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ANTIBIOTICS PRODUCING BACTERIA ISOLATED FROM FARMLANDS

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ABSTRACT

The need for new antibiotics has been highlighted recently with the increasing pace of emergence of drug resistance pathogens. Emerging strains of bacteria resistant to most advanced antibiotics have become issues of very important public health concern. Modification of existing antibiotics with the addition of side chains or other chemical group and genomics based drug targeting have been the preferred method of drug development at the corporate level in recent years. In this regard, soil samples were collected from farmlands located in Ibadan in Oyo state, Ago – Iwoye, and Ikenne in Ogun state, Nigeria. Two putative *Streptomyces* strains and *Bacillus* strains isolated from the 16 selected farmland soils were characterized and assessed for antibiotic production and activity against a wide range of bacteria including *Klebsiella pneumonia*, *Serratia marscens*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*.
The extracts of the putative obtained from *Streptomyces somaliensis*, *Streptomyces anulatus*, *Bacillus megaterium*, and *Bacillus subtilis* showed activities against minimum of 3 and maximum of the 4 of the 7 tested bacteria. Inhibition zones were found to range between 2.0 - 25.0 mm diameters at a concentration of 1ml. The minimum inhibitory concentrations (MICs) of the crude extracts against the tested organisms ranged from 50% and above.
Bacillus megaterium, and *Streptomyces somaliensis* were found to inhibit all the pathogenic bacteria, while *S. anulatus* was unable to inhibit *Proteus vulgaris* and *Staphylococcus aureus*, and *B. Subtilis* was unable to inhibit *Enterococcus faecalis.*

Keywords: Antibiotics, *Bacillus*, Bacteria, Soil, *Streptomyces*

INTRODUCTION

The term soil refers to the outer loose material of the earth crust (**Certini and Ugolini, 2013**). Living portion of the soil body includes small animals and microorganisms such as fungi and bacteria, but it is generally considered that it's microorganisms that play the important role in soil **(Arifuzzaman *et al*., 2010**). Actinomycetic bacteria are best known for their ability to produce antibiotics and the *Streptomyces* are the dominant group (**Pidot *et al*., 2014**). The genus *Streptomyces* is responsible for the formation of more than 60% of known antibiotics (**Gad et al., 2015; Rashad et al., 2015**). Another important species of bacteria known to produce antibiotics is the *Bacillus* spp. Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, its production is mainly by microorganisms that live in the soil (**Jamil et al., 2007**). Resistance by pathogenic bacteria has become a major health concern, many Gram positive bacteria and Gram negative opportunistic pathogens were becoming resistant to virtually every clinically available drugs **(Redgrave *et al*., 2014**). The use of antimicrobial drugs for prophylactic or therapeutic purposes in human and veterinary or for agricultural purposes, have provided the selective pressure favouring the survival and spread of resistant organisms. Selective pressure favouring the survival and spread of vancomycin-resistant enterococci (VRE) was the consequence of the use of antibiotics in food and agricultural practices. Vancomycin-resistance is often associated with multiple-drug resistance (**Chang *et al*., 2015**).
Another cause of great concern is the Gram-negative antibiotic-resistant opportunistic pathogens. These bacteria, like *Pseudomonas aeruginosa*, are common environmental organisms, which act as opportunistic pathogens in clinical cases where the defense system for patient is compromised (**Sanjar *et al*., 2016**). For instance, over 80 % of cystic fibrosis (CF) patients become chronically infected with *P. aeruginosa*. In addition, other intrinsically antibiotic resistant organisms such as *Burkholderia cepacia* and *Stenotrophomonas maltophilia* are emerging as opportunistic pathogens (**Chung *et al*., 2013**). The appearance of multi resistant pathogenic strains have caused a therapeutic problem of enormous proportions (**Barsby *et al*., 2001**). For instance, they cause substantial morbidity and mortality especially among the elderly and immunocompromised patients. With increasing misuses of antibiotics, the serious problems of antibiotic resistance are developing at an alarming rate. Thus, new therapeutic drugs and approaches are needed to improve the management of these diseases and overcome these problems (**Taylor *et al*., 2002**).
Hence, intensive search for new antibiotics has become imperative worldwide especially from natural sources such as soil which is known as the greatest source of antibiotics. New microbial metabolites are permanently needed due not only to the increase in resistant pathogens, but also to the evolution of novel diseases and toxicity of currently used compounds **(Parugao *et al*., 2008**).
This study aims at exploring the antibiotics production potentials of some indigenous soil bacteria isolated from farmlands in some selected locations in Oyo and Ogun state, South Western part of Nigeria. The objectives include: (i) to isolate antibiotic producing bacteria from farmland (ii) to characterize to species level the isolated bacteria (iii) to determine and estimate the antimicrobial efficiency of each of the antibiotics produced by the bacteria.

MATERIALS AND METHOD

Study area

The soil samples were collected from farmlands located in Ibadan, Ago – Iwoye, and Ikenne. Seven samples were collected from individual farmlands in Ibadan which is the capital of Oyo state, while five samples were collected from government farmland located in Ago – Iwoye, and four other samples were collected from IITA research station located in Ikenne, both located in Ogun state.

Media used

The media used were nutrient agar, potato starch casein agar, starch casein agar, and Mac Conkey agar. They were prepared according to the manufacturer's instruction. The media were left to cool to about 45^0c after sterilization before being poured aseptically into the Petri dish.
Serial dilution of soil samples: The soil samples were labeled according to the area of collection. Then 1g was weighed from each soil samples for serial dilution where the10^{-2} to 10^{-4} dilutions were plated.

Plating of the samples

The diluted soil samples were inoculated onto the appropriate agar medium using the streaking method, and they were labeled accordingly. Nutrient agar was used for isolating bacteria, while potato starch casein agar was used for isolating actinomycetes. After inoculation, the plates were incubated at 37^0C over night for bacteria, and while in the case of actinomycetes incubation lasted for 4 days at a temperature of 30^oC before morphological changes can be seen.

Sub culturing

After the completion of the incubation period, the culture media were checked for the growth of organisms, colonies were seen on nutrient agar indicating the presence of bacteria, while potato starch casein agar also show the presence of colonies. Upon this observation, Mac-Conkey agar, and starch casein agar were both prepared aseptically by autoclaving and following the manufacturer's instruction. Using a sterile inoculating loop, morphologically different colonies were picked from the mixed culture of bacteria in the nutrient agar into a new medium (Mac-Conkey agar) under an aseptic condition with proper labeling and it was incubated overnight at 37^0 C, while morphologically different colonies were also picked from potato starch casein agar using a sterile inoculating loop under an aseptic condition into a medium of starch casein agar and it was incubated at 30ºC for 4 days.

Pure culturing

Nutrient agar medium was prepared in a conical flask and sterilized. The nutrient agar medium is poured into sterile vials and allowed to solidify by placing in a slanting manner in the laminar air flow. Pure isolates were carefully collected and streaked on the slant aseptically. The vials were properly closed by cotton plugs and kept in the refrigerator as stock culture.

Biochemical tests

Gram reaction, spore staining, catalase test, gelatin hydrolysis test, casein test, tyrosine test, xanthine test, urease test, starch hydrolysis test, growth in lysozyme, lactose fermentation, cellobiose test, xylose test, mannitol fermentation test, lipid hydrolysis test, glucose fermentation test, and voges proskauer (vp) test were conducted on the isolated organisms and the results were recorded.

Preparation of suspension

Colonies of the pure stocked culture were carefully picked and aseptically suspended into nutrient broth and they were incubated at 35ºC for 24 hours and 4 days for bacteria and *Streptomyces* respectively according to **Biradar *et al***. (2016).

Primary screening for antibiotic production

After the incubation period, the broth cultures were centrifuged for 15 minutes at 4000 rpm to obtain the supernatant of their metabolites which will be used to test some organisms. The supernatants were filtered and were aseptically transferred to a Mac-Cartney bottle and they were labeled and stored at a temperature of -4ºC following the method of **Rai *et al*.** (2016).

Preparation of test organisms

The test organisms used for the screening were clinical samples, and they include; *Klebsiella pneumonia*, *Serratia marscens*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis*. All the test bacteria were grown in 4.50 ml nutrient broth for 12 hours, and then standardized to Mac farland standard of 0.1 at optical density (OD) 600nm before use as described by **Othman *et al***. (2011).

Screening for antibacterial activity

The supernatants gotten were screened for antibacterial activity using the cup well agar method as described by **Valan *et al***. (2012). For this purpose sterilized molten Mueller Hinton agar (MHA) was poured into a Petri dish and allowed to solidify under the laminar air flow, then 50µl of the standardized bacteria was swirl gently and aseptically spread on the medium. Sterile cork borer (6mm diameter) was used to bore a hole in the plate. Then 100 µl of the antibiotic extract was carefully dispensed into the bored holes. This was done without allowing the extract to overflow the well. Extracts were allowed to diffuse for about 2 hrs before incubating. Plates were incubated at 37ºC for 24 hours. The presence of zone of inhibition around each well was indicative of antibacterial activity.

RESULTS

A total number of 85 organisms belonging to six different species of bacteria were isolated from the soil samples. *S. Somaliensis*, *B. subtilis*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* were found to be the most predominant of the isolated microorganisms with prevalence rate of 18.82%, this was closely followed by *B. megaterium* and *S. anulatus* with prevalence rate of 12.94% and 11.77% respectively as shown in (Table 1).

Table 1 Prevalence of microorganisms in the examined soil samples

Organisms	N	%
S. somaliensis	16	18.82
S. anulatus	10	11.77
B. subtilis	16	18.82
B. megaterium	11	12.94
Proteus. vulgaris	16	18.82
Pseudomonas. Aeruginosa	16	18.82
TOTAL	**85**	**100**

The Gram reaction and the biochemical characteristics of the organisms isolated from the farmlands examined indicated that *Streptomyces* spp was the only isolated actinomycetes. The isolated *Streptomyces* were *S. somaliensis*, and *S. anulatus*. These two organisms were differentiated based on their cell morphology on plates or cultural characteristics (casein utilization, tyrosine utilization, xanthine utilization, urea utilization, xylose utilization, cellubiose utilization, gelatin hydrolysis, starch hydrolysis, growth in lysozyme, and lactose fermentation). *S. somaliensis* produces cream to brown colour on starch casein agar, while *S. anulatus* was found to be white, chalky, velvety colour on the same medium. *S. anulatus* was positive to almost all the tests except gelatin hydrolysis test, while *S. somaliensis* was unable to utilize xanthine, urea, xylose, and cellubiose. Also it didn't hydrolyse gelatin, and was found to be a non lactose fermenter as depicted by (Table 2).

Table 2 Gram reaction and biochemical characteristics of actinomycetes isolated from different farmlands

Gr	Cm	Ca	Ty	Xa	Ur	Ge	St	Gi	La	Xy	Ce	Probable Organism
+	Cream to brown	+	+	-	-	-	+	+	-	-	-	*S.somaliensis*
+	Chalky, white, or velvety	+	+	+	+	-	+	+	+	+	+	*S.anulatus*

Key: Xa = Xanthine Utilization, Ur = Urea Utilization, Gr = Gram Reaction, Ge = Gelatin Hydrolysis, Cm = Cell Morphology, St = Starch Hydrolysis, Ca = Casein Hydrolysis, Gi = Growth in Lysozyme, Ty = Tyrosine Utilization, La = Lactose Fermentation, Xy = Xylose Utilization, Ce = Cellobiose Utilization.

The other bacteria isolated from this study apart from *streptomyces* were *Bacillus subtilus*, *Bacillus megaterium*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. *Bacillus. subtilus* and *B. megaterium* were differentiated based on the size of their spore, ability to lyse lipids and Voges proskauer test. *B. megaterium* has large spore while *B. subtilus* has small spore. On the other hand, *B. subtilus* is positive to lipolysis test, while *B. megaterium* is negative. Also *B. subtilis* is positive to VP test, while *B. megaterium* is negative to the test. In general, both species of bacillus are Gram positive, spore containing, catalase positive, non-lactose fermenters with ability to ferment mannitol and glucose, hydrolyse both gelatin and starch, and can't utilize urea.

Consequently, both *Pseudomonas aeruginosa* and *Proteus vulgaris* were found to be Gram negative rod, catalase positive, non-lactose fermenters, non-spore forming, negative to starch hydrolysis, but positive to gelatin hydrolysis and

negative to VP test. But they are differentiated by mannitol test, lipid hydrolysis test, glucose fermentation test, and ability to utilize urea as shown in (Table 3).

Table 3 Gram reaction and the biochemical characteristics of the other isolated organisms.

Gr	Cm	La	Sp	Ma	Li	Ca	Ge	St	Gl	Ur	Vp	Probable Organism
+	Rod	-	+ (small)	+	+	+	+	+	+	-	+	*Bacillus. subtilis*
+	Rod	-	+ (large)	+	-	+	+	+	+	-	-	*Bacillus. megaterium*
-	Rod	-	-	+	-	+	+	-	-	-	-	*P. aeruginosa*
-	Rod	-	-	-	+	+	+	-	+	+	-	*Proteus. Vulgaris*

Key;
Gr = Gram Reaction, Ge = Gelatin Hydrolysis,Cm = Cell Morphology, La = Lactose Fermentation, Sp = Spore Staining, Ma = Mannitol Fermentation,Li = Lipid Hydrolysis, Ca = Catalase, St = Starch Hydrolysis, Gl = Glucose Fermentation, Ur = Urea Utilization,VP = Voges Proskauer.

Pseudomonas. aeruginosa is a mannitol fermenter, while *Proteus vulgaris* can't ferment mannitol. *P. vulgaris* can hydrolyse lipid, while *P .aeruginosa* is negative to the test. Also *P. aeruginosa* is negative to both urea and glucose test, while *P. vulgaris* is positive to both.

The antibacterial activity of the biomolecule from the antibiotic producing bacteria. The biomolecule extracted from each of the antibiotic producing organisms were challenged against seven different bacteria pathogens viz; *Klebsiella pneumonia*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Serratia marscens*, *E. coli*, and *Enterococcus faecalis* as displayed in (Table 4).

Table 4 Antibacterial activity of the biomolecule extracted from the antibiotic producing organisms.

	Zone of inhibition (mm)			
Organisms	**SS**	**SA**	**BM**	**BS**
K. pneumonia	18.00	19.00	9.50	11.50
Proteus vulgaris	21.00	0.00	20.50	8.00
S. marscens	16.50	18.50	20.00	10.50
P .aeruginosa	3.00	6.50	14.50	2.00
E. coli	22.50	18.00	16.50	2.50
S. aureus	12.50	0.00	25.00	19.00
E. faecalis	23.50	25.00	20.00	0.00
Average total of zone of inhibition	**16.60**	**12.40**	**17.90**	**7.60**

Key:
SS= *Streptomyces somaliensis*, SA = *Streptomyces anulatus*, BS= *Bacillus subtilis*, BM = *Bacillus megaterium.*

The biomolecule extracted from *B. megaterium* was found to be the most efficacious of all the tested biomolecules and it was closely followed by *S. somaliensis*. The biomolecule from *S. anulatus* was also effective against some organisms except that it has no effect on *S. aureus* and *Proteus vulgaris*. The extract from *B. subtilis* also tends to be effective, but it has no activity against *Enterococcus faecalis*.

DISCUSSION

With the increasing demand for new antibiotics and novel bioactive compounds, it is necessary to find new strategies that can increase the effectiveness of the search. This present study was carried out to study the production of antibiotic from isolated soil organisms. Different bacteria were isolated from soil and identified as *Bacillus* and *streptomyces*. All showed antibacterial activity against various pathogens.

The antibiotic producing microorganisms isolated from the soil samples taken from various farm lands in Oyo and Ogun state were *Bacillus* spp and *Streptomyces* spp of all the bacterial species originally isolated. The results conform to many reviews of literature that *Bacillus* spp and *streptomyces* spp are known to produce bioactive secondary metabolites (antibiotic). **Al-Ajlani and Hasnain** (2010) in a research discovered the production of bacitracin and subtilin by *Bacillus* spp. **Ayitso and Onyango** (2016) reported that bacitracin produced by *Bacillus* spp inhibits *Escherichia coli* and *Staphylococcus aureus* which corroborated the result in this present study.

Many of the strains evaluated in the present study showed bioactivity against one or more of pathogenic microorganisms. It is becoming increasingly evident that the taxonomic and metabolic diversity encompassed by *Streptomycetes* and *Bacillus* are remarkable, as new and putatively novel *Streptomyces* species are being continuously isolated from under-researched habitats.

Of all the pathogenic organisms tested with the extract, both *Pseudomonas* and *Proteus* spp have lower zones of inhibition compared to their Gram positive counterpart. This observation corroborate with that of **(Zgurskaya and Nikaido**, 2000).

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