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Research Article

SCREENING FOR ANTIBACTERIAL ACTIVITY OF S*TREPTOMYCES* SPECIES ISOLATED FROM ZANJAN PROVINCE, IRAN

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ABSTRACT

Introduction*:* The *Streptomyces* genus is well studied owing to its capacity in producing more than 70% of antibiotics. This study was undertaken to characterize *Streptomyces* strains occurring in soils of Zanjan province, Iran as well as to evaluate their potential to produce antimicrobial compounds.

Methods*:* Samples were collected from agriculture and Non- agriculture zones. In primary screening by agar overlay method, *Streptomyces* strains were assessed for antibiotic production and activity against four bacteria including *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Then active isolates were selected for secondary screening by microplate MIC. Then, 16S rRNA PCR was carried out for confirmation of isolates. **Results**: In primary screening among all of the isolates, 154/229 (67.24%) isolates were active against at least one of the test organisms and 20/229 (8.7 %) strains exhibited a broad-spectrum activity against both test bacteria. The minimum inhibitory concentrations (MICs) of the ethyl acetate extracts ranged from 0.78-200 µg/mL and the least minimum bactericidal concentration (MBC) demonstrated was 12.5 µg/ml against strain *Staphylococcus aureus*. 16S rRNA gene for two more active strains was sequenced and submitted to GenBank. **Conclusion:** These results highlight the importance of *streptomyces* isolates in antibiotic production

Keywords: *Streptomyces* species, Antibacterial activity, 16S rRNA, PCR, MIC.

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INTRODUCTION

Soil is the most extensively studied ecological niche. Organisms such as bacteria present in these environments are good sources of bioactive metabolites[1-3]. One of the most important and well acknowledged groups of microorganisms in the soil is the actinomycetes[4-5]. The actinomycetes especially Streptomyces strains are the most common microorganisms found in soil that produce antimicrobial agents[5]. They produce over two-thirds of the clinically useful antibiotics of natural origin, such as aminoglycoside, anthracyclines, chloramphenicol, β -lactams, macrolides and tetracyclines[6].

Screening directed towards novel antibiotics from Streptomyces has been intensively pursued for many years by researchers. Each year screening of Streptomyces strains as source of new antimicrobial compounds are directed by many pharmaceutical companies[3,7]. Although different bioactive compounds have been isolated from Streptomyces, these are thought to represent only a small fraction of the repertoire of bioactive metabolites produced[4,6,3]. Previous studies show that this group of microorganisms still remains a rich source of important antibiotics[8]. About 50% of all Streptomyces isolated from different sources able to produce

antibiotics[4]. Decreasing in exploring of new Streptomyces strains in recent years, has increased the probability of discovering new compounds from these microorganisms[6,9]. In the future screening of actinobacteria, especially Streptomyces may be lead to the discovery of nearly 100,000 new compounds with different applications[10].

The search for next generation of pharmaceutical agents has increased the exploration of unexplored habitats for new Streptomyces strains[11-12], especially with the increasing of antibiotic-resistant pathogens and the pharmacological limitations some previous marketed antibiotics[13]. Considerable research is being done in order to find new chemotherapeutic agents isolated from soil[14-16]. A few studies have been done so far using Iranian soils to screen for new Streptomyces for new bioactive compounds[17].

This report describes the isolation of Streptomyces strains producing antibacterial secondary metabolites from soil samples collected from different places around Zanjan province, Iran.

METHODS

Soil sample

From 2011 to 2012, 46 soil samples were collected from various locations in Zanjan province of Iran and its surroundings. Several diverse habitats in different areas were selected for the isolation of Streptomyces strains. These habitats included the agricultural soil, preserved areas, mountains soil, waterfall, desert, caves, and mines soil. The samples were taken up from 5-15 cm depth into sterile polyethylene bags, air dried for 3 days at room temperature to reduce the bacterial flora with no harms to the growth of Streptomyces while noting sampling region's explicit features such as pH and altitude[18-19].

Isolation of streptomyces

Isolation and enumeration of Streptomyces colonies performed by soil dilution plate technique using ISP-2 agar (Malt extract 10 g, Yeast extract 4 g, Dextrose 4 g, Agar 15-20 g, Distilled water 1 L) complemented with nystatin (50 μg/ml)[20]. One gram of dried soil was suspended in 9 ml of distilled water then preheated in a shaker incubator at 28 °C, 200 rpm for 30 min. Mixtures were allowed to settle, and serial dilutions up to 10^{-6} were prepared using distilled water and agitated with the vortex at maximum speed. Then 0.1 ml of third concentration was applied for inoculation of ISP-2 agar and the plates were incubated at 28 °C for 7-14 days[19].

These plates were used for isolation of some representative colonies of Streptomyces. Selected colonies (typically pigmented, dry, and powdery) were aseptically transferred from mixed culture of the plates onto respective agar plates and incubated onto at 28 °C for other 7 days. Plates containing pure cultures were stored at 4° C until further examination. After antimicrobial activity screening, the incongruent colonies were kept in 15% glycerol (v/v) at -80°C and tagged as a discrete isolate based on their sampling location and order of colony isolated from same soil sample[19,21].

Test organisms

Four bacteria, including one Gram positive (Staphylococcus aureus ATCC 25423) and three Gram negative (Escherichia coli ATCC 8739, Pseudomonas aeruginosa ATCC 9027 and Salmonella typhi ATCC 5762) were used to assay the antimicrobial activity of the Streptomyces strains. All these test microorganisms were obtained from the department of biotechnology, school of pharmacy, Zanjan University of medical science (Zanjan, Iran).

Antibacterial bioassay

Antibacterial activity of the isolated strains was evaluated against test organisms by double-layer agar method[22] on ISP-2 medium[10,23]. All pure streptomyces strains were spot inoculated on ISP-2 agar medium for six days at 28°C. After grow, 6 mm in diameter, colonies were overlaid with 7 mL of sloppy-nutrient (0.6%) layer containing 100 μL of seeded microbes and incubated for 5 h at 37°C (optical density of 0.6 at 520 nm). Plates were kept at 4°C for at least 2 h to allow the diffusion of produced antimicrobial metabolites. Zones of inhibition around the colonies were examined after 24 h and 48 h incubation at 30 or 37 °C.

Fermentation and extraction of antibacterial metabolites

Based on the results of preliminary screening, 30 putative Streptomyces isolate were selected for the fermentation and assessment of antibiotic production. A loopful of selected strain was inoculated into a 250 mL Erlenmeyer containing 10 mL of ISP-2 broth (seed broth) and incubated on a rotary shaker at 150 rpm, 28 °C. After 48 h of incubation, the seed culture (10% v/v) was transferred to a 1000 mL Erlenmeyer containing 100 mL of the fermentation medium (ISP-2 broth) and incubated on a rotary shaker (200 rpm) at 28°C for 7-14 days.

After fermentation the supernatant from each flask separated from the mycelium by centrifugation at 10,000 rpm for 20 min, and then whole broth was extracted three times with ethyl acetate. Ethyl acetate was added to the filtrate in the ratio 1:3 (v/v) and shacked for 1 h for complete extraction. Two layers separated the aqueous layer and organic layer which contained the solvent ant antimicrobial agent. All the three ethyl acetate phase that were concentrated and evaporated to dryness in water bath at 80-90 °C to obtain crude extracts.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

TheMIC and MBC was determined using the broth micro dilution method using test bacteria that have been standardized to optical density of 0.05 at 660 nm and presence of standard chloramphenicol (50 mg/ml). Extracts were re-suspended in DMSO at a concentration of 50 mg/mL. Using a 96-well microtitre plate, 100 µL nutrient broth was dispensed into all the 96 wells. 50 µL of extract was added into well 3, mixed carefully using pipette and diluted serially along the row to row 11. Addition of the standardized test bacteria into the 96-well microtitre plate was incorporated. Plates were covered and incubated at 37 °C for 18-24 hr. Control wells were loaded with nutrient broth and extract with no bacteria added (wells 1 A-H), broth and bacteria with no extract (wells 12 A-H). Each extract was assayed in duplicate against test bacteria.

After incubation for 18-24 h at 37°C, MIC values were recorded as the lowest concentration of the extract that inhibits the growth of the test organisms. The MBC was determined as the lowest concentration yielding negative subculture after incubation for 48 h.

Molecular study

Among these 229 isolates tested, 30 isolates which were found to be promising were subjected to molecular studies. The genomic DNA was extracted according to previously study[24] and stored at -20 °C.

Each strain is identified to the genera by amplification fragment of the gene 16S rDNA resulting of the polymerase chain reaction (PCR) by using specific primers of StrepB (5' - ACAAGCCCTGGAAACGGGGT - 3') and StrepE (5' - CACCAGGAATTCCGATCT - 3')[25]. The 519 bp fragment of 16S rDNA was amplified in a thermocycler (i-cycler, Bio-Rad) under the following condition: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 60 sec, 57 °C for 30 sec, 72 °C for 2 min and final extension at 72°C for 7 min.

The PCR reaction mixtures (25 µl) contained 1 µL of each StrepB / StrepE primers, 12.5 µl PCR master mix (Cinagen, Iran), 2 µl bacterial genomic DNA as the template DNA and PCR grade water. Streptomyces coelicolor PTCC 1187 was used as standard strain. The PCR products were analyzed using electrophoresis on 1% agarose gel and marked using 1 kb DNA ladder (Fermentas Co.) as the size marker. After migration gels were exposed to ultraviolet light (UV) to locate the amplified bands.

For sequencing of 16S rDNA (Bioneer; Korea), amplification carried out using 27f (TAGAGTTTGATCCTGGCTCAG) and 1392R (GACGGGCGGTGTGTACA) primers. The sequences were compared for similarity with reference sequences in genomic databases using BLAST available at http://blast.ncbi.nlm.nih.gov/.

RESULT AND DISCUSSION

Rapidly emerging strains of bacteria resistant to most advanced antibiotics have become issues of very important public health concern. The screening of microbial natural products continues to represent an important route to the discovery of novel chemicals for the development of new therapeutic agents. Microorganisms belonging to the Actinomycetaceae family, and specifically to the Streptomyces genus, have been proven as a potential source of bioactive compounds and richest source of secondary metabolites[26].

However in the past two decades, there has been a decrease in the discovery of new lead compounds from common soil-derived actinomycetes. For this reason, Research currently directed towards unexploited environments presents a vast potential for new compounds that could be able to safely and effectively target resistant species. This study was undertaken with an aim of highlighting the presence of Streptomyces in these ecosystems and selecting the strains with antibacterial activity. Different scientific researches were carried out on the antibiotic-producing actinomycetes in various ecosystems of Iran, but this study gives the first hand information on the antimicrobial activity of putative indigenous Streptomyces isolated from the Zanjan province, Iran environment.

Isolation of Streptomyces from soil samples

Using the selective medium supplemented with appropriate antibiotic and cultivation conditions described previously a total of 229 Streptomyces, were isolated from 46 soil

samples collected from agriculture and Non-agriculture different zones of Zanjan province, Iran. These results confirm the abundance of Streptomyces in compared to the other genera of Actinomycetes.

The colonies slow growing, aerobic, glabrous or chalky, heaped, folded and with aerial and substrate mycelia of different colors were isolated as Streptomyces. In addition, all colonies possessed an earthy odor. A confirmatory identification to genus was based on acid-fastness, Gram-stain and molecular study. Most of isolates tend to grow in alkaline soils which are an important characteristatic feature of streptomyces species[27].

Antimicrobial activity

Primary screening by agar overlay method revealed that among the 229 tested isolates, 154 strains were active against at least one of the tests-bacteria including Escherichia coli ATCC 8739, Salmonella typhi ATCC 5762, Staphylococcus aureus ATCC 25423 and Pseudomonas aeruginosa ATCC 9027. Among 38.9% and 8.7% of active isolates produced antibacterial substances against gram-positive and gram-negative bacteria respectively (Figure 1).

In former studies, it was shown that the isolation rate of actinomycetes with antimicrobial activity is higher than 40%[28] and in others less than 10%[29]. In this study, the rate of isolation of actinomycetes with antibacterial activity was 67.3% (figure 1). This percentage is higher than those described by Barakate et al. studying on the activity of Moroccan soil Streptomyces[30]. These results were also different from those of other authors showing 16% in soil of Turkey[19] and 44.5% in soils of South-Eastern Serbia[31]. The antibacterial spectrum exhibited by all extracts highlights their potentials and suggests that they could be important candidates for antibiotics in this regard.

According to antibacterial activity and spectrum broadness, thirty of the isolates those were active against all test bacteria selected for recovery antimicrobial compound using ethyl acetate solvent and further study. In former studies, it was concluded that the most of the antimicrobial compounds are extracted using ethyl acetate[32].

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The biological activities (MIC and MBC) of the crude extracts emphasizes that the extracts are active against Gram-positive and Gram-negative bacteria. The MICs values exhibited by all extracts in this study ranged between 0.78-200 µg/ml (Table 1). This was relatively higher than the MIC values obtained from Streptomyces strains against B. subtilis ATCC 6051, S. aureus ATCC 6538 and M. luteus with MICs of 0.0036 mg/ml, 0.0008 mg/mL and 0.002 mg/mL respectively[33]. However, the MIC is not a constant for a given agent, since it is influenced by a number of factors. These factors include the nature of the test organism used, the inoculum size, and the composition of the culture medium, the incubation time, and aeration.

During the screening of the novel secondary metabolite, Streptomycetes isolates are often showed good antimicrobial activity in agar medium but not in broth culture[34]. The investigation of putative isolates using primary screening and secondary screening revealed different results: some of the active isolates did not show the activity in the secondary screening while some showed little activity and some showed improved activity[35].

Gram negative bacteria are inherently more resistant to antimicrobials than Gram positive organisms and this has been ascribed to the combined exclusion of antimicrobial compounds by double membrane barrier and transmembrance efflux present in this group of organisms[36]. The result of primary and secondary screening revealed that most of the active isolates were active against gram positive bacteria (S. aureus) rather than gram negative bacteria. The differences in the susceptibilities of Gram positive and Gram negative bacteria to the study Streptomyces extracts have been observed by previous workers[31].

Molecular assay

The study of morphological, physiological and biochemical characters as well as the parietal components, in particular the amino acids and the glucides provides very distinctive specific information. However, these characters in certain cases fail to classify an isolate in only one genus[37]. Therefore molecular studies are one of the strangest and easiest methods to identification.

In this study we used the molecular tests to conform identification the isolated bacteria. PCR amplification using two specific designed primers for 16S rDNA gene from Streptomycetes was led to amplification of a fragment of 519 bb of 16S rDNA gene (Figure 2). The 16S rDNA gene of two more active strain deposited in the GenBank database of NCBI under accession numbers JX142139 for Fs030 and JX142140 for Sm042.

CONCLUSION

The present finding highlights the importance for further investigation towards the goal of obtaining novel antimicrobial agent from the putative Streptomyces from this untapped habitat. The emergence and dissemination of antibacterial resistance is well documented as a serious problem worldwide. It is anticipated that due to the antibacterial profile and characterization of the crude extracts, putative Streptomyces isolated from Zanjan soils promises to be useful in the discovery of novel antibiotics.

It is therefore recommended that further investigation should address the relationship between the structure of the active component of the extracts and the broad spectrum activity, as well as a rapid method for large scale production and purification and whether this group of antibiotics has any application in managing human infectious disease.

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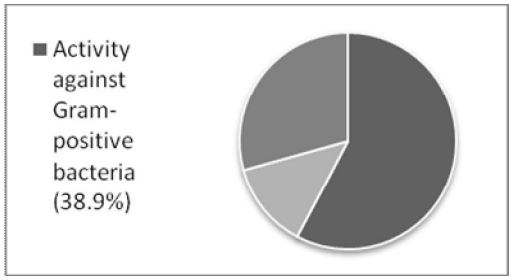

Fig. 1: Primary screening of soil isolates for antimicrobial activity by agar overlay technique

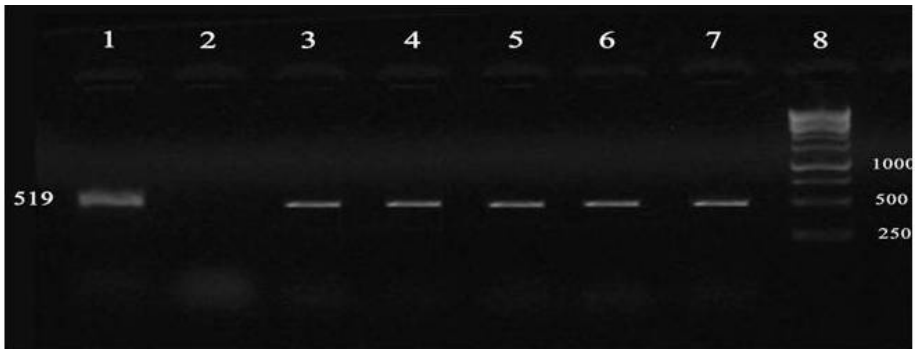

Fig. 2: 1; Positive Control: *Streptomyces coelicolor* PTCC 1187 (519 bp)، 2; Negative Control، 3; Am036 ،4; Fs030، 5; Sm042، 6; Kz008، 7; Gv045, 8; Marker (1Kb)

Table 1: MIC and MBC of the crude extracts of test *Streptomyces* against test bacteria

Test *Strepomyces*	Test bacteria	MIC (µg/ml)	MBC (µg/ml)
KH001	*S. aureus*	3.12	50
	S. typhi	25	200
KH003	*P. aeruginosa*	50	200
	S. aureus	3.12	100
	S. typhi	6.25	50
Sm042	*E. coli*	12.5	200
	S. aureus	0.78	12.5
CM004	*E. coli*	50	NA

	S. aureus	3.12	12.5
	S. typhi	25	NA
VZ003	*S. aureus*	25	100
	S. typhi	50	NA
YZ004	*P. aeruginosa*	50	50
	S. aureus	6.25	25
	S. typhi	12.5	50
MA3201	*P. aeruginosa*	12.5	100
KH008	*S. aureus*	6.25	50
	S. typhi	25	>200
Gv045	*E. coli*	50	200
	P. aeruginosa	12.5	25
	S. aureus	1.56	12.5
	S. typhi	6.25	25
Sz1002	*E. coli*	50	200
	P. aeruginosa	12.5	100
	S. aureus	3.12	25
Az1104	*E. coli*	50	200
	P. aeruginosa	25	50
	S. aureus	3.12	25
	S. typhi	6.25	50
Mv1203	*S. aureus*	12.5	50
Mz1501	*E. coli*	25	100
	P. aeruginosa	25	>200
	S. aureus	12.5	25
	S. typhi	3.12	50
Mz1602	*S. aureus*	1.56	12.5
Mz1703	*S. aureus*	3.12	25
Mz1902	*P. aeruginosa*	50	100
	S. aureus	6.25	100
	S. typhi	6.25	50
SO024	*S. aureus*	50	200
Am3605	*P. aeruginosa*	50	100
	S. aureus	1.56	25
	S. typhi	25	25
DE026	*S. aureus*	50	200
TM032	*E. coli*	50	NA
	S. aureus	6.25	25
Fs030	*E. coli*	50	200
	P. aeruginosa	12.5	25
	S. aureus	3.12	25
	S. typhi	0.78	25
DR033	*S. aureus*	6.25	50
	S. typhi	50	200
DY034	*S. typhi*	50	100
MA3502	*E. coli*	3.12	50
	P. aeruginosa	12.5	100
	S. aureus	6.25	50
MA3503	*S. aureus*	25	50
	S. typhi	25	100
Am036	*E. coli*	25	25
	P. aeruginosa	25	100
	S. aureus	1.56	12.5
	S. typhi	6.25	25
Am049	*P. aeruginosa*	3.12	25
	S. typhi	12.5	50
Rm037	*E. coli*	50	NA
	P. aeruginosa	6.25	50
	S. aureus	25	100
	S. typhi	50	200
Gv4504	*E. coli*	50	NA
	S. aureus	50	200
	S. typhi	12.5	50
Kz008	*E. coli*	6.25	50
	P. aeruginosa	12.5	100
	S. aureus	3.12	25
	S. typhi	6.25	50

NA, Represent not susceptible activity; MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration.

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