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Antimicrobial Metabolites Profile and Inhibitory Activity of *Streptomyces xinghaiensis*-OY62 Isolated from Soil against Indicator Strains

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Authors' contributions

This work was carried out in collaboration between both authors. Author AAO designed the study. Author OMA performed the experiments, collected and analyzed the data, wrote the first draft of the manuscript and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The aims of this study were to screen for potential broad-spectrum antimicrobial-producing actinomycetes from the tropical rain forest of Oyo State, Nigeria, to assess the effects of cultural conditions on antimicrobial metabolites, characterize the metabolites and determine its antimicrobial activity against indicator strains.
Place and Duration of the Study: Department of Microbiology, University of Ibadan, Ibadan, between April 2014 and August 2016.
Methodology: Ten soil samples were purposively collected between April and June 2014 for the isolation of Actinomycetes. The isolated strain was identified culturally and molecularly using 16S rDNA. The effect of cultural parameters on antimicrobial activity was done by a standard method. The antimicrobial metabolites were produced by submerged fermentation. Partial purification was carried out by column chromatography. Chemical characteristics of the metabolites were determined

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by Fourier transformed infra-red spectrometer (FTIR) and gas chromatography coupled with a mass spectrometer. The antimicrobial activity was done by agar well diffusion and macro broth dilution.
Results: Isolate OY62 had broad-spectrum antimicrobial activity and it was identified using its 16S rDNA gene as *Streptomyces xinghaiensis*-OY62 (KU934248). The highest antimicrobial activity against indicator strains was recorded between pH 7 and pH 8, 0.8% (w/v) sodium chloride, at elevated temperature 55°C, casein+KNO_3 as a nitrogen source, starch or absence of carbon source and incubation period of fifteen days. Aliphatic alkene, hydroxyl, carboxylic acids, amides and carbonyls were functional groups detected while thirteen antimicrobial metabolites were characterized. The MIC against indicator strains was between 6.25 mg/L to 12.5 mg/L. *Streptomyces xinghaiensis*-OY62 exhibited broad-spectrum activity against indicator strains.
Conclusion: The observed results showed that potential broad-spectrum antimicrobial-producing strains of *Streptomyces* could be isolated from the soil of southwestern Nigeria, which could be useful in the production of antimicrobials that can inhibit the growth of resistant pathogens, reduce microbial infections and death.

Keywords: Streptomyces xinghaiensis-OY62; submerged fermentation; bioactive antimicrobial metabolites; bis (2-ethylhexyl) phthalate; minimum inhibitory concentration.

1. INTRODUCTION

Streptomyces are Gram-positive filamentous bacteria found predominantly in the soil. They have a morphology cycle that is very complex [1]. They are known to produce a wide range of secondary metabolites including antimicrobial compounds [2]. Most of these secondary metabolites are bioactive in nature. They have unique structures and their formations are regulated by fermentation medium, growth rate, feedback control, enzyme inactivation and induction [3]. They are not useful in the growth or reproduction of the producing microorganism. They are usually synthesized in a consortium of similar metabolites that are chemically different [4]. Examples of secondary metabolites are phenol 2, 4-bis (1, 1-dimethyl ethyl and dibutyl phthalate that had been reported to possess antimicrobial property [5,6]. In addition, aliphatic compounds such as nonadecene and tetradecene have been identified in the solvent extract of different strains of *Streptomyces* [7]. Furthermore, Silber et al. [8] showed that bis (2-ethylhexyl) phthalate possessed inhibitory property against *Micrococcus luteus*, *Vibrio harveyi* and *Pseudoaltermones piscida*. Many of these secondary metabolites are antibacterial, antifungal, anti-tumour in nature and they have found applications in the treatment of human infections [9].

Bioactive compounds have a tremendous impact on human health during the second half of the century [10]. They have been reported to aid mineral absorption, oxidative stress protection, glucose metabolism, lowering of cholesterol, antithrombic, hypotensive, antimicrobial as well as immunomodulatory agents [11]. The relevance of bacteria as natural sources of bioactive compounds has been a subject of interest to pharmaceutical industries [11].

Therefore, due to continuous emergence and re-emergence by pathogenic microorganisms to commercially available antimicrobial drugs, there is a need to search and screen for more antimicrobial metabolites from actinomycetes. It is estimated that these group of bacteria produce more than seven thousand metabolites [12]. Hence, the general objective of the study was to screen for antimicrobial-producing actinomycetes from the uncultivated tropical rain forest of Oyo State, southwestern Nigeria, to profile antimicrobial metabolites produced by *Streptomyces xinghaiensis*-OY62 and to assess its inhibitory activity against some indicator strains.

2. MATERIALS AND METHODS

2.1 Soil Samples Collection

Ten soil samples were purposively collected at a depth of 10-15 cm at different sampling points across Oyo State, Nigeria (7°21'38'N 3°52'22'E, 7°22'18'N 3°48'46'N, 7°54'48'N 3°35'40'E, 7°47'40'N 3°30'32'E, 7°25'08' 3°50'16'E, 7°50'10' 3°52'18'E, 7°52'37'N 3°30'26'E. 7°41'23'N 3°34'20'E, 7°42'15'N 3°40'18'E and 8°42'08' 3°24'36'E). They were transported in a sterile polythene bag to the Microbial Physiology and Biochemistry Laboratory, Department of Microbiology, University of Ibadan, Ibadan, Nigeria.

2.2 Isolation of Actinomycetes

Ten-fold serial dilution was carried out on each of the soil samples using the description of Harrigan and McCance [13]. The isolation was carried out using Pour Plate technique with media that included glycerol asparagine agar, tyrosine agar and starch casein agar [14,15]. The plates were incubated at room temperature (28°C-30°C) for seven days.

2.3 Screening and Indicator Strains

The following indicator strains *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9077, *Enterococcus faecalis* ATCC 29212, *Campylobacter jejuni* ATCC 33291 were used for agar well diffusion assay.

Furthermore, *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 8309, *Salmonella typhimurium* ATCC 13311 and *Pseudomonas aeruginosa* ATCC 9077 were used to determine the MIC and MBC.

2.4 Screening for Antimicrobial Activity of the Actinomycete Isolates

The antibacterial activity of the actinomycete isolates against test strains was done by cross-streaking technique following the procedure of Oskay [16].

2.5 Cultural and Morphological Studies of the Selected Actinomycetes Isolate

The cultural and morphological characteristics of the selected actinomycetes were carried out following the procedures described in International *Streptomyces* Programme ISP by Shirling and Gottlieb [15].

2.6 Molecular Identification Using the 16S rRNA Gene Sequence

Chromosomal deoxyribonucleic acid DNA was extracted using the versatile quick-prep method for genomic DNA [17]. The 16S rRNA analysis of the isolated strains was carried out using the method described by Pospiech and Newmann [17]. The 16S rRNA was amplified using universal primers FC27 (5' to 3') AGAGTTTGATCCTGGACTT and RC1492 (3'to5') ACGGCTACCTTGTTACGACTT, one forward and one backwards. The PCR reaction was done using PCR beads (Qiagen). The PCR mixture of the final volume of 50 µL contained PCR beads, 2 µL for each primer and 10µL template DNA and it was made up to the final volume by distilled water. The PCR programme involved initial denaturation at 94°C primer annealing was programmed at 60°C and primer extension and the cycle of 35 for 60 sec at 72°C. The obtained amplified PCR product of 1.5 µL was sequenced using Big Dye Terminator V3.1. The phylogenetic tree was constructed by the neighbour-joining method using MEGA version 5 software package [18].

2.7 Effect of Cultural Conditions (pH, Sodium Chloride Concentration, Temperature, Carbon and Nitrogen Sources and Incubation Period) on the Antimicrobial Activity of *Streptomyces xinghainesis*-OY62

Starch casein broth (g/l) (soluble starch 15.0, potassium phosphate dibasic 2.0, Potassium nitrate 2.0, Sodium chloride 2.0, Casein 0.30, Magnesium sulphate heptahydrate 0.05, Calcium carbonate 0.02, Iron II sulphate heptahydrate 0.01 at pH 7.2) was used as basal medium [19].

The influence of pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), sodium chloride concentration (w/v) (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0), temperature (°C) (4, 28, 35, 45, 55 and 60), carbon source (w/v) (glucose, galactose, maltose, sucrose, glycerol, starch and control), nitrogen source (ammonium sulphate, peptone, yeast extract, malt extract, casein, potassium nitrate, casein+ potassium nitrate and control) as well as production period in days (3, 5, 7, 10 and 15) on the inhibitory activity of *Streptomyces xinghainesis*-OY62 was assessed by inoculating 500 mL of basal medium with 5.0% (v/v) 24 h old culture of *Streptomyces xinghainesis*-OY62 followed by incubation at 30°C. However, the best-supporting fermentation condition of a previously determined parameter was kept constant in subsequent parameter under study.

The inoculated flasks were placed on an Orbital Shaker (Platform Shaker MSZ-100A) at 150 rpm and incubated for seven days, after which the fermentation was terminated. The fermented broth was filtered using Whatman No. 1 and treated with 50% (w/v) ammonium sulphate, so as to remove interfering proteinous materials

from the antimicrobial extract. The treated filtrate was centrifuged at 5000 rpm for 20 min (Wincom 80-2). Additionally, an equal volume (1:1) of the supernatant and ethyl acetate (Loba Chemicals, India) were strongly shaken together for 30 min in separating funnel to effect the extraction of the antimicrobial metabolites by the solvent. The separating funnel with the mixture was further allowed to stand for another 30 min. The supernatant, solvent contained extract was collected and concentrated at 60°C using Rotary Evaporator (Rotatory Evaporator, RE-52A) [9]. The crude antimicrobial extract was used for the assessment of the cultural conditions using agar well diffusion assay.

2.8 Agar Well Diffusion

The effect of cultural conditions on the antimicrobial activity of the *Streptomyces xinghaiensis*-OY62 against the following indicator strains: *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Campylobacter jejuni* ATCC 33291 and *Pseudomonas aeruginosa* ATCC 9077 was carried out. A well of 6.0 mm diameter was made at the centre of Petri dishes containing Mueller Hinton agar, which had been previously seeded with a 24 h culture of the indicator strains, which had their turbidity adjusted to 0.5 McFarland standards ($1.5x10^8$ CFU/mL). Each well was filled with crude extract (50.0 µL) and the plates were incubated at 37°C (Memmert 854, Schlabach, Germany). The zones of inhibition were read in triplicate using a ruler. The 6.0 mm of the well was subtracted from the values obtained.

2.9 Partial Purification and Characterization of the Antimicrobial Compounds Produced by *Streptomyces xinghaiensis*-OY62

Ten litres of sterile starch casein broth was inoculated with 5.0% (v/v) 24 h seed inoculum of *Streptomyces xinghaiensis*-OY62 and incubated at 55°C. This was followed by separation of the mycelial debris from the fermented broth by filtration using Whatman No.1 filter paper. The filtrate was mixed with ammonium sulphate, 50% (w/v). The ammonium sulphate treated filtrate was centrifuged at 5000 rpm for 20 min, after which the supernatant was mixed with an equal volume (1:1) of ethyl acetate (Loba Chemicals, India) and shaken in a separating funnel for 30 min for the extraction of the antimicrobial metabolites. The separating funnel with the mixture was allowed to stand for another 30 min. The supernatant containing the antimicrobial metabolites was pooled together and concentrated at 60°C using a Rotary evaporator (Rotary evaporator, RE-52A) [9]. The crude antimicrobial extract was transferred to a water bath at 50°C to dry off the°remaining ethyl acetate.

Furthermore, one gram of the crude antimicrobial extract was mixed with 3.0 mL of ethyl acetate. The dissolved crude antimicrobial extract was loaded into a silica gel (100-200 mesh) column chromatography, column dimension 2.0 cm inner diameter x 25 cm length. The column was eluted with n-hexane and ethyl acetate (1:4 v/v) and thirty fractions, 3.0 mL each was collected. All fractions collected were observed for the presence of antimicrobial activity and fractions that exhibited inhibitory activity against indicator strains were pooled together and concentrated at 60°C using Rotary evaporator (Rotary evaporator, RE-52A). The partially purified antimicrobial extract was used for the chemical characterization as well as the minimum inhibitory concentration (MIC) assay against indicator strains.

2.9.1 Chemical characterization of the antimicrobial compounds produced by *Streptomyces xinghaiensis*-OY62

The functional groups present in the partially purified antimicrobial compounds produced by *Streptomyces xinghaiensis*-OY62 was determined using Fourier transformed infrared (FTIR) spectrometer. The partially purified extract was mixed with potassium bromide (KBr). The spectrum obtained from the partially purified antimicrobial compound was recorded on Shimadzu AUX220 spectrophotometer that was in the range of 4000 cm^{-1} to 400 cm^{-1} [20]. Additionally, the chemical structures, formulae and molecular weight of the antimicrobial compounds were determined by gas chromatography-mass spectrometer (GC-MS) Shimadzu QP 2010 [21].

2.9.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration of the partially purified antimicrobial compounds

The Minimum Inhibitory Concentrations (MICs) of the partially purified antimicrobial extract of

Streptomyces xinghaiensis-OY62 was carried out using macro broth dilution method as described by Andrews [22]. The tubes without turbidity after MIC was recorded were streaked on nutrient agar for indicator strains. The streaked plates were incubated at 37°C and were observed for growth after 24 h. The petri dish without growth that was inoculated with the lowest concentrations of the antimicrobial extract was recorded as the minimum bactericidal concentrations (MBCs) of the partially purified antimicrobial extract.

2.9.3 Statistical analysis

Data were collected in triplicates and were subjected to Duncan Multiple Range Test for Mean ± SD.

3. RESULTS

3.1 Isolation, Identification and Antimicrobial Screening against Screening Microorganisms

Seventy-five actinomycetes were isolated from ten soil samples collected purposively across Oyo State, Nigeria. Ten of the actinomycetes exhibited antimicrobial activity, out of which isolate OY62 was observed to have broad-spectrum antimicrobial activity. Table 1 showed that isolate OY62 grew abundantly on all the four media used, aerial and reverse side colours were between brown to golden brown. It produced no diffusible pigment and it was Gram-positive. The 16S rRNA nucleotide sequence of the isolate showed that it has 99% similarity with *Streptomyces xinghaiensis* at NCBI Genbank. The 16S rRNA gene sequence has been deposited at NCBI GenBank and assigned accession number KU934248.

3.2 Effect of pH on Antimicrobial Activity

Table 2 showed that antimicrobial activity was low at extreme pH 4 and 9, with lowest activity against *Pseudomonas aeruginosa* ATCC 9077 (15.3 mm) and *Bacillus subtilis* ATCC (10.7 mm) respectively but reached its best antimicrobial activity between pH 7 and 8. The highest antimicrobial activity was 35.7 mm against *Enterococcus faecalis* ATCC 29212.

3.3 Effect of Sodium Chloride on Antimicrobial Activity

The influence of sodium chloride concentration as presented in Table 3 revealed that a concentration of 0.8% (w/v) gave the highest antimicrobial activity against indicator strains, with *Campylobacter jejuni* ATCC 33291 most sensitive with a zone of inhibition of 28.3 mm and *B. subtilis* ATCC 6633 least sensitive with zone of inhibition of 20.0 mm.

3.4 Effect of Temperature on Antimicrobial Activity

The effect of temperature on inhibitory activity on indicator strains showed that a temperature of 55°C was most suitable for the antimicrobial production and activity by *Streptomyces xinghaiensis*-OY62 against indicator strains (Table 4). A zone of inhibition of 32.7 mm was recorded against *Campylobacter jejuni* ATCC 33292. Low antimicrobial activity was recorded at extremes temperatures of 4°C and 60°C.

3.5 Effect of Carbon Source on Antimicrobial Activity

The result presented in Table 5 revealed that all the carbon sources used in the course of this study gave good antimicrobial activity by *S. xinghaiensis*-OY62 against test strains. However, the production medium without any carbon source also supported the production of antimicrobial compounds and activity with zones of inhibition that ranged between 26.3 mm and 31.7 mm were recorded (Table 5).

Table 1. Cultural characteristics of *Streptomyces xinghaiensis*-OY62

Medium	Growth	Aerial colour	Reverse side colour	Diffusible pigment	Gram's reaction
*ISP2	Abundant	Brown	Golden brown	No	Positive
ISP4	Abundant	Brown	Golden brown	No	
ISP5	Abundant	Golden brown	Brownish-white	No	
SCNA	Abundant	Golden yellow	Brown	No	

**ISP2: Yeast extract malt extract agar, ISP4: Inorganic starch salt agar*
ISP5: Glycerol asparagine agar, SCNA: Starch casein nitrate agar No: No production

3.6 Effect of Nitrogen Source on Antimicrobial Activity

It was observed that the combination of potassium nitrate and casein gave the best antimicrobial activity against indicator strains (Table 6). The antimicrobial activity against indicator strains measured in the zone of inhibition with casein+KNO_3 was between 28.3 mm and 37.7 mm. The activity was very poor in the absence of nitrogen source.

3.7 Effect of Incubation Period on Antimicrobial Activity

The recorded incubation period showed that *Streptomyces xinghaiensis*-OY62 had the highest antimicrobial activities against test strains by the fifteen days of incubation (Table 7). The zones of inhibition against the indicator strains were between 30.0 mm and 37.7 mm by the fifteenth day of fermentation.

3.8 Chemical Characterization of the Partially Purified Antimicrobial Compounds

The IR spectrum of the antimicrobial compounds synthesized by *Streptomyces xinghaiensis*-OY62 had peaked at wave numbers 609.89 cm^{-1}, 653.89 cm^{-1}, 704.04 cm^{-1}, 742.62 cm^{-1} showing the detection of aromatic rings in the compounds (Fig. 2). Also, aliphatic compounds at wave numbers 1039.67 cm^{-1}, 1074.39 cm^{-1} and 1124.54 cm^{-1}, unsaturated alkyl group wave number 1600.97 cm^{-1}, carbonyl functional group at wave number 1828.28 cm^{-1} and amide at wave numbers 3493.20 cm^{-1} and 1728.28 cm^{-1} (Fig. 2).

The antimicrobial metabolites profile of *Streptomyces xinghaiensis*-OY62 according to the GC-MS spectral analysis showed that thirteen compounds were present in the partially purified solvent extract (Table 8). Some of these compounds are Bis (2-ethylhexyl) phthalate, dibutyl phthalate, 1, 2-benzene dicarboxylic acid, bis (2-ethylhexyl) ester, phenol, 2, 4 –bis (1, 1-dimethyl ethyl, 9-Octadeceneamide, (Z) and 9-Octadecenoic acid, methyl ester, (E)- and 1-Nonadecene (Fig 4a-4j).

3.9 Minimum Inhibitory and Bactericidal Concentrations

The MIC and MBC of the partially purified antimicrobial extract against *Bacillus cereus* ATCC 10876 and *Staphylococcus aureus* ATCC 700699 indicator strains was 6.25 mg/L and 12.5 mg/L respectively. However, *E. coli* ATCC 35218, *Salmonella typhimurium* ATCC 13311, *Klebsiella pneumoniae* ATCC 8309 and *Pseudomonas aeruginosa* ATCC 9077 had MIC 12.5 mg/L and MBC 25.0 mg/L respectively (Table 9).

4. DISCUSSION

The morphological and cultural characteristics of *Streptomyces xinghaiensis*-OY62 are similar to the report of Oskay [16] (Table 1). The partial sequence of the 16S rRNA gene of isolate OY62 showed that the isolate had 99% similar nucleotide sequence to other strain of *Streptomyces xinghaiensis* (Fig 1). The genetic and physiological factors of the strains could be responsible for the similarity observed.

The observed pH 7 agrees with the previous reports of Bundle et al. [23], Sarad et al. [24] who had earlier reported optimum antimicrobial production and activities at pH 7. The similarity could be attributed to the structural stability of the enzymes and antimicrobial metabolites at pH 7. Hydrogen ion concentration is believed to influence the activity of enzymes and secondary metabolites production [25].

Sodium chloride has been reported to exert osmotic pressure on the production strain that results to increase in the number of metabolites excreted into the production medium. The observed result is in agreement with the report of Reddy et al. [26]. The similarity could be attributed to the high osmotic pressure which could aid the excretion of antimicrobial compounds from the cells.

The observed elevated temperature was higher than previous reports of Rakesh et al. [27] and Rehman and Mahesh [28] who had previously reported a temperature of 45°C. The difference could be due to different strains used for production, the thermo-stability of the metabolizing enzymes as well as the stability of the antimicrobial metabolites produced at elevated temperature. At such an elevated temperature, the tendency for mesophilic contaminants to survive in the production vessel is reduced and the rate of biochemical reaction is increased thereby reducing the production time.

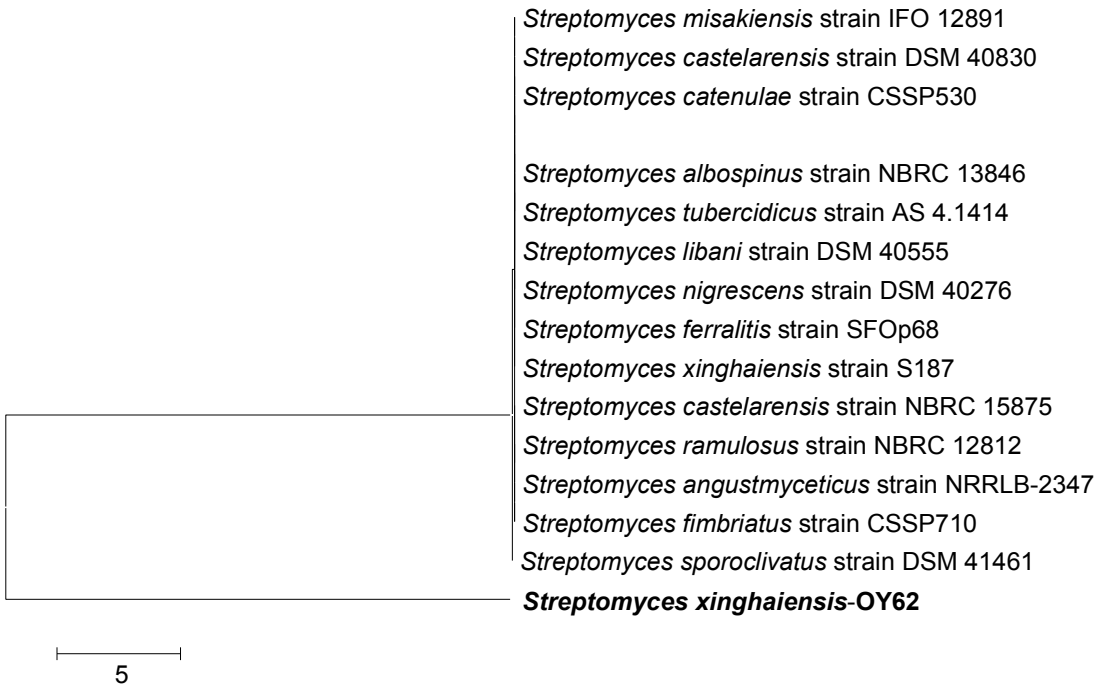

Fig. 1. Phylogenetic tree showing *Streptomyces xinghiaensis*-OY62 relationship to other strains based on the 16S rRNA gene sequence

Table 2. Effect of pH on the antimicrobial activity by *Streptomyces xinghaiensis*-OY62 against indicator strains pH / Zone of inhibition (mm)

Indicator strains	4.0	5.0	6.0	7.0	8.0	9.0
Enterococcus faecalis ATCC 29212	*22.7±0.5^{a}	25.3±0.8^{a}	25.7±0.4^{b}	35.7±0.5^{a}	35.7±0.5^{a}	28.7±0.5^{a}
Campylobacter jejuni ATCC 33291	18.3±0.6^{b}	25.7±0.7^{a}	29.7±0.8^{a}	32.3±0.6^{b}	31.7±0.6^{c}	25.7±0.3^{b}
Bacillus subtilis ATCC 6633	16.7±0.8^{c}	19.7±0.6^{c}	22.3±0.6^{c}	31.0±0.9^{b}	34.3±0.8^{b}	10.7±0.8^{d}
Pseudomonas aeruginosa ATCC 9077	15.3±0.5^{d}	23.0±0.9^{b}	22.3±0.5^{c}	24.3±0.6^{c}	26.3±0.5^{d}	20.0±0.7^{c}

**Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan's multiple range test*

Table 3. Effect of sodium chloride concentration on the antimicrobial activity by *Streptomyces xinghaiensis*-OY62 against indicator strains Sodium chloride concentration (% w/v) / zone of inhibition (mm)

Indicator strains	0.0	0.2	0.4	0.6	0.8	1.0
Enterococcus faecalis ATCC 29212	*12.7±0.7^{c}	12.3±0.3^{d}	11.7±0.4^{d}	14.7±0.5^{c}	27.7±0.4^{a}	22.7±0.6^{b}
Campylobacter jejuni ATCC 33291	10.7±0.6^{d}	13.3±0.8^{c}	14.7±0.5^{c}	26.3±0.3^{a}	28.3±0.5^{a}	23.3±0.4^{a}
Bacillus subtilis ATCC 6633	15.3±0.5^{b}	15.0±0.2b	16.7±0.7^{b}	17.7±0.8^{b}	20.0±0.6^{c}	14.3±0.8^{c}
Pseudomonas aeruginosa ATCC 9077	18.0±1.0^{a}	18.3±0.5^{a}	18.7±0.8^{a}	18.7±0.5^{b}	24.3±0.8^{b}	23.3±0.5^{a}

**Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan's Multiple Range Test*

Table 4. Effect of temperature on the antimicrobial activity by *Streptomyces xinghaiensis*-OY62 against indicator strains temperature (^oC) / zone of inhibition (mm)

Indicator strains	4	28	35	45	55	60
Enterococcus faecalis ATCC 29212	*15.3±0.6^a	23.0±1.0^a	26.3±0.7^a	27.2±0.3^a	29.3±0.4^b	24.3±0.8^a
Campylobacter jejuni ATCC 33291	13.3±0.8^b	18.6±0.3^b	24.7±0.6^b	25.3±0.9^b	32.7±0.2^a	22.7±0.5^b
Bacillus subtilis ATCC 6633	7.3±0.3^c	16.3±0.7^c	21.3±0.4^c	27.7±0.4^a	27.3±0.1^c	13.3±0.3^c
Pseudomonas aeruginosa ATCC 9077	15.7±0.5^a	16.3±0.8ab	26.3±0.2^a	27.3±0.2^a	32.3±0.8^a	13.7±0.4^c

**Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan's multiple range test*

Table 5. Effect of carbon source on the antimicrobial activity of *Streptomyces xinghaiensis*-OY62 against indicator strains Carbon source / zone of inhibition (mm)

Indicator strains	Control	Glucose	Galactose	Maltose	Sucrose	Glycerol	Starch
Enterococcus faecalis ATCC 29212	*32.3±0.6^a	29.7±0.7^a	30.3±0.2^a	32.7±0.5^a	29.0±0.9^a	28.7±0.5^a	37.7±0.5^a
Campylobacter jejuni ATCC 33291	30.7±0.5^b	20.7±0.5^c	20.7±0.8^d	22.3±0.6^c	26.7±0.4^c	19.3±0.8^c	30.3±0.4^b
Bacillus subtilis ATCC 6633	26.3±0.4^c	20.7±0.5^c	22.7±0.6^c	20.7±0.4^d	24.0±0.4^d	20.7±0.6^b	28.3±0.3^c
Pseudomonas aeruginosa ATCC 9077	31.7±0.4^{ab}	23.7±0.6^b	23.3±0.4^b	28.7±0.3^b	30.0±0.7^a	20.3±0.4^b	30.7±0.5^b

**Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan's Multiple Range Test*

Table 6. Effect of nitrogen source on the antimicrobial activity by *Streptomyces xinghaiensis*-OY62 against indicator strains Nitrogen source / zone of inhibition (mm)

0	Control	KNO_3	NH_4NO_3	Yeast extract	Malt extract	Peptone	Casein	Casein+KNO_3
Enterococcus faecalis ATCC 29212	*5.3±0.03^b	14.3±0.8^a	19.7±0.6^a	21.7±0.5^a	32.7±0.5^a	18.3±0.8^a	22.0±0.0^a	37.7±0.6^a
Campylobacter jejuni ATCC 33291	5.7±0.6^b	13.3±0.5^b	13.7±0.7^c	13.3±0.4^c	25.0±0.3^c	13.7±o.6^c	17.0±0.9^b	30.3±0.08^b
Bacillus subtilis ATCC 6633	1.7±0.05^c	12.7±0.2^c	12.3±0.6^d	10.7±0.2^d	22.3±0.8^d	14.3±0.3^b	13.3±0.7^d	28.3±0.3^c
Pseudomonas aeruginosa ATCC 9077	6.3±0.3^a	12.0±1.0^c	17.3±0.8^b	15.7±0.6^b	27.7±0.5^b	14.7±0.5^b	15.0±0.5^c	30.7±0.5^b

**Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan's Multiple Range Test*

Table 7. Effect of incubation period on the antimicrobial activity of *Streptomyces xinghaiensis*-OY62 against indicator strains Incubation period / Zone of inhibition (mm)

Indicator strain	Day3	Day5	Day7	Day10	Day15
Enterococcus faecalis ATCC 29212	*21.7±0.8^{a}	24.3±0.7^{a}	24.7±0.4^{a}	27.7±0.5^{b}	37.7±0.3^{a}
Campylobacter jejuni ATCC 33291	13.0±0.2^{d}	18.3±0.5^{c}	24.0±1.0^{a}	29.0±0.8^{a}	30.3±0.7^{b}
Bacillus subtilis ATCC 6633	17.3±0.3^{c}	18.7±0.4^{c}	20.7±0.4^{c}	26.7±0.6^{c}	30.7±0.5^{b}
Pseudomonas aeruginosa ATCC 9077	20.7±0.6^{b}	21.3±0.7^{b}	23.3±0.9^{b}	27.7±0.2^{b}	30.0±0.8^{b}

**Values are means of triplicate determinations±Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan's Multiple Range Test*

Table 8. GC-MS spectrum of the profile of antimicrobial compounds produced by *Streptomyces xinghaiensis*.-OY62

S/No	*R. Time	Area %	Height %	Molecular weight	Chemical formula	Name
1	8.239	1.31	1.45	210	$C_{15}H_{30}$	1-Pentadecene
2	10.037	2.07	2.12	206	$C_{14}H_{22}O$	Phenol, 2, 5- bis (1,1 dimethylethyl
3	10.623	2.13	2.29	238	$C_{17}H_{34}$	1-Heptadecene
4	12.794	2.04	2.15	266	$C_{19}H_{38}$	1-Nonadecene
5	15.826	5.73	4.54	278	$C_{16}H_{22}O_4$	1, 2 Benzene dicarboxylic acid, bis (2-methylpropyl ester
6	16.351	12.56	11.52	278	$C_{16}H_{22}O_4$	Dibutyl phthalate
7	16.510	3.64	3.08	292	$C_{17}H_{24}O_4$	Phthalic acid, isobutyl 2 pentl ester
8	16.887	1.20	0.95	278	$C_{16}H_{22}O_4$	Dibutyl phthalate
9	16.987	6.53	5.44	334	$C_{20}H_{30}O_4$	1, 2-benzenedicarboxylic acid butyl octyl ester
10	17.259	2.34	1.98	296	$C_{19}H_{36}O_2$	9-Octadecenoic acid, methyl ester (E)-
11	17.409	1.58	1.35	396	$C_{27}H_{56}O$	1-Heptacosanol
12	17.723	1.42	1.42	404	$C_{25}H_{40}O_4$	Phtahlic acid, butyl tridecyl ester
13	17.858	1.72	1.09	404	$C_{25}H_{40}O_4$	Phthalic acid, butyl tridecyl ester
14	20.050	1.61	1.78	281	$C_{18}H_{35}NO$	9-Octadecenamide, (Z)-
15	20.947	44.50	49.30	390	$C_{24}H_{38}O_4$	Bis (2-ethylhexyl) phthalate

**R.time: Retention time*

Table 9. MIC, MBC of partially purified antimicrobial compounds produced by *Streptomyces xinghaiensis*-OY62 and the control (Gentamicin) against indicator strains

	Antimicrobial extract		Gentamicin	
	MIC (mg/L)	MBC (mg/L)	MIC (mg/L)	MBC (mg/L)
Bacillus cereus ATCC 10876	6.25	12.5	0.78	1.56
S. aureus ATCC 700699	6.25	12.5	0.78	1.56
Escherichia coli ATCC 35218	12.5	25.0	3.12	6.25
Klebsiella pneumoniae ATCC 8309	12.5	25.0	0.78	1.56
Pseudomonas aeruginosa ATCC 9077	12.5	25.0	0.78	1.56
Salmonella typhimurium ATCC 13311	12.5	25.0	0.78	6.25

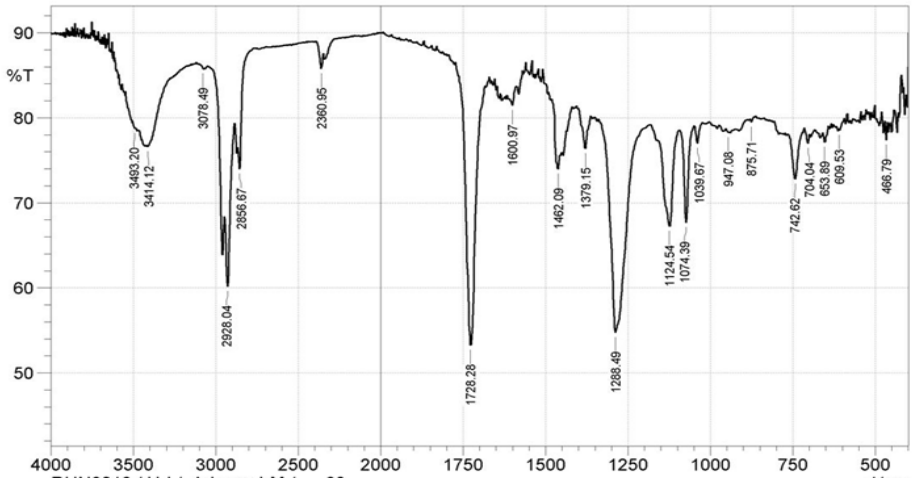

Fig. 2. FTIR spectral analysis of antimicrobial compound produced by *Streptomyces xinghaiensis*-OY62

% T: Percentage transmittance cm^{-1}

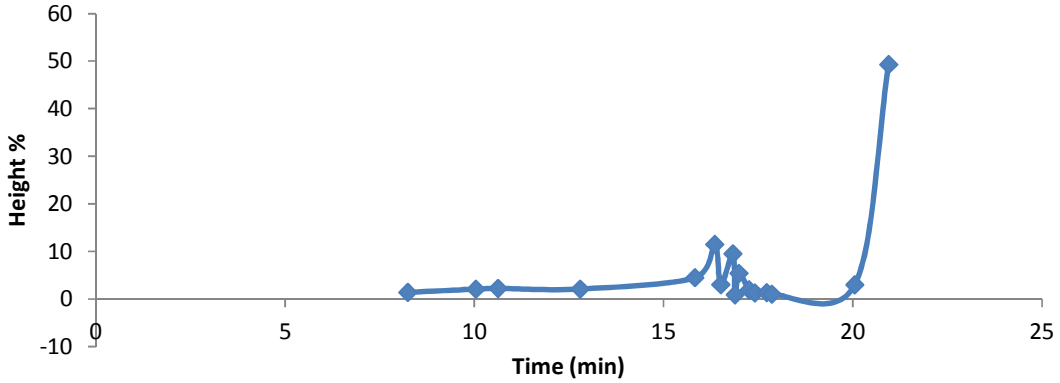

Fig. 3. Separation by GC-MS analysis of antimicrobial compounds produced by *Streptomyces xinghaiensis*-OY62

Furthermore, starch and absence of carbon source supported good antimicrobial activity against indicator strains (Table 5). Starch has been reported to support good antimicrobial production and activity against test microorganisms [29]. However, the production of antimicrobial metabolites in the absence of a carbon source could be due to the possibility of

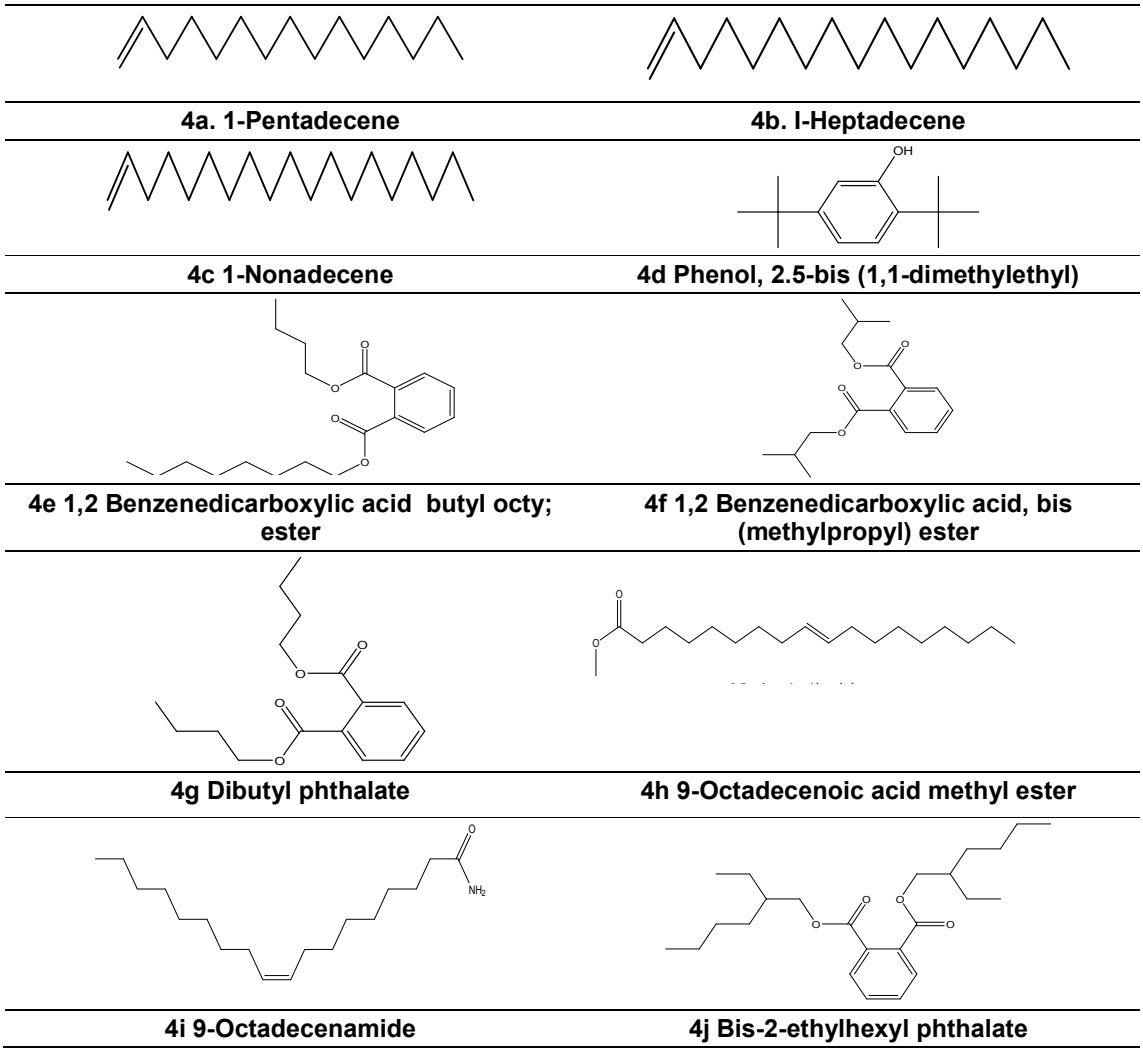

Fig. 4a-4j. Chemical structures of ethyl 5acetate extract of antimicrobial compounds produced by *Streptomyces xinghaiensis*-OY62

Streptomyces xinghaiensis-OY62 might have utilized other organic nutrients in the medium as carbon and energy sources. The use of amino acids such as alanine and valine has been reported to supply carbon skeleton needed in antimicrobial biosynthesis by actinomycetes [30].

The observation in nitrogen source requirement was different from previous reports of Bundle et al. [23] and Sarad et al. [24]. The differences observed could be attributed to a preference for a particular nitrogen source that optimally supported antimicrobial metabolites biosynthesis.

The suitable incubation period for the synthesize of antimicrobial compounds by *Streptomyces xinghaiensis*-OY62 was observed at day fifteen of fermentation as shown in Table 7. This, however, was higher than previous submissions of Bundale et al. [23] and Khattab et al. [31] who had earlier reported between five and ten days respectively. The difference obtained could be as a result of differences in the strains used and the nature of the nitrogen and carbon sources used for fermentation.

The IR spectrum analysis of the partially purified antimicrobial compounds synthesized by *Streptomyces xinghaiensis*-OY62 had

peaked at various wave numbers indicating the presence of functional groups such as unsaturated alkenes, aromatic rings, amide, hydroxyl as well as carbonyl groups (Fig. 2). These are in agreement with earlier reports of Ayari et al., Coates [32,33]. However, the differences observed in wave numbers even, though indicating the same functional groups could be attributed to the spatial arrangement of the molecules and their stretch during vibration [33].

Antimicrobial metabolites such as Bis (2-ethylhexyl) phthalate, dibutyl phthalate, 1, 2-benzene dicarboxylic acid, bis (2-ethylhexyl) ester, phenol, 2, 4 –bis (1, 1-dimethyl ethyl, 9-Octadeceneamide, (Z) and 9-Octadecenoic acid, methyl ester, (E)- and 1-Nonadecene compounds were present in the partially purified solvent extract. Most of these metabolites had been previously reported in the solvent extract of fermented broth of different strains of *Streptomyces* to possess inhibitory properties [6, 21, 34] (Table 3).

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of partially purified antimicrobial compounds produced by *S. xinghaiensis*-OY62 against the test strains ranged between 6.25 mg/L and 12.5 mg/L respectively (Table 4). The results are higher than the previous report of Al-Bari et al [34], Arasu et al. [35]. The differences observed could be due to different strains of indicator organisms and level of purity of the antimicrobial compounds.

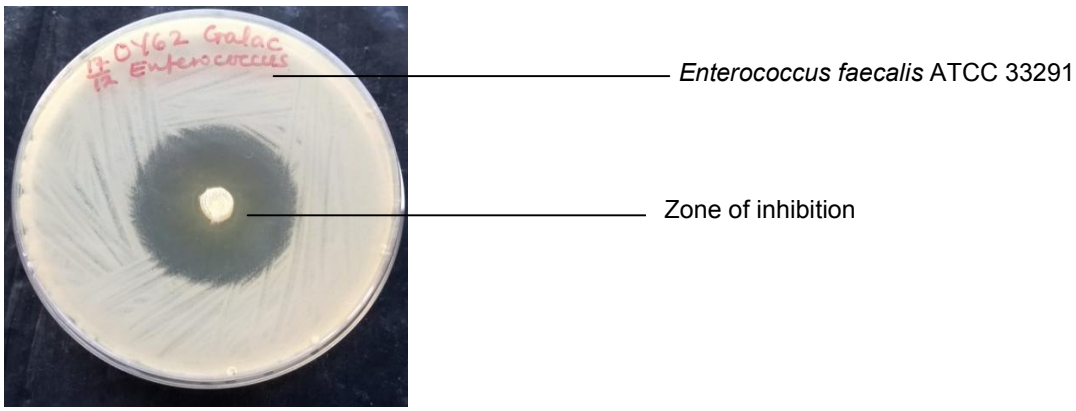

Plate 1. Inhibition of *Enterococcus faecalis* ATCC 33291 by crude antimicrobial compounds produced by *Streptomyces xinghaiensis*-OY62

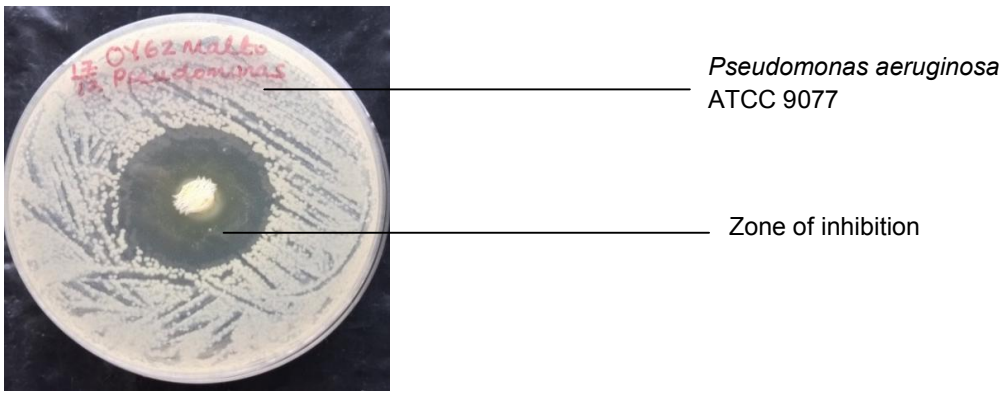

Plate 2. Inhibition of *Pseudomonas aeruginosa* ATCC 9077 by crude antimicrobial compounds produced by *Streptomyces xinghaiensis*-OY62

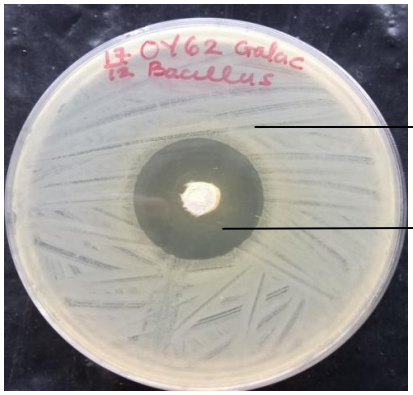

Plate 3. Inhibition of *Bacillus subtilis* ATCC 6633 by crude antimicrobial compounds produced by *Streptomyces xinghaiensis*-OY62

5. CONCLUSION

The outcome of this research study showed that *Streptomyces xinghaiensis*-OY62 (KU934248) was isolated from soil of tropical rain forest of Oyo State, Nigeria. The strain exhibited broad-spectrum antimicrobial activity against indicator strains. Hence, it may be useful in the production of antimicrobial agents which may find application in the in the treatment of microbial infections.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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