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Microbial Isolation and Screening of Antibiotic Producing Bacillus Species from Soil Samples in Okitipupa, Nigeria

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Abstract

The present study aimed to isolate soil bacteria and determine their antibiotic sensitivity susceptibilities. A total of sixty (60) Bacillus species were isolated from the soil samples around the Olusegun Agagu University of Science and Technology (OAUSTECH), Okitipupa, Nigeria. The antibiotic sensitivity test was carried adopting the clinical laboratory standard procedure for agar diffusion method. The test microorganisms were Streptococcus pyogenes, Staphylococcus aureus and Gram negative bacteria such as Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumonia obtained from the Microbiology Laboratory of the university in Okitipupa. Bacillus isolates that produced large inhibition zones were characterized and identified based on their colonial morphology and biochemical reactions. The bacteria isolated include Bacillus subtilis, B. licheniformis, B. pumilus B. megaterium and B. sphaericus. Out of the total 60 Bacillus species screened, 50% of the isolates showed no antibiotic sensitivity and with 0.0 mm clear zone, 26.7% of the population exhibited slight inhibition between 1.0 to 5.0 mm clear zones, 15% showed average of between 5.0 to 15 mm zone of inhibition, while 8.3% exerted high antibiotic sensitivity ranging from 15.0 mm to 27.0 mm wide zones of inhibition respectively. The varied antimicrobial activity exhibited by the isolated microorganisms on the test microorganisms demonstrated the potentials of the Bacillus isolates as antimicrobial source. Bacillus subtilis and B. licheniformis showed very large activities against three of the five test microorganisms with activities against both Gram positive and Gram negative bacteria. Bacillus subtilis G8 isolates inhibit Streptococcus pyrogenes, E. coli and K. pneumoniae. It inhibits Streptococcus pyrogenes with up to 27 mm zone of inhibition. Thus, it can be a source of antimicrobials in the treatment of bacteria human pathogens.

Keywords: Soil, Test Microorganisms, Antibiotic Production, Screening, Pathogenic Bacteria.

Introduction

The term soil refers to the outer loose material of the earth crust regarded as a three phase system consisting of solids, liquids and gases that are dispersed to form a heterogeneous matrix [1]. On the whole the soil is composed of five major components such as minerals, water, organic matter, air and living organisms [2]. The various components of the soil environment constantly changed and the quantity of these constituents are not the same in all soils but vary with locality [1]. Living portion of the soil body includes small animals and microorganisms but it is generally considered that its microorganisms that plays the most important role in the release of nutrient and carbon dioxide for plant growth [3]. Soil bacteria can be very diverse, including rods (bacilli), cocci (spherical), spirilla (spirals), etc. Bacillus are more numerous than others bacteria. They are one of the major groups of soil bacteria population and are very widely distributed [4].

The number and type of bacteria present in a particular soil would be greatly influenced by geographical location, soil temperature, soil type, soil pH, organic matter content, cultivation, aeration and moisture content [5]. Bacillus species produce many antibiotics such as polymyxin, colistin, circulin, bacitracin, pumulin

and gramicidin from different species which are active against Gram positive bacteria such as staphylococci, streptococci, Corybnebacter, etc.[6]. Streptomyces species produce antibiotic like callus tetracycline, chloramphenicol, vancomycin, gentamycin which are active against Gram negative bacteria and Lactobacillus species produce antibiotic like nisin which is produce by Lactobacillus lactis [7].

Bacillus is a rod shaped bacterium found at different locations. Some of the Bacillus species reported to be producing antibiotics are B. subtilis, B. polymyxa, B. brevis, B. licheniformis, B. circulans and B. cereus [8]. Polypeptide antibiotics produced by Bacillus and useful in medical treatments are bacitracin, gramicidin S, polymyxin and tyrotricidin. Antibiotics produced by the Bacillus species are more effective for Gram-positive bacteria; however, the production of large spectrum and anti-fungal antibiotics that are effective for Gram-negative bacteria is relatively less. The Bacillus species have a wide range of antimicrobial activities since they are used as antifungal agents, antiviral agent's anti-ameobocytic agents and anti-mycoplasma agents.

In the light of the aforementioned the present study investigated the antimicrobial activities of Bacillus species isolated from the southern part of Nigerian soil against selected pathogens and other test bacteria. It is hoped that this study will lead to the discovery of novel antibiotics from our environment with potential to control the now very prevalent antibiotic multi resistant microorganisms. There are limited efforts on the isolation of antibiotic producing microorganisms from this part of Nigeria before now [9].

Materials and Method

Collection and Preparation of Soil Samples

Four (4) grams of soil samples were collected with sterile spatula from different locations within the campus of Olusegun Agagu University of Science and Technology (OAUSTECH), Okitipupa, Nigeria. The locations were namely, stagnant water behind OAUSTECH bakery, OAUSTECH farm nursery, OAUSTECH botanical garden, earthen pond in the fishery section, poultry section of the farm, rhizosphere soil zone and the dumping site soil at the school. The samples were taken from 6-12 cm soil depth into sterile plastic bags which were tightly closed immediately after collection and transported aseptically to the microbiology laboratory of the University. Analyses were carried out within 8 h of collection. The samples were homogenized, spread in sterile trays and cleaned of extraneous materials before analyses [10].

Collection of Test Microorganisms

The test microorganisms used in this study are Pseudomonas aeruginosa, Escherichia coli, Streptococcus pyogenes, Staphylococcus aureus and Klebsiella pneumomiae. The test isolates were collected on agar slants from Microbiology laboratory, Olusegun Agagu University of Science and Technology, Okitipupa. Ondo State [11].

Isolation and Identification of Bacillus Species

The method of was used for the isolation of soil Bacillus species. Ten grams of soil sample were diluted in 100 ml of physiological saline solution (0.85% NaCl) in a conical flask and heated at 60oC for 60 mins in a water bath to destroy vegetative forms of the microbes. The samples were then shaken in an orbital shaker at 200 rpm for 30 mins. and then allowed to settle. From each solution, 1 ml was transferred aseptically to a test tube containing 9 ml of sterile physiological saline and mixed well to make a dilution of 10-1. Serial dilutions were continued up to 10-6 for each soil sample. Aliquots of l ml each from the appropriate dilutions were inoculated on Tryptic Soy Agar (Oxoid) plates incubated at 30oC for 24-48 h. After incubation, the cultured plates were observed for physical appearance of the colonies. The physical observations include appearance, shape, elevation, consistency and pigment colourations. The isolates were also evaluated using biochemical analyses to confirm the isolates identities [12] .

Cultivation of Test Organisms for Antimicrobial Screening

The test organisms such as Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pyogenes, Klebsiellap pneumonia, and Escherichia coli, obtained from the Microbiology Laboratory were transferred from Agar slants onto freshly prepared Nutrient agar plates using streaking plate method and the plate was incubated at 37oC for 24 hours. Distinct colonies were isolated and sub-cultured on a Nutrient Agar and kept at 4oC before the plates were used for identification purposes [13].

Antibiotic Susceptibility Testing of Target Bacteria by Disc Diffusion Method

The test organisms were subjected to antibiotic susceptibility using the disk diffusion method as originally described by. All bacteria were cultured on Tryptic Soy Broth (TSB) medium and incubated at the appropriate temperature for 24 h. Nutrient Agar medium (10 ml) as poured into each sterile petri dish (100 mm diameter) and allowed to solidify. Five (5) sterile swabs were used to pick the test organisms and each organism was spread uniformly on petri dish respectively. Multiple disk (Whatman no. 1 filter) was soaked with Tryptic Soy Broth (TSB) containing the "Target bacteria" and was placed on the petri dish aseptically with the aid of sterile forceps and the procedure was repeated for the remaining Bacillus isolates. The petri dishes were then incubated at 37oC for 24 hours in an inverted position. Diameters of zones of inhibition around each antibiotic disc were measured with Caliper and recorded in millimeters. The isolates that showed large zones of inhibition were chosen and further tests were carried out on them [14].

Biochemical Test

Important biochemical and morphological tests and observations were carried out as indicated in the result. Some of the tests carried out included Gram staining, catalase, motility test using tube method, spore staining, oxidase, Indole, lactose fermentation. The tests were carried out as a confirmation of the microbial identity of the test organisms as supplied and the Bacillus isolates [15].

Results

The total Bacillus population count of the soil samples later used for the screening of antibiotic Bacillus producers during this research were shown in Table 1. Microbial analysis reveals that the bacteria load for each soil varies and the total bacteria count of soil sample from the Rhizosphere zone has the highest microbial load (4.40 x 106cfu/g), while the soil sample from the poultry has the lowest microbial load (8.60 x 10 5 cfu/g). Most of the other soil samples have Bacillus population of ranged be-

tween 1.60 x 106 and 3.80 x 106 cfu/g. The test microorganisms was collected from the Microbiology Laboratory of OAUSTEC, Okitipupa, Ondo State and were confirmed using basic morphological and biochemical analyses as shown in Table 2 below.

Sixty Bacillus species were obtained from soil samples collected from OAUSTECH campus and are screened for antimicrobial activity. Out of the sixty isolates, thirty isolates (50%) produced no zones of inhibition, sixteen isolate (26.7%) produce little zones of inhibition of mean 3.36 mm withranges of between 1 and 5 mm. Nine isolates (15%) produced inhibition zones of 5-15 mm ranges and average inhibition zone of 12 mm. The total of five (5) isolates (8.3%) produced the largest inhibition zones with a mean value of 18.8 mm and range of 15 to 27 mm has shown in Table 3. The five Bacillus species that shows large inhibition zones were identified as Bacillus subtilis (G8), Bacillus pumilus (S4), Bacillus licheniformis (S5), Bacillus megaterium (S1), Bacillus sphaericus (EP1) as shown in (Table 4).

The photographic image of Bacillus sphaericus EP1 inhibiting Ps. aeruginosa and E. coli are shown in Plate 1. Bacillus licheniformis (S5) inhibits the Gram positive bacteria (Staphylococcus aureus, Streptococcus pyogenes) and Gram negative bacteria (Klebsiella pneumonia), 3 isolates in all. Bacillus megaterium S1 is only active against Gram negative bacteria (Pseudomonas aeruginosa), Bacillus pumilus S4 is only active against Gram positive bacteria (Staphylococcus aureus, Streptococcus pyogenes), Bacillus subtilis G8 inhibits three of the test microorganisms namely Gram positive bacteria (Streptococcus pyogenes) and Gram negative bacteria (Escherichia coli and Klebsiella pneumonia) as shown in Table 5. Bacillus subtilis G8 shows the most promise of the isolates by indicating zones of inhibition of 27, 17 and 19 on test microorganisms on Streptococcus pyrogens, E. coli and K. pneumoniae, respectfully. B. licheniformis also inhibiting 3 test microorganisms and large zones equally shows good capability and potentials.

Discussion

The total Bacillus count of the soil samples reveals that although Bacillus are present in all the sample soils, they may however, slightly differ from location to location. This might be due to the soil type and composition, and also environmental factors such as pH, temperature and moisture content, exposure to fire and exposure to flood. Morphological and biochemical characteristics shows the bacteria belong to Bacillus spp. as described by. The isolates screened reveals that Bacillus species isolates with antibiotics properties are present in the soil samples screened. This is also in agreement with. who reported that Bacillus are the predominant soil bacteria because of their resistant endospore formation and their ability to produce antibiotics of medical importance. The Bacillus species identified as Bacillus megaterium, Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis and Bacillus sphaericus being observed as predominant of the Bacillus species in soil samples was also reported. Also reported that in a competitive environment, inhibition zones are developed by both organic acid producers and antibiotic producers. The organic acid producers are eliminated by growing them on calcium carbonate medium because they develop clear zones around them on calcium carbonate (CaCO3) medium. Organic acids react with CaCO3 and dissolved to calcium oxide (CaO) and carbon dioxide (CO2). On CaCO3 medium only organic acid producers develop a clear zone. However, demonstrated that Bacillus isolates with large zones of inhibition are not organic acid producers but antibiotic producers, hence Bacillus megaerium, Bacillus subtilis, Bacillus pumillus, Bacillus licheniformis, Bacillus sphaericus reported one this study with large zones most likely produce substances with antibiotic properties.

Bacillus species isolated from OAUSTECH soil shows moderate inhibition against Gram positive Streptococcus pyogenes and Staphylococcus aureus and Gram negative Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumoniae.This is in agreement with who reported the inhibition of Gram positive and Gram negative bacteria by Bacillus species, the highest zone of inhibition is 27 mm which is Bacillus subtilis against Staphylococcus pyogenes. The present study is a preliminary work with potential findings which could be elaborate in future to produce novel isolates antibiotics.

The study revealed that, bacteria with the potential to produce antibiotics are present in the campus soil. Though a large list of antibiotics is commercially available, the search for the most effective one is still on, and this work may contribute in providing preliminary information on the antibiotic producing microorganisms especially in the light of the prevalence of the antibiotic multi-resistance bacterial pathogen in health care [16].

Further work should be carried out on the isolates, while additional exploratory research could be made by checking other soil around OAUSTECH campus in Okitipupa for more novel antibiotic producing bacteria. In addition, the physiological study to establish the optimal condition for antibiotic production and its activity as well as purification and application can be further exploited in the future.

Table 1: Total Bacillus Population Count in Different Soil Samples From Oaustech campus

Sample location	Sample code	*Cfu/g
Stagnant water	S	3.4×10^6
Earthen pond	EP	3.1×10^6
Earthen pond 2	EQ	3.8×10^6
Bakery compost	BC	3.8×10^6
Garden	G	1.6×10^6
Poultry	P	8.6×10^5
Rhizopore zone	R	4.4×10^6
Farm	F	2.2×10^6
Nursery	N	1.9×10^6

Dumping site	DS	3.1 x 10^6

* These values are the means of triplicate values

Table 2: Confirmation of Identities of Test Microorganisms Used for Screening

Isolates	Colony morphology	Gram stain	Indole	Coagulase	Catalase	Methyl red	Oxidase	Lactose	Possible isolate
S1	Metallic green colonies	-	-	-	+	-	+	_	Pseudomonas aeruginosa
S2	Large circular smooth white colonies	-	+	ND	+	+	-	+	Escherichia coli
S3	Small smooth yellow colonies with glistening surface	+	+	+	+	+	-	+	Staphylococcus aureus
S4	Slimy grayish white colonies	-	-	ND	+	-	-	+	Klebisella pneumoniae
S5	Spherical and in chain	+	-	+	-	+	-	+	Streptococcus pyogenes

+ Positive, - Negative, Nd - Not Developed

Table 3: Antimicrobial Production Pattern of Different Bacillus Isolate from Soil

Antibiotic production groups	Zone of inhibition level	Number isolates	Mean zone of inhibition (mm)	% Microorganism frequency
None	0	30	0	50
Slight	1-5	16	3.36	26.7
Average	5-15	9	12	15
Large	15-25	5	18.8	8.3
Total isolate screened	60	60	0.57	100

Plate 1: Antimicrobial effects of Bacillus sphaericus against two test organisms.
A: Pseudomonas aeruginosa; B: Escherichia coli

Tables 4: Morphological Characteristics and Biochemical Features of Major Antibiotic Producing Isolates with Large Inhibition Zone

Isolate code	Colony morphology	Gram stain	Cell shape	Spore stain	Catalase	Motility	Oxidase	Indole	Glucose	Lactose	Probable bacteria
S1	Grey, opaque, raised with round shape colony	+	Rod	+	+	+	-	-	A	-	Bacillus magaterium
S4	White, flat. Undulate with irregular transparent colony	+	Rod	+	+	+	-	-	+	+	Bacillus pumillus

S5	White ,Round, irregular, wrinkled colony	+	Rod	+	+	+	-	-	+	+	Bacillus licheniformis
G8	Grey, round, raised with translucent colony	+	Rod	+	+	+	-	-	+	+	Bacillus subtilis
EP1	Yellow partially transparent with irregular, flat, undulate colony	+	Rod	+	+	+	-	-	+	+	Bacillus sphaericus

Key: + positive, - negative, A: acid production

Table 5: Zones of Inhibition of the Isolated Antibiotic Producing Bacillus Isolates

Antibiotic producing isolates	Test microorganisms (Zones of inhibition, mm)				
	Staphylococcus aureus	Streptococcus pyogenes	Pseudomonas aeruginosa	Escherichia coli	Klebisella pneumonia
Bacillus. megaterium	0	0	20	0	0
Bacillus pumillus	15	16	0	0	0
Bacillus licheniformis	15	17	0	0	26
Bacillussubtilis	0	27	0	19	17
Bacillus sphaericus	0	0	16	0	0

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