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Microbial Analysis of Antibiotics Producing Actinomycetes Species Isolated from Soils in Ikwo LGA of Ebonyi State, Nigeria.

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Abstract

The microbial analysis of soil samples for the presence of antibiotics - producing actinomycete species from Ikwo Local Government Area of Ebonyi State, Nigeria was carried out. Isolation of actinomycete species was done using actinomycete isolation agar. 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 test organisms. The isolates characterized and identified were all S*treptomyces spp*. The antimicrobial activity of *Streptomyces sp* (SSE4) from Enyibichiri was highest with a value of 30 mm in the zone of inhibition against *Klebsiella spp* while it was least in *Streptomyces* sp (SSE 6) with 3 as the zone of inhibition against *Pseudomonas aeruginosa*. In Noyo, the antimicrobial activity of *Streptomyces* spp (SSN 1 and SSN 2) had a highest value of 22 against *S.aureus* and K*lebsiella sp respectively*.

Keywords: Screening, Inhibitory diameter,Actinomycetes, MacFarland standard

INTRODUCTION

The Actinomycetales are an order of Actinobacteria. A member of the order is often called an Actinomycete. Actinomycetes 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 (Sivercumer, 2001).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 (Jeffrey,2008). Streptomycin, actinomycin and streptothricin are all medically important antibiotics isolated from Actinomycetes bacteria (Waksman *et al.*, 2013). Almost two – thirds of the natural antimicrobial drug compounds used currently are produced by different species of Actinomycetes (Bently *et al.*, 2002). These bacteria are therefore extremely relevant to Scientists, Pharmaceutical industries and agricultural industries. 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 (Kalyani *et al.*, 2012). The two major groups of soil Actinomycetes that serve as important sources of antibiotics are *Streptomyces* and *Micromonospora*. The Streptomycetes account for about 80% of the total antibiotic products (Arifuzzaman *et al.*, 2010). Other Actinomycetes that produce bioactive compounds, but on a lower scale includes *Saccharopolyspora*, *Amycolatopsis* and *Actinoplanes* (Mukesh *et al.*, 2014).This aim of this study was to isolate, screen and characterize actinomycetes species from soil samples from Ikwo LGA.

MATERIALS AND METHODS

Materials

Study Area

This study was carried out at Enyibichiri and Noyo communities of Ikwo Local Government Area of Ebonyi State. A very large local government area located on latitude 5° and 6˚ North, longitude 7^0 and 8˚ East with annual mean temperature of 25˚C - 39˚C and rainfall of 1800mm – 2300 mm per annum.

The area has a population of 247,270 people which are mostly famers, petty traders and few civil servants (NPC, 2017). Ikwo is known for high production of the popular Abakaliki rice, yams, cassava and top palm wine. Ikwo community is also a rural settlement with few health care facilities and dispensaries. Source of water supply in the area includes boreholes, dams, well, streams and rain water stored in tanks. Defecation on open fields and farm lands is a common practice observed in this area.

Sample Collection:

A total of six soil samples were collected between the months of October to December, 2018 from different locations in Enyibichiri and Noyo communities of Ikwo L.G.A and were immediately transported to the Microbiology Laboratory of Ebonyi State University for analysis. Each soil sample was collected by clearing a minor part of the soil with a sterile spatula before it was inserted into the soil with the aid of sampling tube to the depth of 4 inches and the top soil was collected. The tube was emptied into a plastic bag, closed and labeled with a marker pen (Stephen, 2014).

Sample 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°C, and their contents was designated as stock cultures.

Isolation of Actinomycetes from Soil Samples

Serial dilution and plating techniques was carried out using actinomycete isolation agar medium. Distilled water (9ml) was taken in each of the 7 test tubes and labeled 1 to 7. 1ml of the supernatant liquid from the dissolved soil sample was transferred into the test tubes to serially dilute the sample. Next, 1 ml volume of the last dilution (10^{-7}) was measured into the Petri-dish before pouring 15ml of

the sterilized Actinomycete isolation agar unto it. The plate was shaken and allowed to gel at room temperature. All pour plates were labeled and incubated at 28˚C for 84 hours (Augustine *et al*., 2014) with multiple streaking, the pure colonies were obtained.

Characterization of Isolates

The isolates were characterized based on the Gram staining, morphology and biochemical tests.

This was further characterized by aerial mass colour (Mukesh *et al*., 2014); production of melanoid pigments (Sonia *et al*.,2011).Reverse side pigments and soluble pigments were observed according to Abebe *et al* 2013 and Mukesh *et al*.,2014.

Slide Culture Technique

A 10mm agar block (actinomycetes agar) was cut with sterile scalpel and lifted to the centre of the slide in the petri-dish. A very small quantity of the actinomycete culture was inoculated at the four corners of the agar block on the slide. The cover slip was placed over the inoculated agar block and incubated at 30^{o}C for 96 hours. The slide was examined under the microscope (x10) where undisturbed conidiophores, conidial ontogeny and conidia were observed. A drop of 95% alcohol was poured on the slide and then stained with 1% cotton blue in lactophenol and mounted for observation (Kalyani *et al.,* 2012).`

Screening of the Actinomycetes for Antimicrobial Activity.

Screening of the isolates for antimicrobial activity was done by cross streaking according to the method of (Kalyani *et al.*,2012). Each of the actinomycete isolates were streaked as a straight line on Mueller Hinton Agar and starch casein agar medium respectively. The media were incubated at 27˚C for 6 days (144h). On 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˚C for 24h. The zone of inhibition was measured to the nearest millimeter with meter rule (Kalyani *et al.,* 2012).

RESULTS

Cultural, Morphological and Biochemical Characterization of Isolates from Enyibichiri

The results of cultural, morphological and biochemical characterization of Actinomycete species from soil from Enyibichiri community as presented in Table 1. The result of the aerial mass colour showed that the common colours shown by the species were white and gray. A total of four species were not distinctive (0) for reverse side pigments production and two were distinctive (1). Colours observed for not distinctive were yellow, olive or yellowish brown colour marked as (0). The spore chain morphology indicates that most of the species were spiral and a few appeared as biverticillus-spiral and retinaculum-apertum. All the species were positive for gram staining. They were also negative for acid fast staining. The results of the biochemical tests indicates that all the Actinomycete isolates tested positive for catalase, urease and Voges proskauer tests respectively. All the isolates tested negative for methyl red test. Isolate EN 1 tested negative for indole test and the rest were positive. Isolates EN 5 tested negative for citrate utilization while two isolates tested negative for oxidase test. The results of the carbohydrate utilization tests indicates that all the isolates tested positive for dextrose, sucrose, starch and fructose utilization respectively. Isolate EN 1 and EN 2 tested negative for mannitol utilization.

Cultural, Morphological and Biochemical Characterization of Isolates from Noyo

The results of cultural, morphological and biochemical characterization of Actinomycete species from soil from Noyo community as presented in Table 2. The result of the aerial mass colour shows that the colours shown by the species were white, yellow and gray. Two species were not distinctive (0) for reverse side pigment production and one was distinctive (1). Colours observed for not distinctive were yellow, olive or yellowish brown colour marked as (0). The spore chain morphology indicates that all the species were spiral. All the species were positive for gram staining. They were also negative for acid fast staining. The results of the biochemical tests indicates that all the Actinomycete isolates tested positive for catalase, urease and Voges proskauer tests respectively. All the isolates tested negative for methyl red test. All the isolate tested positive for indole test. The isolates tested negative for oxidase test and positive for citrate utilization test. The results of the carbohydrate utilization tests indicates that all the isolates tested positive for dextrose, sucrose, mannitol, starch and fructose respectively.

Antimicrobial Activity of *Streptomyces* species from Enyibichiri against Clinical Isolates.

The results of antimicrobial activity of *Streptomyces* spp. from soil from Enyibichiri community against the clinical isolatesas presented in Table 3. Results showed that *Streptomyces* spp. 1 inhibited the growth of *Pseudomonas aeruginosa*, *Klebsiella* spp., and *Enterobacter* species while there was no inhibition on the growth of *Staphylococcus aureus* and *Escherichia coli*. *Streptomyces* spp. 2 inhibited only the growth of *Escherichia coli* while it did not inhibit the growth of *Pseudomonas aeruginosa*, *Klebsiella* spp., *Enterobacter* species and *Staphylococcus aureus. Streptomyces* spp. 3 inhibited the growth of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Enterobacter* species while it did not inhibit the growth of *Klebsiella* species. *Streptomyces* spp. 4 inhibited the growth of *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* species while it did not inhibit the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, *Streptomyces* spp. 5 inhibited the growth of *Escherichia coli* and *Klebsiella* species while it did not inhibit the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterobacter* species. *Streptomyces* spp. 6 inhibited the growth of *Pseudomonas aeruginosa* while it did not inhibit the growth of *Staphylococcus aureus*, *Klebsiella* species, *Enterobacter* species and *Escherichia coli*.

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

SN	Sample code	Cultural Characteristics			Microscopic Characteristics			Biochemical Characteristics							Carbohydrate utilization						Probable species
		AMC	RSP	MP	SCM	GS	AFS	CIT	OX	CAT	IND	MR	VP	UR	DX	MAL	SUC	MAN	FRU	ST	
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	Spiral	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp. 3
4	EN 4	G/W	1	0	SR	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp. 4
5	EN 5	G/W	1	1	Spiral	+	-	-	-	+	+	-	+	+	+	+	+	+	+	+	*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 = maltose, 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 Characterization of Actinomycete species from Noyo Community of Ikwo L.G.A, Ebonyi State.

SN	Sample code	Cultural Characteristics			Microscopic Characteristics			Biochemical Characteristics							Carbohydrate utilization						probable species
		AMC	RSP	MP	SCM	GS	AFS	CIT	OX	CAT	IND	MR	VP	UR	DX	MAL	SUC	MAN	FRU	ST	
1	NO1	White	1	1	spiral	+	-	+	-	+	+	-	+	+	+	-	+	+	+	+	*Streptomyces* spp. 1
2	NO 2	Yellow	0	0	spiral	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp. 2
3	NO 3	Gray	0	1	spiral	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	*Streptomyces* spp. 3

KEY: NO = Noyo, 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 = maltose, SUC = sucrose, MAN = mannitol, FRU = fructose, ST = starch.

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

SN	Species code	Clinical Isolates and IZ (mm)				
		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 = No Inhibition,SSE = *Streptomyces* spp. Enyibichiri, IZ= Inhibition zone, mm= millimeter

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

SN	Isolate code	Clinical Isolates and IZ (mm)				
		Staphylococcus aureus	***Escherichia coli***	***Pseudomonas aeruginosa***	***Klebsiella* species**	***Enterobacter* species**
1	SSN 1	22	NI	NI	NI	NI
2	SSN 2	NI	18	NI	22	12
3	SSN 3	15	NI	19	NI	NI

KEY: NI = No Inhibition, SSN = *Streptomyces* species, Noyo, IZ= Inhibition zone, mm= millimeter

Antimicrobial Activity of *Streptomyces* species from Noyo against Clinical Isolates.

The results of antimicrobial activity of *Streptomyces* spp. from soil from Noyo community of Ikwo L.G.A against the clinical isolates as presented in Table 4. Results showed that *Streptomyces* spp. 1 inhibited only the growth of *Staphylococcus aureus* while it did not inhibit the growth of *Pseudomonas aeruginosa*, *Klebsiella* spp., *Enterobacter* species and *Escherichia coli*. *Streptomyces* spp. 2 inhibited the growth of *Escherichia coli, Klebsiella* spp. and *Enterobacter* species while it did not inhibit the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus. Streptomyces* spp. 3 inhibited the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* while it did not inhibit the growth of *Escherichia coli, Enterobacter* species and *Klebsiella* species.

DISCUSSION

This research revealed that the dominant antibiotic producing Actinomycetes in the soil were the *Streptomyces* species (tables 1 and 2). This is consistent with the work of Kalyani *et al*., 2012 and Pandy *et al*., 2011 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 ,2014 which stated that 1g of soil when plated, harbours up to 10 billion microorganisms, of which about 2.15×10^6 CFU/g(dry weight) were accounted for by the *Streptomyces* species. This study observed a range of 0 to 25mm inhibition zone diameter while Kalyani *et al*., 2012 and Gurung *et al*., 2009 reported an inhibition zone Diameter (IZD) of 0 to 12 mm and 0 to 18 mm respectively. Results of the antimicrobial activity of S*treptomyces spp*. from soil in Noyo Community of Ikwo L.G.A showed that *Streptomyces spp1* inhibited the growth of *S.aureus* and did not for *P.aeruginosa, Klebsiella spp, Enterobacter spp* and *E.coli.* However, *Streptomyces spp* 2 inhibited the growth of *E.coli, Klebsiella spp.* and *Enterobacter spp* but did not for S.*aureus* and *P.aeruginosa while Streptomyces spp3* inhibited the growth of *S. aureus* and *P.aeruginosa* while its did not inhibit the growth. *Streptomyces* spp 1 only inhibited the growth of *Staphylococcus aureus* while it did not inhibit the growth of *P.aeruginosa*, *Enterobacter spp,E.Coli and Klebsiella spp.* This corroborates the works of Kalyani *et al.*, 2012 and Gurung *et al*., 2009.

CONCLUSION

Till date there has not been any 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 and different types of antibiotics that could fight against antibiotics resistant pathogens.

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