Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Isolation and Characterization of Antibacterial Metabolites Produced by *Streptomyces* species from Escravos River, Delta State, Nigeria

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ARTICLE INFO

Article history:
Received 29 June 2017
Revised 09 July 2017
Accepted 12 July 2017
Published online 15 July 2017

Keywords:
Streptomyces,
bioactive compounds ,
test bacteria ,
crude extract ,
antibacterial

ABSTRACT

The isolation of *Streptomyces* sp that yields bioactive molecule is of significant importance in the advancement of new metabolite to combat pathogen. Water, soil and sediment samples were obtained from Escravos River, Delta State. The analysis was carried out using standard culture-based method and polymerase chain reaction approach for identification of the isolates. Of the thirty-six putative *Streptomyces* sp screened using ethyl acetate crude extracts, five showed varying levels of antimicrobial activities against test bacterial isolates used. This five putative *Streptomyces* were further identified by partial sequencing the 16S rDNA amplified polymerase chain reaction (PCR) products. The BLAST analysis revealed 99-100% *Streptomyces* sp similarity in the GenBank nucleotide database. The five *Streptomyces* sp were ESC003 (*Streptomyces griseus*), ESC012 (*Streptomyces griseus*), ESC021 (*Streptomyces* sp.), ESC022 (*Streptomyces fulvissimus)* and ESC030 (*Streptomyces* sp.). Physiological conditions for optimum biomass yields were found to be pH 7 temperature at 28°C incubation days (7-10) and specific rate of metabolite production at day 6 (0.004-0.0045 day^{-1}) from the 5 *Streptomyces* sp characterized. The MIC values unveiled by all the extracts ranged from 0.016 to 0.056 mg/mL. The minimum bactericidal concentration (MBC) of the extracts ranged between 2.5 and >10 mg/mL. On the time kill assay, the utmost reductions in cell population's density were revealed by ESC021 and ESC030 against *Staphylococcus aureus* ATCC 25823. Findings from this study revealed that extracts of the *Streptomyces* isolates from Escravos River have antimicrobial properties with the potential for novel drugs discoveries.

Introduction

Actinomycetes have been described as the basis which forms the significant number of novel antibiotic drugs, with its use in recycling of organic matter and in the treatment of ailment [1-4]. Actinomycetes comprise an extensive amount of the populace of lakes, soil, marine water, fresh water and river muds [5]. Naturally, actinomycetes have been recovered from lithosphere sources. However, the principal documentation of mycelium producing actinomycetes obtained from marine sediments surfaced decades ago. Marine sediments have been described as pioneer origin for the recovery of novel actinomycetes producing new yields and are acknowledged as potential sources of new anticancer and antibiotic agents [6]. Secondary by-products with anticellular activity formed by actinomycetes harbours a broad range of biological activities [7-8].

Streptomyces are regarded as a group of Gram positive filamentous bacteria that are found in diverse natural environmental sources [9-11]. The genus *Streptomyces* alone accounts for a remarkable number of antibiotics. It possesses a massive biosynthetic significance that remains unrivalled without a meaningful competitor among other microbial populations [12]. *Streptomyces* are especially prolific, most economically valuable prokaryotes and can express many antibiotics as well as other class of secondary metabolites that are biologically active with wide spectrum of activity [13-15].

These microorganisms express possibly the utmost diverse, unique and unparalleled, occasionally very complex compounds with excellent antimicrobial potentials and frequently low venomousness [16].

A significant motive for determining new and novel secondary metabolites is to circumvent the menace of resistant pathogenic clones, which are no longer active to the currently used antibiotics [2, 17-18]. The risk associated with the health care system is as a consequence of the persistent and prompt dissemination of the numerous antibiotic resistant bacteria resulting in life threatening diseases and thus the mandate for novel antibiotics continues to heighten [19]. The presence of multiple drug resistant bacteria clones causing extensive mortality and morbidity especially amongst the aged and immunocompromised individuals is worrisome. To outlive this predicament, there is a growing curiosity to develop or discern new class of antibiotics with diverse spectrum of activity and mode of action globally [20]. Though, substantial advancement has been achieved towards the aspect of engineered biosynthesis and chemical synthesis of antibacterial metabolites for new antibiotics, natural sources remain the best and the most resourceful [21].

The increasing numbers of new microorganisms and their by-products obtained from under-explored catchments of the globe such as Jordan [22], Antarctica [23], Atlantic Ocean and certain biotope of Manipur [24] infer that a vigilant examination of novel natural sources might prove to be advantageous. The detection of microorganisms that expresses potent antibacterial metabolites is of significant concern to the advancement of novel drugs to combat the growing number of pathogens, especially with

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https://doi.org/10.26538/tjnpr/v1i1.5

the persistent emergence of intrinsic and acquired antibiotic resistance. This study was extensively carried out in the under-explored Escravos River located in the lowlands of Delta State, Nigeria, with possibilities of isolating Actinomycetes that can produce a secondary metabolite that can add significant value to health care delivery. This study was aimed at characterizing and elucidating the antimicrobial potential of *Streptomyces* species from the aquatic milieu towards test bacteria isolates.

Materials and Methods

Study Area

The Escravos River is situated in the South-South geopolitical zone of Nigeria, which flows for 57 km joining at the Bight of Benin of the Gulf of Guinea where it runs into the Atlantic Ocean.

Sample Collection

Water, Soil and sediments samples were obtained from the Escravos River located in the lowlands of the South-South geographical region of Nigeria. Water samples were obtained from depths at the knee level as tides pushes the water in from the ocean into sterilized 4 L bottles, while sediment samples were obtained from 2 ft deep dug-outs with the aid of a sediment grab and transferred into sterile polythene bags. No specific permits were required for these field studies. All samples were conveyed in ice packets to the laboratory for analyses at the University of Benin, Benin City, Nigeria.

Isolation of the Actinomycetes

The crowded plate method was used as previously described by Singh *et al*. [16] with modification. Each sample (Water, sediment and soil) was diluted to obtain 10^{-1} to 10^{-6} dilutions. One hundred microliter (100µL) of each diluent from each sample was placed in Petri dishes containing solidified Starch Casein Agar (SCA) prepared with the addition of 1.0 mg/mL of sodium penicillin and 5.0 mg/mL of Griseofulvin. Plates were incubated at 28 ± 2ºC for 14 d.

Identification of Streptomyces Isolates

The actinomycetes isolates were phenotypically categorized based on dryness, roughness, colour, regularity of margin, convex colonial shapes and sizes, spore formation, hard leathery colonies, (and once existent), aerial mycelia and diverged asexual mycelia as defined by Ghanem *et al*. [25] Other parameters investigated include colour of substrate mycelium, aerial mycelium, melanin pigment formation, reverse side colour, and formation of diffusible pigmentation were used for morphological identification.

Characterization of the Streptomyces Isolates

Biochemical characterization carried out on the Streptomyces isolates were Voges Proskauer reaction, citrate utilization, catalase production, production of H_2S from triple sugar iron (TSI) agar, indole production, oxidative/fermentative utilization of glucose, urease, methyl red and sugar fermentation. These tests were conducted in accordance with the methods of Cheesbrough [26]. Other test adopted include lipid hydrolysis, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, melanin formation, nitrate reduction, 3%, 5%, 7%, 9% and 11% NaCl tolerance and utilization of nitrogen sources.

Test Bacteria isolates

The test bacteria isolates used in this study comprise of both Gram positive and Gram negative isolates. They were obtained from culture collection bank in the Applied Microbial Processes & Environmental Health Research Group Laboratory, Department of Microbiology, University of Benin, Benin City. They include *Staphylococcus aureus* ATCC 12600, *Staphylococcus aureus* ATCC 25823, *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* ATCC 7644, *Listeria monocytogenes* Scott A, *Staphylococcus aureus* ATCC 12598, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 29214, *Proteus vulgaris* ATCC 15315, *Salmonella enterica* serovar Typhimurium ATCC 14028 and *Shigella flexneri* ATCC 120222.

Preparation of Test Bacteria Inocula

All test bacteria were cultivated on Tryptone Soy broth (TSB) and incubated for 24 h at 37ºC. Thereafter, a total of 10 mL of the cultivated strain in TSB was centrifuged to remove the cell debris, washed twice with sterilized normal saline and the suspension accustomed to attain an optical density of 0.1 at 600nm which corresponds to cell population equivalent to 10^6 cells/mL on the McFarland turbidity standard [27]. Prepared bacterial suspensions were kept in test tubes with screw caps and store at 4ºC until ready for use. The respective test bacteria suspensions were tested for viability 24 h prior to usage and in instances where cell concentrations are brought down underneath the 10^6 cells/mL, fresh customary suspension are organized.

Determination of Antimicrobial Potential of Streptomyces Spot Inoculation on Agar Medium

The antimicrobial potential was conducted using the spot inoculation technique on Yeast Malt Extract agar as described by Singh *et al*. [16] with modification. The petri plates were spot inoculated with the *Streptomyces* isolate and incubated for a period of seven (7) days at 28°C and thereafter upturned for 40 min over chloroform in fume cupboard. *Streptomyces* colonies were then enclosed with a 0.6% agar layer of tryptone soy agar (TSA) previously sown with bacterial suspension of 1.5×10^8 colony forming units (CFU/spores)/ml in sterilized physiological saline [28] of the bacterial strains of interest and then incubated for 24 h at 37°C. The clear zones of inhibition were observed after incubation. Thereafter, five (5) *Streptomyces* isolates that exhibited maximum effective action centred on clear zones of inhibition were carefully chosen for this study. The isolates were implied as follows; ESC003, ESC012, ESC021, ESC022, ESC030.

Effect of Temperature, pH and Incubation Period on Growth and Production of Antimicrobial Agent

The influence of temperature on proliferation and assembly of antimicrobial material was investigated on yeast malt extract broth at varying temperatures (25 to 37°C) at pH 7. Five millilitre (5 mL) of spore suspension from the *Streptomyces* isolate was introduced into 100 mL of yeast malt extract broth in 250 mL Erlenmeyer flask and incubated in a shaker sustained for 7 d at 150 rpm as previously described by Singh *et al*. [16] with modification. The antimicrobial activity was elucidated against the test bacteria via agar well diffusion method [29]. Likewise, the influence of pH on the antimicrobial agent produced was studied at dissimilar pH (5, 6, 7, 8 and 9) using yeast malt extract broth incubated for 7 d at 28°C. The effect of incubation time on the antimicrobial metabolite produced was also evaluated by incubating the culture for 1 to 10 d at 28°C using yeast malt extract broth at pH 7.

Estimation of Growth Potential of the Streptomyces species

The cells from the cultivated leftovers separated through centrifugation was moved to a pre-weighed moisture-free filter paper with the aid of a sterilized spatula and then kept in a hot air oven overnight at 50°C to attain a specified weight. Growth with respect to biomass accumulated was reported as mg/mL of cultivated medium.

Specific Rate of Product Formation (qp)

The specified production ratio of the antimicrobial agent (qp) was estimated in accordance to the succeeding equation below:

$$qp = \frac{1}{X}\left(\frac{dp}{dt}\right)$$

Where X equates concentration of biomass or cell (mg/mL), t equates time and p equates concentration of antimicrobial agent respectively. The dp/dt was estimated in accordance to the process adopted by Le Duy and Zajic [30].

Extraction of Genomic DNA, PCR Amplification of streptomycete 16S rDNA and sequencing

Presumptively identified *Streptomyces* genomic DNA was extracted from the 5 potent *Streptomyces* isolates by applying the genomic DNA extraction kit (Biobasic Inc. Canada) as described by the manufacturer's protocol. The amplification procedure for the *Streptomyces*-specific primers StrepB/StrepF was elucidated in 50 µL volumes which contains: 0.25 µL of the forward and reverse primer, 25 µL of Econo Taq PLUS GREEN 2× Master Mix, 22.5 µL DNAse free water and 2 µL (10 ng) of template DNA as previously elucidated by Rintala et al. [31]. The primer pairs StrepB 5'-ACAAGCCCTGGAAACGGGGT-3' (forward) StrepF 5'-ACGTGTGCAGCCCAAGACA-3' (reverse) amplified 1070 bp fragments, nucleotides 139–1212 of the 16S rDNA gene *S. ambofaciens*; numbering by Pernodet et al. [32]. PCR was carried out in a Peltier-Based Thermal Cycler (Bio-Separation System, Shanxi, China), with an initial denaturation for 5 min at 98°C, denaturation (30 cycles) for 1 min at 95°C, primer annealing for 40 s at 58°C and primer extension for 2 min at 72°C; with a final extension for 10 min at 72°C. The amplified products were separted on agarose gel 1.2 % w/v in 1× TBE buffer (pH 8.3), stained with ethidium bromide (0.5 µg/mL), for 60 min at 100 V and imaged under a UV transilluminator (EBOX VX5, Vilber Lourmat, France). The PCR

amplified 16S rDNA gene products were cleaned up by QIAquick PCR cleanup kit (Qiagen) according to manufacturer's protocol. The 16S rDNA gene was sequenced by using the PCR products directly as sequencing templates with the following primers, StrepB/StrepF by Rintala *et al*. [31]. All sequencing reactions were sequenced with an Automated DNA sequencing Analyzer (ABI 3730X). The comparison of the 16S rDNA gene sequences of the isolated actinomycete was determined by Basic Local Alignment Search Tool (nucleotide BLAST) according to the method of Altschul *et al*. [33], for similarity searching of GenBank nucleotide database.

Preparation of Streptomyces Suspension

The *Streptomyces* suspensions were primed by re-suspending a loop-full of purified *Streptomyces* isolate on 2 mL sterilized physiological saline, homogenize the mixture by vortex and kept at 4ºC until ready for use. This suspension served as *Streptomyces* inoculums in all cultivations where applicable.

Fermentation and Preparation of Crude Ethyl Acetate (EtOAc) Extracts

Fermentation for antibiotic production and successive extraction of the antibiotics from the potent *Streptomyces* sp was conducted as designated by Ilic et al. [34] with slight modification. Twenty (20) mL of prepared yeast malt extract broth (YMB) was allotted into 100 mL capacity sterilized Erlenmeyer flask, and left to cool. It was thereafter inoculated with the initially prepared 0.5 mL *Streptomyces* suspension, incubated for 48 h at 28ºC for 230 rpm. Five hundred (500) mL of YMB was then introduced in 1L Erlenmeyer flask, injected with the *Streptomyces* pre-culture that is 48 h old and incubated at 230 rpm and 28°C for 10 d. After the incubation period, the culture was reaped via centrifugation for 15 min at 14000 rpm. The supernatant was removed twice and added with *equivalent* volumes of ethyl acetate (1:1 v/v) and evaporated to aridity at 50°C in a rotary evaporator. The carefully obtained extract was re-suspended in 50 % filter decontaminated ethyl acetate to acquire the anticipated concentration at each screening stage.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Extracts

The MIC and MBC was carried out with the aid of the broth micro dilution method as previously exemplified by Eloff [35]. Moreover, to balance this assay, the growth of the bacterial was confirmed with the addition of 50 µL of 0.2 mg/mL solution comprising 2[4-iodophenyl]3[4-nitrophenyl] -5-phenyl-2H-tetrazolium chloride (INT) in individual test well, the plate were incubated for additional 1h at 37°C to guarantee sufficient colour improvement. The least concentration at which a definite reduction in colour was observed was considered as the MIC of the potent extract to the corresponding test bacterium. The MBC was elucidated by streaking on TSA plates from wells that revealed growth reduction/inhibition. Streaking was carried out in triplicates per well that revealed growth reduction/inhibition to improve susceptibility and precision. The concentration of potent extract in individual wells where there was no growth on plate was regarded as the MBC.

Determination of Rate of Kill

The procedure of Spangler et al. [36] was followed with slight modification. For the time-kill assay, susceptible bacteria were selected. Viability of the test bacteria was determined first. Test inocula were organized from 100 µL aliquots of the test bacteria suspension in sterilized physiological saline and confirmed by carrying out colonial counts. Volume of suspension of about 80 µL of acknowledged cell density of prepared carefully chosen test bacteria was incorporated to 10 mL of TSB in Bijou bottles of respective amount relative to the MIC of individual extracts. Time kill assay of individual extract towards the respective carefully chosen test bacteria were carried out following the concentrations of the extract: MIC, 2 × MIC, 3 × MIC and 4 × MIC and the reactants bottles were incubated at 37°C. The time kill kinetics was carried out at 0, 3 and 6 h. Approximately 0.5 mL volume of the reaction combination was taken at the right time and conveyed to 4.5 mL of TSB recovery medium encompassing 3 % "Tween-80" to nullify the activity of the extracts surpluses from the test bacterial suspensions and serially diluted in sterile TSB. Exactly 100 µL aliquots of each diluent were plated out for viable counts by spread plate method. Each procedure was carried out in duplicate, while the mean of respective indistinguishable results was estimated. Only plates which produce an average of 300 colonies and less were carefully chosen for calculation. Growth data were recorded as $\log_{10}$ cfu/mL. McCartney bottles which contains broth and the test bacteria devoid of extract was adopted as growth control in corresponding procedures.

Results and Discussion

Preliminary Screening of Tentative Isolates of Streptomyces for Antibacterial Activity, and Characterization of the Streptomyces spp.

Terrestrial and aquatic environments (comprising of soils, rivers, lakes) have been the main areas of research for the isolation of antimicrobial compounds and have been an important source of bioactive compounds. Multidrug resistance has continuously been a challenge that has led to researches on new improved drugs from various sources [2]. Antimicrobial resistance has posed a major challenge to medicine; it has cost the pharmaceutical industries a lot of finance in research and development [29]. Great contributions of new drugs from natural sources have come from countries like Japan, United States, India, China and Germany; with little report from Africa.

Antimicrobial metabolites have been a major interest in medicine and science as a whole and it involves the production of drugs that have the potential of inhibiting the growth of pathogenic organism. Apart from their ability to inhibit the growth of pathogenic organisms, they also have therapeutic functions like protection against lethal photo-oxidation, environmental stress and cofactors. This source of metabolite is simpler to obtain when compared to others and there is more opportunity for gene manipulation [37].

The present investigation was to assemble a detailed record of the *Streptomyces* sp found in Escravos River and its bioactive metabolites, from the aquatic milieu. The putative *Streptomyces* species isolated in the study was 36 and was coded accordingly. The cultural, biochemical and morphological characterization of the *Streptomyces* sp isolates is shown in Table 1. The findings of preliminary screening are presented in Table 2. Of the thirty-six (36) putative *Streptomyces* species screened, five revealed fluctuating levels of antibacterial activities towards the eleven test organisms which included *S. aureus* ATCC 25823, *S. aureus* ATCC 12600, *S. aureus* ATCC 29213, *S. aureus* ATCC 12598, *B. cereus* ATCC 14579, *E. coli* ATCC 29214, *P. vulgaris* ATCC 15315, *L. monocytogenes* ATCC 7644, *L. monocytogenes* Scott A, *S. enterica* serovar Typhimurium ATCC 14028 and *S. flexneri* ATCC 120222. ESC003 and ESC012 showed activity against *P. vulgaris* ATCC 15315, while ESC012, ESC022, revealed activity towards *S. aureus* ATCC 29213. Isolate ESC021 and ESC022 unveiled activity towards *S. aureus* ATCC 12600. Isolate ESC021 and ESC030 was active against *S. flexneri* ATCC 120222 and *S. aureus* ATCC 25823.

The five (5) *Streptomyces* sp were further identified using polymerase chain reaction (PCR) and sequenced. The gel electrophoresis of the 5 potent *Streptomyces* species using *Streptomyces*-specific primer targeting the 16S rDNA sub region is shown in Figure 1. Comparison and BLAST analysis of the 16S rDNA gene sequences substantiated that these isolates were meticulously or wholly identical (99% to 100%) to previously recounted *Streptomyces* in the Genbank database. The 16S rDNA gene sequences of the five isolates revealed their % distinctiveness. ESC003 revealed 100% distinctiveness with *Streptomyces griseus*, ESC012 100% with *Streptomyces griseus*, ESC021 showed 99% with *Streptomyces* sp., ESC022 99% with *Streptomyces fulvissimus*, and ESC030 shown 100% with *Streptomyces* sp. ESC003 was isolated from water samples while ESC012, ESC021, ESC022, and ESC030 were isolated from sediment samples (Table 3).

The proportion of antimicrobial activity of the potent extracts in this study is lesser than those previously described [34, 38-40]. However, similar findings were observed from other authors [41-42]. On a similar note, antibacterial activity was revealed by extracts of marine *Streptomyces* [43-44]. The spectrum of activity unveiled by all extracts pinpoints their capabilities and proposes that they could be functional as antibiotics in this capacity. Generally, this research provides the principal facts on the antibacterial property of native *Streptomyces* detected from the Escravos River.

The modifications in the sensitivities of Gram negative and Gram positive bacteria strains in the description of extracts from *Streptomyces* have been elucidated by previous researchers [34]. Gram negative bacteria have been reported to be more inherently resistant to antibiotics compared to their Gram positive counterparts. It has been credited to the collective segregation of antimicrobial metabolites by transmembrane efflux pumps and double membrane obstruction present in this group of microorganisms [45]. Zarina and Nanda [46] reported that Gram positive bacterial isolates were more susceptible to metabolites from actinomycetes than Gram negative strains in both primary and secondary screening.

Table 1: Cultural, biochemical and morphological characterization of *Streptomyces* spp isolated from Escravos river

Test	*Streptomyces* spp.
Substrate mycelium	Dark yellow
Aerial mycelium	Dull white
Pigmentation	Nil
Colour of aerial mycelium	Coffee brown
Spore morphology	Spiral
Spore mass	Dark grey
Colour of substrate mycelium	Dark grey
Oxidase	-
Catalase	+
Melanin production	-
Urease	+
Starch hydrolysis	+
Gelatin hydrolysis	+
3% NaCl tolerance	++
5% NaCl tolerance	++
7% NaCl tolerance	+++
9% NaCl tolerance	-/+
11% NaCl tolerance	-
Nitrate reduction	-
Lipid hydrolysis	+
Voges proskauer	-
Casein hydrolysis	+
Indole production	-
Methyl red	-
H_2S production	-
Citrate utilization	+
Starch	++++
Mannitol	++
Maltose	+++
Dextrose	++
Raffinose	-
Cellulose	+
Rhamnose	+
Sucrose	+
Fructose	+
Xylose	+
Galactose	+
Mannose	+
L-tyrosine	+++
L-arginine	+
D-alanine	++
L-phenylalanine	+++

Legend: + = present/positive; ++ = moderately present; +++ = highly present; - = negative; -/+ = low present

Antibacterial potential of crude product from the Streptomyces sp

Extract from ESC003 revealed antibacterial properties towards 1 test bacteria recording 16±0.12 mm as zone of inhibition, while ESC012 and ESC021 extracts were active towards 2 and 3 test bacteria with zones of inhibition ranging from 14±0.01 to 21±0.05 mm respectively. ESC022 and ESC030 extracts were active towards 2 test bacteria each recording zones of inhibition within the range of 12±0.14 to 20±0.13 mm respectively. In addition, the streptomycin antibiotics showed zones of inhibition measuring between 10.0±0.01 and 18.0±0.04 mm (Table 4). Generally, some metabolites of respective putative *Streptomyces* harbour antibacterial potentials towards Gram positive bacteria strains, with staphylococci inclusive. Staphylococci in this regard are amongst frequently faced etiological agents in human and veterinary medicine principally *S. aureus*. *S. aureus* is known to be a pioneer cause of food poisoning, hospital infections, pyoarthritis, toxic shock syndrome, endocarditis, osteomyelitis, and other disorders [47]. At present, a worrisome upsurge in staphylococcal infections has been documented by strains that are multidrug resistant. Currently, this menace has translated to the assessment of methicillin resistant staphylococcal pathogens in food animals that potentially harbours resistant and virulent determinants [48].

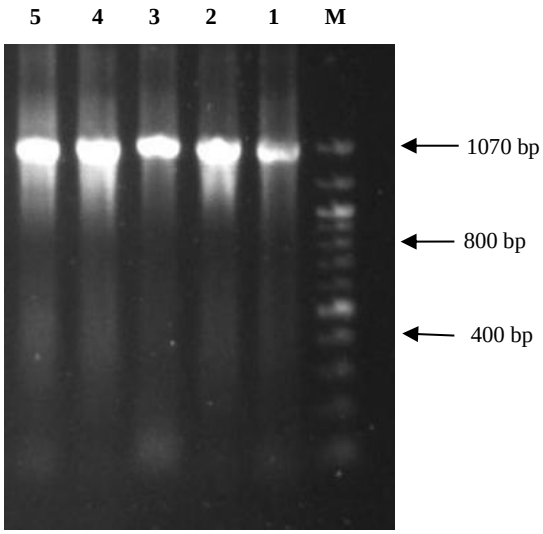

Figure 1: Amplified PCR product of *Streptomyces* species 16S rDNA gene on 1.2% agarose gel. M: Size maker DNA Ladder (100 bp). Line 1=ESC003, Line 2=ESC012, Line 3=ESC021, Line 4= ESC022, Line 5= ESC030 which represents the potent *Streptomyces* species.

Hence, extracts from this study have strong potentials in applications that might circumvent the ever-increasing antibiotic resistance associated with infectious diseases.

Effect of pH, Incubation Period and Temperature on the Putative Streptomyces species

The effect of pH, incubation period, and temperature on YMB was studied. The *Streptomyces* species were observed to be mesophilic in nature as optimum antibiotic activity and zones of inhibition was observed at the mesophilic range (28°C) (Figure 2a,b). More so, the maximum antibiotic production was observed at pH close to neutrality where zones of inhibition were highest pH 7 (Figure 3a,b). With respect to the incubation days, optimum antibiotics were produced by the potent *Streptomyces* species between day 7 to day 10 (Figure 4a,b). The effect of NaCl on *Streptomyces* growth revealed that optimum growth was observed at 7% NaCl. The maximum values for the specific rate of antibiotic produced (0.004-0.0045 day^{-1}) were observed at day 6 (Figure 5). Other researchers also reported that optimum antibiotic production was observed at pH 7 near neutrality [16, 49], temperature between 28 and 37°C [16, 49-50], and antibiotic production optimum at day 6 [16]. Song *et al*. [49] however revealed that antibiotic formation time was at day 4; contrary to the observation of this study.

Minimum Inhibitory Concentration (MIC) of the Streptomyces sp on Test Bacterial Isolates

The MIC values unveiled by all the extracts ranged from 0.016 to 0.056 mg/mL (Table 5). The minimum bactericidal concentration (MBC) of the extracts ranged between 2.5 and >10 mg/mL (Table 6). The MIC and MBC of the crude extracts highlights that the extracts are potent towards Gram-positive and Gram-negative bacteria strains. The MICs unveiled by all extracts in the present study stretched from 0.016 mg/mL to 0.056 mg/mL. This was somewhat greater than the MIC standards reported by El-Gendy et al. [51] towards *B. subtilis* ATCC 6051 (0.0036 mg/mL), *S. aureus* ATCC 6538 (0.0008 mg/mL) and *M. luteus* (0.002 mg/mL); and within range for Sparsomycin produced by *Streptomyces* sp. It was also higher than the MIC values obtained by Singh *et al*. [16] which stretched from 0.5 µg/mL to 3 µg/mL. Ambavane et al. [4] also reported less MIC values ranging from 0.39 µg/mL to 1.56 µg/mL. AZ-NIOFD1 recovered from the Nile River, Egypt [52] had MIC range from 0.0117 mg/mL to 0.03125 mg/mL. However, Pandey *et al*. [53] mentioned that the MIC is not a persistent factor for a particular agent, as it is subject to different factors which range from the nature of the size of the inoculum, the strain tested, the duration of incubation, the content of the aeration adopted, and the cultivation medium. The screening procedures also contribute significantly towards the MIC observed. More so, it must be noted that the respective extracts are in their crude forms and thus reveals a strong potential of the discovery of new antibiotics.

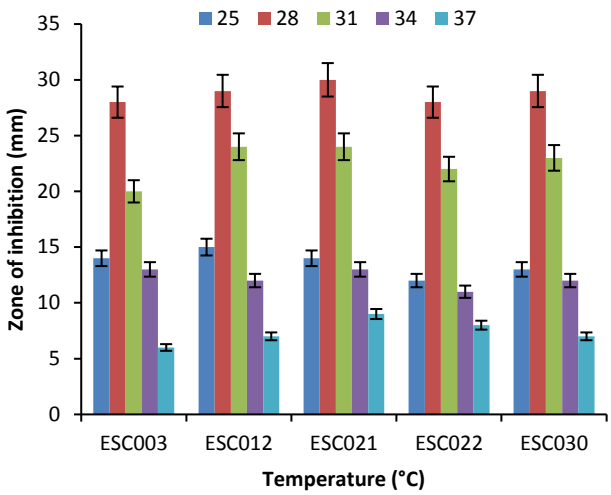

Figure 2a: Effect of incubation temperature on antimicrobial agent produced from the *Streptomyces* sp

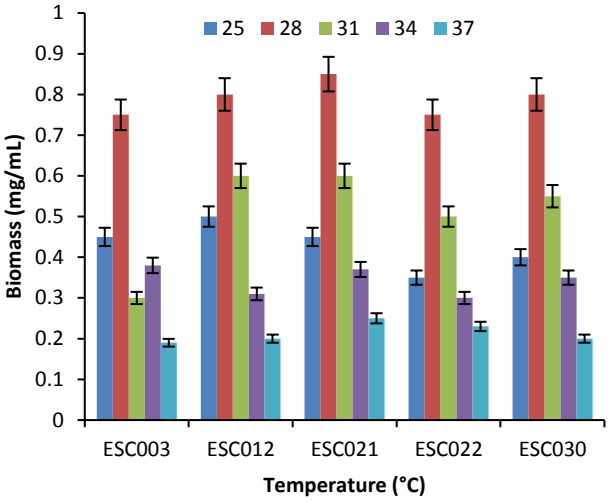

Figure 2b: Effect of incubation temperature on growth by the *Streptomyces* sp

Table 2: Primary evaluation of the antibacterial profile of the *Streptomyces* species against some selected bacteria of clinical isolates.

Test Streptomyces	**Antibacterial activity**										
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**
ESC001	-	-	-	-	-	-	-	-	-	-	-
ESC002	-	-	-	-	-	-	-	-	-	-	-
ESC003	-	-	-	-	-	-	+	-	-	-	-
ESC004	-	-	-	-	-	-	-	-	-	-	-
ESC005	-	-	-	-	-	-	-	-	-	-	-
ESC006	-	-	-	-	-	-	-	-	-	-	-
ESC007	-	-	-	-	-	-	-	-	-	-	-
ESC008	-	-	-	-	-	-	-	-	-	-	-
ESC009	-	-	-	-	-	-	-	-	-	-	-
ESC010	-	-	-	-	-	-	-	-	-	-	-
ESC011	-	-	-	-	-	-	-	-	-	-	-
ESC012	-	-	+	-	-	-	+	-	-	-	-
ESC013	-	-	-	-	-	-	-	-	-	-	-
ESC014	-	-	-	-	-	-	-	-	-	-	-
ESC015	-	-	-	-	-	-	-	-	-	-	-
ESC016	-	-	-	-	-	-	-	-	-	-	-
ESC017	-	-	-	-	-	-	-	-	-	-	-
ESC018	-	-	-	-	-	-	-	-	-	-	-
ESC019	-	-	-	-	-	-	-	-	-	-	-
ESC020	-	-	-	-	-	-	-	-	-	-	-
ESC021	+	+	-	-	-	-	-	-	-	-	+
ESC022	-	+	+	-	-	-	-	-	-	-	-
ESC023	-	-	-	-	-	-	-	-	-	-	-
ESC024	-	-	-	-	-	-	-	-	-	-	-
ESC025	-	-	-	-	-	-	-	-	-	-	-
ESC026	-	-	-	-	-	-	-	-	-	-	-
ESC027	-	-	-	-	-	-	-	-	-	-	-
ESC028	-	-	-	-	-	-	-	-	-	-	-
ESC029	-	-	-	-	-	-	-	-	-	-	-
ESC030	+	-	-	-	-	-	-	-	-	-	+
ESC031	-	-	-	-	-	-	-	-	-	-	-
ESC032	-	-	-	-	-	-	-	-	-	-	-
ESC033	-	-	-	-	-	-	-	-	-	-	-
ESC034	-	-	-	-	-	-	-	-	-	-	-
ESC035	-	-	-	-	-	-	-	-	-	-	-
ESC036	-	-	-	-	-	-	-	-	-	-	-

A= *Staphylococcus aureus* ATCC 25823, **B=** *Staphylococcus aureus* ATCC 12600, **C=** *Staphylococcus aureus* ATCC 29213, **D=** *Staphylococcus aureus* ATCC 12598, **E=** *Bacillus cereus* ATCC 14579, **F=** *Escherichia coli* ATCC 29214, **G=** *Proteus vulgaris* ATCC 15315, **H=** *Listeria monocytogenes* ATCC 7644, **I=** *Listeria monocytogenes* Scott A, **J=** *Salmonella enterica* serovar Typhimurium ATCC 14028, **K=** *Shigella flexneri* ATCC 120222. + ve = extract active against test bacteria; -ve = extract inactive against test bacteria.

Table 3: The sequence analysis of PCR amplified products with primer pairs StrepB/StrepF, and the sequence similarities of known *Streptomyces* 16S rDNA sequences

Sample	Site location	Isolate Code	% Sequence	Sequence similarity
Water	L 3	ESC003	I00% M76388.1.	*Streptomyces griseus*
Bottom sediment	L 6	ESC012	100% JQ806119.1	*Streptomyces griseus*
Bottom sediment	L 1	ESC021	99% AJ971883.1	*Streptomyces* sp.
Bottom sediment	L 2	ESC022	99% CP005080.1	*Streptomyces fulvissimus*
Bottom sediment	L 4	ESC030	100% CP020555.1	*Streptomyces* sp

Table 4: Antibacterial profile of crude products from the selected *Streptomyces* species

Test Bacteria isolates	Gram reaction	Antibacterial action (zone of clearance in mm) in mean ± standard deviation of the mean					
		ESC003	ESC012	ESC021	ESC022	ESC030	Streptomycin (0.002 mg/mL)
Staphylococcus aureus ATCC 25823	+	3.0±0.00	5.0±0.00	14.0±0.13	0.0±0.00	13.0±0.05	12.0±0.12
Staphylococcus aureus ATCC 12600	+	0.0±0.00	3.0±0.00	17.0±0.04	12.0±0.14	3.0±0.00	18.0±0.04
Staphylococcus aureus ATCC 29213	+	5.0±0.00	15.0±0.21	2.0±0.00	16.0±0.13	5.0±0.00	16.0±0.03
Staphylococcus aureus ATCC 12598	+	0.0±0.00	3.0±0.00	5.0±0.00	3.0±0.00	3.0±0.00	14.0±0.04
Bacillus cereus ATCC 14579	+	4.0±0.00	2.0±0.00	2.0±0.00	5.0±0.00	2.0±0.00	13.0±0.02
Escherichia coli ATCC 29214	-	0±0.00	5.0±0.00	3.0±0.00	5.0±0.00	5.0±0.00	14.0±0.01
Proteus vulgaris ATCC 15315	-	16.0±0.12	14.0±0.01	5.0±0.00	4.0±0.00	5.0±0.00	11.0±0.02
Listeria monocytogenes ATCC 7644	+	0±0.00	3.0±0.00	5.0±0.00	2.0±0.00	3.0±0.00	13.0±0.13
Listeria monocytogenes Scott A	+	3.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	15.0±0.02
Salmonella enterica serovar Typhimurium ATCC 14028	-	0.0±0.00	4.0±0.00	3.0±0.00	3.0±0.00	2.0±0.00	10.0±0.01
Shigella flexneri ATCC 120222	-	2.0±0.00	0±0.00	21.0±0.05	4.0±0.00	20.0±0.13	16.0±0.14

Table 5: Minimum inhibitory concentrations (MICs) of the ethyl acetate (EtOAc) crude extracts

Test Bacteria Isolates	Gram reaction	MIC (mg/mL)				
		ESC003	ESC012	ESC021	ESC022	ESC030
Staphylococcus aureus ATCC 25823	+	n/a	n/a	0.044	n/a	0.044
Staphylococcus aureus ATCC 12600	+	n/a	n/a	0.046	0.054	n/a
Staphylococcus aureus ATCC 29213	+	n/a	0.054	n/a	0.056	n/a
Staphylococcus aureus ATCC 12598	+	n/a	n/a	n/a	n/a	n/a
Bacillus cereus ATCC 14579	+	n/a	n/a	n/a	n/a	n/a
Escherichia coli ATCC 29214	-	n/a	n/a	n/a	n/a	n/a
Proteus vulgaris ATCC 15315	-	0.016	0.056	n/a	n/a	n/a
Listeria monocytogenes ATCC 7644	+	n/a	n/a	n/a	n/a	n/a
Listeria monocytogenes Scott A	+	n/a	n/a	n/a	n/a	n/a
Salmonella enterica serovar Typhimurium ATCC 14028	-	n/a	n/a	n/a	n/a	n/a
Shigella flexneri ATCC 120222	-	n/a	n/a	0.045	n/a	0.046

Legend: n/a – Not Applicable

Table 6 Minimum bactericidal concentrations (MBCs) of the ethyl acetate (EtOAc) crude extracts

Test Bacteria	Gram reaction	MBC (mg/mL)				
		ESC003	ESC012	ESC021	ESC022	ESC030
Staphylococcus aureus ATCC 25823	+	n/a	n/a	>10	n/a	>10
Staphylococcus aureus ATCC 12600	+	n/a	n/a	>10	>10	n/a
Staphylococcus aureus ATCC 29213	+	n/a	>10	n/a	>10	n/a
Staphylococcus aureus ATCC 12598	+	n/a	n/a	n/a	n/a	n/a
Bacillus cereus ATCC 14579	+	n/a	n/a	n/a	n/a	n/a
Escherichia coli ATCC 29214	-	n/a	n/a	n/a	n/a	n/a
Proteus vulgaris ATCC 15315	-	2.5	>10	n/a	n/a	n/a
Listeria monocytogenes ATCC 7644	+	n/a	n/a	n/a	n/a	n/a
Listeria monocytogenes Scott A	+	n/a	n/a	n/a	n/a	n/a
Salmonella enterica serovar Typhimurium ATCC 14028	-	n/a	n/a	n/a	n/a	n/a
Shigella flexneri ATCC 120222	-	n/a	n/a	>10	n/a	>10

Legend: n/a – Not Applicable

Time Kill Assay of the Streptomyces sp on Test Bacterial Isolates

The time-kill kinetics of the crude ethyl acetate extracts from the *Streptomyces* species are revealed in Table 7. Three of the respective extracts exhibited both varying degrees of bacteriostatic and bactericidal properties which depend on the test bacterial isolates. A remarkable reduction in mean viable cell amount was detected at respective time studied. Results are interpreted as Log_{10} CFU/mL. Mean Log decrease in viable cell count for ESC012 vary from 3.15 $Log_{10,}$ to 3.09 Log_{10}, 2.56 Log10 and 2.36 Log10 CFU/mL post 3 h activity; and from 2.80 $Log_{10,}$ to 2.61 Log_{10}, 2.48 Log_{10} and 2.18 Log_{10} CFU/mL post 6 h activity in MIC, 2 × MIC, 3 × MIC and 4 × MIC where applicable on *Staphylococcus aureus* ATCC 29213. ESC021 Log reduction in viable cell count changed from 3.08 Log_{10} to 2.65 Log_{10}, 2.65 Log_{10} and 2.01 Log_{10} CFU/mL after 3 h of activity at MIC, 2 × MIC, 3 × MIC and 4 × MIC where applicable; whereas at 6 h activity it was observed as 3.04 Log_{10}, 1.38 Log_{10}, 0.36 Log_{10} to -1.0 Log_{10} at MIC, 2 × MIC, 3 × MIC and 4 × MIC towards *Staphylococcus aureus* ATCC 25823. As regards ESC030 extract, the Log decrease in viable cell populations varied from 3.46 Log_{10} to -2.81 Log_{10}. The utmost decrease in cell populations were obtained by ESC021 and ESC030 against *Staphylococcus aureus* ATCC 25823 with an average decrease in viable cells of -1.0 Log_{10} and -2.81 Log_{10} individually at 6 h for 4 × MIC respectively.

Time–kill kinetics has been applied to elucidate activities of diverse antibacterial metabolites. The technique is frequently applied as the basis for *in vitro* research into pharmacodynamics of drug activities [29]. It yields expressive (qualitative) data on the pharmacodynamics of antibacterial peptides [10]. It is also used to provide clinically relevant assay of the activity of antimicrobial agent [11]. The time kill kinetics of the extracts from the S*treptomyces* species produced variable observations amongst sensitive test bacterial isolates. Both bactericidal and bacteriostatic properties were established by the extracts. The extracts demonstrated a somewhat concentration-dependent inhibiting/killing profile. Though literatures on time kill assays from marine *Streptomyces* species are limited, time kill assays from a marine bacterium towards clinical *Staphylococcus aureus* that are methicillin resistant has previously been recounted by Isnansetyo and Kamei [54] where bactericidal properties was revealed to be significantly higher compared to vancomycin.

Extracts of ESC021 and ESC030 had bactericidal activity using 4 × MIC towards *Staphylococcus aureus* ATCC 25823 at 3 h with a little upsurge in activity at 6 h interface respectively. A bacteriostatic profile was detected at reduced concentrations. Time kill assay emanating from the synergistic action of gentamicin, with a steady derivative "thienamycin N-Formimidoyl' thienamycin (MK0787)", obtained from *Streptomyces cattleya* or with tobramycin towards enterococci have been reported [55]. From their findings, a synergistic action of gentamicin with N-Formimidoyl' thienamycin or applying tobramycin revealed over 95% bactericidal activity towards forty-seven *Streptococcus faecalis* strains tested. Consequently, extracts of ESC021 and ESC030 metabolites propose significant use towards infections emanating from staphylococci. ESC012 revealed bacteriostatic activity towards *Proteus vulgaris* ATCC 15315 at respective concentration adopted. More so, bacteriostatic action was detected with increased concentrations (3 × MIC and 4 × MIC) and lengthier time.

ESC021 showed bacteriostatic action at 2 × MIC at 3 h as well as 6 h towards *Shigella flexneri* ATCC 120222. More so, bactericidal action was established as regards 4 × MIC at 3 h and 6 h activity. Infection emanating from *Shigella* is a significant public health menace in resource poor countries [56]. Humans have been reported as natural reservoirs, though other primates might be colonized and/or infected [57-63]. No original food product contains intrinsic *Shigella* species [64]; though an extensive diversity of foods might be adulterated leading to shigellosis. Shigellosis is disseminated via routes of faecal-oral spread [58]. Other means of dissemination comprise consumption of adulterated water or food [61]. *Shigella flexneri* has been documented not to be sensitive to dapsone, but sensitive to nalidixic acid, ampicillin, trimethoprim/sulfamethoxazole, and ciprofloxacin [56-57, 59-63, 65]. Though ESC030 had a bacteriostatic spectrum towards *Shigella flexneri* ATCC 120222, ESC021 and ESC030 proved bactericidal with 4 × MIC concentrations. ESC021 offers to be a noteworthy agent towards infections that arises from *Shigella flexneri*.

Anti-*Proteus* activity was demonstrated by ESC012, the anti-bacteremic and sepsis time kill assay of marine *Streptomyces* is restricted, this seem to be one of the principal report of anti- sepsis and bacteremic time kill assay of extracts of marine *Streptomyces* from Escravos River (Table 7). This extract (ESC012), seems to be a significant drug candidate, for the advancement of anti- sepsis and bacteremic antibiotics with potential application in therapy of human diseases. Thus, it is suggested that further research to unravel the relationship amongst the assembly of bioactive metabolite of the extracts and their spectrum of action. Also, a speedy system for production and purification of the extract in large scale and elucidating the structure of the bioactive components of interest is herein advocated.

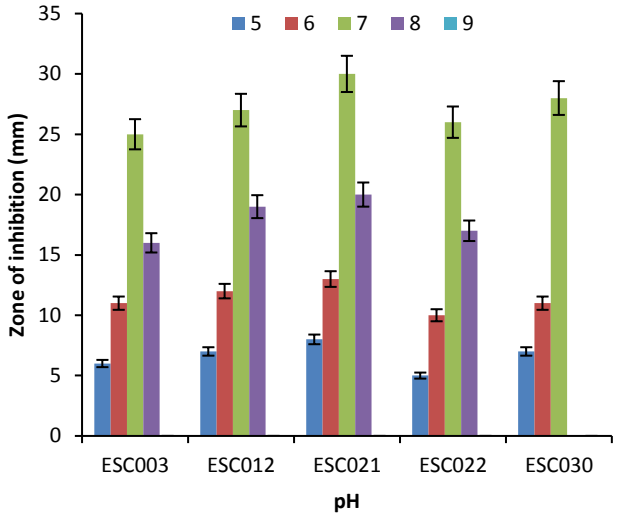

Figure 3a: Effect of pH on antimicrobial agent produced by the *Streptomyces* sp

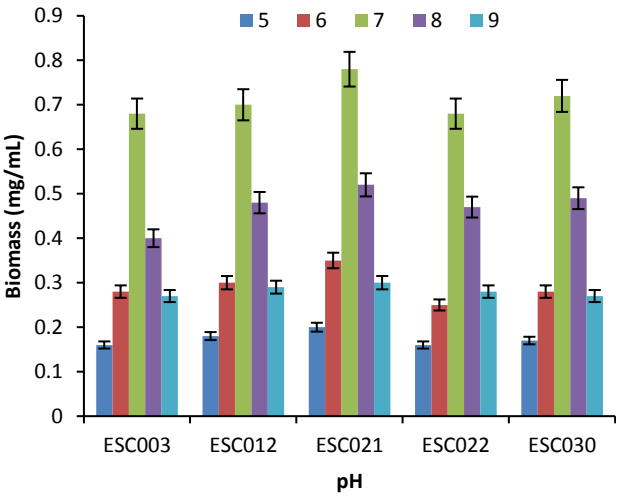

Figure 3b: Effect of pH on growth of the *Streptomyces* sp

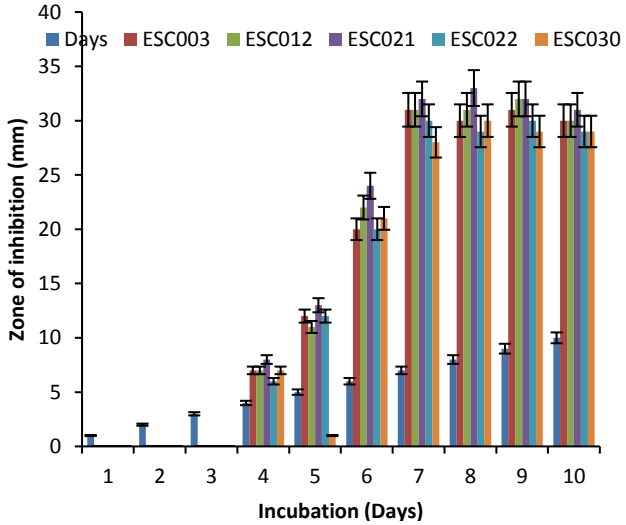

Figure 4a: Effect of incubation periods on antimicrobial agent produced from the *Streptomyces* sp

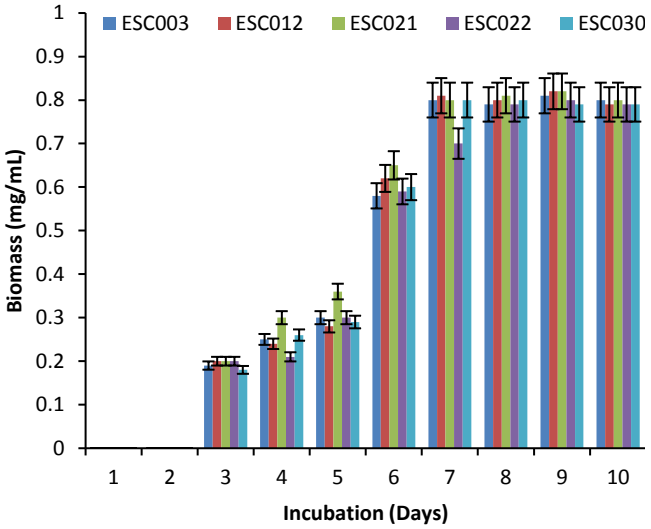

Figure 4b: Effect of incubation periods on growth of the *Streptomyces* sp

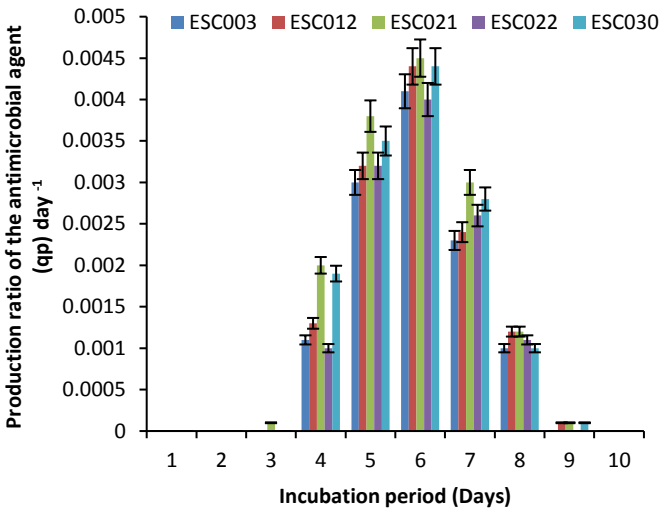

Figure 5: Specific rate of antimicrobial production by the *Streptomyces* sp

Table 7: Time-kill kinetics of the ethyl acetate (EtOAc) crude extracts

Extracts	Test bacteria	Gram reaction	MIC Log_{10} Kill			2 × MIC Log_{10} Kill			3 × MIC Log_{10} Kill			4 × MIC Log_{10} Kill		
			0 h	3 h	6 h	0 h	3 h	6 h	0 h	3 h	6 h	0 h	3 h	6 h
ESC012	*Staphylococcus aureus* ATCC 29213	+	3.19	3.15	2.80	3.21	3.09	2.61	2.62	2.56	2.48	2.41	2.36	2.18
	Proteus vulgaris ATCC 15315	-	2.92	2.80	2.55	2.69	2.57	2.16	2.01	1.97	1.76	1.72	1.43	1.06
ESC021	*Staphylococcus aureus* ATCC 25823	+	3.12	3.08	3.04	2.93	2.65	1.38	2.91	2.65	0.36	2.91	2.01	-1.0
	Staphylococcus aureus ATCC 12600	+	2.84	2.63	1.97	1.95	1.78	1.65	1.73	1.55	1.16	1.93	1.14	-0.89
	Shigella flexneri ATCC 120222	-	3.19	3.15	3.05	2.72	2.69	2.67	2.74	2.53	1.70	2.70	2.48	0.62
ESC030	*Staphylococcus aureus* ATCC 25823	+	3.61	3.46	2.64	3.44	3.01	1.27	1.61	1.47	- 0.74	1.60	1.39	-2.81
	Shigella flexneri ATCC 120222	-	2.73	2.58	2.11	2.73	2.50	2.04	2.56	2.38	1.73	2.32	2.26	1.20

Conclusion

The findings from this study highlight the significance for advance investigation concerning the objective of tracking down new antibacterial metabolites from detected *Streptomyces* from underexplored habitat. The Escravos River seems to be a habitat that is under- or unexplored in this regard, with distinctive biological functions and biodiversity. The biodiversity of the Escravos River needs to be explored further so as to obtain superb benefit from the valuable bio-resource, especially as it concerns emerging drug resistant bacterial strains. The emergence and widespread of multiple antibiotic resistance is well recognized as a public health menace globally. Consequently, it is projected that as a result of the antibacterial silhouette and portrayal of the crude extracts, recognized marine *Streptomyces* detected from Escravos River underscores the usefulness of finding novel antibiotics in unexplored environments. Currently, the focal aspect of our research group is the mechanism by which these extracts inhibit/kill bacterial strains and elucidation of the bioactive molecule.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We are grateful to the "International Foundation for Science" (IFS), Sweden (Grant No. F/5081-2) and co-financed by the Committee on Scientific and Technological Cooperation (COMSTECH) for providing the financial support for this research.

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