The Journal of Microbiology (2012) Vol. 50, No. 3, pp. 459–468 DOI 10.1007/s12275-012-2095-4
Copyright ⓒ 2012, The Microbiological Society of Korea

# Molecular Screening of *Streptomyces* Isolates for Antifungal Activity and Family 19 Chitinase Enzymes

**Youssuf Gherbawy[1,2*], Hesham Elhariry[1,3], Abdulla Altalhi[1], Bahig El-Deeb[1], and Ghada Khiralla[4]**

[1]*Department of Biology, Faculty of Science, Taif University, P.O.Box: 888-Taif, Kingdom of Saudi Arabia (KSA)*
[2]*Permanent address: Botany Department, Faculty of Science, South Valley University, Qena, Egypt*
[3]*Permanent address: Department of Food Science, Faculty of Agriculture, Ain Shams University, POB 68-Hadayek Shoubra, Cairo 11241, Egypt*
[4]*Department of Biotechnology, Faculty of Science, Taif University, P.O.Box: 888-Taif, Kingdom of Saudi Arabia (KSA)*

(Received February 21, 2012 / Accepted April 3, 2012)

**Thirty soil-isolates of *Streptomyces* were analyzed to determine their antagonism against plant-pathogenic fungi including *Fusarium oxysporum*, *Pythium aristosporum*, *Colletotrichum gossypii*, and *Rhizoctonia solani*. Seven isolates showed antifungal activity against one or more strain of the tested fungi. Based on the 16S rDNA sequence analysis, these isolates were identified as *Streptomyces tendae* (YH3), *S. griseus* (YH8), *S. variabilis* (YH21), *S. endus* (YH24), *S. violaceusniger* (YH27A), *S. endus* (YH27B), and *S. griseus* (YH27C). The identity percentages ranged from 98 to 100%. Although some isolates belonged to the same species, there were many differences in their cultural and morphological characteristics. Six isolates out of seven showed chitinase activity according to a chitinolytic activity test and on colloidal chitin agar plates. Based on the conserved regions among the family 19 chitinase genes of *Streptomyces* sp. two primers were used for detection of the chitinase (*chiC*) gene in the six isolates. A DNA fragment of 1.4 kb was observed only for the isolates YH8, YH27A, and YH27C. In conclusion, six *Streptomyces* strains with potential chitinolytic activity were identified from the local environment in Taif City, Saudi Arabia. Of these isolates, three belong to family 19 chitinases. To our knowledge, this is the first reported presence of a *chiC* gene in *S. violaceusniger* YH27A.**

***Keywords***: *Streptomyces*, family 19 chitinase, 16S rRNA, phytopathogenic fungi

## Introduction

*Streptomyces* is one of the most attractive sources of biologically active substances such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors (Omura, 1986; Bonjar, 2004). Soil *Streptomyces* are of the major contributors to the biological buffering of soils and have roles in decomposition of organic matter conducive to crop production (Keiser *et al.*, 2000). Studies even show that use of *Streptomyces* enhances growth of the crop plants (Bonjar, 2004). The search for new principles in biocontrol of plant pathogens different from the classically used fungicides, is of worldwide interest (McSpadden-Gardener and Fravel, 2002). Biological control of plant diseases is slow and gives few quick profits, but can be long-lasting, inexpensive, and harmless to life (Zarandi *et al.*, 2009). A collection of 53 antibiotic-producing *Streptomyces* isolated from soils from Minnesota, Nebraska, and Washington were evaluated by Xiao *et al.* (2002) for their ability to inhibit plant pathogenic *Phytophthora medicaginis* and *Phytophthora sojae in vitro* and to control Phytophthora root rots on alfalfa and soybean. *Streptomyces* isolates have significantly reduced root rot severity in alfalfa and soybean caused by *P. medicaginis* and *P. sojae*, respectively (Xiao *et al.*, 2002). In Turkey, Yücel and Yamaç (2010) reported that out of 290 *Streptomyces* isolates in a screening program, 180 isolates (62%) exhibited antimicrobial activity against a panel of four bacteria, two yeasts and four filamentous fungi. In Morocco, Errakhi *et al.* (2010) evaluated 10 antibiotic-producing *Streptomyces* spp. isolated from Moroccan soils for their ability to inhibit *Sclerotium rolfsii* development *in vitro*. Four isolates having the greatest pathogen inhibitory capabilities were subsequently tested for their ability to inhibit sclerotial germination in sterile soil (Errakhi *et al.*, 2010). They stated that treatment with biomass inoculum and culture filtrate gave the highest inhibition of sclerotia compared with the treatment with the spore suspension of the same isolates.

Chitin is one of the most abundant natural renewable polysaccharides in the world and is present in fungi, alga, insects and marine invertebrates. Chitin is hydrolyzed by two main enzymes, Chitinase (E:C:3.1.1.14) and ß-N-acetyl hexosaminidase (E.C.3.2.1.52) (Patil *et al.*, 2000). *Streptomyces* species are saprophytic soil bacteria and are well known as decomposers of chitin, which is hydrolyzed by chitinase (EC 3.2.1.14). Many chitinase genes of *Streptomyces* have been cloned and sequenced (Robbins, *et al.*, 1988; Miyashita *et al.*, 1991; Robbins *et al.*, 1992; Blaak *et al.*, 1993; Fujii and Miyashita, 1993; Miyashita and Fujii, 1993; Tsujibo *et al.*, 1993; Ohno *et al.*, 1996; Miyashita *et al.*, 1997; Saito *et al.*, 1998). Chitinases have a wide range of biotechnological applications such as: generation of fungal protoplasts (Vyas and Deshpande, 1989), crustacean chitin waste management (Suresh and Chandrasekaran, 1998), production of single cell protein (Vyas and Deshpande, 1991) and biocontrol of fun-

*For correspondence. E-mail: Youssufgherbawy@yahoo.com; Tel.: +00966-553993906

gal pathogens (Ordentlich *et al*., 1988). Chitinases so far sequenced are classified in two different families (namely, families 18 and 19) in the classification of glycosyl hydrolases based on amino acid sequence similarities (Henrissat, 1991; Henrissat and Bairoch, 1993). Family 18 contains chitinases from bacteria, fungi, viruses, and animals as well as class III and V chitinases from plants. On the other hand, class I, II, and IV plant chitinases belong to family 19, and this family solely comprises chitinases that are phylogenetically of plant origin. The two different families of chitinases display no sequence similarities with each other and have different three-dimensional structures (Davies and Henrissat, 1995).

A few studies have focused on the use of family 19 chitinase genes as molecular markers indicating the biocontrol abilities of saprophytic bacteria. The chitinases obtained from *Streptomyces* have been reported to have antifungal activity (Tsujibo *et al*., 2000). Taechowisan *et al.* (2004) have investigated the chitinolytic enzyme of the endophyte *Streptomyces aureofaciens* CMUAc130, which can be used for fungal cell wall degradation.

Due to its rapidity, sensitivity and specificity, PCR-based finger printing techniques have proved extremely useful in assessing the changes in microbial community structure. Such techniques can yield complex community profiles and can also provide useful phylogenetic information. Al-Kahtani *et al.* (2008) reported that *Streptomyces* species isolated from the Riyadh region, Saudi Arabia, have a high degree of genomic diversity and that the RAPD procedure can be a useful method to distinguish *Streptomyces*. Also, their results revealed that the RAPD technique appears to be a simple, quick and sensitive technique for the characterization of other local *Streptomyces*. Ribosomal RNAs are the molecules most widely employed in phylogenetic studies, with 16S rDNA used in prokaryotes and 18S rDNA used in eukaryotes (Weisburg *et al*., 1989). Over several years, the 16S rRNA gene (16S rDNA) has been sequenced in many prokaryotes, and the importance of this molecule has increased since its use in the current phylogenetic classification of prokaryotes in the Second Edition of Bergey's Manual of Systematic Bacteriology. Currently, the sequencing of 16S rDNA must be performed before the description of any bacterial species can be published. Moreover, the identification of bacteria, especially fastidious species, in human, animal and plant tissues is one of the most important applications of the 16S rDNA molecule in clinical diagnosis. Since the beginning of 16S rDNA sequencing, many studies have been performed in which the use of this molecule in phylogeny of animal pathogenic bacteria are described (Weisburg *et al*., 1991).

The aim of the present study is to screen and molecularly identify family 19 chitinase-containing *Streptomyces*. Their potential antagonism against some phytopathogenic filamentous fungi was also investigated.

## Materials and Methods

### Isolation of antagonistic strains

Soil samples were collected from a number of cultivation areas in the Taif region. One gram of sample was diluted ($10^2$ to $10^5$) with sterile water and plated on Gause's synthetic agar containing 0.01% potassium dichromate in order to minimize fungal contamination. The plates were then incubated for 14 days at 28°C. Single colonies obtained were purified by the quadrant streaking technique (5–6 times) on the same medium. For further work, the purified single cultures were maintained on Gause's synthetic agar slants at 4°C.

### Antagonistic activity

A 3 ml inoculum of each isolated strain, precultured in nutrient broth for 24 h at 28°C, was transferred to 30 ml YS medium [2.6% yeast extracts, 4.0% starch, 0.5% dextrin, 0.05% $K_2HPO_4$, 0.25% $MgSO_4{\cdot}7H_2O$, 0.4% $(NH_4)_2SO_4$, and 0.3% $CaCO_3$, pH 7.0]. Inoculated media were incubated at 28°C for 4 days on a rotary shaker at 180 rpm. The mature medium was then centrifuged at 3,000×g and the cell-free supernatant fluid was assessed for antifungal activities. Fungal strains (including *Fusarium oxysporum* ATCC 695; *Pythium aristosporum* ATCC 11101b; *Colletotrichum gossypii* ATCC 12788; and *Rhizoctonia solani* ATCC 16120) were used as model pathogenic fungi. Antagonistic isolates, which manifested significant inhibitory activity against tested fungal strains, were selected for further work. Antifungal activity was estimated on PDA medium using the hyphal extension-inhibition assay of Roberts and Selitrennikoff (1986).

### Classical identification of *Streptomyces* isolates

The morphological and cultural characteristics of *Streptomyces* strains were assessed in accordance with the method described by Shirling and Gottlieb (1966). Cultural characteristics were observed on International *Streptomyces* Project media (ISP-2, ISP-3, ISP-4, and ISP-5) according to the methods of Shirling and Gottlieb (1966). These media were: yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3), inorganic salt starch agar (ISP-4), and glycerol asparagine agar (ISP-5). Morphological characteristics were assessed via light microscopy after 7 days of culturing at 28°C. These characteristics, included color of aerial mycelium, color of reverse side of colony, color of soluble pigment and growth density. Microscopic features were observed after 7 and 14 days of culturing at 28°C on ISP-4 medium.

### DNA-extraction

Antagonistic isolates were grown on MGYP medium [containing (g/L): malt extract 3.0, glucose 5.0, yeast extract 3.0, peptone 5.0, agar 18.0, distilled water; pH 7.0]. For DNA isolation the organism was grown with shaking for 40 h at 30°C in baffled flasks containing 50 ml YEME medium [containing (g/L): yeast extract 3.0, malt extract 3.0, peptone 3.0, glucose 10.0, sucrose 340.0, distilled water; pH 7.2] supplemented with 5 mM $MgCl_2$ and 0.5% (w/v) glycine. The method described by Tripathi and Rawal (1998) was applied for extraction of DNA from the antagonistic isolates. Cultures were centrifuged at 8,000×g for 10 min at 4°C and the cells washed twice with STE buffer (0.3 M sucrose, 25 mM Tris·HCl, and 25 mM $Na_2EDTA$, pH 8.0); 1.0 g (wet/wt) mycelium was resuspended in 8.55 ml STE buffer and 950 ml lysozyme (20 mg/ml STE buffer) was then added. Protoplast formation took about 20–30 min at 30°C. 500 ml 10% SDS

(w/v) and 50 ml Pronase (20 mg/ml) were added and the mixture was held at 37°C for 1 h. 1.8 ml 5 M NaCl were added with gentle mixing to avoid shearing the DNA. 1.5 ml 10% (w/v) CTAB in 0.7 M NaCl (CTAB/NaCl solution) were added and incubated for 20 min at 65°C. After the addition of CTAB, all the steps were carried out at room temperature. The lysate was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol) and centrifuged at 12,000×g for 10 min. The aqueous phase was finally extracted with chloroform/isoamyl alcohol (24:1, v/v) and transferred to a fresh tube. 0.6 volume of propan-2-ol was added and DNA was spooled out after 10 min. Alternatively, it was recovered by centrifugation at 12,000×g for 10 min. The pellet was washed twice with 70% (v/v) ethanol, vacuum dried and dissolved in 2 ml TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0). RNaseA (50 mg/ml) was added and the mixture was incubated at 37°C for 2 h. The sample was again extracted with phenol as described above.

**DNA sequencing**

The PCR-amplified 16S rDNA fragments were amplified using two universal primers; forward: AGA GTT TGA TCC TGG CTC AG; reverse: ACG GCT ACC TTG TTA CGA CTT (Weisburg *et al*., 1991). The reaction mix was composed of: x µl Template DNA, 2 µl BigDye-Mix, 1 µl Specific primer (10 µmol/L), and HPLC water to a final volume of 10 µl. The amount of template DNA applied is dependent on the concentration of target sequences to obtain about 10 ng DNA in the final mix. The PCR program was as follows; initial denaturation at 96°C for 2 min (1 cycle), denaturation at 96°C for 10 sec (30 cycles), annealing at 45°C for 5 sec (30 cycles), extension at 60°C for 4 min (30 cycles), and then cooling at 4°C. The PCR product was purified as recommended by the manufacturer and then sequenced. PCR fragments were analyzed by cycle sequencing, using the BigDye terminator cycle sequencing kit (Applied Biosystems, UK). These fragments were sequenced in both directions.

**Sequence analysis**

Sequences were aligned using ClustalX software (Thompson *et al*., 1997). Sequence divergence was computed in terms of the number of nucleotide differences per site between pairs of sequences according to Kimura's two-parameter model (Kimura, 1980) using Geneious software. Idles were excluded from pair-wise sequence comparisons. The distance matrix for all pair-wise sequence combinations were analyzed with the neighbor-joining (NJ) method (Saitou and Nei, 1987). The phylogenetic tree was constructed with 1,000 bootstrap replicates using Geneious software.

**Molecular screening of family 19 chitinase-producing *Streptomyces***

According to the conserved regions among the family 19 chitinase genes of *Streptomyces* sp. two oligonucleotide primers, 5′-TTGACCCAGTGGTCCAGACC-3′ (forward primer) and 5′-GTGTGCTGCTCACGCCAG-3′ (reverse primer), were designed by Taechowisan *et al*. (2004). A standard PCR was performed in a total volume of 100 ml, containing 100 mM (each) deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 50 pmol of each PCR primer, 1.5 U of DNA polymerase and 50 ng of genomic DNA from *Streptomyces* sp. Each PCR utilized melting 94°C 5 min, then 35 cycles of 94°C 30 sec, 55°C for 1 min, and 72°C extension 1 min, with extension at 72°C for 10 min after the last cycle. The purified PCR products (10 µg) were separated on 1% agarose gel, stained with 1% ethidium bromide solution and then visualized by UV-transillumination.

**Determination of chitinase activity**

Twenty milliliters of Luria-Bertani (LB) liquid medium (Sambrook *et al*., 1989) were inoculated with germinated spores and incubated at 30°C for 48 h with shaking at 150 rpm. The culture was divided into three aliquots. Mycelia in each aliquot were harvested by centrifugation and washed with 10 ml of YE medium (0.7 g of $K_2HSO_4$, 0.3 g of $KH_2SO_4$, 0.5 g of $MgSO_4$, 0.01 g of $FeSO_4$, 0.3 g of $NH_4NO_3$, and 1.0 g of yeast extract per L). The mycelia were resuspended in an equal volume of YE medium and YE medium with 0.05% (w/v) colloidal chitin. The protein content of 1 ml of culture was measured as described by Bradford (1976), and the inocula were adjusted to about 100 mg of protein/ml. The cultures were grown at 30°C with shaking. A 0.5-ml portion of the culture fluid was sampled periodically 24, 48, 72, 96, and 120 h and then stored at -20°C. The samples were thawed and centrifuged, and chitinase activity was measured in the culture supernatant as described by Miyashita *et al.* (1991) using the fluorogenic substrate 4-methylumbelliferyl-N,N9,-diacetyl chitobioside [4MU-(GlcNAc)2] (Sigma). Three independent experiments were done and the average of chitinase activity was expressed in units per µg mycelial protein.

**Extracellular chitinolytic assay**

This assay was performed with the culture filtrates of the seven selected *Streptomyces* isolates using the well diffusion method. To prepare the colloidal chitin, 10 g chitin (Sigma) was treated with 100 ml phosphoric acid ($H_3PO_4$) according to Kim and Ji (2001). One loop of the pure culture of the seven tested isolates was inoculated into 100 ml nutrient broth containing 0.5% colloidal chitin and incubated for 48 h at 30°C and 150 rpm in a rotary shaker. One milliliter of the activated culture with 0.5 $OD_{570}$ was inoculated into 100 ml minimal medium containing 1% colloidal chitin and incubated for 48 h at 30°C and 150 rpm in a rotary shaker. The cultures were harvested, centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was collected (as a source of extracellular chitinase) Shanmugaiah *et al*. (2008). Colloidal chitin (0.5%) agar plates were prepared and wells were made using a 9 mm sterile cork borer. Culture filtrate (100 µl) of each isolate was placed in each well and incubated at 30°C. After 12 h, the development of any clear zone around the well was observed by staining with 1% Iodine solution.

**Statistical analysis**

The data for chitinase activity obtained from three replicates were analyzed by a two-way ANOVA using 'Proc Mixed' (SAS 8.2, Cary, NC). The level of statistical significance was performed with $P<0.05$ or $P<0.01$.

### Accession numbers

The nucleotide sequence data have been deposited at the EMBL nucleotide sequence database under accession nos. HE604197, HE604198, HE604199, HE604200, HE604201, HE604202, HE604203.

## Results and Discussion

### Isolation of *Streptomyces* strains and their antagonistic activity

Using the selective medium and cultivation conditions described previously, 30 *Streptomyces* isolates were obtained from 10 soil samples collected from different locations in Taif city, Kingdom of Saudi Arabia during the period of April–June 2011. All Isolates were tested for their antagonistic assay against *Fusarium oxysporum* ATCC 695; *Pythium aristosporum* ATCC 11101b; *Colletotrichum gossypii* ATCC 12788 and *Rhizoctonia solani* ATCC 16120 (Table 1). Of the thirty isolates only seven showed antifungal activity against one or more of the tested fungi. These isolates were YH3, YH8, YH21, YH24, YH27A, YH27B, and YH72C. The isolate YH8 and YH27C showed antifungal activity against the four tested fungi. YH21 and YH27A showed antifungal activity against only one; *Colletotrichum gossypii* and *Pytdhium aristosporum*, respectively. The other *Streptomyces* isolates showed antifungal activity against two of the tested fungi (Table 1), that differed for each isolate. The average range of the inhibition zone was 3±0.06 to 12.5±0.25 mm. In the present

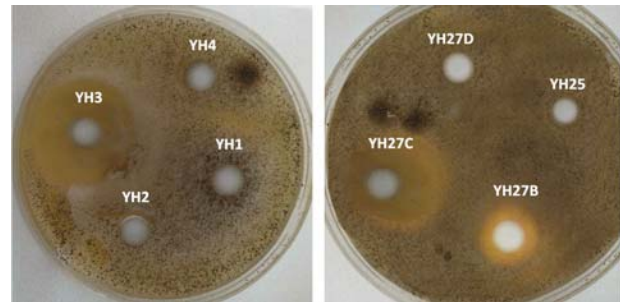


**Fig. 1.** Representative photograph of antagonistic action of the crude extract of some *Streptomyces* isolates against *Colletotrichum gossypii* ATCC 12788 grown for 4 days on PDA.

**Table 1.** Antifungal activities of *Streptomyces* isolates

| Isolates[a] | Antifungal activity of isolates against the following fungi | | | |
|---|---|---|---|---|
| | *Fusarium oxysporum* | *Pythium aristosporum* | *Colletotrichum gossypii* | *Rhizoctonia solani* |
| YH1 | - | - | - | - |
| YH2 | - | - | - | - |
| YH3 | - | + | +++ | - |
| YH4 | - | - | - | - |
| YH5 | - | - | - | - |
| YH6 | - | - | - | - |
| YH7 | - | - | - | - |
| YH8 | ++ | ++ | ++ | +++ |
| YH9 | - | - | - | - |
| YH10 | - | - | - | - |
| YH11 | - | - | - | - |
| YH12 | - | - | - | - |
| YH13 | - | - | - | - |
| YH14 | - | - | - | - |
| YH15 | - | - | - | - |
| YH16 | - | - | - | - |
| YH17 | - | - | - | - |
| YH18 | - | - | - | - |
| YH19 | - | - | - | - |
| YH20 | - | - | - | - |
| YH21 | - | + | - | - |
| YH22 | - | - | - | - |
| YH23 | - | - | - | - |
| YH24 | + | - | - | + |
| YH25 | - | - | - | - |
| YH26 | - | - | - | - |
| YH27A | - | + | - | - |
| YH27B | ++ | - | + | - |
| YH27C | ++ | ++ | +++ | ++ |
| YH27D | - | - | - | - |

[a] Based on their antifungal activity (as a diameter of inhibition zone); isolates were classified into four categories of no (-), weak (+), moderate (++) or strong (+++) activity.

**Table 2.** **Cultural characteristics, on different media, of *Streptomyces* isolates that had antifungal activity**

| Isolate No. | Yeast extract-malt extract (ISP-2) medium | | | |
|---|---|---|---|---|
| | Color of aerial mycelium | Color of reverse side of colony | Color of soluble pigment | Growth |
| YH3 | Yellowish white | Pale yellow | Beige | Heavy |
| YH8 | White | Grayish brown | Beige | Moderate |
| YH21 | Yellowish white | Pale yellow | Beige | Moderate |
| YH24 | Light yellow | Reddish yellow | Ivory | Scant |
| YH27A | Yellowish white | Pale yellow | Ivory | Moderate |
| YH27B | White | Yellowish white | Ivory | Moderate |
| YH27C | White | Grayish brown | Light grayish brown | Moderate |
| | Oatmeal (ISP-3) medium | | | |
| | Color of aerial mycelium | Color of reverse side of colony | Color of soluble pigment | |
| YH3 | White | Pale yellow | Beige | Moderate |
| YH8 | White | Pale yellow | Beige | Moderate |
| YH21 | White | Pale yellow | Beige | Moderate |
| YH24 | Pale yellow | Pale yellow | Beige | Moderate |
| YH27A | White | Pale yellow | Ivory | Moderate |
| YH27B | White | Pale yellow | Ivory | Moderate |
| YH27C | White | Pale yellow | Ivory | Moderate |
| | Inorganic salts-starch iron (ISP-4) medium | | | |
| | Color of aerial mycelium | Color of reverse side of colony | Color of soluble pigment | |
| YH3 | White | Pale yellow | Beige | Moderate |
| YH8 | Grayish white | Light olive brown | Light gray | Moderate |
| YH21 | Yellowish white | Yellowish white | Light brown | Moderate |
| YH24 | White | White | Beige | Moderate |
| YH27A | White | Yellowish white | Ivory | Moderate |
| YH27B | White | White | Beige | Moderate |
| YH27C | Grayish white | Light olive brown | Light gray | Heavy |
| | Glycerol-asparagine (ISP-5) medium | | | |
| | Color of aerial mycelium | Color of reverse side of colony | Color of soluble pigment | |
| YH3 | Yellowish white | Yellowish white | Ivory | Moderate |
| YH8 | White | Yellowish gray | Ivory | Moderate |
| YH21 | Yellowish white | Yellowish white | Ivory | Moderate |
| YH24 | Grayish white | Reddish yellow | Beige | Moderate |
| YH27A | White | Pale yellow | Ivory | Moderate |
| YH27B | Grayish white | Grayish brown | Ivory | Moderate |
| YH27C | Grayish white | Grayish brown | Ivory | Moderate |

study, we classified the isolated *Streptomyces* according to their antifungal activity (diameter of inhibition zone) into four categories of no (-), weak (+), moderate (++) or strong (+++) activity. The size range of the inhibition zone of these categories were, 0; 1 - < 4, 4 - < 8, > 8 mm, respectively.

According to these categories the *Streptomyces* isolate YH3 and YH27C had strong antifungal activity against *Colletotrichum gossypii* (Table 1 and Fig. 1), whereas, isolate YH27B showed weak antifungal activity. On the other hand, isolate YH27C had moderate activity against the other three tested fungi. The observed antifungal activity of the tested isolates may be due to production of chitinase or another antimicrobial agents such as secondary metabolites. In the earlier studies, chitinases from *Streptomyces* showed activity against fungi such as *Aspergillus* sp., *Phycomyces blakesleeanus*, and *Trichoderma reesei* (Williams *et al*., 1983). Hoster *et al.* (2005) reported chitinase activity against *A. nidulans* and phytopathogens such as *Botrytis cinerea*, *Fusarium culmorum*, *Gulgnardia bidwellii*, and *Sclerotia sclerotiorum*.

## Morphological and cultural characteristics of *Streptomyces* isolates

In general, the taxonomic classification and identification of *Streptomyces* are based on morphological and biochemical characterization. In the present study, some morphological and cultural characteristics of the seven active isolates were studied on four different ISP media (ISP-2, ISP-3, ISP-4, and ISP-5). The color of the aerial mycelium, color of the reverse side of the colony, color of soluble pigment and growth density are summarized in Table 2. Generally, the selected seven *Streptomyces* isolates grew on all tested media, but at different growth densities. Most of the *Streptomyces* isolates showed moderate growth on the four tested media. The abundance and the color of the mycelium depended on the medium composition and the age of the culture. It was noted that the color differed significantly for all isolated strains when ISP-2, ISP-4, or ISP-5 were used. However, few differences were observed when ISP-3 medium was used.

**Table 3.** Identification of antifungal-active *Streptomyces* isolates by partial sequencing of 16S rRNA gene

| Isolate No. | Database accession No. | Similarity according to EMBL BLAST (%) | Proposed identify |
|---|---|---|---|
| YH3 | HE604197 | 100 | *S. tendae* YH3 |
| YH8 | HE604198 | 98 | *S. griseus* YH8 |
| YH21 | HE604199 | 99 | *S. variabilis* YH21 |
| YH24 | HE604200 | 99 | *S. endus* YH24 |
| YH27A | HE604201 | 99 | *S. violaceusniger* YH27A |
| YH27B | HE604202 | 100 | *S. endus* YH27B |
| YH27C | HE604203 | 99 | *S. griseus* YH27C |

### Analysis of 16S rRNA gene sequence of *Streptomyces* isolates

The molecular methods, such as 16S rRNA gene sequence analysis, are more rapid and convenient than classical identification methods based on phenotypic characteristics, thus the molecular methods represent an important and alternative approach for identification of *Streptomyces* (Park *et al.*, 2006). The seven *Streptomyces* isolates obtained in the present study were identified based on partial sequencing of 16S rDNA. The obtained nucleotide sequence data have been deposited at the EMBL nucleotide sequence database under accession nos. HE604197, HE604198, HE604199, HE604200, HE604201, HE604202, and HE604203. According to the EMBL BLAST, the similarity percentages ranged from 98 to 100% (Table 3). Based on the BLAST results, the isolate YH8 and YH27C belonged to the same species, *Streptomyces griseus*, with similarity percentage 98 and 99%, respectively. Also, the isolate YH24 and YH27B belonged to species *Streptomyces endus*, with a similarity percentage of 99 and 100%, respectively (Table 3). Although the isolate YH24 and YH27B belonged to the same species, the cultural characteristics showed that there were differences between these two isolates. For example, the studied cultural characteristics were similar for these two isolates only on the ISP-4 medium, whereas they were dissimilar on the other ISP media (Table 2). Similar findings were recorded for the isolate YH8 and YH27C.

The phylogenetic tree based on the partial sequences of the 16S rRNA gene showed that the *Streptomyces* isolates grouped into two main clusters (Fig. 2). *S. griseus* YH8 and YH27C belonged to the same cluster, whereas, the other five isolates belonged to another cluster, which could be divided into two sub-clusters. The first one included the isolate *S. tendae* YH3 and *S. variabilis* YH21, whereas, the second included *S. endus* YH24, *S. violaceusniger* YH27A, and *S. endus* YH27B (Fig. 2). Because morphological characteristics of *S. antimycoticus* were unavailable, we cannot compare it with the cultural characteristics of *S. violaceusnigi* obtained in the present study; however, these two strains belonged the same branch in the same sub-cluster (Fig. 2).

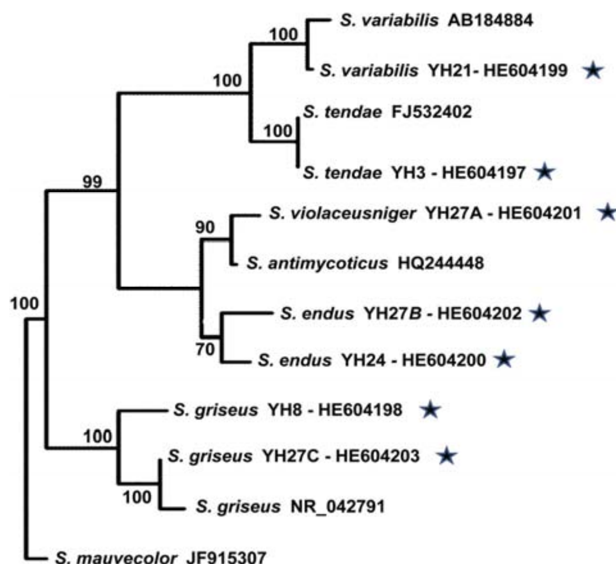


**Fig. 2.** Phylogenetic tree based on the partial sequences of 16S rDNA gene comparisons of the *Streptomyces* strains isolated in this study (marked with stars) and other known *Streptomyces* strains in the GenBank Database, using the neighbor-joining method.

### Determination of chitinase activity

The chitinase activity of the seven *Streptomyces* isolates was studied by determining the chitinase activity of each isolate at intervals from 24 h to 120 h (Fig. 3). All tested strains started production of chitinase after 24 h except the isolates YH27B and YH27C. The optimal time for obtaining the maximum chitinase activity differed according to the tested isolate. For example, the maximum chitinase activity was recorded after 72 h for isolate YH21, YH24, and YH27A, and 96 h for isolate YH3, YH8, YH27B, and YH27C (Table 4). In previous studies, the optimal time for chitinase production has also differed according to the tested strains. Narayana and Vijayalakshmi (2009) found that chitinase production by *Streptomyces* sp. ANU 6277 started after 24 h of incubation and reached maximum levels after 60 h of cultivation, whereas Kim and Ji (2001) found that *Streptomyces griseus* HUT6037 reached to the maximum level after 72 h of cultivation. In the present study, the highest chitinase activity was recorded for the isolate *S. griseus* YH27C and the lowest activity was observed by the isolate *S. tendae* YH3. Statistically, there are no significant differences ($P<0.05$ or $P<0.01$) among YH8, YH21, YH27A, and YH27C. Therefore, the area under curve of each diagram in Fig. 3 was calculated and presented in Table 4. The area under the curve for each isolate indicates the stability of chitinase activity during the experimental period (120 h). According to the calculated area under curve, the isolate *S. variabilis* YH21 had the highest stability, followed by *S. griseus* YH8, *S. endus* YH24, *S. griseus* YH27C and *S. violaceeusniger* YH 27A. The lowest stability of chitinase activity was seen for *S. endus* YH27B.

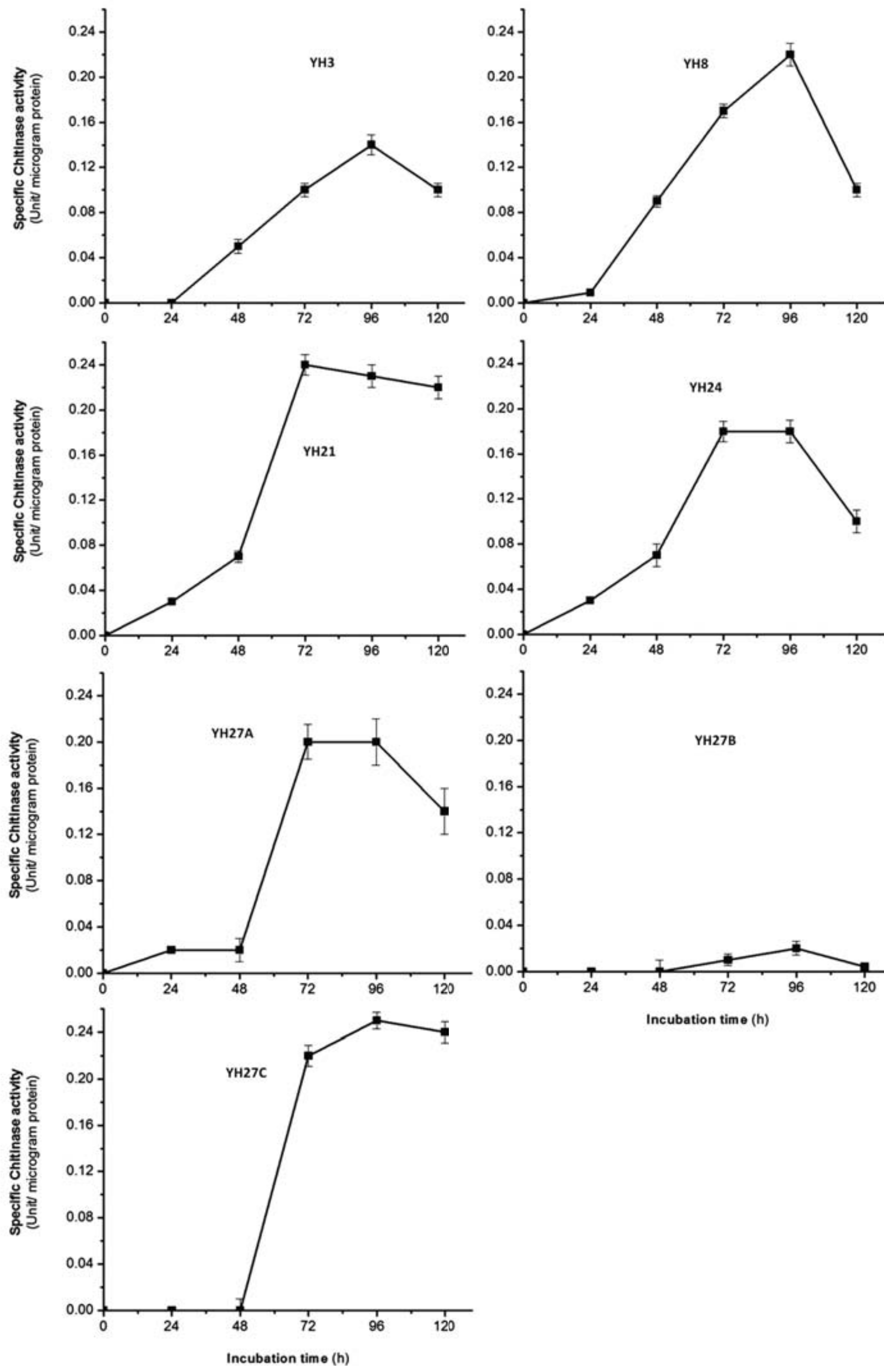


**Fig. 3. Chitinolytic activity of seven tested *Streptomyces* isolates for 120 h at 30°C.** The tested isolates were *S. tendae* YH3, *S. griseus* YH8, *S. variabilis* YH21, *S. endus* YH24, *S. violaceusniger* YH27A, *S. endus* YH27B, and *S. griseus* YH27C.

## Extracellular chitinolytic assay

The extracellular chitinolytic activity of the examined isolates was determined on colloidal chitin agar plates (Fig. 4). It was clearly observed that all tested isolates had extracellular chitinolyic enzymes except isolate YH27B, which did not show a clear zone around the well. According to the diameter of the clear zones, the isolate YH3 had the lowest activity compared with the other tested isolates. Results of the extracellular chitinase activity test on the colloidal chitin agar plates demonstrated that this is an adequate test, especially when iodine solution was used for visualizing the clear zone. The diameter of the clear zone could be considered as a good indicator for chitinase activity. There are many studies that used this test for indicating chitinolytic activity (Shanmugaiah *et al*., 2008; Narayana and Vijayalakshmi, 2009). Also, extracellular secretion of chitinase had been demonstrated previously for 13 strains of *Actinobacteria* (Kawase *et al*., 2004).

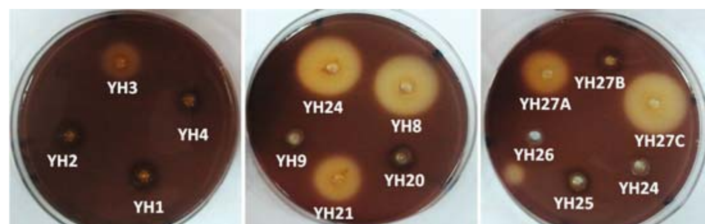


**Fig. 4. Extracellular chitinolytic activity of the crude extract (supernatant) on 0.5% colloidal chitin agar plates.** The crude extract (100 μl) was placed in the 9 mm wells and the plates incubated at 30°C for 12 h. Plates were flooded with 1% iodine solution for color development. Preparation of the crude extract was described in detail in the Methods section.

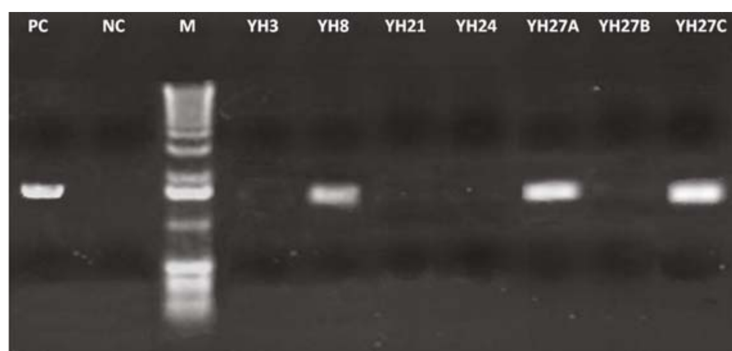


**Fig. 5. Agarose gel electrophoresis of PCR-amplified products from several *Streptomyces* DNAs using two PCR-primers targeting a region of the catalytic domain of family 19 chitinase genes.** PC, reference strain of *S. griseus* ATCC 12475 as a positive control (fragment with 1.4 kb); NC, *B. subtilis* ATCC 6633 as a negative control; M, 1 kb leader DNA marker; YH3–YH27C, seven *Streptomyces* isolates that showed an antagonistic effect against some pathogenic fungi.

**Molecular screening of *chiC*-gene-containing *Streptomyces***

Plant family 19 chitinases are thought to be part of a defense mechanism against fungal pathogens, and some plant chitinases exhibit antifungal activity *in vitro* (Leah *et al*., 1991; Collinge *et al*., 1993; Heitz *et al*., 1994). Since chitinases of some *Streptomyces* species share significant sequence similarity to plant family 19 chitinases in the catalytic domain, the antifungal activity of ChiC was examined and a remarkable ability of ChiC to inhibit hyphal extension of *Trichoderma reesei* was demonstrated by Itoh *et al.* (2002).

The seven *Streptomyces* strains that showed antifungal activity were screened for the presence of a *chiC* gene belonging to the family 19 chitinases. A DNA fragment of 1.4 kb was observed only for the isolates *S. griseus* YH8, *S. violaceusniger* YH27A, and *S. griseus* YH27C (Fig. 5). The other isolates did not show the target DNA fragment. The isolate YH27B showed antifungal activity, although it did not show chitinolyic activity. This means that the isolate TH27B produced an antifungal agent such as an antibiotic. Kawase *et al.* (2006) compared enzymatic properties of family 19 chitinases and family 18 chitinases produced by *Streptomyces coelicolor*. The four chitinases (Chi18bA, Chi18aC, Chi18aD, and Chi19F), whose genes are expressed at high levels in the presence of chitin, were produced in *Escherichia coli* and purified. Their results indicated that only Chi19F exhibited significant antifungal activity. Chitinase C (*chiC*) from *Streptomyces griseus* HUT6037, described in 1997, was the first family 19 chitinase found in an organism other than higher plants (Watanabe *et al*., 1999). In addition, the presence of genes similar to *chiC* of *S. griseus* HUT6037 have been suggested in at least 13 strains of *Actinobacteria* (Kawase *et al*., 2004). In the present study, the *chiC* gene was detected in two different strains of *S. griseus*. We also detected this gene in the *S. violaceusniger* strain YH27A. To our knowledge this is the first report on the presence of this gene in *S. violaceusniger*.

## Acknowledgements

This work was supported by a grant (Contract No. 1023-432-1) from Taif University, Kingdom of Saudi Arabia. The authors expresses their gratitude to Dr. Sayed Sadek (Department of English language, Faculty of Arts, University of Taif) for English revision of the manuscript.

**Table 4. Specific chitinase activity of seven *Streptomyces* isolates that showed antifungal activity**

| Isolate No. | Proposed name | MCA[a] (U/μg protein)[b] | Optimal time for MCA (h) | Area under curve ($mm^2$) |
|---|---|---|---|---|
| YH3 | *S. tendae* YH3 | $0.14^{Bb}$ ± 0.011 | 96 | 5.28 |
| YH8 | *S. griseus* YH8 | $0.22^{Aa}$ ± 0.014 | 96 | 9.096 |
| YH21 | *S. variabilis* YH21 | $0.24^{Aa}$ ± 0.013 | 72 | 10.92 |
| YH24 | *S. endus* YH24 | $0.18^{Bb}$ ± 0.016 | 72 | 8.88 |
| YH27A | *S. violaceusniger* YH27A | $0.20^{Aa}$ ± 0.014 | 72 | 8.16 |
| YH27B | *S. endus* YH27B | $0.02^{Cc}$ ± 0.006 | 96 | 0.48 |
| YH27C | *S. griseus* YH27C | $0.26^{Aa}$ ± 0.014 | 96 | 8.28 |

[a] MCA, Maximum Chitinolytic Activity.
[b] values with the same capital or small letters were not significantly different at probability levels of 0.05 or 0.01, respectively.

## References

**Al-Kahtani, M., AI-Khalil, A., AI-Kadeeb, S., and Hassan, H.Z.** 2008. Molecular genetic fingerprint of some *Streptomyces* isolated from Riyadh city. *Saudi J. Biological Sci.* **15**, 243–251.

**Blaak, H., Schnellmann, J., Walter, S., Henrissat, B., and Schrempf, H.** 1993. Characteristics of an exochitinase from *Streptomyces olivaceoviridis*, its corresponding gene, putative protein domains and relationship to other chitinases. *Eur. J. Biochem.* **214**, 659–669.

**Bonjar, S., Fooladi, M.H., Mahdavi, M.J., and Shahghasi, A.** 2004. Broadspectrim, a novel antibacterial from *Streptomyces* sp. *Biotechnol.* **3**, 126–130.

**Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

**Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., and Vad, K.** 1993. Plant chitinases. *Plant J.* **3**, 31–40.

**Davies, G. and Henrissat, B.** 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* **3**, 853–859.

**Errakhi, R., Bouteau, F., Lebrihi, A., and Barakate, M.** 2010. Evidences of biological control capacities of *Streptomyces* spp. against *Sclerotium rolfsii* responsible for damping-off disease in sugar beet (*Beta vulgaris* L.). *World J. Microbiol. Biotechnol.* **23**, 1503–1509.

**Fujii, T. and Miyashita, K.** 1993. Multiple domain structure in a chitinase gene (*chiC*) of *Streptomyces lividans*. *J. Gen. Microbiol.* **139**, 677–686.

**Heitz, T., Segond, S., Kauffmann, S., Geoffroy, P., Prasad, V., Brunner, F., Fritig, B., and Legrand, M.** 1994. Molecular characterization of a novel tobacco pathogenesis-related (PR) protein: a new plant chitinase/lysozyme. *Mol. Gen. Genet.* **245**, 246–254.

**Henrissat, B.** 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **280**, 309–316.

**Henrissat, B. and Bairoch, A.** 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **293**, 781–788.

**Hoster, F., Schmitz, J.E., and Daniel, R.** 2005. Enrichment of chitinolytic microorganisms: isolation and characterization of a chitinase exhibiting antifungal activity against phytopathogenic fungi from a novel *Streptomyces* strain. *Appl. Microbiol. Biotechnol.* **66**, 434–442.

**Itoh, Y., Kawase, T., Nikaidou, N., Fukada, H., Mitsutomi, M., Watanabe, T., and Itoh, Y.** 2002. Functional analysis of the chitin-binding domain of a family 19 chitinase from *Streptomyces griseus* HUT6037: substrate-binding affinity and cis-dominant increase of antifungal function. *Biosci. Biotechnol. Biochem.* **66**, 1084–1092.

**Kawase, T., Saito, A., Sato, T., Kanai, R., Fujii, T., Nikaidou, N., Miyashita, K., and Watanabe, T.** 2004. Distribution and phylogenetic analysis of family 19 chitinases in *Actinobacteria*. *Appl. Environ. Microbiol.* **70**, 1135–1144.

**Kawase, T., Yokokawa, S., Saito, A., Fujii, T., Nikaidou, N., Miyashita, K., and Watanabe, T.** 2006. Comparison of enzymatic and antifungal properties between family 18 and 19 chitinases from *S. coelicolor* A3(2). *Biosci. Biotechnol. Biochem.* **70**, 988–998.

**Keiser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A.** 2000. General introduction to actinomycete biology, pp. 1–21. *In* Practical *Streptomyces* genetics. The John Innes Foundation, England.

**Kim, K. and Ji, H.-S.** 2001. Effect of chitin sources on production of chitinase and chitosanase by *Streptomyces griseus* HUT 6037. *Biotechnol. Bioprocess Eng.* **6**, 18–24.

**Kimura, M.** 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120.

**Leah, R., Tommerup, H., Svendsen, I., and Mundy, J.** 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* **266**, 1564–1573.

**McSpadden-Gardener, B.B. and Fravel, D.R.** 2002. Biological control of plant pathogens: Research, commercialization and application in the USA. http://www.apsnet.org/online/feature/biocontrol/top.html.

**Miyashita, K. and Fujii, T.** 1993. Nucleotide sequence and analysis of a gene (*chiA*) for chitinase from *Streptomyces lividans* 66. *Biosci. Biotechnol. Biochem.* **57**, 1691–1698.

**Miyashita, K., Fujii, T., Watanabe, W., and Ueno, H.** 1997. Nucleotide sequence and expression of a gene (*chiB*) for a chitinase from *Streptomyces lividans*. *J. Ferment. Bioeng.* **83**, 26–31.

**Miyashita, K., Fujii, T., and Sawada, Y.** 1991. Molecular cloning and characterization of chitinase genes from *Streptomyces lividans* 66. *J. Gen. Microbiol.* **137**, 2065–2072.

**Narayana, K.J.P. and Vijayalakshmi, M.** 2009. Chitinase production by *Streptomyces* sp. Anu 6277. *Braz. J. Microbiol.* **40**, 725–733.

**Ohno, T., Armand, S., Hata, T., Nikaidou, N., Henrissat, B., Mitsutomi, M., and Watanabe, T.** 1996. A modular family 19 chitinase found in the prokaryotic organism *Streptomyces griseus* HUT 6037. *J. Bacteriol.* **178**, 5065–5070.

**Omura, S.** 1986. Philosophy of new drug discovery. *Microbiol. Rev.* **50**, 259–279.

**Ordentlich, A., Elad, Y., and Chet, I.** 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. *Phytopathology* **787**, 84–88.

**Park, H.S., John, J., and Kilbane, I.I.** 2006. Rapid detection and high-resolution discrimination of the genus *Streptomyces* based on 16S rRNA spacer region and denaturing gradient gel electrophoresis. *J. Ind. Microbiol. Biotechnol.* **33**, 289–297.

**Patil, R.S., Ghormade, V., and Deshpande. M.V** 2000. Chitinolytic enzymes: an expoloration. *Enzyme Microb. Technol.* **26**, 473–483.

**Robbins, P.W., Albright, C., and Benfield, B.** 1988. Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli*. *J. Biol. Chem.* **263**, 443–447.

**Robbins, P.W., Oberbye, K., Albright, C., Benfield, B., and Pero, J.** 1992. Cloning and high-level expression of chitinase-encoding gene of *Streptomyces plicatus*. *Gene* **111**, 69–76.

**Roberts, W.K. and Selitrennikoff, C.P.** 1986. Isolation and partial characterization of two antifungal proteins from barley. *Biochim. Biophys. Acta* **880**, 161–170.

**Saito, A., Fujii, T., Yoneyama, T., and Miyashita, K.** 1998. glkA is involved in glucose repression of chitinase production in *Streptomyces lividans*. *J. Bacteriol.* **180**, 2911–2914.

**Saitou, N. and Nei, M.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

**Sambrook, J., Fritsch, E.F., and Maniatis, T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA.

**Shanmugaiah, V., Mathivanan, N., Balasubramanian, N., and Manoharan, P.T.** 2008. Optimization of cultural conditions for production of chitinase by *Bacillus laterosporous* MML2270 isolated from rice rhizosphere soil. *Afr. J. Biotechnol.* **7**, 2562–2568.

**Shirling, E.B. and Gottlieb, D.** 1966. Methods for characterization of *Streptomyces* species. *Inst. J. Syst. Bacteriol.* **16**, 313–340.

**Suresh, P.V. and Chandrasekaran, M.** 1998. Utilization of prawn waste for chitinase production by the marine fungus *Beauveria bassiana* by solid state fermentation. *World J. Microbiol. Biotechnol.* **14**, 655–660.

**Taechowisan, T., Peberdy, F.P., and Lumyong, S.** 2004. PCR cloning and heterologous expression of chitinase gene of endophytic *Streptomyces aureofaciens* CMUAc130. *J. Gen. Appl. Microbiol.* **50**, 177–182.

**Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G.** 1997. The Clustal X windows interface: flexible

strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. **24**, 4876–4882.

**Tripathi, G. and Rawal, S.K.** 1998. Simple and efficient protocol for isolation of high molecular weight DNA from *Streptomyces aureofaciens. Biotechnol. Tech.* **12**, 629–631.

**Tsujibo, H., Endo, H., Minoura, K., Miyamoto, K., and Inamori, Y.** 1993. Cloning and sequence analysis of the gene encoding a thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520. *Gene* **134**, 113–117.

**Tsujibo, H., Okamoto, T., Hatano, N., Miyamoto, K., Watanabe, T., Mitsutomi, M., and Inamori, Y.** 2000. Family 19 chitinases from *Streptomyces thermoviolaceus* OPC-520: Molecular cloning and characterization. *Biosci. Biotechnol. Biochem*. **64**, 2445–2453.

**Vyas, P. and Deshpande, M.V.** 1989. Chitinase production by *Myrothecium verrucaria* and its significant for fungal mycelia degradation. *J. Gen. Appl. Microbiol.* **35**, 343–350.

**Vyas, P. and Deshpande, M.V.** 1991. Enzymatic hydrolysis of chitin by *Myrothecium verrucaria* chitinase complex and its utilization to produce SCP. *J. Gen. Appl. Microbiol.* **37**, 267–275.

**Watanabe, T., Kanai, R., Kawase, T., Tanabe, T., Misutomi, M., Sakuda, S., and Miyashita, K.** 1999. Family 19 chitinase of *Streptomyces* species: characterization and distribution. *Microbiology* **145**, 3353–3363.

**Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J.** 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**, 697–703.

**Weisburg, W.G., Tully, J.G., Rose, D.L., Petzel, J.P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T.G., Van Etten, J., and *et al.*** 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. *J. Bacteriol*. **171**, 6455–6467.

**Williams, S.T., Goodfellow, M., Alderson, G., Wellington, E.M.H., Sneath, P.H.A., and Sackin, M.** 1983. Numerical classification of Streptomyces and related genera. *J. Gen. Microbiol.* **129**, 1743–1813.

**Xiao, K., Kinkel, L.L., and Samac, D.A.** 2002. Biological control of phytophthora root rots on alfalfa and soybean with *Streptomyces*. *Biol. Control*. **23**, 285–295.

**Yücel, S. and Yamaç, M.** 2010. Selection of *Streptomyces* isolates from turkish karstic caves against antibiotic resistant microorganisms. *Pak. J. Pharm. Sci.* **23**, 1–6.

**Zarandi, M.E., Shahidi Bonjar, G.H., Dehkaei, F.P., Moosavi, S.A.A., Farokhi, P.R., and Aghighi, S.** 2009. Biological control of rice blast (*Magnaporthe oryzae*) by use of *Streptomyces sindeneusis* isolate 263 in greenhouse. *Am. J. Appl. Sci.* **6**, 194–199.