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PROTEASE ACTIVITY OF SOME MESOPHILIC STREPTOMYCETES ISOLATED FROM EGYPTIAN HABITATS

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Summary

Different streptomycetes (317 isolates) were obtained from several sources and areas in Egypt and were screened for proteolytic activity. Thirty nine of them produced proteases and were subjected to identification. Streptomyces anulatus formed the most abundant portion of the isolates. This species deserves special attention because it is a good candidate for biotechnological applications.

Key words: *Egypt, identification, isolation, protease, streptomycetes.*

Introduction

Proteases are one of the three largest groups of industrial enzymes. They account for nearly 60 % of the total enzyme sales covering about 20 % of the world market, and they are used mainly in detergents [15, 26, 37]. Proteases possess high catalytic activity and substrate specificity. They can be industrially produced in large quantities and are economically essential for the detergent, protein, brewing, meat, photographic, leather, and dairy industries.

Proteases are common enzymes in plant and animal tissues, fungi, and bacteria. Microorganisms are the preferred proteases producers, as they grow rapidly, require small cultivation space, and can easily be subjected to genetic manipulation. Bacterial proteases are industrially the most significant compared to animal and fungal proteases [37].

The possible use of streptomycetes for enzyme production has recently been investigated, and proteases have been obtained from various species [2, 21]. The streptomycetes comprise Gram-positive bacteria of high G+C content and unusual morphological complexity, which develop in their life cycle substrate and aerial mycelia, sporophores and spores [5]. Several proteases were obtained from streptomycetes and were biochemically characterized, such as serine protease produced by *Streptomyces pactum*, metallo- and serine proteases from *Str. exfoliatus*, and aminopeptidase from *Str. rimosus* [3, 17, 18, 36]. These enzymes are involved in the assimilation of proteinaceous nitrogen sources, degradation of aerial mycelium sporulation process, as well as in antibiotic production [16, 19]. In Egypt, high quantities of proteases are imported from abroad and it was found of great interest and of economic importance to produce industrially these enzymes by use of microorganisms.

The aim of the present investigation was to isolate and characterize mesophilic streptomycetes strains and test them for proteolytic activity. The *Streptomyces* isolates that showed considerable proteolytic activity were taxonomically identified.

Materials and Methods

Sampling and sampling sites. Samples were collected from three different types of sources from five Egypt governorates. The industrial source was taken from Cairo Tanneries sewage (T) and soil around it (Ts), the agricultural soil from Plant Island at Aswan (As) and agricultural field at El-Sharkia (Es). The water sources were obtained from a fish farm at Port Said (Fw), sediments with neutral (Fs) and alkaline pH (Fs*), and Qaroun Lake water (Q) at El Fayoum.

Isolation of streptomycetes. The serial dilution method was applied for isolation of streptomycetes, and each sample was diluted to 10^{-6} [12]. Eight agar media were used for isolation as follows: starch-nitrate [24], malt-yeast extract [29], Difco actinomycetes isolation, brain-heart infusion (BHI) [22], modified glucose-aspartic acid-ammonium nitrate, modified starch–aspartic acid-ammonium nitrate [8], lactose-peptone [6] and maltose-leucine-lycine [20]. 0.1 ml inoculum of the appropriate dilution was placed on each plate. The plates were incubated at 28 ^{o}C for 7-14 days to allow the slow growing forms to develop. Streptomycetes were isolated based on their specific morphological characteristics and then subjected to purification.

Screening for proteolytic activity. The streptomycete isolates were screened for their ability to produce protease. The enzyme activity was determined by three different methods. Extracellular protease production was assessed by the size of the clear zone as poor (≤1), fair (≤2), good (≤3) and very good (>3) on 3 % brain heart infusion supplemented with gelatin [22] and on egg-yolk agar [25]. The ability of the isolates to liquefy gelatin was rated as well [10].

Identification of streptomycetes. The active streptomycetes isolates were taxonomically identified through morphological, physiological and chemotaxonomical investigations. The following tests were carried out: colony and micromorphological characteristics, pigment production, lecithinase, lipolysis, proteolysis, hydrolysis of pectin, chitin, hipurate, degradation of xanthine, elastine, arbutin, utilisation of sucrose, I-inositol, D-fructose, xylose, galactose, glucose, L-arabinose, rhamnose, D-mannitol, raffinose, NO_3^- reduction, H_2S production, whole cell sugar pattern and cell wall chemotype [13, 33, 34, 38].

Statistical analysis. The streptomycetes isolates showing proteolytic activity were subjected to all the tests listed. The obtained data were examined for normality and homogeneity of variance. Analysis was done using the SPSS software package for the dendrograms generation [31], similarity calculations were based on simple matching (S_{SM}). The UPGMA algorithm was applied for dendrogam generation.

Results and Discussion

A wide range of methods can be applied to detect proteases using gelatin as a substrate [11]. Another approach is based on substrate hydrolysis as an evidence for gelatinase-like proteases production [4]. Egg yolk is routinely used in proteolytic activity detection test systems [1].

A total of 317 streptomycete isolates were obtained on the eight different isolation media from the various collected samples. All of them were screened for protease production on 3 % BHI supplemented with gelatin, gelatin and egg-yolk agar medium. 39 isolates demonstrated proteolytic activity (Table 1). They originated from all the studied samples except those from Cairo Tanneries sewage (T). The activity was the best on BHI with gelatin, and it was moderate on the other two media what conformed to the results of Vermelho et al. [35] who reported that streptomycetes hydrolyzed preferentially gelatin incorporated in BHI. As actinomycetes are known to be good protease producers [9, 27], the protein substrate and the composition of the medium could markedly influence the extracellular protease production [35]. Differences in the ability to utilize various protein substrates may be due to substrate specificity of the produced enzyme [28]. Gelatin, being a type 1 collagen, is the most effective protease inducing substrate. It is possible that the gelatin, as a high molecular weight protein, increases the protease production to degrade the substrate to a suitable form for the microorganisms.

The methods described above are quite relevant for detecting extracellular protease directly in the culture medium. The use of gelatin in the culture medium provides a qualitative assay, which is a simple, inexpensive, straightforward method to assess the proteolytic activity of a given microbial colony. Thus, in addition, substrate selection is comfortable since low

Table 1. Proteolytic activity of some mesophilic streptomycetes isolated on different media.

Medium	Number of strains and activity			
	Poor	Fair	Good	Very good
3 % BHI + Gelatine	*2* Es 48, 112	*10* Es 4, 24, 37, 127 As 81; Fs 1, 19 Fw 13, 14 Q1	*18* Es 5, 81, 119, 130 As 8, 10, 45, 49, 53, 71, 75, 77 Fs 3, 13, 16 Fw 11; Fs*4 Ts 3	*9* Es 22, 117 As 22, 94 Fs 35; Fs*5 Ts 5, 6, 9
Gelatine medium	*9* Es 24, 37, 48, 112, 117 As 8, 81 Fs* 4, 5	*23* Es 4, 5, 22, 81, 119, 127, 130 As 10, 22, 49, 53, 71, 77, 94 Fs16 Fw 11, 13, 14 Ts 3, 5, 6, 9 Q1	*7* As 45, 75 Fs 1, 3, 13, 19, 35	*None*
Egg-yolk agar	*11* Es 22, 81, 112, 130 As 8, 22, 45, 75 Fw 11, 13 Ts3	*15* Es 4, 117, 119 As 10, 49, 53, 71, 81 Fs 13, 19 Fw14 Ts 5, 6, 9 Q1	*13* Es 5, 24, 37, 48, 127 As 77, 94 Fs 1, 3, 16, 35 Fs* 4, 5	*None*

molecular weight (egg yolk) or high molecular weight (gelatin) proteins could be used. Consequently, simplicity is the greatest advantage of these methods, although they cannot be used for quantitative analyses.

The isolates that showed proteolytic activity were characterized and identified. The results of the morphological, physiological and chemotaxonomical tests presented in Tables 2 and 3 were subjected to statistical analysis (SPSS) and a dendrogram of the active streptomycetes isolates was plotted (Fig. 1). Using the determinative keys [13, 33, 34, 38] different *Streptomyces* species were identified. The cell walls of all the strains contained the diagnostic amino acid L-DAP and did not contain diagnostic sugars. This is characteristic for the species of genus *Streptomyces*.

The most frequently isolated streptomycetes (43.6 %) were identified as *Str. anulatus* (Fig. 2). The strains in this group belonged to the yellow and grey colour series, and formed spores with smooth surface arranged in rectiflexibiles or occasionally spiral chains. As a rule, they did not produce melanoid pigments, with a few positive exceptions, particularly on tyrosine agar. This phenotypically variable species is widespread in nature where decaying organic matter is present. Based on the phenotypic scheme of Williams et al. [38], *Str. alboniger*, *Str. aureofaciens* and *Str. griseus* are nomen species of *Str. anulatus*, and all they produce extracellular proteases [20, 23, 32].

It is intriguing that the closest phenotypic relative of *Str. anulatus*, *Str. albidoflavus* was also detected (10.3 %). The two clusters were connected at 80 % S_{SM}, and that was in accordance with the results of Williams et al. [38] who reported the level of similarity at 77.5 %. Most strains in this group had rectiflexibiles spore chains and smooth spore surface. Melanoid pigments were rarely produced. The spore colour was yellow or sometimes white. Proteases have been detected in cultures of *Str. albidoflavus* [16, 30]. The genome sequencing of *Str. coelicolor*, subjective synonym of *Str. albidoflavus*, reveals a multide of putative protease genes [14].

Five strains (12.8 %) were identified as *Str. microflavus*. Their sporophores were rec-

tiflexibiles or spiral, the spore surface was smooth and the spore mass was grey. Most strains produced melanoid pigments.

Another cluster was identified as *Str. exfoliatus* (5.1 %). These strains also had rectiflexibiles spore chains, smooth spore surface, and the spore mass was grey. Some strains produced melanoid pigments. Kim et al. has mentioned that *Str. exfoliatus* produces proteases. Single member phenons were also detected as *Str. lydicus* (5.1 %), *Str. chromofuscus* (5.1 %), and *Str. lavendulae* (2.6 %). Two small clusters were identified as *Str. atroolivaceus* and *Str. violaceus* (5.1 % each). Only two isolates (5.1 %) could not be identified at species-level and remained to be refferred as *Streptomyces* sp. All of the *Streptomyces* species studied possessed rectiflexibiles or spiral spore chains, the spore surface was smooth, spiny or hairy, and the spore mass was yellow or grey. Melanoid pigments were produced by all but few of the strains. All the identified *Streptomyces* species are well known as active producers of proteases [7, 16].

Table 2. Morphological characteristics of some streptomycetes isolates.

Characteristics	Number of positive strains	
Spore chain morphology		
Closed spiral	*1*	Es4
Flexible	*15*	Es 5, 37, 81, 112, 117, 130; As 49, 53, 75; Fs 3, 13, 16, 19, 35; Fw14
Flexible, straight	*12*	Es24; As 8, 71, 77, 81, 94; Fw13; Ts 3, 5, 6, 9; Q1
Straight	*4*	Es 22, 48, 127; As45
Straight, flexible	*3*	Es119; As10; Fs*4
Hooks	*4*	As22; Fs1; Fw11; Fs*5
Spore surface ornamentation		
Smooth	*34*	Es 4, 5, 22, 24, 37, 48, 81, 127, 130; As 8, 10, 22, 45, 49, 71, 77, 81, 94; Fs 1, 3, 13, 16, 19, 35; Fw 11, 13, 14; Fs* 4, 5; Ts 3, 5, 6, 9; Q1
Spiny	*4*	Es 112, 117, 119; As75
Hairy	*1*	As53
Colour of spore mass		
Medium grey	*4*	Es 4, 5, 48; As81
Light grey	*11*	Es 24, 81, 112, 117; As 10, 45, 77; Fs 1, 13,19; Fw11
Dark grey	*3*	As 8, 22, 53
Grey	*5*	Es37; As71; Fs16; Ts 3, 6
Yellow	*4*	Es 22, 130; Fs 3, 35
Green	*3*	Es 119, 127; Fw14
White	*4*	As49; Ts 5, 9; Q1
Blue	*1*	As 75
Pink	*4*	As94; Fw13; Fs* 4, 5
Pigmentation of substrate mycelium		
Yellow brown	*36*	Es 4, 5, 22, 24, 37, 48, 81, 112, 117, 127, 130; As 8, 10, 22, 45, 49, 53, 71, 75, 77, 81; Fs 1, 3, 13, 16, 19, 35; Fw 11, 13, 14; Fs* 4, 5; Ts 5, 6, 9; Q1
Yellow	*1*	Es119
Dark yellow brown	*1*	As94
Dark brown	*1*	Ts3
Diffusible pigments		
Yellow	*6*	Es22; As 71, 75; Fs 3, 35; Ts9
Citrin yellow	*1*	Es119
Orange	*1*	Fw11
Citrin orange	*1*	Es130
Orange citrin	*3*	As 49, 94; Fs16

Table 3. Physiological characteristics of streptomycetes isolates.

Characteristics	Number of strains	
	Positive	Negative
Utilization of carbon sources		
L-Arabinose	*22* Es 4, 24, 37, 48, 81, 112, 117; As 10, 22, 45, 53, 71, 77 Fs 1, 13, 16, 35; Fw13; Ts 3, 5, 6, 9	*17* Es 5, 22,119, 127, 130; As 8, 49, 75, 81, 94; Fs 3, 19 Fw 11, 14; Fs* 4, 5; Q1
D-Fructose	*28* Es 4, 5, 22, 24, 37, 81, 112, 127, 130; As 10, 22, 45, 49, 53, 71, 75, 81; Fs 1, 3, 13, 16, 19, 35; Fw 11, 14; Fs*5; Ts3; Q1	*11* Es 48, 117, 119; As 8, 77, 94; Fw13; Fs*4; Ts 5, 6, 9
Sucrose	*27* Es 4, 5, 24, 37, 81, 112, 117, 130; As 8, 10, 22, 49, 53, 71, 75, 77, 81; Fs 13, 16, 35; Fw 11, 13; Ts 3, 5, 6, 9; Q1	*12* Es 22, 48, 119, 127; As 45, 94; Fs1, 3, 19; Fw14; Fs* 4, 5
Rhamnose	*23* Es 4, 5, 24, 37, 81, 117, 119, 127, 130; As 8, 10, 22, 45, 53, 71, 75, 77, 81; Fs 1, 16, 19; Fw13; Ts3	*16* Es 22, 48, 112; As 49, 94; Fs 3, 13, 35; Fw 11, 14; Fs* 4, 5 Ts 5, 6, 9; Q1
D-Mannitol	*33* Es 4, 5, 22, 24, 37, 48, 81, 117, 119, 127; As 8, 10, 45, 49, 53, 71, 75, 77, 81, 94; Fs 1, 3, 13, 16, 19, 35; Fw 11, 14 Ts 3, 5, 6, 9; Q1	*6* Es 112, 130; As22; Fw13; Fs* 4, 5
D-xylose	*28* Es 4, 22, 24, 37, 48, 112, 117, 119, 127; As 8, 10, 22, 49, 53, 71, 75, 77, 81, 94; Fs 16, 35; Fw 11, 13, 14; Fs* 4, 5; Ts3; Q1	*11* Es 5, 81, 130; As45; Fs 1, 3, 13, 19; Ts 5, 6, 9
Raffinose	*13* Es 37, 48, 81, 119; As 8, 10, 22, 71, 75, 77; Fs19; Fw11; Ts3	*26* Es 4, 5, 22, 24, 112, 117, 127, 130; As 45, 49, 53, 81, 94 Fs 1, 3, 13, 16, 35; Fw 13, 14; Fs* 4, 5; Ts 5, 6, 9; Q1
I-inositol	*15* Es 4, 5, 24, 81, 112, 117, 130; As 22, 53, 75, 77, 81 Fs1; Fw11; Ts3	*24* Es 22, 37, 48, 119, 127; As 8, 10, 45, 49, 71, 94; Fs 3, 13, 16, 19, 35; Fw 13, 14; Fs* 4, 5; Ts 5, 6, 9; Q1
Galactose	*37* Es 4, 5, 22, 24, 37, 48, 112, 117, 119, 130; As 8, 10, 22, 45, 49, 53, 71, 75, 77, 81, 94; Fs 1, 3, 13, 16, 19, 35; Fw 11, 13, 14 Fs* 4, 5; Ts 3, 5, 6, 9; Q1	*2* Es 81, 127
H_2S production	*31* Es 4, 5, 22, 24, 37, 48, 81, 112, 117, 119, 127, 130; As 8, 22, 49, 53, 71, 75, 81, 94; Fs 3, 13, 16, 19, 35; Fw 13, 14 Ts 3, 5, 6, 9	*8* As 10, 45, 77; Fs1; Fw11; Fs* 4, 5; Q1
Nitrate reduction	*31* Es 5, 22, 24, 37, 112, 119, 127; As 8, 10, 22, 49, 71, 77, 81, 94; Fs 1, 3, 13, 16, 19, 35; Fw 11, 13, 14; Fs* 4, 5 Ts 3, 5, 6, 9; Q1	*8* Es 4, 48, 81, 117, 130; As 45, 53, 75

Table 3. Continued.

Characteristics	Number of strains	
	Positive	Negative
Degradation of		
Xanthine	*16* Es 4, 5, 81, 119; As 10, 22, 53, 75, 81; Fs1; Fw11 Fs* 4, 5; Ts 5, 6, 9	*23* Es 22, 24, 37, 48, 112, 117, 127, 130; As 8, 45, 49, 71, 77, 94 Fs 3, 13, 16, 19, 35; Fw 13, 14; Ts3; Q1
Elastine	*None*	*All*
Arbutin	*36* Fs 4,15, 22, 24, 37, 81, 112, 117, 119, 127, 130; As 8, 10, 22, 45, 49, 53, 71, 75, 77, 81, 94; Fs 1, 3, 13, 16, 19, 35 Fw 11, 14; Fs* 4, 5; Ts 5, 6, 9; Q1	*3* Es48; Fw13; Ts3
Pectine	*13* Es 4, 5, 24, 37, 48; As 8, 49, 53, 71; Fs 1, 13, 16, 35	*26* Es 22, 81, 112, 117, 119, 127, 130; As 10, 22, 45, 75, 77, 81, 94 Fs 3, 19; Fw 11, 13, 14; Fs* 4, 5; Ts 3, 5, 6, 9; Q1
Chitin	*None*	*All*
Enzyme activity		
Protease	*38* Es 4, 5, 22, 24, 37, 48, 81, 112, 117, 119, 127, 130; As 8, 10, 22, 45, 49, 53, 71, 75, 77, 81, 94; Fs 1, 3, 13, 16, 19, 35 Fw 11, 13, 14; Fs* 4, 5; Ts 3, 5, 6, 9	*1* Q1
Lipase	*18* Es 4, 37, 130; As 10, 22, 45, 49, 71, 81; Fs3, 35 Fw 11, 13, 14; Ts 3, 5, 6; Q1	*21* Es 5, 22, 24, 48, 112, 117, 119, 127; As 8, 53, 75, 77, 94 Fs 1, 13, 16, 19; Fs* 4, 5; Ts9
Lecitinase	*27* Es 4, 5, 24, 48, 81, 112, 127, 130; As 10, 45, 49, 53, 71, 77, 81 Fs 1, 3, 13, 16, 19, 35; Fw14; Fs* 4, 5; Ts 3, 6, 9	*12* Es 22, 37, 117, 119; As 8, 22, 75, 94; Fw 11, 13; Ts5; Q1
Melanine production on		
Iron agar	*16* Es 22, 48, 112, 119, 130; As 8, 22, 45, 49, 75; Fs 1, 3, 16, 35 Fw 13, 14	*23* Es 4, 5, 24, 37, 81, 117, 127; As 10, 53, 71, 77, 81, 94 Fs 13, 19; Fw11; Fs* 4, 5; Ts 3, 5, 6, 9; Q1
Tyrosine agar	*6* Es 22, 112, 119, 130; As75; Fs35	*33* Es 4, 5, 24, 37, 48, 81, 117, 127; As 8, 10, 22, 45, 49, 53, 71, 77, 81, 94; Fs 1, 3, 13, 16, 19; Fw 11, 13, 14; Fs* 4, 5; Ts 3, 5, 6, 9; Q1

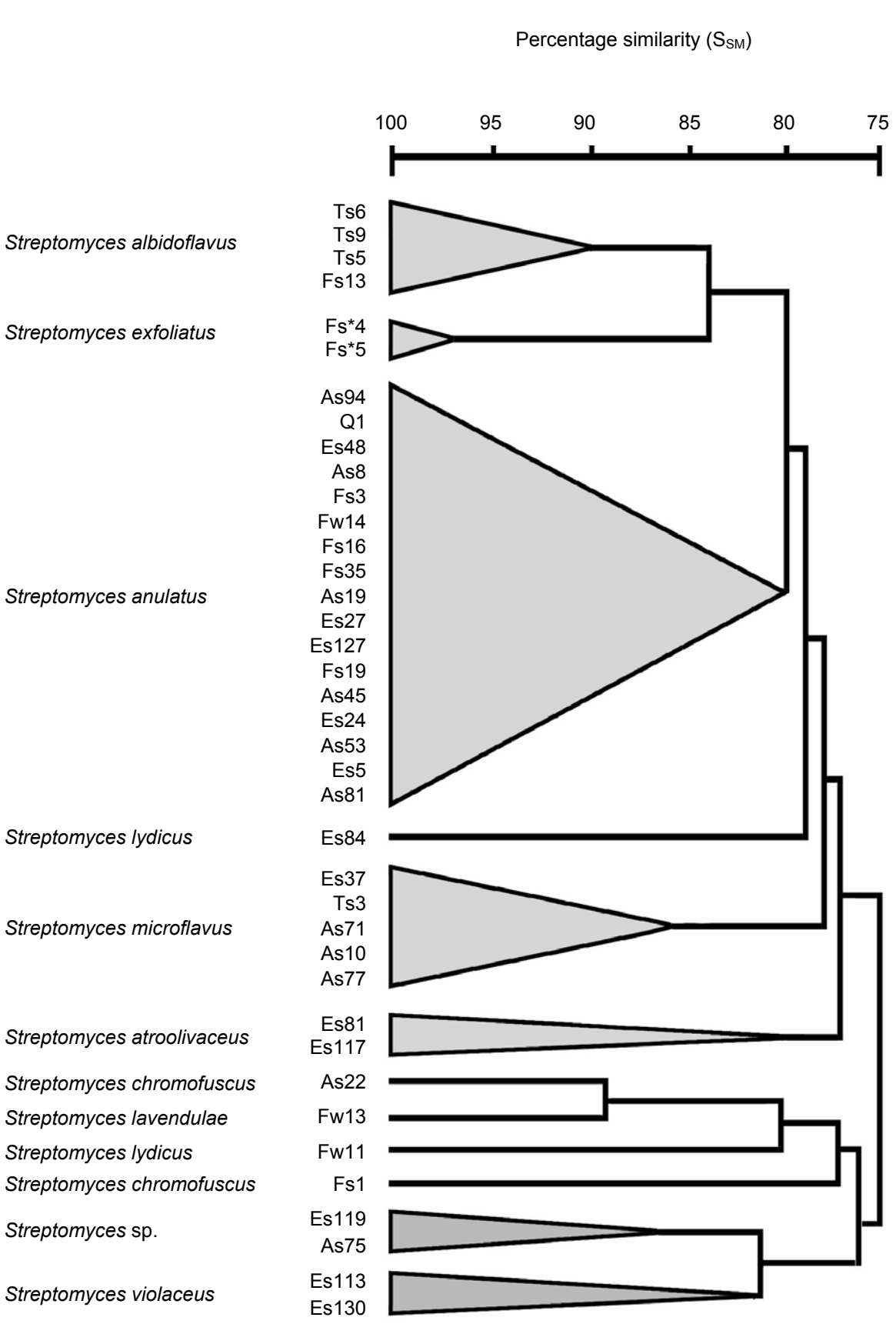

Fig. 1. Phenogram of *Streptomyces* species, producers of proteolytic enzymes.

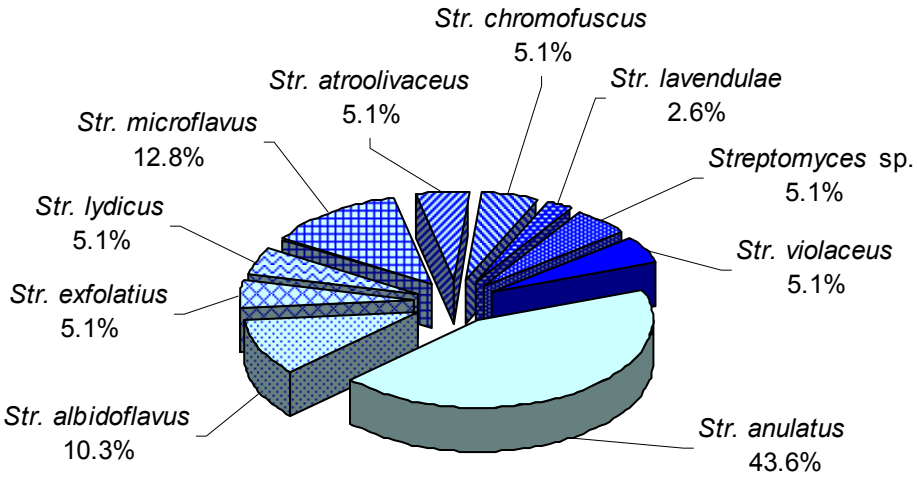

Fig. 2. Distribution of *Streptomyces* species, producers of proteolytic enzymes.

Conclusions

The present study demonstrated the production of multiple extracellular proteolytic enzymes by different *Streptomyces* species isolated from several sources, which were coordinate during growth. More than 300 streptomycetes strains were screened for protease activity in three assay media and 39 of them actively produced proteases. Rapid, sensitive detection and qualitative assay of streptomycetes proteases were highly desirable. The production of proteases was much more sensitively detected on 3 % BHI containing gelatin than on gelatin or egg yolk agar media. The streptomycetes isolates that were active in protease production were subjected to identification. The obtained results displayed *Str. anulatus* as the major source for proteases. This species is widely acknowledged as a vast reservoir of natural products with different activities. The diverse metabolic capacities of *Str. anulatus* strains and their specific growth characteristics, i. e. mycelium formation and relatively rapid colonization of selective substrates, facilitate their collecting as suited proteases producers. Beside *Str. anulatus*, different *Streptomyces* species contribute to the production of proteases. These organisms grow in various environments. The use of indigenous strains for proteases production is an alternative approach, since the organisms have been already adapted to the habitat.

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ПРОТЕАЗНА АКТИВНОСТ НА МЕЗОФИЛНИ СТРЕПТОМИЦЕТИ, ИЗОЛИРАНИ ОТ РАЗЛИЧНИ МЕСТООБИТАНИЯ В ЕГИПЕТ

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Резюме

Триста и седемнадесет стрептомицетни култури са изолирани от различни източници и области в Египет и са изпитани за протеолитична активност. От тях 39 продуцират протеази и те са определени до вид. Най-много изолати са отнесени към Streptomyces anulatus. Този вид заслужава особено внимание поради големия си потенциал за приложение в биотехнологиите.