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Full Length Research Paper

Antifungal and antibacterial compounds from *Streptomyces* strains

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Sixteen antibiotic-producing *Streptomyces* sp. isolated from the North Cyprus soils were evaluated for their ability to inhibit *in vitro* against six filamentous fungi including human and plant pathogens. One promising strain, designed as KEH23 with strong antifungal activity, was selected for further studies. This isolate was identified as *Streptomyces* sp. based on a great variety of morphological, cultural, physiological and biochemical characteristics. Its antifungal activity was confirmed in batch culture. In order to standardize the antibiotic production some cultural conditions like different incubation temperatures (20.0, 25.0, 30.0 and 37.0°C), carbon sources (glucose, glycerol, starch and sucrose), pH (6.0, 7.0, 7.5, 8.0 and 9.0) and incubation time in hours (24, 48, 72, 96 and 120) were determined. During fermentation, growth, pH and antibiotic production were monitored at 12 h intervals. *Penicillium* sp. was most sensitive to the produced compound(s) followed by *Candida albicans*, *Cladosporium oxysporum* and *Alternaria alternata* with an inhibition zone 28.0, 20.0, 16.0 and 15.0 mm, respectively. In addition, antibacterial activity of this strain was determined against human pathogenic bacteria such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Kocuria rhizophila*, *Escherichia coli*, *Salmonella typhimurium* and methicillin-resistant *Staphylococcus aureus* (MRSA). The present results indicate that isolate KEH23 is a potential antibiotic producer agent for the biocontrol of plant and human pathogens.

Key words: Antifungal activity, biocontrol, characterization, fermentation, isolation, North Cyprus, pathogenic fungi, *Streptomyces*, soil.

INTRODUCTION

Fungal phytopathogens cause serious problems worldwide in agriculture and food industry by destroying crops and economically important plants in the field and during storage, especially in the subtropical and tropical regions (Pohanka, 2006). In addition, many also produce mycotoxins, which are harmful to humans and livestock. To a certain extent, chemical fungicides but also antifungal antibiotics and compounds based on natural products can control fungal pathogens. Many of synthetic compounds can keep fungal infections at an acceptable level.

Nevertheless, they are associated with several drawbacks such as their lack of specificity, accumulation if biodegradation is slow or even missing, and others are toxic not only to fungi but also to other beneficial life forms as well, including humans. They also have led to environmental pollution and development of pathogen resistance (Zaitlin et al., 2004; Dahiya et al., 2006; Gohel et al., 2006; Pohanka, 2006). Against these worsening problems in fungal disease control, biodegradable antifungal agents are preferred as they are free from polluting residues and represent a reduced likelihood of developing resistant fungal strains. Therefore, microbe-based biocontrol methods are one alternative way to control diseases in place of agrochemicals. This means that there will probably never be a perfect antifungal agents and new ways of controlling fungal pathogens (Bressan, 2003; Ismet et al., 2004; Dahiya et al., 2006; Jayasinghe and Parkinson, 2008).

Various microbial antagonists have been investigated as potential antifungal biocontrol agents of plant diseases. Many species of actinomycetes, especially those belonging to the genus *Streptomyces* (Gram-positive, mycelia-forming soil bacteria), are well known as biocontrol agents that inhibit or lyse several soilborne and airborne plant pathogenic fungi (Augustine et al., 2005; El-Tarabily, 2006; Og et al., 2008; Silva Sousa et al., 2008).

It is well known that *Streptomyces* sp. can produce industrially useful compounds, notably wide spectrum of antibiotics, as secondary metabolites, and continues to be screened for new bioactive compounds (Hayakawa et al. 1996).

The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds (El-Tarabily et al., 2000; Lee and Hwang, 2002; Augustine et al., 2005; Pohanka, 2006; El-Tarabily, 2006) and may be related to chitinase production (Ismet et al., 2004; Dahiya et al., 2006; Gohel et al., 2006).

Important systemic pathogenic fungi on humans are *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus.* The polyenes derived from *Streptomyces* sp. have a broad *in vitro* spectrum of activity against a wide range of fungi including the *Aspergillus* sp. and *Candida* sp. (Hay, 2003). The main polyenes are amphotericin B, nystatin, and natamycin. They are widely used for the treatment of candidiasis, coccidiodal meningitis, cutaneous dermatophytes and histoplasmosis. Wide spread use of the limited numbers of antifungal agents for control of mycotic diseases has led to the development of drug resistance. Much attention has been focused to over come resistance but the development of new classes of antifungal drugs are likely to have the most significant future impact (Gupte et al., 2002; Iznaga et al., 2004; Augustine et al., 2005; Gohel et al., 2006).

The antifungal potential of extracellular metabolites of *Streptomyces* strains against some fungi was previously reported from different locations of the world. However, there is not documented information on antifungal activities of *Streptomyces* sp. isolated from the North Cyprus habitats as a novel source for the discovery of new bioactive compounds. Such unexplored or under-exploited environments may be crucial for new strains of streptomycetes being wild types showing rich source of useful metabolites. Therefore, the study reported herein was undertaken to determine the antifungal potential of *Streptomyces* against some pathogenic fungi, the taxonomy of the antibiotic producing strain as well as detailed production optimization.

MATERIALS AND METHODS

Microorganisms and culture conditions

Antibiotic producing *Streptomyces* sp. strains isolated different habitats of the North Cyprus soils were obtained from our culture collection in Celal Bayar University, Department of Biology. They were cultured by streak plate technique on glycerol yeast extract agar, glycerol-asparagine agar (ISP 5), yeast malt extract agar (ISP 2) or casein starch agar plates as previously described (Shirling and Gottlieb, 1966; Waksman, 1961) and incubated at 27.0 - 30.0°C for 1 - 2 weeks before use. Selected colonies of streptomycetes were transferred from the plates onto respective agar slants and incubated at 28°C for 14 days. Slants containing pure cultures were stored at 4.0°C until further examinations. The fungal strains *Fusarium oxysporum*, *Aspergillus niger*, *Alternaria alternata*, *Trichoderma hamatum*, *Cladosporium oxysporum*, *Penicillium* sp. and *Candida albicans* ATCC 10231, were kindly provided by Dr. F. Kalyoncu. All fungi were cultured on potato dextrose agar (PDA, Oxoid) plates and incubated at 27.0°C for one week. *Candida albicans* was cultured in yeast extract malt extract broth at 30.0°C for 48 h and maintained on the PDA slants. The bacteria; *Staphylococcus aureus* ATCC 6538P, methicillin-resistant *Staphylococcus aureus* ATCC 95047 (MRSA), *Escherichia coli*, *Kocuria rhizophila* ATCC 9341, *Bacillus cereus* CM 99, *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium* CCM 5445, *Pseudomonas fluorescens, Serratia marcescens* CCM 583, *Staphylococcus epidermidis* ATCC 12228 and *Enterococcus faecalis* ATCC 29212 were obtained from the Department of Biology, Ege University (Izmir/Turkey). Cultures of these bacteria were grown in Mueller-Hinton broth (Oxoid) at 37.0°C for 24 h and stored in nutrient agar slants at 4.0°C.

Screening of strains for antagonistic activity

Primary and secondary screening

Preliminary screening for antibiotic production was done by conventional spot inoculation method (Yamamura et al., 2003) with some modifications. Pure streptomycetes strains were spot inoculated on PDA medium and incubated at 28.0°C for three days, and then inverted over 1.5 ml chloroform for 45 min in fume hood. Killed colonies were overlaid with 10 ml of sloppy glucose yeast extract agar, previously inoculated with one of the target organisms ($1X10^6$ spores/ml fungi and $1X10^4$ yeast/ml). The antifungal activity around the colonies was recorded after 2 and 5 d of incubation at 28.0°C for fungi and at 30.0°C for *Candida albicans*. The antibacterial activity of *Streptomyces* sp. KEH23 was determined by the streak method. Mueller Hinton agar (MHA, Oxoid) plates were prepared and inoculated in straight line on plates of 90 mm diameter and incubated at 28.0°C for six days. The test organisms were inoculated by a single streak at a 90^o angle to streptomycetes colony. The plates were incubated at 37.0°C for 24 h. At the end of the incubation, the antibacterial activity was observed by determining the distance of inhibition between target bacteria and streptomycetes colony margins.

Based on the zone of inhibition, secondary antimicrobial screening and further analysis of promising isolate (KEH23) were done under submerged fermentation conditions by agar well diffusion assay. Selected isolate was grown on four different medium at 28.0°C in a rotary shaker (150 rpm) for 5 d. To obtain the cell-free supernatant, the culture broth was centrifuged at 8000 rpm for 10 min. The wells (6.0 mm diameter, 2.0 cm apart) were cut using a sterile cork borer and 60.0 µl of clear supernatant was loaded into each well for the assay of antagonistic activity against different pathogens. The dishes were preincubated at 4.0°C for 2 h to allow uniform diffusion into the agar. After preincubation, the plates were incubated at 37.0°C for 24 h for bacteria and at 30°C for 48 h incubation for *Candida albicans*. The antimicrobial activity was evaluated by measuring the inhibition zones diameter observed.

Negative controls included only liquid culture media. Each experiment was repeated three times and the mean of inhibitory zones recorded.

Determination of chitinase activity

A selective medium, chitosanase detection agar (Cheng and Li, 2000) containing 10.0 g chitosan (Sigma, 86%, N-acetyl), 1.3 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 0.24 g $MgSO_4.7H_2O$, 1.0 g NH_4Cl, 20.0 g agar, 1000 ml distilled water, pH 6.5-7.0, was spot inoculated in duplicates and incubated at 28.0°C for 3 days. After incubation, chitinase-producing strains were identified by a pale clearing zone surrounding the colony. Clear zone diameters were measured (mm) and used to indicate the chitinase activity of each isolate.

Identification of promising strain KEH23

Color determination

Aerial mass color on ISP3 and ISP4, substrate mycelia color and diffusible soluble pigments on ISP5, melanin production on ISP6 and ISP7 were observed at 27.0°C after 21 days using a reference color key (Prauser, 1964).

Cultural and morphological observations

Cultural and morphological features of KEH23 were characterized following the directions given by the International *Streptomyces* Project (ISP) (Shirling and Göttlieb, 1966) and the Bergey's Manual of Systematic Bacteriology (Cross, 1989; Williams et al., 1983a). Cultural characteristics of pure isolates in various media (ISP2–7) were recorded after incubation at 27.0°C for 7 - 14 days. Morphology of spore bearing hyphae with entire spore chain was observed with a light microscope (Model SE; Nikon) using cover-slip method in ISP media (ISP 3 - 6).

Physiological and biochemical characteristics

Carbon utilization was determined on plates containing ISP basal medium 9 to which carbon sources were added to a final concentration of 1.0%. The plates were incubated at 27.0°C and growth was read after 7, 14, 21 days using glucose as positive control and using carbon source free medium as negative control (for compareson more suitable). The ability to utilize nitrogen sources was determined in a basal medium containing glucose 10.0 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, K_2HPO_4 1.0 g, NaCl 0.5 g, agar 3.0 g and distilled water 200 ml; after 15 days using L-asparagine or L-proline as positive control and a medium without nitrogen source was used as a negative control. Ability to grow in the presence of different antibiotics (the discs were placed on the surface of MHA medium plates seeded with a loopful of 14 day ISP 2 broth culture and inhibition zones observed at 27.0°C after 2 and 5 days of incubation) and different concentration of chemical inhibitors [4.0%, 7.0%, 10.0% and 13.0% (w/v) NaCl, 0.01% sodium azide, 0.1% phenol, 0.001% potassium tellurite and 0.0001% crystal violet] were determined in Bennet's agar after 7, 14 and 21 days. Hydrolysis of hippurate, gelatin, starch and nitrate reduction was examined as described by Williams et al. (1983a). Degradation of elastin, arbutin, tyrosine and xanthine, the ability to produce H_2S, urease, DNase, caseinase, xylanase, chitosanase and carboxy methyl cellulase were also determined. Other physiological and biochemical characteristics were determined using the method described by Williams et al. (1983a). All tests were performed at 27.0°C.

Chemotaxonomic analyses

Colony of KEH23 was scraped from the ISP2 medium and processed for the isomers of diaminopimelic acid (*LL*-DAP or *meso*-DAP) and whole cell sugar patterns by thin layer chromatography (TLC) using silica gel plates (20X20, 60 F_{254}, Merck, Darmstadt, Germany) by the method of Lechevalier and Lechevalier (1970), with some modifications. Shortly, for determination of DAP, one ml of 6 N HCl was added to 50.0 mg dried mycelia in a screw-capped vial and heated 12 h at 100.0°C. After cooling, the mixture was washed with 2.0 ml of distilled water and then dried off. This step was repeated several times to remove HCl. The final dried material was dissolved in 0.2 ml of distilled water. 5.0 µl of the sample and 3.0 µl of standard DAP (Sigma D-1377) were separately spotted onto a TLC silica plate. The plate was developed in the solvent system; methanol: distilled water: 6 N HCl: pyridine (80: 26: 4: 10) for 2 h 40 min, then dried in a room temperature for 30 min and sprayed with 0.1% ninhydrin, followed by heating at 110.0°C for 10 min on a hot plate.

For analysis of whole-cell sugars, 1.0 ml of one N H_2S04 was added to 50.0 mg of freeze-dried mycelia in a vial with a screw cap and heated at 100.0°C for 2 h. The mixture was centrifuged at 3000 rpm for 10 min. The supernatant was adjusted to pH 5.0 with saturated barium hydroxide, followed by centrifugation at 8000 rpm for 10 min. The supernatant was removed and the filtrate lyophilized and dissolved in 0.1 ml of distilled water. 5.0 µl of the sample and 3.0 µl of standard 1.0% sugar solution (D-glucose, D-mannose, L-arabinose, D-fructose, D-xylose and L-rhamnose) were spotted separately (20 mm apart) onto a TLC plate. The plate was developed in the solvent; n-buthanol: distilled water: pyridine: toluene (10: 6: 6: 1), for 3 h 20 min. The plate was dried at room temperature for 2 h and sprayed with aniline phthalate, followed by heating at 100.0°C for 4 min on a hot plate.

Taxonomic analyses

For identification of unknown strain; the morphological, physiological and biochemical data contained in the major and minor clusters of Williams et al. (1983a, b) were analyzed using Minitab-pc software package (version 13.20, Minitab Inc., 2000) with the simple matching coefficient (S_{SM}). A dendrogram obtained by comparison of the unknown isolate with major clusters constructed using the single linkage method. The Willcox probability was used to assign an identity to an unknown where scores of 0.8 and above indicated a positive identification.

Optimization of temperature, pH and carbon sources concentration for antifungal metabolite production

The effect of carbon sources (glucose, glycerol, starch or sucrose) was investigated to standardize the antibiotic production medium. Culture optimization was performed for the isolate KEH23 at different pH values (6.0, 7.0, 7.5, 8.0 and 9.0), different incubation temperatures (20.0, 25.0, 30.0, and 37.0°C), and incubation time in hours (24, 48, 72, 96 and 120) in replicates. Fermentation studies were carried out in three stages. For sporulation (a), selected isolate was grown in ISP2 medium by streak plate method at 28.0°C for 14 days. To prepare inocula (b), a loopful spore from a well-sporulated plate added each 40.0 ml respective seed medium in 250 ml erlenmeyer flasks and incubated at 28.0°C for 48 h on a rotary shaker (150 rpm). After optimization of the fermentation parameters, 2.0 ml of the seed culture (5.0%, v/v) was transferred to 250 ml Erlenmeyer flask containing 40.0 ml of the production medium. Fermentation (c) was carried out in a basal medium containing glycerol 10.0 g, meat extract 3.0 g, yeast extract 1.0 g, bacteriologic peptone 5.0 g, $CaCO_3$ 1.0 g, K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, trace element solution (Shirling and Gottlieb, 1966) 1.0 ml and distilled water 1000 ml for 120 h at 30.0°C with agitation (150 rpm) and the pH was adjusted to 7.5 with 0.1 N NaOH or 0.1 N HCl prior to sterilization. As maximum antibiotic production was observed on the 5 day of incubation and fermentation was terminated.

During fermentation, various parameters were monitored at 12-h intervals. The pH was measured on a digital pH meter (Hanna pH 211). Growth was monitored by determining the dry cell weight (DCW). Fermentation broth was taken aseptically and the cells were separated from the culture filtrate by centrifugation at 8000 rpm for 10 min and dried at 100.0°C for 24 h. DCW was calculated by the difference in the weights.

Effect of shelf - life and high temperature on antimicrobial activity

To determine the effect of temperature on stability of the producing

Table 1. Antifungal activity of selected *Streptomyces* strains against plant pathogens and *C. albicans.*

Streptomyces strains	Target fungi and inhibition zones[a]													
	Time (day)	FOX		AN		AA		TH		COX		P		CA	
		2	5	2	5	2	5	2	5	2	5	2	5	2	5
	KVK30	-	-	-	-	-	-	10	5	-	-	-	-	-	-
	KEH23	8[b]	6[±]	5	6[±]	20	15	10	5	8	16	28	26	20	20
	KGG13	6	-	-	-	-	-	7	6[±]	-	-	6	15	-	-
	KMY10	-	-	10	8[±]	13	10[±]	12	15[±]	10	10[±]	14	13[±]	10	-
	KAK35	-	-	8	8[±]	13	8[±]	-	-	-	10	14	10	12	-

[a]FOX, *Fusarium oxysporum*; AN, *Aspergillus niger*; AA, A*lternaria alternata*; TH, *Trichoderma hamatum*; COX, *Cladosporium oxysporum*; P, *Penicillium* sp; CA, *Candida albicans.* [b]Diameter of inhibition zone in mm; [±], partially inhibition;. -, not active.

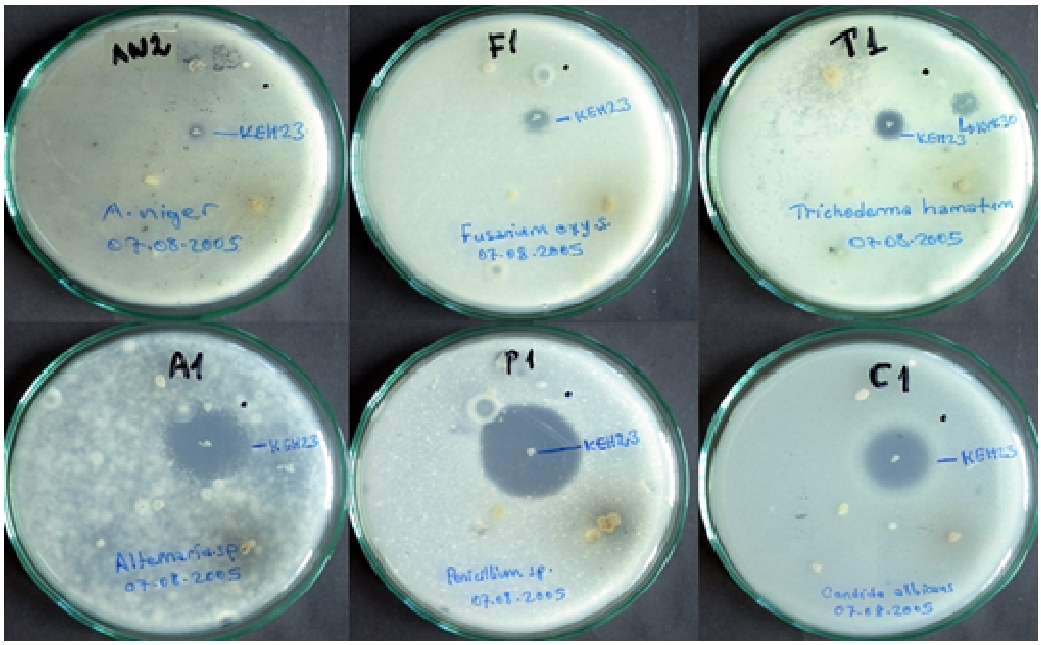

Figure 1. Antifungal activities of *Streptomyces sp*. KEH23 against different test organisms (test medium PDA).

antibiotic, the culture supernatant kept at high temperatures (60.0, 70.0, 80.0, 90.0°C and autoclaving at 121.0°C) in glass tubes for different times and used for antimicrobial studies against to *S. aureus*, *E. coli* and *C. albicans*.

The shelf-life (storage time) on antimicrobial activity of the culture supernatant of KEH23 was determined by storing the supernatant at 4.0°C in tubes for different time periods (1, 3, 5, 7, 9 and 12 months). After the specified period, 60.0 µl from each tube was added to wells of MHA or PDA plates already swabbed with test organisms.

RESULTS

Antagonistic activities – Primary and secondary screening

The results of the primary antifungal screening assays indicated that, 5 of 16 strains (31.25%) had some antagonist activity against filamentous fungi including plant pathogens (Table 1). Results showed that most of the strains suppressed in different degree the test fungi with an inhibition zones ranging from 6 to 28 mm. Most of them were not active against pathogenic fungi such as *A. niger, F. oxysporum* and *Cladosporium oxysporum* but repressed the growth of *Penicillium* sp, *A. alternata* and *C. albicans* in a significant extent (Figure 1). The most antifungal activity on *Penicillium* sp. selected as test organisms were showed by four strains and most active strain was KEH23 with an inhibition zone 28 mm, end of the second day. Three strains possessed activity against pathogenic yeast *C. albicans*.

KEH23 was the most antagonistic streptomycete strain with an inhibition zones ranged from 7-30 mm against

Table 2. Comparative antibacterial activity of *Streptomyces* sp. KEH23 with some standard antibiotics.

Antibiotic	Test bacteria[a]										
	SA	**BC**	**KR**	**EC**	**PF**	**BS**	**STYP**	**SM**	**SE**	**EF**	**MRSA**
Streptomyces sp. KEH23	15	26	20	20	7	30	12	10	-	20	22
Nalidixic acid (30 µg/disc)	20[b]	28	10[R]	26	30	32	6[R]	30	6[R]	30	24
Novobiocin (30 µg/disc)	32	25	28	6[R]	20	13[R]	40	6[R]	26	28	34
Ampicillin (10 µg/disc)	15[R]	6[R]	26	6[R]	6[R]	10[R]	6 [R]	6[R]	16	16	16[R]
Penicilline G (10 i.u./disc)	24	10[R]	20[R]	6[R]	6[R]	8[±R]	6 [R]	6[R]	11[R]	24	6[R]
Vancomycin (30 µg/disc)	12	15	15	6[R]	6[R]	6[R]	28	6[R]	15	16	14
Chloramphenicol (30 µg/disc)	20	28	30	26	12[R]	30	40	30	25	30	25

[a]*SA* - *Staphylococcus aureus* ATCC 6538P, *BC* -*Bacillus cereus* CM 99, *KR* - *Kocuria rhizophila* ATCC 9341, *EC* - *Escherichia coli*, *PF* - *Pseudomonas fluorescens*, *BS* - *Bacillus subtilis* ATCC 6633, *STYP* - *Salmonella typhimurium* CCM 5445, *SM* - *Serratia marcescens* CCM 583, *SE* - *Staphylococcus epidermidis* ATCC 12228, *EF* - *Enterococcus faecalis* ATCC 29212, *MRSA* - methicillin-resistant *Staphylococcus aureus* ATCC 95047.
[b]Diameter of inhibition zone in mm, including disc diameter (6 mm)
[R]resistant, ± partially inhibition, 6 - no activity.

eleven human pathogenic bacteria (except *S. epidermidis*) that are resistant to some antibiotics. It shows promising antibacterial activity (*in vitro*) that is comparable to that of nalidixic acid as well as novobiocin. It is found to be more potent than penicillin G and vancomycin against human pathogens (Table 2).

The antimicrobial activity of the strain KEH23 from the liquid culture of four different medium was also tested against *S. aureus, E. coli* and *C. albicans* at 12 h intervals (Table 3). The obtained broad-spectrum inhibition was verified during secondary screening on MHA medium. Antimicrobial production started at 16 h of fermentation. The highest antimicrobial production of KEH23 occurred from fermentation medium 3 (FM3) including glycerol, at 72 - 96 h against test microorganisms. Whereas, the strain was little active from fermentation medium 4 (FM4) including sucrose.

Based on the antimicrobial activity of streptomycete strains, KEH23 was selected as the final candidate for antibiotic production, because it was only the isolate that has satisfied intense and broad antifungal and antibacterial spectrum.

Characteristics of the strain KEH23 selected for fermentation

A great variety of morphological, cultural, physiological, biochemical and other features of the strain KEH23 were studied. Colonies of strain KEH23 are grey or pale brown and wrinkled on ISP2 agar revealed the presence of the vegetative hyphae (Figure 2). Growth, the aerial and substrate mycelium color and formation of soluble pigments were observed in nine different media after 14 days. The *Streptomyces* sp. KEH23 is a gram positive filamentous bacteria with extensively branched aerial mycelia, aerobic, mesophilic and grows readily on most of the nutrient media described (Waksman, 1961; Locci, 1989).

During observation with light microscopy on the ISP 3 - 6, spore-bearing hyphae of the strain were spirale chain (Figure 3a and b). The number of the spores was higher than 20, which indicated they refer to the long chains of spores. On the other hand, the vegetative hyphae was branched but not fragmented. Verticils, synnemata, sclerotia, or sporangia were not detected. It showed different color of aerial and substrate mycelia; produced yellow to brown diffusible pigments when grown on the ISP4 and glucose-nitrate agar (Table 4). The strain did not produce melanoid pigment on both ISP 6 and ISP 7. KEH23 did not grow on substrates like D-fructose, xylitol, D-mellibiose and adonitol. Some differences occurred in utili-sitation of nitrogen sources; L-cysteine, L-valine, L- lysine and DL-α-n-butyric acid were not used. KEH23 hydrolyses the gelatin and starch; degrade xanthine, reduced nitrates, whereas H_2S was not produced. Growht at three different temperatures (4.0, 37.0 and 45.0°C), different concentrations of inhibitors in the media was stu-died. Temperature for growth ranged from 15.0 to 45.0°C and an optimal temperature was 30.0°C, but not grew at 4.0°C. It grew at 4.0% and 7.0% NaCl. There was no growth detected at higher concentrations of NaCl but also with sodium azide (0.01), phenol (0.1) and crystal violet (0.0001). Other physiological and biochemical features of KEH23 are summarized in Table 5. The compositions of DAP and sugar components from the whole-organism hydrolysates were detected. DAP existed as isomers with LL types. Cells contained no diagnostic sugar components.

The taxonomic status of the KEH23, according to morphological observations and *LL*-DAP determination indicated that it is a bacterium belonging to the genus *Steptomyces*. However, additional results such as absence of diagnostic sugars in whole-cell hydrolysates, the presence of DAP (wall chemotype I), together with other cultural, physiological and biochemical properties could not

Table 3. Antimicrobial activity of fermentation culture broth of the strain KEH23 (mm).

Time (h)	TM*	Fermentation medium			
		FM1	FM2	FM3	FM4
16	*SA*	-	-	6	
	EC	-	-	-	ND
	CA	-	-	-	
24	*SA*	-	-	10	
	EC	-	-	-	ND
	CA	-	-	10	
32	*SA*	10	8	16	
	EC	8	6	8	ND
	CA	12	10	12	
44	*SA*	14	14	16	
	EC	10	10	10	ND
	CA	12	10	12	
56	*SA*	12	10	14	
	EC	10	-	$10^{\pm}$	ND
	CA	10	10	12	
64	*SA*	16	12	14	
	EC	6	-	-	ND
	CA	10	10	10	
72	*SA*	16	12	16	8
	EC	$12^{\pm}$	10	$10^{\pm}$	-
	CA	8	4	12	-
84	*SA*	10	10	16	10
	EC	-	-	-	-
	CA	10	8	12	-
96	*SA*	10	8	14	7
	EC	-	-	$12^{\pm}$	8
	CA	12	10	13	-
108	*SA*	14	10	14	13
	EC	$10^{\pm}$	6	$10^{\pm}$	15
	CA	8	8	12	-
120	*SA*	10	8	12	14
	EC	8	4	$10^{\pm}$	16
	CA	10	8	10	-

*; TM; Test Microorganisms, SA; *S. aureus*, EC; *E. coli*, CA; *C. albicans.*
$^{\pm}$; partially inhibition FM1; Fermentation medium 1 (carbon source, glucose), FM2; Fermentation medium 2 (carbon source, starch) FM3; Fermentation medium 3 (carbon source, glycerol), FM4; Fermentation medium 4 (carbon source, sucrose).
- negative, ND; not determined, inhibition zones not including well diameter (6 mm).

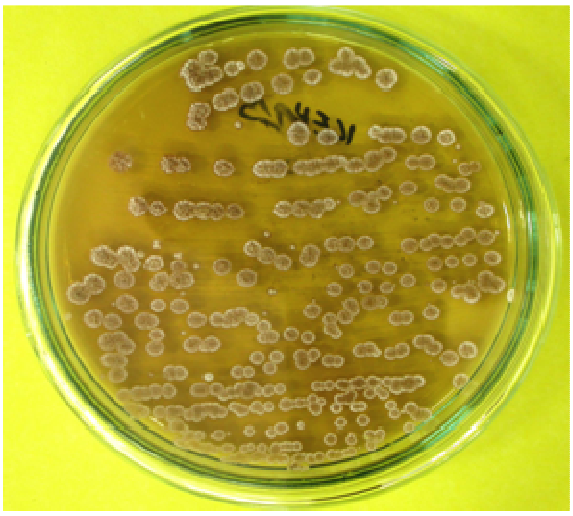

Figure 2. Colonies of *Streptomyces* sp. KEH23 on yeast malt extract agar (ISP2).

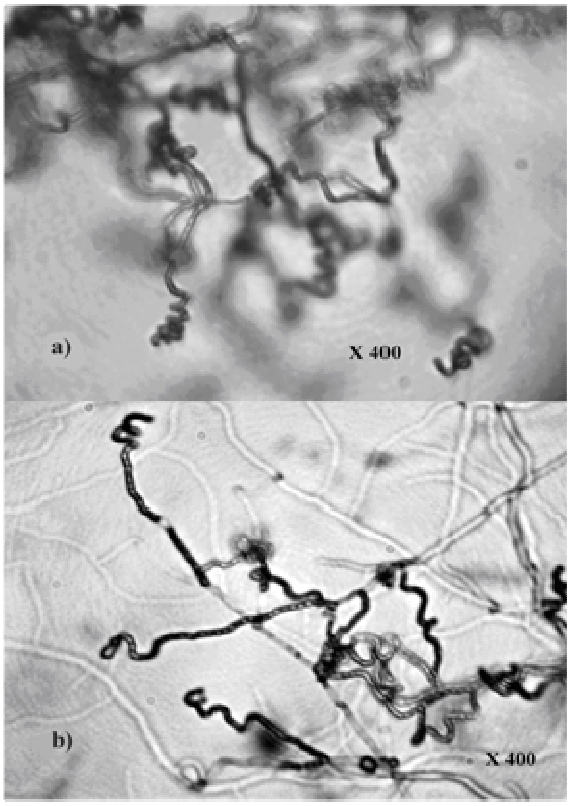

Figure 3. Sporulating aerial mycelium of strain KEH23 (a) oat meal agar (ISP3), (b) inorganic salt-starch agar (ISP4).

place it in the already known species (Williams et al., 1983a). These results suggest that KEH23 isolated from the North Cyprus habitats, may be new species, not yet described before.

Optimization of cultural conditions and fermentation studies

The preliminary screening revealed that the *Streptomyces* sp. isolate KEH23 was a good antibacterial and mo-

Table 4. Cultural characteristics of potential biocontrol agent *Streptomyces* sp. KEH23.

Medium	Growth*	HMC	SMC	DP
Yeast malt extract agar (ISP2)	+++	Grey / brown	brown	+/-
Oat meal agar (ISP3)	+++	Grey / violet	Pale brown	-
Inorganic salt-starch agar (ISP4)	+++	Grey	Cream	Brown
Glycerol-asparagine agar (ISP5)	++	Grey	Grey	-
Peptone yeast extract iron agar (ISP6)	++	Grey	Yellow/brown	-
Tyrosine agar (ISP7)	++	Grey	Grey	-
Starch-asparagine agar	+++	Grey	Grey	-
Glucose-nitrate agar	+++	Cream	Cream	Yellow
Glucose-asparagine agar	++++	White	Grey	-

* HMC; aerial mycelium color, SMC; substrate mycelium color, DP; diffusible pigments other than melanin, +; sparse growth, ++; poor growth, +++; moderate growth, ++++; abundant. +/-; variable, -; negative.

derate antifungal compound producer. Optimization of antimicrobial compound production was carried out in batch culture. The strain was able to grow in all tested carbon sources. However, maximum growth and antibiotic production were observed in glycerol as the sole source of carbon followed by glucose (Figure 4). KEH23 was cultivated at different initial pH values (6.0-9.0) and temperature (20.0 - 37.0°C) in shake flask cultures. Our results indicated that biomass and antibiotic production was affected by initial pH and temperature of the medium. The highest biomass and antibiotic production was observed when initial pH of culture medium set at pH 7.5 (Figure 5). Consequently, the optimal temperature for maximum antimicrobial was found to be 30.0°C (Figure 6) and an incubation time of 96 h.

During fermentation, various parameters; growth, pH and antibiotic production were monitored at 12 h intervals. To find out screen a suitable carbon source, pH and temperature, the strain was incubated in basal medium containing various carbon sources at a concentration of 1.0% (w/v). The maximum yield of cell growth of *Streptomyces* sp. KEH23 (as DCW) was 1.5 g/100 ml in medium containing glycerol obtained at 30.0°C after 72 h of incubation and this medium gave higher antimicrobial activity (18 mm). Higher spectrum of broadness observed after 96 h of incubation (14, 12 and 13 mm against *S. aureus, E. coli* and *C. albicans*, respectively). However, after 108 h, the diameter of the inhibition zone dropped slowly (Table 3).

Effect of shelf - life and high temperature on antimicrobial activity

The stability of the supernatant was tested by heat treatment. Heat-treated culture supernatant of KEH23 when compared to the its fresh supernatant indicated that *E. coli*, *S. aureus* and *C. albicans*, showed sensitivity with 10, 14 and 12 mm, respectively. However, the antibiotic lost its antifungal activity completely after autoclaving at 121.0°C for 15 min. The antibiotic was stable for a period of 12 months at 4.0°C. Thus, the supernatant from KEH23 retained its antimicrobial activity even after 12 months of storage.

DISCUSSION

The use of microorganisms or their secretions to prevent plant pathogens and insect pests offers an attractive alternative or supplement for the biological control of plant diseases. Therefore, biological control tactics have become an important approach to facilitate sustainable agriculture (Dahiya et al., 2006). Fungal plant diseases are one of the major concerns to agricultural production. It has been estimated that total losses because of plant diseases reach 25% of the yield in western countries and almost 50% in developing countries. Of this, one third is due to fungal infections (Gohel et al., 2006). Therefore, there is a pressing need to control fungal diseases that reduce the crop yield to ensure a steady and constant food supply to ever-increasing world population. Conventional practice to overcome this problem has been the use of chemical fungicides, which have adverse environmental effects causing health hazards to humans and other non-target organisms, including beneficial life forms (Dahiya et al., 2006; Gohel et al., 2006). Hence, there is increasing concern towards the toxicity and biomagnification potential of these chemicals in agriculture. Currently, actinomycetes, and more specifically, streptomycetes that produce various bioactive natural products including antibiotics, are being used as pharmaceuticals and agrochemicals (Lazzarini et al., 2001; Watve et al., 2001; Sajid et al., 2008).

The *in vitro* tests indicated that, 5 of 16 *Streptomyces* strains were antagonists against most of the test filamenttous fungi. Some actinomycete strains had broad spectra of antifungal activity (example, KEH23, KMY10, KAK35)

Table 5. Morphological, physiological and biochemical characteristics of *Streptomyces* sp. KEH23.

Characteristics[a]	KEH23	Characteristics	KEH23
Aerial mycelium	+	Resistance to:	
Spore chain morphology[b]:		Neomycin (50 µg/ml)	R
Rectinaculiaperti	-	Rifampicin (5 µg/ml)	R
Rectiflexibiles	-	Penicillin G (10 IU)	R
Spirals	+	Growth at 45°C	+
Verticillat	-	37°C	+
Spore mass color:		4°C	-
Red	-	Growth with (% w/v) :	
Grey	+	NaCl (%4)	+
Mycelium pigment red-orange	-	NaCl (%7)	+
Diffusible pigment produced	+	Sodium azide (0.01)	-
Diffusible pigment yellow-brown	+	Phenol (0.1)	-
Melanin production on:		Potassium telluride (0.001)	+
Peptone yeast iron agar	-	Crystal violet (0.0001)	-
Tyrosine agar	-	Utilization of nitrogen sources:	
Mycelium fragmentation	-	DL-α-n-butyric acid	-
Substrate mycelium sporulation	-	L-Cysteine	-
Antibiosis against to:		L-Valine	-
B. subtilis	+	L-Phenylalanine	+
K. rhizophila	+	L-Histidine	+
C. albicans	+	L-Hydroxyproline	+
S. cerevisiae	-	KNO_3	+
A. niger	+	L-Proline (positive control)	+
Enzyme activity:		L-Lysine	-
Lecithinase	+	L-Tyrosine	+
Lipolysis[c]	+	L-Asparagine (positive control)	+
Nitrate reduction	+	L-Arginine	+
H_2S production	-	Utilization of carbon sources:	
Starch reduction	+	Sucrose	+
Gelatin reduction	+	*Meso*-Inositol	+
DNase	+	Mannitol	+
Urease	-	L-Rhamnose	+
Xylanase	-	Raffinose	+
CMC	-	D-Melesitose	+/-
Proteolitic activity	-	Adonitol	-
Degradation:		D-Mellibiose	-
Hippurate	-	Dextran	+/-
Elastin	-	Xylitol	-
Xanthine	+	Lactose	+
Arbutin	-	Xylose	+
Oxalate	-	D-Fructose	-
Chitosanase	-	Glucose (positive control)	+

[a]CMC; carboxy methyl cellulase, IU; international units, [b]result on ISP 4 medium after 14 days incubation, [c]egg-yolk medium results, +; positive, -; negative, +/-; variable, S; sensitive, R; resistant.

while others inhibited a limited number of fungal species (example, KGG13, KVK30). About 68.75% (11) of the streptomycete isolates did not show any antifungal activity towards the test fungi on PDA as medium of production. Nevertheless, it is probable that they produce other useful compounds for which they were not screened in

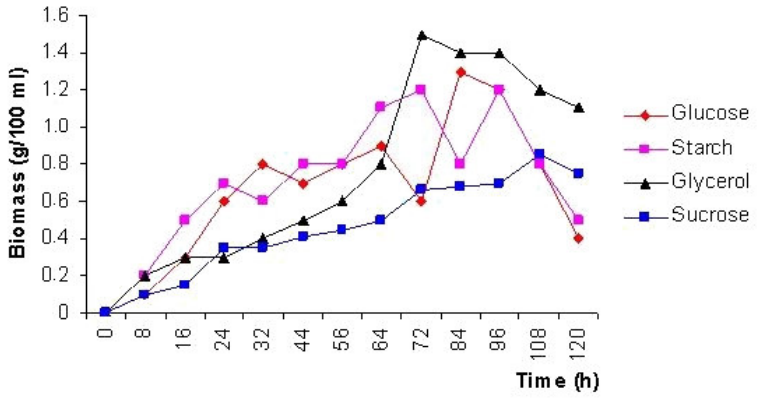

Figure 4. Effect of different carbon sources and incubation time for biomass of KEH23 (pH: 7.5, temperature: 30.0°C).

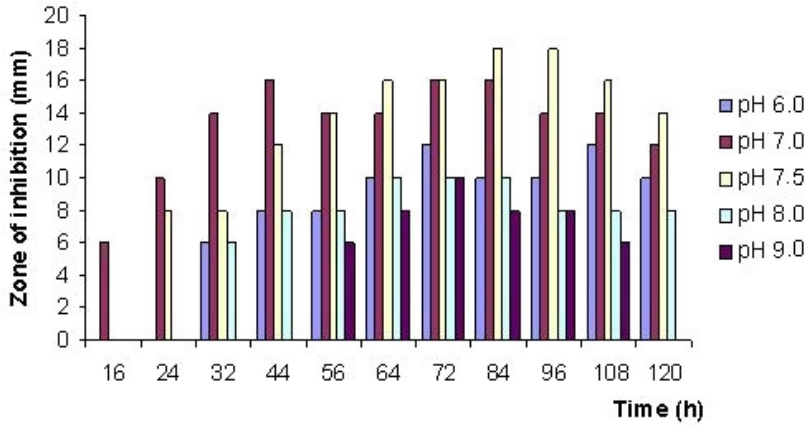

Figure 5. Effect of pH for production of antimicrobial compound of strain KEH23 (Temperature: 30.0℃, carbon source: glycerol, test organism: *S. aureus*, test medium: MHA).

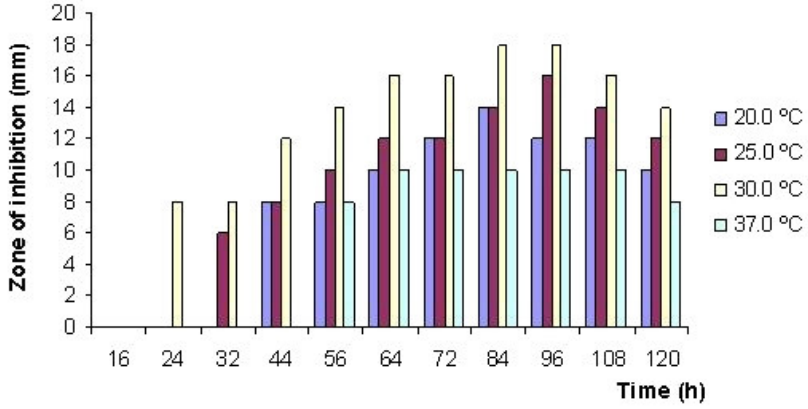

Figure 6. Effect of temperature for production of antimicrobial compound of strain KEH23 (pH: 7.5, carbon source: glycerol, test organism: *S. aureus*, test medium: MHA).

this study. This is similar to the work of Zaitlin et al. (2004), who reported 4 out of 10 actinomycete strains isolated being active against fungal pathogens *in vitro*. In the current study, strain KEH23 that seem to be strong

fungal inhibitor, showed antibiosis against all highly resistant fungal species (example, *F. oxysporum*, *A. niger, A. alternata*, *Trichoderma hamatum*, *C. oxysporum*, *Penicillium* sp.). This suggests that a single streptomycete strain will be providing broad-spectrum to control diverse plant pathogenic fungi. In addition, it is interesting to note that, of the test fungi, fast growing genera (example, *Alternaria*, *Penicillium*, *Trichoderma*,) were more tolerant to streptomycete antagonism than slow and moderately slow-growing genera (example, *Cladosporium*, *Fusarium*). While many studies have focused on soil actinomycetes in general, the streptomycetes are most frequently isolated and have a widespread distribution.

Many of these have been shown to lyse or inhibit soil fungi (Zaitlin et al., 2004). However, the inhibitory effect of these isolates on the growth of soil-borne fungal pathogens and disease development is probably derived from more than one mechanism. Although the exact mechanisms by which these actinomycetes isolates operate to reduce disease incidence is not elucidated, one possibility is that these biocontrol agents exert a direct inhibitory effect on hyphal growth and structure of fungal pathogens (Zakalyukina and Zenova, 2007; Loqman et al., 2009).

The ability to inhibit fungi may be related to chitinase production (Gupte et al., 2002; Dahiya et al., 2006; Gohel et al., 2006), and chitinase extracted from a *Streptomyces viridodiasticus* has been shown to suppress the growth of *Sclerotinia minor*, a pathogen causing basal drop disease of lettuce (El-Tarabily et al., 2000). In the *in vitro* tests done here, the *Streptomyces* strains were incapable of producing detectable levels of chitinase.

Many reports refer to the role of actinomycetes, mostly *Streptomyces* strains, in plant protection and are antagonists against most of the phytopathogenic fungi. Bressan (2003) reported the effectiveness of *Streptomyces* sp. strains previously isolated from maize rhizosphere and found that two *Streptomyces* sp. strains inhibited seed pathogenic fungi, *Aspergillus* sp. and *Fusarium subglutinans* whereas they did not suppress the development of *Penicillium* sp. In the present study, the *Penicillium* sp. was strongly affected by *Streptomyces* strains. The potential antagonistic activity of the streptomycetes found in this study against plant pathogenic fungi highlights their potential in bio-control programs and explains why all previous studies on actinomycetes were mainly focused on their abilities as biocontrol agents (Augustine et al., 2005; El-Tarabily, 2006; Mutitu et al., 2008). More recently, mycelium growth of *Pythium ultimum*, *Fu-sarium oxysporum* f. sp. *albedinis*, *Sclerotium rolfsii*, *Verticillium dahliae* and *Botrytis cinerea* was also inhibited by the streptomycete cultures, when grown in PDA medium plates (Loqman et al., 2009).

It has been reported that nutritional requirements of *Streptomyces* play an important role during metabolite synthesis process. Amongst various nutritional requirements, antifungal substance production has been known to be influenced by media components and cultural conditions, such as aeration, agitation, pH, temperature, carbon, nitrogen source and incubation time, which vary from organism to organism (Dahiya et al., 2006; Asha Devi et al., 2008; Yu et al., 2008). Cultural conditions were found to affect antifungal metabolite production by *Streptomyces* sp. KEH23. The change in initial pH of the culture medium that affected maximum antibiotic production was obtained at 7.5. This strain was able to grow in all the tested carbon sources (Table 2). Amongst the four kinds of carbon sources, sucrose was not suitable both growth and antimicrobial metabolite production by the strain KEH23. However, maximum antibiotic production was obtained in cultures supplemented with glycerol as a sole carbon source followed by cultures containing glucose. Hassan et al. (2001) found similar results with *Streptomyces violatus* in batch cultures.

Streptomycetes have been determined largely because of morphological and biochemical criteria, resulting in the arrangement of strains into cluster groups (Williams et al., 1983a). Analysis of the morphological and some cultural characteristics of the KEH23, allowed us to determinate its probable taxonomic classification in genus *Streptomyces*, according to the directions given in Williams et al. (1983a). Results analyzed by the computer program to determine hierarchical cluster could not identify this strain. KEH23 was confirmed as a strain of *Streptomyces* (Locci, 1989; Williams et al., 1989; Anderson and Wellington, 2001) and named *Streptomyces* sp. KEH23.

This is the first study of streptomycetes on their antifungal activities from the North Cyprus soil habitats. The results indicated that *Streptomyces* sp. strains provides a significant inhibitory activity against pathogenic fungi and have potential as biological control agents. Although *Streptomyces* sp. KEH23 showed significant control of pathogenic fungi, further studies will have to be made to evaluate the efficacy of control under fields and laboratory conditions before it becomes an agricultural practice.

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