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Production of Streptothricin D by a Rhizospheric *Streptomyces* sp. Isolated from the Medicinal Plant *Mentha longifolia*

Fatemeh Alinejad, Gholam Khodakaramian*

Department of Plant Protection, Faculty of Agriculture, Bu-Ali Sina University, Hamadan, Iran

***Corresponding author: khodakaramian@basu.ac.ir**

Introduction

Most antibiotics used today are derived from Actinomycetes, particularly *Streptomyces* genus (Barka et al., 2015). The members of this genus have 20-60 gene clusters responsible for synthesizing secondary metabolites in their genome, making them a potential source for discovering new antimicrobial compounds (Challis, 2008). Genomic studies have shown that closely related *Streptomyces* from different environments possess different secondary metabolite biosynthesis gene clusters (Ian et al., 2014). Genomic differences are related to genomic islands (GIs) on the chromosome, which are usually the result of horizontal gene transfer (Penn et al., 2009). The rhizosphere area is influenced by the root exudates and creates an environment conducive to microbial growth (Dennis et al., 2010). The rhizosphere of wild medicinal plants is one of the rich and unique sources for isolating and identifying *Streptomyces* bacteria (Bekiesch et al., 2020; Oberhofer et al., 2019). *Streptomyces* are the most successful group of microorganisms to colonize this special niche due to their ability to produce a wide range of antimicrobial compounds and develop resistance mechanisms (Schlatter et al., 2009). This study aims to isolate and identify *Streptomyces* bacteria from the rhizosphere of wild medicinal plants in Hamedan province and investigate their potential to produce secondary metabolites and antimicrobial compounds.

Material and Methods

Isolation of Streptomyces and screening antimicrobial activity: Soil samples were collected from the rhizosphere of wild medicinal plants, in Avarzaman, Hamedan province. The samples were air dried and Streptomyces cultures were isolated by serial dilution plating technique (Seong et al.,

2001; Shirling & Gottlieb, 1966). Primary screening and antibacterial activity of the *Streptomyces* isolates against gram-positive and gram-negative bacteria were tested by cross streak method (Lemos et al., 1985). Active isolates with inhibition of growth activity were selected for secondary screening, and their ability to produce bioactive metabolites in the liquid medium was investigated too (Kibret et al., 2018; Rajeswari et al., 2015). The crude extracts from the liquid cultures were evaluated for their antimicrobial activity against indicator bacteria using the disk diffusion method (Bauer et al., 1966). Molecular identification of isolates was performed using 16S rRNA gene sequence and polymerase chain reaction (PCR). Genomic DNA was extracted from fresh cultures of isolates on ISP4 culture medium using alkaline lysis buffer and amplification of 16 rRNA sequences was performed with pA and pH primers (Edwards et al., 1989).

Purification and identification of bioactive compounds: The active extract was dissolved in acetonitrile and ammonium formate and fractionation was carried out on a HPLC system. The fractions were collected and dried, then their antimicrobial activity was investigated using the disk diffusion method. The bioactive fractions were identified by measuring the growth inhibition of indicator bacteria. The analysis of active fractions was performed using electrospray ionization mass spectrometry in positive ion mode. The active fractions were dissolved in acetonitrile and formic acid and loaded into the mass spectrometer. The flow rate and voltage were adjusted, and scans ranged from m/z 100 to 2000 with a 5 min scan time. Precursor ions were selected for MS/MS analysis and collision energy was optimized.

Result and Discussion

Isolation of Streptomyces and screening antimicrobial activity: In total seven different isolates of *Streptomyces*, based on morphology, were isolated from the rhizosphere of wild medicinal plants Averzaman, Hamedan Province, Iran. In the primary screening for antimicrobial activity using the cross streak method, isolate 3Z collected from the rhizosphere of *Mentha longifolia* (L.) showed strong antimicrobial activity against both gram-positive and gram-negative indicator bacteria. BPM3 culture medium was used to investigate the ability of this isolate to produce antimicrobial compounds in liquid medium, and crude extracts obtained from the broth culture inhibited the growth of indicator bacteria in the secondary screening using the disk diffusion method. Based on 16s rRNA nucleotide sequence blast, *Streptomyces enissocaesilis* was the closest species to isolate 3Z (GenBank accession no. OR501904) with 99.60 % sequence similarity.

Purification and identification of bioactive compound: *Streptomyces* sp. 3Z extracts were fractionated by RP HPLC in gradient mode to separate compounds responsible for the antibacterial activity against indicator bacteria. In total of 17 fractions were collected, and the first fraction (collected at 7-8.2 min) was identified as the active fraction responsible for the antimicrobial activity that prevented the growth of indicator bacteria. The active fraction was analyzed using

ESI-MS. The 3Z active fraction analysis in positive ion mode displays m/z 759.49 ($[M + H]^+$) as the main peak. MS/MS spectrum of this precursor ion was analyzed and compared with public data available from the library of *Streptomyces* compounds (Streptome DB), GNPS spectrum library, and information available in the literature. Based on this information, it was determined that this molecular mass is related to streptothricin type D, which is responsible for the antimicrobial activity (Ji et al., 2008; Ji et al., 2007; Van Tamelen et al., 1961). Streptothricin group antibiotics have a broad spectrum of antimicrobial activity, but their usage is limited due to their cytotoxicity. They are characterized by the presence of streptolidine lactam, carbamoyl-D-gulosamine, and a β-lysine chain in their structure. Different types of streptothricin antibiotics can have varying numbers of β-lysine units (Haupt et al., 1980; Van Tamelen et al., 1961; Kusumoto et al., 1982).

Recently, a new isomer of streptothricin D, called 12-Carbamoylstreptothricin D, was reported. This isomer has a carbamoyl group attached to C-12 of D-gulosamine instead of C-10. It has a similar molecular mass and antimicrobial activity as streptothricin D (Ji et al., 2008; Ji et al., 2007). Consequently, it becomes challenging to determine which isomer of streptothricin D is produced by *Streptomyces* sp. 3Z. Considering the high genomic potential of *Streptomyces* bacteria to produce antimicrobial compounds, genomics studies can help to induce and better express the gene clusters responsible for the synthesis of secondary metabolites in these bacteria. (Rutledge & Challis, 2015).

Conclusions

In this study, the potential of *Streptomyces* isolates isolated from the rhizosphere of wild medicinal plants in Hamedan province was studied, and based on bioassay testing and using mass spectrometry analysis, streptothricin D production by one isolate collected from the rhizosphere of *Mentha longifolia* (L.) was confirmed, but its isomer type was not determined.

Keywords: Streptomyces, Antibiotic, Streptothricin, Antibacterial activity, Rhizosphere, Mass spectrometry.

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Declaration of conflict of interest

The authors declare that they have no conflicts of interest.