Identification and characterization of antifungal active substances of Streptomyces hygroscopicus BS-112

ARTICLE *in* WORLD JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY (FORMERLY MIRCEN JOURNAL OF APPLIED MICROBIOLOGY AND BIOTECHNOLOGY) · MARCH 2013

Impact Factor: 1.78 · DOI: 10.1007/s11274-013-1307-3 · Source: PubMed

World J Microbiol Biotechnol (2013) 29:1443–1452
DOI 10.1007/s11274-013-1307-3

ORIGINAL PAPER

Identification and characterization of antifungal active substances of *Streptomyces hygroscopicus* BS-112

Nan Zhang · Zhen Song · Yuhua Xie · Ping Cui · Hongxia Jiang · Tao Yang · Ruicheng Ju · Yuhua Zhao · Jinyu Li · Xunli Liu

Received: 24 December 2012 / Accepted: 28 February 2013 / Published online: 7 March 2013

Abstract An antifungal *Actinomyces* BS-112 strain, with *Aspergillus flavus* as the target pathogen, was isolated from soil in the forest land of Mountain Tai. This strain showed a strong antagonistic activity against various mold fungi in food and feed. Strain BS-112 was identified as *Streptomyces hygroscopicus* based on its morphologic, cultural, physiological, biochemical characteristics, cell wall components and 16S rDNA sequence. Four active components were separated and purified from strain BS-112. These four antifungal components were identified as tetrins A and B and tetramycins A and B using spectroscopic analysis including mass spectrometry and nuclear magnetic resonance spectroscopy. Tetrins A and B and tetramycins A and B strongly inhibited the growth of *A. flavus*, *A. alutaceus*, *A. niger*, and *A. fumigatus* in vitro.

N. Zhang · Y. Xie
College of Life Sciences, Neijiang Normal University, Neijiang 641112, People's Republic of China

N. Zhang · Z. Song · H. Jiang · T. Yang · R. Ju · Y. Zhao · J. Li · X. Liu (✉)
College of Forestry, Shandong Agricultural University, Daizong Street, Taishan District, Tai'an 271018, People's Republic of China
e-mail: y090122@sdau.edu.cn; nan2007006@163.com

N. Zhang
Key Laboratory of Regional Characteristic Agricultural Resources, Department of Education, Neijiang 641112, People's Republic of China

P. Cui
Department of Chemistry and Life Science, Hechi University, Yizhou 546300, People's Republic of China

Keywords *Streptomyces hygroscopicus* BS-112 · Identification · Antifungal active substance · Isolation and purification · Structure identification

Introduction

Polyene macrolide antibiotics are a large group of antifungal agents. Their structures consist of macrocyclic rings closed by a lactone bond (so-called lactone ring), with one or more sugars attached glycosidically to the macrolide ring. The polyene subgroup is characterized by a lactone ring of 26–38 atoms, a polyene chromophore consisting of a series of four to seven alternating double bonds that form part of the macrolide ring, and one amino sugar moiety (Martin 1977). According to the number of alternating double bonds in the polyene chromophore, the polyene macrolides were divided into triene, tetraene, pentaene, hexaene, and heptaene, and each class has a specific u.v. absorption peak. The u.v. absorption (291 ± 2, 302 ± 2, and 320 ± 3 nm) is specific to tetraene antibiotics. All of these possess antifungal abilities, including more than 50 different members discovered since the early 1950s (Lin et al. 2008). Tetraene antibiotics have been widely used in the fields of medicine, food and agriculture. For example, natamycin has a broad-spectrum activity against yeasts and molds, with a low toxicity against mammalian cells (Levinskas et al. 1996). Therefore, it is used as a food preservative and has been approved as a generally regarded as safe (GRAS) product by the Food and Drug Administration (FDA) for use in food manufacturing (Liang et al. 2008). Likewise, tetramycin has been well used in preventing and treating *Cytospora* canker and black spot diseases of forest trees (Ren and Li 1996; Hu et al. 1995). Tetramycin promotes healing of impaired tissue,

development of weak seedling root, and regeneration of aging root, and improves the resistance of trees to diseases; and tetramycin has several advantages such as having a low toxicity, it is safe to use in human beings and animals, nuisance free, and does not cause environment pollution (Tang and Zhang 2010). Thus, tetramycin became a new type of agricultural antibiotic. Most of the reported antibiotics with antifungal activity have a polyene macrolide structure (Volpon and Lancelin 2002). Pathogenic fungi have difficulty in generating resistance to polyene antibiotics, thus, polyene antibiotics are commonly used in clinical medicine as antifungal agents (Vilar et al. 2006).

Microbial contamination is one of the major causes of food deterioration during its processing and storage. Nearly, 30 % people in the world suffer from food borne diseases every year caused by microbes (Ashok et al. 2010). *Aspergillus flavus* and *Aspergillus parasiticus* are important contaminants of certain foods and animal feeds because of their ability to produce aflatoxins (Eduardo et al. 2005). Food contamination with these fungi and the presence of aflatoxin is a major concern, which has received worldwide attention due to their deleterious effects of aflatoxin on human and animal health as well as their importance in international food trade (Ashok et al. 2010). The undesirable effects of chemical preservatives and restrictions forced by the food industries on their application as food additives have renewed interest to search for alternative antimicrobial agents to be used in food-processing and post-harvest storage technology (Mishra and Das 2003; Ashok et al. 2010). During the course of screening antimicrobial agent producers, strain BS-112 was isolated from the forest land of Tai Mountain, China. This bacterial strain showed a broad-spectrum resistance to *Aspergillus* in food and feed, such as *Aspergillus flavus*, *Aspergillus alutaceus*, *Aspergillus niger*, and *Aspergillus fumigatus*. However, strain BS-112 also exhibited a strong antagonistic activity against pathogenic bacteria, such as *Escherichia coli*, *Staphylococus aureus*, *Salmonella typhimurium,* and especially a strong inhibitory effect on *Bacillus bombysepticus* (data not shown).

In this paper, we identified the BS-112 stain through its morphologic, cultural, physiological, and biochemical characteristics, cell wall components, and 16S rDNA sequence. Moreover, the antifungal active substances from strain BS-112 were extracted and purified by X-5 macroporous resin, silica gel column chromatography and high performance liquid chromatography (HPLC). The chemical structures of antifungal components were identified using spectroscopic analysis including mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). Their minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against *Aspergillus* were determined.

Materials and methods

Microorganism

The BS-112 strain was isolated from the forest land of Mountain Tai. The strain was stored at the China general microbiological culture collection (CGMCC) and assigned with accession number CGMCC 3504. The *A. flavus* (CGMCC 3.2890), *A. niger* (CGMCC 3.6478), *A. alutaceus* (CGMCC 3.1409), and *A. fumigatus* (CGMCC 3.3552) strains were obtained from the Academy of State Administration of Grain. The four *Aspergillus* strains and BS-112 strain were maintained on a modified potato dextrose agar (PDA) slant at 4 °C and were either transferred every 3 months or kept in a glycerol suspension (20 %, v/v) at −70 °C for 1 year. The modified PDA medium contained potato (200.0 g), glucose (20.0 g), $(NH_4)_2SO_4$ (1.0 g), $MgSO_4$ (1.0 g), and agar (17.5 g) in 1 l distilled water.

Reagents and apparatus

X-5 macroporous adsorption resin (non-polar, 0.3–1.25 mm particle size, 500–600 m^2/g surface area; Tianjin Nankai Hecheng S&T Co., Ltd., China), silica gel (200–300 mesh size; Qingdao Jiyida silica reagent factory, China), methanol (Yongda Chemical Factory, Tianjin, China), distilled water; Lab Tech LC600 (Beijing, China), and Ultra performance liquid chromatography (UPLC) (Waters, Shanghai, China).

Phenotypic characters

The medium used for morphological studies was yeast extract-malt extract agar (ISP2) (Shirling and Gottlieb 1966) and the incubation time of the pure culture was 7–15 days at 29 °C. Morphological properties were examined by light microscopy (Olympus microscope BH-2). Cultural and Physiological characteristics of strain BS-112 were determined according to the methods proposed by Shirling and Gottlieb (1966) and Williams et al. (1983). Color determination was done with color chips from the ISCC-NBS COLOR CHARTS standard samples no. 2106 (Kelly 1964).

Chemotaxonomy

The cell wall fraction was purified and analysed by the methods of Lechevalier and Lechevalier (1980).

DNA G+C content determination

The DNA G+C base content of strain BS-112 was determined by the thermal denaturation method (Mandel and Marmur 1968).

16S rDNA sequence determination and phylogenetic analysis

The chromosomal DNA of bacterial isolates was extracted according to the procedures of Kieser et al. (2000). The polymerase chain reaction (PCR) amplification of 16S ribosomal DNA (16S rDNA) was carried out with universal primers: 16Sf (5′-ACGGCTACCTTGTTACGACT-3′) and 16Sr (5′-AGAGTTTGATCCTGGCTC AG-3′) in a thermal cycler (Bio-Rad, USA). The PCR reaction conditions were as follows: 4 min at 94 °C for one cycle, followed by 30 cycles of 1 min each at 90 °C 1 min at 54 °C and 2 min at 72 °C, and finally one cycle for 10 min at 72 °C. The amplified PCR product (expected size was 1,500 base pairs) was purified using a TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit (Takara, Japan), ligated into the pUCm-T cloning vector (Sangon, Shanghai, China), and then transformed into the chemically competent *E. coli* Competent Cells DH5α. Sequencing was performed with M13 primers at Sangon Biotech (Shanghai) Co., Ltd., China. The sequence was compared with similar 16S rDNA gene sequences retrieved from the DNA databases using the BLAST search program in the National Center for Biotechnology Information (NCBI). The neighbor-joining phylogenetic tree was constructed using the MEGA 4.0 program (Center for Evolutionary Functional Genomics, USA) based on the 16S rDNA gene sequences.

Fermentation broth preparation

The BS-112 strain was transferred from a slant culture into an Erlenmeyer flask (250 ml) containing 50 ml of seed medium, which was similar to the modified PDA medium (without agar), for the production of the inoculum. The seed cultures were grown at 29 °C on a rotary shaker incubator at 200 rpm for 48 h. The inoculum (10 %, v/v) was transferred into a 250 ml Erlenmeyer flask containing 55 ml of the fermentation medium and incubated at 29 °C for 84 h on a rotary shaker at 200 rpm. The fermentation medium was composed of (g/l): corn meal, 5.00; glucose 18.61; soybean powder, 31.15; KH_2PO_4, 0.38; and $MgSO_4$, 0.85. The culture broth was harvested after 84 h of incubation, which coincided with the maximum culture activity, and then heated at 100 °C for 10 min. The cooled supernatant was collected by centrifugation at 9,500×*g* for 10 min and was used for purification. All media was autoclaved at 121 °C for 20 min.

Analytical methods

The Oxford plate assay system was used for the quantification analysis of the antifungal active substances, with *A. flavus* as the indicating microorganism (National Pharmacopoeia Committee 2005). The concentration of spores was controlled at 2×10^4–4×10^4 colony-forming units/ml (c.f.u./ml) in the mixed-germs-plate. The diameter of the inhibition spot was measured by cross transposition after the mixed-germs-plate was incubated at 28 °C for 18 h with an oxford cup placed regularly (200 µl/cup). The titer was then counted through a standard equation that has been validated: $Y = 10^{[(x + 21.153)/15.577]} \times n$ [$R^2 = 0.9982$, where Y is the titer of the antifungal active substances (µg/ml), x is the diameter of the inhibition spot (9.3 mm < x < 19.2 mm), and n is the dilution multiple of the antifungal active substances] (Wang and Liu 2007; Song et al. 2012).

Antifungal active substance purification

The antifungal active substances from fermentation broth of strain BS-112 were extracted using column chromatography with X-5 macroporous adsorption resin. The X-5 resin needed to be pretreated and activated prior to use. Firstly, they were rinsed with distilled water and filtered with nylon filter cloth to retain those with a particle diameter larger than 0.3 mm. They were then soaked overnight in 2 bed volumes (BV) of 95 % ethanol. After soaking, the resins were introduced into a glass column and rinsed with a further 2 BV of 95 % ethanol. Subsequently they were rinsed with 2 BV of distilled water to dispel the ethanol 1 BV of 4 % (v/v) HCl sodium hydroxide 2 BV of distilled water 1 BV 4 % (w/v) NaOH, and finally by distilled water until the pH of the eluent became neutral. The pretreated hydrated resin (10 g) was placed into an Erlenmeyer flask (500-ml) containing 300 ml of the fermentation broth supernatant. The flasks were then shaken at 120 rpm for 2.5 h at 25 °C. The collected resins (20 g) were packed in glass columns (40 cm × 2.6 cm). The bed volume of resin was 35 ml. The column was first washed with 105 ml distilled water, and then eluted using an ethanol–water (75:25, v/v) solution under a flow rate of 0.5 ml/min. Every 10 ml of elution was collected into a sample tube with a flow rate of 0.5 ml/min. The antifungal titer of each sample tube was determined using the Oxford plate assay system. All the sample tubes with antifungal activities were pooled, concentrated using a rotary evaporator under reduced pressure at 40 °C, and then freeze-dried using the freeze dryer. The antifungal extract was stored in a refrigerator at −20 °C until further use.

The antifungal extract (2 g) was dissolved in 5 ml methanol. The liquid was loaded on the top of the silica gel column (60 × 2.6 cm) after filtration. Approximately, 45 g of the silica gel was packed into the column and the bed volume was 110 ml. The column was eluted with methanol-chloroform (3:2, v/v) at a flow rate of about 1 ml/min, and the effluent was then collected in each 15 ml fraction.

The antifungal titer of each fraction was determined using the oxford plate assay system. All fractions with antifungal activities were pooled, concentrated using a rotary evaporator under reduced pressure at 35 °C, and then freeze-dried using the freeze dryer. The residuals were dissolved in methanol and filtered through a 0.22 μm filter for subsequent HPLC purification.

The antifungal fraction collected from the silica gel column chromatography was purified via preparative HPLC using a Venusil XBP C18 column (20 mm × 250 mm 10 μm) at 30 °C. The mobile phase was methanol and water in the gradient mode as follows: 0–24 min, 62 % methanol and 24–40 min, 66 % methanol. The flow rate was 5 ml/min and the effluent was continuously monitored at 305 nm. About 6 ml of the sample solution containing 30 mg of the antifungal active substances was injected through the injection value after the mobile phase front emerged and hydrodynamic equilibrium was established in the column. Peak fractions were collected according to the elution profile. The antifungal activity of each fraction was determined using the oxford plate assay system. The antifungal fraction was concentrated using a rotary evaporator under reduced pressure at 35 °C, and then freeze-dried using the freeze dryer. Each antifungal fraction was dissolved in the methanol and water solution (62:38, v/v) and filtered through a 0.22 μm filter for purity analysis by ultra-performance liquid chromatography (UPLC), using a BEH C18 column (2.1 mm × 50 mm, 1.7 μm) at 30 °C. The mobile phase was methanol and water in the gradient mode as follows: 50:50–70:30 in 15 min. The flow rate was 0.2 ml/min and the effluent was continuously monitored at 305 nm. About 0.2 μl of the sample solution containing 0.05 μg of the antifungal fraction was injected through the injection value.

Structural identification of antifungal fractions

The mass spectrometer equipped with an electro-spray ionization (ESI) source was applied and the ion source was operated in positive (ESI^+) and negative ion modes (ESI^-). The antifungal fractions were dissolved in DMSO-d_6 for structural identification by nuclear magnetic resonance spectroscopy (NMR). ^1H NMR and ^{13}C NMR spectra were measured on a Varian INOVA 600 spectrometer, with tetramethylsilane (TMS) as the internal standard.

Antifungal activity assay

According to NCCLS references M38A (NCCLS 2002), the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined using the macrodilution broth method. The doubling of the serial dilution of antifungal substances was prepared in RPMI 1640 media (σ). Fresh cultures of *A. flavus*, *A. niger*, *A. alutaceus*, and *A. fumigatus* were used to prepare the spore suspension, which was adjusted to 2×10^4–4×10^4 c.f.u./ml. The 96-well microplates were incubated aerobically at 28 °C, and the results were then read after 48 h. The MIC was defined as the lowest drug concentration that showed a visual turbidity of less than or equal to 80 % inhibition compared with that produced by the growth control well. MFC was assessed by plating the entire volume of the broth from each well above the MIC for each organism. The lowest concentration of the drug that killed ≥99.9 % of the initial inoculum was defined as the MFC end point (Sader et al. 2010). The tests were performed in triplicate.

Antifungal activity on peanuts under storage conditions

The inhibitory effect on *A. flavus* was determined according to the method of Zhang et al. (2008), with a slight modification. The antifungal active substances were obtained by silica gel column chromatography. Antifungal fraction was freeze-dried, weighed, and then dissolved in 50 mM Tris–HCl with a pH of 7.5. Peanuts were surface-sterilized with 1 % NaOCl solution and rinsed in three successive changes of sterile distilled water. Fifteen grams of peanuts were distributed in each conical flask (100 ml) and 2 ml of each concentration of compounds was added to each flask. For the control 2 ml of 50 mM Tris–HCl (pH 7.5) was added to 15 g peanuts in conical flasks. The flasks were kept with thorough agitation for 24 h so that all of the solution can be absorbed by the peanuts. The flasks were then inoculated with 100 μl of *A. flavus* spore suspension (2–6 × 106 c.f.u./ml). After incubation at 30 °C for 7 days with vigorous shaking for 5 min daily, the growth of *A. flavus* was evaluated macroscopically. The experiments were carried out at least in triplicate.

Results

Strain BS-112 identification

The physiological and biochemical characteristics of strain BS-112 are indicated in Table 1.

The cell wall peptidoglycan of strain BS-112 contained only LL-diaminopimelic acid and glycine, indicating that strain BS-112 has a chemotype cell wall type I (Lechevalier and Lechevalier 1970a, b).

The partial 16S rDNA gene sequence of the BS-112 strain, which was 1,492 bp in length, was deposited in the GenBank nucleotide database (Accession number EU529841) at the NCBI website. Phylogenetic relationships can be inferred through the alignment and cladistic analysis of homologous nucleotide sequences of known bacteria

Table 1 Some phenotypic properties of strain BS-112

Characteristic	BS-112	Characteristic	BS-112
Colony color on ISP2	Gray	Utilization of:	
Spore shape	Oval	Maltose	+
Spore chain morphology	Spare spiral	Lactose	−
Melanoid pigment	+	Glucose	+
Gelatin liquefaction	+	Xylose	+
Milk coagulation	+	Mannose	+
Milk peptonization	+	Raffinose	−
Growth at 7 % NaCl	−	Arabinose	−
Starch hydrolysis	+	Galactose	+
Production of diffusion pigment	+	Inositol	+
Starch hydrolysis	+	Mannitol	+
Fiber hydrolysis	+	Sorbitol	+
G+C mol %	72.6	Sodium acetate	−

Symbols: +, postive; −, negative

[a] Data for reference *S. hygroscopicus* was taken from Group of Actinomycetes Taxonomy Institute of Microbiology Chinese Academy of Science (1975)

(11 strains). The approximate phylogenetic position of the strain BS-112 is shown in Fig. 1. Based on the analysis of the 16S rDNA gene sequence, this strain was found to be similar to *Streptomyces* sp. 32(2) (EF063464) (homology, 99.8 %) and identified to belong in the genus *Streptomyces*. Strain BS-112 was identified as *Streptomyces hygroscopicus* based on its morphologic, cultural, physiological, and biochemical characteristics and cell wall components.

Antifungal active substance extraction by macroporous resin chromatography

X-5 macroporous adsorption resin was used to extract the antifungal active substances from the fermentation broth of strain BS-112. Water was first used to remove hydro soluble chemicals, such as pigments and polysaccharides, which had no or little retention on X-5. Subsequently, 75 % ethanol was used to elute most target compounds, in preparation for further silica gel column chromatography. Finally, 95 % ethanol was used to activate the resin for another use. Consequently, the elution with antifungal activity was determined in the 2–12 sample tubes (from 0.6 BV to 3.4 BV). The recovery rate of the antifungal active substances on the X-5 resin was calculated as 85.27 %. Therefore, the X-5 macroporous resin was a suitable resin in extracting antifungal active substances because it exhibited strong adsorption abilities to antifungal active substances and no adsorption abilities to antibacterial substances.

Crude extract purification by silica gel column chromatography

The dark brown extract from the fermentation broth of strain BS-112 was sequentially purified through silica gel column chromatography. The titers of the antifungal extract in the eluate were determined, and one peak fraction with antifungal activity was collected for further purification. The antifungal active substances were in the 25–35 sample tubes (from 1.4 BV to 2.1 BV), and the recovery of antifungal active substances on the silica gel was calculated as 83.62 %. The collected antifungal fraction was concentrated and then freeze-dried. The sample appeared as a light yellow powder. The impurities decreased significantly after silica gel column chromatography.

Preparative HPLC separation

The antifungal fraction from silica gel column chromatography was purified using preparative HPLC. Seven completely separate fractions were obtained (Fig. 2). All peaks present were collected. The four main fractions (with retention times of 9.35, 15.78, 19.13, and 31.73 min) exhibited antifungal activities on *A. flavus* and were named as I, II, III, and IV, respectively. The three minor fractions (named as V, VI, and VII, with retention times of 10.71, 12.37, and 22.53 min, respectively) did not show any antifungal activities, thus were not investigated further. The ratio of the peak area as determined by HPLC, the components I, II, III, and IV in the co-products were 5.54, 11.39, 37.78, and 41.69 %, respectively. The purities of components I, II, III, and IV analyzed by UPLC were 99.91, 99.83, 99.31, and 99.13 %, respectively (Fig. 3).

Identification of the separated components

The chemical structures of components I, II, III, and IV separated by preparative HPLC were identified according to their ESI–MS, ^{1}H NMR, ^{13}C NMR, gCOSY, gHMBC, and gHMQC data. The data of each peak component were given as follows.

Component I: light brown amorphous powder (methanol). The u.v. spectrum in methanol showed a characteristic polyene spectrum similar to that of nystatin, with maximum values at 290, 303, and 318 nm. The ESI–MS showed molecular ion peaks at m/z 696.78 $[M - H]^-$ and 720.78 $[M + Na]^+$. The molecular formula of component I was established as $C_{34}H_{51}NO_{14}$ based on the ESI–MS, ^{1}H NMR, and ^{13}C NMR analyses. Component I was identified as tetrin B based on the above analysis and comparison with literature data (Rinehart et al. 1971).

Component II: light yellow microcrystalline powder (methanol). The u.v. spectrum in methanol showed

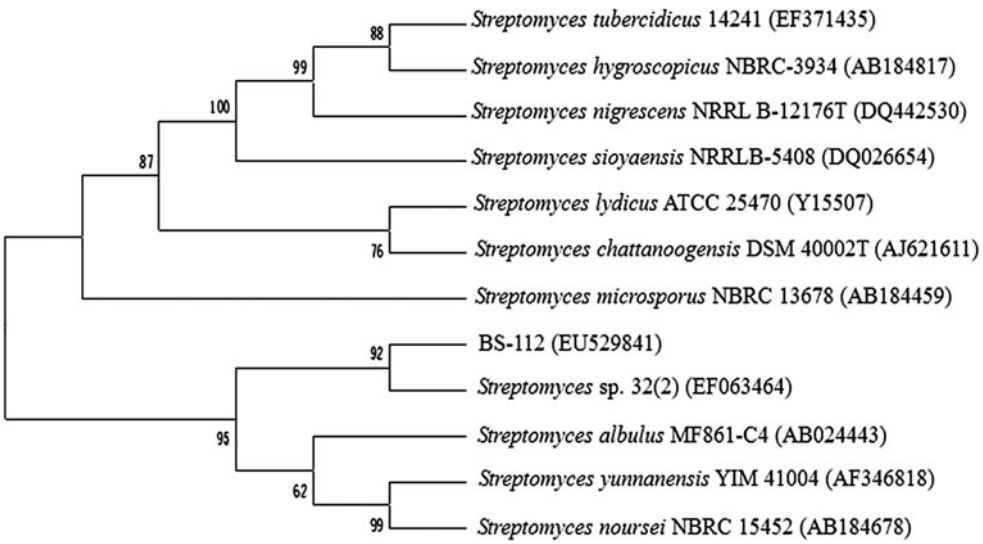

Fig. 1 Phylogenetic tree of strain BS-112 based on its 16S rDNA sequence by neighbor-joining method. The *numbers* on the tree indicate the percentages of bootstrap based on 1,000 replication and are shown for branches with more than 50 % support

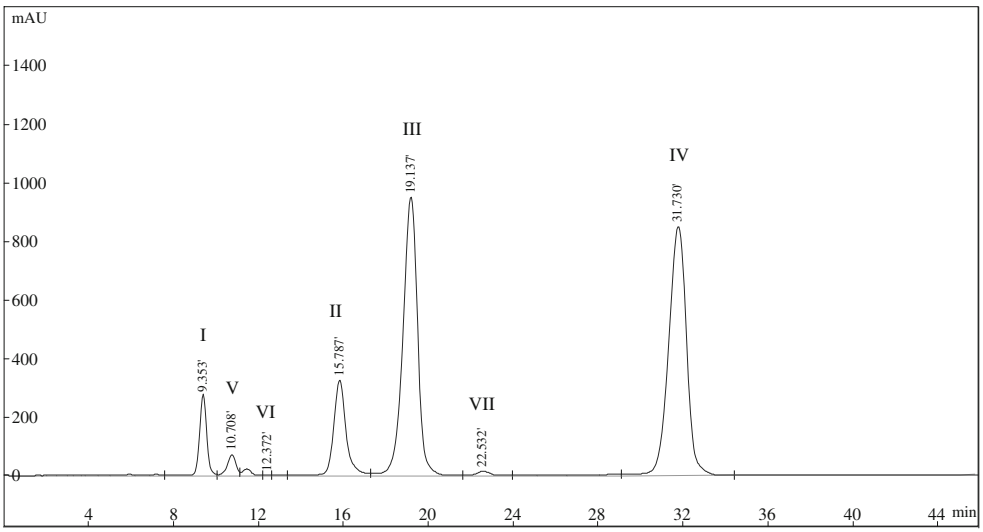

Fig. 2 HPLC spectrum of antifungal substance extracted by silica gel column chromatography. Peaks *I*, *II*, *III* and *IV* correspond to tetrin B, tetramycin B, tetrin A and tetramycin A, respectively; peaks V, VI and VII: without antifungal activities on *A. flavus*

maximum values at 292, 304, and 318 nm. The ESI–MS showed molecular ion peaks at m/z 710.81 $[M - H]^-$ and 734.81 $[M + Na]^+$. The molecular formula of component II was established as $C_{35}H_{53}NO_{14}$ based on the ESI–MS, ^1H NMR, and ^{13}C NMR analyses. Component II was identified as tetramycin B based on the above analysis and comparison with literature data (Ryu et al. 1999).

Component III: colorless needles (methanol). The u.v. spectrum in methanol showed maximum values at 290, 303, and 318 nm. The ESI–MS showed molecular ion peaks at m/z 680.78 $[M - H]^-$ and 704.78 $[M + Na]^+$. The molecular formula of component III was established as $C_{34}H_{51}NO_{13}$ based on the ESI–MS, ^1H NMR, and ^{13}C NMR analyses. Component III was identified as tetrin A based on the above analysis and comparison with literature data (Pandey et al. 1971).

Component IV: Pale yellow powder (methanol). The u.v. spectrum in methanol showed maximum values at 290, 304, and 318 nm. The ESI–MS showed molecular ion peaks at m/z 694.81 $[M - H]^-$ and 718.81 $[M + Na]^+$. The molecular formula of component IV was established as $C_{35}H_{53}NO_{13}$ based on the ESI–MS, ^1H NMR, and ^{13}C NMR analyses. Component IV was identified as tetramycin A based on the above analysis and comparison with literature data (Ryu et al. 1999).

MIC and MFC determination

Four *Aspergillus* species of grain mold were used in the antifungal test (Table 2). Table 2 shows that tetrins A and B and tetramycins A and B exhibited significant activity against *A. flavus* and *A. fumigatus*, with MIC values of

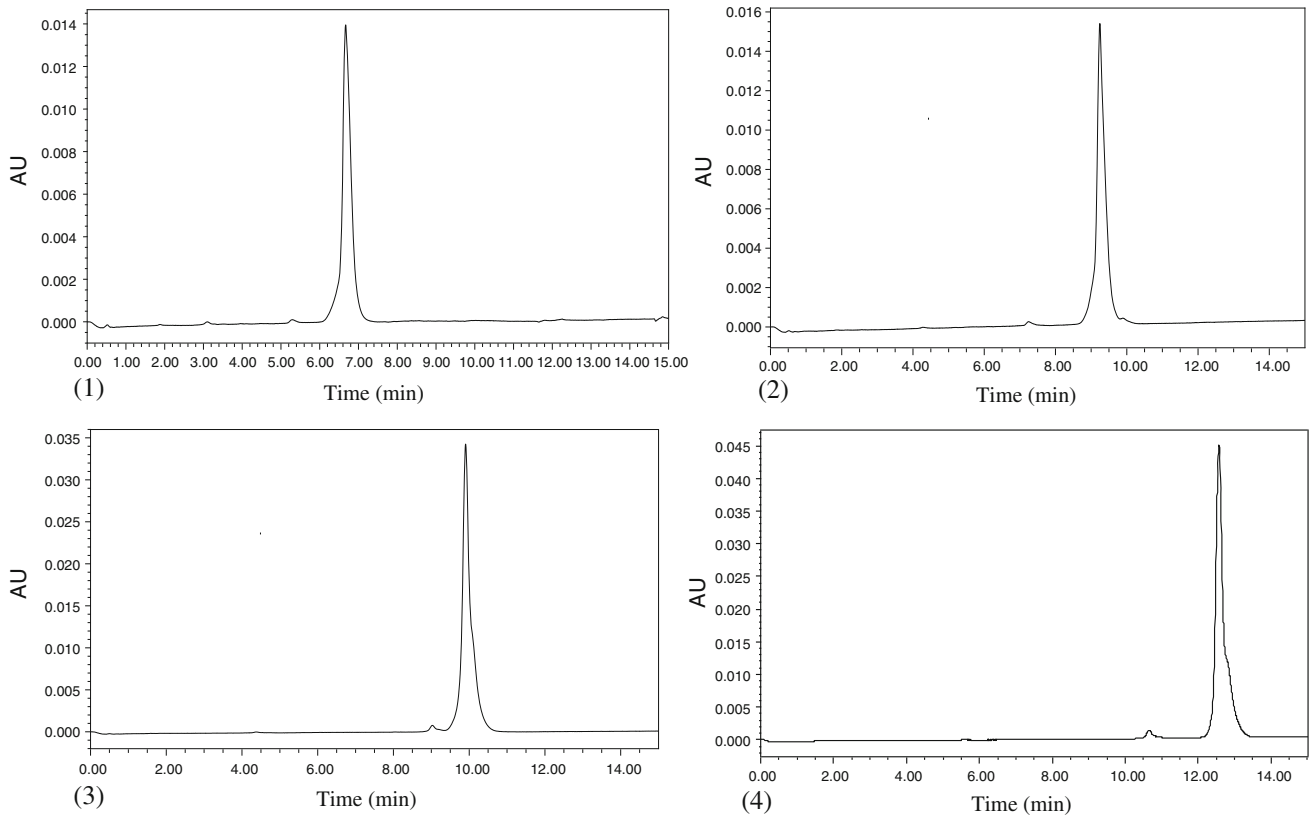

Fig. 3 UPLC spectrum of the antifungal fractions purified by preparative HPLC. **1** HPLC fraction I (tetrin B), **2** HPLC fraction II (tetramycin B), **3** HPLC fraction III (tetrin A), **4** HPLC fraction IV (tetrin A)

3.13, 12.56, 1.56, and 6.25 μg/ml and MFC values of 6.25, 25.0, 3.13, and 12.56 μg/ml, respectively. *A. alutaceus* and *A. niger* were more sensitive to tetrins A and B and tetramycins A and B with MIC values of 2.83, 11.25, 1.42, and 5.63 μg/ml and MFC values of 5.63, 22.5, 2.83, and 11.25 μg/ml, respectively.

Inhibitory activity in vivo

The contamination of *A. flavus* on peanuts was examined after incubation for 7 days in the presence of antifungal substance (Fig. 4). The growth of *A. flavus* decreased with increasing active compound concentration. Compared with the blank control 10 μg/g of the antifungal substance significantly reduced mycelial growth. The growth of *A. flavus* was completely inhibited at 30 μg/g of antifungal substance.

Discussion

The 16S rDNA sequencing approach is usually used in identifying microorganisms. It provides genus identification in most cases (90 %), but less so with regard to species (65–83 %), with 1–14 % of the isolates remaining unidentified after testing (Drancourt et al. 2000; Mignard and Flandrois 2006). Therefore, strain BS-112 was identified to belong in the genus *Streptomyces* by 16S rDNA gene sequencing. The strain was then identified as *Streptomyces hygroscopicus* based on its morphologic, cultural, physiological, and biochemical characteristics and cell components. Both the antibacterial and antifungal substances were produced by *S. hygroscopicus* BS-112. The antibacterial substance was a novel neutral extracellular polysaccharide with an average molecular weight of approximately 2,284 Da (measured by high-performance gel permeation chromatography), which mainly consisted of β-D-glucopyranose (Zhang 2010). The antibacterial substance had no acute toxicity to mice. It also had antioxidant effect and could scavenge free radicals of superoxide anion, hydroxyl, and DPPH (2,2-diphenyl-1-picrylhydrazyl) (Zhang 2010). The antifungal substances were tetrins A and B and tetramycins A and B. To the best of our knowledge, this study was the first to report information pertaining to the production of an antibacterial polysaccharide and four teraene macrolide antibiotics using *S. hygroscopicus*. The fermentation characteristics of strain BS-112 are well defined and the yield and activity of

Table 2 Activity of Tetrin A, Tetrin B, Tetramycin A and Tetramycin B against *Aspergillus* strains

Fungi tested	Tetrin A (μg/ml)		Tetrin B (μg/ml)		Tetramycin A (μg/ml)		Tetramycin B (μg/ml)	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. flavus	3.13	6.25	12.56	25.0	1.56	3.13	6.25	12.56
A. niger	2.83	5.63	11.25	22.5	1.42	2.83	5.63	11.25
A. alutaceus	2.83	5.63	11.25	22.5	1.42	2.83	5.63	11.25
A. fumigatus	3.13	6.25	12.56	25.0	1.56	3.13	6.25	12.56

[a] *MIC* minimum inhibitory concentration, *MFC* minimum fungicidal concentration

[b] MIC and MFC were determined by a macrodilution method and expressed in μg/ml (W/V)

[c] The results (MIC and MFC) are means of three independent triplicate experiments

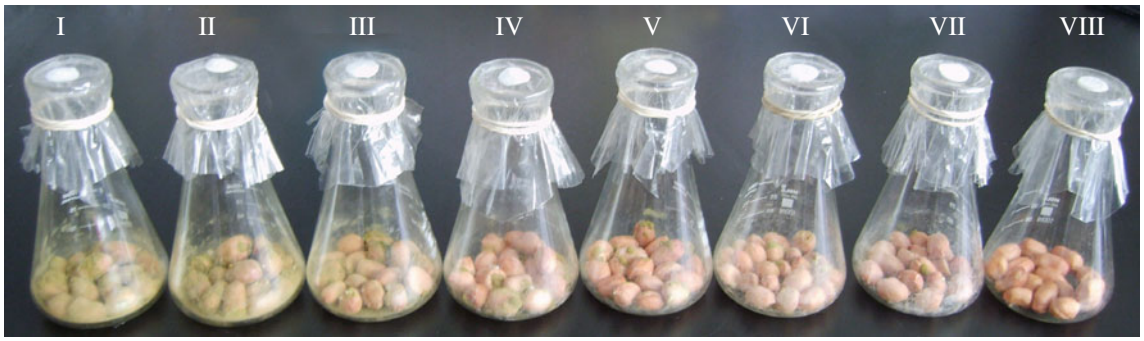

Fig. 4 The antifungal substance extracted by silica gel column chromatography inhibit *A. flavus* on peanuts at a series of concentration: (*I*) blank control; (*II*) 2.5 μg/g; (*III*) 5 μg/g; (*IV*) 10 μg/g; (*V*) 15 μg/g; (*VI*) 20 μg/g; (*VII*) 25 μg/g; (*VIII*) 30 μg/g

antifungal substances produced by this strain are both high and stable, respectively (Zhang et al. 2012). Together, these characteristics are critical for further applications.

The X-5 resin was selected for the extraction of the antifungal active substances from the fermentation broth of strain BS-112. The saturated absorption capacity of the resin was 73.37 mg/g, and only took 150 min to achieve saturation adsorption at 25 °C. The optimal elution solution was 75 % ethanol with a desorption rate that can reach 93.68 %. The separation of antifungal active substances can be primarily and effectively achieved via adsorption and desorption on the X-5 resin. Therefore, X-5 resin can be used in the industrial extraction of antifungal active substances.

Silica gel column chromatography afforded only one main peak. This result indicated that it was difficult to separate the four antifungal components via silica gel column chromatography because the four components have the same polarity due to their similar structures. However, silica gel column chromatography can remove a large number of impurities and pigments in the crude extract.

HPLC methods for the separation of antifungal active substances were initially established. The appropriate elution system and flow rate were then investigated and optimized. The mobile phase of 62 % methanol was used during 0–24 min. The mobile phase was then changed to 66 % methanol after 24 min to save solvent and time. Reducing the flow rate can improve separation to some extent, but more time and mobile phase are needed and the chromatogram peaks must be extended. Hence, the mobile phase flow rate was set to 5 ml/min. Furthermore, sample loading was also optimized on the HPLC test. About 5 mg/ml was chosen as the optimum concentration because peaks I and V would overlap and the purities of the separated peaks would be significantly reduced when the sample concentration was more than 5 mg/ml. Figure 2 shows that the four antifungal components were well separated and separation time was acceptable. Their purities were all above 99.1 % (Fig. 3). Therefore, preparative HPLC was successful in separating and purifying antifungal active substances from stain BS-112 under the above optimized conditions.

The antibiotic tetrin was first reported in 1960 (Gottlieb and Pote 1960). It was isolated from *Streptomyces* sp. (No. 155-2) and immediately recognized as a member of the family of antibiotics containing an isolated tetraene chroniophore. Rinehart et al. (1963) later reported that the antibiotic consists of two closely related components, namely, tetrins A and B. Tetrins A and B exhibited strong inhibitory effect on the growth of plant pathogenic fungi such as *Pythium ultimum*, *Rhizoctonia solani*, *Alternaria solani*, *Glomerella cingulata*, and *Verticillium albo-atrum* (Van Etten and Gottlieb 1967). Tetrin A was 2–8 times

more active than tetrin B. Tetrin A also inhibited the growth of *Saccharomyces cerevisiae* at much lower concentrations than tetrin B. Contrary to most polyene antibiotics that cause rapid lysis of mammalian erythrocytes even at low concentrations, tetrins A and B only caused partial lysis of calf red cells after long exposures to high concentrations (Van Etten and Gottlieb 1967). Tetrin A exhibited little effect on calf red cells even at 0.2 mg/ml and after 6 h of incubation, whereas pimaricin and nystatin caused complete lysis of the cells under the same conditions (Van Etten and Gottlieb 1967).

Tetramycin is an antifungal, tetraene macrolide antibiotic produced by *Streptomyces noursei* Hazen and Brown, 1950 var. jenesis nov. var. JA 3789 (Dornberger et al. 1979). The structures of tetramycins A and B were determined using high-field NMR spectroscopy (Radics et al. 1982). Aside from their structural similarities with natamycin, tetramycins A and B are also related to lucensomycin and differ from tetrins A and B merely in the substituent group at C-24. The structural similarity is most certainly due to the common biosynthetic pathway of these molecules. The concentrations of tetramycins A and B that are necessary for the complete inhibition of the growth of *Saccharomyces cerevisiae* JH, *Fusarium solani* JP, *Penicillium notatum* JP, and *Scopulariopsis* sp. JP 25 were determined by Radics et al. (1982). The four strains were insensitive to both tetramycins even at 25 and 12.5 μg/ml of tetramycins A and B, respectively. Tetramycins A and B showed nearly the same antifungal activities against the selected spectrum of test organisms (Radics et al. 1982). Therefore, tetramycins A and B were proven to be broad-spectrum, highly effective antifungal antibiotics.

In previous reports, the activities of tetrins A and B and tetramycins A and B were mainly tested against plant pathogenic fungi (Van Etten and Gottlieb 1967; Radics et al. 1982). However, they are sensitive to sunlight, ultraviolet light, and extreme pH values because of their structures that consist of four alternating double bonds. They became microbiologically inactive after 48 h of exposure to sunlight, thus, they cannot be widely used in the field of crop farming. In the present paper, we found that tetrins A and B and tetramycins A and B showed a strong antagonistic activity against *Aspergillus*, particularly against *A. flavus*. *A. flavus* is responsible for spoilage of many foods and feeds, and causes decay on stored fruits damaged by insects, animals, early splits, and mechanical harvesting. Its produced aflatoxin has been classified as a class 1 human carcinogen by the International Agency for Research on Cancer (IARC 1993). Therefore, it is important to find a practical, cost effective, and non-toxic method to prevent fungal deterioration of stored food and feed. Biological control is one of the more promising techniques among the several research approaches used to reduce and ultimately eliminate *A. flavus* contamination, particularly for the near-term (Norner 2004). Purified antifungal compounds from stain BS-112 could inhibit the growth of *A. flavus* on peanuts. Tetrins A and B and tetramycins A and B exhibited MIC values of 0.313 μg/ml to 12.56 μg/ml against *A. flavus* (Table 2), whereas the MIC ratio of the four components was the same with that of natamycin (Sun et al. 2005). A comparison with previous studies clearly showed that they were more effective than Bacillomycin D and extracts of *Agave* species (Eduardo et al. 2005; Zhang et al. 2008). Although tetrins A and B and tetramycins A and B are not widely used in agriculture, they have the potential to be developed as biocontrol agents against *A. flavus* in food storage.

In conclusion, tetrins A and B and tetramycins A and B produced by *S. hygroscopicus* BS-112 exhibited a strong antifungal activity against *Aspergillus* in food and feed. Further research is needed on the breeding of strain BS-112 and batch fermentation optimization.

References

Ashok K, Ravindra S, Priyanka S, Nawal KD (2010) Chemical composition, antifungal and antiaflatoxigenic activities of *Ocimum sanctum* L. essential oil and its safety assessment as plant based antimicrobial. Food Chem Toxicol 48(2):539–543

Dornberger K, Thruma H, Radics L (1979) The structure of tetramycin, a new polyene macrolide antibiotic. Tetrahedron 35 (15):1852–1856

Drancourt MC, Bollet A, Carlioz R, Martelin JP, Gayral RD (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J Clin Microbiol 38(10):3623–3630

Eduardo S, Norma H, Santos G (2005) Inhibition of growth and mycotoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* by extracts of *Agave* species. Int J Food Microbiol 98(3):271–279

Gottlieb D, Pote HL (1960) Tetrin, an antifungal antibiotic. Phytopathology 50(11):817

Group of Actinomycetes Taxonomy Institute of Microbiology Chinese Academy of Science (1975) Identifying manual of *Streptomycetes*. Science Press, Beijing, pp 3–15

Hu YL, Zhang HL, Zong YL, Zhang XH, Bao XF, Chen S, Wang Z (1995) Study on mutation breeding of Wuningmeisu producing strain. J Microbiol 15(2):40–44

IARC (1993) IARC monographs on the evaluation of carcinogenic risks to humans: some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Int Agency Res Cancer (Lyon) 56:489–521

Kelly KL (1964) Inter-society color council-national bureau of standards color-name charts illustrated with centroid colors. US Government Printing Office, Washington, DC

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, pp 161–167

Lechevalier HA, Lechevalier MP (1970a) A critical evaluation of the genera of aerobic actinomycetes. In: Prauser H (ed) The Actinomycetes. Gustav Fischer Verlag, Jena, pp 393–405

Lechevalier MP, Lechevalier HA (1970b) Chemical composition as a criterion in the classification of aerobic actinomycetes. Int J Syst Bacteriol 20(4):435–443

Lechevalier MP, Lechevalier HA (1980) The chemotaxonomy of actinomycetes. In: Dietz A, Thayer DW (eds) Actinomycete taxonomy. Society for Industrial Microbiology, Virginia, pp 22–291

Levinskas GJ, Ribelin WE, Shaffer CB (1996) Acute and chronic toxicity of pimaricin. Toxicol Appl Pharmacol 8(1):97–109

Liang JL, Xu ZN, Liu TF, Lin JP, Cen PL (2008) Effects of cultivation conditions on the production of natamycin with *Streptomyces gilvosporeus* LK-196. Enzyme Microb Tech 42(2):145–150

Lin N, Li W, Sha Y, He JY, Jiang QH, Li X (2008) Structure elucidation of polyene macrol ide antibiotic lucensomycin. Chinese J Magn Reson 25(4):514–522

Mandel M, Marmur J (1968) Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. Methods Enzymol 12B:195–206

Martin JF (1977) Biosynthesis of polyene macrolide antibiotics. Ann Rev Microbial 31:13–38

Mignard S, Flandrois JP (2006) 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. J Microbiol Methods 67(3):574–581

Mishra HN, Das C (2003) A review on biological control and metabolism of aflatoxin. Crit Rev Food Sci Nutr 43(3):245–264

National Pharmacopoeia Committee (2005) Pharmacopoeia of the people's Republic of China (Part II). Chemical Industry Press, Beijing, pp 181–182

NCCLS (2002) Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved Standard M38-A. Wayne, PA

Norner JW (2004) Biological control of aflatoxin contamination of crops. J Toxicol Toxin Rev 23(2):425–450

Pandey RC, German VF, Nishikawa Y, Rinehart KL (1971) Polyene antibiotics. II. The structure of Tetrin A. J Am Chem Soc 93(15):3738–3747

Radics L, Incze M, Dornberger K, Thruma H (1982) Tetramycin B, a new polyene macrolide antibiotic: the structure of tetramycins A and B as studied by high-field NMR spectroscopy. Tetrahedron 38(1):183–189

Ren BJ, Li YH (1996) Control of Valda mail with Wuning-mycin. Chin J Biol Control 2:89

Rinehart KL, German VF, Tucker WP (1963) Isolierung und eigenschaften der Tetrine A und B. Justus Liebigs Annalen der Chemie 688(1):77–86

Rinehart KL, Tucker WP, Pandey RC (1971) Polyene antibiotics. III. The structure of tetrin B. J Am Chem Soc 93(15):3747–3751

Ryu G, Choi WC, Hwang SJ, Yeo WH, Lee CS, Kim SK (1999) Tetrin C, a new glycosylated polyene macrolide antibiotic produced by *Streptomyces* sp. GK9244. J Nat Prod 62(6):917–919

Sader HS, Becker HK, Moet GJ, Jones RN (2010) Antimicrobial activity of daptomycin tested against *Staphylococcus aureus* with vancomycin MIC of 2 μg/mL isolated in the United States and European hospitals (2006–2008). Diagn Microbiol Infect Dis 66(3):320–331

Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* sp. Int J Syst Bacteriol 16(3):313–340

Song Z, Liu QX, Guo H, Ju RC, Zhao YH, Li JY, Liu XL (2012) Tostadin, a novel antibacterial peptide from an antagonistic microorganism *Brevibacillus brevis* XDH. Bioresour Technol 111:504–506

Sun QH, Cui SY, Li AP, Done F, Chen L (2005) Study on ant putrefactive and bacteriostatic efficacy of natamycin. Chin J Disinfect 22(4):435–436

Tang W, Zhang CX (2010) Study on the high yield breeding of tetramycin strain. J Liaoning Agric Coll 12(5):1–4

Van Etten JL, Gottlieb D (1967) Studies on the mode of action of Tetrin A. J General Microbiol 46(3):377–387

Vilar VJP, Botelho CMS, Boaventura RAR (2006) Equilibrium and kinetic modeling of Cd(II) biosorption by algae Gelidium and agar extraction algal waste. Water Res 40:291–302

Volpon L, Lancelin JM (2002) Solution NMR structure of five representative glycosylated polyene macrolide antibiotics with a sterol-dependent antifungal activity. Eur J Biochem 269(18): 4533–4541

Wang ZW, Liu XL (2007) Medium optimization for antifungal active substances production from a newly isolated *Paenibacillus* sp. using response surface methodology. Bioresour Technol 99(17): 8245–8521

Williams ST, Goodfellow M, Alderson G, Wellington EMH, Sneath PHA, Sakin MJ (1983) Numerical classification of *Streptomyces* and related genera. J Gen Microbiol 129:174–1813

Zhang N (2010) Fermentation of *Streptomyces hygroscopicus* BS-112 and its antibiotic substances purification and characterization. Shandong Agricultural University, Shandong, pp 165–169 (data not shown)

Zhang T, Shi ZQ, Hu LB, Cheng LG, Wang F (2008) Antifungal compounds from *Bacillus subtilis* B-FS06 inhibiting the growth of *Aspergillus flavus*. World J Microbiol Biotechnol 24(6): 783–788

Zhang N, Qi ZM, Duan HG, Xie YH, Yu J, Lu CX, Liu XL (2012) Optimization of medium composition for production of antifungal active substance from *Streptomyces hygroscopicus* BS-112. Afr J Microbiol Res 6(1):71–80