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Antibacterial activity of a new species of *Streptomyces* and optimization its antibacterial compounds

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ABSTRACT

Streptomyces are capable of producing secondary metabolites including antibiotics and the main environment of these microorganisms is soil. The purpose of this study was to isolate and evaluate *Streptomyces* producing antibacterial compounds from different east parts of Gilan province and optimizing antibacterial compounds produced by them. After isolation and purification of *Streptomyces*, its antibacterial activity against pathogenic microorganisms including *Micrococcus luteus* PTCC 1408, *Bacillus cereus* PTCC 1154, *Staphylococcus aureus* PTCC 1189, *Pseudomonas aeruginosa* PTCC 1310, *Salmonella typhi* PTCC 1609 and *Proteus mirabilis* PTCC 1776 was investigated. *Streptomyces* was identified based on morpholigaical, biochemical, physiological, 16SrRNA gene sequencing and phylogenetic analysis. Optimization of various factors in the content of production of antibacterial compounds was investigated. In this study, 16SrRNA gene sequencing revealed that the isolate belonged to *Streptomyces* genus and with the highest similarity (95.70%) to *Streptomyces malachitospinus* indicating significant differences at species level and it can be introduced as a new species. This isolate showed significant activity against pathogenic microorganisms. The optimum culture medium, pH, temperature, carbon and nitrogen sources and incubation time for the maximum antibacterial compounds production were ISP2, 7, 28°C, glycerol, yeast extract and 7 days, respectively. The results show that the soils of the eastern regions of Gilan province are a rich source of antimicrobial compounds, which due to the resistance of the pathogenic microorganisms to antimicrobial drugs, is essential for the production of antimicrobial compounds from natural origin.

1. Introduction

Streptomyces are identified as gram- positive, aerobic, chemoorganotrophic, filamentous bacteria (Dehnad et al., 2010) and produce substrate mycelium and aerial hyphae (Luo et al., 2017). They found in all ecosystems such as soil, water, marine sediment and hot water but their main environment is soil and it is made of a large population of natural flora (Shantikumar & Bora, 2006; Bredhold et al., 2008) and have an important role in recycling organic materials in soil (Rotich et al., 2017). They are responsible for the stability of soil texture (Dehnad et al., 2010). Some of them act as biological control (Hasani et al., 2014). Distribution of *Streptomycetes* in water and soil depends on some factors such as temperature, pH, moisture, salinity, soil texture, food stress and climate (Hasani et al., 2014). This genus can produce a

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various range of bioactive compounds, such as antibiotics and hydrolitic enzymes (Charousova et al., 2016), herbicides, anti-cancer drugs, growth factors like vitamin B12 (Hasani et al., 2014). Genetically changes or attainment of resistance factors and some physiological state (such as biofilm formation) are responsible for antibiotic resistance (Hasani et al., 2014). To overcome multidrug resistant pathogenic its necessary to discovering novel class antibiotics that have different mechanisms of action (Gebreyohannes et al., 2013) and that is important for industrial application and human health care (Maleki et al., 2013).

The aim of this study was evaluation of antibacterial activity of *Streptomyces* isolated from soils of eastern Gilan province and optimization of antibacterial compounds produced by it.

2. Materials and Methods

2.1. Soil sampling

Soil samples were collected from different eastern parts of Gilan province at a depth 5-10 cm from the surface of the soil and transferred to the laboratory under sterile conditions. Then, were air dried at room temperature for 7 days.

2.2. Isolation of Streptomyces

Serial dilution up to 10^{-7} were prepared and 0.1 ml of each dilution was inoculated on Starch Casein Agar (SCA) medium and plates were incubated at 28° C for 21 days. Suspected colony with dry appearance, powder, umbrella shape, wrinkled and leathery were isolated. It was transferred subsequently to the new culture medium for purification several times. For long storage, 20% (v/v) glycerol was added to pure colonies and stored at –20 °C.

2.3. Pathogenic microorganisms

The pathogenic microorganisms; *Micrococcus luteus* PTCC 1408, *Bacillus cereus* PTCC 1154, *Staphylococcus aureus* PTCC 1189, *Pseudomonas aeruginosa* PTCC 1310, *Salmonella typhi* PTCC 1609 and *Proteus mirabilis* PTCC 1776 were purchased from the Iranian Scientific and Industrial Research Organization.

2.4. Screening for antibacterial activity

Isolates were screened against pathogenic microorganisms by primary and secondary screening. Primary screening method was done by cross streak technique (Valli et al., 2012; Chaudhary et al., 2013; Thirumalairaj et al., 2015). Nutrient agar media was used and plates were streaked with isolates at the center of the plates and incubated at 30 °C for 7 days. Later, sub-cultured pathogenic microorganisms were streaked perpendicular to the isolates and incubated at 37 °C for 24 h and the diameter of the inhibition zones was measured. Secondary screening method was done by Agar well diffusion technique (Gebreyohannes et al., 2013). Submerged state fermentation method was used to produce crude extracts. Isolate was inoculated into Yeast extract malt extract broth medium (ISP2) and incubated at 28° C in shaker incubator at 150 rpm for 7 days. Then the broth was filtered through Whatman No. 1 filter paper and centrifuged at 4000 rpm for 15 min to extract the antimicrobial compounds. Pathogenic microorganisms were inoculum by sterile swabs on the surface of mueller hinton agar. The wells were prepared by using sterile cork borer and a volume of 100 µL of crude extract was dispensed into wells. Then, plates were incubated for 24h at 37°C and the diameter of the inhibition zones was measured.

2.5. Morphological, Biochemical and Physiological Characterization

Pure isolate was characterized morphologically, biochemically and physiologically. Pure isolate was on the characterized basis of morphological features (colony properties, the color of aerial and substrate mycelium and pigmentation) on various media including Starch casein agar (SCA), Tryptone yeast extract agar (ISP1), Yeast extract malt extract agar (ISP2), Inorganic salts starch agar (ISP4), Peptone yeast extract iron agar (ISP6) and Raffinose histidine agar. Antibiotic resistance of the strain was done using different antibiotic discs and Physiological characteristics such as NaCl tolerance and temperature range were done.

2.6. Molecular identification of isolate using 16SrRNA sequencing and phylogenetic analysis

DNA extraction was done with boiling method (Ntsaluba et al., 2011; Zamani et al., 2013). Pure sub-cultures of the isolate were cultured in SCA medium and incubated at 28°C for 7 days. A loop full colony was picked for use in extraction of DNA and suspended in 100 ml of sterile distilled water in a 1.5 ml suspension and were boiled in hot water for 10 minutes and finally were centrifuged at 10000 round for 10 minutes. The 16S rRNA gene was amplified by using primers 27F (5'AGA GTT TGA TCC TGG CTC AG3') and 1492R (5'GGT TAC CTT GTT ACG ACT T3'). A final reaction volume of 25 µl was prepared containing; 1 µl of genomic DNA, 2.5 µl of 10X buffer, 1 µl of 20 pmole each primer, 0.5 µl dNTPs, 1.5 µl of $MgCl_2$, and 0.5 µl of Taq polymerase. Amplifications were carried out according to the following steps: denaturation of the target DNA at 95˚C for 2 min followed by 35 cycles of 95˚C for 30 sec, annealing at 52˚C for 30 sec and elongation at 72˚C for 1 min (Alijani et al., 2017). After multiying the fragment 1500 bp, 16SrRNA gene, electrophoresis was done on agarose gel 1% containing 1X TE buffer for almost 1 hour at 72 voltage. The PCR product was sent to Bioneer in South Korea for sequencing. The result sequences in the existing sequences were checked at NCBI database using BLAST software. The phylogenetic tree was derived using the neighbor-joining method.

2.7. Optimization of bioactive metabolites production

Nutrition factors and growth conditions have a strong influence on the production of secondary metabolites by microbes. In this study the optimal conditions required for maximum production of secondary metabolites by *Streptomyces* isolate were investigated.

2.8. Effect of Media

The isolate was inoculated in different culture media such as Inorganic Salt Agar (ISP4), Starch Casein Broth (SCB), Mueller Hinton Broth (MHB), Tryptone Yeast Extract Broth (ISP1) and Yeast Extract Malt Extract Broth (ISP2) and incubated in shaker incubator at 28°C for 7days. Then, the appropriate culture medium to produce the highest level of antimicrobial metabolites was identified and the maximum antimicrobial activity was calculated.

2.9. Effect of pH

To study the influence of pH level, the isolate was inoculated in the optimum medium with different pH values including 5, 6, 7, 8, 9, 10, 11 and incubated at 28 °C for 7 days on the shaker incubator at 150 rpm. The optimum pH level of culture medium to produce maximum antimicrobial metabolites production was used for subsequent study.

2.10. Effect of temperature

Various temperatures including 25, 28, 32, 37 and 45°C were used for bioactive metabolite production and the cultures kept in a shaker incubator at 150 rpm for 7 days. The optimum temperatures for maximum antimicrobial copmpounds production were investigated by measuring diameter of inhibition zone against the pathogenic microorganisms in well diffusion agar technique.

2.11. Effect of carbon and nitrogen sources

The influence of various carbon sources (glucose, glycerol, arabinose, sucrose and maltose at a concentration of 1% v/v) and nitrogen sources (peptone, malt extract, yeast extract and casein at a concentration of 1% v/v) were investigated to produce the highest level of the antimicrobial metabolites. The cultures incubated at 150 rpm for 7 days. Carbon-free and nitrogen-free flasks were also used as controls.

2.12. Effect of incubation period

The optimum incubation period for bioactive metabolite production was examined at 24, 48, 72, 96, 120, 144, 168 and 192 hours. Maximum antimicrobial compounds production was investigated by measuring diameter of inhibition zone against the pathogenic microorganisms in well diffusion agar technique.

3. Results

The isolated *Streptomyces* from the soils of eastern Gilan province was identified according to colony morphology, sporulation and pigment production, the color of aerial and substrate mycelium (Table1). The colony had dry appearance, powder, umbrella shape, wrinkled and leathery and was transferred subsequently to the new culture medium for purification several times. The pure colony appearance on SCA medium is shown in figure1 and the biochemical and physiological features of isolate are shown in Table 2. Primary and secondary screening of isolate SN5 was showed a broad spectrum antimicrobial activity against pathogenic microorganisms. As it is shown in tables 3 and 4, the most sensitive microorganism against antimicrobial activity of this isolate, is *M. luteus* with diameter of inhibition zone of 22±0.5 mm, while the most resistant microorganism is *P. aeruginosa* with a diameter of inhibition zone of 8±0.5 mm. For molecular identification, extracted DNA was evaluated by using agarose gel 1%. The presence of clear and transparent bands and lack of smear indicated appropriate and acceptable quality of extracted DNA for PCR usage. Agarose gel electophoresis PCR product confirmed the position of the amplified fragment at the target site (1500bp) using a marker. The result of the PCR is presented in figure 2. The results obtained from nucleotide sequence based on 16SrRNA gene sequencing in genetic database (NCBI), showed that the isolate belonged to *Streptomyces* genus and with the highest similarity (95.70%) to *Streptomyces malachitospinus* indicating significant differences at species level. This result molecularly confirms that it can be introduce as a new *Streptomyces* species.

The optimization results showed that the maximum production of bioactive metabolites was observed in Yeast extract malt extract broth (ISP2), followed by Starch casein broth, Mueller hinton broth, Inorganic Salt Agar (ISP4), Tryptone Yeast Extract Broth (ISP1) and antibacterial activity was noticeable against *B. cereus* (fig4). The isolate SN5 exhibited maximum antibacterial production at pH 7.0 and showed the highest antibacterial activity against *M. luteus* (fig5). The highest metabolite production was revealed at 28°C with maximum antibacterial activity against *S. aureus* (fig6). Glycerol and Yeast extract were found to be optimal for maximum antibacterial production as a carbon and nitrogen sources (fig7 and 8). An incubation period for maximum bioactive compound production was found at 168 h against *B. cereus* (fig9). Before 168 h incubation time a decreased amount of antibacterial production was observed.

Figure1. The isolate SN5 on SCA media

Table 1. Cultural characteristics of the isolate SN5 on different media after 7 Days of Incubation at 28˚C

Medium	Growth	Aerial Mycelium	Substrate Mycelium	Diffusible Pigment
Starch casein agar	Excellent	Gray	Whitish gray	Light brown
Tryptone yeastextract agar (ISP1)	Moderate	Light yellowish	Yellow brown	None
Yeast extract malt extract agar (ISP2)	Excellent	Gray	Light gray	Light Brown
Inorganic salts starch agar (ISP4)	Excellent	Cream	Whitish gray	Greenish yellow
Peptone yeast extract iron agar (ISP6)	Moderate	None	White	None
Raffinose histidine agar	Poor	None	None	None

Table 2. Biochemical and physiological characteristics of the isolate SN5

Characteristic	Result
Gram reaction	+
Acid-fast reaction	-
Catalase production	+
Oxidase production	+
Strach hydrolysis	+
Urease production	+
H_2S production	-
Methyl red test	-
Vogues proskauer test	-
Indole production test	-
NaCl tolerance (%, w/v)	1-3%
Range of temperature for growth:	
4°C	-
25°C	+
37°C	+
45°C	-
Optimum temperature for growth	28°C
Antibiotic resistance:	
Resistance to Chloramphenicol	+
Resistance to Tetracycline	-
Resistance to Penicillin	+

Table 3. Antimicrobial activity of the isolate SN5 by using Cross Streak method, Zone of inhibition (mm)

Isolate	*M. luteus*	*B. cereus*	*S. aureus*	*S. typhi*	*P. mirabilis*	*P. aeruginosa*
SN5	17±0.5	14±0.3	13±0.2	9±0.3	11±0.5	8±0.5

Table 4. Antimicrobial activity of the isolate SN5 by using Well Diffusion Agar method. Zone of inhibition (mm)

Isolate	*M. luteus*	*B. cereus*	*S. aureus*	*S. typhi*	*P. mirabilis*	*P. aeruginosa*
SN5	22±0.5	18±0.2	16±0.5	11±0.5	14±0.3	9±0.5

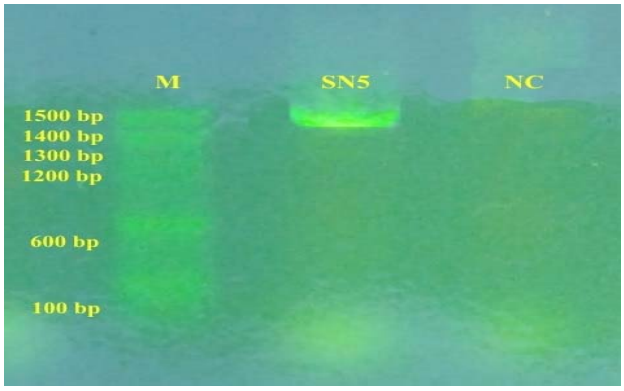

Figure 2. Electrophoresis of PCR product
Column M: marker (1500bp), NC: Negative Control

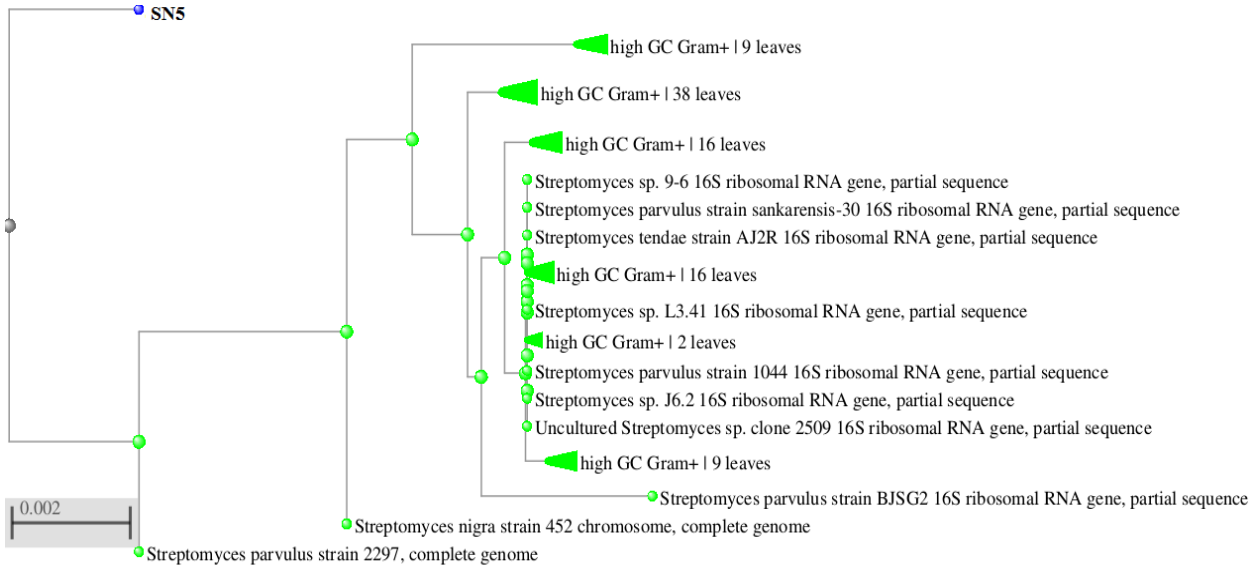

Figure 3. Phylogenetic relationships based on neighbour joining analysis of 16SrRNA gene sequence of the isolate SN5 and other *Streptomyces* species

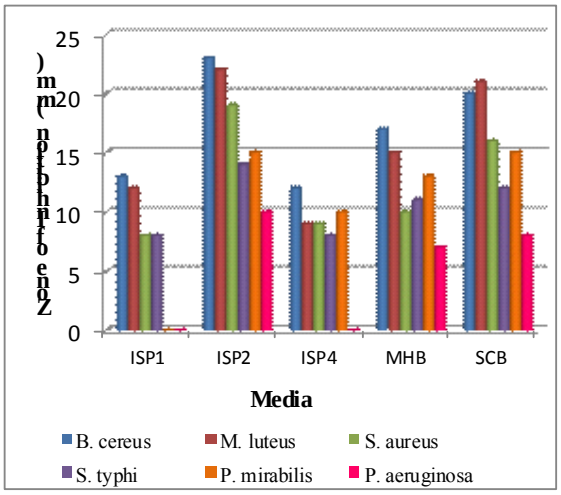

Figure 4. Effect of different media on antimicrobial compound production of SN5

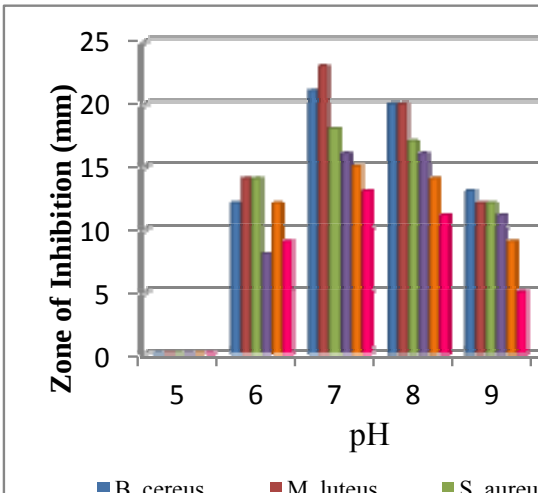

Figure 5. Effect of different pH on antimicrobial compound production of SN5

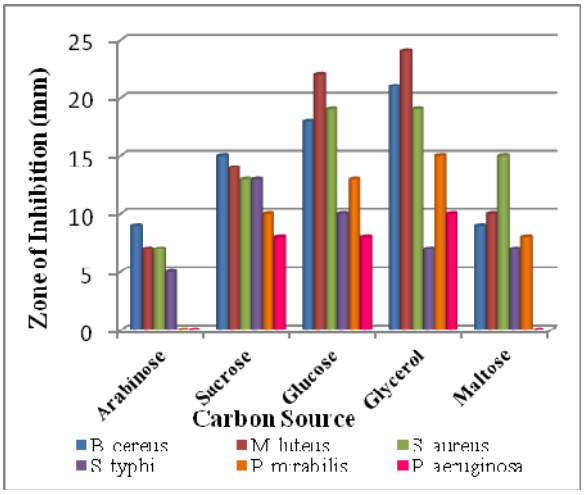

Figure 6. Effect of different carbon sources on antimicrobial compound production of SN5

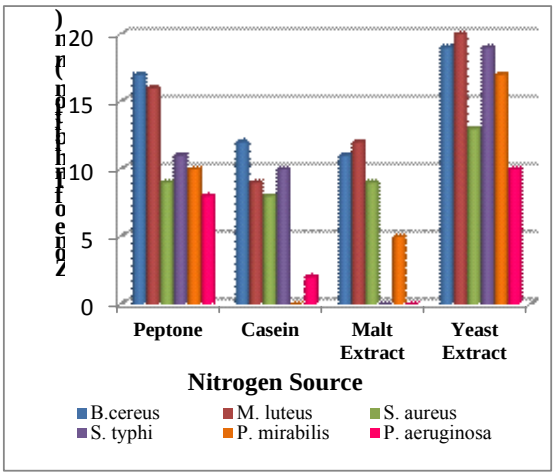

Figure 7. Effect of different nitrogen sources on antimicrobial compound production of SN5

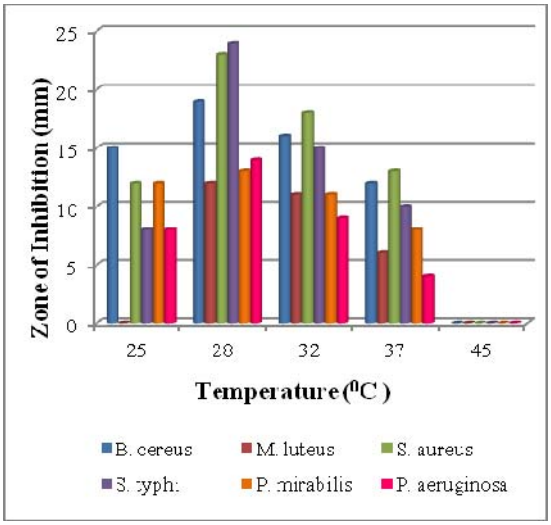

Figure 8. Effect of different temperature on antimicrobial compound production of SN5

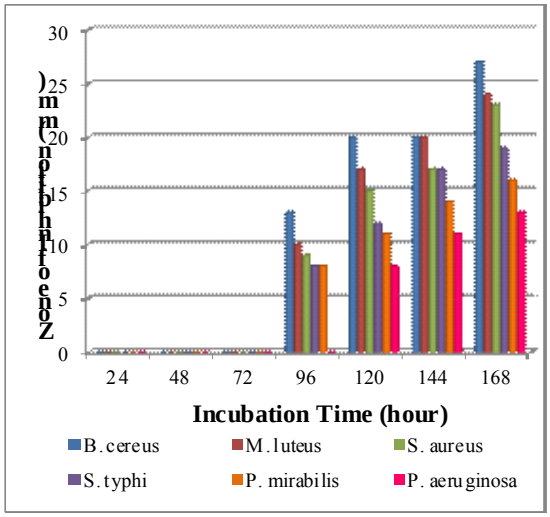

Figure 9. Effect of different incubation time on antimicrobial compound production of SN5

4. Discussion

Diagnosing new antibiotic agents are very important because of existence of new pathogens and antibiotic resistance (Axenov-Gibanov et al., 2016; Monciardini et al., 2014). *Streptomyces* attracted the attention of many researchers because they are able to produce biological active metabolites (Gontang et al., 2010; Ravikumar et al., 2010). In the present study, soil samples were collected from different eastern parts of Gilan province and the isolate SN5 was isolated and selected for screening its antimicrobial activity and optimization of its antimicrobial metabilites. The isolate SN5 was identified through morphological, biochemical, physiological and molecular techniques. The isolate showed strong antimicrobial activities against pathogenic bacteria in the primary and secondary screening methods.

Previous study showed that agricultural soils have a wide variety of *Actinomyces* (Oskay et

al., 2004) that are in agreement with the results. Previous studies found that *Streptomyces* usually prefer alkaline and neutral acidity for growth and are aboundant in such dry and moderate humidity (Locci 1998). In the crop soils, the population diversity of *Actinomyces* is high compared to the other soils due to the production of secondary metabolites by cultivated plants, which leads to the diversification of *Streptomyces* (oskay et al., 2004).

SCA medium was identified as the best medium for high growth of *Actinomycetes* and was introduced as a more suitable medium than other media (Remya et al., 2008; Senthilkumar et al., 2005) which is consistent with the results of this study.

The previous researches showed that gram-negative bacteria in comparison with gram-positive bacteria have more resistance to antimicrobial impact that can be compared with different structure of outer membrane of gram negative bacteria that contains lipopolysacharide structure that leads to being impenetrable (Gebreyohannes et al., 2013). Valli et al., reported that *Actinomyces* isolates showed more active antimicrobial activity against gram positive bacteria than gram negative bacteria (Valli et al., 2012), this was similar to the present findings. In previous study, investigation the antimicrobial effect of *Streptomyces* showed that *P. aeruginosa* has a high resistance to the antimicrobial effects of *Streptomyces* (Harounabadi et al., 2015) that is in accordance with this research.

In this study, 16S rRNA gene was sequenced and analyzed by BLAST software. 16SrRNA gene sequencing revealed that the isolate belonged to *Streptomyces* genus and with the highest similarity (95.70%) to *Streptomyces malachitospinus* indicating significant differences at species level and it can be introduced as a new species. The results confirm that the nature of the constituents of the culture medium and environmental conditions had a remarkable effect on the antimicrobial compounds produced by the isolate SN5, which is consistent with studies by other researchers on different *Streptomyces* spp. (Khandan Dezfullyet al., 2015). In previous study has shown that the ability of bacteria to produce antimicrobial under different environmental conditions can be increased or decreased (Khattab et al., 2016). The pH level influences the cellular metabolism and biosynthesis of secondary metabolites in *Streptomyces* species (Bhavana et al., 2014). Previous study showed that very high level of pH is undesirable in terms of secondary compounds (Khattab et al., 2016). In the present study, it was found that the pH level 7-8, in order to produce the maximum metabolites. Narayana et al., reported that optimum pH for maximum antimicrobial production was pH 7.0 (Narayana et al., 2008). The results confirm that optimum incubation time was 7day but previous study revealed that the optimum incubation time for the production of antimicrobial compounds was 10 days (Khandan Dezfully et al., 2015), whereas Kathiresan et al., reported that the maximum antimicrobial compounds obtained at 5 days (Kathiresan et al., 2005). This is in contrast with the current study. Yadav et al., reported that the highest concentration of antibiotics production was found at 28^{o} C (Yadav et al., 2009) that is in accordance with this research. In another study maximum antimicrobial production was found at 35^{o} C (Narayana et al., 2008) and also Viana et al., reported that the highest concentration of antibiotics production was found at 32^{o} C (Viana et al., 2010) that is in contrast with the current study.

In this study glycerol was introduced as carbon source that is in accordance with previous research (Viana et al., 2010). Another previous study showed that maltose was the best carbon source for maximum antimicrobial production (Narayana et al., 2008) that is in contrast with the current study. For highest antimicrobial production soybean flour (Viana et al., 2010) and soybean meal (Narayana et al., 2008) was interoduced as a nitrogen source that are in contrast with the current study.

Conclusion

The results of this study show that soils from eastern Gilan province are valuable for antimicrobial metabolites production and it could be important for pharmaceutical industry.

Refereces

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