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Evaluation of the Antibiotics Producing Potentials of *Actinomycetes* species Isolated from the Soil from Ikwo, South Eastern Nigeria.

Onwa Ndubuisi Collins and Elom Emeka Elom

Department of Applied Microbiology Ebonyi State University, Abakaliki, Nigeria.

ABSTRACT

The microbial analysis of soil samples for the presence of antibiotic-producing Actinomycete species from Ikwo Local Government Area of Ebonyi State, Nigeria was carried out. A total of six soil samples were collected from different locations in Enyibichiri and Noyo communities of Ikwo L.G.A and were immediately sent to he microbiology laboratory for analysis. Isolation Actinomycete species was done using Actinomycete isolation agar and carried out under standard microbiological procedure. Identification and characterization were done using morphological, physiological, cultural and biochemical tests. Screening of the isolates for their antimicrobial activity was done by the cross streak method against the following clinical isolates: *pseudomonas aeruginosa, E. coli, Klebsiella* spp., *Enterobacter* spp and *Staphyloccus aureus.* Comparison of the antimicrobial activity of the Actinomycetes isolates to commonly used antibiotics was carried out using the Kirby Bauer agar well diffusion method. The optimum growth temperature and pH for *Actinomycetes* species that showed activity against the test isolates were determined. Results showed that 6 species of Actinomycete from Enyibichiri soil showed activity against one or more of the test organisms and 3 species from Noyo soil showed activity against one or more of the test organisms. Characterization of the isolates that showed activity against one or more of the test isolates revealed that all of them were *Streptomyces* species. The *Streplomyces* species Noyo I produced the highest activity against *Pseudomonas aeruginosa*, with inhibition zone diameter (IZD) of 19mm while *Streptomyces* species Enyibichiri 2 (SSE2) produced the highest activity against *Escherichia coil* with IZD of 26mm. SSE4 produced the greatest activity against *Klebsiella* spp. with IZD of 30mm while SSE3 produced the greatest activity against *Enterobacter* spp. with IZD of 25mm. *Streptomyces* species Noyo I produced the highest activity against *Staphylococcus aureus* with IZD of 22mm. Results of the comparative antimicrobial activity showed that the activities of *Streptomyces* species were less for all the test organisms. Also the optimum growth temperature for all the *Streptomyces* species was 30^0C while the optimum pH was 8. This study indicates that the *Streptomyces* species that had activities against the test organisms may have potentials as sources of antimicrobial agents.

Keywords: Actinomycetes, Soil, Antibiotics, Antimicrobial activity.

INTRODUCTION

The Actinomycetales are an order of Actinobacteria. A member of the order is often called an Actinomycete. Actinomycete are classified as a group of gram-positive bacteria that are unique for their spore forming abilities and formation of mycelia structures (Stephen, 2014). It produces branching mycelium which may be of two kinds: substrate mycelium and aerial mycelium [1]. The colonies of Actinomycetes have pastel colours, soil-like odour. Hard and stuck into agar. Many species of Actinomycetes produce anti-microbial compounds under certain conditions and growth media [2]. Streptomycin, actinomycin and streptothricin are all medically important antibiotics isolated from Actinomycetes bacteria [3]. Almost two – thirds of the natural antimicrobial drug compounds used currently are produced by different species of Actinomycetes [4].

These bacteria are therefore extremely relevant to Scientists, pharmaceutical industries and agricultural industries. Recent report show that this group of microorganism still remains vital source of antibiotics [5]. As a result of the increasing prevalence of antibiotic resistant pathogens and the pharmacological limitation of antibiotics, there is an exigency for new antimicrobial substances from bacteria [6].

The two major groups of soil Actinomycetes that serve as important sources of antibiotics are *Streptomyces* and *Micromonaspora*. The Streptomycetes account for about 80% of the total antibiotic products [7]. Othere Actrinomycetes that produce bioactive compounds, but on a lower sacle includes *Saccharopolyspora, Amycolatopsis* and *Actinoplanes* [8].

Nature especially soil and marine environments harbor a large number/population of microorganisms with bioactivity that are yet to be discovered [9]. Actinomycetes are well known for their ability to produce bioactive compounds especially antibiotics [10]. Screening of soils from Ikwo LGA of Ebonyi State for antibacterial activity by Actinomycetes is yet to be carried out. New/novel antibiotics will definitely reduce mortality and morbidity burden in our health institutions.

Soil is a unit entity that's inhabits varieties of micro-organisms and the microbial community is an integral part of the soil. Actinomycetes are primarily soil inhabitants and are also very widely distributed in nature [11]; [12]. They are also well known as soil saprophytes and are responsible for the distinctive earthy odour of freshly ploughed soil due to the production of geosmin. The most dominant Actinomycetes in soil is the genus *Streptomyces* although, others like *Norcardia Microbispora, Micromonospora, Actinmyces Actinoplanes* and *Streptosporangium* has also been isolated from the soil. The number and variety of Actinomycetes present in any soil sample would be significantly influenced by geographical location, soil temperature, soil type, soil pH, organic matter content, agricultural activities, aeration, nutrient availability, moisture content and soil vegetation [13]. Actinomycetes have been isolated from diverse soil types and locations such as arid, tropical forest, mining, cave, swamp, desert and savannah. They are particularly abundant in slightly alkaline soil rich in organic matters and produce several structurally diverse secondary metabolites of pharmaceutical and agricultural importance. Actinomycetes play an important ecological role in the recycling and mineralization of nutrients in the soil. They help to recycle nutrients by degrading vast numbers of organic matter in the soil and are found most common in compost. They act as plant growth promoters by helping in nitrogen fixation, solubilization of nutrients, immobilization of nutrients, siderophpores production, biological control and soil structure maintenance [14]; [15]; [16]; [17]. Actinomycetes are of great practical important in nature and seem to be ultimately involved in soil ecology [18].

This research is aimed at isolation of actinomycetes with potentials for antibacterial activity from soil samples form Ikwo LGA. There is a need to search for new and efficient antibiotics because of the increasing multiple resistance of microorganisms to available antibiotics.

Statement of Problem

The project was conceived as a result of the increasing pathogen resistance to commonly used antibiotics [19]. Multidrug resistance strains of pathogens are also emerging [20]. There is a need for new antibiotics to cure emerging and re-emerging infections (WHO, 2001b). it has been reported that treatment cost of infectious diseases is increasing because of the increasing resistance of pathogens to antibiotics (WHO, 2001a).

Methods

Sample Collection

A total of six soil samples were collected between the months of October to December, 2017 from different locations in Enyibichiri and Noyo communities of Ikwo L.G.A and were immediately transported to the Microbiology laboratory for analysis. Samples that were not processed on the day of collection

were stored in the refrigerator at 4^0C. Each soil sample was collected by clearing a minor part of the soil with a sterile spatula before inserting a soil sampling tube (apple core) up to the depth of 4 inches to collect the top soil. The tube was subsequently emptied into a plastic bag, closed and labeled with a marker pen [21].

Sampling processing

The soil slurry was made by suspending 10g of the collected dry soil in 90ml distilled water. The slurry was vortexed for 2 minutes in an orbital shaker incubator at 27^0C, and their contents was designated as stock cultures.

Isolation of Actinomycetes from Soil Samples

Isolation of Actinomycetes was done by serial dilution and plating technique (pour plate) using Actinomycetes isolation agar medium. Distilled water (9ml) was taken in each of the 7 test and labeled 1 to 7. Iml of the supernatant liquid from the dissolved soil sample was transferred into the test tubes as to achieve the serial dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} ml of the dilution sample. Next, 1 ml volume of the least dilution (10^{-7}) was measured into the petri-dish before pouring 15ml of the sterilized Actinomycetes isolation agar unto it. The plate was shaken and allowed to gel at room temperature. All pour plates were labeled and incubated at 28^0C for 48hours [22], [23] [24].

Pure Cultures

Isolated colonies were further purified by multiple streaking methods using Actinomycetes isolation agar. The distinct colonies were labeled sand inoculated into Actinomycetes agar slants. The labeled Actinomycetes isolates were used for further studies.

Characterization of Isolates

The isolates were characterized using morphological, cultural, biochemical and physiological tests

Cultural Characterization

The cultural characteristics was examined by incubating the isolates for 10 days at 30 ^{0}C on Actinomycetes isolation agar. After incubation, the growth, aerial mass colour and melanoid pigment production were recorded [25].

Aerial Mass Colour

The colour of the mature sporulating aerial mycelium was recorded in a simple way (White, grey, red, green, blue and violet). When the aerial mass colour falls between two colour series, both the colours are recorded. If the aerial mass colour of any isolate shows inter mediate tints, then also, both the colour series are noted [2].

Melanoid Pigment

The grouping was made on the production of melanoid pigments (i.e greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. The isolates are grouped as melanoid pigment produced (+) and not produced (-) [7].

Reverse Side Pigments

The species were divided into two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely, distinctive (+) and not distinctive or none (-). In case, a colour with low chroma such as pale yellow, olive or yellowish brown occurs, it is included in the latter group (-) [12].

Soluble Pigments

The species were divided into two groups based on their ability to produce soluble pigments other than melanin: namely, produced (+) and not produced (-). The colour is recorded (red, orange, green, yellow, blue and violet) [20].

Microscopic Observation

Gram staining, acid fast staining was performed to check the morphology of the cells and spore chain morphology was identified by slide culture technique [22].

Gram Staining Test

A smear of culture was made on a clean grease-free slide and was allowed to air dry before being heat fixed with the aid of a Bunsen burner flame and the smear was covered with crystal violet for 30 seconds and washed off with clean water. The smear was flooded with klugols iodine (a mordant) and allowed to stand for 60 seconds before rinsing the slide with clean water. It was immediately decolorized with drops of acetone and the smear was covered with safranine reagent for 30 seconds. The slide was rinsed

slowly under running tap and was examined microscopically using X 100 objective lens. Gram positive bacteria appeared purple while the *Escherichia coli* (Gram negative) which was used as a control appeared red [25].

Acid Fast Stain Method

A smear of culture was made on clean grease-free slide and was allowed to air dry before being heat fixed with the aid of a Bunsen burner flame. The slide was then flooded with carbol fuchsin and allowed to stand for 30 seconds. The slide was then heated to dry and rinsed off in tap water. The slide was then flooded with a 1% solution of sulphuric acid alcohol (methanol) and allowed to stand for 15 to 20 seconds, thus, removing the stain from the cells that are unprotected by a waxy lipid layer. Thereafter, the cells was stained (counter stain) in methylene blue for 30 seconds, and excess stain was rinsed with slow running tap water. The slide was then viewed with the microscope using the oil immersion lens. Acid-fast bacteria retain Carbol Fuchsin so they appeard red, while non-acid-fast bacteria pick up the counter-stain and become blue. This test distinguishes between the *Streptomyces* and non-*Streptomyces* Actinomycetes [16].

Slide Culture Technique

A 10mm agar block (Actinomycetes agar) was cut with sterile scalpel ad lifted to the centre of the slide in the petri-dish. A very small quantity of the Actinomycetes culture was inoculated at the four corners of the agar block on the slide. The cover slip was then placed over the inoculated agar block and incubated at 30^0C for 96 hours (4 days). The slide was then examined under the microscope (x 10) where undisturbed conidiophores, conidial ontogeny and conidia were observed. A drop of 95% alcohol was poured unto the slide and then stained with 1% cotton blue in lactophenol and mounted for observation [9].

Spore Chain Morphology

With regard to spore chains, the isolates were grouped into 'sections'. The species belonging to the genus *Streptomyces* are divided into three sections (Lingakumar *et al.,* 2011), namely rectiflexibles (RF), retinaculiaperti (RA) and Spirales (S). When an isolate forms two types of spore chains, both are noted (e.g. SRA and BIV-S).

Biochemical Characterization

Actinomycetes isolates were characterized using citrate utilization, starch hydrolysis case in hydrolysis, urease production, indole production, methyl red, Voges-proskauer, catalase and oxidase production tests [11].

Oxidase Test

A 1.1g oxidase reagent (tetramethyl-p-phenyldiaminedihydrochloride) was dissolved in 100ml of distilled water. Drops of the solution were poured unto a filter paper in a petri dish. Using a glass rod, the test sample was smeared unto the filter paper and observed for purple coloration. This is a positive result [5].

Indole Test

The test organism was inoculated into the bijou bottle containing 3ml of sterile peptone water and incubated for 48hrs at 30^0C. Then, indole production was tested by adding 0.5ml of Kovac's reagent, shake gently and observed for a red colour on the surface layer within 10 minutes [8].

Catalase Test

A sterile wire loop was used to pick a colony of the test organism and immersed into 2 drops of 3% hydrogen peroxide and observed for immediate bubbles [23].

Citrate Utilization Test

Five (5) ml of the media was dispensed into 16mm test tubes and autoclaved at 121^0C at 15psi for 15 minutes. The tubes were cooled in a slanted position. The slants were inoculated by stabbing with a straight wire loop and incubated at 30^0C for 48 hours. Growth with color change from green to intense blue indicates a positive reaction, otherwise negative [7].

Urease Test

The entire surface of a Christensen's urea slope was inoculated with the test organism and incubated at 37^0C for 48 hours. After the incubation period, they were observed for red-pink colouration [19].

Methyl Red Test

Five (5) ml of the broth was dispensed into each of the test tube and sterilized by autoclaving at 15psi (121^0C) for 15 minutes. The test isolates were then inoculated into each of the test tubes and

incubated at 30^{0}C for 4 days. After incubation one drop of methyl red reagent was added to the test tubes and observed for a color change to red coloration, which indicates positive production of mixed acid [7].

Voges-Proskauer (VP)

The MR-VP broth was prepared according to the manufacturers instruction, 5ml of the broth was dispensed into each of the test tube and sterilized by autoclaving at 15 psi (121^{0}C) for 15 minutes. The test isolates was then inoculated into each of the test tubes and incubated at 30^{0}C for 48 hours. After incubation 0.6ml of alpha-naphthol (Barrit's solution A) followed by 0.2ml of 40% KOH (Barrit's solution B) were added to the test tubes, gently shook and allowed to stand for 15 minutes. After which the test tubes were observed for a colour change to pinkishs red colouration, which indicates positive production of acetion and 2,3-butanediol.

Screening of the Actinomycetes for Antimicrobial Activity

Screening of the isolates for antimicrobial activity was done by cross streaking according to the method of [14]. Each of the Actinomycetes isolates were streaked as a straight line on Mueller Hinton Agar and starch casein agar medium respectively. The media were incubated at 27^{0}C for 6 days (144h). After the 6th day, clinical isolates of *Pseudomonas aeruginosa, E. coli, Klebsiella* spp., *Enterobacter* spp and *Staphylococcus aureus* were streaked at right angle, but not touching each other, and then incubated at 37^{0}C for 24h. The zone of inhibition was measured to the nearest millimeter with meter rule [9].

Comparing the Activity of the Antimicrobial Agents Produced by Actinomycete Isolates to Commonly used Antibiotics

Starch casein broth was prepared according to the manufacturer's instruction and 10ml taken in a bijou bottle, sterilized and inoculated with 0.1ml of 0.5 McFarland standard of Actinomycete culture. It was incubated in a shaker flask with intermittent shakings at 250 rpm for seven days at a temperature of 30^{0}C. After seven days, the supernatant was centrifuged at 2000rpm for 5 minutes and 0.5 ml taken into a hole that was bored with a cock borer at the center of the Petri-dish containing Mueller Hinton agar. Initially, 1ml of 0.5 McFarland of 24 hour old culture of the clinical isolates was poured on the prepared MHA and spread unto the surface with a swab. It was allowed to stand for 15 minutes before the hole was made at the center of the petri-dish with a sterile cock borer. Following the Kirby Bauer disc diffusion method according to the recommendations of the Clinical Laboratory Standard Institute (CLSI) (CLSI, 2015), the antibiotic disks which includes Erythromycin (15 µg), Ofloxacin (5 µg), Bacitracin (10 µg), Amoxicillin Clavulanic Acid (30 µg), Cefoxitin (30 µg) and Amikacin (30 µg) that were produced by Oxoid UK was aseptically placed on the surface of the inoculated petri-dish using sterile forceps. The plate was incubated at 37^{0}C for 24 hours and the zone of inhibition of both the antibiotics and the supernatant from the actinomycetes broth culture were measured to the nearest millimeter with a meter rule and the results recorded.

Carbohydrate Utilization Test

The isolated organism were tested for its ability to utilize carbon sources such as dextrose (glucose). Fructose, maltose, mannitol, starch and sucrose.

Procedure:

The preparation of the carbohydrate fermentation broth was done as follows; 1g of trypticase, 0.5g of carbohydrate, 0.5g of sodium chloride and 0.0189mg of phenol red were measured and dissolved in 100ml of distilled water in a conical flask. This repeated for each of the carbohydrate listed above. The prepared phenol red broth was poured into a screw capped fermentation tubes and each tube was inserted an inverted Durham tube. The Durham tube was fully filled with broth. The tubes were sterilized at 115^{0}C for 15 minutes. The tubes were allowed to cool before inoculating the broth with bacterial culture using the sterile wire loop. The tubes were incubated at 37^{0}C for 48 hours and observed.

1. If the medium changes from red to yellow; it is an indication that the organism has fermented the given

carbohydrate and produced organic acids that reduced the pH of the medium to acidic.
2. If the medium turns yellow with gas bubbles in the inverted Durham tube. This shows that the organism has fermented the given carbohydrate and gas was produced.
3. If the broth retains the red color, it is an indication that the organism cannot utilize the particular carbohydrate in the broth.

Physiological Characterization

Temperature

The ability of the isolate to grow at various temperatures (20 to 40^{0}C) was evaluated to determined the optimum growth temperature of the isolated actinomycetes.

Procedure: The actinomycetes isolation broth was prepared and 5 ml of it taken into each of the bijou bottle. 0.1ml of 0.5 McFarland of the actinomycetes broth was poured into the bijou bottles and incubated differently at the temperatures of 20^{0}C of 20^{0}C, 25^{0}C, 30^{0}C, 35^{0}C and 40^{0}c respectively for 92 hours (4 days) after which the turbidity (microbial cell numbers) was estimated using the psectrophotometric method at 600nm. The spectrophotometer measures turbidity directly.

Range of pH

The ability of the actinomycetes isolate to grow at various range of pH (7-9) in medium was tested.

Procedure: The actinomycetes isolation broth was prepared and poured into three beakers, the pH was then measured using a pH meter and adjusted to 7,8 and 9 respectively by the addition of 1Molar NaOH or 1 Molar HCL while shaking it. It was then sterilized at 121^{0}C/15 minutes and 5 ml of it poured into each of the Bijou bottle. 0.1ml of 0.5 McFarland of the actinomycete culture was poured into the bijou bottle and incubated at 28^{0}C for 92 hours (4 days) after which the turbidity (microbial cell numbers) was estimated using the spectrophotometric method at 600nm.

Physiological Characterization

Temperature

The ability of the isolate to grow at various temperatures (20 to 40^{0}C) was evaluated to determine the optimum growth temperature of the isolated actinomycetes.

Procedure: The actinomycetes isolation broth was prepared and 5 ml of it taken into each of the bijou bottle, 0.1ml of 0.5 McFarland of the actinomycetes broth was poured into the bijou bottles and incubated differently at the temperatures of 20^{0}C, 25^{0}C, 300C, 35^{0}C and 40^{0}C respectively for 92w hours (4 days) after which the turbidity (microbial cell numbers) was estimated using the spectrophotometric method at 600nm. The spectrophotometer measures turbidity directly.

Range of pH

The ability of the actinomycetes isolate to grow at various range of pH (7-9) in medium was tested.

Procedure: The actinomycetes isolation broth was prepared and poured into three beakers, the pH was then measured using a pH meter and adjusted to 7, 8 and 9 respectively by the addition of 1 Molar NaOH or 1Molar HCL while shaking it. It was then sterilized at 121^{0}C/15 minutes and 5 ml of it poured into each of the Bijou bottle. 0.1ml of 0.5 McFarland of the actinomycete culture was poured into the bijou bottle and incubated at 28^{0}C for 92 hours (4 days) after which the turbidity (microbial cell numbers) was estimated using the spectrophotometric method at 600nm.

Statistical Analysis

The results obtained from the study was presented using tables while relevant data were statistical analyzed with the use of Analysis of Variance (ANOVA) on SPSS version 22.

RESULTS

Table 1: Cultural, Morphological and Biochemical Characterization of Actinomycete species from Enyibichiri Community of Ikwo L.G.A, Ebonyi State

		Cultural Characteristics			Microscopic Characteristics			Biochemical Characteristics						Carbohydrate Utilization							
SN	Sample code	AMC	RSP	MP	SCM	GS	AFS	CTT	OX	CAT	IND	MR	VP	UR	DX	MAL	SUC	MAN	FRU	ST	Probable species
1	EN 1	White	0	0	Spiral	+	-	+	-	+	+	-	+	+	+	+	+	-	+	+	*Streptomyces* spp 1
2	EN 2	Gray	0	0	B/S	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	*Streptomyces* spp 2
3	EN 3	W/G	0	1	Sprial	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp 3
4	EN 4	G/W	1	0	SR	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp 4
5	EN 5	G/W	1	1	Sprial	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp 5
6	EN 6	White	0	1	Spiral	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp 6

Key: EN = Enyibichiri, AMC = Aerial Mass Colour, RSP = reverse side pigment, MP = Melanoid production, SCM = spore chain morphology, GS = gram stain, AFS = acid fast stain, CIT = Citrate, OX = oxidase, CAT = catalase, IND = indole, MR = Methyl red, VP = Voges-Proskauer, UR = urease, DX = dextrose, MAL = maltoase, SUC = sucrose, MAN = mannitol, FRU = fructose, ST = starch, W/G = white and gray, G/W = gray and white, B/S = biverticillus-spiral, SR = STRAIGHT Rectus.

Table 2: Cultural, Morphological and Biochemical Characteristics of Actinomycete species from Noyo Community of Ikwo L.GA, Ebonyi State.

		Cultural Characteristics			Microscopic Characteristics			Biochemical Characteristics						Carbohydrate Utilization							
SN	Sample code	AMC	RSP	MP	SCM	GS	AFS	CTT	OX	CAT	IND	MR	VP	UR	DX	MAL	SUC	MAN	FRU	ST	Probable species
1	NO 1	White	1	1	Spiral	+	-	+	-	+	+	-	+	+	+	-	+	+	+	+	*Streptomyces* spp 1
2	NO 2	Yellow	0	0	Spiral	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp 2
3	NO 3	Gray	0	1	Sprial	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp 3

Key: NO = Noyo, AMC = Aerial Mass Colour, RSP = reverse side pigment, MP = Melanoid production = spore chain morphology, GS = gram stain, AFS = acid fast stain, CIT = citrate, OX = oxidase, CAT = catalase, IND = indole, MR = Methyl red, VP = Voges=Proskauer, UR = urease, DX = dextrose, MAL = naltose, SUC = sucrose, MAN = mannitol, FRU = fructose, ST = starch.

Table 3: Antimicrobial activity of *Streptomyces* species from Enyibichiri against the clinical isolates

		Clinical Isolates and Diz (mm)				
S/N	Species code	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella* species	*Enterobacter* species
1	SSE 1	NI	NI	10	20	20
2	SSE 2	NI	26	NI	NI	NI
3	SSE 3	10	17	12	NI	25
4	SSE 4	NI	20	NI	30	15
5	SSE 5	NI	9	NI	15	NI
6	SSE 6	NI	NI	3	NI	NI

KEY: NI Inhibition, SSE = *Streptomyces* spp, Enyibichiri IZ = Inhibition zone, mm = millimeter

Table 4: Antimicrobial activity of *Streptomyces* species from Noyo against the clinical isolates

		Clinical Isolates and Diz (mm)				
S/N	Species code	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella* species	*Enterobacter* species
1	SSE 1	22	NI	NI	NI	NI
2	SSE 2	NI	18	NI	22	12
3	SSE 3	15	NI	19	NI	NI

KEY: NI Inhibition, SSN = *Streptomyces* species, Noyo, IZ = Inhibition zone, mm = millimeter

Table 5: Inhibition Zone Diameter (IZD) Produced by *Streptomyces* species from Enyibichiri community and the commonly used Antibiotics against the clinical isolates (mm)

		***Streptomyces* from Enyibichiri**						**Conventional Antibiotics**					
SN	Clinical isolates	SSE 1	SSE 2	SSE 3	SSE 4	SSE 5	SSE 6	Ak(30µg)	Ak (15µg)	FOX (30µg)	B(10µg)	AMC(30µg)	OFX (30µg)
1	*Staphylococcus aureus*	7	NI	25	7	NI	NI	20	NI	23	35	NI	19
2	*Escherichia coli*	NI	NI	NI	NI	12	NI	15	12	30	NI	NI	7
3	Pseudomona aeruginosa	NI	8	NI	NI	NI	NI	23	7	NI	NI	NI	NI
4	*Klebsella* species	10	NI	NI	15	7	NI	19	NI	NI	NI	10	10
5	*Streptococcus* spp	NI	NI	NI	7	NI	7	24	NI	NI	NI	10	NI
6	*Proteus* spp	NI	NI	NI	NI	NI	NI	NI	9	11	NI	NI	10
7	*Enterobacter*	10	NI	8	7	NI	NI	24	10	20	30	NI	NI

Key: SSE = *Streptococcus* species, Enyibichiri, E = Erythromycin (15µg), OFX = Ofloxacin (5µg), B = Bacteriacin (10µg), AM = Amoxicillin Clavulanic Acid (30µg) , FOX = cefoxitin (30µg), AK = Amikacin (30µg), NI= Ni Inhibition

Table 6: inhibition zone diameter (IZD) Produced by *Streptomycess* from Noyo and antibiotic against the clinical isolates (mm)

		***Streptomyces* From Enyibichiri**			**Conventional Antibiotics**					
SN	Clinical isolate	SSN 1	SSN 2	SSN 3	AK(30µg)	E(15µg)	FOX(30µg)	B(10µg)	AMC(30µg)	OFX(30µg)
1	*Staphylococcus aureus*	15	NI	NI	20	NI	23	35	NI	19
2	*Escherichia coli*	NI	7	NI	15	12	10	NI	NI	7
3	*Pseudomonas aeruginosa*	7	NI	9	23	7	NI	NI	NI	NI
4	*Klebsiella* species	NI	15	NI	10	NI	NI	NI	10	10
5	*Streptococcus* spp	NI	NI	NI	24	NI	NI	NI	10	NI
6	*Proteus* spp	NI	NI	11	NI	9	11	NI	NI	10
7	*Enterobacter*	NI	7	NI	24	10	20	30	NI	NI

Table 7: Growth of *Streptomyces* species from Enyibichiri at different Temperatures.

	Streptomyces	Cell Number (cells/m) at Different Temperatures				
SN	Species	20^0C	25^0C	30^0C	35^0C	40^0C
1	SSE 1	$1.2X10^7$	$8.39X10^7$	$5.99X10^8$	$1.05X10^8$	$9.68X10^7$
2	SSE 2	$7.19X10^7$	$1.52X10^8$	$5.9X10^8$	$1.07X10^8$	$9.92X10^7$
3	SSE 3	$7.38X10^7$	$1.44X10^8$	$7.26X10^8$	$6.19X10^8$	$3.79X10^8$
4	SSE 4	$2.85X10^7$	$1.08X10^8$	$4.74X10^8$	$9.94X10^7$	$8.98X10^7$
5	SSE 5	$7.39X10^7$	$1.54X10^8$	$2.11X10^8$	$1.6X10^8$	$1.52X10^8$
6	SSE 6	$6.98X10^7$	$1.5X10^8$	$7.7X10^8$	$1.26X10^8$	$1.17X10^8$

KEY: SSE = *Streptomyces* species from Enyibichiri

Table 8: Growth of *Streptomyces* species from Noyo at different Temperatures.

	Streptomyces	Cell Number (cells/m) at Different Temperatures				
SN	Species	20^0C	25^0C	30^0C	35^0C	40^0C
1	SSE 1	$3.44X10^7$	$9.12X10^7$	$1.14X10^8$	$1.11X10^8$	$8.09X10^7$
2	SSE 2	$6.75X10^7$	$1.47X10^8$	$6.17X10^8$	$4.97X10^8$	$4.1X10^8$
3	SSE 3	$2.2X10^7$	$1.02X10^8$	$6.85X10^8$	$1.14X10^8$	$1.05X10^8$

Key: SSE = *Streptomyces* species from Noyo

Table 9: Growth of *Streptomyces* species from Enyibichiri at different pH .

		Cell Number (cells/m) at different pH		
S/No	*Streptomyces* species	7	8	9
1	SSE 1	$1.41X10^8$	$8.57X10^8$	$1.36X10^8$
2	SSE 2	$1.28X10^8$	$1.48X10^8$	$1.13X10^8$
3	SSE 3	$1.33X10^8$	$1.59X10^8$	$1.31X10^8$
4	SSE 4	$1.27X10^8$	$1.40X10^8$	$1.25X10^8$
5	SSE 5	$1.04X10^8$	$1.23X10^8$	$1.02X10^8$
6	SSE 6	$1.27X10^8$	$1.4X10^8$	$1.17X10^8$

KEY: SSE = *Streptomyces* species Enyibichiri

Table 10: Growth of *Streptomyces* species from Enyibichiri at different pH .

		Cell Number (cells/m) at different pH		
S/No	*Streptomyces* species	7	8	9
1	SSE 1	$1.3X10^8$	$6.81X10^8$	$1.21X10^8$
2	SSE 2	$1.20X10^8$	$1.37X10^8$	$1.17X10^8$
3	SSE 3	$1.31X10^8$	$4.26X10^8$	$1.9X10^8$

KEY: SSE = *Streptomyces* species Noyo

DISCUSSION

Antibiotics are the most important bioactive compounds for the treatment of infectious diseases. Thus, due to the burden for high frequency of multidrug resistant pathogens in the world, there has been increasing interest for searching effective antibiotics form soil Actinomycetes in diversified ecological niche [4].

The research revealed that the dominant antibiotics producing Actinomycetes in the soil were the *Streptomyces* species (tables 1 and 2). This is consistent with the work of [3] [4] which stated that the richest source of antibiotic producing *Streptomyces* species is the soil. The presence of *Streptomyces* species in the soil as found in this study is also in line with the work of Stephen Kugbere (2014) which stated that 1g of soil when plated, harbours up to 10 billion microorganisms, of which about $2.15x10^6$ CFU/g (dry weight) are accounted for by the *Streptomyces* species.

This study observed a range of o to 25 mm inhibition zone diameter while [14] [15] reported an inhibition zone Diameter (IZD) of 0 to 12mm and 0 to 18mm respectively. [23] reported 0 to 40mm (including the combination effect of extracts).

Absence of clear zones found in some of the Actinomycetes isolates during final examination of Mueller Hinton agar plates (table 2), shows that no antibiotics were produced in such colonies. Although, the other isolates were able to inhibit the growth of indicator bacteria, however the inhibition was not observed in all tested indicator bacteria. This therefore means that there was a low secretion of antibiotics by such Actinomycetes isolates. A probable explanation for these results could be that the culture method used in this study, did not provide ideal conditions that should have enabled the growth of large numbers of Actinomycetes and secretion of high amounts of antibiotics. Furthermore, experiments with this technique (culture-dependent methods) are quite limited in detecting the broad population of uncultured microorganisms in the soil ecosystem [8].

In all aspects, strain SSE showed broad spectrum of antimicrobial activity and it inhibits all groups of microbes used in our study.

This work recorded activity against *E. coli* and *Pseudomonas* spp. contrary to other reports that observed total resistance to the antimicrobial agents from *Streptomyces* broth by gram negative bacteria. [12] observed that antimicrobial agents were resisted by gram negative bacteria (*E. coli* and *P. aeruginosa*) and therefore stated that the reason for different sensitivity between gram positive and gram negative bacteria could be explained to the morphological differences between these microorganisms, gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, but the gram positive showed more susceptibility having only an outer peptidoglycan layer which is not an effective permeability barrier.

The antimicrobial agents showed less activity when compared to the commonly used antibiotics. The less activity of antimicrobial agents when compared to commonly used antibiotics was also reported by [5] [6]. It is also necessary to note that the antimicrobial drugs in the open market have undergone the highest possible purification while the supernatant from the broth culture of the *Streptomyces* used in this study is yet to be subjected to such series of purification and concentration.

The optimum growth (table 7and 8) of *Streptomyces* species that showed antimicrobial activity against the test isolates was recorded at the temperature of 30^0C. This agreed with the work of [17], [18] [19] that recorded peak growth for antibiotic producing, *Strptomyces* species at the temperature of 30^0C.

It was also observed that optimum growth occurred at pH 8 (table 9 and 10). This is consistent with the work of [6], [7] [8] that recorded optimum growth at pH 8.

The antimicrobial agents showed less activity when compared to the commonly used antibiotics. The less activity of

antimicrobial agents when compared to commonly used antibiotics was also reported by [21] [22]. It is also necessary to note that the antimicrobial drugs in the open market have undergone the highest possible purification while the supernatant form the broth culture of the *Streptomyces* used in this study is yet to be subjected to such series of purification and concentration.

The optimum growth (table 7 and 8) of *Streptomyces* species that showed antimicrobial activity against the test isolates was recorded at the temperature of 30^{0}C. This agreed with the work of [14], [15] [16] that recorded peak growth for antibiotic producing *Streptomyces* species at the temperature of 30^{0}C.

It was also observed that optimum growth occurred at pH 8 (Table 9 and 10). This is consistent with the work of [8], [9] [10] that recorded optimum growth at pH 8.

CONCLUSION

Till date there is no scientific report on Actinomycetes producing antimicrobial compounds from Ikwo Local Government Area of Ebonyi State, Nigeria. Based on the results obtained from this work, it can be seen that the soil samples from Ikwo have proven to be an eminent source of antimicrobial compounds from Actinomycetes. Therefore, isolation and screening of Actinomycetes from the area under study may contribute to the discovery of new antibiotics that could fight against antibiotics resistance pathogens.

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