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ORIGINAL PAPER

Isolation and characterization of wastewater sand filter actinomycetes

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Abstract One hundred twenty-two different actinomycete strains were isolated from sample collected from several depths of the Marrakech wastewater infiltration-percolation system. To evaluate the antimicrobial effect of the different actinomycetes recovered, eleven wastewater-associated micro-organisms known as human potential pathogens were used. Results showed that 44 isolates had an in vitro inhibitory effect toward at least seven of the indicator microorganisms while only five active strains inhibited all these pathogens. All five selected active isolates belonged to the genus *Streptomyces*. Three were identified as *Streptomyces violaceorubidus*. These isolates showed the broad activity spectrum against a wastewater-associated pathogenic yeast (*Candida albicans*), Gram-negative (*Salmonella* sp. CCMM B_{17}) and Gram-positive (*Staphylococcus aureus* CCMM B_3). These findings indicate the potential involvement of antagonistic actinomycetes in the removal of wastewater-associated pathogens.

Keywords Actinomycetes · Antimicrobial activities · Infiltration-percolation system · Screening · Wastewater

Introduction

Wastewater effluent treatment by soil infiltration and percolation has long been used as simple, low-cost means of wastewater management throughout the world (Wotton 2002). In Marrakech city (Morocco), an infiltration-percolation system was provided for treatment of wastewater of a tourist complex (Palmariva). In this domestic wastewater treatment, based on the purification capacity of sand filter, the mean removal of faecal coliforms is 99% (Hassani et al. 1999). Bacterial removal mechanisms in infiltration systems are a combination of physical, chemical and biological factors (Bomo et al. 2003). The studies of Bomo et al. (2003) indicated that biological wastewater filters are dynamic systems and biological factors (i.e., negative interactions between microorganisms) can be of major importance in these systems. Among soil microorganisms, actinomycetes are known to be good producers of bioactive secondary metabolites including antibacterial (Barakate et al. 2002), antifungal (Ouhdouch et al. 2001; Paul and Banerjee 1986; Hamdali et al. 2008) and antiparasitic agents (Dieter et al. 2003). The actinomycetes are very widespread in the soil except in sites exposed to extreme conditions (Elliot Juhnke et al. 1987). They represent part of the microbial population of the soil from the surface which has reached more than a meter of depth (Breton et al. 1989) and were met in large variety of natural substrates (Bignell et al. 1980). Wastewater treatment by infiltration percolation systems presents an unexplored environment. In this context, Moroccan wastewater treatment by sand filter might be a rich source of actinomycetes producing antimicrobial compounds and/or involved in the removal of wastewater-associated pathogens. However, this habitat has not been investigated. The main objectives of this study were to isolate antagonistic actinomycetes originating from wastewater treatment by sand filter and to characterize the selected strains through some genotypic and phenotypic features.

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Materials and methods

System description and sampling

The Palmariva infiltration-percolation system is equipped with an anaerobic pond (320 m^2) and one accumulation tank (98 m^3) used for sectional alimentation of sand filtration basins. The infiltration is done over five sand filtration basins of 300 m^2, each constituted by a bed 2 m thick of sand drained at its base. At all seasons, sand filters are fed with the decanted wastewater, of tourist complex Palmariva, loaded at about 15 cm per day (33 m^3) until clogging; and then they were allowed to dry. Three samples (S1, S2 and S3) were collected using the Pochon and Tardieux method (1962) from several depths (10–30, 30–50 and 50–70 cm) respectively. The sample from each depth was taken with an auger after removing approximately 10 cm of the soil surface for S1. Samples were placed in sterile polyethylene bags, closed tightly and stored in the refrigerator at 4°C until use.

Isolation of actinomycetes

Samples from each depth were first mixed, suspended in sterile distilled water (10 g in 100 ml) homogenized by vortexing and finally treated 10–15 min by sonication according to Ouhdouch et al. (2001). All treated samples were serially diluted up to 10^{-6} and spread (0.1 ml) in three replicates over the surface of two soil extract agar plates (SEI) enriched with 1% arginine, (SEII) enriched with 1% KNO_3 (Barakate et al. 2002) and Actinomycetes Isolation Agar (Olson 1968; 5% glycerol, 0.2% sodium caseinate, 0.01% L-asparagine, 0.4% sodium propionate, 0.05% K_2HPO_4, 0.0001% $FeSO_4$ and 1.5% agar Difco). The three media were supplemented with 40 μg actidione/ml to inhibit the development of fungi (Olson 1968), and 10 μg nalidixic acid/ml to inhibit bacteria capable of swarming without affecting the growth of actinomycetes (Nonomura and Hayakawa 1988; Bulina et al. 1997). The plates were incubated at 28°C and the number of colonies was determined for total bacteria after 21 days. Actinomycetes were recognized on the basis of morphological features following directions given by International *Streptomyces* Project (ISP; Shirling and Gottlieb 1966). All observed colonies were isolated, purified and conserved in 20% glycerol at −20°C.

Screening for antagonistic activity

The antibacterial activity of total actinomycetes isolates was tested by the confrontation test. Isolates were suspended in distilled water and inoculated on the surface of nutrient agar by streaking the tested isolate. After 72 h of incubation at 28°C, the following bacteria: *Bacillus subtilis* ATCC 9524(Bs), *Micrococcus luteus* ATCC 10240 (Ml), *Bacillus cereus* ATCC 14579 (Bc), *Vibrio cholerae* non O1 (Vc), *Aeromonas hydrophila* ATCC 7966 (Ah), *Aeromonas sobria* (As), *Aeromonas caviae* (Ac), *Escherichia coli* ATCC 94 (Ec1), *Escherichia coli* (Ec2), *Salmonella typhimurium* ATCC 13314 (St1) and *S. typhimurium* (St2) were inoculated in perpendicular directions of the tested isolate and incubated at 28°C for 2 days (Highley and Ricard 1988). The antagonistic activity is expressed by the inhibition zones. A control plate, without actinomycetes, was prepared and similarly inoculated.

Antimicrobial activity

Secondary screening was performed by the agar block diffusion method against the pathogenic standard test organisms (Table 1). Active actinomycetes isolates were grown on Bennett medium (beef extract (Merck, Germany) 1 g l^{-1}; glucose (Merck) 10 g l^{-1}; peptone (Merck) 2 g l^{-1}; yeast extract (Merck) 1 g l^{-1} and agar (Difco) 15 g l^{-1}) for 7 days at 28°C. Agar cylinders (10 mm in diameter) were taken with sterile cork borers and deposited on the surface of the Mueller–Hinton and Sabouraud media, which had previously been seeded with the test organisms (Parente et al. 1994).

Table 1 Microorganisms used for antimicrobial activity

Gram-positive bacteria	Gram-negative bacteria	Yeast
Arthrobacter aurescens DSM200116 ATCC13344 (A.a)	*Vibrio cholerae* non O1 (V.c)	*Candiada albicans* (C.a)
Staphylococcus aureus CCMM B_3 (S.a)	*Klebsiella peumoniae* (K.p)	*Candida krusei* (C.k)
Micrococcus luteus ATCC10240 (M.l)	*Enterobacter cloacae* (E.c)	*Candida glabrata* (C.g)
Mycobactérium phlei DSM750 (M.p)	*Salmonella* sp. CCMM B_{17} (S.sp.)	*Candida parapsilosis* (C.p)
Bacillus subtilis ATCC 9524 (B.s)	*Pseudomonas fluorescens* DSM 50090 ATCC13525 (P.f)	
Bacillus cereus ATCC 14579 (B.c)	*Escherichia coli* K12 (W1130) (E.c3)	

Morphological, physiological and chemotaxonomic characterization of selected actinomycete isolates

The morphological, cultural, physiological and biochemical characteristics of the selected isolates were evaluated as described in the International Streptomyces Project (ISP; Shirling and Gottlieb 1966). Cultural characteristics were observed on yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salts-starch agar (ISP4) media at 30°C from 7 to 21 days and the colour series were determined according to the system proposed by Nonomura (1974). The assimilation of carbohydrates was studied by using the medium ISP9, containing 16 different carbohydrates at a concentration of 1% (w/v) as sole carbon source. The chemical analyses of the diaminopimelic acid isomer were performed as described by Becker et al. (1964). Spore chain morphology and spore shapes were observed on the same media using light microscopy.

Amplification and sequencing of the 16S rDNA of the selected strains

The purified selected actinomycete isolates were grown for 2 days at 28°C with agitation in 500-ml flasks containing 100 ml of Hickey-Tresner medium containing 1 g l^{-1} yeast extract, 1 g l^{-1} beef extract, 2 g l^{-1} NZamine A, 10 g l^{-1} dextrin, 20 mg l^{-1} $CoCl_2 \cdot 6H_2O$ (Hopwood et al. 1985). Biomass was harvested by centrifugation (8,000*g* for 10 min) and washed twice with double-distilled water. 200 mg of mycelium was used for DNA extraction as described in Liu et al. (2000). The 16S rDNA was amplified using the PCR method with *Taq* DNA polymerase (Qiagen, USA) and universal primers PA (5′-AGAGTTT GATCCTGGCTCAG-3′) and PH (5′-AAGGAGGTGATC CAGCCGCA-3′). Amplification was carried out in 50 µl reaction mixture containing 1.5 U of AmpliTaq Gold *Taq* polymerase (Applied Biosystems), 10 µl of 5× AmpliTaq Gold reaction buffer (Applied Biosystems), 2.5 mM of each dNTP, 1 µM of each primer and 100 ng of genomic DNA. Reaction conditions were: 97°C for 4 min, (97°C for 45 s, 52°C for 45 s and 72°C for 45 s) × 35 cycles followed by an incubation at 72°C for 10 min. The amplified products were visualized on a 0.8% (w/v) agarose gel stained with ethidium bromide. Sequencing reactions were performed by Macrogen (Seoul, Korea). The sequences obtained were compared for similarity with sequences present in the genomic database banks, using the 'NCBI BLAST' program available at the ncbi.nlm.nih.gov web site.

Statistical analysis

The results are presented in the form of averages ± SEM. The comparison of the averages was made by ANOVA and analysed using SPSS 10.0 for Windows. The differences are considered significant at $P < 0.05$.

Results and discussion

Isolation of actinomycetes

In wastewater treatment by sand filter actinomycetes can particularly contribute to the treatment processes, as they are able to degrade a wide variety of complex organic compounds and recalcitrant hydrocarbons (Hatano et al. 1999). In this study, actinomycetes strains were isolated from soil samples collected from the domestic wastewater treatment by infiltration-percolation system (Palmariva), using two soil extract media agar (SEI and SEII) and the standard Isolation Actinomycetes Agar (Olson 1968). Determination of the actinomycetes shows that their number ranged from 2.6 to 63 × 10^5 c.u.f/g of sand. The variation of actinomycete abundance according to depth is made in the same way whatever the media used (Table 2). Soil extract media agar SEI seems to be specific and sensitive for actinomycetes (Barakate et al. 2002). Since all isolation media used contained glycerol that most actinomycetes use as carbon source, only the nitrogen sources of (SEI) and (SEII) were different. The best isolation rates were obtained using SEI medium. The number of actinomycetes in the bottom was maximum in the sample taken from a depth of 50–70 cm (Table 2), was slightly less in the sample from a depth of 10–30 cm, and very weak in the depth of 30–50 cm. Curiously, in the top layers of the sand filter, less actinomycete abundance is reproducible, despite actinomycetes being able to produce spores, a form of dissemination and resistance to many adverse conditions (Goodfellow and Williams 1983; Chater 1993). Furthermore, the filamentous nature of these bacteria and ability to

Table 2 Abundance of actinomycetes

	Samples	S1	S2	S3
	Depth (cm)	10–30	30–50	50–70
Actinomycetes	Olson (×10^5 c.f.u/g of sand)	3.3 ± 0.1	2.6 ± 0.3	23.3 ± 1.7
	SEI (×10^5 c.f.u/g of sand)	37.0 ± 0.2	4.0 ± 0.7	63.0 ± 1.1
	SEII (×10^5 c.f.u/g of sand)	13.6 ± 0.7	4.0 ± 0.2	17.6 ± 0.5

adhere to solid particles of sand might increase their presence in the top layer and slow down their elimination (Thirup et al. 2001).

Antibacterial activity of isolates

Properly designed biological filters or infiltration systems have the capacity to significantly reduce effluent concentrations of pathogenic microorganisms in wastewater (Bomo et al. 2003; Hassani et al. 1999; Rafouk 2005). The antimicrobial activities of sand filter strains could be involved in microbial removal mechanisms in infiltration systems which are a combination of physical, chemical and biological factors. In this study, a total of 122 actinomycetes subjected for primary screening process and the distribution of active isolates is shown in Table 3. Despite the low actinomycete abundance observed at the depth between 30 and 50 cm, the highest percentage of active strains (90%) was shown for this sampling point. For the other depths, the percentage of active strains was over 60%. This percentage is in line with those described by Barakate et al. (2002) studying the activity of actinomycetes from some Moroccan soils. Only 44 strains were screened for their activity against more than seven Gram-positive and Gram-negative bacteria tested. Of the 44 active isolates, 15 strains were active against both Gram-positive and Gram-negative organisms, 16 against Gram-positive and only 2 against Gram-negative bacteria. The investigation of the activity against the eleven microorganisms tested allowed the possibility to divide the 44 active isolates into several groups using their spectrum of activity. However, the reported results were anticipated because earlier studies showed an importance of the constituents of the screening media and the temperature under which the producing microorganisms were cultivated (Iwai and Omura 1992). Although nutrient availability is a major factor controlling activity of soil actinomycetes, other factors such as temperature may play an important role (Goodfellow and Williams 1983).

In the secondary screening performed by agar blocks (Figs. 1, 2), out of the 44 active isolates, 48% were active against (B.s), 43% against (A.a), 41% against (S.a), 31% against (V.c), 23% against (M.p), 18% against (B.c), 11% against (Ml), 4% against (*E.c*3) and (S.sp), and only 2% against (Kp) and 43% against *Candida* sp. strains (Fig. 3). Using the agar block method, strains 40S2, 44S2, 32S3, 15S3 and 31S1 showed a broad activity spectrum against

Table 3 Antibacterial activity of actinomycetes isolates from several depths

Depth (cm)	10–30	30–50	50–70
Number of actinomycete	43	47	33
Number of active isolates	26	43	17
Purcentage of active isolates	60.46	91.49	62.96
Test bacteria			
Vibrio cholerae non O1	23	25	11
Aeromonas hydrophila ATCC 7966	8	13	4
Aeromonas sobria	7	11	2
Aeromonas caviae	9	10	1
Escherichia coli ATCC 94	3	6	1
Escherichia coli	7	14	2
Salmonella typhimurium ATCC 13314	5	10	2
Salmonella typhimurium	5	10	0
Bacillus subtilis ATCC 9524	17	41	17
Micrococcus luteus ATCC10240	10	27	10
Bacillus cereus ATCC14579	14	31	9

Antagonism was estimated by local confrontation between actinomycetes and test bacteria

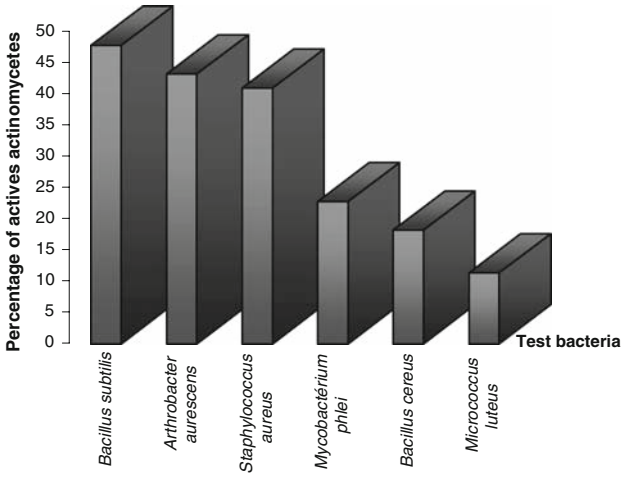

Fig. 1 Activity spectrum of 44 active actinomycete isolates against Gram-positive bacteria (*Active strains belong to S1, S2 and S3)

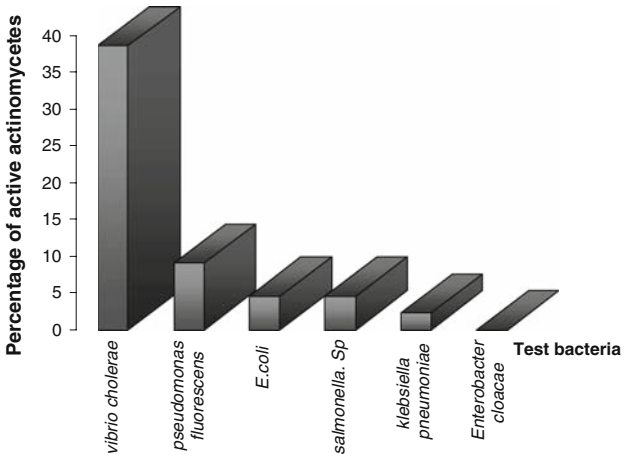

Fig. 2 Activity spectrum of 44 active actinomycete isolates against Gram-negative bacteria (*Active strains belong to S1, S2 and S3)

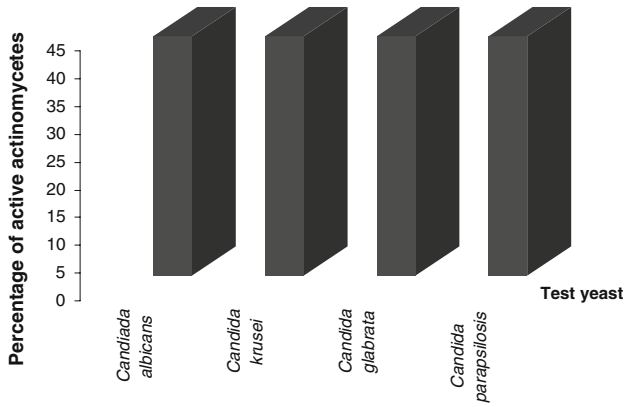

Fig. 3 Activity spectrum of 44 actinomycete isolates against yeasts (*Active strains belong to S1, S2 and S3)

bacteria and yeast (Table 4). As regards the behaviour sensitivity/resistance of bacteria tested, (B.s), (M.l), and (S.a) were the most susceptible strains. They were inhibited by all active strains. (E.c3) and (E.c) exhibited resistance to all active actinomycete strains; whereas, (S.sp) did not exhibit susceptibility to all active strains. The removal of microorganisms during infiltration can be attributed to the combination of straining, adsorption and inactivation and the actinomycete antagonistic proprieties in this system. However, in order to optimize retention and elimination of bacteria in infiltration systems, it is necessary to understand how design and maintenance affect individual biotic and abiotic factors and their interactions Stevik et al. (2004). The studies of Hassani et al. (1999) and Rafouk (2005) of antibiotic resistance among faecal coliform bacteria isolated from wastewater before and after treatment by an experimental sand filter showed that *E. coli* was abundant in raw wastewater, whereas, in treated effluents, the proportions of *K. pneumoniae*, *Ent. cloacae* and *C. freundii* were higher. At all sampling points, these three latter species showed a higher percentages of drug resistance than *E. coli* (Hassani et al. 1999).

Table 4 Antimicrobial activity selected isolates using agar blocks methods

Test bacteria	Selected isolates[a]				
	40S2	44S2	32S3	15S3	31S1
E. coli K12 (W1130)	0	0	0	0	0
Enterobacter cloacae	0	0	0	0	0
Salmonella sp. CCMM B17	0	15	0	0	0
Klebsiella pneumoniae	0	0	0	0	15
Pseudomonas fluorescens DSM50090 ATCC13525	20	0	0	0	30
Vibrio cholerae Non-O1	23	28	14	15	15
Mycobactérium phlei DSM 750	0	30	0	0	0
Micrococcus luteus ATCC 10240	18	17	0	0	18
Bacillus subtilis ATCC	23	30	24	24	33
Bacillus cereus ATCC 14579	22	30	22	23	30
Arthrobacter aurescens DSM200116 ATCC13344	26	30	22	22	28
Staphylococcus aureus CCMM B3	20	30	17	19	27
Candida glabrata	0	0	16	13	14
Candida albicans	0	0	18	12	15
Candida parapsilosis	0	0	17	13	14
Candida krusei	0	0	12	12	16

[a] The activity was estimated by measuring the diameters (mm) of in vitro inhibition zones

Taxonomic characterization of the selected isolates

Identification of isolated actinomycetes may follow one of several methods of classification. Major works on the identification of actinomycetes have been published in a review by in Holt et al. (1994). Identification to genus can usually be accomplished by using a combination of morphological and chemical properties (Goodfellow 1989), but characterization to species is often more difficult. 16S rRNA sequencing has also been used to differentiate between genera of actinomycetes, which generally agree with morphological and chemical taxonomy, although there are some differences (Embley and Stackebrandt 1994). In this study, the five selected strains were tested for taxonomical diversity using morphological, cultural, physiological and biochemical criteria as well as other features (Table 5). Morphology of the actinomycete colonies was determined on the media used for their isolation. The aerial and substrate mycelium colour was determined on media ISP2, 3, 4 and 6. The five strains showed different abilities to assimilate 17 carbon sources tested. Strains 40S2, 32S3, 15S3 and 31S1 were able to use all tested carbon sources whereas inositol, D-raffinose, sorbitol, rhamnose, galactose, and xylose were not used by 44S2. The analysis of cellular constituents of the nine isolates revealed the presence of the L-diaminopimelic acid (DAP). All the five selected isolates were predicted to belong to the genus *Streptomyces*.

The sequencing of the 16S RNA of these strains (Table 6) confirmed this classification. 40S2 and 44S2 isolates exhibited 99.3 and 99.5% sequence identity to *Streptomyces coeruleofuscus* and *Streptomyces rectiverticillatus* respectively. 32S3, 15S3 and 31S1 isolates

Table 5 Biochemical and morphological characteristics of the five active isolates

Characteristics	Strains origin				
	40S2	44S2	32S3	15S3	31S1
ISP3	+++	++	++	++	+++
ISP4	+++	−	++	++	+++
ISP6	+	−	+	+	+
Aerial spore mass	Grey	Grey	White	White	White
Colony reverse	Grey	Grey	Cream	Cream	Cream
Soluble pigment	−	Violet	Deep yellow	Deep yellow	Deep yellow
Spore morphology	RF	RF	SS	SS	SS
DAP-isomer	LL	LL	LL	LL	LL
Gram staining	+	+	+	+	+
Tyrosin hydrolysis	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Catalase	+	+	+	+	+
C. source utilization					
Sucrose	+	+	+	+	+
Fructose	+	+	+	+	+
Glucose	+	+	+	+	+
Glycerol	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Melibiose	+	+	+	+	+
Mannose	+	+	+	+	+
Citrate	+	+	+	+	+
Mannitol	+	+	+	+	+
Galactose	+	−	+	+	+
Inositol	+	−	+	+	+
Rhamnose	+	−	+	+	+
Xylose	+	−	+	+	+
D-Raffinose	+	−	+	+	+
Sorbitol	+	−	+	+	+
Arabinose	−	−	+	+	+

+, tested positive/utilized as substrate; −, tested negative/not utilized as substrate; RF, rectiflexible; SS, spiral

Table 6 Percentage of sequence identity to the sequence of 16S RNA of other Actinomycetes strains

Selected isolate	Sequence identities (%)	Actinomycetes strains
40S2	99.3	*Streptomyces coeruleofuscus*
44S2	99.5	*Streptomyces rectiverticillatus*
32S3	99.5	*Streptomyces violaceorubidus*
15S3	99.5	*Streptomyces violaceorubidus*
31S1	99.5	*Streptomyces violaceorubidus*

The alignments were made with 1,400–1,500 bp long DNA fragments

exhibited 99.5% sequence identity to *Streptomyces violaceorubidus*. Although 32S3, 15S3 and 31S1 belong to the same species using partial 16SrDNA gene, the isolates produced different active compounds expressed by their biological activity spectrum. The inhibition zones between the different isolates express their diversity in producing different antibiotics (Raaijmakers et al. 2002; Anibou et al. 2008). The results obtained are in line with those reported in the literature. Indeed, the *Streptomyces* family is the most common among the actinomycete isolates from water (Zaitlin and Watson 2006), polluted water habitats (Naidenova and Vladimirova 2002) and from constructed wetland for industrial effluent treatment (El-Shatoury et al. 2004).

In conclusion, sand filters for wastewater treatment habitats might be a rich source of actinomycetes species producing antimicrobial compounds. Comparing these results with those of other authors, it could be said that members of *Streptomyces* family are the most common among the isolates from polluted regions. This is, probably, due to their remarkable resistance to bad environmental conditions and to different pollutants and also to their

antagonist activity against pathogen microorganism as shown for *Streptomyces rectiverticillatus* (44S2) against S*almonella* sp. The involvements of actinomycetes of the sand filter in bacterial removal as well as purification of compounds produced are under investigation.

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