Extraction and identification of bioactive compounds (eicosane and dibutyl phthalate) produced by *Streptomyces* strain KX852460 for the biological control of *Rhizoctonia solani* AG-3 strain KX852461 to control target spot disease in tobacco leaf

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**Abstract**

*Streptomyces* strain KX852460 having antifungal activity against *Rhizoctonia solani* AG-3 KX852461 that is the causal agent of target spot disease in tobacco leaf. The aim of the study was to determine the antifungal activity of *Streptomyces* strain KX852460 extract against *R. solani* AG-3 and to identify bioactive antifungal compounds produced by strain KX852460. Crude substance was produced by submerged fermentation process from *Streptomyces* strain KX852460. Various solvent was used to extract the culture filtrate. Among all, ethyl acetate extracted supernatant showed great potency against *R. solani* AG-3 KX852461. The active fractions were purified by silica gel column chromatography having 52 mm zone of inhibition against *R. solani* AG-3 KX852461. The purified fractions were identified by gas chromatography–mass spectrometry technique. Twenty-seven compounds were identified and most of the compounds were the derivatives of aromatic compounds. Eicosane (C20H42) and dibutyl phthalate (C16H22O4) were found antifungal compounds in this study. While morphinan, 7,8-didehydro-4,5-epoxy-17-methyl-3,6-bis[(trimethylsilyl)oxy]-, (5.Alpha. 6.Alpha)—(C23H35NO3Si2), cyclononasiloxane, octadecamethyl—(C18H54O9Si9) and benzoic acid, 2,5-bis(trimethylsiloxy) (C16H30O4Si3) were the major compounds with highest peak number. These results suggested that *Streptomyces* strain KX852460 had good general antifungal activity and might have potential biocontrol antagonist against *R. solani* AG-3 KX852461 to cure the target spot in tobacco leaf.

**Keywords:** *Streptomyces* strain KX852460, *Rhizoctonia solani* AG-3, Gas chromatography–mass spectrometry, Aromatic compounds, Target spot

**Introduction**

In the Liaoning province of China in 2006 target spot disease of tobacco was investigated, *Rhizoctonia solani* was the causal agent and caused heavy economic loss regarding to the production and quality of the tobacco (Wu et al. 2012). Anastomosis AG-3 of the *R. solani* is the causal agent of target spot in tobacco (Johnk et al. 1993). To conflict with phytopathogens biocontrol is most potent and environment friendly practice (Castano et al. 2013). Against the *R. solani* biocontrol is better strategy even though environmental conditions affected its efficacy (dos Reis Almeida et al. 2007). *Actinomycetes* are extensively present microorganisms in the environment that have potential to produce bioactive compounds against the phytopathogens (Xue et al. 2013; Zeng et al. 2013). Among the *Actinomycetes*, *Streptomyces* is the
largest genus and belongs to the family Streptomycetaceae (Kämpfer 2006). Streptomyces have potential to produced curial compounds for antibiotics and agro-antibiotics (Demain 2009). Streptomycetes species are the source of 75% of antibiotics (Bhavana et al. 2014). Different solvents and elucidation utilized to extract the secondary metabolites and their structure can be finding by different techniques for example gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), and nuclear magnetic resonance (NMR) (Tiwari et al. 2015). Volatile and semi volatile compounds with lower molecular mass can be separated by using GC–MS (Snyder et al. 2012). The GC–MS is novel technology for isolation of the compounds that present in the secondary metabolites. Recently antibacterial (Khattab et al. 2016), antifungal compound against Lycopersici and Fusarium oxysporum (Jalaluldeen et al. 2015), antifungal compounds against Pyricularia oryzae (Awla et al. 2016), and broad spectrum pharmaceutical compounds (Narasaiha et al. 2014) were extracted by GC–MS. The main objective of this study was extraction, purification and identification of bioactive antifungal compounds produced from Streptomyces strain KX852460 grown under submerged fermentation.

Materials and methods

Microorganism

Streptomyces strain was isolated from soil and identified by 16S rRNA gene sequence technology and sequence was submitted to Gene bank under accession number of KX852460 and also submitted to Chinese general microbial collection center (CGMCC4.7384). The strain was used for the production of antifungal compounds in submerged fermentation. Rhizoctonia solani AG-3 was obtained from naturally infected tobacco leaves in Dan-dong of China which was also identified by 16S rRNA gene sequence technique and sequence obtained was submitted to the Gene bank and under Accession Number of KX852461 and also submitted to Chinese general microbial collection center (CGMCC3.18223). Other test pathogens obtained from the plant pathology lab of college of plant protection, Shenyang agricultural University China. Fungus pathogens were stored on potato dextrose agar (PDA) at 4 °C.

Preparation of inoculum

Fermentation was performed in two stages, seed growth and production of active antifungal substance. Streptomyces Strain KX852460 was grown on plates of Gause’ s synthetic agar medium at 28 °C for 5 days after spore production used in liquid fermentation medium. Two spore cakes (5 mm) were used to inoculate a 250 ml flask having medium volume of 40 ml and then incubated at 28 °C with agitation speed of 160 rpm for 48 h.

Fermentation technique

For the production of antifungal compounds, 40 ml of fermentation medium [47 g soluble starch, 3 g yeast extract, 22 g peanut meal, 2.7 g (NH4)2 SO4, 2.7 g NaCl, 2.7 g CaCO3 dissolved in 1 L distilled water and pH was adjusted to 6.8–7.2] was taken in 250 ml flask and sterilized. After sterilization, the medium was inoculated with 5% (v/v) seed culture and incubated at 28 °C in rotatory shaker with agitation speed of 160 rpm for 96 h. After the termination of fermentation process, the culture was centrifuged and the supernatant was stored at −4 °C for further work (Gao et al. 2015).

Antifungal activity

Antifungal activities were determined by oxford cup method (Wang et al. 2010a, b) and measured the inhibition zone.

Stability test of the cultural filtrate of Streptomyces KX852460

Thermal stability, pH stability, illuminated light stability, and UV light stability were performed according to Zhao and Wu (2006). All the experiment were performed in triplicates and antifungal activity determined by oxford cup method mentioned above.

Extraction of the culture filtrate

The culture filtrate (500 ml) was extracted two times with ethyl acetate as solvent. The solvent was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 20 min. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase using separating funnel. Ethyl acetate layer was concentrated by evaporating to dryness at 50 °C and residue obtained was purified using methanol to (1.8 g) brown crude extract (Ahmed 2007).

Purification and identification of the compound

The purification of the antimicrobial compound was carried out using silica gel column chromatography as described by Atta et al. (2009). Ethyl acetate was used as eluting solvent. The column was packed with silica gel (60–120 mesh). The sample to be separated was loaded on the packed column and eluted with the solvent at the flow rate of one drop per minute. A conical flask was placed at the bottom of the column to collect the eluted fractions. Antifungal activity was checked and most active fractions were used for further analysis. The antifungal compounds
were identified by using gas chromatography–mass spectrometer technique (GC–MS). Agilent technologies 6890–5973 N with capillary column TG-5 ms Phenyl Methyl Siloxane (30 m x 250 μm x 0.25 μm) system were used. Mass detector used in split mode, and helium gas with flow rate of 1.0 ml/min was used as a carrier. Injector was operated at 230 °C and oven temperature for initial setup was 60 °C for 2 min, ramp 10/min to 280 °C for 8 min.

Results

Streptomyces strain KX852460 was isolated from the soil and screened against the R. solani AG-3 that is the causal agent of target spot in tobacco leaf. This strain had great potency against the pathogen. Twenty litter fermentation broth was produced by the Streptomyces strain KX852460 and broth was active against different plant pathogens including R. solani AG-3 having inhibition zone diameter of 45.78 mm. The strain also showed strong activity against Sclerotinia sclerotiorum with inhibition zone diameter of 50.4 mm (Table 1). Antifungal activity of the fermentation broth was found stable at various temperature levels from 60 to 90 °C, while at 100 °C activity was decreased (Fig. 1a). At different pH values antifungal activity of fermentation broth was observed, having peak activity at pH 6, while extreme pH conditions (pH 2, 14) resulted decreased antifungal activity (Fig. 1b). Fermentation broth treated with illuminated light showed stability in the activity against the pathogen (Fig. 1c). Under UV light treated broth was affected within duration of treatment and activity decreased abruptly (Fig. 1d). Solvent extraction method used to extract the bioactive compounds with several organic polar and non-polar solvents. All the extracts showed some inhibition effect against the pathogen ranging from 1.43 to 44.36 mm inhibition zone. But extract with ethyl acetate showed strong antifungal activity against the R. solani AG-3 KX852461 (Fig. 2). For further work based on this result ethyl acetate was selected and resulted antifungal activity against R. solani AG-3 KX852461 (Fig. 3). Crude extract dried at 50 °C and brown color powdery substance obtained that further purified by silica gel column chromatography by using ethyl acetate as eluent. Several fractions were obtained and most active fractions against the R. solani. In (Fig. 4) fraction number 8 had strongly inhibited the R. solani AG-3 with diameter of inhibition zone 52 mm. Active fraction was further analyzed by gas chromatography–mass spectrometer (GC–MS). GC–MS analysis detected 27 bioactive compounds (Table 2). By comparison of mass spectra of the constituent with NIST library twenty-seven peaks obtained (Fig. 5a). Among 27 different compounds, 16 compounds were the constituent of aromatic compounds while others were derivatives of different hydrocarbons. Eicosane (C_{20}H_{42}) and dibutyl phthalate (C_{12}H_{22}O₄), having retention time of 13.641 (Fig. 5b) 18.852 (Fig. 5c) respectively were two antifungal compounds identified.

Discussion

In this study Streptomyces strain KX852460 was screened against the R. solani AG-3 that is the causal agent of target spot disease in tobacco. Strain KX852460 belongs to Streptomyces which strongly inhibits the pathogen and could be the target spot in tobacco. The effects of the R. solani diseases are very severe throughout the world and affected the quality and yield of the several crops. For the control of R. solani, bacterial antagonist could be an environment friendly substituent. Various bacterial antagonists against the R. solani, including Bacillus subtilis CA32 in eggplant (Abeyesinghe 2009), Pseudomonas fluorescens In5 (Michelsen and Stougaard 2011), Burkholderia cepacia TIA-2B and Pseudomonas sp. T4B-2A in tomato (De Curtis et al. 2010), inocum of GB7 and 3Re4-18 in lettuce (Grosch et al. 2012), endophytic Streptomyces damping off growth promotion in tomato (Goudjal et al. 2014) have been reported as effective biological control agents. Antagonist activity of Streptomyces sp. CACIS-1.16CA against different phytopathogens including R. solni was also investigated by Evangelista-Martínez (2014).

In this study stability of the active cultural filtrate was determined at various temperatures, pH values, and treated with illuminated light and UV light. At 60–90 °C the antifungal activity of cultural filtrate of the Streptomyces strain KX852460 remained same, however above these conditions, the activity become decreased. pH values remained stable between 5.0 and 8.0 pH, while pH values above and lower beyond this were not stable. Uddin et al. (2013) reported stability of antimicrobial filtrate at different temperature and pH values. Treated with illumination light cultural filtrate was stable and showed good activity. Treated with UV light for long

Table 1 Antimicrobial effects of the cultural filtrate

| Test pathogens     | Inhibition spectrum (mm) of cultural filtrate |
|--------------------|-----------------------------------------------|
| Alternaria alternata| 33.96                                         |
| Botrytis cinerea    | 40.16                                         |
| Alternaria solani   | 38.62                                         |
| Rhizoctonia solani AG-3 | 45.78                                 |
| Fusarium oxysporum | 24.78                                         |
| Sclerotinia sclerotiorum | 50.4                              |
| Bipolaris maydis    | 33.72                                         |
| Colletotrichum capsici| 25.48                                       |
Fig. 1  Effect of temperature (a), pH (b), illuminated light (c) and ultra violet light (d) on the stability of fermentation broth. Ck represents control of each treatment.

Fig. 2  Effect of different solvents on activity of bioactive compound produced from Streptomyces strain KX852460
time was not stable, while treated for 30 min and for 1 h
was stable. Stability of antimicrobial cultural filtrate from
Streptomyces was also reported by Zhao and Wu (2006).

The crude extract obtained by solvent extraction with
ethyl acetate showed strong activity against the R. solani.
Isolation of crude extract by solvent extraction is very
important phenomenon, to find a good solvent that have
the potential to extract high yield and most potent bio-
active compounds. Studies demonstrated that the extract
of ethyl acetate have wide antimicrobial spectrum against
the bacterial and fungus pathogens (Khamna et al. 2009;
Kobayashi et al. 1994). Extract from Streptomyces
EF37141 contains 27 different organic compounds. GC–
MS analysis showed that the majority of the compounds
were derivatives of the aromatic compounds. These
compounds were antimicrobial and antifungal. Volatile
organic compounds and polycyclic aromatic derivatives
have the antifungal potential (Müller et al. 2009; Memić
et al. 2011).

GC–MS is a novel technique to identify the second-
ary metabolites from the Streptomyces fermentation
broth and analysis of GC–MS is very reliable to iden-
tify the compound in complex biochemical product.
From current study some compounds were reported
antifungal. Eicosane reported as antifungal compound
(Karanja et al. 2012; Nandhini 2015) and dibutyl phtha-
late also reported antifungal compound (Nandhini 2015;
Roy et al. 2006). Morphinan, 7,8-didehydro-4,5-epoxy-
17 methyl-3,6-bis[(trimethylsilyloxy)]–(5.alpha,
6.alpha)–, cyclononasiloxane, octadecamethyl- and
1,2,4-benzenetricarboxylic acid, 4-butyl 1,2-dime-
thyl ester showed highest peaks and the area percent
of these compounds also more than other compounds.
On the base of GC–MS analysis highest peak number
and area percent indicated that these three compounds

Fig. 3  Activity of solvent extracted supernatant with ethyl acetate
against R. solani AG-3
| Peak # | Retention time | Area % | Name of the compound | Chemical formula | Molecular weight |
|-------|---------------|--------|----------------------|------------------|------------------|
| 1     | 4.786         | 3.92   | Benzoic acid, 2-methoxy-, methyl ester | C₉H₁₀O₃ | 166 |
| 2     | 8.024         | 5.36   | Undecane             | C₁₁H₂₂       | 156 |
| 3     | 8.106         | 1.8    | Undecane             | C₁₁H₂₂       | 268 |
| 4     | 11.373        | 0.98   | Cyclohexasiloxane, dodecamethyl- | C₁₂H₃₆O₆Si₆ | 444 |
| 5     | 13.641        | 0.98   | Eicosane             | C₂₀H₄₂       | 282 |
| 6     | 13.87         | 0.72   | Phenol, 2,4-bis(1,1-dimethylethyl) | C₁₆H₂₂O       | 206 |
| 7     | 13.935        | 1.02   | Butylated hydroxytoluene | C₁₅H₂₂O       | 220 |
| 8     | 16.009        | 0.74   | N-(Glycyl)alanine    | C₅H₁₀N₂O₃    | 146 |
| 9     | 16.955        | 1.98   | 1,3-Diphenyl-4H-1,2,4-triazoline-5-thione | C₁₄H₁₁N₃S  | 253 |
| 10    | 17.378        | 1.09   | Cyclononasiloxane, octadecamethyl- | C₁₉H₃₈O₆Si₆ | 666 |
| 11    | 17.901        | 5.9    | 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester | C₁₆H₃₆O₄ | 278 |
| 12    | 18.406        | 4.67   | Hexadecanonic acid, methyl ester | C₁₆H₃₂O₂     | 270 |
| 13    | 18.852        | 6.44   | Diphenyl phthalate   | C₁₆H₁₄O₄     | 278 |
| 14    | 18.929        | 1.63   | Cyclodecasiloxane, eicosamethyl- | C₂₀H₄₀O₁₀Si₁₀ | 740 |
| 15    | 19.264        | 2.04   | 1-Phenylethanol      | C₁₂H₁₄O      | 242 |
| 16    | 19.74         | 1.1    | p-Dicyclohexylbenzene | C₁₆H₁₄O      | 242 |
| 17    | 20.092        | 3.44   | 9,10-Anthracenedione, 2-ethyl- | C₁₉H₂₂O₂     | 236 |
| 18    | 20.357        | 5.95   | Cyclodecasiloxane, eicosamethyl- | C₂₀H₄₀O₁₀Si₁₀ | 740 |
| 19    | 20.515        | 1.4    | Octahydrotriphenylene | C₁₉H₂₄     | 236 |
| 20    | 21.649        | 5.35   | 7-Chloro-10-ethyl-1-[[2-[2-hydroxyethyl (amino) ethyl] amino]-3-[(4-...| | 503 |
| 21    | 22.689        | 1.48   | Hexanedioic acid, bis(2-ethylhexyl) ester | C₉H₁₀O₄     | 370 |
| 22    | 22.836        | 6.56   | Morphinan, 7,8-didehydro-4,5-epoxy-17-methyl-3, 6-bis [[trimethylsilyl]oxy], (5.alpha. 6. Alpha.)- | C₁₉H₁₄NO₃Si₂ | 429 |
| 23    | 23.947        | 14.46  | Cyclononasiloxane, octadecamethyl- | C₁₉H₃₈O₆Si₆ | 666 |
| 24    | 25.11         | 7.55   | Benzoic acid, 2,5-bis(trimethylsilyloxy)-, trimethylsilyl ester | C₁₉H₂₆O₃Si₃ | 370 |
| 25    | 26.497        | 8.32   | 1,2,4-Benzencarboxylic acid, 4-butyl 1,2-dimethyl ester | C₁₉H₂₆O₆ | 294 |
| 26    | 28.23         | 4.15   | Cycloptol, hexamethyl- | C₁₉H₃₂Si₅ | 222 |
| 27    | 30.468        | 0.97   | Cycloptol, hexamethyl- | C₁₉H₃₂Si₅ | 222 |

(See figure on next page.)

**Fig. 5** Gas chromatography-mass spectrometer (GC–MS) analysis of the purified active fraction (a), detection of eicosane (b) and dibutyl phthalate (c) from purified active fraction.
were major in the extract of *Streptomyces* EF37141. These compounds consider active substances against the *R. solani*. Extract from the *Streptomyces* shows the same effectiveness as the oxine benzoate and fungicide (Sabalatnam and Traquair 2002).
Wang YH, Fang XL, Li YP, Zhang X (2010b) Effects of constant and shifting dissolved oxygen concentration on the growth and antibiotic activity of Xenorhabdus nematophila. Biorec Technol 101:7529–7536
Wu YH, Zhao YQ, Fu Y, Zhao XX, Chen JG (2012) First report of target spot of flue-cured tobacco caused by Rhizoctonia solani AG-3 in China. Plant Dis 96:1824
Xue L, Xue Q, Chen Q, Lin C, Shen G, Zhao J (2013) Isolation and evaluation of rhizosphere actinomycetes with potential application for biocontrol of Verticillium wilt of cotton. Crop Prot 43:231–240
Zeng Q, Huang H, Zhu J, Fang Z, Sun Q, Bao S (2013) A new nematicidal compound produced by Streptomyces albogriseolus HA10002. Antonie Van Leeuwenhoek 103:1107–1111
Zhao G, Wu YH (2006) Antimicrobial spectrum and stability of the fermentation broth of Streptomyces. J Agrochem 8:806