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Original Research Paper

Characterization of a Bioactive Compounds Produced by *Streptomyces phaeochromogenes* NRRL B-2123

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ABSTRACT

Streptomyces is the largest genus of Actinobacteria and includes aerobic, Gram-positive filamentous bacteria with high G+C content. Although, few species of *Streptomyces* are pathogens, they could be characterized by production of the beneficial metabolites viz., antibiotics, antifungal, antiparasitic, and immunosuppressant compounds. The present study was conducted to isolate *Streptomyces* from soil and characterize their bioactive compounds. In total 85 soil samples were collected and assessed for production of the bioactive compounds by Agar Well Diffusion method against antagonistic microorganisms such as *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus*, *Aspergillus niger* and *Candida albicans*. Of all the *Streptomyces* isolates, one strain exhibited potent activity against *Staphylococcus aureus, Bacillus cereus* and *Aspergillus niger*. The promising strain identified using 16SrRNA gene sequencing and recognized as *Streptomyces phaeochromogenes* strain NRRL. Then, the growth phase of production and Arbitrary Unit (AU) of the bioactive compounds were determined, and the cured bioactive compound was purified and subjected to 1H NMR, 13C NMR and FTIR analysis for determination of the structural formula. The results obtained illustrated that high level of the bioactive compounds produced at stationary phase with Arbitrary Unit 32 AU. On the other hand, the data obtained from 1H NMR, 13C NMR and FTIR analysis exhibited a straight chain with a possible structural formula $C_{13}H_{15}NO_3$ for the bioactive compound.

INTRODUCTION

The genus *Streptomyces* includes Gram-positive filamentous bacteria with potent activity for production of bioactive compounds and enzymes (Lam 2006). These bacteria are living in the environment such as soil and water as microflora (Dehnad et al. 2010). Nowadays, *Streptomyces* are characterized by production of several useful metabolites and therefore, high ability of *Streptomyces* for surviving in different environments could be depended on their potential for production of several metabolites.

However, the metabolites produced by *Streptomyces* exhibited different properties, most of them considered secondary metabolites. In general, secondary metabolites are not absolutely required for the survival of the organism, but they can help organisms to survive in various environments.

Currently, *Streptomyces* is characterized by production of complex secondary metabolites (Berdy 2005). The secondary metabolites are produced by bacteria mainly during stationary phase. Indeed these metabolites produce at stationary phase during reduction of the nutrients and increase the waste compounds in the bacterial environments (Bibb 2005).

On the other hand, existence of antibiotic-resistant bacteria with high frequency culminated in demand for the novel antimicrobial agents (Bharti et al. 2010). Hence, in order to achieve this goal many researchers have begun to investigate on this area. Output of these investigates were introducing more than 50 different antibiotics, which provide most of the world's antibiotics (Emerson de Lima Procópio et al. 2012, Ibrahim Mabrouk 2012). Parallel with them the present study was undertaken to survey on isolation of soil origin *Streptomyces* and the evaluation of their antimicrobial compounds in order to increase information concerning to the bioactive compound producing *Streptomyces* in our geographical area.

MATERIALS AND METHODS

Soil sample collection: In total, 85 soil samples were collected from different areas in the north, center and south of Iran. All samples were taken from depth of 10-15 cm soil and dried in air. Then 10g of each sample was added into 90 mL sterile distilled water and kept in a shaker at 150 rpm for 30 min. Afterward, the suspensions were full cultured on the trypticasein soy agar plates (TSA) and incubated at 28-30°C for 5-6 days (Ningthoujam et al. 2009).

Screening and Identification of bioactive producing *Streptomyces*: Preliminary identification of *Streptomyces* isolates was carried out using macroscopic and microscopic characters of each isolate viz., type of colony and vegetative hyphae. After phenotypic identification, production of bioactive compounds by the isolates was evaluated using the Agar Well Diffusion method. The assay was performed by cultivation of each isolate in TSB and shaken in a shaker incubator with 150 rpm at 25-30°C for 48-72 h. Then, each broth medium was centrifuged at 10,000 rpm for 10 min and filtered by Watchman No.1 paper. Afterward, the antimicrobial activity of the filtrate was assessed against *Escherichia coli* (PTCC 1330), *Pseudomonas aeruginosa* (PTCC 1074), *Bacillus cereus* (PTCC 1015), *Staphylococcus aureus* (PTCC 1112), *Aspergillus niger* (PTCC 5012) and *Candida albicans* (PTCC 5027) using Muller Hinton Agar (MHA) (Vimal et al. 2009, Bharti et al. 2010). To perform the experiment each bacterium was fully cultured on MHA, then wells were made in the medium using sharp borer. 100 µL of the supernatant was added to each well and the plates incubated at 37°C for 24 h. Subsequently, a zone of inhibiting growth was considered as the antimicrobial effect of the metabolite, and the size was measured and recorded (Voravuthikunchai et al. 2006).

Identification and authentication of bioactive producing *Streptomyces*: Of all the *Streptomyces* isolates, one strain showed potent activity in production of bioactive compounds. In order to molecular identification of promising strain, DNA extraction was carried out using DNA PCR kit (Roche-Germany). Then the purity of extracted DNA was assessed by absorbance at 260 and 280 nm. The extracted DNA with ratio (260/280nm) more than 1.9 was used for Polymerase Chain Reaction (PCR). Amplification of 16SrRNA gene was performed using Forward and Reverse primers with sequences of 5'-CAACGAGCGCAACCCT-3'and 5'-GGTTACCTTGTTACGACTT-3' respectively. Each reaction tube was containing 18 µL of water (Sigma Aldrich Company Ltd.), 2.5 µL of 10 × PCR buffer (Cinnagen-Iran), 1 µL of each forward and reverse PCR primers, 0.5 µL of a 10 mM dNTPs (Cinnagen-Iran), 0.25 µL of Smar taq polymerase (Cinnagen-Iran), 0.75 µL of 50mM $MgCl_2$ (Cinnagen-Iran) and 5 µL of DNA template. PCR conditions of thermocycler (Cleaver, England) were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 60 s, 56°C for 45s, and 72°C for 60s, with a final extension at 72°C for 5 min and storage at 4°C. The PCR product was run on a 1.5% (w/v) agarose gel. PCR product was electrophoresed at 90V for 20 min and then DNA band was virtualized after staining with ethidium bromide. Finally, the PCR product with pure DNA band has been sent to Macrogen in South Korea (http://www.macrogen.com/) for DNA sequencing. The 16S rRNA sequenced data were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) to identify respective 16S rRNA gene amplicon (Baserisalehi & Bahador 2012).

Bioactive compounds extract by different solvents: The extraction of bioactive compounds was performed using different solvents viz., ethyl acetate, chloroform, acetone and ethanol. The solvents separately were added to the filtered supernatant in 1:1 proportion, and mixed by homogenizer for 45 min. Then, solvents were centrifuged at 5000 rpm for 15 min and the solvent parts were separated and evaporated at 70 and 80°C. The dark, brown separated and gummy compounds from each solvent were subjected for determination of antimicrobial activity (Dehnad et al. 2010).

Arbitrary Unit (AU) of bioactive compound: To determine Arbitrary Unit of bioactive compounds produced by *Streptomyces* isolate, the bacterial culture was serially diluted (1^{-2}, 1^{-4}, 1^{-8}, 1^{-16}, 1^{-32}, 1^{-64}, 1^{-128} and 1^{-256}), then 100 µL of each dilution was added into the wells of seeded Muller Hinton agar by *Bacillus cereus* PTCC1015. The plates were incubated at 37°C for 24 hrs and Arbitrary Unit of each bioactive compound was determined by reciprocal of highest dilution exhibiting the antimicrobial effect (Voravuthikunchai et al. 2006).

Determination of the growth phase of bioactive compound production: To obtain the phase of bioactive compound production, the *Streptomyces* isolate was inoculated in TSB and incubated in a shaker incubator at 150 rpm at 28°C for 48-72 h. The optical density (OD) of suspension was determined at 680 nm in time interval of 12 h. At the same time antimicrobial activities of each sample was determined as explained above (Moshafi et al. 2011).

Nuclear magnetic resonance (NMR) and Fourier transform infrared spectra (FTIR) analysis of the bioactive compound: The bioactive compound was purified by Column chromatography (Augustine et al. 2005) and the pure bioactive compound was dissolved in acetone and subjected to 1HNMR and 13CNMR (300 MHz, Brucker Biospin, Swit-

Table 1: Antimicrobial activity of the compound produced by *Streptomyces* sp. isolate.

Microorganisms	Inhibition Zone Diameter (mm) of bioactive compound
Escherichia coli	-*
Pseudomonas aeruginosa	-
Staphylococcus aureus	15
Bacillus cereus	20
Aspergillus niger	12
Candida albicans	-

*, no zone

zerland). In addition, FTIR analysis of the pure bioactive compound was carried out at 400 to 4000 cm^{-1} spectrum range. The spectrum was obtained by potassium bromide pellet technique. The pellet was prepared using 100mg dried potassium bromide and 1mg of bioactive compound. At the end the spectrum was plotted as intensity versus wave number. All the data obtained from this part of investigation were subjected to NCBI, PubChem Search Structure to determine possible bioactive compound structure.

RESULTS

Isolation of bioactive producing strains: In total, 6 strains of bioactive producing *Streptomyces* could produce bioactive compounds. Out of all, one strain with potent activity for production of antimicrobial compound was selected for further study. This strain showed antimicrobial activity against Gram-positive as well as fungi. As shown in Table 1 *Bacillus cereus, Staphylococcus aureus* and *Aspergillus niger* were sensitive, but *Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans* were resistant to the bioactive compound.

Identification of bioactive producing *Streptomyces*: The results obtained from phenotypic identification of the bioactive producer exhibited isolation of *Streptomyces* sp. In addition, alignment analysis of 16SrRNA genes of the bacterial strain showed 98% identical to *Streptomyces phaeochromogenes* strain NRRL B-2123 (No.1) with accession number gb|EU594477.1| (Fig. 1).

Bioactive compound extraction and arbitrary unit: The result obtained from extraction of the bioactive compound by different solvents indicated that acetone followed by chloroform, and ethanol extracted the bioactive compound relatively more. In addition, Arbitrary Unit obtained for the bioactive compound was 32 AU.

Production of bioactive compound at different growth

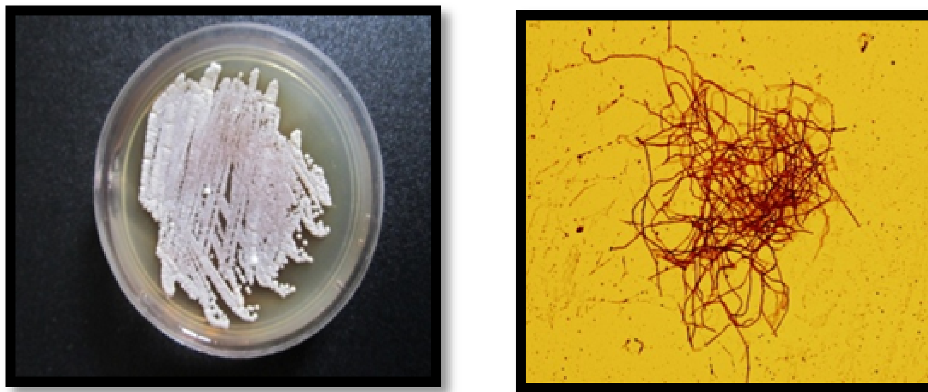

Fig. 1: Macroscopic and microscopic characters of *Streptomyces phaeochromogenes*.

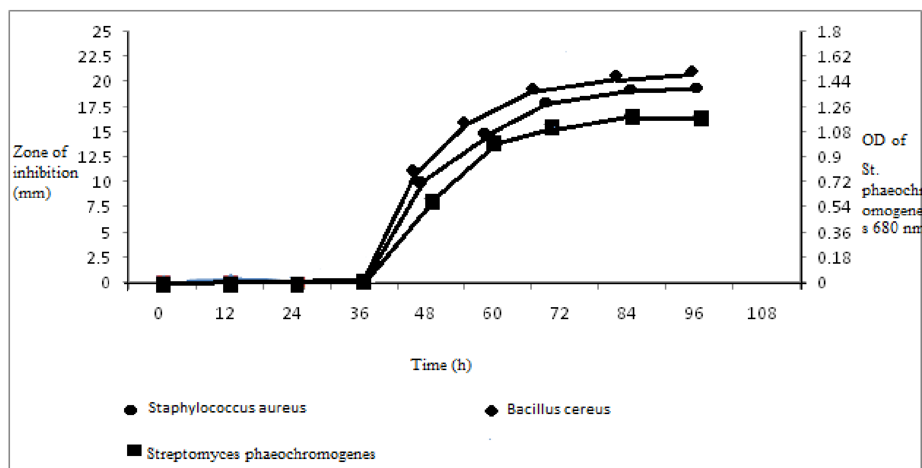

Fig. 2: Growth curves and their relation with bioactive compound production.

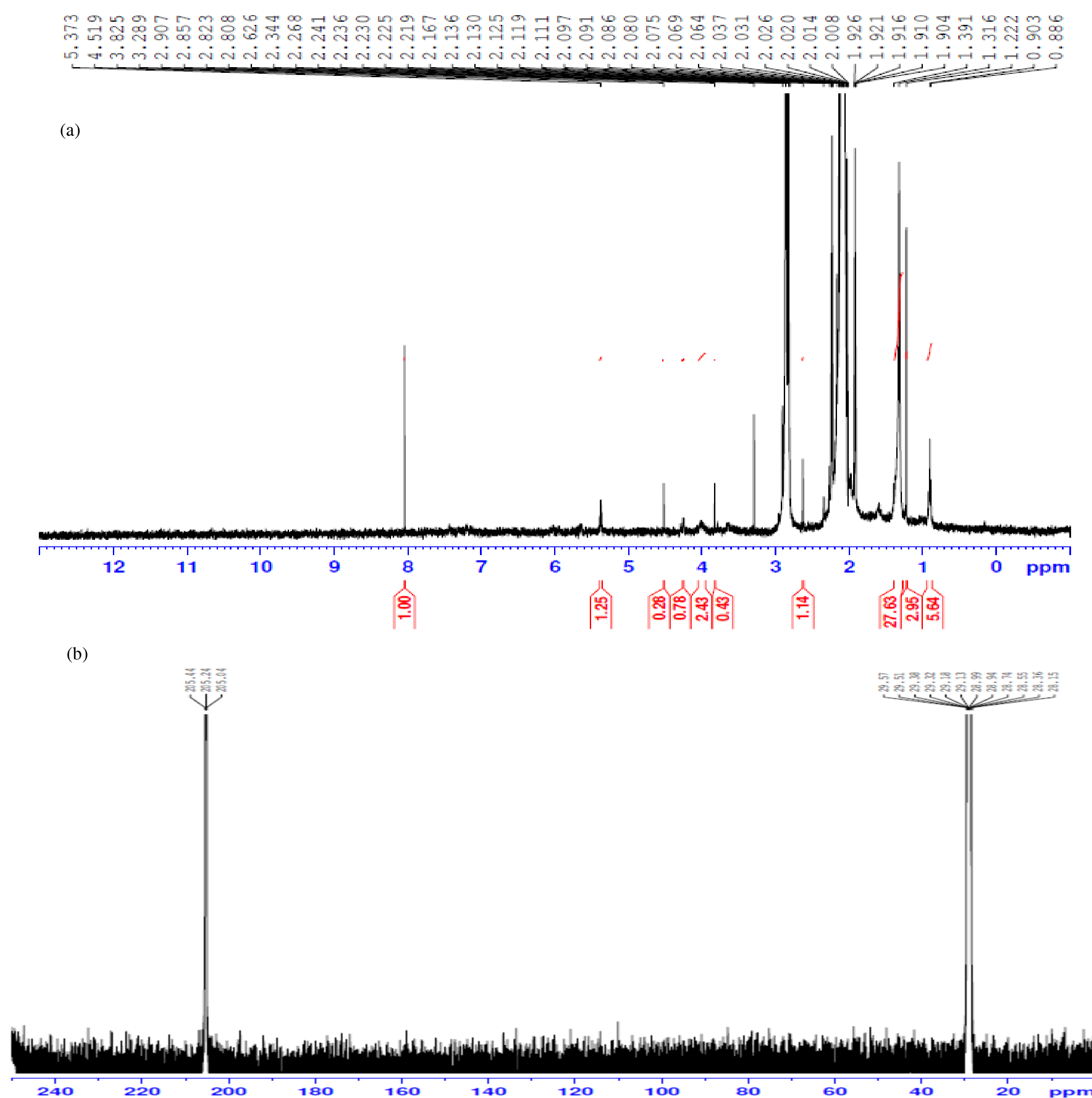

Fig. 3: 1H (a) and 13C (b) NMR (300 MHz) spectrum of the bioactive compound produced by *Streptomyces phaeochromogenes.*

phases of *Streptomyces* isolate: The results obtained from the production of bioactive compound during growth of *Streptomyces phaeochromogenes* indicated that the production of the compound was started on 48th hour and reached to maximum level on 84th hour of the bacterial growth. It means that stationary phase was considered a phase of production and therefore the bioactive compound produced by the isolate is secondary or basic metabolite (Fig. 2).

Structural analysis of the bioactive compound produced by *Streptomyces phaeochromogenes*: The results obtained from 1H NMR, 13C NMR spectral data (proton, carbon) and FTIR exhibited the existence of methoxy, carbonyl, carboxyl, aldehyde and amide groups within structure of the compound. In addition, the data showed the existence of two cycles in straight chain of the bioactive compound. Furthermore, all the data obtained from interpretation of 1H NMR, 13C NMR and FTIR were subjected to NCBI, PubChem Structure Search in order to determine possible bioactive compound

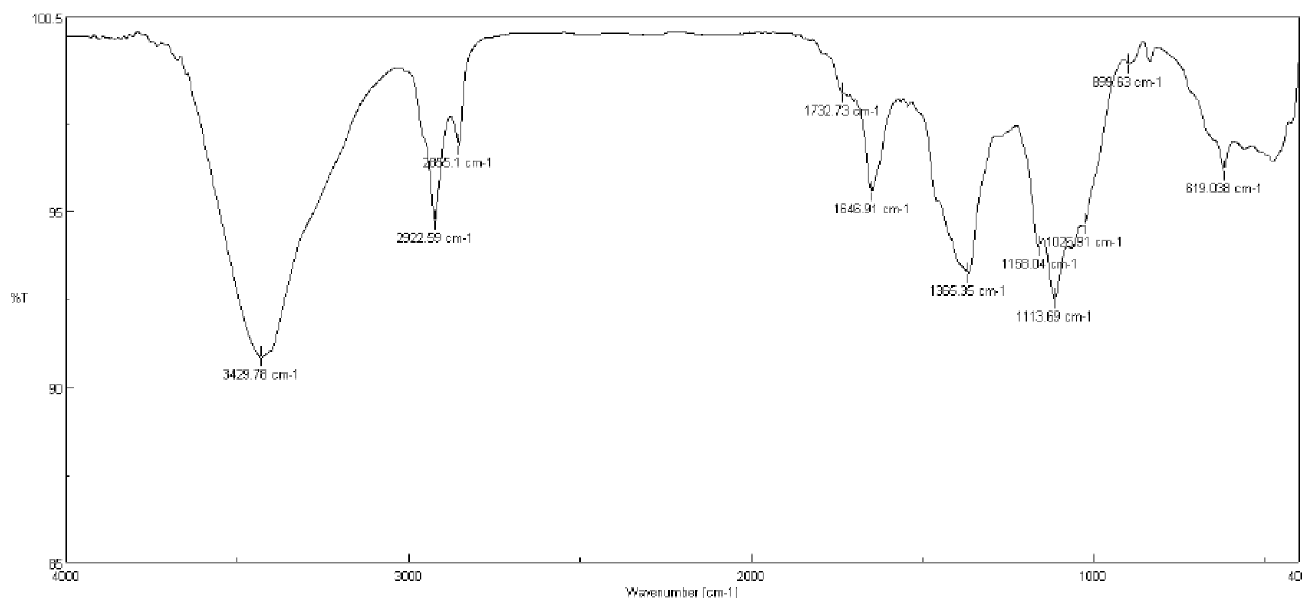

Fig. 4: FTIR analysis of the bioactive compound produced by *Streptomyces phaeochromogenes.*

structure. The result obtained suggested a structural formula $C_{13}H_{15}NO_3$ for the bioactive compound produced by *Streptomyces phaeochromogenes* (Figs. 3 and 4).

DISCUSSION

Nowadays, pharmaceutical industries introduce the new antimicrobial components with potent activity against pathogenic bacteria, viruses, etc. (Dehnad et al. 2010, Mageshwaran et al. 2011, Gontang et al. 2007). Therefore, the new sources and strains could be considered for achieving the compounds with broad spectrum against antibiotic-resistant microorganisms (Basik et al. 2003).

Recently, frequency of occurrence of antibiotic-resistant bacteria has increased. Therefore, to eliminate this phenomenon, pharmaceutical industries made attempts to introduce the new antimicrobial components (Dehnad et al. 2010, Mageshwaran et al. 2011, Gontang et al. 2007). Of all microorganisms, *Streptomyces* has shown high potential for production of bioactive compounds. Therefore, drug industries attempt to isolate or make new strains by genetic engineering. This is confirmed that *Streptomyces* strains could produce different antibiotics, for instance, *Streptomyces griseus* produce Streptomycin, and *Streptomyces venezuaele* produce Chloramphenicol (Akagawa et al. 1975, Distler et al. 1987). In this study, *Streptomyces phaeochromogenes* showed different character, which earlier has been reported. In this regard, production of anti-inflammatory polyketides, bromoperoxidase, isomerase, and dehydrogenase by *Streptomyces phaeochromogenes* were reported by many scientists (Graziani et al. 2005, Fukui & Tanaka 1982). However, our isolated strain of *Streptomyces phaeochromogenes* showed an antimicrobial property. On the other hand, 1H and 13C NMR and FTIR analysis of this compound exhibited straight chain with a possible structural formula $C_{13}H_{15}NO_3$, which it is differed from phaeochromycin $C_{13}H_{12}O_3$ (Li et al. 2008). Furthermore, the bioactive compound was produced in stationary phase, hence it might be considered antibiotic. In general, based on foregoing evidence, detected bioactive compound produced by *Streptomyces phaeochromogenes* might be a new bioactive compound; however, it needs more evaluations.

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