically retard or inhibit the growth of the fungal pathogens. This fractionation study will be continued in future work to confirm whether the above result was indeed due to a nutritional component or due to synergy of multiple compounds that have been separated.

Overall, this present study shows that thermophilic streptomycetes have potential bioactivities that may be exploited. More specific characterizations of the bioactive metabolites, however, are required in order to determine the antifungal activities of the enzymes and antibiotics they produce. This study shows that thermophilic *Streptomyces* strains AC4 and AC7 are worthy of further investigation. Future work will be focused on separation and characterization of the antifungal metabolites and enzymes.

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