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Isolation and Screening of Streptomyces spp from Soil Samples of Ekiti State University Nigeria for Antibacterial Activity

Kehinde Caleb Omidoyin[1]*, Titilayo Olufunke Femi-Ola[2]

[1] Department of Microbiology, University of Ibadan, Ibadan, Nigeria
[2] Department of Microbiology, Ekiti State University, P.M.B. 5363, Ado-Ekiti, Nigeria

***Corresponding author e-mail:** omidoyinkehindecaleb@gmail.com

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ABSTRACT

Actinomycetes are a versatile group of procaryotes that exist in all habitats and are widely distributed in all types of the ecosystem including the soil, water, and sediments obtained from deep sea. This group of gram-positive bacteria, especially the *Streptomyces* is widely known to produce a variety of secondary metabolites, including antibiotics and other therapeutically useful bioactive molecules. Therefore, the isolation and identification of new strains is crucial for the production of desirable antibiotics. The aim of this study was therefore to isolate and identify novel strains of *Streptomyces* exhibiting a high antibacterial activity. To achieve this objective, 60 actinomycetes colonies were isolated in pure cultures from different soil samples within Ekiti State University, Nigeria, and 12 isolates which showed a high antibacterial activity against test organisms were selected. The selected actinomycetes isolates were characterized for morphological and biochemical identifications. Analysis of morphological and biochemical characteristics indicated that all the 12 selected isolates exhibiting antibacterial properties belonged to the genus *Streptomyces.* Five (41.7%) of the *Streptomyces* isolates produced diffusible pigments of different colors. Moreover, screening of the isolates with regard to their antimicrobial activity against test bacteria indicated that all the *Streptomyces* isolates exhibited antimicrobial activity against at least two of the test organisms while eight isolates (66.7%) were found to have moderate to high antimicrobial activity. Two (16.7%) of the *Streptomyces* isolates were active against all the test organisms while the highest antibacterial activity based on zone of inhibition was observed in *Klebsiella* spp. with an inhibition zone of 18mm. Since 20% of the actinomycetes isolates in this study showed inhibitory effects against indicator bacteria, it was suggestive that soil of Ekiti State University, Nigeria could be used as an interesting source to be explored for novel *Streptomyces* strains with high potency of antimicrobial production.

Keywords: isolation; actinomycetes; *Streptomyces;* antibacterial activity.

1. INTRODUCTION

The soil remains a reservoir source for the isolation of new microbial strains with the potentiality to produce active secondary metabolites. Among such microbial strains, actinomycetes are of special interest, since they are known to produce chemically diverse compounds with a wide range of unique and biologically active metabolites (Bawazir & Shantaram, 2018), of commercial importance in medical and agricultural applications (Rotich *et al.*, 2017).

The genus *Streptomyces* are known to provide wide variety of new antibiotics more than any other genus; hence, it is of the foremost importance for both industrial application and human health care (Baniya *et al.*, 2018). They produce a large number of secondary metabolites and particularly antibiotics that are in favor of pharmaceutical companies nowadays, resulting in conduction of widespread investigation towards discovery of new antibiotics. Additionally, *Streptomyces* is one of the model systems for the evaluation of various bacterial characteristics (Worrall & Vijgenboom, 2010). The demand for new antibiotics continues to grow due to the rapid emergence of multi-drug resistant pathogens. Actinomycetes are free-living, saprophytic, gram positive filamentous bacteria, with high G+C content. They are found in soil, fresh and marine water environments (Tandale *et al.*, 2018).

Soil as their main natural habitat is nutritionally, biologically and physically complex and variable. As a result, they are able to perform a broad range of metabolic processes and to produce an immense diversity of bioactive secondary metabolites (Baniya *et al.*, 2018). The present study was conducted to isolate and identify novel soil actinomycetes. As a part of our investigation, we have selected the unexplored area of the southwest Nigeria i.e. Ekiti State University for the isolation of potent actinomycetes. Ekiti State University, Nigeria is mainly an upland zone, rising over 250 meters above sea level. It lies on an area underlain by metamorphic rock. Due to its vegetation and geographical distribution, there are promising chances of diversity of microorganisms with antibiotics-producing potentials. The present study dealed with isolation, screening and identification of potent actinomycetes from soil samples collected from Ekiti State University, Nigeria.

2. METHODS

2.1. Isolation of bacterial strain

Soil samples were collected from different locations within Ekiti State University, Nigeria from 10-15 cm depth. The collected soil samples were air-dried for about 14 hours (overnight) and subsequently oven-dried at 50°C for 15 minutes and kept at 4^{o}C until microbial assays be performed on them. The serial dilution process was used in the process of isolation. One gram (1g) of each soil sample was placed in a sterile test tube, in which 9 mL of sterile distilled water was added and mixed thoroughly. Each of the sample was serially diluted i.e. 1 mL from the stock sample was transferred to the first test tube containing 9 mL of sterile distilled water. This was serially diluted to 10^6 dilution. Each time the contents were vortexed to form a uniform suspension. The pour plate method was used as the method of isolation. A sterilized media of malt-extract-yeast-extract-agar was prepared according to the manufacturer's standard. To minimize the bacterial and fungal growth, chloramphenicol suspension (125 mg/mL) and nystatin (100,000 IU/mL) were used. One millimeter (1 mL) of 10^6 dilution was inoculated on sterile petri dishes, and then the sterilized media was poured aseptically on the plates and the plates incubated for 7 days at 28^{o}C. The identical colonies were scored out and the selected colonies were sub-cultured on malt-extract-yeast-extract-agar slants and incubated at 28°C for 7 days. Morphological features of colonies such as colony pigmentation were used for preliminary classification of the bacterial population. Sixty disparate isolates with different morphological characteristics were kept at 4^{o}C and tagged as a distinct isolate based on their sampling location and the order of colony isolated from same soil sample (Kreuze *et al.*, 1999; Oskay *et al.*, 2004).

2.2. Screening for antibiotics-producing Streptomyces strains

The screening for antibacterial activity was done using the cross-streak method. The antimicrobial activities of the isolates were tested by cross streak plate method employing nutrient

agar medium for bacteria. The media were sterilized by autoclaving at 121 °C for 15 min. Then cooled into 40-45 °C and then poured into Petri plates and allowed to solidify. Each plate was streaked with one isolate of actinomycetes at the center and incubated at 20 °C for 7 d (Ganesan *et al.*, 2017). After incubation, test organisms were streaked perpendicular to the growth of the isolate; 24 h culture of bacteria. The test organisms included four gram-positive bacteria; *Staphylococcus aureus, Bacillus* spp*, Proteus* spp, *Pseudomonas aeruginosa*, and two gram-negative bacteria - *Escherichia coli* and *Salmonella typhi.* The inhibitory effect was evaluated after 48 hours of incubation at 28^{o}C and the results were evaluated by the measurement of inhibition zone diameter.

2.3. Morphological and biochemical characterization

The actinomycetes isolates showing inhibitory effects on test organisms were selected and the morphological characteristics of the 7-day-old culture isolates on malt extract-yeast extract agar which include the shape, size, surface etc. was studied and recorded. The coverslip technique was used to observe hyphae and spore chain morphology by light microscopy. The color of the aerial mycelia, substrate mycelia and pigment production by the selected isolates was determined on malt-extract-yeast-extract-agar after 7 days of incubation at 28°C. Further characterization of the selected isolates was performed with the using various biochemical tests.

3. RESULTS AND DISCUSSION

A total of 60 actinomycetes strains were isolated from different soil samples obtained from 8 sample locations within Ekiti State University, Nigeria. The soil sample from sample location FacAg with a pH of 8.0 showed the highest number of actinomycetes colonies isolated with 25% of the total isolated colonies in all the soil samples collected while the soil sample from FacLaw with a pH of 6.5 showed the lowest number of colonies isolated with a percentage of 3.33% (Table 1).

The result of screening based on the effect of intact bacteria revealed that of the 60 actinomycetes colonies isolated, only 12 potent isolates showed antimicrobial activities against indicator bacteria with varying zones of inhibition (Table 2). Based on the results presented in Table 2, EKGLA 2 and EKGLA 12 were discovered to have a broad-spectrum activity as the isolates were able to inhibit all the test organisms, including the gram-positive and gram-negative bacteria. EKGLA 11 was also observed to have a broad spectrum of activity but could not inhibit the growth of *Escherichia coli.* All the isolates possessed an earthy odor and were observed to be slow growing, aerobic, glabrous or chalky, folded and with aerial and substrate mycelia of different colors. Five (5) of the selected isolates produced diffusible pigments with colors ranging from yellow to brown (Table 3). The physiological characteristics of the isolates revealed that they all reacted positively to gram's reaction and were all positive to catalase test. However, only 6 of the isolates were able to hydrolyze starch and lipid. None of the selected isolates was positive for indole test, motility test, MR test and VP test (Table 4). The sugar fermentation tests of the selected actinomycetes isolates showed that all the isolates were capable of fermenting glucose and galactose, whereas no isolate could ferment lactose and mannitol. All the selected isolates could ferment sucrose and arabinose except EKGLA 9 and EKGLA 6, respectively.

Table 1. The microbial load of the soil samples collected from different locations within Ekiti State University

Sample collection site code	Number of actinomycetes colonies (CFU/gram)	pH of soil samples
FacAg	1.5×10^5	8.0
NFCom	9.0×10^6	7.5
PTPCom	1.1×10^5	7.3
OFScB	4.0×10^6	7.4
FacLaw	2.0×10^6	6.5
SchLib	7.0×10^6	6.9
HCA	5.0×10^6	7.0
GHOF	7.0×10^6	7.2

Table 2. Zone of inhibition of the actinomycetes isolates on the test microorganisms (mm)

Isolates	*Bacillus* spp.	*Klebsiella* spp.	*E.coli*	*Proteus* spp.	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
EKGLA 1	4	0	0	3	0	0
EKGLA 2	6	18	16	6	15	5
EKGLA 3	3	0	0	0	13	0
EKGLA 4	0	0	0	2	2	0
EKGLA 5	0	15	0	3	12	0
EKGLA 6	4	0	6	0	0	3
EKGLA 7	8	2	2	0	0	0
EKGLA 8	0	0	0	12	3	8
EKGLA 9	0	2	0	2.5	2	1.5
EKGLA 10	14	9	6	0	0	0
EKGLA 11	8	3	0	4	10	6
EKGLA 12	5	11	3	12	8	5

Table 3. Morphological characteristics of the *Streptomyces* spp. Isolates

Isolate	Growth degree	Colony form	Color of aerial mycelia	Color of substrate mycelia	Diffusible pigment
EKGLA 1	Abundant	Rhizoid, flat surface	White	Gray	ND
EKGLA 2	Abundant	Oval, flat, and leathery	Light green	Yellow	Golden yellow
EKGLA 3	Moderate	Round, concave, and leathery	Pale yellow	Deep yellow	ND
EKGLA 4	Abundant	Rhizoid, flat surface	Gray	Gray	ND
EKGLA 5	Abundant	Round, concave, and tough	Greenish brown	Blackish green	Brownish yellow
EKGLA 6	Few	Round, concave, and wooly	Snow white	White	ND
EKGLA 7	Moderate	Tough, leathery and Oval	Reddish brown	Brown	ND
EKGLA 8	Abundant	Rhizoid, flat surface	Snow white	Pale yellow	ND
EKGLA 9	Abundant	Oval and leathery	Green	Yellow	Golden yellow
EKGLA 10	Moderate	Tough, leathery, and complex	Reddish brown	Brown	ND
EKGLA 11	Moderate	Oval and wooly	Brownish black	Darky	Brown
EKGLA 12	Abundant	Round, flat and leathery	Pale green	Yellow	Yellow

ND- Not detectable

Table 4. Physiological and Biochemical characteristics of the *Streptomyces* spp. Isolates

Isolates	Gram staining	Catalase test	Starch hydrolysis test	Lipid hydrolysis	Gelatin hydrolysis	Esqulin hydrolysis	Indole test	MR test	VP test	Motility test
EKGLA 1	+	+	-	+	-	-	-	-	-	-
EKGLA 2	+	+	+	+	-	+	-	-	-	-
EKGLA 3	+	+	-	-	+	+	-	-	-	-
EKGLA 4	+	+	-	-	+	+	-	-	-	-
EKGLA 5	+	+	-	-	+	+	-	-	-	-
EKGLA 6	+	+	+	+	-	-	-	-	-	-
EKGLA 7	+	+	-	-	-	-	-	-	-	-
EKGLA 8	+	+	-	-	+	+	-	-	-	-
EKGLA 9	+	+	+	-	-	+	-	-	-	-
EKGLA 10	+	+	+	+	-	-	-	-	-	-
EKGLA 11	+	+	+	+	-	+	-	-	-	-
EKGLA 12	+	+	+	+	+	-	-	-	-	-

The soil is a natural ecological treasury with many microorganisms living together, and many of these organisms produce useful natural bioactive compounds, including antibiotics. This study was performed with the aim of isolating *Streptomyces* strains with antibacterial activities using the selective isolation media. *Streptomyces* strains from different soil samples within Ekiti State University, Nigeria were isolated, characterized and screened for their ability to produce antibacterial agents.

From the result in Table 1, it was observed that the soil sample from sample site FacAg with pH 8.0 had the highest number of actinomycetes colonies isolated accounting for 25% of the total isolated colonies in all the samples, hence having the highest microbial load of $1.5x10^5$ cfu/gram. However, the soil sample from sample site FacLaw with a pH of 6.5 was observed to have the lowest number of colonies isolated with a percentage of 3.33% which represented the lowest microbial load of $2.0x10^6$ cfu/gram. The soil sample with the highest microbial load was collected from a recently cleared land which was still covered by dead and decaying plants and not very far from a heavily-loaded dump site. The pH of the soil was 8, and this may probably be the reason for the highest count in the soil sample. This was in agreement with the results achieved by Oksay *et al.* (2004). It could be deduced that soil pH tending to the alkaline scale was the best candidate for exploration studies on *Streptomyces* regarding their antibacterial activity.

The test microorganisms were four gram-negative bacteria and two gram-positive bacteria. The gram-positive bacteria were *Bacillus* spp and *Staphylococcus aureus* while the gram-negative bacteria were *Klebsiella* spp*, Escherichia coli*, *Proteus* spp and *Pseudomonas aeruginosa.* As shown in Table 2, 12 *Streptomycetes* isolates showed an antibacterial activity against at least two of the test organisms. However, the isolates exhibited different inhibitory patterns against the test organisms. It was shown that *Bacillus* spp*, Proteus* spp and *Staphylococcus aureus* were inhibited by 8 of the 12 isolates (66.7%), *Klebsiella* spp was inhibited by 7 of the 12 isolates (58.3%), *Escherichia coli* was inhibited by 5 of the 12 isolates (41.7%), while *Pseudomonas aeruginosa* was inhibited by 6 of the 12 isolates (50%). Isolates EKGLA 2 and EKGLA 12 were discovered to have a broad-spectrum activity as the isolates were able to inhibit all the test organisms, including the gram-positive and gram-negative bacteria. Isolate EKGLA 11 was also observed to have a broad-spectrum activity but could not inhibit the growth of *Escherichia coli.* Isolate EKGLA 3 could only inhibit the gram-positive bacteria among the test organisms, which were *Bacillus* spp and *Staphylococcus aureus* but could not inhibit any of the gram-negative test bacteria.

The highest zone of inhibition was observed in *Klebsiella* spp. with a zone of inhibition of 18 mm by the isolate EKGLA 2. In a

study from the soil samples of Karanjal regions of Sundarbans of Bangladesh, about 55 actinomycetes of different genera were isolated and screened for the antibacterial activity (Arifuzzaman *et al.*, 2010). In their screening work, they found that 20 isolates (36.3%) were active against the test organisms. In another study, 356 *Streptomyces* isolates were obtained from soils in the Aegean and East Black Sea regions of Turkey, and 36% of the isolates were found to active against tested microorganisms (Denizci, 1996). In another study performed in 2010 by Dehand *et al.* (2010) the antibacterial activity of *Streptomyces* isolates from soil samples of West Iran was investigated. Out of 150 isolates, only 20 isolates (13.30%) showed an activity against the test bacteria.

From Table 3, it was shown that the *Streptomyces* isolates with antibacterial activity exhibited different growth degrees, ranging from few, moderate to abundant. Of the twelve (12) isolates, 58% grew abundantly, 33% grew moderately while only 8% showed a few growths. Table 2 also showed the colony morphology of the isolates. Over 66% of the isolates were round to oval in shape while others showed a rhizoid growth. The predominance of round to oval shape was also noted by Nonoh *et al.* (2010), where 60% of the isolates were round to oval in shape. It was also observed from Table 3 that *Streptomyces* colonies had different colors of both the aerial mycelia and substrate mycelia on malt-extract-yeast-extract-agar. The varying color indicated that the *Streptomyces* isolates were of different species and strains. It was observed that some of the isolates were able to produce diffusible pigments into the medium while others could not produce diffusible pigment. Of the twelve (12) different isolates, only 41.7% were found to produce diffusible pigments. This result varies with the study carried out in 2010 by Md. Ajijur *et al. (*2011) in which out of 30 isolates only two isolates (6.7%) produced diffusible pigments. The pigments were of different colors which were golden yellow, brownish yellow, brown and yellow. It was also discovered that for isolates able to produce pigments, the color of their substrate mycelia was very similar to the color of the pigment produced.

It could be deduced from Table 3 that all the *Streptomyces* isolates were positive to Gram's reaction which implies that they are all gram-positive bacteria which was in accordance to the description of genus *Streptomyces* in the 9th edition of Bergey's Manual of Systemic Bacteriology (Aryal, 2019). Also, all the isolates were positive to catalase test which signified that all the *Streptomyces* isolates possessed the enzyme catalase which would breakdown hydrogen peroxides (H_2O_2) to release free oxygen gas. However, not all the *Streptomyces* isolates could hydrolyze starch. From Table 3, it was shown that only 50% (6 out of 12) could hydrolyze starch, lipid and esqulin. In the work of Nonoh *et al.,* (2010), 66% of the isolates were positive to starch hydrolysis test.

Table 4 showed the sugar fermentation pattern by the *Streptomyces* isolates. It could be observed that all isolates could ferment glucose, galactose, and sucrose except isolates EKGLA 4 and EKGLA 9. Arabinose sugar was fermented by all the isolates except isolate EKGLA 6 whereas lactose and mannitol were not fermented by the isolates. Result from Table 3 showed that the morphological properties of isolates EKGLA 2 and EKGLA 12 were almost the same. Results from Table 3 and 4 also showed that the physiological and biochemical characteristics as well as the sugar fermentation pattern of these isolates, EKGLA-2 and EKGLA-12 were very much similar. However, it was discovered from the data in Table 2 that these two isolates exhibited a broad spectrum of activity against all the test organisms, including the gram-positive and gram-negative bacteria. Also, these two isolates produced diffusible pigments of similar color. Therefore, it could be suggested that these two isolates were of the same species but different strains.

4. CONSLUSIONS

Comparing the above-mentioned results with this study, it could be concluded that the soil samples obtained from Ekiti State University, Nigeria were rich sources of *Streptomyces* spp. which produced metabolites inhibitory to bacteria pathogens. However, further characterization of the isolates was needed to identify the isolates to the strain level. Also, further investigations were needed in order to determine the active metabolites produced by these isolates.

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