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Molecular Characterization and Bioassay of Soil *Actinomycetes* Strains on Multidrug Resistant Bacteria

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Abstract
This study was undertaken for molecular characterization and bioassay of soil Actinomycetes strains on multidrug resistant bacteria. A total of Nine (9) uncultivated soil samples from different area in Calabar, Cross river State were randomly collected from 6-12 inches depth into tightly sealed sterile polyethylene sample bags with P^H and moisture content taken respectively. The soil samples were analyzed using standard microbiological protocol for isolation and identification. Further characterization was performed using VITEK 2 System and 16S rRNA sequence primer for Polymerase Chain Reaction (PCR) analysis. Extraction of Soil Actinomycetes strain was carried out using submerged fermentation technique and bioassay was performed using Agar well Diffusion Method. The result revealed P^H ranges from 3.3-7.1 and moisture content from 17.5-76.9 in the soil sample. PCR analysis with specific primer on selected strain revealed the presence of ten (10) different genera of soil Actinomycetes strain comprising of Leucobacter chironomi (2), Jatrophihabitan stelluris (2), Leucobacter populi (1), Rhodococcus tukisamuensis (1), Leucobacter triazinivorans (1), Nesterenkoniamas siliensis (1), Leucobacter denitrificans (1), Leucobacter kyeonggiensis (1) with their pairwise identity assess by the BLAST genomic program. All soil Actinomycetes strain extract had no inhibitory activity on MDR P. aeruginosa while antimicrobial activity ranging from 11-31mm was exhibited against E. coli, P. aeruginosa, S. typhi, Methicillin Resistant Staphylococcus aureus and K. pneumoniae. Difference in zones of inhibition produce by soil Actinomycetes strain was statistical significance at $p \leq 0.05$. The result from this study is a pointer to the strain from underexploited environments that could be a very fruitful source of novel bioactive secondary metabolites. This strains were found to be potent antimicrobial producers. Further determination of the active metabolites of these isolates which could be harness by biopharmaceutical industries is necessary.

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I. Introduction

Soil *Actinomycetes* strains are free-living filamentous, sporulating Gram-positive bacteria that are saprophytic, and occasionally exist as plants colonizing forms (Emamiet al., 2017).In the ecosystem, soil Actinomycetes strains are found in all marine sediments, hot springs, soil, and, water (Emamiet al., 2017). The presence of soil *Actinomycetes* strain in the ecosystem depends on the soil ecology such as amount of organic material, pH and humidity of the environment. Evidence abound that their proliferation remain prominent in prairie and grasslands than agricultural lands (Emamiet al., 2017). The occurrence of soil *Actinomycetes* strain has been reported in several countries including Nigeria, Turkey, India Egypt, and Malaysia (Unaoguet al., 2003; Thakur et al., 2007; Pimenta-Rodrigues et al., 2008; Jeffrey et al., 2007).

These strain are recognized to play important role in decomposition of organic matters, etiologic of plant disease, antibiotic production and so on (Emamiet al., 2017). The DNA of soil *Actinomycetes* strains is highly compose of guanine+cytosine content of 55 % (Ventura et al., 2007) and about 45% of bioactive moleculesare secreted by soil *Actinomycetes* strain. In recent time, these bioactive compounds have been proven as novel active secondary metaobolite (Abdi et al., 2011; Andalibiet al., 2015).

Earlier study as posited the high capacity of Soil Actinomycetes strains to synthesize secondary metabolites such as herbicides, enzymes, anticancer drugs, antibiotics and other useable industrial compound. In the context of antibiotic synthesis, these strain account they more than 75% of antibiotics and various classes of antimicrobial compounds (Emamiet al., 2017). Clinically important antibiotics use in agricultural husbandry, veterinary medicine, human medicine and allied industries are produce from these strains (Basilioet al., 2003; Saadoun, 2003). According to Pandey et al. (2004) about 80% of antibiotic medication are produce by soil *Actinomycetes* strain mostly from genera *Micromonospora* and *Streptomyces*; this strain remains the most economically and biotechnologically useful microbes (Pandey et al., 2004).

However, emergence and dissemination of antimicrobial resistant including multidrug-resistant strain, is on the increasing, affecting larger population in a community. Moreover, the reported incidence of MDR has become a serious problem due to prolong hospital stay due to treatment failure and there’s need for urgent attention. As such, this study intends to isolate and characterized soil *Actinomycetes* strain from soils sample in Calabar, Cross river state Nigeria and evaluate their antimicrobial activity as a panacea for treatment infection cause by MDR bacteria.

II. Method

Sample collection and Isolation of soil *Actinomycetes* strain

About nine (9) random uncultivated soil samples from different area in Calabar located at latitude 4° 58’ 36’ N and longitude 8° 20’ 18’ E. The samples were named AC1, AC 2, AC 3, AC 4, AC 5, AC 6, AC 7, AC 8, AC 9 accordingly. Each sample were collected from 6-12 inches depth with the P^H and moisture content of respective soil taken before sample collection. The samples were placed in tightly sealed sterile polyethylene sample bags, and immediately transported to Biotechnology laboratory unit of University of Calabar for Bacteriological Analysis. The soil *Actinomycetes* strain in the soil samples was isolated using 10fold serial dilution as described by Emami et al. (2017). An aliquot of 0.5 ml of each dilution 10^{-4} was taken and inoculated by streaking on sterile solidified *Actinomyces* Isolation Agar (AIA) medium. The inoculated agar plates were incubated at 30°C for 24hours. After overnight incubation, macroscopic characterization of the isolated soil *Actinomycetes* strain revealed submerged mycelium, aerial mycelium and diffusible pigments as described in Microbiology Practical Handbook (Iroha et al., 2019). Selection of ten (10) isolates was performed on the basis of their distinct pigmentation and mycelium for further confirmatory test using VITEK 2 System (bioMerieux, France) according to manufacturer’s guideline.

Clinical Strains Screening

Pure colony of non-repeated and non-duplicated MDR clinical bacteria; *E. coli*, *P. aeruginosa, S. typhi*, Methicillin Resistant *Staphylococcus aureus*, and *K .pneumoniae* were collected from University of Calabar Teaching Hospital, Cross River State, Nigeria. The clinical strains were confirmed using VITEK 2 System (bioMerieux, France) according to manufacturer’s guideline.

Strain Extraction.

The ten selected soil *Actinomycetes* strain, were subjected to submerged fermentation as described by Sapkota et al. (2020). Equal volume of supernatant was mixed with ethyl acetate and incubated for 24hours. After overnight, the upper layer of ethyl acetate was separated and evaporated at 40°C. The concentrated solvent was used for bioassay (Sapkota et al., 2020).

Bioassay of Antibacterial Activity

The test was performed using Agar Well Diffusion Technique as described by Osuntokun et al., (2018). A single well was bore at the center of Solidified Muellar-Hinton agar plate using a sterile cork-borer of 6mm diameter. Exactly 0.5 MacFarland Standard with an equivalent approximate density of MDR bacteria population of 10^6 colony forming unit per milliliter (Cfu/ml) of test isolate was streaked evenly on the agar plate surface and 1mL of crude extract supernatant was poured in the wells respectively and the inoculated plate incubated at 37°C for 24-48 hours. Inhibition Zone was measured and recorded in millimeters

16S rRNA sequencing of soil Actinomycetes strain

DNA extraction and purification

The ZR bacterial DNA Mini prep was use for all genomic DNA extracted and purified using as describe by Egwu et al. (2021). PCR sequencing of the 16S rRNA gene with forward primer (16S rRNA Actino Specific Forward Primer -5'-CGTATTACACATGCAAGTCGA-3' and Actino Specific Reverse primer-5'CGTATTACCGCGGCTGCTGG-3') and all the protocols were implemented following the methods previously described by (Salim et al., 2017; Egwu et al., 2021).The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator V3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis.

Statistical Analysis

One-way ANOVA was use to estimate difference in zones of inhibition produce by soil *Actinomycetes* strain**.** The statistical significance level was expressed at $p \leq 0.05$.

Table 1: Physical Characteristics of Soil samples from uncultivated farmlands within Calabar metropolis

Location/Source	Soil color	Texture	pH	Moisture content
AC1	Dark brown	Clay and silt	6.1	76.9
AC2	Reddish	Sand and Clay	5.5	61.8
AC3	Light brown	Clay and silt	5.8	53.6
AC4	Black	Loam	3.3	37.9
AC5	Black	Clay and Loam	3.3	27.5
AC6	Black	Loam and silt	3.5	17.8
AC7	Pale brown	Silt and clay	7.1	17.5
AC8	Brown	Clay	6.9	23.6
AC9	Dark brown	Loam and clay	6.2	20.3

Table 2: Antibacterial Activity of Soil Actinomycetes strain on Multidrug resistant bacteria

Identified Strain	Inhibition Zone					Antibiotic (μg)	
	S. aureus	*P. aeruginosa*	*E. coli*	*K. pneumoniae*	*S. typhi*	Streptomycin (10)	p-*value**
Leucobacter chironomi	23	0	0	9	12	30	
Leucobacter chironomi	13	0	12	15	0	27	
Jatrophihabitan stelluris	18	0	17	10	25	30	0.000019
Leucobacter populi	0	0	11	0	17	29	
Rhodococcus tukisamuensis	11	0	21	11	10	28	
Leucobacter triazinivorans	21	0	8	19	14	30	
Nesterenkoniamas siliensis	34	0	14	12	9	26	
Leucobacter denitrificans	25	0	19	0	10	28	
Jatrophihabitan stelluris	30	0	11	0	13	29	
Leucobacter kyeonggiensis	31	0	12	24	20	30	

Key: NI-No inhibition,**One-Way ANOVA**; $p < 0.05$

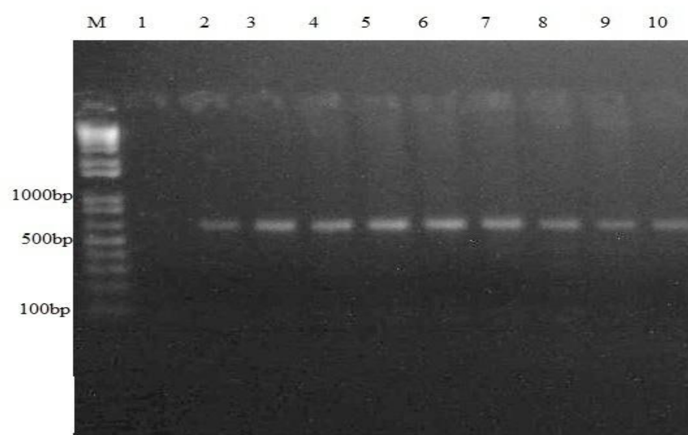

Figure 1. Agarose Gel Electrophoresis of 16S rRNA Gene Amplification of Soil *actinomycetes* isolates (band size = 550kb)

Key: Lane M= molecular size markers (100 bp); Lanes 1-10= Number of isolates

Table 3: Pairwise Identity Corresponding to the Gene Sequences of the Isolated soil *Actinomycetes strain*

S/No.	Description	E value	Pairwise Identity	Accession number
A1	*Leucobacter kyeonggiensis* strain F3-P9 16S ribosomal RNA, partial sequence	0.0	85.89%	NR_132679.1
A2	*Leucobacter chironomi* strain MM2LB 16S ribosomal RNA, partial sequence	0.0	86.05%	NR_044475.1
A3	*Jatrophihabitan stelluris* strain N237 16S ribosomal RNA, partial sequence	0.0	82.66%	NR_163611.1
A4	*Leucobacter populi* strain 06C10-3-11 16S ribosomal RNA, partial sequence	2e-163	87.28%	NR_144591.1
A5	*Rhodococcus tukisamuensis* strain Mb8 16S ribosomal RNA, partial sequence	7e-161	81.71%	NR_028629.1
A6	*Leucobacter triazinivorans* strain JW-1 16S ribosomal RNA, partial sequence	1e-84	85.16%	NR_159263.1
A7	*Nesterenkoniamas siliensis* strain NP1 16S ribosomal RNA, partial sequence	0.0	81.73%	NR_144709.1
A8	*Leucobacter denitrificans* strain M1T8B10 16S ribosomal RNA, partial sequence	2e-137	83.09%	NR_108568.1
A9	*Jatrophihabitan stelluris* strain N237 16S ribosomal RNA, partial sequence	0.0	82.42%	NR_163611.1
A10	*Leucobacter kyeonggiensis* strain F3-P9 16S ribosomal RNA, partial sequence	0.0	87.79%	NR_132679.1

III. Result and Discussion

In this current study, antibiotic-producing soil *Actinomycetes* strain were identified from soil samples in Calabar metropolis. Ten strains were selected based on their distinct pigmentation, aerial mycelium, and submerged mycelium. The degree of DNA similarity of the strain were assess by The BLAST genomic program (www.ncbi.nlm.nih.gov/blst) which reveal the different genera of soil *Actinomycetes* species comprising of *Leucobacter chironomi* (2), *Jatrophihabitan stelluris* (2), *Leucobacter populi* (1), *Rhodococcus tukisamuensis* (1), *Leucobacter triazinivorans* (1), *Nesterenkoniamas siliensis, Leucobacter denitrificans* (1), *Leucobacter kyeonggiensis* (1). The distribution of the isolate support the fact stated in earlier study that one gram of soil when plated, harbors up to 10 billion microorganisms, of which about 4.2×10^6 CFU/g (dry weight) are accounted for by bacteria species (Torsvik and Ovreas, 2002). The genera of soil *Actinomycetes* identified in this study differs from the common strain reported in other studies; Salim et al., (2017) reported *Nocardia alba* in soil and water sample while Sapkotaet al. (2020) reported Streptomyces 19.5%, *Micromonospora* species 43.34%and *Nocardia*s pecies 9.5% of *actinomycete* isolates. However, this current study opine our findings to variation in altitude and soil type and their contents, which may elucidate the possibility of observing similar

soil *Actinomycete* strain, which conjectures to vary the distribution of antimicrobial producing *Actinomycetes* (Gurunget al., 2009).

It's also suggestive of the fact that exposure of the soil to sun rays, inadequate vegetation covering of most soil sample, and steepness of the soil terrain may have resulted in leaching of commonly reported soil *Actinomycetes* and therefore have partly contributed to the observed difference in *Actinomycetes* genera documented in this study. Additionally, the difference observed from other studies may result from fact that the environmental factor of the sample source such as diversity of plants species grown on that particular soil, pH and humidity may influence the growth rate of different *Actinomycetes* genera (Singh et al., 2009). According to Emami et al. (2017) most *Actinomycetes* are mostly isolated from soils with pH values around 6 – 7.5 and 15% humidity, as the author further reported detectable association between the pH and humidity of soil samples and the difference in *actinomycetes* genera. Additionally, the aforementioned strain in our study are putative organisms that encompasses the production of antibiotics in this work.

In vitro bioassay of soil *Actinomyces* genera revealed zone of inhibition ranging from 11-31mm against *S. aureus,* 11-21mm to *E. coli*, 9-24mm to *K. pneumoniae,* 9-25mm to *S. typhi***.** There was no statistical difference in zones of inhibition produce by soil *Actinomycetes* strain ($p \leq 0.05$)**.** Our observation agrees with findings from various studies; Aparanji et al. (2013) also reported that all the 42 *Actinomycete* strains tested demonstrated antibacterial activity corresponding to our current findings. Elbendary et al. (2018) found inhibition zones of 23mm against *S. aureus* which support data obtained in this study and also similar to report in Egyptian Soils (Mabrouk and Saleh, 2014). In yet another study, crude extracts obtain from submerged state fermentation at a concentration of 500 µg/ml displayed antimicrobial activity against *Escherichia coli* measuring 11mm (Salim et al., 2017) which therefore support inhibition diameter recorded against *E. coli* in our study. Previous study recorded Minimum Inhibitory Concentration (MIC) of 2.5 mg/ml against *E. coli* (Hotam et al., 2013). It's worth noting that, the morphological features of MDR *S. aureus* i.e., the outer peptidoglycan matrix which is not an effective permeability barrier may have resulted in the high antibacterial activity observed against *S. aureus* in the current study. During the bioassay, *P. aeruginosa* exhibited high level of resistant to all antibiotic producing strain. Similar observation has been reported elsewhere (Sapkota et al., 2020)**.** However, such observation is linked to an outer polysaccharide membrane component of the bacteria which make the cell wall impermeable to lipophilic solutes. In the context of displayed *P. aeruginosa* resistant, another explanation for these results could be linked to the crude extract methodology employed in this current study, which may not have provided an ideal conditions that should have enable the recovery of high amounts of natural antibiotic molecule.

Interestingly, the high antibacterial activity of the antibiotic producing strain in our study may be linked the solvent (ethyl acetate) used, resulting in bioactive compound solubilization in the solvent which in turn enhances the increase in concentration of the secondary metabolite or bioactive compound in the extracted mixture.

To the best of our knowledge, this is the first reported bioassay of soil-actinomycetes strains (*Leucobacter chironomi*, *Jatrophihabitan stelluris*, *Leucobacter populi, Rhodococcus tukisamuensis*, *Leucobacter triazinivorans*, *Nesterenkoniamas siliensis*, *Leucobacter denitrificans*, *Leucobacter kyeonggiensis*) isolated from this settings in Nigeria. The result from this study is a pointer to the strain from underexploited environments that could be a very fruitful source of novel bioactive secondary metabolites. This strains were found to be potent antimicrobial producers. Further determination of the active metabolites of these isolates which could be harness by biopharmaceutical industries is necessary, while different molecular analysis methods such as PCR-based fingerprinting techniques and DNA re-association may help broaden the scope about the total genetic and clonal diversity among soil *Actinomycetes* community. Wherein, such methods could enhance and improve isolation of antibiotic producing soil *Actinomycetes* strains reported in this current study.

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