Production and Partial Characterization of Bioactive Metabolites Isolated From Halophilic Actinomyces

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Abstract

Aim: The purpose of this study was isolation of halophilic *Actinomyces* and evaluation of their potential for producing antimicrobial metabolites.

Materials and methods: To isolate antimicrobial metabolites producing *Actinomyces*, culture supernatant of 51 halophilic *Actinomyces* isolates were assessed against *Escherichia coli* (PTCC 1330), *Pseudomonas aeruginosa* (PTCC 1074), *Bacillus cereus* (PTCC 1015), *Staphylococcus aureus* (PTCC 1112), *Candida albicans* (PTCC 5027) and *Aspergillus niger* (PTCC 5012) using Well Diffusion Agar (WDA) method. Then promising strains were identified by API kit (bio Merieux) and 16 SrRNA gene sequencing. The antimicrobial production was optimized at different temperatures, pHs and carbon and nitrogen-sources. Then structural analysis of metabolites was carried out by UV-visible, FT-IR and GC mass spectrometry.

Results: Of 51 halophilic Actinomyces isolates, three strains showed potent activity for production of antimicrobial metabolites. *Bacillus cereus*, *Staphylococcus aureus* and *Candida albicans* were sensitive to all and *E.coli* was sensitive to bioactive compounds produced by Bm18 and Ba8. *Pseudomonas aeruginosa* was resistance to all antimicrobial metabolites. Phenotyping and molecular identification of antimicrobial metabolites producing strains recognized them as *Streptomyces* sp., *Streptomyces roseoviolaceus* and *Streptomyces turgidiscabies*. Optimal temperature, pH and C, N-sources for growth of these strains were 27°C, 8 and fructose, xylose and yeast extract and peptone respectively. In addition, The UV-visible, FT-IR and GC mass spectrometry suggesting the peptide nature of compounds. However chloroacetate, ethylcholoroacetate and 4 chloro 3 hydroxybutyronitrite groups could be probably linked to structures, but complete structure elucidations were not fully recognized.

Conclusion: Our finding probably introduces new antimicrobial metabolites produced by halophilic *Actinomyces* however it needs more evaluation.

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Introduction

Actinomycetes are aerobic filamentous gram-positive bacteria belonging to Actinomycetales. These bacteria are similar to Corynebacteria and Mycobacterium and have high level Guanine-cytosine content (GC content) (55-75 %) (Ningthoujam *et al*., 2009a, Mageshwaran *et al*., 2011, Shouvik *et al*., 2012). Natural habitats of Actinomycetes because of their ability to produce several metabolites viz., enzymes, vitamins, hormones, antibiotics and pigments are soil, water and plants (Aghamirian and Ghiasian, 2009, Bharti *et al*., 2010, Radhakrishnan *et al*., 2011). These bacteria can grow in the artificial media and produce sticky colonies with different colors (Pragya *et al*., 2012). Recently, 12000 antibiotics are recognized and it is proved that more than 70 % of them were produced by Actinomyces family. Usually these antimicrobial compounds are secondary metabolites and they are belonging to aminoglycosides, beta lactam, macroloides and tetacyclines antibiotic groups (Jang and chang, 2005, Gontang *et al*., 2007, Bharti *et al*., 2010, Hogan, 2010). Although, *Actinomyces* family could produce several secondary metabolites, *Sterptomyces* have occupied a prominent position for production of pharmaceutical drugs such as antibacterial, antifungal and antitumor compounds (Monisha *et al*., 2011). On the other hand, natural habitats of these bacteria impacted on activity of the antimicrobial metabolites produced by *Streptomyces*. For example the production of antimicrobial metabolites depended on alkaline, acidic and salty environments (Bhattacharya *et al*., 2007, Dehnad *et al*., 2010). However, it should be noted that many environments still remain unexplored and need evaluation for a greater diversity of novel *Actinomyces*. Hence, the present study conducted to isolate halophilic *Actinomyces* and evaluate their potential for production of antimicrobial metabolites. In addition, the antimicrobial metabolites were chemically analyzed for determining their chemical structures.

Materials and Methods

Sample Collection and Phenotype Identification

In total, 51 salt sediment samples were collected from Maharlu Lakes in south of Iran. To collect samples, yellow, orange and brown color regions of each Lake selected and the sample was taken from depth of 10-15 cm. Then the samples transferred to the laboratory within 2 hours subjected for microbiological analysis. The collected samples added into trypticase soy broth with various salt concentrations of 3, 4, 5, 6, 7 and 8 % NaCl and incubated at 30°C. After 72 hours a loop of each culture streaked on the trypticase soy agar with similar salt concentration and incubated at 30°C for 72 hours. Then brown and white pigmented colonies with rough and crumbly texture (similar to *Actinomyces*) picked up and subjected to the Gram stain and microscopy examination. Finally, the potential of each suspected *Actinomyces* evaluated for the production of the bioactive metabolites (Dhananjeyan *et al*., 2010, Nanjwade *et al*., 2010, Baskaran *et al*., 2011, Tian *et al*., 2013).

Screening Of Bioactive Metabolite Producing Actinomyces

After initial screening of halophilic *Actinomyces*, the production of antimicrobial metabolites was evaluated using Well Agar Diffusion method. To perform the test each *Actinomyces* colony was cultured in trypticase soy broth with 5% NaCl and shacked in an orbital shaker at 25-30°C with 150 rpm for 48-72 hours. The suspension centrifuged at 10,000 rpm for 10 minute and filtered through sterile Whatman paper No.1 (Ningthoujam *et al*., 2009b). Then antimicrobial activity of the filtrate assessed against Escherichia coli (PTCC 1330), *Pseudomonas aeruginosa* (PTCC 1074), Bacillus cereus (PTCC 1015), Staphylococcus aureus (PTCC 1112), Candida albicans (PTCC 5027) and Aspergillus Niger (PTCC 5012) using Muller Hinton Agar (MHA) and Well Agar Diffusion (Citarasu *et al*., 2013). The experiment carried out by full culture of each antagonistic microorganism on the MHA and then wells were made in the agar medium by sharp borer. Afterward 100 µl of the supernatant added to each well and the plates incubated at 37 °C for 24 hours. After this period growth inhibition zone around wells considered the antimicrobial effect of the bioactive metabolites produced by the isolates and the size measured (mm) and recorded (Ningthoujam *et al*., 2009b).

Authentication of Bioactive Metabolite Producing Actinomyces Isolates

The isolates identified using biochemical tests viz., oxidase, catalase, Oxidative/Fermentative, casein and starch hydrolysis and API coryne kit (bio Merieux). Afterwards the bioactive producing *Actinomyces* authenticated by 16 SrRNA Gene sequencing. To perform the test DNA extraction of the isolates was done using DNA extraction kit (Roach Germany). The forward and reverse primers of 16 SrRNA genes were obtained from tug Kompnhagn (Denmark) (Table 1).

Table 1: Forward and reverse primer sequences for the 16SrRNA gene.

Primers	**5'→3'**

1000F16	CAACGAGCGCAACCCT
1492 R	GGTTACCTTGTTACGACTT

Master Mix of PCR with 25 µL volume, 18 µL of sterile water, 2.5 µL of PCR buffer at a concentration 10 times, 0.75 µL $MgCl_2$, 0.5 µL dNTP, 0.25 µl of the enzyme polymerase, 1 µ primers forward and reverse (with a concentration of 10 mol / µL) added and 1 µL of DNA template were mixed, and finally the PCR by thermocycler (Eppendorf Germany), was performed. The thermocycler was adjusted on 95 °C for 3 minutes followed by 95 °C for 1 minute, 56 °C for 45 seconds and 72°C for1 minute. The final extension was performed for 5 minutes 72°C (Dehnad *et al*., 2010). To ensure replication genes 16 SrRNA, electrophoresis was performed. Then for sequencing of 16 SrRNA gene, 50 µL of the PCR product was sent to the Makrogen Korea Company. Then Gene sequences achieved by using BLAST software available on the NCBI website (Bamzadeh *et al*., 2013).

Culture Optimization for Bioactive Metabolites Production

Culture optimization for production of antimicrobial metabolites carried out by different temperatures, pHs and carbon and nitrogen sources. The experiment performed by inoculation of the isolates into trypticase soy broth with 5% NaCl and incubated at temperatures of 25, 30, 35 and 40°C for 72 hours. Then 100 microliters of each supernatant (centrifugation at 10000 rpm, 20 min) added into the wells embedded in Muller Hinton agar cultured with Bacillus cereus (PTCC 1015) and incubated at 37°C. After 24 hours, the effect of temperature on production of the bioactive compounds was evaluated based on inhibition zone diameters (mm). The best pH for production of secondary bioactive metabolites determined using trypticase soy broth with 5% NaCl adjusted to various pHs of 5, 6, 7 and 8. Then the isolates inoculated in the medium and incubated at 30°C for 72 hours. Afterwards, *Bacillus cereus* (PTCC1015) fully cultivated on the Muller Hinton Agar and well made using sterile sharp borel and 100 µL of each supernatant (centrifugation at 10000 rpm, 20 min) added into the wells and incubated at 37°C for 24 hours. The effect of pHs on production of the bioactive metabolites was evaluated based on inhibition zone diameters (mm). To determine the best carbon and nitrogen sources glucose, lactose, maltose, sucrose, fructose, starch, glycerol, peptone, yeast extract and trypton were used. To perform the experiment all the carbon and nitrogen sources sterilized separately and added at 1% concentration into with 5% NaCl. Then, the isolates were separately inoculated to with 5% NaCl mixed with carbon and nitrogen sources and incubated in a rotator shaker incubator at 150 rpm at 30°C for 48-72 hours. Optimization of TSB with carbon and nitrogen sources was evaluated against *Bacillus cereus* (PTCC 1015) as mentioned above (Dhanasekaran *et al*., 2005).

Extraction of Bio Active Metabolites

Extraction of the bioactive compounds was done by cultivation of the strains in Trypticase soy broth (salt concentration was determined based on the isolated strains) and incubated in an orbital shaker incubator at 150 rpm at 30°C for 48-72 hours. After three days the fermented broth centrifuged at 10,000 rpm for 10 min. Then the mycelia biomass was separated by sterile filter paper Whatman No. 1 (Ningthoujam *et al*., 2009). For the extraction of antimicrobial metabolites different solvents viz., ethyl acetate, chloroform, acetone and ethanol were used. The solvents added to the filtered supernatants in 1: 1 proportion, and then were mixed and agitated for 45 min with homogenizer. The ethyl acetate and chloroform were separated from the aqueous phase by separating funnel (Dhananjeyan *et al*., 2010). The solvents were centrifuged at 5000 rpm for 15 minutes to separate traces of aqueous phase. Then, the solvents were evaporated at 70 and 80 °C. Solutions of the dark brown gummy compounds that obtained were used for determination of antimicrobial activities (Augustine *et al*., 2005).

Determination of Arbitrary Units of the Bioactive Metabolites

Arbitrary Unit (AU) of the bioactive metabolites determined based on Voravuthikunchai and his colleagues (Voravuthikunchai *et al*., 2006). To determine AU, 100 µL of various dilutions of each metabolite was poured into the Muller Hinton agar cultivated by Bacillus cereus (PTCC 1015). The plates incubated at 37°C for 24 hours. Arbitrary Unit of each metabolite was determined by reciprocal of highest dilution showing antimicrobial effect (Voravuthikunchai *et al*., 2006).

Bio Structural Analysis of the Bioactive Metabolites Produced By Actinomyces isolates

Biological structures of the bioactive compounds were evaluated by GC-mass spectrometry, UV- visible and Fourier Transform Infra-Red spectroscopy (FT-IR). To identify of the structures 5 mL of each bioactive metabolite used for GC-mass spectrometry (Muhanned *et al*., 2015). GC- mass spectrometry was performed using methanolic extract of bioactive metabolites. Helium was the carrier gas at the rate of 1.0 ml/min. Ionization voltage was 70 eV and ion source temperature was 230°C. All compounds were partially recognized using available data in the GC-MS library in the literatures. For UV- visible and FT-IR spectrophotometer analysis, the pure bioactive metabolites diluted to 1: 5 with the same solvent. The bioactive compounds were scanned in the wavelength ranging from 260-900 nm using spectrophotometer and the characteristic peaks were detected. FT-IR analysis for the bioactive metabolites performed using spectrophotometer system, which was used to detect the peaks in ranging from 400-4000 cm-1 and their functional groups. The peak values of the UV- visible and FT-IR were recorded (Augustine *et al*., 2005).

Results

Isolation of Bioactive Metabolites Producing Halophilic Actinomyces

In Total 51 *Actinomyces* isolates were assessed for the production of antimicrobial metabolites. Of all, three strains could produce antimicrobial metabolites. Table 2 showed the results of antimicrobial assay of the metabolites produced by the isolates against *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus, Candida albicans* and *Aspergillus niger*. As shown in this table *Bacillus cereus*, *Staphylococcus aureus* and *Candida albicans* were sensitive to all and *E.coli* was sensitive to the bioactive metabolites produced by Bm18 and Ba8. *Pseudomonas aeruginosa* was resistance to all bioactive metabolites produced by the isolated strains.

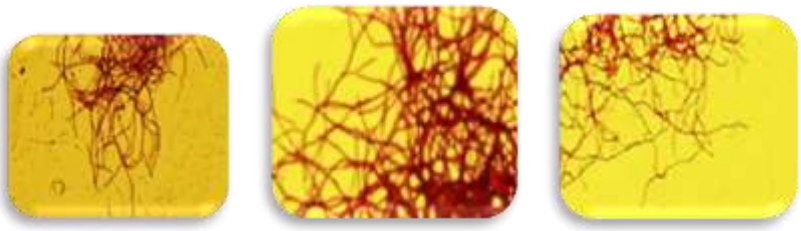
Table 2: Antimicrobial property of the bioactive compounds produced by presumptive halophilic Actinomyces isolates

Antagonistic	**zone of inhibition (mm)**		
Microorganisms	Bm18	Ba11	Ba8
Escherichia coli	21	-	14
Pseudomonas aeruginosa	-	-	-
Staphylococcus aureus	23	19	18
Bacillus cereus	15	14	16
Candida albicans	18	16	21
Aspergillus niger	-	11	13

* No zone the numbers represented in the table are average of three replicates

Identification and Authentication of the Bioactive Metabolite Producing Halophilic Actinomyces

As seen in plate 1 colony characters and the cell properties of the isolates were similar to Actinomycetes family. In addition the results obtained from phenotypic identification of the isolates illustrated that antimicrobial producing bacteria were *Actinomyces* spp. However, Alignment analysis of 16 SrRNA genes of the bacterial strains exhibited 99%, 100% and 99% identical to *Streptomyces* sp. 287, *Streptomyces roseoviolaceus* strain ISP 5277and *Streptomyces turgidiscabies* isolate TU. 1C respectively (Table 3).



A: Ba8 B: Ba11 C: Bm18

Plate 1: Morphological character of the bioactive compound producing *Actinomyces* isolates (100X)

Table 3: Alignment analysis of 16 SrRNA genes of bioactive metabolite producing *Actinomyces*

Strains identified	**similarity %**	**accession number**
Ba8 *Streptomyces* sp. 287	99%	AY582725.1
Ba11 *Streptomyces roseoviolaceus* strain ISP 5277	100%	NR_025493.1
Bm18 *Streptomyces turgidiscabies* isolate TU.1C	99%	KF782311.1

Extraction of the Bioactive Metabolites by Different Solvents

The result obtained from extraction of the bioactive compounds indicated that the best solvents for all were chloroform followed by acetone, ethylacetate and ethanol.

Structural Analysis of the Bioactive Metabolites Produced By Halophilic Actinomyces

The UV-visible absorption spectrum (UV 1601, Shimadzu Japan) of the antimicrobial compound was examined between 200 and 900 nm. The compounds showed absorption maxima at 264 and 269 nm were corresponding to characteristic absorption of peptide bonds. A shoulder at 268 nm indicated the protein nature of the compounds (Fig 1). The FT-IR (Fourier Transform Infrared) spectrum (FT-IR) (8400, Simadzu, Japan) exhibited characteristic absorption valley at 1024.02, 1024.98 Cm-1 (carboxyl group), valley at 667.25 and 668.214 Cm-1 (Gaussian amide bonds) and valley at 3327.57 and 3329.5 Cm-1 (hydrogen bonded OH groups). All results indicated that substance contains peptide bonds (Fig 2). The O-H stretching was indicted by valley at 3138. All above characteristics valleys indicated peptide-

based structure of the compound. As shown in Fig 3 Chromatogram GC-MS analysis of the methanolic extract of bioactive compounds showed the presence of three major peaks and the components corresponding to the peaks were determined as follows: The peaks of similar abundance, at 34, 36 and 39 m/z are likely to be L-alanine, methyl ester, L-(+)- Alanine and N-acetyl. In general, biological analysis of all metabolites illustrated that these compounds closely resemble to peptide type antibiotics in the antibiotic library and probably they have C_3H_5ClO, $C_4H_6ClO_2$ and C_4H_6ClNO but not exactly. Therefore, based on foregoing evidence and antibiotic library the compounds must be new molecules.

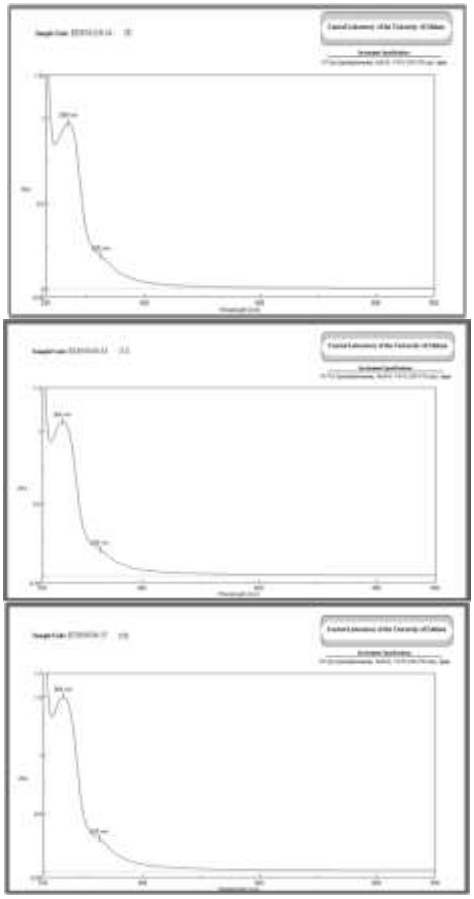
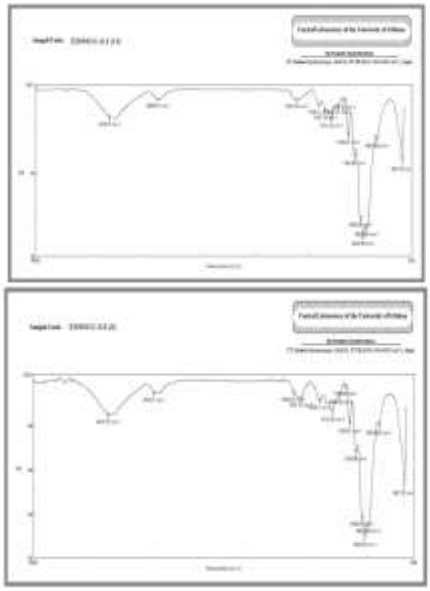


Figure 1: UV-visible absorption spectrum of the bioactive metabolites produced by *Streptomyces* isolates



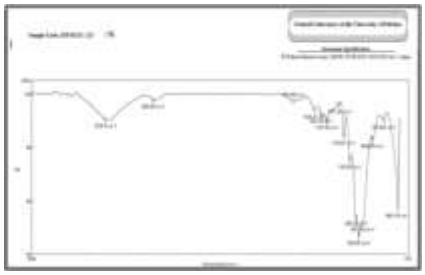


Figure 2: Fourier Transform Infrared (FT-IR) spectrum of the bioactive metabolites produced by *Streptomyces* isolates

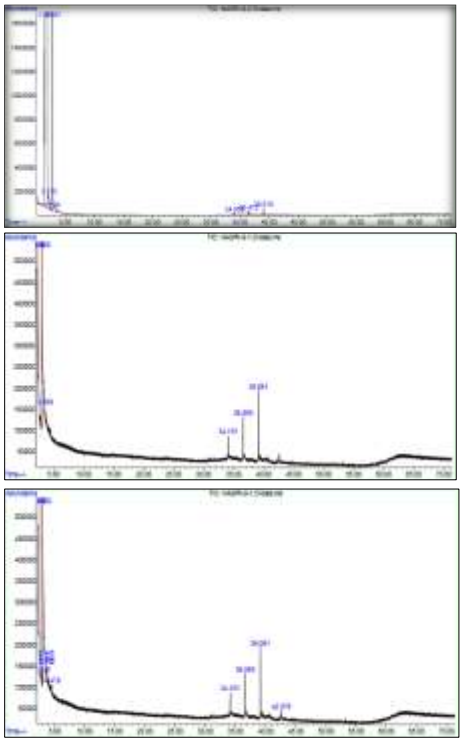


Figure 3: Gas chromatography-mass spectrometry spectrum of bioactive compounds produced by *Streptomyces* isolates

Discussion

Actinomycetes are considered major sources for production of secondary metabolites such as antibiotics and other bioactive compounds (Kekuda *et al*., 2010). Of all Actinomyetes, the genus Streptomyces has been reported to contribute almost 70% of antibiotics and anticancer metabolites (Bibb, 2005). Nowadays, several antibiotics are introduced to increase their activities and decrease the rate of occurrence of antibiotic resistant bacteria. Pharmaceutical industries attempted to find the new sources of the antimicrobial metabolites with high activity against pathogenic microorganisms (Gontang *et al*., 2007, Dehnad *et al*., 2010, Mageshwaran *et al*., 2011). Among all microorganisms, Actinomycetes considered a special target by the drug companies because of their ability for producing of the antimicrobial compounds (Eccleston *et al*., 2008). Several reports illustrated the production of antimicrobial compounds could be depended on geographical areas. In this regards, Atika and Djinni and their colleagues separately in 2011 and 2012 verified the production of different antimicrobial metabolites by Halophilic *Actinomyces* isolates from soil samples in Aljazeera. These reports confirmed detection of different antimicrobial metabolites produced by *Actinomyces* isolates even in one country. Citarasu *et al*., in 2013 opined that the antimicrobial effect of the metabolites produced by *Actinomyces* could be changed based on their natural habitats. Several reports in Australia, Egyp, china, Germany and India have shown the potent activity of anti-microbial metabolite isolated halophilic microorganisms (Maskey *et al*., 2003, Saadoun *et al*., 2003, Mayuran *et al*., 2006, Eccleston *et al*., 2008, Furtado and Velho Pereira, 2012, Todkar *et al*., 2012, Tian *et al*., 2013). In the present study antimicrobial metabolites of halophilic *Actinomyces* considered as area of investigation. Our finding indicated that out of 51 *Actinomyces* isolates 3 strains could produce antimicrobial compound. These isolates were *Streptomyces* sp. 287, *Streptomyces roseoviolaceus* strain ISP 5277 and *Streptomyces turgidiscabies* isolate TU.1C. Optimal temperature, pH and C, N-sources for growth of the strains were 27°C, 8 and fructose, xylose and yeast extract and peptone respectively and the best solvents for all were chloroform followed by acetone. These results supported by many reports (Rajendra *et al*., 2003, Augustine *et al*., 2005). Generally, secondary metabolites produced by

Actinomycetes comprise various chemical moieties, such as polyketide backbones, amino acid derivatives and sugars (Donadio *et al*., 2007). Thus, based on foregoing evidence the structure of antimicrobial metabolites and probably antimicrobial spectrum of these compounds are different. The results obtained for the determination of possible structure of bioactive compounds produced by *Streptomyces* isolates opined that UV-visible, FT-IR and GC-mass spectrometry suggesting the peptide nature of the compounds chloroacetate, ethylcholoroacetate and 4 chloro 3 hydroxybutyronitrite groups were probably linked to the structures. But complete structure elucidation was not fully recognized and needs more evaluation. However, based on foregoing evidence and antibiotic library the compounds must be new molecules.

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