Journal of Infection and Public Health 13 (2020) 125–130

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Isolation and characterization of polyketide drug molecule from *Streptomyces* species with antimicrobial activity against clinical pathogens

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ARTICLE INFO

Article history:
Received 9 May 2019
Received in revised form 20 June 2019
Accepted 1 July 2019

Keywords:
Streptomyces sp.
Antimicrobial activity
Minimum inhibitory concentration
Ketoacyl synthase gene

ABSTRACT

Background: Natural products derived from marine microbes have more potential toward to treatment of various diseases. Among the microbes, the secondary metabolites recovered from the marine actinomycetes were more added values.
Objective: A promising antimicrobial metabolite producing filamentous actinomycete SCA-7 recovered from Alkhobar marine region was investigated for its potential to inhibit Gram positive *Enterococcus* sp. In addition to the chemical characterization, the polyketide gene cluster of the actinomycete SCA-7 was sequenced.
Results: Among the 8 actinomycetes isolated from the marine sample, the isolate SCA-7 produced significant antimicrobial activity against *Enterococcus* sp. The biochemical, physiological and morphological characteristics and fermentation assimilation pattern confirmed that the isolate belonged to the genus *Streptomyces*. The 16S rRNA gene amplification and sequencing results showed 99% sequence similarity to *Streptomyces felleus*. The antimicrobial activity of the crude ethyl acetate extract was performed by disc diffusion method. The spectral characterization was done by ^{13}C NMR and ^{1}H NMR. The compound was polyketide in nature. The Minimum Inhibitory Concentration (MIC) of the polyketide compound against *Enterococcus* sp. was 25 μg/mL. Among the agro-industrial waste materials, wheat bran showed increased secondary metabolite production. Antibacterial activity was found to be high when the isolate SCA-7 was grown in wheat bran substrate and maximum zone of inhibition (22 mm) was recorded in it. Among the carbon and nitrogen sources, lactose enhanced the production of secondary metabolites and the zone of inhibition against *Enterococcus* sp. was 25 mm. The amplification and sequencing of the ketoacyl synthase gene clearly indicated that it was type I polyketide synthase (PKS) gene of *Streptomyces* species.
Conclusion: Overall, the therapeutic drug molecule isolated from the marine *Streptomyces* species might be used for the treatment of disease causing microbial clinical pathogen.

Introduction

Actinomycetes are a group of organisms considered to be intermediates between bacteria and fungi. They are classified as Gram-positive bacteria [1]. They are widely distributed in man-made and natural environments. Actinomycetes are mainly distinguished from other bacteria by their morphology, nucleic acid sequencing, guanine and cytosine content in DNA and pairing studies. Actinomycetes are mainly characterized by having a high G+C content (>55%) in their DNA. They are found in large numbers in fresh water, soils, river bottoms, lake, composts, manures and dust as well as on damaged food products and plant residues [2]. Actinomycetes have no nuclear membrane and have cell wall. They reproduce by spores either by conidia or by fissions. Actinomycetes are one of the important constituents of soil microbes, together with other bacteria as well as some fungi. Among actinomycetes, *Streptomyces* are dominant in nature. Most of the actinomycetes are free living in nature [3]. Some actinomycetes inhabit marine habitats while other groups live in mangrove sediments. Actinomycetes play a critical role in decomposition of organic materials such as chitin and cellulose and thereby play an important part in carbon cycle ad organic matter turnover [4].

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https://doi.org/10.1016/j.jiph.2019.07.002

Actinobacteria are noteworthy as important antibiotic producers, making three quarters of all well known commercially available antibiotics. They are highly responsible for the production of many bioactive secondary metabolites, particularly antibiotics, antitumor agents, immune suppressive agents and various enzymes. Among the actinobacteria, *Streptomyces* accounted for more than 80% of the total antibiotic products, followed by *Actinopolyspora* and *Micromonospora* which are rare actinobacteria with less than one-tenth of *Streptomyces* population [5]. It was estimated that about 42% of commercial metabolites are known to be produced by various actinomycetes, 16% by fungal strains and the remaining from bacterial source. These organisms produce secondary metabolites and release into the environment. They have broad spectrum biological activities such as antifungal, antibacterial, antiparasitic, antiviral, immunosuppressive, insecticidal, antitumor, antioxidant, anti-inflammatory, diabetogenic and enzyme inhibition [6]. The discovery of antibiotics had a significant impact on the control of various infectious diseases and the development of pharma industry. The search for novel antibiotics continues in order to combat various evolving pathogens, naturally resistant fungi and bacteria, and previously susceptible microbes that have developed resistance against existing antibiotics, to combat tumors, viruses and parasites, to improve pharmacological properties, and to discover more potent, safer and broad spectrum secondary metabolites [7]. The present study was aimed to isolate and characterize actinomycetes from the marine region of Saudi Arabia and to investigate the activity of the identified compounds against Gram-positive bacteria. Additionally, the polyketide type I gene cluster of the isolate was characterized and sequenced.

Materials and methods

Isolation and purification of actinomycetes from estuary environment

The samples were collected from various marine regions of Alkhobar, Saudi Arabia. Isolation of actinomycetes was carefully performed by serial dilution and plating on starch casein agar medium with suitable antibacterial and antifungal agents [8]. For the isolation, one gram of the soil sample was initially suspended in 10 ml of sterile double distilled water in an Erlenmeyer flask, stirred continuously with the help of a glass rod and left for a while. The sample was serially diluted up to 10^{-7} and poured in starch casein agar medium aseptically. All plates were incubated at room temperature ($28 \pm 2\,^{\circ}C$) for 7–10 days. Based on the colony morphology, pigment production capability and spore structure arrangement, the isolates were selected and further purified using starch casein agar medium.

Screening of actinomycetes for antimicrobial activity

The selected actinomycetes were screened for activity against *Enterococcus* sp. by cross streak method [9]. Briefly the actinomycetes isolates were grown on the starch casein agar medium and incubated at 28 °C for four days and then the bacterial pathogen was streaked perpendicular to the actinomycetes and evaluated for the inhibition property.

Identification and characterization of the active actinomcyetes

Among the eight actinomycetes isolated from the sample, isolate SCA-7 exhibited effective antimicrobial activity. Therefore, the isolate SCA-7 was further identified by biochemical, physiological and morphological characteristics. The morphological characteristics of the isolate were performed by growing the isolate in different cultivation media to note their morphological patterns. Carbohydrate fermentation pattern of the isolate was assessed by following the standard method.

Molecular level identification by sequencing 16S rRNA gene fragment

16S rRNA gene of the isolate SCA-7 was amplified by using the forward primer (5′ AGA GTT TGA TCG TGG CTC AG 3′) and reverse primer (3′ GGT TAC CTT GTT ACG ACT T 5′) using Taq polymerase enzymes. The amplification conditions were as follows; initially at 95 °C for 5 min, at 95 °C for 2 min and annealing at 55 °C for 2 min and final extension at 72 °C for 5 min. The overall conditions were maintained for 35 cycles. After that the amplified PCR products were rechecked using 1% agarose gel by electrophoresis and the gene product was sequenced by Macrogen Co. Ltd. (Seoul, Korea). The resulted sequences were checked for their similarity in NCBI, BLAST program by online search and deposited in the Genbank with individual accession number.

Fermentation and extraction of the antimicrobial metabolites

The spore suspension of the isolate SCA-7 was collected from the freshly cultivated batch and used for the bioreactor cultivation in 5 l scale. Briefly, 3.5 l of the fermentation medium was sterilized and 10 percentage of the spore suspension was transferred aseptically into the bioreactor and incubated for 10 days. After incubation the mycelia were separated using the centrifuge and the cell free spent medium was mixed with equal volume of ethyl acetate and chloroform mixture (50:50). The obtained solvents were distilled using vacuum evaporator and the crude extracts were checked for the antimicrobial activity by disc diffusion method. Further the extract was purified by silica gel column chromatography using methanol and ethyl acetate mixture and 12 fractions were collected. Among the 12 fractions studied, fraction 5 revealed good antimicrobial activities. The fraction 5 was subjected to purity check by thin layer chromatography and the obtained compound was analyzed by spectroscopic techniques.

Screening of agro-industrial wastes for the antimicrobial compound production

To screen the potency of agro-industrial wastes for the production of secondary metabolites by *Streptomyces* sp. the agro wastes such as apple peel, pineapple peel, orange peel, wheat bran and rice bran were collected and dried in an oven at 70 °C for one day. The selected agro-wastes were powdered and used as substrate. Five grams of selected substrate were taken individually in an Erlenmeyer flask and moistened with 0.1M sodium phosphate buffer at 75% level (v/w) and mixed thoroughly. This was sterilized for 30 min and cooled, and then 0.5 ml of inoculum from the potent actinobacterial isolate was added and incubated at room temperature for 7 days. After that, 50 ml of methanol was added and extracted as described previously. Differences observed among used substrates were determined using one way- analysis of variance (ANOVA) and significance was calculated ($p < 0.05$).

Optimization of carbon and nitrogen sources for antimicrobial compound production

Among the selected substrates used in this study, wheat bran showed better metabolite production. Hence, this substrate was used for optimization of secondary metabolite production. Five grams of wheat bran were moistened with 0.1 M sodium phosphate buffer (pH 7.0) at 75% level (w/v) and mixed gently. To study the effect of carbon source on secondary metabolite production,

various carbon sources (1%, w/w/) (Xylose, Glucose, Sucrose and Lactose) were added individually with solid substrate. To study the effect of nitrogen source on secondary metabolite production, various nitrogen sources (1%, w/w/) (Caesin, Glycine, Skimmed milk and Gelatin) were added. This was sterilized for 30 min and cooled, and then 0.5 ml of each inoculum was added and incubated at room temperature for 7 days. To the control experiments, carbon/nitrogen source was not added. After that 50 ml of methanol was added to each of the fermented medium and kept on a rotary shaker (30 min, 200 rpm). It was further centrifuged (10,000 rpm for 10 min) and the supernatant was used as the source of secondary metabolites. The sample was dried, suspended in Dimethyl sulfoxide (DMSO) and used for antimicrobial studies. Differences observed among used nutrient sources were determined using one way- analysis of variance (ANOVA) and significance was calculated ($p < 0.05$).

Antimicrobial activity of the compound obtained from the isolate SCA-7

The antimicrobial activity of the compound was assessed by micro dilution technique [10]. The minimum inhibitory concentrations and minimum bactericidal concentrations of the identified compounds were determined by standard methods. The antimicrobial activity of the compound was compared with the commercial standard antibiotic streptomycin.

Amplification, cloning and sequencing of ketoacyl synthase gene

The genes involved in the biosynthesis of polyketide group were amplified using the forward primer 5′-TSG CST GCT TGG AYG CSA TC-3′ and reverse primer 5′-TGG AAN CCG CCG AAB CCT CT-3′ [11]. The primers were designed based on the polyketide synthase type I genes. The PCR amplified products were checked for its purity by agarose gel electrophoresis and the obtained amplified products were sequenced by Macrogen Co. Ltd. (Seoul, Korea). The obtained sequences were checked for similarity in NCBI, BLAST program and submitted in Genbank with individual accession numbers.

Results

Isolation and screening of antimicrobial actinomycetes

Actinomycetes produce various bioactive compounds with potent commercial value and it is important to screen for novel bioactive substances. In this study, actinomycetes were isolated from marine environment to characterize novel antimicrobial substances. The actinobacterial isolates were isolated by serial dilution technique using casein agar plates. Eight actinomycetes isolates showed noticeable antimicrobial activity against Gram positive *Enterococcus* sp. by streak plate method and well diffusion method. Among the eight isolates, the isolate SCA-7 revealed comparatively better activity against the tested pathogen. The antimicrobial activity ranged from 4 mm to 12 mm zone of inhibition (Fig. 1). Therefore, the SCA-7 was further characterized at biochemical, physiological and molecular levels.

Identification and characterization of antimicrobial actinomycetes

The promising isolate SCA-7 recovered from the sediment exhibited comparatively effective activity against Gram-positive pathogen. The morphological characteristics of the isolate SCA-7 on different cultivation media suggested that the isolate was growing well in the MNGA, SCA, and PDA media (Fig. 2). After seven days of incubation, the isolate showed crowded filamentous appearance with aerial mycelium. On the other hand the isolate was not able

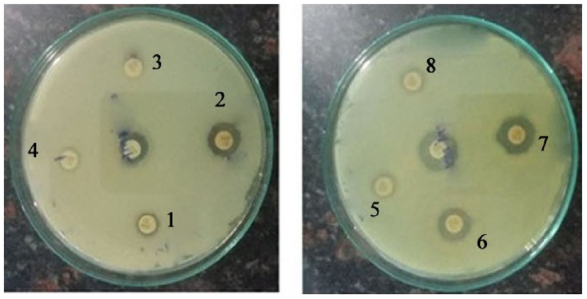

Fig. 1. Antimicrobial activity of actinomycetes sp.SCA-7 from the marine soil samples. *Enterococcus* sp. was grown on MHA plates and crude sample was loaded and the plates were incubated for 24 h. Antibacterial activity appeared as zone of inhibition.

Fig. 2. Morphological characteristics of actinomycetes sp.SCA-7 on PDA media isolated from the marine soil samples. PDA plate was incubated for 7 days.

to grow well in the nutrient agar medium and Muller–Hinton agar medium. Interestingly the isolate was able to produce diffusible pigment when grown in SCA, MNGA and PDA cultivation media. Biochemical features suggested that the isolate was Gram-positive and able to utilize wide level of sugars such as glucose, fructose, maltose, sucrose and others. The isolate exhibited less growth in arabinose. Also, the growth of the isolate under different nitrogen sources was interesting and exhibited good growth in all the nitrogen sources. Physiological characteristics suggested that the isolate was capable of producing extracellular enzymes such as amylase, protease, geleatinase, cellulase and lipase respectively. Also the growth under different pH and different temperatures from 25 °C to 45 °C revealed its wide level of adaptability. The isolate was sensitive to different antibiotic such as streptomycin, gentamycin and kanmycin. Over all the 16S rDNA gene sequence suggested that the isolate SCA-7 showed similar characteristics of *Streptomyces* species (Fig. 3).

Purification and identification of the antimicrobial compound

The crude extract was subjected to column chromatography which yielded 12 individual fractions. Among them, fraction 5 revealed promising antibacterial activity against Gram-positive pathogen. After confirming the purity of the fraction, the compound was subjected to spectroscopic analysis. The IR spectrum of the compound showed strong peak at 2678 and 1478 cm^{-1} which indicated carbonyl, hydroxyl group and methyl group closely similar to the polyketide antibiotic (4aR,7R,8R,8aS)-2-heptylhexahydropyrano[3,2-d][1,3]dioxine-6,7,8-triol (Fig. 4). The

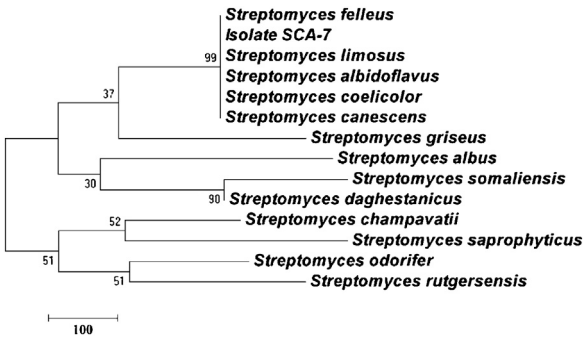

Fig. 3. Phylogenetic analysis of actinomycetes sp.SCA-7 from the marine soil samples.

molecular weight of the compound was confirmed as 290.35 after checking the MASS spectrum of the compound. ^{1}H NMR (300 MHz) spectrum exhibited signals at 5.19, 7.71, 9.61, 11.54, and 12 corresponding to methylene group. The ^{13}C NMR spectrum showed signals at 103, 284.3, 150.1 and 127.7. Signals at δ C 181 and 189.3 indicated the presence of carbonyl group. The carbon and hydrogen NMR data clearly evidenced the molecular structure of the compound as $C_{14}H_{26}O_6$.

Effect of carbon and nitrogen sources on antibacterial secondary metabolite production by Streptomyces sp.

In the present study carbon and nitrogen sources were supplemented with wheat bran to enhance the production of secondary metabolites. Among the carbon sources supplemented, lactose enhanced the production of secondary metabolites and the zone of inhibition against *Enterococcus* sp was 25 mm and it was statistically significant. Sucrose also found to increase antibiotics production followed by control. Among the nitrogen sources, glycine enhanced the production of secondary metabolites, followed by skimmed milk. One-way ANOVA revealed that nitrogen sources significantly influenced on secondary metabolites production ($p < 0.05$) (Figs. 5 and 6).

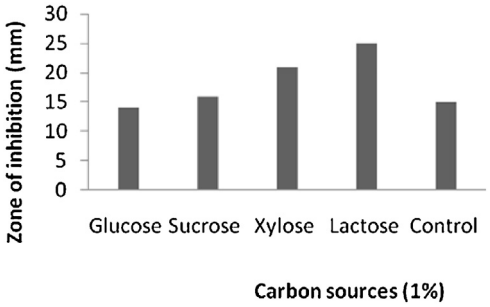

Fig. 5. Effect of various carbon sources on the production of polyketide antibiotics. Wheat bran was used as the substrate and 1% (v/w) carbon source was supplemented and inoculated with *Streptomyces* sp. and incubated for 7 days.

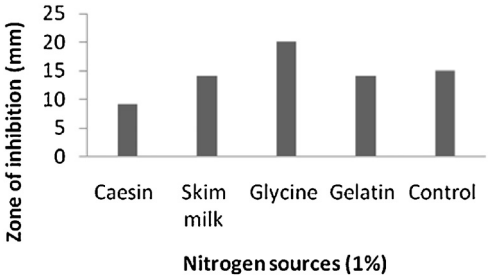

Fig. 6. Effect of various nitrogen sources on the production of polyketide antibiotics. Wheat bran was used as the substrate and 1% (v/w) nitrogen source was supplemented and inoculated with *Streptomyces* sp. and incubated for 7 days.

Production of the antimicrobial compound using agro-industrial residues

To screen the potent agro-wastes for the production of secondary metabolites, the substrates such as orange peel, apple peel, wheat bran, pineapple peel, rice bran and black gram husk were used as the substrate. Antibacterial activity was found to be high when the isolate was grown on wheat bran substrate and maxi-

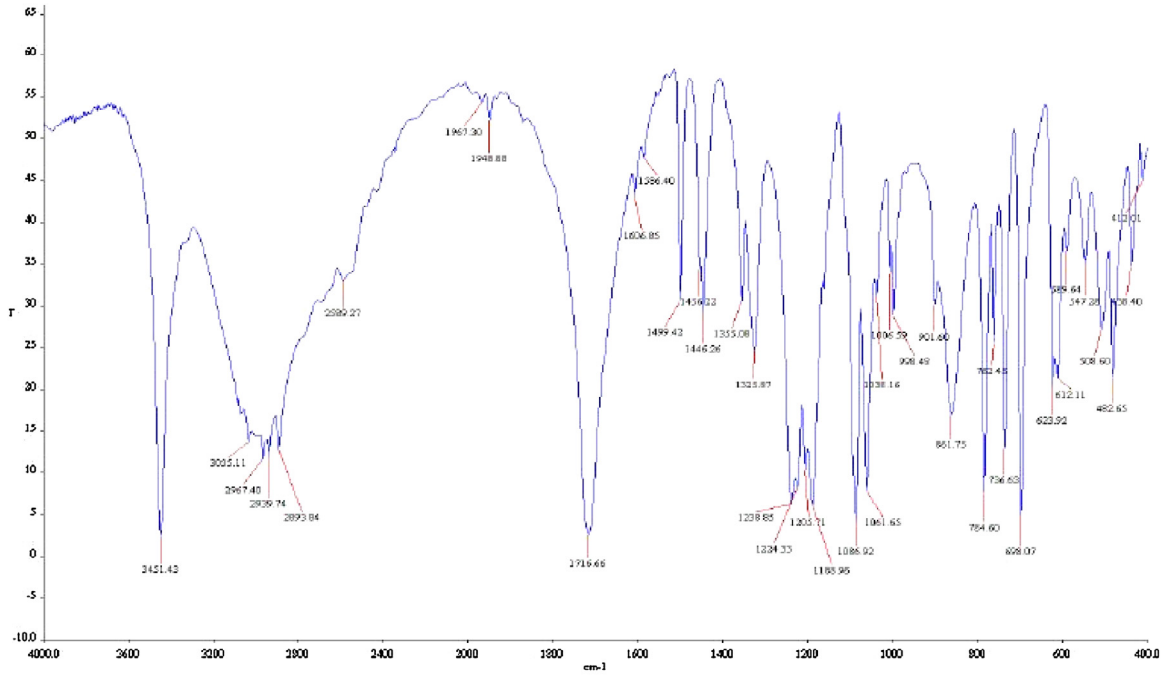

Fig. 4. IR spectrum of the isolated compound.

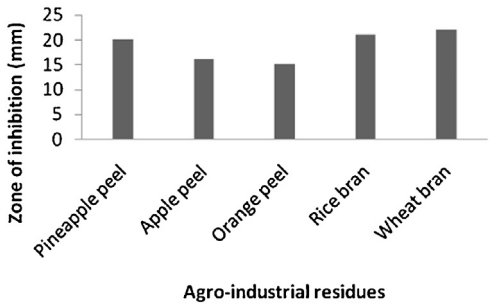

Fig. 7. Antibacterial activity of actinomycetes sp.SCA-7 grown on various agro-wastes. The selected substrates were moistened with 0.1 M sodium phosphate buffer (75%, v/w) and sterilized. After that, *Streptomyces* sp. was inoculated and incubated for 7 days.

mum zone of inhibition (22 mm) was obtained (Fig. 7). The selected agro-wastes significantly influenced on secondary metabolite production and was found to be statistically significant ($p < 0.05$).

Antimicrobial activity of the compound

The disc diffusion study confirmed that the compound revealed promising activity at 100 μg level. Therefore, the minimum inhibitory concentration (MIC) values were checked by keeping 100 μg as the higher level. The results indicated that the polyketide compound showed MIC values for *Enterococcus* sp at 24 μg. The positive control streptomycin was also checked for the MIC values.

Cloning and sequencing of ketoacyl synthase gene

For confirming the ketoacyl synthase gene, the ketoacyl synthase type I gene was amplified and the sequencing results confirmed the presence of 850 base pairs. The obtained base pairs were checked for similarity using NCBI, Blast program. The results clearly confirmed that the type I gene of polyketide synthase from the isolate SCA-1 showed close similarity towards *Streptomyces felleus*. The isolate revealed 100 percent identify and 99% similarity towards *Streptomyces felleus* (Fig. 8). The phylogentic analysis of the isolate was compared with other polyketide antibiotic producing actinomycetes such as *Streptomyces vietnamensis, S. blastmyceticus, S. hygroscopicus* and *Micromonospora* species. These strains were known for the production of polyketide antibiotics.

Discussion

Identification and characterization of novel molecules from natural sources is a continuous process to cope with the demand for the treatment of various infectious diseases. Many molecules from various biological sources have been used to treat medically associated infectious diseases and also for the inhibition of plant associated microbial pathogens. It is very important to characterize the antimicrobial metabolite producing microbial strains for the inhibition of various microbial pathogens. Few reports claimed that the Actinomycetes from marine origin were ideal sources of novel secondary molecules with diverse chemical diversity [12]. Therefore, recently the attraction towards the characterization of novel secondary metabolites with promising bio-applications from marine origin has increased [13]. The characterization of actinomycetes from marine samples is well documented; yet the proportion of these filamentous antimicrobial actinomycetes which represents the indigenous marine micro-flora remains unclear; there are many regions from which the study of novel molecules is limited. Therefore, the present study focused on the isolation and characterization of novel actinomcycetes from marine region. The extraction of novel bioactive molecules from the crude extracts by column chromatography techniques was performed. In addition, the molecular biosynthetic class of the identified compound was investigated by gene amplification and sequencing. The activity of extracted compound was comparable with standard streptomycin. Many reports suggested that the actinomycetes could be isolated using starch casein agar medium supplemented with some antibiotics such as actidione and nalidixic acids [14]. In the present study the promising actinomycete was isolated by using starch casein agar medium. The isolate was Gram-positive, spore forming, filamentous and dry in their appearance on various cultivation media. Also, the biochemical and physiological characteristics suggested that the isolate was able to grow well at 30 °C and able to withstand different pH and NaCl concentration. The wide level growth pattern of the isolate in various carbon sources revealed its characteristics. These biochemical and other morphological characteristics and properties coincided with the report of Williams et al. [15] and Tresner et al. [16]. Mukherjee and Sen [17] and Roes and Meyer [18] proved that the spore morphology of the actinomycetes is an important criterion for the identification of the actinobacteria in their species level. The 16S rRNA gene analysis confirmed that the isolate SCA-7 shared close (99%) similarity towards the *Streptomyces* species and therefore was considered as *Streptomyces* sp. SCA-7.

Interestingly, the isolate SCA-7 exhibited antimicrobial activity in both solid and liquid cultivation media. The antimicrobial activity of the isolates on both solid and liquid fermentation media suggested that the antibiotic was extracellular in nature. It was reported that most of the promising antimicrobial metabolites were extracellular in nature either active in solid or liquid medium respectively [19]. The purification and characterization of the crude extracellular component yielded a promising compound (4aR,7R,8R,8aS)-2-heptylhexahydropyrano[3,2-d][1,3]dioxine-6,7,8-triol. The chemical characteristics clearly showed that the identified molecule belonged to the class polyketide. Polyketides are generally synthesized by the group of metabolic enzymes called PKS [20,21]. In the present study the 16S rRNA gene and the polyketide synthase gene of isolate SCA-7 shared similar phylogentic pattern towards the *Streptomyces* strain [22]. Interestingly, the obtained compound showed effective antimicrobial activity against the tested Gram positive bacteria.

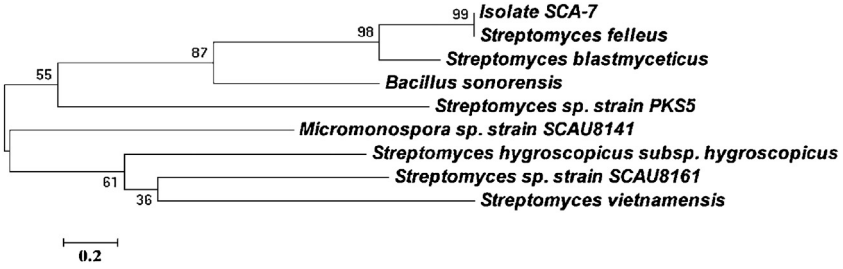

Fig. 8. Phylogenetic analysis of ketoacyl synthase gene from the actinomycetes sp.SCA-7.

The results demonstrated that the optimal nitrogen source for antibiotic production was skimmed milk. Different carbon and nitrogen sources were evaluated for maximum production of antibiotics. It was found that lactose (carbon source) and skimmed milk (nitrogen source) supported more secondary metabolites production. Optimization of culture medium is required to enhance the metabolite yield. This can be obtained by using a wide range of techniques from classical "one-variable-at-a-time" approach. The ability of microbes to produce antibiotics is not a constant property but can be influenced or completely lost under various conditions of nutrients or culture conditions [23]. The physiochemical parameters influenced quantitatively the biosynthesis of antibiotics from *Streptomyces* sp. [24]. Himabindu and Jetty [25] reported that starch was found to be best carbon source for gentamicin production. Kathiresen et al. [26] showed that among the carbon sources, glycerol, glucose, lactose and maltose were the best sources. Effect of nitrogen sources on production of secondary metabolite was detected by fermentation media with selected *Streptomyces* sp. at different nitrogen sources.

Conclusion

In summary, novel actinomycetes with promising antibacterial activity against Gram positive microbial pathogen was isolated from the marine sample. The biochemical, physiological and molecular characterization clearly showed that the isolate SCA-7 belonged to *Streptomyces* species. The bulk level production and further characterization of the novel molecule by various downstream processing helped to identify the promising pure compound. Results clearly indicated that the identified compound shared close similarity with the polyketide antibiotics. Interestingly, the MIC values of the polyketide compounds were comparable with the reported standard antimicrobial compounds. In addition, the functional classes of the antibiotics were determined by gene amplification and sequencing. Overall, the marine actinomycete SCA-7 is an ideal source to prevent infectious disease causing Gram positive microbial pathogens.

Funding

No funding sources.

Competing interests

None declared.

Ethical approval

Not required.

Acknowledgement

This work was funded by Deanship of Scientific Research of King Faisal University, (Nashir track, project number:186039)

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