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Isolation and Identification of Halophilic Actinomyces with Antimicrobial Activity and Partial Characterization of their Bioactive Compounds

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Research Article

Abstract

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

Methodology and results: To isolate antimicrobial metabolites producing Actinomyces, culture supernatant of 51 halophilic Actinnomyces 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 the promising strains were identified by API kit (bioMerieux) and 16S rRNA gene sequencing. The antimicrobial production was optimized at different temperatures, pHs, and variety of carbon, nitrogen-sources. Then the structural analysis of the metabolites carried out by UV-visible, FT-IR and 1H NMR and 13C NMR. Out of 51strainsof halophilic Actinomyces isolates, three strains showed potent activity for the production of antimicrobial metabolites. *Bacillus cereus*, *Staphylococcus aureus* and *Candida albicans* were sensitive to all and *E. coli* was sensitive to the bioactive compounds produced by Am18 and Aa8. *Pseudomonas aeruginosa* was resistance to all antimicrobial metabolites produced by the isolates. Phenotyping and molecular identification of the antimicrobial metabolites producing strains recognized them as *Streptomyces* sp. Ahbb4, *Streptomyces flavidofuscus* strain HBUM1740 and *Streptomyces olivoviridis* strain CGMCC. 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 1H NMR and 13C NMR spectrum suggesting the peptide nature of the compounds. However chloroacetate, ethylcholoroacetate and 4 chloro 3 hydroxybutyronitrite groups could be probably linked to the structures, but complete structure elucidations were not fully recognized. Conclusion, significance and impact of study: Our finding probably introduces new antimicrobial metabolites produced by halophilic Actinomyces however it needs more evaluation.

Keywords: Halophilic Actinomyces; Antimicrobial metabolites; Biostructres.

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%) [1-3].

Natural habitats of Actinomycetes because of their ability to produce several metabolites *viz*., enzymes, vitamins, hormones, antibiotics and pigments are soil, water and plants [4-6]. These bacteria can grow in the artificial media and produce sticky colonies with different colors [7]. 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 [5,8-11]. 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 [12]. 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 [13,14]. 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.

2. Materials and Methods

2.1 Sample collection

In total, 51 salt sediment samples were collected from AranBidgol and Maharlu Lakes in center and south

of Iran. To collect the 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 h a loop of each culture streaked on the trypticase soy agar with similar salt concentration and incubated at 30°C for 72 h. 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 valuated for the production of the bioactive metabolites [15-18].

2.2 Screening and Identification of producing bioactive metabolite Actinomyces

After initial screening of halophilic Actinomyces, the production of antimicrobial metabolites was evaluated using the Well Agar Diffusion method. To perform the test each Actinomyces colony cultured in trypticase soy broth with 5% NaCl and shacked in an orbital shaker at 25-30°C with 150 rpm for 48-72 h. The suspension centrifuged at 10,000 rpm for 10 min and filtered through sterile Whatman paper No.1 [1]. 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 [19].

The experiment carried out by full culture of each antagonistic microorganism on the MHA and then the 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 h. 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 [10].

2.3 Identification and authentication of the bioactive metabolite producing Actinomyces isolates

The isolates identified using biochemical tests viz., oxidase, catalase, Oxidative/Fermentative, casein and starch hydrolysis and API coryne kit (bioMerieux). Afterwards the bioactive producing Actinomyces authenticated by 16S rRNA 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 16S rRNA genes were obtained from tugKompnhagn (Denmark) (Table 1).

MasterMix of PCR with 25 µL volume, 18 µL of sterile water, 2.5 µL of PCR buffer at a concentration10 times, 0.75 µL $MgCl_2$, 0.5 µL dNTP, 0.25 µl of the enzyme polymerase, 1 µL 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 min followed by 95°C for 1 min, 56°C for 45 s and 72°C for 1 min. The final extension was performed for 5 min 72°C [14]. To ensure replication genes 16S rRNA, electrophoresis was performed. Then for sequencing of 16S rRNA 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.

Table 1. Forward and reverse primer sequences for the16S rRNA gene.

Primers	5'→3'
1000F16	CAACGAGCGCAACCCT
1492 R	GGTTACCTTGTTACGACTT

2.4 Optimization of culture for production of the bioactive metabolites

Optimization of culture for maximum production of antimicrobial metabolites carried out by changing 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 h. Then 100 µL of each supernatant (centrifugation at 10000 rpm, 20 min) added into the wells embedded in Muller Hinton agar cultured with *Bacillus cereus* (PTCC1015) and incubated at 37°C. After 24 h, 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 h. 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 h. 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 h. Optimization of TSB with carbon and nitrogen sources was evaluated against *Bacillus cereus* (PTCC 1015) as mentioned above [20].

2.5 Extraction of the bioactive metabolites produced by Actinomyces isolates

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 h. 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 [1]. 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 [15]. The solvents were centrifuged at 5000 rpm for 15 min 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 [21].

2.6 Arbitrary Unit of the bioactive metabolites

Arbitrary Unit (AU) of the bioactive metabolites determined based on Voravuthikunchai and his colleagues [22]. 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 h. Arbitrary Unit of each metabolite was determined by reciprocal of highest dilution showing antimicrobial effect.

2.7 Determination of the bioactive metabolites produced by Actinomyces isolates

Biostructure of the bioactive compounds determined by Nuclear Magnetic Resonance (NMR), UV-visible and *Fourier Transform Infra-Red* Spectroscopy (*FT-IR*). To identify of the structures 5 mL of each bioactive metabolite used for 1H NMR, 13C NMR (300 MHz, BruckerBiospin, Switzerland). The metabolites dissolved in 5 mL of acetone and subjected to Nuclear Magnetic Resonance (NMR) (300 MHz, BruckerBiospin, Switzerland) analysis.

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.

3. Results

3.1 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 Am18 and Aa8. *Pseudomonas aeruginosa* was resistance to all bioactive metabolites produced by the isolated strains.

3.2 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 16S rRNA genes of the bacterial strains exhibited 97%, 95% and 99% identical to *Streptomyces* sp. Ahbb4, *Streptomyces flavidofuscus* strain HBUM1740 and *Streptomyces olivoviridis* strain CGMCC, respectively (Table 3 and Supplementary Figure 1).

3.3 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.

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

Antagonistic Microorganisms	Zone of Inhibition (mm)		
	Am18	Aa11	Aa8
Escherichia coli	20		15
Pseudomonas aeruginosa			
Staphylococcus aureus	22	20	17
Bacillus cereus	14	14	15
Candida albicans	17	17	20
Aspergillus niger	-*	10	12

*, no zone
The numbers represented in the table are average of three replicates

Strains Identified	Similarity %	Accession Number
Aa8 *Streptomyces* sp. Ahbb4	97%	gb/KM214828.1
Aa11 *Streptomyces flavidofuscus* strain HBUM1740	95%	gb/EU841706.1
Am18 *Streptomyces olivoviridis* strain CGMCC	99%	gb/JQ924395.1

Table 3. Alignment analysis of 16S rRNA genes of bioactive metabolite producing Actinomyces.

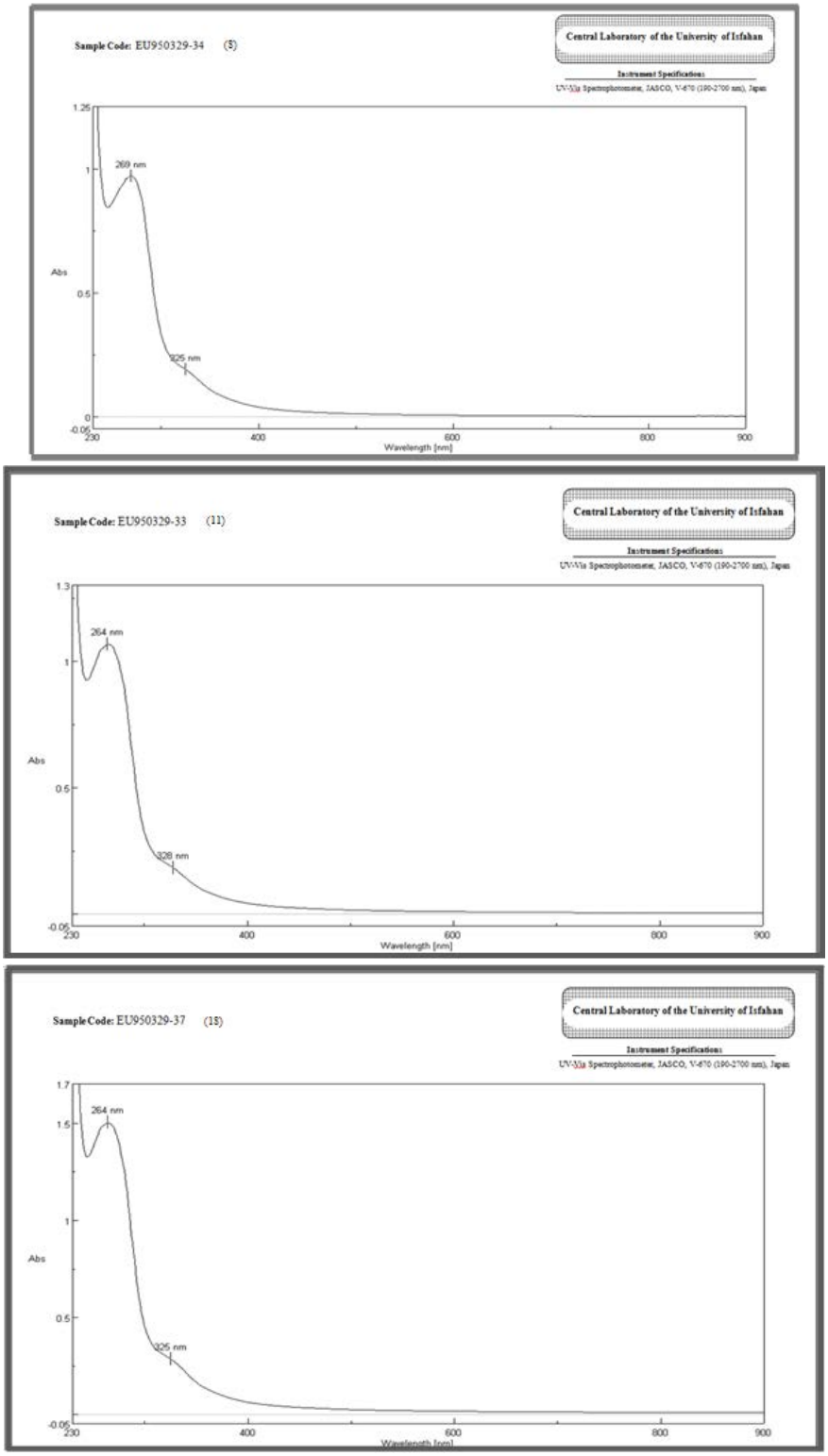

Figure 1. UV-visible absorption spectrum of the bioactive metabolites produced by Streptomyces isolates.

3.4 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 (Figure 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 (Figure 2). The O-H stretching was indicted by valley at 3138. All above characteristics valleys indicated peptide-based structure of the compound.

The 1H NMR (Nuclear magnetic resonance, 500 MHz) 13C NMR spectra of the antimicrobial compound in D_2O indicated presence of large peaks in the regions 1, 3.6, 4.6 and small peaks in regions 1.6 probably indicates existence of CH_3, RCH_2Cl, $R_2C=CH_2$ protons in the structure of the compound. Regarding to The 13C NMR (300 MHz) showed $R\text{-}CH_3$ at region 10 to 25, RCH_2OH at region 60 ppm (Figure 3). The analysis of all major peaks indicated that the compounds closely resemble to peptide type antibiotics in the antibiotic library and probably

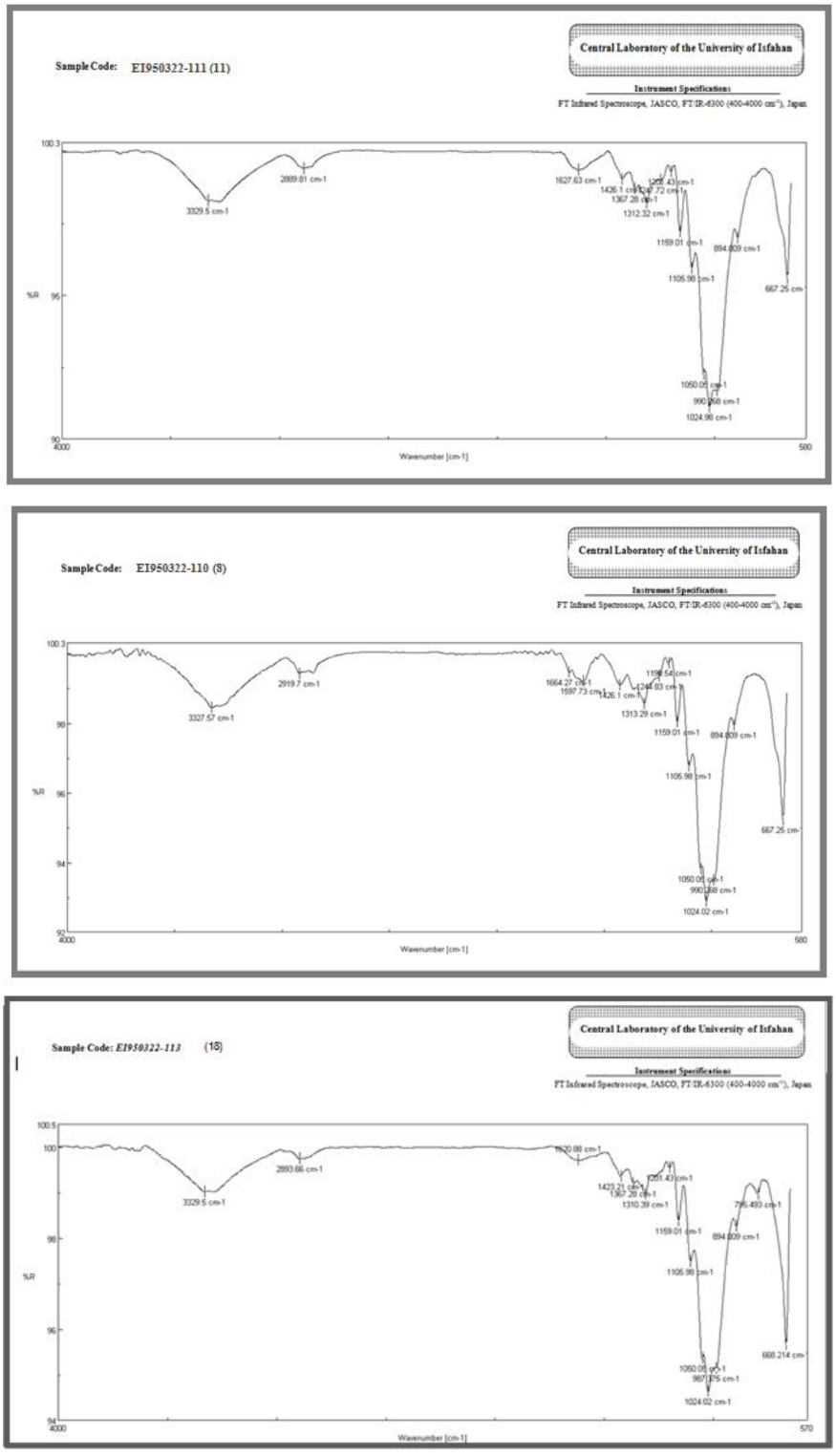

Figure 2. Fourier transform infrared (FT-IR) spectrum of the bioactive metabolites produced by Streptomyces isolates.

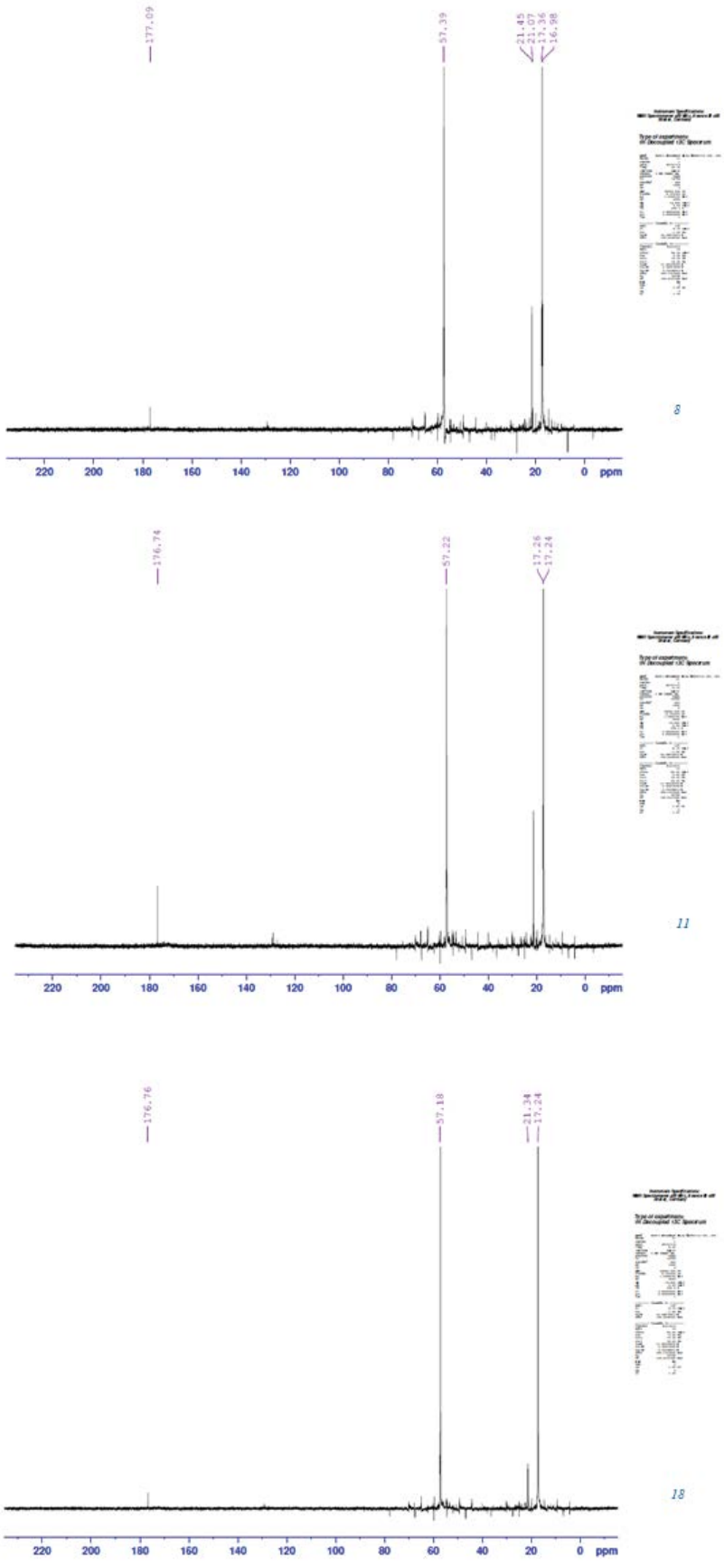

Figure 3. 13C NMR (300 MHz) spectrum of the bioactive metabolites produced by Streptomyces isolates.

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.

4. Discussion

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 [2,9,14]. Among all microorganisms, Actinomycetes considered a special target by the drug companies because of their ability for producing of the antimicrobial compounds [23]. Several reports illustrated the production of antimicrobial compounds could be depended on geographical areas. In this regards, Atika et al. [24] 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. Many scientists such as Eccleston et al. [23]; Citarasu et al. [19] supported this idea that the antimicrobial effect of the metabolites produced by Actinomyces changed based on their natural habitats. Several reports in Australia, Egypt, china, Germany and India have shown the potent activity of antimicrobial metabolite isolated halophilic microorganisms [18,23,25-29]. 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. Ahbb4, *Streptomyces flavidofuscus* strain HBUM1740 and *Streptomyces olivoviridis* strain CGMCC. 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. This results supported by many reports MAskey et al. [25]; Augustine et al. [21]. In addition, The UV-visible, FT-IR and 1H NMR and 13°C NMR spectrum 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.

5. Conclusion

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

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