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Contributed Paper

Thin Layer Chromatography-Bioautography Assay for Antibacterial Compounds from *Streptomyces* sp. TBRC 8912, a Newly Isolated Actinomycin D Producer

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Abstract

Based on a primary screening for antibacterial agents using the agar disc diffusion method, among one hundred and sixty-one actinobacterial strains isolated from herbicide sprayed soil in agricultural areas, a newly isolated *Streptomyces* sp. TBRC 8912 exhibited antibacterial activity against *Xanthomonas axonopodis* pv. *citri* (Xac) a pathogen that causes canker disease in kaffir lime. The strain TBRC 8912 was cultivated in the International *Streptomyces* Project (ISP) 2 and modified Wickerhams Antibiotic Test Medium (WATM) in liquid and solid fermentations. Ethyl acetate crude extracts from ISP2 and WATM cultures showed antibacterial activity against Xac by causing an inhibition zone diameter with a range of 15.75±0.48-17.38±0.24 mm on bioassay plates when tested using the paper disc diffusion method. The ethyl acetate extract obtained from the WATM liquid culture, was separated by column chromatography over Sephadex LH-20 (dichloromethane/methanol, 1:1) and silica gel (dichloromethane/methanol). Subsequently, bioassay-guided fractionation was used to select active fractions, and then a bioautographic technique was employed to isolate a mixture of compound **1** and **2** (10 mg). The active fraction was further investigated using liquid chromatography-electrospray ionization-quadrupole-time-of-flight mass spectrometry (LC-ESI-Q-TOF-MS). ESI-MS of the isolated compounds showed pseudomolecular ion peaks at m/z 1255.6 $[M+H]^+$ and 1277.6 $[M+Na]^+$ at t_R 28.44 and 28.84 min corresponded to the molecular mass of 1254 which matches to actinomycin D and its isomer. The 16S rDNA gene sequence of the strain TBRC 8912, exhibited 99.11 % identity with the *Streptomyces panaciradicis* strain 1MR-8^T. This is the first report of a representative of *Streptomyces panaciradicis* capable of producing actinomycins. Further study is recommended for evaluation of the persistence and biodegradation of plant tissue and soils in a greenhouse, before application in the environment.

Keywords: herbicide sprayed soil, soil extract agar, bioassay-guided fractionation, actinomycin D

1. INTRODUCTION

Actinobacteria are valuable and available sources of secondary metabolites. Among actinobacteria members, the Streptomycetes are reported to produce 80 % of antibiotics used for medicinal and agricultural applications [1]. *Streptomyces* spp. are gram-positive filamentous bacteria that grow in various habitat such as, soil [2], plant tissue [3] and the marine environment [4]. This includes herbicide sprayed soil where biological processes are performed by microorganism after chemical spray application [5]. Several newly isolated strains have been reported to be known natural product producers [6-8].

Actinomycins are a group of chromopeptide lactone antibiotics first isolated from cultures of *Actinomyces antibioticus* by Waksman and Woodruff in 1940 [9], and actinomycins were quickly shown to be antibiotics with both bacteriostatic and bactericidal properties [10]. However, in antibiotic therapy, actinomycins are limited in use apart from applications in anti-tuberculosis treatment, but they are highly effective in the treatment of Wilm's tumor and other cancers [11-12]. Among the known natural actinomycins, actinomycin D has been employed as a tool in molecular biology research due to its chemical and biological properties [6]. To date, natural product screening programs have identified at least 30 species of actinomycin producing strains, some of which have been reported as novel sources for further study [13].

Nowadays, natural product discovery from *Streptomyces* strains relies on biological activity guided isolation methods [14-15] nevertheless, chemical screening methods are needed. To avoid time-consuming isolation procedures, the dereplication method has been used for the rapid identification of known metabolites present in complex mixtures [16]. In 1990, the term of dereplication was introduced by Beutler and colleagues [17], and dereplication procedures are a combination of bioassay, separation, spectroscopic methods, and database searches for the rapid detection and identification of compounds [18]. Frequently, dereplication workflow involves acceleration of bioassay- guided fractionation by using paper disc diffusion assay or thin layer chromatography (TLC)-bioautography, followed by an isolation step [19]. In addition, mass spectrometry is commonly used to reduce the time required due to its accurate sample detection.

Canker is a devastating disease caused by *Xanthomonas axonopodis* pv. *citri* (Xac) which occurs on citrus plants, such as kaffir lime (*Citrus hystrix*), lime (*C. aurantiifolia*) and pomelo (*C. maxima*). During the monsoon season, the disease is spread widely, helping the bacteria to colonizes the plant surface, and subsequently to enter the leaves through wounds or stomata. Currently citrus canker is controlled worldwide by spraying copper-based bactericides to decrease the pathogenic bacteria population on the surfaces of plants. However, multiple applications have led to the development of copper-resistant strain [20-21], therefore, the search for alternative bactericides is important. Currently, only a few studies have employed bioassay-guided fractionation to isolate antibacterial agents against target phytopathogens [15,22]. This study aimed to search for new antibacterial agents against Xac using the method of dereplication for rapid identification of antibacterial compounds obtained from crude extracts under different cultivation conditions.

2. MATERIALS AND METHODS

2.1 Actinobacteria Strains

One hundred and sixty-one actinobacteria strains were isolated from herbicide sprayed soil collected at agricultural areas in Kamphaeng Phet province, Thailand (August, 2016). Serial dilution was used to obtain single colonies on soil extract agar. Based on an agar disc diffusion screening method, the actinobacteria strain TBRC 8912 was selected for further investigation regarding its antibacterial activity against Xac. A stock culture is maintained at the Thailand Bioresource Research Center, National Science and Technology Development Agency, Thailand.

2.2 Amplification of 16S rDNA and Phylogenetic Analysis

Primers 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' were used for PCR amplification of 16S rDNA gene. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 µl reaction mixture using a EF-Taq (SolGent, Korea) as follows: 95 °C for 2 minutes, followed by 35 cycles of 95 °C for 1 minute, 55 °C, and 72 °C for 1 minute, finishing with a 10-minute step at 72 °C. Sequencing reactions were performed using a PRISM Big Dye Terminator v3.1 Cycle sequencing kit and were analyzed on an ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). 16S rDNA gene sequence of related strains were obtained from GenBank, and evolutionary history was determined using the Neighbor-Joining method. Evolutionary analyses were conducted using MEGA7 [23].

2.3 Cultivation and Preparation of Crude Extracts

2.3.1 Cultivation

The strain TBRC 8912 was cultivated in 250 ml Erlenmeyer flasks containing 50 ml International *Streptomyces* Project (ISP) 2 liquid medium for seed inoculum preparation [24]. The flasks were incubated on a shaking incubator at 30°C for 48 hours at 165 rpm. Antibacterial compound production was investigated in shake flasks and solid fermentation using ISP2 medium and modified Wickerhams Antibiotic Test Medium(WATM) [25] (Supplementary File 1). For liquid fermentation, nine 1 litre Erlenmeyer flasks containing of 400 ml liquid medium were inoculated with 10% seed inoculum and shaken on a rotary shaker (165 rpm) at 30 °C for 5 days. The cultivation on solid fermentation was prepared by transferring 100 µl of seed inoculum onto 1 litre ISP2 agar plates and WATM agar plates (90 cm diameter) with growth at 30 °C for 3 weeks.

2.3.2 Preparation of crude extracts

The whole culture broth of ISP2 and WATM were extracted using ethyl acetate at a 1:1 ratio (v/v) with vigorous shaking for 30 min for complete extraction. The ethyl acetate extracts were evaporated to dryness using a rotary evaporator. The dried crude extracts were weighed and stored at 4 °C. Meanwhile, each solid culture was harvested after incubation for 3 weeks. The agar was cut into squares and soaked overnight in ethyl acetate. The ethyl acetate mixture was filtered, and the filtrate was concentrated using a rotary evaporator. The dried crude extracts were weighed and stored at 4 °C.

2.4 Antibacterial Activity Assay

Antibacterial activity assay against the plant pathogenic bacterium, Xac was undertaken using a paper disc diffusion assay as described in a previous study [26]. For bioassay plate preparation, bacterial suspensions were adjusted to approximately 10^8 colony forming units/ml. The culture was inoculated on to the surface of nutrient agar plates. Sterile paper discs (6 mm in diameter) were then loaded with 20 µl of the crude extract solutions (10 mg/ml), air-dried thoroughly, and placed on the surface of bioassay plates. These plates were then incubated for 24 h at 30 °C. Each experiment was carried out in four replicates. Chloramphenicol (1 mg/ml) was used as a control. The data were recorded by measuring the inhibition zone around the discs (in mm) and are expressed as means ± SD.

2.5 Separation

Ethyl acetate crude extract obtained from 2.7 litre of liquid culture of WATM (530 mg) was applied to a Sephadex LH-20 column (dichloromethane/methanol, 1:1) (diameter 2.5 cm x 100 cm). Fractions (8 ml) were collected every 5 minutes to obtain 6 fractions. Bioassay-guided fractionation was conducted using a paper disc diffusion assay to select active fractions [22]. The active fraction (Fraction 2) (100 mg) was applied to a silica gel column

(0.4 cm x 15 cm). Fractionation was undertaken using increasing concentrations of methanol in dichloromethane (100% dichloromethane, 4:96, 5:95, 10:90, 20:80, 50:50, 70:30 and 50% methanol). All the fractions (fraction 1–fraction 12) were tested using a bioautographic assay against Xac [27]. The chromatograms were developed in chloroform: acetone (9:1), and bands were first observed under UV at 254 and 366 nm, and then anisaldehyde/sulphuric acid reagent was sprayed on the chromatograms before heating. The separated compounds from fraction 2 (35 mg) were further purified by preparative thin-layer chromatography (PTLC, 20x20 cm) using chloroform: acetone (9:1). Antibacterial agents in a mixture of compounds were detected using bioautography.

2.6 LC-ESI-Q-TOF-MS/MS Conditions

A 6540 ultrahigh definition accurate mass Q-TOF (Agilent Technologies, Palo Alto, CA) was coupled to an Agilent 1260 infinity high performance liquid chromatography instrument (Agilent, Waldbonn, Germany) via an ESI interface. Analysis parameters were set using both negative and positive ion modes with spectra acquired over a mass range of m/z 100–1,700 amu. The ESI-MS condition parameters were as follows: capillary voltage, +3,500 V; dry gas temperature, 350 °C; dry gas flow, 10 L/min; nebulizer pressure, 30 psig; and spectra rate, 4 Hz. Fragmentations were performed using auto MS/MS experiments with collision energies at 10 v, 20 v, and 40 v. Nitrogen (UHP) was used as a collision gas. Chromatographic separation was performed on a phenomenex Luna C-18(2) column (5 μm, 150 × 4.6 mm internal diameter) Phenomenex Inc., Torrance, CA). The mobile phase consisted of 0.1% formic acid in water v/v (Solvent A) and 0.1% formic acid in acetonitrile v/v (Solvent B). The linear gradient started from 5% to 95% of Solvent B for 30 minutes and hold on 10 min then post run 5 min before the next injection. The injection volume was 5 μl. The flow rate was set at 0.5 ml/min.

2.7 Confirmation of Actinomycin D

To confirm the structure of actinomycin D and its isomer, ^{1}H NMR spectra of the compound was measured using a Fourier Transform Nuclear Magnetic Resonance Spectrometer 500 MHz (Avance III HD, Bruker). Moreover, the mixture of compound **1** and **2** was tested in reactions with 2N NaOH and concentrated H_2SO_4.

3. Results

3.1 Phylogenetic Analysis of Strain TBRC 8912

A screen of one hundred and sixty-one actinobacteria strains that were isolated from herbicide sprayed soil collected at agricultural areas in Kamphaeng Phet province, Thailand for antibacterial activity against Xac identified strain TBRC 8912 as warranting further study. The 16S rDNA region from strain TBRC 8912 was amplified by PCR and sequenced. By comparing the 16S rDNA gene sequence of this strain with the sequences in GenBank, this strain was found to be 99.11 % identical to *Streptomyces panaciradicis* 1MR-8^T (GenBank accession no. KF971876). In a phylogenetic tree analysis, strain TBRC 8912 clustered in a clade with *S. durhamensis* NRRL B-3309^T (GenBank accession no. AY999785), *S. filipinensis* NBRC 12860^T (GenBank accession no. AB184198), *S. Yaanensis* Z4^T (GenBank accession no. JQ307192), *S. niveiscabiei* S78^T (GenBank accession no. AF361786), *S. sasae* JR-39^T (GenBank accession no. HQ267987) and *S. panaciradicis* 1MR-8^T (GenBank accession no. KF971876). The results suggest that this strain should be classified as a conventional species of *S. panaciradicis* on the basis of the phylogenetic analysis (Figure 1).

3.2 Antibacterial Activity Assay

Ethyl acetate crude extracts (200 μg/disc) obtained from 4 different culture methods were evaluated for their antibacterial activity against Xac using a paper disc diffusion method (Table 1). The results showed that WATM solid culture exhibited the maximum antibacterial activity

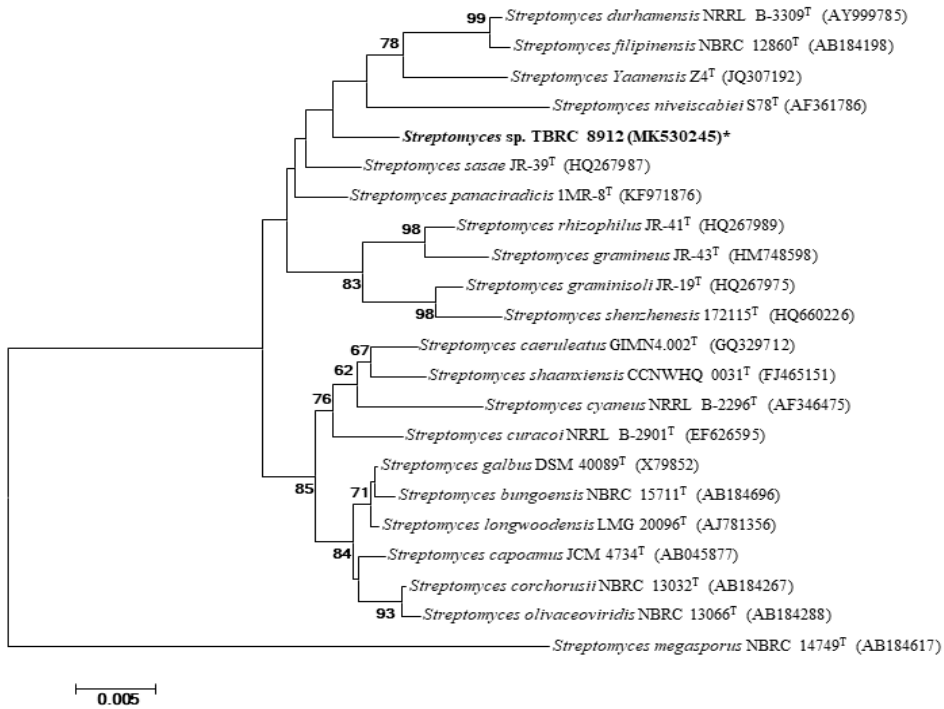

Figure 1 Neighbor-joining tree based on 16S rDNA sequences (1374 nt), showing the relationship between strain TBRC 8912 and recognized members of the genus *Streptomyces*. *Streptomyces megasporus* NBRC 14749^T was used as an outgroup. Bootstrap values based on 1,000 replications are shown at branch nodes. Bar, 0.005 substitutions per nucleotide position.

(zone of inhibition 17.38 mm), whereas crude extracts obtained from the ISP2 solid culture, had the smallest zone of inhibition (15.75 mm). In this study, we also found that solid cultivation provided higher amount of crude extract than liquid cultivation (Table 1), and higher yields of crude extract were observed in WATM medium when cultivated in both liquid and solid medium, as compared to ISP2 medium (Table 1).

Table 1 Cultivation conditions, yield of crude extracts, and presence of antibacterial activity against *X. axonopodis* pv. *citri*.

Culture medium	Cultivation condition	Zone of inhibition (mm)*	Yield of crude extract (mg/L)
WATM	Liquid	17.00 ± 0.35	363
	Solid	17.38 ± 0.24	658
ISP2	Liquid	17.00 ± 0.58	54
	Solid	15.75 ± 0.48	340
Control	-	25.25 ± 0.25	-

*Inhibition zone includes the diameter of the disc (6 mm); control: Chloramphenicol (20 μg/disc). Values are mean of four readings (mean ± SD).

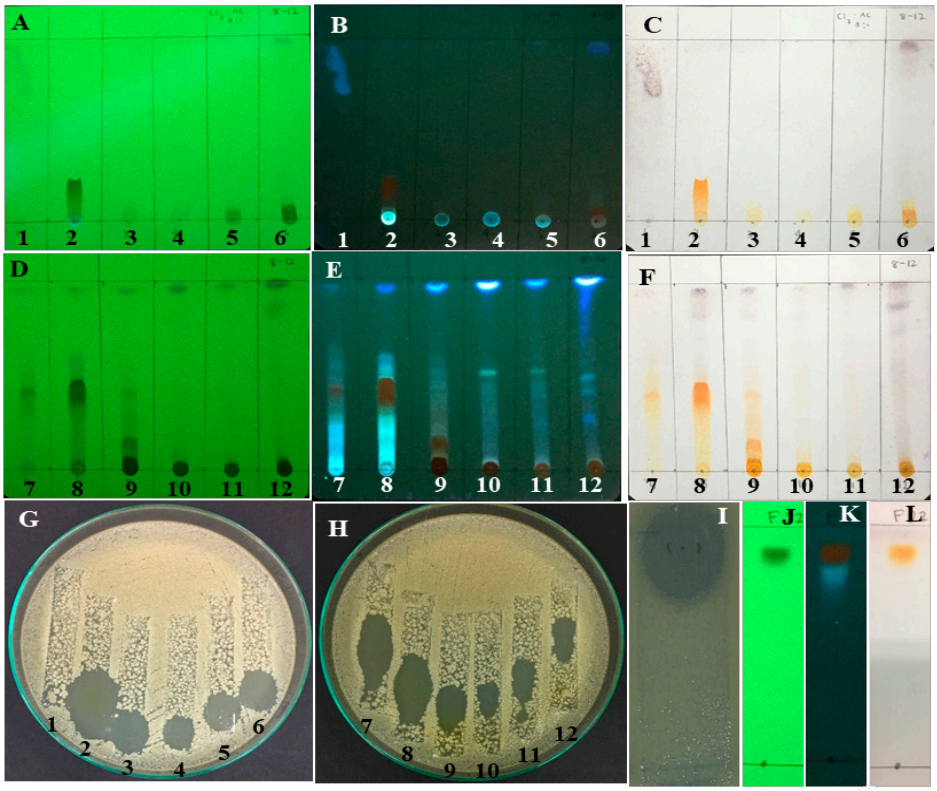

Figure 2 Isolation of metabolites from *Streptomyces* sp. strain TBRC 8912; A-F = Thin Layer Chromatography (TLC) of fractions obtained from silica gel column chromatography, TLC developed in chloroform: acetone (9:1), G-H = Bioautographic assay of 12 derived fractions showed a zone of inhibition (40 µg/spot), I= A mixture of compound **1** and **2** exhibited a clear zone when tested against Xac using a bioautographic assay, J = mixture of compound **1** and **2** was observed on the TLC plate using ultraviolet light at 254 nm, K = TLC plate was observed using an ultraviolet light at 366 nm, L = *p*-anisaldehyde-sulphuric acid stain was visualized after being heated to 105°C.

3.3 Separation, Bioassay-guided Fractionation and TLC-bioautography

The results of the antibacterial activity assay showed that TBRC8912 was able to produce antibacterial agents under all cultivation conditions, therefore WATM liquid medium was selected for further processing based on the yield of crude extract, and time effectiveness. Based on the bioautographic assay results (Figure 2, G-H), fraction 2 (35 mg) was further selected for fractionation by planar thin layer chromatography (PTLC) and fractions were eluted with chloroform: acetone (9:1) to yield a mixture of compounds **1** and **2** (10 mg) (Figure 2, J-K).

The mixture appeared as an orange-yellow powder, which gave a characteristic orange color reaction with anisaldehyde/sulphuric acid upon developing the TLC plates (Figure 2, L). The mixture reacted with concentrated sulphuric acid and turned to a red colour, but no colour change was observed after reaction with 2N NaOH (supplementary Figure S1). The ^{1}H NMR spectrum of the mixture in $CDCl_3$-*d* was identical to actinomycin D and its isomer as previously reported [28] (Figure 3).

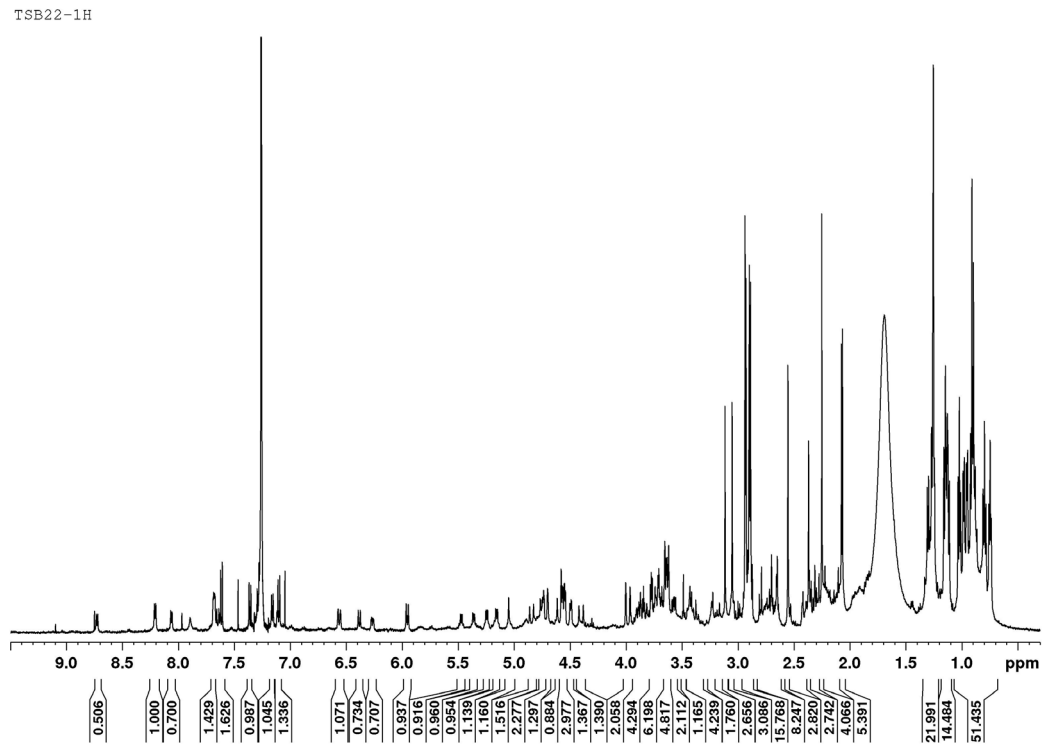

Figure 3 ^{1}H NMR spectrum of actinomycin D and its isomer in $CDCl_3$-*d* at 500 MHz.

3.4 Identification Using ESI-Q-TOF-MS

Electrospray ionization mass spectrometry (ESI-MS) spectra showed molecular ion peaks at m/z 1255.6 $[M+H]^+$ and 1277.6 $[M+Na]^+$ at t_R 28.44 (A) and 28.84 (B) min (Figure 4). A search of this data (Chemspider) confirmed the mixture of compounds to be actinomycin D ($C_{62}H_{86}N_{12}O_{16}$) and its isomer in comparison with previously reported data [6, 29-30].

4. Discussion

Soil is a rich source of natural products with biologically active molecules. In a natural product screening program, soils were collected and screened for chemicals with potential against Xac, resulting in identification of strain TBRC8912. On the basis of a phylogenetic analysis, *Streptomyces* sp. TBRC8912, is a newly isolated strain that was found to be 99.11 % identical to *Streptomyces panaciradicis* 1MR-8^T. In the past, fermentation conditions for novel isolates were optimized for actinomycin production, however those methodologies produce only small quantities of actinomycins in fermentation broth [7, 31-32]. This is the first report of a representative of *Streptomyces panaciradicis* that can produce actinomycin D derivatives under solid state and liquid fermentation. Some studies have suggested that solid state fermentation is simple and cost effective for antibacterial agent production [33], especially in commercial production as agricultural and industrial wastes can be used as substrates in the fermentation process.

Various *Streptomyces* species have been reported as producers of actinomycins [30, 34-35]. Actinomycin D is the most intensively studied because of its remarkable chemical and biological activities [6]. In a screening program for new bioactive metabolites such as anti-tuberculosis, antimicrobial and anti-cancer drugs from marine derived culture,

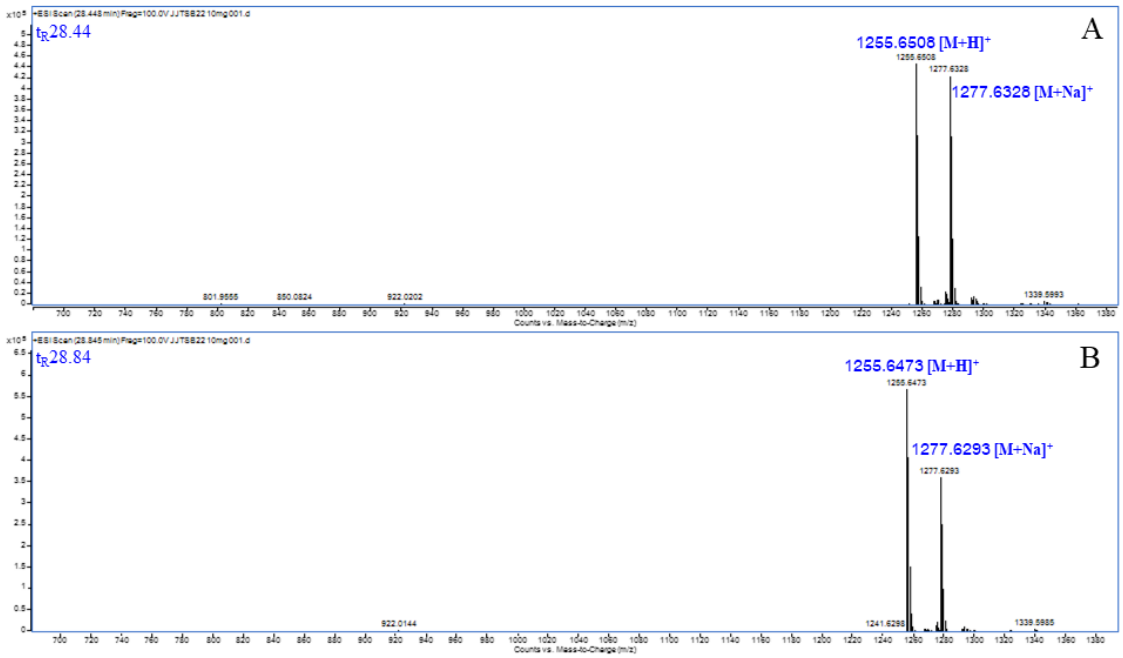

Figure 4 ESI-MS spectra of actinomycin D and its isomer shown molecular ion peaks at m/z 1255.6 $[M+H]^+$ and 1277.6 $[M+Na]^+$ at t_R 28.44 (A) and 28.84 min (B).

Streptomyces capillispiralis MTCC10471 was found to produce 30 mg/l of crude antibiotic under non-optimized cultivation. The compound was identified as actinomycin D using NMR and mass spectrometry techniques [36]. These techniques have also been employed to identify actinomycin $X_{o\beta}$, X_2 and D when cultivated under optimized condition in culture broth from marine-derived *Streptomyces heliomycini.* Actinomycin X_2 exhibited greater antibacterial activity than actinomycin D when tested against *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis* and *B. cereus* [37]. Actinomycin D has been shown to inhibit biofilm formation from *Staphylococcus aureus* strains [38], and the pathogenic bacterium that causes citrus canker, Xac can produce biofilms on citrus leaves to facilitate infection [39]. In this regard the crude extract obtained from *Streptomyces* sp. TBRC 8912 should be examined for activity against Xac biofilm formation.

Both fruits and leaves of kaffir lime are used in folk medicine, and as ingredient for cosmetics and cookery, and citrus canker often damages kaffir lime leaves and spreads widely in the rain season. *Streptomyces* strains found to be potential biocontrol agent are used in Taiwan and China [40]. A few previous reports have shown that plant extracts helped in reducing disease damages when measured in green house experiments [41-42]. Likewise, little is known of the use of microbial extracts and their chemical diversity in combating Xac, and limited knowledge of controlling Xac is a challenge for natural product discovery for agricultural use. Moreover, powerful tools for the rapid and target-directed isolation of antibacterial molecules are needed. In this work, bioassay-guided fractionation and TLC- bioautography were used to select active mixtures from crude extracts of *Streptomyces* sp. TBRC 8912 and liquid chromatography-electrospray ionization-quadrupole-time-of-flight mass spectrometry (LC-ESI-Q-TOF-MS) was employed to identify the bioactive molecules. The strain from this study can be a potential commercially applicable actinomycin D

producer, which could offer significant value to the bacteriocide market. Furthermore, biodegradation on plant tissue and soil should be determined, as well as appropriate method for application in citrus orchards.

5. Conclusion

This study has shown that a newly isolated *Streptomyces* sp. TBRC 8912 can produce antibacterial compounds in culture broth and solid fermentation which are able to inhibit the growth of the phytopathogenic bacteria, *Xanthomonas axonopodis* pv. *citri* (Xac), and the active compound was identified as actinomycin D and its isomer. The 16S rDNA sequence of strain TBRC 8912 showed that it is closely related to *Streptomyces panaciradicis* 1MR-8^{T}. This is the first report of a representative of *Streptomyces panaciradicis* that can produce actinomycins.

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