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# Isolation and characterization of *Streptomyces alboflavus* SC11 producing desertomycin A

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**An actinomycete, isolated from soil samples of western Sichuan Plateau of China, was identified as *Streptomyces alboflavus* SC11 by 16S rDNA analysis, and also cultural, morphological and physiological characteristics of the strain were recorded. From the fermentation broth of the strain, an antimicrobial ingredient was isolated and elucidated as desertomycin A, mainly by analysis of electrospray ionization tandem mass spectrometry (ESI-MS/MS), $^{1}$H nuclear magnetic resonance (NMR) and $^{13}$C NMR spectra data. Desertomycin A exhibited good antimicrobial potential against the pathogenic bacteria and fungi. Minimum inhibition concentration (MIC) values of desertomycin A were 3.9, 7.8 and 7.8 µg/ml against *Bacillus cereus*, *Bacillus subtilis* and *Staphyloccocus aureus*, respectively.**

**Key words:** *Streptomyces alboflavus* SC11, Desertomycin A, 16S rDNA, Antimicrobial activities.

## INTRODUCTION

Soil microorganisms provide a huge and excellent resource for the isolation and identification of agricultural and medicinal important natural products (Lin and Liu, 2010). Particularly, the class actinomycetes are an important group of filamentous, gram-positive bacteria producing metabolites, in which, the members of the genus *Streptomyces* alone contributes more than half of the discovered natural metabolites up to date. Besides, 60% of antibiotics developed and applied for agricultural pur-pose were isolated from the *Streptomyces* genus (Berdy, 2005; Junker et al., 2009), such as Blatididin S (Takeuchi et al., 1958), polyoxins (Suzuki et al., 1965; Isono et al., 1965), Validamycin (Iwasa et al., 1970, 1971a, 1971b) etc. Thus, those promising progress have emphasized that it is necessary to continue the research on screening of new antibiotics from various regions of soils.

In order to obtain strong antimicrobial compounds, a number of microorganisms from various soil samples from western Sichuan Plateau of China were screened using traditional and sensitive procedures. Fortunately, a strain numbered SC11, exhibiting strong and stable antimicrobial activity against agricultural pathogenic fungi and bacteria, was selected for further study.

In this present study, the aims were to identify the strain based on morphological, physiological, biochemical characteristics and 16S rDNA gene sequence analysis, as well as the isolation, identification and biological evaluation of the active compounds from the secondary

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metabolites of the strain.

## MATERIALS AND METHODS

### Strain isolation

Strain SC11 was isolated by the dilution agar plating method from soils collected in western Sichuan Plateau of China. After heating the soil to 55°C for 30 min in order to eliminate bacterial growth, the dry soil samples were suspended in sterile distilled water and diluted. Aliquots (0.1 ml) of serially diluted samples were inoculated by spreading Petri plate onto starch casein agar (SCA) medium (Composition: soluble starch, 10 g; $K_2HPO_4$, 2 g; $KNO_3$, 2 g; casein, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 15 g; filtered water,1000 ml and pH, 7.0±0.1), which was supplemented with cyclohexamide at 25 µg/ml to minimize contamina-tion with fungi and 10 µg/ml nalidixic acid to minimize contaminant bacteria growth (Takizawa et al., 1993; Ravel et al., 1998), respec-tively. Plates were incubated for two weeks under dark at 28 ±1°C and then the colonies with a tough or powdery texture, dry or folded appearance and branching filaments with or without aerial mycelia were subcultured and transferred on SCA plates or slants without antibiotic substance (Mincer et al., 2002). After isolation, strain SC11 was stored at 4°C on agar slants of SCA medium for further use (Das et al., 2008).

### Morphological and physiological characteristics of strain SC11

Strain SC11 was inoculated on SCA medium for seven-day in an incubator under dark at 28±1°C. The micro-morphology was observed by optics microscopy (Olympus, Japan). Colors of aerial and substrate mycelia were determined with the ISCC-NBS centroid color charts (US National Bureau of Standard 1976). The study on physiological characteristics of strain SC11 was carried out following the methods recommended by the International Streptomyces Projects (ISP, Shirling et al., 1966) and Waksman and Henrici (1943), and the utilization of carbon sources was tested according to the growth condition on plates containing different sugar source.

### DNA extraction, polymerase chain reaction (PCR) amplification and sequence analysis

The strain was grown on 100 ml of SCA liquid medium under the constant shaking at 28±1°C for 2 days. Spores were obtained by centrifugation and filtering. Approximately 300 mg of spores were used for genomic DNA extraction as follow: The sample was whetted with liquid nitrogen and dispersed in 10 ml of the lysis solution (Tris–HCl, 100 mM; pH, 7.4; ethylenediaminetetraacetic acid (EDTA), 20 mM; NaCl, 250 mM; sodium dodecyl sulphate (SDS0, 2%; lysozyme, 1 mg/ml; $H_2O$ qsp, 100 ml), 500 µl of RNAase (50 mg/ml) was added and the samples were incubated at 37°C for 1 h. Then, 120 µl of proteinase K solution (20 mg/ml) was added, and the lysis solution was re-incubated at 65°C for 30 min. The lysate was extracted twice with an equal volume of phenol, centrifuged and re-extracted with chloroform (1:1, v/v) to remove residual phenol. DNA was precipitated by adding NaCl solution (150 mM) and 95% cool ethanol. After centrifugation, the DNA was cleaned with 500 µl of 70% ethanol, centrifuged, and then resuspended in 500 µl of Tris EDTA (TE) buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA purity and quantity were checked by agarose gel electrophoresis and spectrophotometer at 260 and 280 nm, respectively.

PCR amplification of 16S rDNA was performed using universal genus -specific primers: 27f (5'-AGAGTTTGATCCTGGCTCAG-3'), and 1492r (5'-GGTTACCTTGTTACGACTT-3'). The reaction mixture was prepared in a total volume of 50µl containing 10×PCR reaction buffer 5µl (Tris–HCl 100 mM; KCl 50 mM; pH 9.0; $MgCl_2$ 1.5 mM, Triton X-100 0.1% (w/v); Taq DNA polymerase (Finnzyme) 2.5 U; dNTP 200 µM; forward primer 2 µM; reverse primer 2 µM) and template DNA 100 ng. The complete reaction mixture was incubated in a gradient thermocycler (Eppendorf, German). The PCR temperature profile was 94°C for 3 min, then 30 cycles consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and an extension step at 72°C for 10 min. The PCR product was detected by agarose gel electrophoresis and visualized by ultraviolet (UV) fluorescence after ethidium bromide (EB) staining. Then, the PCR product was submitted to Shanghai Biological Technology Co. Ltd. (Shanghai, China) for sequence analysis. The determined sequence was compared for similarity level with the reference species contained in GenBank database, using the ''NCBI Blast'' available at the ncbi.nlm.nih.gov web site. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 software. The 16S rDNA sequence were aligned using the CLUSTAL program against corresponding nucleotide sequences of representatives of the genus bacterial retrieved from Gene-Bank. Evolutionary distance matrices were generated and a phylogenetic tree was inferred by the neighbor-joining method. Tree topologies were evaluated by bootstrap analysis based on 1000 re-samplings of the neighbor-joining data set (Galeano and Martinez, 2007; Garrity et al., 2007; Lin and Liu, 2010).

### Cultivation and fermentation

The seed medium consisted of millet extract, 1%; glucose, 1%; peptone, 0.5%; NaCl, 0.25%; $(NH_4)_2SO_4$, 0.1% and $CaCO_3$, 0.05% (pH 7.0). A 500-ml Erlenmeyer flask, containing 150 ml of the seed medium, was incubated with a stock culture of the producing strain. After incubation for 16 h at 28±1°C on a rotary shaker set at 180 rpm, every 10 ml of the seed culture was transferred to each of the 250 ml Erlenmeyer flasks containing 90 ml of the production medium, which was consisted of millet extract, 1%; glucose, 1%; peptone, 0.3%; NaCl, 0.25%; $(NH_4)_2SO_4$, 0.1% and $CaCO_3$, 0.1% (pH7.0). The fermentation was carried out at 28±1°C for four days on a rotary shaker at 180 rpm.

### Extraction and isolation

The fermentation broths were harvested and filtered with cheesecloth to remove the medium and culture liquid. The filtrate, which exhibited strong antibacterial activity by agar diffusion method, was absorbed onto a macroporous resin HPD400 (Cangzhou Baoen, Co. Ltd, Cang Zhou City, China), followed by elution with methanol. The methanol fractions were evaporated at 40°C in vacuum to yield extract. The crude extract was subjected to a Sephadex LH20 column (2.8 × 80 cm) and eluted with methanol. The eluents were collected in 10 ml test tubes for each fraction. The antimicrobial fractions were purified by reverse phase-high performance liquid chromatography (HPLC) (Hypersil ODS2, 10×250 mm, 5 µm) with methanol/water/Trifluoroacetic acid (TFA) (67: 32.9:0.1, v/v/v) as mobile phase with detecting wave of 230 nm and flowing rate of 2.5 ml/min. Finally, active fractions were yield by an active-guided bioassay method.

### Identification of desertomycin A

Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was measured on a Bruker APX500 analytical spectrometer (Bruker, Shanghai, China). $^{1}H$- and $^{13}C$-nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AV-500 spectrometer, with dimethyl sulfoxide (DMSO)-$d_6$ as solvent and TMS as an internal

**Table 1.** The cultural and physiological characteristics of strain SC11.

| Culture Property | | Utilization of carbon source | |
|---|---|---|---|
| **Media name** | **Growth, pigment** | D-dextrose | + |
| Gause's synthetic agar | G, NSP | Sucrose | + |
| Starch casein agar | G, NSP | Lactose | + |
| Czapek's agar | G, NSP | D-galactose | + |
| Glucose asparagine | M, NSP | D-ribose | — |
| Calcium malate agar | G, NSP | D-xylose | — |
| Yeast-mall extract agar (ISP2) | — | L-arabinose | + |
| Oatmeal agar (ISP3) | G, NSP | Maltose | — |
| Inorganic salts-starch (ISP4) | G, NSP | Inositol | + |
| Glycerol asparagine (ISP5) | G, NSP | D-fructose | + |
| Yeast peptone agar (ISP6) | M, NSP | D-mannitol | + |
| **Physiological characteristic** | | | |
| Liquefaction of gelatin | — | | |
| Hydrolysis of starch | + | | |
| Use of cellulose | — | | |
| Production of $H_2S$ | + | | |
| Coagulation of milk | — | | |
| Peptonization of milk | — | | |

G, Good growth; M, moderate growth; NSP, no soluble pigment; +, positive reaction; -, negative reaction.

standard. All solvents and chemicals were of analytical grade.

#### Antimicrobial activity of desertomycin A

The tested gram-positive bacteria were *Bacillus cereus* 1.1846, *Bacillus subtilis* 1.88, *Staphyloccocus aureus* 1.89 and the gram-negative bacteria were *Escherichia coli* 1.1636, *Pseudomonas aeruginosa* 1.2031, which were purchased from China General Microbiological Culture Collection Center (CGMCC).

The tested phytopathogenic fungi *Fusarium graminearum*, *Alternaria solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Verticillium dahliae*, *Alternaria alternate* and *Colletotrichum gloesporioides* were obtained from department of plant pathology, Northwest A & F University of China.

Antibacterial activity was measured by the micro-broth dilution method in 96-well culture plates using the Mueller-Hinton (MH) broth (Hangzhou Microbial Reagent Co. Ltd, Hangzhou City, China), according to the Standard of National Committee for Clinical Laboratory (2006). Ampicillin (Sigma, Shanghai, China) was used as positive control. The tested bacteria were incubated in the MH broth for 12 h at 30 ± 1°C at 180 rpm, and the spore concentration was diluted to approximately $1\times10^6$ CFU with MH broth. After incubation for 24 h at 30 ±1°C, the minimum inhibition concentrations (MICs) were simply examined.

Antifungal activities were tested by paper method. Briefly, 5 mm diameter disc of the tested phytopathogens were cut from the periphery of four days old culture on potatoes dextrose agar (PDA) plates to place mycelia surface down in the center of the plates and a serial of 5 mm diameter sterile paper discs containing desertomycin A (40, 20, 10, 5, 2.5µg/disc) were equably inoculated on opposite edges of the PDA plates. The plates were incubated in the dark at 28±1°C for three days.

## RESULTS

### Taxonomic characterization of the strain SC11

The colonial morphology of strain SC11 was observed after cultivating on Gause's synthetic agar for seven days. The front side of the plate was white-orange and the reverse side was yellow-orange. The edge of strain colony was round and prominent in the center while soluble pigment was not observed. Cultural and physiological characteristics of the strain are recorded in Table 1. The strain exhibited good growth on Gause's synthetic agar, starch casein agar (SCA), Czapek's agar, ISP3, ISP4 and ISP5 while the growth was moderate in glucose-asparagine agar and ISP6, but no growth was observed on ISP2. Soluble pigment production by the strain was not found on the culture media tested. The strain utilized D-dextrose, sucrose, lactose, D-galactose, L-arabinose, inositol, D-fructose and D-mannitol as sole carbon sources, but not D-ribose, D-xylose, and maltose (Table 1). Biochemical activities like $H_2S$ production, gelatin liquefaction, coagulation and peptonization of the milk and hydrolysis of starch were observed. The results support the identification of this strain as a member of the genus *Streptomyces*. Furthermore, 16S rDNA sequence

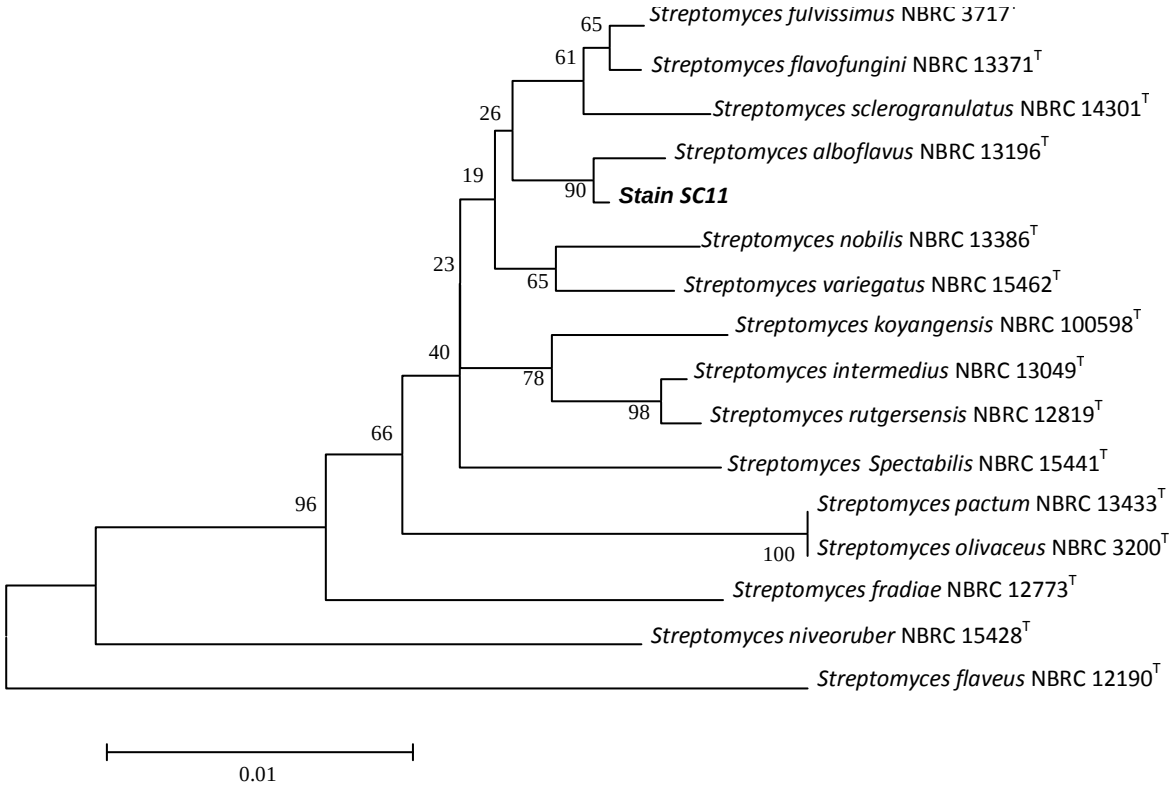


**Figure 1.** Phylogenetic tree showing the position of strain SC11 based on 16S rDNA gene sequence analysis.

(1433 nucleotides) of strain SC11 was determined and registered in the GenBank (accession number: JF807997). The phylogenetic analysis with 16S rDNA database sequences revealed that strain SC11 branched deeply within a member of the gene *Streptomyces* and was most closely related to *Streptomyces alboflavus* NBRC-13196$^T$ (Figure 1). As the sequence similarity was high (99%) and most cultural and physiological characteristics were highly similar, except for the liquefaction of gelatin, use of cellulose and production of $H_2S$, train SC11 might be identified as *S. alboflavus* SC11.

## Structural elucidation of desertomycin A

The active principle of the fifth fraction collected after rp-HPLC purification appeared as yellowish solid, which was soluble in DMSO, MeOH and $H_2O$. The structure of the pure bioactive compound was elucidated by analysis of ESI-MS, $^1$H NMR and $^{13}$C NMR spectral data. In ESI-MS spectra (Figure 2), the compound showed molecular ions at m/z are 1192 $[M+H]^+$ inferring the molecular weight of the compound was 1191.The $^1$H NMR (DMSO-$d_6$, 500 MHz) of the pure bioactive compound displayed proton signals ($\delta$: 6.66; 5.46; 5.43; 5.41; 5.39; 5.37; 5.36; 5.16; 5.05; 4.64; 4.17; 4.13; 4.07; 3.90; 3.89; 3.82; 3.80; 3.79; 3.73; 3.61; 3.61; 3.56; 3.55; 3.54; 3.49/3.65; 3.43; 3.41; 3.26; 3.24; 2.77/2.52; 2.33/2.20; 2.21; 2.17 (2H); 2.09/1.92; 2.07; 1.83; 1.80 (3H); 1.77/1.58; 1.69; 1.59 (3H); 1.56/1.52; 1.54; 1.48/1.26; 1.47/1.28; 1.47; 1.47/1.27; 1.46; 1.43/1.20; 1.41/1.30; 1.35/1.27; 1.29 (2H); 0.99 (3H); 0.88 (3H); 0.84 (3H) 0.83 (3H); 0.79 (3H); 0.66 (3H); 0.65 (3H) ). While $^{13}$C NMR (DMSO, 125 MHz) depicted 65 peaks at C ($\delta$: 167.0; 143.9; 142.6; 138.4; 133.2; 133.1; 131.6; 130.1; 127.6; 123.3; 122.7; 96.4; 81.4; 75.1; 74.9; 74.1; 74.0; 73.9; 73.3; 72.6; 71.5; 71.1; 70.6; 69.9; 69.9; 67.6; 67.2; 67.2; 67.0; 64.2; 63.8; 61.7; 46.4; 46.2; 42.9; 42.7; 42.4; 41.8; 41.4; 41.0; 40.6; 40.5; 40.5; 40.5; 34.9; 33.5; 32.9; 32.6; 30.1; 29.5; 26.6; 24.3; 17.3; 15.8; 12.8; 12.7; 12.1; 11.7; 10.9; 10.7; 10.2). Based on these spectral data and available library data (Uri et al., 1958; Dolak et al., 1983; Dinya et al., 1998; Ivanova, 1997), the compound was identified as desertomycin A (Figure 3) with molecular formula $C_{61}H_{109}NO_{21}$.

## Antimicrobial activity of desertomycin A

The antibacterial activities of desertomycin A were tested by double diffusion assay. The compound exhibited strong antibacterial activities against a wide variety of gram-positive bacteria with MIC values ranging from 1.0 to 10 µg/ml (Table 2). Among the tested bacteria, *B. cereus* exhibited high sensitivity towards desertomycin A followed by *B. subtilis* and *S. aureus*. Desertomycin A had no obvious inhibitory effects against gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* at the concentration of 250 µg/ml.

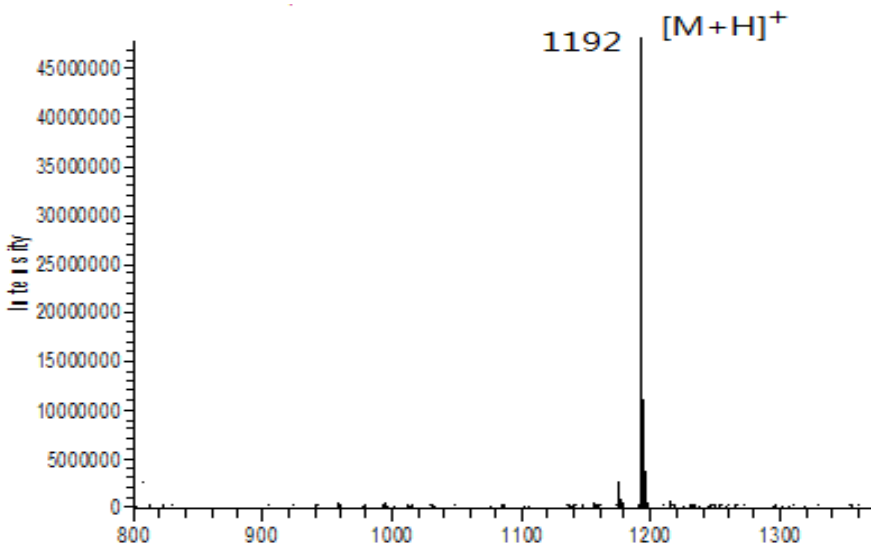


**Figure 2.** ESI-MS of Desertomycin A.

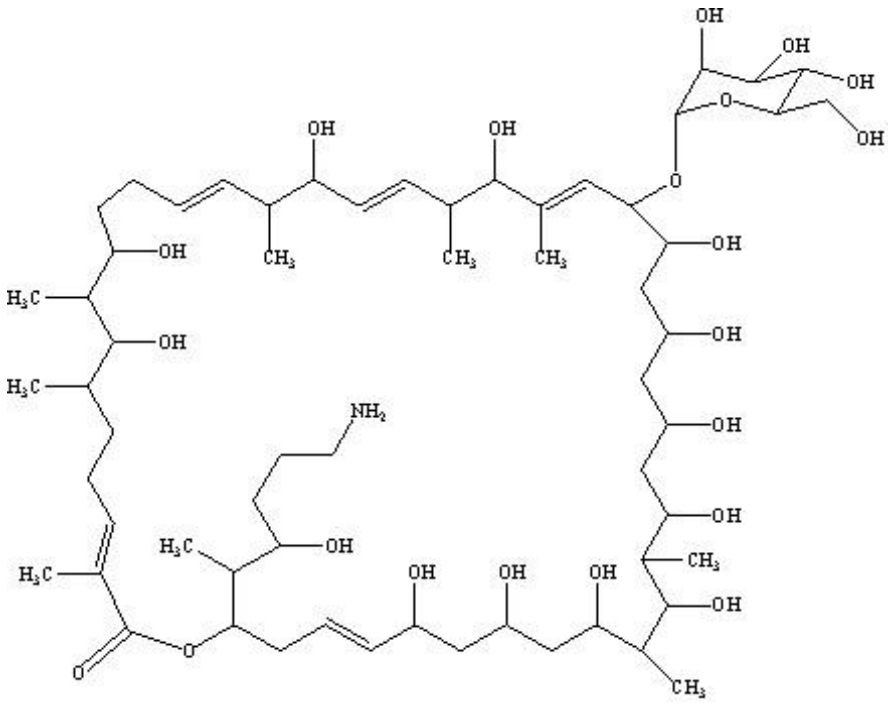


**Figure 3.** Molecular structure of Desertomycin A.

The antifungal spectrum of the bioactive compound, desertomycin A was further evaluated *in vitro*. As shown in Figure 4, desertomycin A inhibited the hypha growth of six plant pathogenic fungi *in vitro* from 2.5 to 40 μg/disc, respectively. Especially, at the dose of 10 μg/disc, desertomycin A exhibited inhibitory effect against *Verticillium dahlia* and *Sclerotinia sclerotiorum* while at the dose of 20 μg/disc, it showed strong activities against *Colleto-trichum gloesporioide*, *Gibberella zeae* and *Alternaria alternata*.

## DISCUSSION

In this study, based on the results of morphological, physiological characterizations and 16S rDNA gene sequence analysis, strain SC11 was been identified as *S. alboflavus* SC11, from which one active constituent was obtained and identified as a known compound

**Table 2.** MICs of desertomycin A against five tested bacteria.

| Test bacteria | MIC(μg/ml) | |
|---|---|---|
| | Desertomycin A | Streptomycin Sulfate |
| *Bacillus cereus* | 3.9 | 1.56 |
| *Bacillus subtilis* | 7.8 | 0.78 |
| *Staphyloccocus aureus* | 7.8 | 0.78 |
| *Escherichia coil* | >250 | 0.78 |
| *Pseudomonas aeruginosa* | >250 | 1.56 |

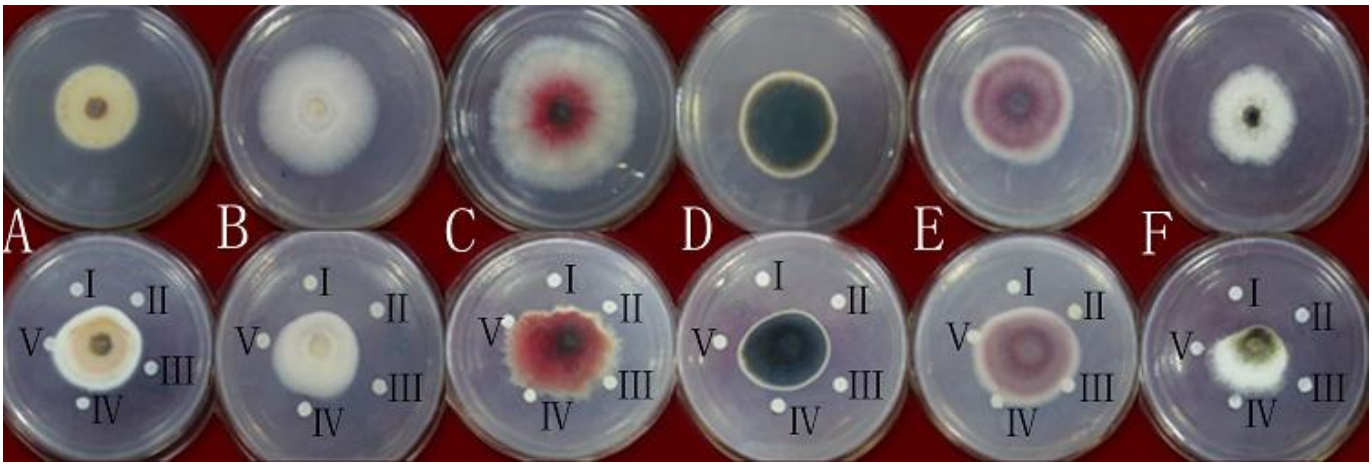


**Figure 4.** Antifungal activities of desertomycin A against test six phytopathogenic fungi. (The top are CK and the bottom are tested. A, *Colletotrichum gloesporioide*; B; *Verticillium dahliae*; C, *Gibberella zeae*; D, *Alternaria alternata*; E, *Fusarium oxysporum*; F, *Sclerotinia sclerotiorum.* The doses of desertomycin A are 40 μg (I), 20 μg (II), 10 μg (III), 5 μg (IV), 2.5 μg (V), respectively).

desertomycin A. *Streptomyces alboflavus* has been reported to produce tetracyclines (Perveen et al., 2006) and a series of cyclic hexapeptides (Guo et al., 2009; Ji et al.,2012) antibiotics. However, it was first reported that *S. albo-flavus* SC11 could metabolite this compound deserto-mycin A. Desertomycins family was first reported to be produced by *streptomyces flavofungini* from African desert sand in 1958 (Uri et al., 1958). The microorganisms producing desertomycin A had been successively reported from soil microorganisms, such as *S. macro-nensis* (Dolak et al., 1983) and S. *spectabilis* (Bastide et al., 1986; Lvanova, 1997). Desertomycin A, a broad spectrum antibiotic consisting of a series of 42-membered macro-lactones with mannose moiety, exhibits *in vitro* inhibitory activity towards various plant pathogenic fungi and bacteria (Uri et al., 1958; Lvanova, 1997). The stereo-chemical structure of desertomycin A was comprehensively investigated using 2D NMR techniques (Bax et al., 1986; Bognar et al., 1988). Unfortunately, it has considerable cytotoxic activity, probably by direct damage on the cell membranes (Dolak et al., 1983; Uri 1986). Besides, in the fermentation broth of *S. alboflavus* SC11, cyclopeptides composition were simultaneously obtained and preliminarily showed strong antimicrobial activities as well. This study reveals that the strain SC11 is a promising microorganism producing antibiotics compounds.

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