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Isolation of Potential Actinomycetes Producing Novel Antibiotics from Landfill Sites in Okitipupa, Ondo State, Nigeria.

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ABSTRACT: Purpose: This study was carried out with intention to isolate, identify, and screen actinomycetes obtained from landfill sites in Okitipupa for possible novel antibiotics production.
Methods: Twenty-four isolates of actinomycetes were obtained from 15 landfill sites samples collected from different locations in Okitipupa township. The isolates were identified on basis of their morphological and biochemical characteristics. The Primary screening for antibiotic activity was carried out against six clinical pathogens obtained from Okitipupa State Specialist Hospital: Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonia aeruginosa, Escherichia coli, Salmonella typhi, and Shigella boydii by cross streaking method. While, secondary screening was carried out by the agar well diffusion method using diethyl ether for the extraction of antibiotic from the Actinomycetes isolates.
Findings: Based on morphological and biochemical characterization of the isolates, **53.3**% of the isolates were identified as Streptomyces spp., 26.7% as Nocardia spp., and 20% as Micromonospora spp. 62.5% of actinomycetes isolates was found to be active against tested bacteria from the primary screening. Also, there is progressive increased in the antibiotic activity of the isolates against tested bacteria in the secondary screening, among which 76.34% were effective against Gram-positive and 80.19% against Gram-negative tested bacteria. Micromonospora spp showed highest (85.9%) broad-spectrum antibiotic activity in this study.
Recommendation: The result from this study could be used for basis of producing novel broad spectrum antibiotics from Micromonospora species. Also, landfill sites can be importance source of isolating novel Actinomycetes producing broad spectrum antibiotics.

KEYWORDS: Actinomycetes, antibiotic, landfill, screening, diethyl ether, pigment

I. INTRODUCTION

Antibiotics resistance has been constituted a serious challenges for public health in this 21st century. As the bacteria evolving to resist antibiotics faster, a newly discovering antibiotic may prove irresistible to bacteria. Every time an antibiotic is used, bacteria are getting to know it a little better. For instance, Staphylococcus aureus that cause most of the human infections has developed antibiotics resistant strains that are resistance to penicillin and its derivatives like methicillin and penicillin G (Enright, 2003). The increase in antibiotic resistance has been attributed to; incomplete and indiscriminant use of antibiotics in people and animals led to increase selective pressure on bacteria. Bacteria capable of resisting antibiotics survive and spread these traits by horizontal gene transfer (Livermore, 2003; Willey, et al., 2008). Therefore, it is crucial to exploit novel antibiotics that can effectively kill or eliminate drug-resistant pathogens. Thus, vast majority of novel antibiotics have been detected by screening of bacteria and fungi isolates obtained from soil and other natural habitats such as rhizosphere of plants, sewage, and marine (Fernando, 2006; Bawazir, et al., 2018). Although a wide taxonomic range of microorganisms have the ability to produce antibiotics, yet from all the known microorganisms the actinomycetes are a fascinating group of microorganisms. They are the major source of most of the antibiotics used in medicine today. They also produce bioactive substances as secondary metabolites that are used as anticancer agents, and drugs that suppress the immune system in patients who have undergone organs transplanting.The ability of actinomycetes to

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produce vast majority of medical useful substances are related to their mode of growth, and this usually occurs when substrate mycelia are developed. Actinomycetes produce wide range of antibiotics, for example, the broad-spectrum antibiotic; Neomycin, a powerfu antibiotic agent against gram-negative bacteria also effective against mycobacteria species that are caused tuberculosis and leprosy is derived from Streptomyces species of actinomycetes (Abebe, et al., 2013; Willey, et al., 2008; Enright, 2003). Actinomycetes are very important from a medical point of view because of their ability to produce and secrete a large variety of secondary metabolites called polyketides.These include aminoglycosides, macrolides, polyenes, tetracyclines, ansamycins, lovastatin, acetogenins and anthracycline. It is estimated that more than 80% of the antibiotics are obtained from Actinomycetes, majorly genus Streptomyces while genus Micromonospora that produces antibiotics such as gentamicin is next but with less than one-tenth Streptomyces. Although antibiotics are natural products derived majorly from bacteria and fungi. But with recent advances in Synthetic Chemistry many synthetic antibiotics such as sulfa drugs, chloramphenicol and quinolones are now produced chemically. Of course several hundreds natural and synthetic antibiotics that have been purified, only a few have been sufficiently non-toxic to be of use in medical practice.

II. MATERIAL AND METHODS

Soil sample collection

A total of 30 soil samples were collected from 15 landfill sites in Okitipupa Township (2 samples each from landfill site). Landfill site was chosen for this study because of its ecological significance to microorganisms. The soil samples were taken from depth of 5 cm and 10 cm by using sterile hand trowel and collected in clean, dry and sterile polyethylene bags. All samples were labeled and taken to Laboratory for analysis with 8 hours.

Isolation of actinomycetes

Samples of the soil collected were serially diluted tenfold in which ten grams of each sample was diluted in 90 ml normal saline water (0.9% of NaCl) followed by homogenization by horizontal and vertical agitations for a few minutes to obtain 10^{-1} dilution. Further tenfold serial dilution was made up to 10^{-4} for colony count. 1 ml of volume of each dilution was spread plated in duplicate on oatmeal agar and Actinomycetes Isolation Agar (AIA) incubated aerobically at 35°C for 10 days. The colonies were counted and recorded, also morphological characteristics were noted.

Preservation of isolates

Actinomycetes isolates were sub-cultured into Nutrients agar and stored at 4^0C for further used.

Screening of antibiotic activity of actinomycetes isolates against test microorganisms

Test microorganisms

The test bacteria used for antibiotics screening were: S. aureus, E. coli, P. aeruginosa, S. typhi, S. boydii, and S. pneumoniae. The test bacteria were all clinical isolates obtained from Okitipupa Specialist Hospital, Nigeria. The test were cultured into Nutrients agar and incubated at 35^0C for seven days prior to their used for primary and secondary antibiotics screening.

Primary screening

All the twenty-four isolates were primarily screened for antimicrobial activity against six tested bacteria according to Pandey et al., (2004). Seven day grown isolates were streaked as a straight line across diameter on Nutrient Agar plates and incubated at 30^0C for 6 days. After 6 days, the test microorganisms, namely, S. aureus, E. coli, P. aeruginosa, S. typhi, S. boydii, and S. pneumoniae were streaked at right angle, but not touching the streaked isolate and incubated at 37°C for 24 hours (Gurung, et al., 2009; Ningthoujam, et al., 2009; Lechevalier, 1989). The formation of clear zone between the actinomycetes isolate and the test organisms was regarded as positive for antibiotic production. The isolates that produced cleared zone against tested bacteria were then picked for further studies.

Secondary screening

Crude antibiotics extraction from actinomycetes isolates:

Three genera of actinomycetes isolates were identified and selected for antibiotic susceptibility. These isolates were grown in small scale submerged fermentation system. 100 ml of starch casein broth was dispensed into 250 ml Erlenmeyer flask, to which a loop full of seven days grown isolates were inoculated and incubated at 30^0C for 10 days (Abebe, et al., 2013; Bawazir and Shantaram, 2018). After ten days of incubation, the content of incubated flask was filtered using Whatman filter paper. Equal volume of 6.9% diethyl ether (1:1) was then added to the culture filtrates and shaken vigorously for 1 h and solvent phase that assumed to have contained antibiotics

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compound was separated from aqueous phase in a separating funnel. The diethyl ether phase that contains antibiotics was heated gently in evaporating tube placed on hot plate stirrer at 100 rpm for 5 mins to concentrate the crude extract.The concentrated crude extract obtained was stored and later used for secondary screening.

Disc diffusion assay:

Antimicrobial activity of the concentrated crude extract of each isolate was tested by using disc diffusion assay. For this purpose, nutrient agar (NA) was inoculated with 0.1 ml overnight culture of each test organism; S. aureus, S. Pneumoniae, P. aeruginosa, E. coli S. boydii and S. typhi. 10 µl of 25 µg/ml crude extract of each isolate was put on sterile Whatman filter paper (3.0 mm) and placed on the inoculated agar plates (Lechevlier, 1989; Willey, et al., 2008). Another filter paper that contained only diethyl ether were also placed on inoculated plates that served as negative control and this was done in duplicates. The Petri dishes were then kept in a refrigerator at 10^0C for 4 hours to allow the diffusion of the extracts in the media. The Petri dishes were then incubated at 37^0C for 24 hours to detect and measure the zone of inhibition around the discs (Lalitha, 2004).

Identification and characterization of selected isolates

All isolates were identified as actinomycetes based on colony morphology and color of mycelium (Morton, 1997; Laidi, et al., 2006). They were also subjected to Gram's staining examined under oil immersion microscope (x100). The Number was assigned (1 to 15) for every entity to confirm the color of aerial mycelium. Biochemical characteristics (sugar utilization, catalase, urease, oxidase and Hydrogen Sulphide test) were further used to assign them into three different genera based on Bergey's manual of Bacteriology classification (Berdy, 1980; Bergey and Holt, 1994; Pridham and Gottlieb, 1948).

Statistical Analysis

Data was entered using SPSS version 20.01v computer software for analysis. Data was analysed using both descriptive and inferential statistics. For the descriptive statistics, frequencies and cross tabulations were generated. The main outcome was to determine susceptibility percentage of the test organisms to the actinomycetes isolates. Other outcome was to compare the isolates antibiotics activity in term of broad spectrum. The strength of association was determined using odds ratio and $p< 0.05$ values at 95% level of confidence. The statistical significance difference between three major genera of actinomycetes isolated was tested by using Mann-Whitney U test ($p<0.05$).

III. FINDINGS AND DISCUSSION

Table1: Number of Actinomycetes isolated from 5cm and 10cm depth of digged landfill sites respectively.

Sampling sites in number	Isolates from 5cm depth in 10^6 cfu/ml	Isolates from 10cm depth in 10^6 cfu/ml
1	2	1
2	1	0
3	2	1
4	0	0
5	0	0
6	5	3
7	0	0
8	1	0
9	0	0
10	1	0
11	0	0
12	2	1
13	2	1
14	1	0
15	0	0

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A total of twenty-four different actinomycetes isolates were recovered from thirty different samples of soil obtained from fifteen landfill sites. Soil samples collected from depth of 5cm contained about seventy-five percent of total isolates compared with the soil collected from depth of 10cm that contained about twenty-five percent of the total isolates. This study was in agreement with previous studies conducted by researchers whose observed that actinomycetes were unevenly distributed in the soil (Geetanjah, 2016; Ghorbani-Nasrabadi, et al., 2013; Hackl, et al., 2004; Santhi, et al., 2010; Selvameenal, et al., 2009).

Table 2: Active isolates in primary screening.

Zone of inhibition is measured in (mm)

Samples	Probable isolates	S.aureus	S. boydii	S. Ssss S. Pneumoniae	P. aeruginosa	S. typhi	E. coli
S1	Streptomyces species	13	12	5	0	19	16
S2	Micromonospora pecies	15	15	15	10	10	12
S3	Streptomyces species.	10	12	14	0	9	7
S4	Micromonospora species	15	14	13	12	12	15
S5	Streptomyces species	14	18	16	0	11	15
S6	Nocardia species	6	9	3	0	7	8
S7	Streptomyces species	15	10	20	12	12	11
S8	Streptomyces species	8	13	0	2	6	4
S9	Nocardia species	8	0	0	0	12	10
S10	Streptomyces species	12	0	7	0	7	6
S11	Streptomyces species	15	17	0	0	6	7
S12	Nocardia species	14	0	0	2	12	14
S13	Micromonospora species	14	12	13	15	15	17
S14	Nocardia species	10	0	10	0	10	8
S15	Streptomyces species	14	13	15	2	12	14

The twenty-four isolates of actinomycetes obtained from sampled landfill sites were th identified as Streptomyces species (13 isolates), Nocardia species(7 isolates), and Micromonospora spp. (4 isolates) on the basis of morphological and biochemical characterization as shown in Tables 4 and 5. Most isolates gave different pigmentation; similar observation was reported by Remya and Vijayakumar (2008), and Santhi, et al., (2010) in their studies. The color of colonies varied accordingly from yellow (48%), brown (10%), grey (13%), and greenish brown (6%) and 3% had blue, greenish yellow, grayish white, light pink, and brownish yellow to blue pigments and this results were in agreement with observations of Abebe, Feleke and Berhanu (2013) and that of Dharmawan, et al., (2009) whose reported different colours of aerial and substrate mycelia in actinomycetes isolates. The identified isolates were then subjected to primary screening as presented in Table 2. Out of 24 pure isolates, 15 isolates showed antimicrobial activity against the tested organisms during primary screening by the perpendicular streaking method (Table 2). It was observed that all the isolates were active against Staphylococcus aureus, Salmonella typhi and Shigella boydii. While Pseudomonas aeruginosa showed resistance to majority of the isolates except isolates, S2, S4, S7 and S13. Isolates S2, S4 and S13 were identified as Monomicrospora species while isolate S7 was identified as Streptomyces species (Table 2). Chitti, et al., (2018) reported that Micromonospora species was found to be active against both Gram-positive and Gram-negative test organisms; this study was similar to their observation as it was showed in Table 2. Among 15 active isolates, 13 isolates showed zone of inhibition against most of the tested organisms. The three major species of

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actinomycetes identified that showed maximum zone of inhibition to the 75% of tested organisms were chosen for secondary screening using disc diffusion assay method.

TABLE 3: Showed antibiotic action of isolates extract against test organisms for secondary screening in disc diffusion assay.

The zone of inhibition is measured in (mm)

Test organisms	Streptomyces spp extract	Nocardia spp extract	Micromonospora spp extract	Diethyl ether without extract
S. aureus	17	16	29	5
E. coli	24	17	20	0
P. aeruginosa	14	4	25	3
S. typhi	23	18	30	1
S. pneumoniae	15	5	27	2
S. boydii	15	15	31	0

The extract from isolates using diethyl ether were subjected to agar well diffusion method assay against test organisms. Antibiotic activity was measured in millimetres in terms of zone of inhibition after 48 hours of incubation. It was observed that Micromonospora species extract showed maximum antimicrobial activity against Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Streptococcus pneumoniae, Salmonella typhi and Shigella boydii (Table 3). Micromonospora extract exhibited significant antibacterial effects against isolates. The results show that the organism produces antibiotics that are effective against both Gram positive and Gram negative bacteria. This is consistent with the findings of Chitti, et al., (2018) and Abebe, et al., (2013 that most strains of Micromonospora produced broad spectrum antibiotics. It was observed in this study that some of tested organisms were fairly resistance to Nocardia extract while only few of the tested organisms were fairly susceptible to Streptomyces extract, while majority of the organisms showed maximum susceptibility. The same observations were reported by previous researchers (Parungao, et al., 2007; Oskay, et al., 2004; Ningthoujam, et al., 2009). Generally, the isolates extract were more active against the tested organisms than the pure isolates used in the primary screening.

Table 4: Morphology characteristics of Actinomycetes isolates as observed on AIA medium and oatmeal agar.

Probable organism	Colonies appearance	Aerial mycelium	Substrate mycelium	Diffusible pigment	Growth pattern	Shape
Micromonospora spp	Greenish grey	Absent	Present; brownish grey	Present; Red-wine	Low to moderate growth	Coccoid
Nocardia spp	Whitish, light yellow	Present; whitish, blue	Yellow	None	Moderate growth	Coccoid
Streptomyces spp	whitish, grey	Present; whitish	Present; Yellowish-brown	None	Extensive growth	Long chain

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Table 5: Biochemical test results of actinomycetes isolates

Probable organism	D-glucose	Arabinose	D-galactose	D-mannitol	Sucrose	Xylose	Melanin pigment	Catalase	Oxidase	Urease	H_2S
Micromonospora spp	++	-	+	-	+	+	D	+	+	+	D
Nocardia spp	++	+	++	+	-	-	+	+	+	+	P
Streptomyces spp	++	-	++	-	++	++	-	+	+	+	P

+= Positive, -= negative, D= diverse, p= produce gas or diffuse pigment, ++= highly positive.

IV. CONCLUSION:

Phenomenon of antibiotic resistance has paved way for the searching of new antibiotics. The simple and rapid methods to do this is by isolating potential microorganisms that producing novel antimicrobial substances as the secondary metabolites from the soil and screening them against pathogenic microbes. So far so good many microorganisms have been evaluated for the production of antimicrobial substance. However the high cost and low yields have been the main problem for its industrial production (Rotich, et al., 2013; Manandhar, et al., 2017). In this study, the actinomycetes isolates with antimicrobial activity from landfill sites were isolated. Among all screened isolates, Micromonospora species metabolite showed maximum inhibition against both gram positive as well as gram negative bacteria.

RECOMMENDATION:

The result from this study could be used for basis of producing novel broad spectrum antibiotics from Micromonospora species. Also, landfill sites can be importance source of isolating novel Actinomycetes producing broad spectrum antibiotics.

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