Antimicrobial activity of moderately haloalkaliphilic Streptomyces roseosporus A3 isolated from saline-alkaline soils of Ararat soils of Ararat plain in Armenia

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ANTIMICROBIAL ACTIVITY OF MODERATELY HALOALKALIPHILIC *STREPTOMYCES ROSEOSPORUS* A3 ISOLATED FROM SALINE-ALKALINE SOILS OF ARARAT PLAIN, ARMENIA

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Abstract
A moderately haloalkaliphilic streptomycete strain with high antimicrobial activity was isolated from the saline-alkaline soils of Ararat Plain, Armenia and phenotypically identified as *Streptomyces roseosporus* A3. The isolate exhibited optimal growth at 5% NaCl and pH 9 at 37°C and had high antimicrobial activity against Gram-positive bacteria and yeasts. Optimum salt and pH value for antibiotic production was 2% NaCl and pH 9, respectively. The antimicrobial compound was extensively synthesized at stationary stage of growth and had high stability against proteinase-K.
Keywords: Saline-alkaline soils, soil *Streptomyces,* alkaliphiles, halophiles, antimicrobial activity

Introduction

Actinobacteria, especially representatives of the genus *Streptomyces,* produce over two-thirds of pharmaceutically useful antibacterial, antifungal, anti-tumor agents and immunosuppressants of natural origin [1, 2]. The isolation of novel actinomycetes from extreme environments have promised a raise in the prospect of discovering new natural compounds that can be developed as a resource for biotechnological research and drug discovery [3, 4]. It is widely accepted that alkaliphilic actinomycetes will provide a valuable resource for novel products of industrial interest, including enzymes and antimicrobial agents [4]. So far only few haloalkaliphilic streptomyces have been explored for their antimicrobial activity [4, 5]. Despite extensive exploration of the actinomycetes for their antimicrobial products in the past, the search for novel molecules having unique therapeutic properties continues being an active area of research.

Hydromorphic saline-alkaline soils are developed in the areas of Ararat Plain, Armenia, where subsoil water is mineralized and located close to the surface (1-2 m). They are specified by strong salinity (total soil content 1-3%), considerable carbonatization, low humus content (<1.0%), high alkaline reaction (pH 9-11) and high absorbed sodium content [6]. In these conditions only alkaliphilic and halophilic bacteria are able to remain viable. In this study we describe the isolation and characterization of a haloalkaliphilic streptomyces identified as *Streptomyces roseosporus* A3, from saline-alkaline soils of Ararat Plain, Armenia and study of their antimicrobial activity.

Materials and methods

Isolation of halophilic streptomyces

The soil samples were collected from 6 different sites of the saline-alkaline soils of Ararat Plain, Armenia and were transferred under sterile conditions to the laboratory and stored at 4°C until use. Soil samples were serially diluted in sterile water and spread plated

over medium containing 0.1% KNO_3, 0.1% $CaCO_3$, 0.05% K_2HPO_4, 0.05% $MgSO_4$ x $7H_2O$, 0.0001% $FeSO_4$ x $7H_2O$, 5% NaCl, 2% glucose, 1.5% agar pH 9.0. After incubation at 37°C for 5 days typical actinomycetes colonies were picked and subcultured until purification [7].

Phenotypic characterization

Actinomycetes preliminarily were recognized by traditional morphological criteria including their characteristic tough leathery colonies, morphology of substrate and aerial hyphae and pigment produced [8]. Sporangiums were observed by light microscopy using the method of intersect lines (90x) [9]. Physiological characteristics of the isolate were tested in a range of pH (5-12), temperature (4-60°C) and salinity (NaCl concentrations 1-20%). Biochemical tests were carried out following the method recommended by Bergey's Manual of Determinative Bacteriology [9], Shirling and Gottlieb [10] and Gause [11].

Antibiotic resistance was tested by using the commercial discs contained (μg): kanamycin (30), stereptomycin (10), gentamycin (10), ampicillin (10), erythromycin (15) and tetracycline (30).

Selection of antimicrobial activity

Isolates were examined for their antimicrobial activity by using agar plug method [7]. As test organisms were used *Saccharomyces cerevisiae, Bacillus subtilis, Staphylococcus sp., Enterococcus faecalis and Escherichia coli E17* strains. After incubating for 24 h at 37°C the intensity of antimicrobial activity was found by measuring the zones of growth inhibition of test organism. In order to determine the effects of temperature, pH and NaCl concentration on production of antimicrobial compound the strain was grown on agar medium supplemented with different values of pH (from 5 to 11), NaCl concentrations (0-12%) and temperature (from 4 to 60°C) separately. In order to find out correlation of in growth and production of antimicrobial substances, the culture was incubated in a liquid medium at optimal conditions (pH 9 and 2% NaCl) with continuous shaking at 120 rpm at 37°C and was tested its antimicrobial activity in various stages of culture growth.

To determine the location of antimicrobial substance previously incubated culture at stationary stage of growth was centrifuged (5000 g) to divide supernatant and the cell mass and their antimicrobial activity was examined separately by using agar plug method [10]. The antimicrobial compound stability was also checked against Proteinase-K (Sigma, USA). The culture was incubated with 10 U of Proteinase-K at room temperature for 30 min [10] and antimicrobial activity was determined as described above.

Results

In total five isolates of actinomycete designed as A3, A5, Sov-1, Sov-2 and Sov-4 were isolated from soil samples collected from different sites of the saline-alkaline soils of Ararat Plain, Armenia. The morphological characteristics like musty odor, spore formation, dimorphic mycelial forms such as aerial and substrate mycelium, and the Gram-positive non-motile nature of the colonies indicated that they belong to the genus *Streptomyces.*

The results of the antimicrobial screening assays indicated that isolated A3 showed significant antimicrobial effect against tested Gram-positive bacteria and yeast (Fig.1).

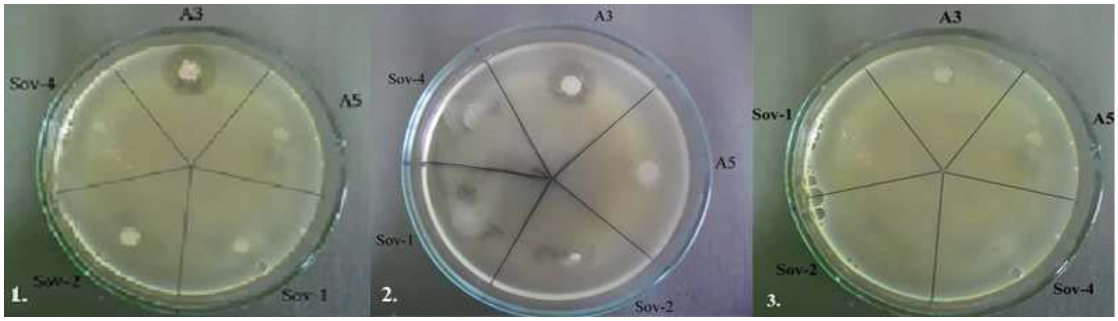

Figure 1. Antimicrobial activity of A3 isolate against *B. subtilis* (1), *S. cerevisea* (2) and *E. coli E17* (3).

A3 isolate was selected and further characterized based on morphological, physiological and biochemical properties. The isolate A3 was Gram-positive, having a long filamentous structure. Aerial mycelium of isolate A3 was pink, with yellowish white substrate mycelium. Sporangiums were well branched (Fig.2).

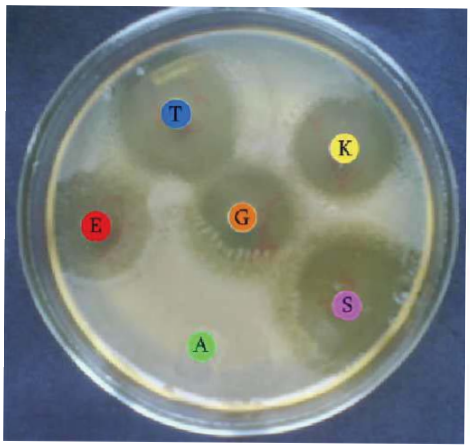

Figure 2. Microscopic examination of the aerial mycelium of isolate *A3* with branch sporangiums (90×)

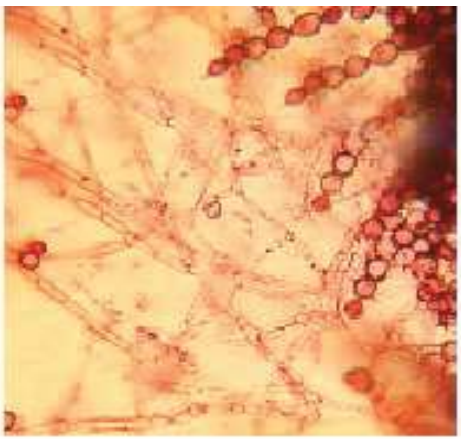

Figure 3. Antibiotic resistance of isolate A3. (T)-Tetracycline, (K)-Kanamycin, (S)-Streptomycin, (G)-Gentamycin, (A)-Ampicillin and (E)-Erythromycin

The isolate exhibited optimal growth at 5% NaCl and pH 9 at 37°C. The Isolate was catalase positive, utilized a wide range of carbon sources: glucose, arabinose, mannitol and xylose. The negative test on MacConkey agar revealed that isolate could't utilize lactose. The utilization of starch and casein showed that these isolates produced the extracellular enzymes amylase and protease to metabolize the polymeric components. Phenotypic characteristics of the isolate are given in Table-1.

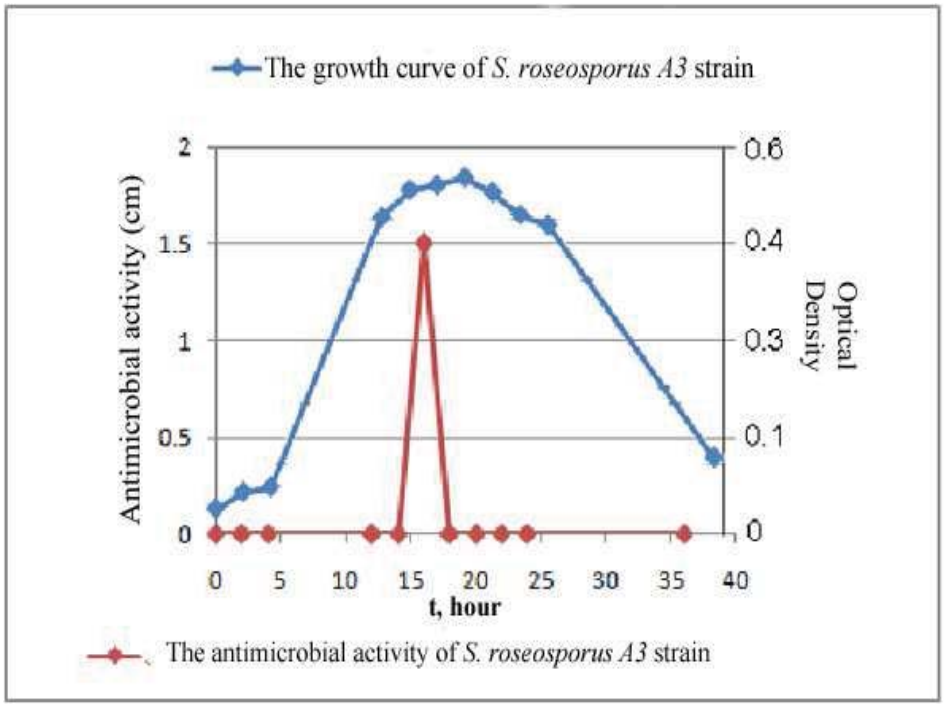

Figure 4. The growth curve and antimicrobial compound production during the growth of *S. roseosporus A3* at optimum conditions.

Table 1. Phenotypic characteristics of the isolate A3.

Test	Result	Test	Result
Gram's stain	+	Growth on NaCl	
Cell shape	Mycelial	2%	+
MacConkey agar growth	-	5%	+
Motility	-	10%	+
Voges-Proskauer test	-	15%	-
Catalase	+	Growth on pH	
Indole production	-	5	-
Acid production from		8	+
Glucose	+	9	+
Arabinose	+	10	+
Mannitol	+	11	+
Xylose	+	12	-
Citrate utilization	-	Growth at T◦C	

Starch hydrolysis	+	4	-
Gelatin hydrolysis	-	21	+
Casein hydrolysis	+	37	+(Optimum)
Anaerobic growth	-	56	+
Nitrate reduction	+	60	-

+, Positive; -, Negative reaction

Antibiotic resistance of isolate A3, tested by using the commercial discs, showed stability towards ampicillin (Fig.3). Following to the criteria of Bergey's Manual of Determinative Bacteriology [9] and Actinomycete identification keys [10, 11] isolate A3 were identified as *Streptomyces roseosporus*.

As for antimicrobial activity test of *S. roseosporus A3* shows high antimicrobial activity against *B. subtilis,* farther studies were carried out with it as test organism. Determination of temperature, pH and NaCl concentration effects on production of antimicrobial compound was revealed that optimum of antibiotic production was 37^0C, pH 9 and 2% NaCl respectively.

To determine the correlation of growth and production of antimicrobial substance, the culture was incubated at optimal conditions under shaking and in different stages of culture growth its antimicrobial activity was tested. This experiment shows that the antimicrobial substance of *S. roseosporus A3* strain is highly produced at the stationary stage of growth (16^{th} h of culture incubation) (Fig. 4). It was shown that antimicrobial substance produced by *S. roseosporus A3* has intracellular location and Proteinase-K did not have any inhibiting effect on it.

Discussion

Apart from normal actinomycetes, the salt-tolerant and alkaliphilic actinomycetes are much less explored for their antimicrobial potential. A moderately haloalkalophilic actinomycete strain designated as *Streptomyces roseosporus* A3 with characteristic antagonistic property against mainly Gram-positive bacteria was isolated from a soil sample collected from Ararat Plain, Armenia. Optimum growth of *Streptomyces roseosporus A3* in moderate salt concentration (5% NaCl) and pH 9 suggests that the organism is moderately halophilic and alkaliphilic one. Although *S. roseosporus* A3 strain optimally growth in medium with 5% NaCl, the optimal conditions for antimicrobial production and activity of strain was 2% NaCl. This result is quite comparable with literature data. Recently, it was shown the antimicrobial production of marine actinomycete isolated from the Sundarbans region of the Bay of Bengal, India, was maximum with 5% NaCl and pH 7-9, while growth optimum of isolate was 20% NaCl [12]. The antimicrobial activity of *S. roseosporus* A3 strain was determined after 16 h incubation of culture, which is common with it stationary stage of growth. Therefore, the result is quite comparable with *Streptomyces sp*. KEH 23, for which stationary stage of growth begins after 2 days of culture growth and antibacterial activity was also exhibited in this stage [13].

Although studied strains phenotypically identified as *S. roseosporus*, this need to be further confirmed by phylogenetic analysis based on 16S rRNA gene sequences, fatty acid analysis, DNA-DNA hybridization. While these results are important for further taxonomic work, positive result on antimicrobial activity is indicative of potential application of isolate.

Conclusions

The distribution of alkaliphilic and halophilc actinomycetes inhabiting in saline-alkaline soils of Ararat Plain, Armenia was studied. In total five haloalkaliphilic actinomycete strains were isolated from soil samples collected from different sites of the saline-alkaline soils of Ararat Plain, Armenia. The morphological characteristics like musty odor, spore formation, dimorphic mycelial forms, the gram-positive non-motile nature of the colonies indicated that they belong to the genus *Streptomyces* of the bacterial community. A moderately haloalkalophilic strain phenotypically identified as *Streptomyces roseosporus* A3 was selected as an active producer of antimicrobial compound mainly against Gram-positive bacteria and yeasts. Effects of temperature, pH and NaCl concentration on antibacterial activity were determined in order to use in further studies. The intracellular antimicrobial substance was highly produced at the stationary stage of strain growth and had high stability against Proteinase-K.

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