Screening, isolation and mechanism of a nematicidal extract from actinomycetes against the pine wood nematode *Bursaphelenchus xylophilus*

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HIGHLIGHTS

- *Streptomyces lactacystinicus* TCS19-048 showed good inhibitory activity against *B. xylophilus*, which was the first report.
- *Streptomyces lactacystinicus* TCS19-048 was identified by multiphase classification.
- The active extract was able to significantly increase the body fluid leakage of *B. xylophilus*.
- The active extract was isolated and had an inhibitory effect on the movement behaviour of *B. xylophilus*.

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Abstract

Actinomycetes are an important microbial resource closely related to human production and life. Approximately 70% of the antibiotics currently used are produced by various actinomycetes. Pine wilt disease (PWD) is caused by the pine wood nematode (PWN) (*Bursaphelenchus xylophilus*), which is a devastating forest pest that can cause the wilting and death of pine trees. The purpose of this work was to screen out, identify, and ferment active actinomycetes with high lethality to pine wood nematodes and study the active compounds in their secondary metabolites. A group of 227 original strains/isolates of actinomycetes was screened, and *Streptomyces* sp. TCS19-048 was selected for further analyses. The taxonomic characteristics and molecular analysis results indicated that the strain was a member of the genus *Streptomyces* and exhibited the highest similarity (99.77%) with the 16S rRNA gene sequences of *Streptomyces lactacystinicus* OM-6519. Furthermore, the active extract was isolated and had an inhibitory effect on the movement behaviour of *B. xylophilus* and was able to significantly increase the body fluid leakage of *B. xylophilus*. This study focused on isolating secondary metabolites from various soil actinomycetes to control PWD and identified an extract with nematicidal activity from a selected strain. The nematicidal effect of extract S-3-1 was confirmed, and the strain *Streptomyces* sp. TCS19-048 showed good inhibitory activity against *B. xylophilus*, which was reported for the first time at home and abroad.
1. Introduction

Pine wilt disease (PWD) is a devastating disease caused by *Bursaphelenchus xylophilus* (pine wood nematode, PWN), and it can result in the rapid wilting and death of pine trees (Suzuki, 2002). *Monochamus alternatus* is the main vector of PWN in East Asia. When the pupae of *Monochamus alternatus* infected by *B. xylophilus* emerge as adults and fly to healthy pine trees to feed or lay eggs, it will spread the spreading forth of pine branches. Therefore, *B. xylophilus* has caused huge economic losses and damage to the environment on a global scale, especially in Japan, China, and South Korea (Seo et al., 2014) in East Asia and Portugal and Spain in Western Europe (Vicente et al., 2012). With the development of international trade, the disease has spread faster and more heavily.

For the control of PWD, physical, chemical and biological methods are generally used, such as felling, fumigation, crushing and burning of pine trees suffering from PWD (Cai and Lang, 2011); reducing the planting quantity of pine forests around the epidemic area; and implementing preventive methods, including injecting chemically synthetic pesticides (emamectin benzoate) (Rys et al., 2011) into the trunk or spraying insecticides (thiacloprid) into the affected area (Shin, 2008; Yu et al., 2016; Zhao et al., 1998). However, burning has the potential to cause uncontrollable forest fires that endanger healthy trees and the use of nematicidal chemicals may have adverse effects on forest ecosystems (Siemann et al., 2008; Yi et al., 2015) as well as human health (Koarn et al., 2014). Moreover, the frequent use of commercial nematicides may increase the risk of nematode resistance (Hague and Gowen, 1987). Biological control mainly relies on microbial control. Microorganisms are widely distributed, reproduce quickly, and have a wide variety of species that can produce abundant secondary metabolites for the control of PWN. The fungal genus *Metarhizium* was isolated from the body of *Scleroderma guani* Xiao et Wu, and it can effectively kill *Monochamus alternatus* (Rys et al., 2011). Burg RW et al. selected mice infected with nematodes as test objects for drug screening and obtained avermectin, which is a highly effective anthelmintic drug (Burg et al., 1979). In addition, *Beauveria bassiana*, toxin-producing fungi, pine wood nematode-killing fungi, and *Actinomycetes* provide for microbiological control, and *Aspergillus vitis* and *Serratia marcescens* can be used against *Monochamus alternatus* to achieve environmentally friendly biological control (Otoguro et al., 1988). However, low-cost and environmentally friendly alternative control agents are still needed, and the development of bionematicides derived from actinomycetes has profound significance for forest protection (Shin et al., 2016).

Actinomycetes are a type of gram-positive bacteria with a high percentage of (G + C) (Guo, 2012). They are closely related to human life, with more than 70% of the widely used antibiotics produced by actinomycetes (Demain, 2014; Liu et al., 2000; Wei et al., 2000), and their metabolites have also shown strong nematicidal potential against *B. xylophilus* (Wu et al., 2008). In terms of nematode control, avermectin produced by *Streptomyces avermitilis* has broad-spectrum and efficient nematicidal activity and nikkomycin produced by *Streptomyces* acts as a natural nucloside antibiotic, has good control activity against nematodes (Zhang and Zhang, 2000) and is safe for humans, animals and the environment, similar to polyoxomycin (Hori et al., 1974; Mrozik et al., 1989). Zheng et al. (2012) screened 206 plant endophytic bacteria and identified LCB-3 as a *Brevundimonas diminuta*, which has strong nematicidal activity. Sun et al. (Sung, 2010) confirmed the ability of *Esteya verrucose* (Liu et al., 1999) to kill *B. xylophilus* within 4–5 days, previously reported by Wang et al. (2009), Liu et al. (2019) screened strain AN091965, which was identified as *Streptomyces spectabilis*, and showed that among 5000 actinomycetes, it had high nematicidal activity against *B. xylophilus*. Kang et al. (2021) screened and identified the actinomycete *Streptomyces* sp. 680560, which was toxic to *B. xylophilus*, and found that its secondary metabolite Teleocidin B4 was not only lethal to *B. xylophilus* but also inhibited egg hatching. Based on their uniqueness, superiority and sustainability, nematicidal actinomycetes and their secondary metabolites have been separated and purified and shown huge research value and development prospects.

This experiment investigated the nematicidal activity of 227 actinomycete strains against *B. xylophilus*. A strain with high mortality to *B. xylophilus*, which was designated as *Streptomyces* sp. TCS19-048, was screened out.

2. Materials and methods

2.1. Instruments and materials

The chemicals and reagents used to extract the fermentation supernatant were analytical grade and purchased from Qingchen Chemical...
Table 2. Mortality of 10 strains of actinomycetes against pine wood nematode.

| Strain     | Mortality (%) | Strain     | Mortality (%) |
|------------|---------------|------------|---------------|
| TCS19-028  | 85.1 ± 0.7 e  | TCS19-144  | 87.1 ± 0.8 d  |
| TCS19-048  | 99.6 ± 0.5 a  | TCS19-147  | 94.5 ± 1.4 c  |
| TCS19-063  | 80.8 ± 0.6 f  | TCS19-148  | 97.2 ± 0.8 b  |
| TCS19-116  | 84.6 ± 1.3 e  | TCS19-166  | 80.7 ± 2.1 f  |
| TCS19-138  | 85.4 ± 0.2 c  | TCS19-168  | 85.3 ± 1.8 e  |

Reagent Factory, Hangzhou, China. The methanol (MeOH) for high-performance liquid chromatography (HPLC) analysis was HPLC grade and purchased from Tedia High Purity Solvents Co., Ltd. Ohio, USA. Column chromatography purification was carried out using silica gel (200–300 mesh).

2.2. Actinomycete strains

The strains used in this experiment were collected from Qinghai and provided by Shanghai Pesticide Research Institute. A total of 227 actinomycete strains were purified and cultured on Gao’s No. 1 medium (20 g soluble starch, 0.5 g NaCl, 1 g KNO₃, 0.5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 20 g agar, 1 L distilled water (DW), and pH 7.4) at 28 °C for 7 days. All strains were fermented in broth medium (20 g soluble starch, 10 g glucose, 25 g soybean meal, 1 g beef extract, 4 g yeast extract, 2 g NaCl, 0.005 g K₂HPO₄, 1 L DW, and pH 7.2) and shaken at 200 rpm for 10 min, and the supernatants were stored at 4 °C for the bioassay tests.

2.3. Collection of B. xylophilus

*B. xylophilus* was isolated and purified from the infected masson pine (*Pinus massoniana* Lamb.) in Ningbo, supplied by the Department of Forest Protection, Zhejiang Agriculture and Forestry University, and maintained on *Botrytis cinerea* cultured on potato dextrose agar (PDA) in the dark at 28 °C for 7 days. Before the nematicidal activity test, *B. xylophilus* was extracted by the Baermann funnel method (Ogura and Nakashima, 2002) and washed with sterile water, and then prepared into the suspension containing 5000–6000 nematodes per milliliter (mL).

2.4. Nematicidal activity assay

Two millilitres of the fermentation supernatant were filtered through a 0.45 μm microporous membrane to obtain the fermentation filtrate. In a 24-well plate, 450 μL of the fermentation filtrate and 50 μL of the nematode suspension, which contained a mixture of juvenile and adult nematodes, were added. Each treatment was tested in 3 repetitions with sterilized water, medium was used as a blank, negative control (CK), and abamectin was used as a positive control. The treated plates were placed at 25 °C in the dark, and the mortality of the nematode was counted by microscopic observation every 24 h after treatment. The nematodes were considered dead if their bodies were motionless and straight, even after mechanical touching when transferred to sterile water (Choi et al., 2007). The nematode mortality was calculated according to the Schneeberger-Orelli formula: corrected mortality (%) = [mortality in treatment (%) – mortality in control (%)]/[100 – mortality in control (%)] × 100. Mortality in the full text refers to the corrected mortality (Püntener, 1981).

Strains that displayed corrected activities over 80% to *B. xylophilus* in this nematicidal activity test were further extracted with an equal volume of ethyl acetate, and the organic phase was collected and dried. The organic phase sample was dissolved in dimethyl sulfoxide (DMSO) and diluted with sterile water to obtain a solution with a concentration of 2 mg mL⁻¹. The nematicidal activity assay was conducted according to the same procedures as those used for the fermentation filtrate, except DMSO (50 μL) was used as the negative control.

2.5. Study on the stability of fermentation broth of active strain

The fermentation supernatant of active strain was heated in a water bath for 30 min at 20 °C, 40 °C, 60 °C, 80 °C and 100 °C respectively. After the treatment, they were naturally cooled to room temperature and the nematicidal activity was tested, with untreated fermentation supernatants were used as CK, and each treatment was repeated for 3 times. After 24 h, the mortality was calculated by microscopic examination.

After that, the fermentation supernatant of active strain was adjusted to pH 4, 7, 10 with HCl and NaOH solution, and the culture solution with pH 4 and 10 was precipitated. The supernatants with three pH values were...
extracted with equal volume of ethyl acetate (EtOAc), and the EtOAc phase and aqueous phase were collected respectively. The precipitate was extracted with 80% acetone by ultrasonic overnight, and the extract was collected. The EtOAc and acetone extract phase were subjected to rotary evaporation to obtain crude extract, which was dissolved in DMSO and diluted into a solution with a concentration of 2 mg mL\(^{-1}\).

**B. xylophilus** was treated with aqueous phase, EtOAc phase and acetone phase at different pH values, and the phases of fermentation supernatant without adjusting pH values were used as CK. Each treatment was repeated for 3 times, and the corrected mortality was calculated after 24 h.

### 2.6. Identification of active strains

The morphological characteristics of the *Streptomyces* sp. TCS19-048 strain were observed by the plate insert method (Park et al., 2004; Yan, 1992). The active strain was streaked on Gao's No. 1 medium, and the sterilized coverslip was inserted at a 45-degree angle with tweezers and incubated at 28 °C for 7 d–14 d. The coverslip was then removed, and the morphology of the bacteria was observed using an optical microscope (Leica DM4 B; Leica, GER) and scanning electron microscope (PW-100-011; PW, NL) (Williams and Davies, 1967).
D-Fructose + D-Xylose + D-Inositol - Potassium nitrate
D-Mannitol - Urea - L-Fucoidol + Salicylic acid - L-Fucose

hexane, and dichloromethane (CH2Cl2), and the mycelia were extracted with the following parameters: 1 min at 95°C sterile distilled water. Polymerase chain reaction (PCR) was carried out according to the manufacturer’s instructions. 16S rRNA was amplified with the universal primer set 27F (5′-AGAGTTTGTATCCTGCGCAAG-3′) and 1492R (5′-GTTACCTTGTTACGACTT-3′) in a 20 μl reaction mixture consisting of 1 μl of DNA template, 4 μl of dNTPs (Takara, Otsu, Japan), 2 μl of Taq DNA polymerase (Takara, Otsu, Japan), 2 μl of primers and 11 μl of sterile distilled water. Polymerase chain reaction (PCR) was carried out with the following parameters: 1 min at 95°C for initial denaturation, followed by 30 cycles of 10 s at 95°C for denaturation, 30 s at 50°C for annealing, and 30 s at 72°C for extension, and 4 min at 72°C for terminal extension. Then, PCR products were purified and sequencing was performed by using an Applied Biosystems BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, USA). The obtained sequences were blasted on NCBI (https://www.ncbi.nlm.nih.gov/) and aligned by CLUSTALW software (Larkin et al., 2007). A phylogenetic tree was established using the neighbor-joining (N-J) method in the MEGA 7 program, with the number of bootstrap trials set to 100 (Saitou and Nei, 1993). When the strain was cultured in a constant temperature incubator at 28°C for 7 d and 14 d, the mycelium colour was evaluated using the Inter-Society Colour Council—National Bureau of Standards (ISCC–NBS) colour chart (Kelly, 1964). Physiological and biochemical tests (Ruan, 1977; Waksman, 1961) for cellulose and starch hydrolysis; milk coagulation and peptonization; melanin, urease, and H2S production; gelatine liquefaction; nitrate reduction; temperature, NaCl, and pH tolerance; and sole carbon source and nitrogen source utilization were also performed for the purpose of evaluating the strain.

Based on the 16S rRNA gene sequence analysis of Streptomyces sp. TCS19-048, a molecular phylogenetic analysis was carried out. Total genomic DNA was extracted directly from mycelium with a Gentra Puregene Yeast/Bact. Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer’s instructions. 16S rRNA was amplified with the universal primer set 27F (5′-AGAGTTTGTATCCTGCGCAAG-3′) and 1492R (5′-GTTACCTTGTTACGACTT-3′) in a 20 μl reaction mixture consisting of 1 μl of DNA template, 4 μl of dNTPs (Takara, Otsu, Japan), 2 μl of Taq DNA polymerase (Takara, Otsu, Japan), 2 μl of primers and 11 μl of sterile distilled water. Polymerase chain reaction (PCR) was carried out with the following parameters: 1 min at 95°C for initial denaturation, followed by 30 cycles of 10 s at 95°C for denaturation, 30 s at 50°C for annealing, and 30 s at 72°C for extension, and 4 min at 72°C for terminal extension. Then, PCR products were purified and sequencing was performed by using an Applied Biosystems BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, USA). The obtained sequences were blasted on NCBI (https://www.ncbi.nlm.nih.gov/) and aligned by CLUSTALW software (Larkin et al., 2007). A phylogenetic tree was established using the neighbor-joining (N-J) method in the MEGA 7 program, with the number of bootstrap trials set to 1000 (Saitou and Nei, 1993). When the strain was cultured in a constant temperature incubator at 25°C for 24 h. the J2s were treated with active extracts at 250, 125, 62.5, 31.25, 15.63, and 7.81 μg mL⁻¹. The DMSO treatment was used as a negative control. Each treatment was repeated three times. The mortality and LC₅₀, LC₉₀, and LC₉₀ values of the active extract were calculated after 24 h of constant temperature culture at 25°C.

Note: +, positive; -, negative.

| Utilize as sole carbon source | Results | Utilize as sole nitrogen source | Results | Physiological and biochemical characteristics | Results |
|-------------------------------|---------|-------------------------------|---------|---------------------------------------------|---------|
| D-Glucose                     | +       | Peptone                       | +       | Coagulation and peptonization of milk       | +       |
| L-Arabinoxose                 | +       | Yeast                         | +       | Production of melanin                      | -       |
| L-Rhamnose                    | -       | L-Proline                     | +       | Liquefaction of gelatin                    | +       |
| D-Sucrose                     | +       | L-Arginine                    | -       | Production of urease                       | -       |
| Lactose                       | +       | L-Tyrosine                    | +       | Production of H₂S                         | +       |
| D-Fructose                    | +       | Casein                        | +       | Hydrolysis of starch                       | -       |
| D-Xylene                      | +       | L-Asparagine                  | +       | Decomposition of cellulose                 | -       |
| L-Rhamnose                    | -       | Ammonium Sulfate             | -       | Reduction of nitrate                       | +       |
| D-Mannitol                    | -       | Urea                          | -       |                                             |         |
| D-Inositol                    | -       | Potassium nitrate            | +       | Growth at/with                             |         |
| L-Fucoidol                    | +       | Sodium nitrate               | +       | Temperature range (°C) 10–40               |         |
| L-Fucose                      | +       | NaCl% tolerance (w/v)        | 0–2     |                                             |         |
| D-Sorbitol                    | -       | pH range                      | 4–12    |                                             |         |
| Salicylic acid                | -       |                               |         |                                             |         |

2.7. Isolating nematicidal extract from the cultures of Streptomyces sp. TCS19-048

Streptomyces sp. TCS19-048 was cultured as described in Section 2.2, and the culture broth was obtained and centrifuged to separate the mycelia and supernatant. The supernatant of Streptomyces sp. TCS19-048 was extracted with an equal volume of EtOAc, chloroform (CHCl₃), n-hexane, and dichloromethane (CH₂Cl₂), and the mycelia were extracted with an equal volume of 80% acetone. The extracted organic phases were collected and stored separately, dried at 40°C and dissolved in DMSO to determine their nematicidal activities as described in Section 2.4. As a result, the EtOAc extract was the most active in the test and was used for further isolation.

The concentrated EtOAc extraction was subjected to silica gel column chromatography using a stepwise solvent system of petroleum ether (PE): EtOAc (8:1 to 1:1; v/v) to generate twelve fractions, which were collected separately and then dried at 40°C on a rotary evaporator to complete the nematicidal activity test at a final concentration of 0.5 mg mL⁻¹. The fraction that showed the highest nematicidal activity was separated on a silica gel column eluted with PE: EtOAc (9:1 to 1:1; v/v) and subsequently on a Sephadex LH-20 column eluted with CH₂Cl₂: MeOH (1:1, v/v) as the mobile phase. The active fraction was further purified by HPLC with 65% MeOH to obtain the final active extract. Preliminary structural identification of the extract was carried out using one-dimensional nuclear magnetic resonance ¹H NMR.

2.8. Nematicidal activity assay of active extract

To investigate the nematicidal activity of the active extract against second stage juveniles (J2) of B. xylophilus, the nematicidal activity assay was conducted according to the method presented in Section 2.3. A large number of nematodes and eggs were obtained in a flat-bottomed glass Petri dish using sterile water. After removing the nematode suspension, the dish was washed three times with sterilized water to remove adult nematodes. Hatched B. xylophilus J2s (approximately 250 J2s) were collected and added to the wells of a 24-well plate after the eggs were incubated at 25°C for 24 h. The J2s were treated with active extracts at 250, 125, 62.5, 31.25, 15.63, and 7.81 μg mL⁻¹, and the DMSO treatment was used as a negative control. Each treatment was repeated three times. The mortality and LC₅₀, LC₉₀, and LC₉₀ values of the active extract were calculated after 24 h of constant temperature culture at 25°C.

2.9. Observation of morphological structure of B. xylophilus

B. xylophilus was treated with active extract diluted into a solution with the LC₉₀ concentration. The status of B. xylophilus was observed and photographed under a microscope (Leica DM4 B; Leica, GERM) after treatment at 25°C for 12 h. Treatment with DMSO for 24 h served as the blank control.

2.10. Movement behavior of B. xylophilus

Thirty B. xylophilus J2s were selected and treated with active extracts at 0.25, 0.125, 0.0625, and 0.03125 mg mL⁻¹ in a 24-well plate. DMSO was used as a negative control. After the J2s were incubated at a constant temperature of 25°C for 1 h, 3 h, 6 h, 12 h,
and 24 h, five of them were randomly selected to count the frequency of body fluctuation (the number of complete wavelengths shifted from head to tail), head swinging (the number of times the head was swung from one side to the other and then back to its original position) and body bending (the number of times the body part was bent relative to the long axis of the body) within 1 min (Tsalik and Hobert, 2003). Three trials with three replicates were performed for the whole experiment.

2.11. Body fluid leakage of B. xylophilus

B. xylophilus individuals were treated separately with active extracts at 0.25, 0.125, 0.0625, and 0.03125 mg mL⁻¹, with DMSO serving as a negative control, and the experiment was repeated 3 times. The leakage of nematode body fluid was measured and recorded by electrical conductivity using a conductivity metre after the nematodes were cultured at 25 °C for 1 h, 3 h, 6 h, 12 h, and 24 h (Bai et al., 2011).

2.12. Statistical analysis

The experimental data were analysed using SPSS 20.0 software (SPSS for Windows), and the LC20, LC50, and LC90 values were determined via regression analysis. Data were evaluated using one-way analysis of variance, and differences were considered statistically significant at P < 0.05. Values are expressed as the mean ± standard deviation (SD). Data were mapped using GraphPad Prism 6.01 software.

3. Results

3.1. Nematode bioassay of strains

In the preliminary screening experiment, a total of 227 actinomycete strains were tested for their nematicidal activity. Among them, the fermentation supernatant of 59 strains had a lethal rate higher than 80% after exposure for 72 h and were selected for secondary screening.

Figure 6. Phylogenetic tree of Streptomyces sp. TCS19-048 constructed based on 16S rDNA by neighbor-joining method.
convex, and the surface was velvety, powdery, dry and opaque (Figure 4(A)). The substrate mycelium did not have a transverse septum and was well developed. Aerial mycelia were abundant and branched (Figure 4(B)), and spores were mostly cylindrical (Figure 4(C)).

*Streptomyces* sp. TCS19-048 grew well on Gao's No. 1, ISP 1 (Figure 5A), ISP 2 (Figure 5B), ISP 3 (Figure 5C), ISP 5 (Figure 5E), and ISP 7 (Figure 5G) media but showed weaker growth on ISP 4 (Figure 5D) and ISP 6 (Figure 5F) media and did not produce aerial mycelium on ISP 6 medium only. The specific colour of the intrabasal mycelia, aerial mycelia, and spores and the production of soluble pigments in the medium are presented in Table 3 and Figure 5.

*Streptomyces* sp. TCS19-048 is a gram-positive bacterium. It was cultured on Gao's No. 1 solid medium for 7 d–14 d at different temperatures. It can grow in the range of 10 °C–37 °C, and 28 °C is the optimum growth temperature. The salt tolerance test showed that *Streptomyces* sp. TCS19-048 could grow in a NaCl concentration range of 0–2% (w/v). The pH tolerance range was 4–12. *Streptomyces* sp. TCS19-048 was positive for milk coagulation and peptonization, gelatin liquefaction, hydrogen sulfide detection and nitrate reduction and negative for melanin production, urease, starch hydrolysis and cellulose hydrolysis. In the carbon source utilization experiment. *Streptomyces* sp. TCS19-048 was shown to utilize glucose, arabinose, sucrose, lactose, fructose, xylose, raffinose, fucitol and fucose as single carbon sources but could not use rhamnose, mannitol, inositol, sorbitol, and salicylic acid as carbon sources in the nitrogen source utilization test. *Streptomyces* sp. TCS19-048 could use peptone, yeast powder, proline, tyrosine, casein, asparagine, potassium nitrate, and sodium nitrate as a single nitrogen source for growth but could not use arginine, ammonium sulfate and urea (Table 4).

### 3.3. Taxonomic characteristics of *Streptomyces* sp. TCS19-048

As shown in Figure 4, *Streptomyces* sp. TCS19-048 developed round convex colonies with abundant aerial mycelium and substrate mycelium on Gao's No. 1 medium. The colonies were small, dense, round and

![Figure 7. Mortality of 12 components of *Streptomyces* sp. TCS19-048 against *B. xylophilus* at concentration of 0.5 mg mL\(^{-1}\).](image)

The EtOAc phase extractions of the 59 strains were tested at a concentration of 2 mg mL\(^{-1}\), and 10 strains led to greater than 80% mortality (Table 2). *Streptomyces* sp. TCS19-048 had the highest activity of 99.6% and was selected for further experimentation.

Different solvents (EtOAc, CHCl_3, n-hexane, and CH_2Cl_2) were used to extract the fermentation supernatant of *Streptomyces* sp. TCS19-048, and the nematicidal activities showed that EtOAc extraction had the highest mortality (Figure 1). The CHCl_3 extract of the supernatant and the acetone extract of the mycelia showed considerable nematicidal activities. However, the nematicidal activities of the hexane and CH_2Cl_2 extracts were extremely low.

### 3.2. Stability analysis of fermentation broth of active strain

The results of the stability of fermentation broth of *Streptomyces* sp. TCS19-048 are shown in Figure 2. After the fermentation supernatant was treated at 20 °C (97.37%) and 40 °C (95.40%), the nematicidal activity did not exhibit significant change compared with CK (96.75%), but when it was treated at above 60 °C (91.97%), the nematicidal activity decreased significantly. This indicated that the nematicidal substances in the fermentation supernatant kept good activity even when the temperature rose to 40 °C, so the influence of temperature on it during storage or transportation could be ignored.

As demonstrated in Figure 3, there was no significant difference between the nematicidal activity of EtOAc phase at pH 7 and pH 10 and CK, which proved that the active substances had good stability under neutral and alkaline conditions. The nematicidal activity of pH 7 (35.50%) and CK (36.82%) aqueous phase was significantly lower than that of EtOAc phase (pH 7: 95.40%, CK: 97.36%), which proved that the active substances existed in EtOAc phase. When the pH value was 4, the nematicidal activity of acetone phase (69.85%) was significantly higher than that of EtOAc phase (28.21%), which indicated that the active substance was located in the sediment of supernatant under acidic conditions. When the pH value was 4, the nematicidal activity of acetone phase (69.85%) was the highest, but it was still significantly lower than that of EtOAc phase in the other three treatment groups (CK: 97.36%, pH 7: 95.40%, pH 10: 88.80%), which indicated that the activity of active substances decreased under acidic conditions.

Based on the 16S rRNA gene sequence analysis of *Streptomyces* sp. TCS19-048, a molecular phylogenetic analysis was carried out. A 16S rRNA gene sequence analysis of *Streptomyces* sp. TCS19-048 was conducted via pairwise comparison with the representative sequences of closely related actinomycetes, and the results of the N-J tree indicated that *Streptomyces lactacystinicus* OM-6519 was the closest neighbor, with 99.77% sequence similarity (Figure 6).

### 3.5. Identification and isolation of the active metabolite from *Streptomyces* sp. TCS19-048

According to the above experimental results, the EtOAc extract, which exhibited the highest activity, was isolated by silica gel column chromatography to obtain 12 fractions. After the nematicidal assay, fraction 3 exhibited the highest mortality of 98.8% at 0.5 mg mL\(^{-1}\)
14.48 min) was ultimately identified at a flow rate of 1 min mL⁻¹ and a wavelength of 220 nm.

3.5. Activity of extract S-3-1

The trend of the electrical conductivity of B. xylophilus (Figure 10) at 0.25, 0.125, and 0.0625 mg mL⁻¹ was basically consistent (Figure 11). The leakage of B. xylophilus body fluids was positively correlated with the test time and concentration. The conductivities of all the concentrations increased fastest within 1 h–6 h, and the rising trend was accelerated within 6 h–24 h. In addition, the conductivity of the group treated at 0.03125 mg mL⁻¹ decreased after 3 h of treatment, although the value was still higher than that of the control group. However, the conductivities of the DMSO-treated nematodes were relatively stable at the same time points in the experiment.

3.6. Activity against B. xylophilus J2 of extract S-3-1

The activity of S-3-1 against B. xylophilus was determined after 24 h of exposure, and 250 μg mL⁻¹, 125 μg mL⁻¹, 62.5 μg mL⁻¹, 31.25 μg mL⁻¹, 15.63 μg mL⁻¹, and 7.81 μg mL⁻¹ led to 92.1%, 77.8%, 68.9%, 42.7%, 26.49% and 11.02% mortality respectively (Figure 8). The LC₉₀, LC₅₀, and LC₂₀ values were 238.669 μg mL⁻¹, 38.524 μg mL⁻¹, and 12.190 μg mL⁻¹, respectively.

3.7. Morphological observation after treatment of extract S-3-1

B. xylophilus was treated with active extract diluted into a solution at the LC₉₀ concentration. The results revealed that a structure similar to vacuoles emerged inside the body wall of B. xylophilus after 12 h of treatment with S-3-1 (Fig. 9A and B), and the enlargement of the vacuole-like structure was observed after 24 h (Fig. 9C and D). Meanwhile, the surface structures of B. xylophilus were intact while the inside was in a shrunken state. Conversely, no vacuole-like structures were observed in the control group.

3.8. Movement behavior after treatment of extract S-3-1

Figure 10 showed the effects of extract S-3-1 on the frequency of body fluctuation, head swinging and body bending of B. xylophilus J2s after different treatments at various times. S-3-1 obviously inhibited the frequency of body fluctuation, and the inhibition process was negatively correlated with the processing time and concentration (Figure 10A). The body fluctuation frequency of nematodes decreased insignificantly compared with the control group at 31.25 μg mL⁻¹ of extract S-3-1 at 1 h and 3 h, and the frequency was not significantly different from that of the DMSO-treated nematodes until 6 h. The frequency was lower than that of the control group thereafter, indicating that the higher concentration of extract S-3-1 had a shorter exertion time (P < 0.05). The effect of S-3-1 on the head swinging frequency (Figure 10B) and the body bending frequency (Figure 10C) were similar to that of the body fluctuation frequency.

3.9. Body fluid leakage of B. xylophilus

The trends of electrical conductivities of B. xylophilus at 0.25, 0.125, and 0.0625 mg mL⁻¹ were basically consistent (Figure 11). The leakage of B. xylophilus body fluids was positively correlated with the test time and concentration. The conductivities of all the concentrations increased fastest within 1 h–6 h, and the rising trend was accelerated within 6 h–24 h. In addition, the conductivity of the group treated at 0.03125 mg mL⁻¹ decreased after 3 h of treatment, although the value was still higher than that of the control group. However, the conductivities of the DMSO-treated nematodes were relatively stable at the same time points in the experiment.

4. Discussion

Actinomycetes are generally abundant natural sources, and some of their metabolites have demonstrated nematicidal activities against B. xylophilus, although the associated antibiotics have not had an effect. This study concerned the nematicidal activity of 227 actinomycetes collected in China, out of which 59 strains displayed over 80% corrected mortality against B. xylophilus. Among them, 10 strains were further selected at a concentration of 2 mg mL⁻¹. Streptomyces sp. TCS19-048 had the highest activity. In addition, the nematicidal activity of the most highly active Streptomyces sp. TCS19-048 extract (EtOAc, acetone, CHCl₃, n-hexane, CH₂Cl₂) was tested at 1, 2, and 3 mg mL⁻¹ to identify the most suitable solvent. After 24 h of treatment with EtOAc extract, the mortality of nematodes was 99.6%. The fermentation supernatant of Streptomyces sp. TCS19-048 has good thermal stability, and its activity has no significant decrease at 40 °C, so the influence of temperature on it during transportation or storage can be ignored. By adjusting the pH value of the fermentation supernatant, it is clear that the active substances exist in the EtOAc phase, but not in the water phase, and keep good activity in the neutral environment and alkaline environment with pH 10, but the activity decreases in the acidic environment. Guo et al.
(2017) found that the ethanol extract of Pomegranate (*Punica granatum* L.) rind had significant nematicidal activity against PWN with the mortality of 71.50% in 72 h at the concentration of 1.0 mg mL$^{-1}$, and the aqueous fraction derived from the ethanol extract was more active with the mortality of 80.07% in 72 h than the EtOAc soluble fraction only with the mortality of 30.47% at the same conditions. It suggests that active compounds from different sources may have different properties. The active substance was abundant in the supernatant of actinomycetes and
Screened and identified as a polyether compound (Fig. S1). The signal in the 1H NMR spectrum was not observed after and could not be integrated and may be saturated hydrogens. However, a approximately 120 are polyether compounds produced by the nearly 10,000 polyketone compounds discovered thus far, approximately 74.5% mortality at 6.25, 12.5, 25, 50, and 100 μg mL⁻¹. A vacuole-like structure was observed inside the body wall of nematodes in the test groups compared with the control groups. Extract S-3-1 had an inhibitory effect on the movement behaviour and was able to significantly increase the body fluid leakage of the nematodes. The concentration and treatment time of S-3-1 were positively correlated with the effects on nematodes. Autopsy shows that hyperaemia and swelling are common among animals that die from polyether compound poisoning (Jiang et al., 1990; Novilla, 1992; Todd et al., 1984), and these animals have a large amount of fluid in the body cavity (Van Vleet et al., 1985). Moreover, animals treated with polyether antibiotics will generally show paralysis (Wagner et al., 1983). Previously, avermectin was successfully used to control PWN (P. xylophilus) from South Korea that was able to effectively prevent and control parasitic nematodes in animals, so an effective alternative drug was needed to fight pine infection. Liu et al. (2019) screened an active compound, which was identified as spectinabilin, and it showed significant nematocidal activity against B. xylophilus, with an LC₅₀ value of 0.84 μg mL⁻¹. Kang et al. (2021) found that telodecin B4 showed nematocidal activity with 41.0, 54.8, 56.7, 63.1, and 74.5% mortality at 6.25, 12.5, 25, 50, and 100 μM, respectively after 24 h after telodecin B4 treatment. Extract S-3-1 showed activity against B. xylophilus, with an LC₅₀ of 39.4 μg mL⁻¹, these results proposed that extract S-3-1, spectinabilin and telodecin B4 were toxic to B. xylophilus and represented some promising biological sources for the control and management of nematodes. This is the first report on the effect of polyether compounds on B. xylophilus, and the findings suggest that these compounds could be used as a PWN control agent.

In this study, the yield of the active substance is low, and only the extract S-3-1 has been preliminarily identified. In the following research, on the one hand, the fermentation medium and fermentation conditions can be optimized, the yield of the target compound can be increased, and the production and application of large-scale fermentor can be realized. On the other hand, more identification methods are used to further identify the substance.
clarify its molecular formula and chemical structure, so as to realize mass production and further structural improvement and optimization by chemical synthesis. In brief, for the pragmatic use of S-3-1 as a potential nematicide, additional studies focused on improving the production of S-3-1 and further structural identification and improvement of S-3-1 and field trials are suggested.

Declarations

Author contribution statement
Jia Shen; Chuang Zhang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. 
Zhang Shaoyong: Performed the experiments; Analyzed and interpreted the data. 
Fei Chen; Fan Pei: Contributed reagents, materials, analysis tools or data. 
Sha Zhou, Ph.D; Haiping Lin: Conceived and designed the experiments.

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The authors declare no conflict of interest.

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