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# ISOLATION AND SCREENING OF ANTIBIOTIC PRODUCING *BACILLUS* SPECIES FROM SOIL SAMPLES IN OKITIPUPA, NIGERIA

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**Abstract**
This study was carried out to isolate soil *Bacillus* species with antibiotic production features and determine their antibiotic sensitivity pattern around the Olusegun Agagu University of Science and Technology (OAUSTECH), Okitipupa, Nigeria. The antibiotic sensitivity test was carried out using Agar diffusion method. The test microorganisms were *Streptococcus pyogenes*, *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* from the Microbiology Laboratory of the University. *Bacillus* isolates were able to produce large inhibition zones, they were characterized and identified based on their colonial morphology and biochemical test. The isolates identified are *Bacillus subtilis*, *B. licheniformis*, *B. pumilus*, *B. megaterium* and *B. sphaericus*. Out of the total 60 *Bacillus* species screened, 50% of the isolates demonstrated no antibiotic sensitivity and with 0.0 mm inhibition zone, 26.7% of the population exhibited slight inhibitions of 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 of 15.0 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 antibiotics or antimicrobial sources. *Bacillus subtilis* and *B. licheniformis* showed very large activities against three each 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. Future studies are required to further confirm and characterized the antibiotic feature of the antimicrobials produced. *Bacillus* species isolates have demonstrated their potentials as a possible source of antibiotics in the treatment of microbial human infections. Future studies are required to further confirm and characterize the antibiotic feature of the antimicrobials produced.
**Keywords:** Antibiotic, *Bacillus* species, test microorganisms, pathogenic bacteria, screening, Soil.

## Introduction

Soil is the outer loose material of the earth crust. It is regarded as a three-phase system consisting of solids, liquids and gases that are dispersed to form a heterogeneous matrix (Needelman, 2013). On the whole the soil is

composed of five major components which are minerals, water, organic matter, air and living organisms (DeGomez et al., 2019). The various components of the soil environment constantly change and their constituents are not the same in all soils but vary with locality (Needelman, 2013). 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 nutrients and carbon dioxide for plant growth ( Tapia-Torres *et al.*, 2016, Jacoby *et al.,* 2017). Soil bacteria can be very diverse, including rods (bacilli), cocci (spherical), spirilla (spirals), etc.

*Bacillus species* are one of the major groups of soil bacterial population and are very widely distributed (Bhagabati *et al*, 2004). 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 (Davies and Williams, 1999). Antibiotics are one of the most important commercially exploited secondary metabolites produced by the bacteria and employed in a wide range of applications (Nester *et al.,* 2009). The British scientist Alexander Fleming was credited with being first to notice that another microorganism could inhibit bacteria growth in 1928 (Tan and Tatsumura, 2015). He noticed that the growth of *Staphylococcus aureus* was inhibited by a mold (fungus) that contaminated his plate. The mold was later identified as *Penicillium notatum* and the antibiotic isolated later was named penicillin (Nester *et al.,* 2009). Several microorganisms abound in nature having potential to produce different classes of antibiotics. *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, corynebacteria (Amin, *et al.*, 2012). *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* (Waites *et al.*, 2008)

*Bacillus* is a rod-shaped bacterium found at different locations. Bacillus species are one of the major groups of soil bacterial population and are very widely distributed (Bhagabati et al.,2004).

Some of the *Bacillus* species reported to be producing antibiotics are *B. subtilis, B. polymyxa, B. brevis, B. licheniformis, B. circulans* and *B. cereus* (Caulier et al., 2019). Polypeptide antibiotics produced by *Bacillus* and useful in medical treatments are Bacitracin, gramicidin S, polymycin and tyrotricidin (Morikawa *et al.,* 1992; Perez *et al.*, 1992; Drablos *et al.*, 1999). Antibiotics produced by the *Bacillus* species are more effective against Gram-positive bacteria; however, the production of large spectrum and anti-fungal antibiotics that are effective against Gram-negative bacteria is relatively less (Morikawa *et al.*, 1992; Perez *et al.*, 1993; Eltem and Ucar,1998). The *Bacillus* species have a wide range of antimicrobial activities since they are used as antifungal agents (Milner *et al.*, 1995), antiviral agents (Steller *et al.*, 1999) anti-ameobocytic agents (Galvez *et al.,* 1994) and anti-mycoplasma agents (Peypoux *et al.*, 1999).

This study therefore aimed at investigating the antimicrobial activity of *Bacillus* species isolated from the southern part of Nigerian soil against some 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.

**Materials and method**

**Collection and preparation of soil samples**

Soil Samples were collected 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 and the dumping site soil at the school. Soil samples of 4.0 g each were collected from the selected locations using sterilized spatula. 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.

**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.

**Isolation and identification of *Bacillus* species**

The method of Collins *et al.* (1989) 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 $60^0$ C 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 $30^0$ C 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 (Collins *et al.*, 1995).

**Cultivation of test organisms for antimicrobial screening**

The test organisms; *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Klebsiella 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 $37^0$C for 24 hours. Distinct colonies were isolated and sub-cultured on a Nutrient Agar and kept at $4^0$C before the plates were used for identification purposes.

**Antibiotic susceptibility testing of target bacteria by disc diffusion method**

The test organisms were subjected to antibiotic susceptibility test using the disk diffusion method as originally described by Baueet *et al.* (1996). 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 swab was used to pick the test organisms (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Klebsiella pneumonia*, and *Escherichia coli*) and each organism was spread uniformly on 5 Petri dishes 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 37 $^{0}$C for 24 hours in an inverted position. Diameters of zones of inhibition around each antibiotic disc were measured and recorded in millimeters. The isolates that showed large zones of inhibition were chosen and further tests were carried out on them.

**Micromorphological and Biochemical test**

*Important biochemical and morphological tests and observations* (Collins *et al.* 1989) 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 microorganisms as supplied and the *Bacillus* isolates.

**Results**

The total *Bacillus* population counts of the soil samples later used for the screening of antibiotic *Bacillus* producers during this research were shown in Table 1. The results of the microbial analysis reveals that the bacterial loads each soil varied with the count bacterial from the Rhizosphere zone (4.40 x $10^{6}$ Cfu/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 between 1.60 x $10^{6}$ and 3.80 x $10^{6}$ Cfu/g. The test microorganisms (*Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Klebsiella pneumoniae*) were confirmed using basic morphological and biochemical analyses as shown in Table 2.

Sixty *Bacillus* species were obtained from soil samples collected from OAUSTECH campus and were screened for antimicrobial activity. Out of the sixty isolates, thirty isolates (50%) did not produce no zones of inhibition, sixteen isolates (26.7%) produced little zones of inhibition of mean 3.36 mm with ranges between 1 and 5 mm. Nine isolates (15%) produced inhibition zones between of 5-15 mm and average inhibition zone of 12 mm. The total of five isolates (8.3%) produced the largest inhibition zones with a mean value of 18.8 mm ranging from 15 to 27 mm (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) (Table 4).

The Photographic image of *Bacillus sphaericus* EP1 inhibiting *Ps. aeruginosa* and *E. coli* was shown in Plate 1. *Bacillus licheniformis* (S5) inhibits the (*Staphylococcus aureus*, *Streptococcus pyogenes*) and (*Klebsiella pneumonia*). *Bacillus megaterium* S1 was only active against (*Pseudomonas aeruginosa*), *Bacillus pumilus* S4 is only active against (*Staphylococcus aureus*, *Streptococcus pyogenes*), *Bacillus subtilis* G8 inhibits three of the test microorganisms namely (*Streptococcus pyogenes*) and (*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 inhibited 3 test microorganisms with large zones of inhibition.

**Discussion**

The total *Bacillus* count of the soil samples revealed that although *Bacillus* species 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 (Oyeleke *et al.*, 2008). Morphological and biochemical characteristics shows that the bacteria belong to *Bacillus* spp. as described by Davies and Williams (1999) as well as Collins *et al.* (2010). The isolates screened reveals that *Bacillus* species isolates with antibiotics properties are present in the soil samples screened. This is also in agreement with Bala (2012) who reported that *Bacillus* species 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 (Oyeleke *et al.*, 2008; Bala, 2012). Bala *et al.* (2012) 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 ($CaCO_3$) medium. Organic acids react with $CaCO_3$ and dissolved to Calcium Oxide ($CaCO_3$) and Carbon dioxide ($CO_2$). On $CaCO_3$ medium only organic acid producers develop a clear zone. However, Chandrashekhara (2010) and Todar *et al.* (2005) demonstrated that *Bacillus* isolates with large zones of inhibition are not organic acid producers but antibiotic producers, hence *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus pumillus*, *Bacillus licheniformis*, *Bacillus sphaericus* reported most likely produce substances with high antibiotic properties.

*Bacillus* species isolated from OAUSTECH soil showed 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 Todar *et al.* (2005) who reported the inhibition of Gram positive and Gram-negative bacteria by *Bacillus* species. The present study is a preliminary work with potential findings which could be elaborate in future to produce novel isolates antibiotics.

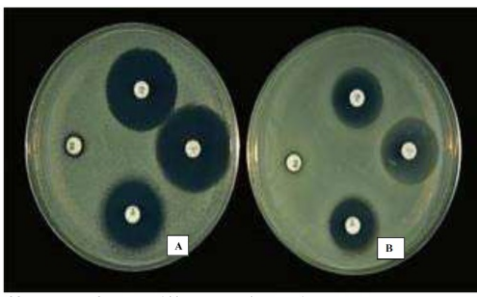


Plate 1: Antimicrobial effects of *Bacillus sphaericus* against two test organisms. A: *Pseudomonas aeruginosa;* B: *Escherichia coli*

**Table 1: Total *Bacillus* population count in different soil samples from OAUSTECH campus**

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

* These values are the means of triplicate values

**Table 2: Confirmation of identities of test microorganisms used for screening**

| Isolation code | Colony Morphology | Gram Stain | Indole | Coagulase | Catalase | Methyl red | Oxidase | Lactose | Probable bacteria |
|---|---|---|---|---|---|---|---|---|---|
| S1 | Metallic green | - | - | - | + | - | + | - | *Pseudomonas aeruginos* |
| S2 | Large circular smooth white colonies | - | + | ND | + | + | - | + | *Escherichia coli* |
| S3 | Small yellow colonies glistening surface | + | + | + | + | + | - | + | *Staphylococcus aureus* |
| S4 | Slimy white colonies | - | - | ND | + | - | - | + | *Klebisella pneumonia* |
| S5 | Spherical in chain | + | - | + | - | + | - | + | *Streptococcus pyogenes* |

**Keys:** + positive, - negative, ND - not determined

**Table 3: Antimicrobial production pattern of different *Bacillus* isolates from soil**

| Antibiotic production categories | Antibiotic production range | Number of antibiotic producers | Average zone of inhibition | Percentage Antibiotic production |
|---|---|---|---|---|
| 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 | 0-25 | 60 | 4.26 | 100 |

* These values are the means of triplicate values

**Tables 4: Morphological characteristics and biochemical features of major antibiotic producing isolates with large inhibition zone**

| Isolation code | Colony Morphology | Gram Stain | Cell Shape | Spore Stain | Catalase | Motility | Oxidase | Indole | Glucose | Lactose | Probable bacteria |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S1 | Grey, Opaque, round shape, colony | + | Rod | + | + | + | - | - | A | - | *Bacillus magaterium* |
| S4 | White, flat, undulate with irregular transparent colony | | | | | | | | | + | *Bacillus pumilus* |
| S5 | White round, irregular, wrinkled colony | + | Rod | + | + | + | - | - | + | + | *Bacillus licheniformi* |
| G8 | Grey, round, translucent colony | + | Rod | + | + | + | - | - | + | + | *Bacillus subtilis* |
| EP1 | Yellow transparent partial with flat, undulate colony | + | Rod | + | + | + | - | - | + | + | *Bacillus sphaericus* |

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

**Table 5: Zones of inhibition of the isolated antibiotic producing *Bacillus* isolates Test microorganisms (Zones of inhibition, mm)**

| Antibiotic Producing Isolates | *Staphylococcus aureus* | *Streptococcus pyogenes* | *Pseudomonas aeruginosa* | *Escherichia coli* | *Klebsiella pneumoniae* |
|---|---|---|---|---|---|
| *Bacillus megaterium* | 0 | 0 | 20 | 0 | 0 |
| *Bacillus pumilus* | 15 | 16 | 0 | 0 | 0 |
| *Bacillus licheniformis* | 15 | 17 | 0 | 0 | 26 |
| *Bacillus subtilis* | 0 | 27 | 0 | 19 | 17 |
| *Bacillus sphaericus* | 0 | 0 | 16 | 0 | 0 |

**Conclusion**

The study revealed that, bacteria with the potential to produce antibiotics are present in OAUSTECH 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.

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.

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