Fjrs.futa.edu.ng
FUTA Journal of Research in Sciences
ISSN: 2315 – 8239 (Print); E-ISSN: 2489 - 0413

FUTA Journal of Research in Sciences, Vol. 15(1), April, 2019: 14-22

ISOLATION AND IDENTIFICATION OF *BACILLUS*, *STREPTOMYCES* AND MOLDS WITH POTENTIALS FOR ANTIBIOTIC PRODUCTION FROM SOILS AT SPECIFIC BIOTOPES IN OKITIPUPA.

***E. E. Nmema, T. E. Adeoye, B. A. Fagbami, A. M. Ilemobayo, E. B. Sam-Omoniyi.**

Department of Biological Sciences, Ondo State University of Science and Technology, Ondo State, Nigeria. Corresponding Author's e-mail: *revivalsprings@gmail.com*

ABSTRACT

The search for new antibiotics remains a priority in the face of challenges posed by increasing antimicrobial resistance among bacterial pathogens. Soil samples were collected from five specific biotopes including rhizosphere, poultry, compost, earthen fish pond and stockyard respectively, in Igodan, Okitipupa Local Government Area of Ondo State. They were analyzed for potential antibiotic producing *Bacillus* and *Streptomyces* species of bacteria. Aliquots of 10^{-4} and 10^{-5} dilutions of soil samples were inoculated on Nutrient agar and modified Streptomyces Isolation agar (MSIA). *Bacillus* isolates were identified using colonial characteristics, Gram-staining and biochemical tests, while *Streptomyces* and molds were identified by appearance of aerial mycelium. Ability of *Bacillus* species to produce antibiotics was tested by their inhibitory effects against five target organisms. The isolates consisted of five *Bacillus* strains (41.7%), three *Streptomyces* strains (25%) and four *Penicillium* strains (33.3%). On MSIA, *Streptomyces coelicolor* strains PgsS1 and RzsS4 were isolated from stockyard and rhizosphere. Incorporation of 250,000 IU of nystatin antifungal per liter of MSIA did not inhibit the growth of *Penicillium* strains growing on the medium. However, 2500 mg/L of rifampicin added to MSIA as an antibacterial completely inhibited all bacterial growth. The findings of the present study reveal abundance of potential antibiotic producers in different biotopes in the locality under study.

Keywords: *Bacillus, Streptomyces coelicolor*, *Penicillium*, Biotopes, antibiotic producers, and photomicrograph.

INTRODUCTION

Antibiotics are secondary metabolites produced naturally by microorganisms to regulate and control microbial populations in soil, compost and water (Cherif *et al*., 2003). Antibiotics important in medicine are mainly produced by *Penicillium*, *Streptomyces*, *Cephalosporium*, *Micromonospora*, and *Bacillus* accounting for more than 5,000 compounds (Berdy, 2012). Most of the antibiotic producers are soil microbes.

Microbes exist in a competitive environment. *Bacillus* species are the predominant soil bacteria because of their resistant endospores and production of vital antibiotics which inhibit the growth of other microorganisms. *Bacillus* species are Gram-positive bacteria which are known for production of endospores, industrially important enzymes, and polypeptide antibiotics. *Bacillus* spp. that produce polypeptide antibiotics that are clinically important include *Bacillus subtilis* (Subtilin, Bacitracin), *B.*

polymyxa (Polymyxin B), *B. brevis* (Gramicidin S, Tyricidine), *B. licheniformis* (Bacitracin), *B. cereus* (Biocerin), and *B. circulans* (Stein *et al*., 2006; Stein, 2005; Kuta *et al*., 2009; Morikawa *et al*., 1992).

Actinomycetes are Gram-positive bacteria and abundant in soils, marine sediments as well as associated with various plants and animals. These bacteria have a widely recognized potential for the production of bioactive secondary metabolites and valuable enzymes (van der Meij *et al*., 2017). One of the most notable characteristics of the actinomycetes is their ability to produce antibiotics, such as streptomycin, neomycin, erythromycin, tetracycline, amphotericin B, and gentamicin, produced by *Streptomyces griseus*, *S. fradiae*, *S. erythreus*, *S. rimosus*, *S. nodosus*, and *Micromonospora purpurea* respectively (Kumari *et al*., 2013).

Although soils have been screened by the pharmaceutical industry for about 50 years, only a small fraction of the surface of the earth has been sampled, and only a small fraction of *Actinomycetes* taxa has been discovered. *Actinomycetes* are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinational chemistry. Additional actinomycete-produced antibiotics are likely to be discovered by subjecting soils and marine sediments to innovative enrichments and whole-cell screening methods (Baltz, 2007).

Actinomycetes of the genus *Streptomyces* are the most versatile producers of bioactive secondary metabolites and they continue to be interesting sources for the discovery of new antibiotics (Demain, 2009). *Streptomycetes* usually possess 20-50 gene clusters dedicated to the biosynthesis of metabolites in their genomes, underscoring their potential for the discovery of new bioactive compounds (Nett *et al*., 2009). Most such clusters responsible for biosynthesis of metabolites remain "silent" in laboratory conditions (Kealey *et al*., 2017).

The mold *Penicillium notatum* produces the antibiotic penicillin which was the first antibiotic discovered in 1929 by Alexander Fleming. Since then, molds that have been used to produce antibiotics have included *P. chrysogenum* (Penicillin), *P. griseofulvin* (Griseofulvin), and *Cephalosporium acremonium* (Cephalosporin) (Lobanovska and Pilla, 2017).

Screening for new natural products remains an important part in the drug discovery process, and investigating new sample sources from underexplored habitats is an important strategy (Goodfellow and Fiedler, 2010). It appears especially beneficial to look into extreme habitats and unique environmental niches (Berdy, 2012; Zotchev, 2012).

The current work was aimed at the isolation of potential antibiotic-producing *Streptomyces* and *Bacillus* species from soils collected at various biotopes in Okitipupa, Ondo South Local Government Council of Ondo State, Nigeria.

MATERIALS AND METHODS

Materials

Soil samples were collected from five specific biotopes in Okitipupa, namely poultry, stockyard, earthen fish pond, rhizosphere zone, and around decomposing plant materials (compost). Nutrient agar was obtained from Lab M (Neogen Corporation, Scotland). Modified Streptomyces Isolation Agar was prepared in the microbiology laboratory of Ondo State University of Science and Technology, Okitipupa.

Preparation of soil samples

Approximately 100 g of each sample was collected using sterilized spatula, from 10 cm depth into sterile plastic bags and closed tightly. The soil samples were labeled according to their source, and transported aseptically to the Microbiology Laboratory (Ondo State University of Science and Technology, Okitipupa), for analysis within 8 h. The

samples were homogenized, spread in sterile trays and cleaned of extraneous materials before analysis.

Isolation of *Bacillus* species

Serial dilution

Ten (10) grams of soil sample were diluted in 100 ml of physiological saline solution (0.85% NaCl) in a conical flask and heated at 60^{o}C for 60 min in a water bath to destroy vegetative forms of microbes. The samples were then shaken in an orbital shaker at 200 rpm for 30 min 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 dilution was continued up to 10^{-5} for each soil sample.

Culture and Identification of *Bacillus* species

Aliquots of 100 µL from 10^{-4} and 10^{-5} dilutions were inoculated on Nutrient Agar medium (Lab M, Scotland). Plates were incubated at 30 ^{o} C for 24-48 h. After incubation, the plates were examined and the suspected colonies were identified by colony characteristics, Gram staining and catalase tests. Gram-positive, rod-shaped, endospore-forming bacilli that were catalase positive were selected for further studies. A B-350 Optica Microscope with camera was used to obtain photomicrographs of *Bacillus* isolates.

Preparation of modified Streptomyces Isolation Agar (MSIA)

In order to isolate *Streptomyces* species from soil samples, a modified Streptomyces Isolation agar was prepared in the laboratory with the following composition (w/v):

Constituent	**Weight/Volume**
Nutrient agar	28 g
Dextrose	4 g
$CaCO_3$	2 g
Distilled water	1 L

The mixture was autoclaved and allowed to cool to 50^{o}C. Then 250,000 (w/v) units of nystatin and 2500 mg of rifampicin (w/v) were added. The mixture was swirled very well to mix the contents and then poured out into plates.

Standardization of target organisms used for antimicrobial screening

The target organisms used to screen the inhibitory effects of *Bacillus* isolates included *Staphylococcus aureus* ATCC 700699, *Shigella flexneri* ATCC 120222, *Salmonella enteritidis* ATCC 1307, *Klebsiella pneumoniae* ATCC 8309, and *Candida albicans* ATCC 10231. Each of the target bacteria was activated by culturing in Nutrient broth (NB) at 37^{o} C for 24 h. *Candida albicans* ATCC 10231 was grown on Sabouraud dextrose agar (SDA). After incubation, 5 mL of each culture in a test tube was adjusted until the turbidity of the cell suspension matched the 0.5 McFarland Standards, containing approximately 1.5 ×10^{8} colony forming units per ml (CFU/ml).

Testing inhibitory effects of *Bacillus* isolates by the agar-well diffusion method

Extracts from *Bacillus* isolates were screened for antimicrobial activity against six target organisms including *Staphylococcus aureus* ATCC 700699, *Shigella flexneri* ATCC 120222, *Salmonella enteritidis* ATCC 1307, *Klebsiella pneumoniae* ATCC 8309, and *Candida albicans* ATCC 10231. All bacteria were cultured on Nutrient Broth and incubated at the appropriate temperatures for 24 h. Nutrient Agar (20 ml) was poured into each sterile Petri dish (100 mm diameter) and allowed to solidify. Suspensions (100 µl) of target bacterial strain cultured for 24 h, and matching the 0.5 McFarland Standards were spread on the plates, and wells of 8 mm diameter were punched in the agar with a sterile steel borer.

The *Bacillus* culture broths were centrifuged at 6000 *g* for 15 min to remove cell debris. After centrifugation, 100 µl of each supernatant sample

was introduced into the wells of agar plates inoculated with target strains. The inoculated plates were incubated for 24 h at 30 ^{o} C.

Isolation of *Streptomyces* species on Modified Streptomyces Isolation Agar (MSIA)

Ten (10) grams of each soil sample were diluted in 100 ml of physiological saline solution (0.85% NaCl) in a conical flask. The samples were then shaken in an orbital shaker at 200 rpm for 30 min 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 dilution was continued up to 10^{-5} for each soil sample. Aliquots of 100 µL from 10^{-4} and 10^{-5} dilutions were taken separately and spread evenly over the surface of modified Streptomyces isolation agar (MSIA). MSIA contained the following constituents: 28g/L of Nutrient agar containing 4 g of Dextrose, 2 g of $CaCO_3$, 250,000 units of nystatin, and 2500 mg of rifampicin. Nystatin and rifampicin were added to inhibit molds and bacteria respectively. Plates were incubated at 30^{o}C, and monitored after 48, 72, 96 h etc, up to 14 days. Actinomycetes were primarily identified based on appearance of aerial mycelium.

DATA ANALYSIS

Data obtained in the study was analyzed using the equation:

$$\text{percentage of spp isolated} = \frac{\text{number of spp isolates}}{\text{total number of isolates}} \times 100$$

(where spp = Bacillus, streptomyces and mold)

RESULTS

Isolation and identification of *Bacillus* species

All the soil samples yielded monocultures of *Bacillus* species on Nutrient agar. *Bacillus* species RzsB4, PtsB2, CpsB3 and EpsB5 and PgsB1 were isolated from rhyzosphere, poultry, compost, earthen fish pond and stockyard repectively. All the *Bacillus* species isolated were Gram-positive, catalase-positive, formed endospores and did not produce pigments, which are biochemical reactions consistent with the *Bacillus* species (Table 1).

Table 1: Biochemical and morphological characteristics of *Bacillus* isolates

Bacillus isolate	Gram reaction	Cell shape	Endospore	Catalase	Pigment	Colony characteristics
RzsB4	+	Rods	+	+	-	Cream coloured, flat and circular with entire and undulated margins
PtsB2	+	Rods	+	+	-	Cream coloured, flat and circular with entire and undulated margins
CpsB3	+	Rods	+	+	-	Cream coloured, flat and circular with entire and undulated margins
EpsB5	+	Rods	+	+	-	Cream coloured, flat and circular with entire and undulated margins
PgsB1	+	Rods	+	+	-	Cream coloured, flat and circular with entire and undulated margins

Plate 1 shows a light micrograph of *Bacillus subtilis* RzsB4 isolated from rhizosphere soil. *B. subtilis* is an endospore-forming rhizobacterium and is able to produce more than two dozen antibiotics including subtilin and bacitracin (Stein, 2005; Stein *et al*., 2006). *Bacillus* species produce

antibiotics during the stationary phase of growth (postlog phase), a phase during which spores are also produced.

Plates 2 is a photomicrograph of *Bacillus* strain PtsB2 isolated from poultry soil. *Bacillus* strains often isolated from poultry include *B. cereus* and *B. licheniformis. B. cereus* strains are known to produce the antibiotics biocerin and kanosamine, an antibiotic against plant pathogens (Milner *et al*., 1996).

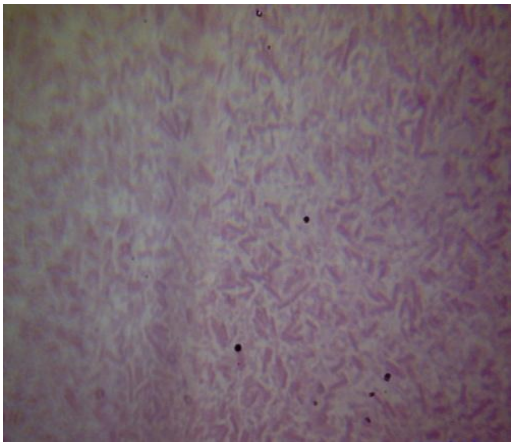

Plate 1: Photomicrograph of *Bacillus* sp. (strain RzsB4) isolated from rhizospere zone. (B-350 Optica. Magnification 100×)

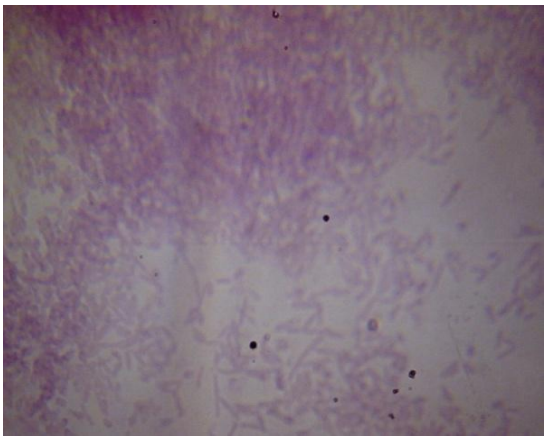

Plate 2: Photomicrograph of *Bacillus* sp. (strain PtsB2) isolated from poultry soil. (B-350 Optica. Magnification 100×)

Inhibitory effects of *Bacillus* isolates by the agar-well diffusion method

Extracts from *Bacillus* isolates were screened for antimicrobial activity against six target organisms including *Staphylococcus aureus* ATCC 700699, *Shigella flexneri* ATCC 120222, *Salmonella enteritidis* ATCC 1307, *Klebsiella pneumoniae* ATCC 8309, and *Candida albicans* ATCC 10231. The results did not show inhibitory effects against any of the target organisms (Plate 3).

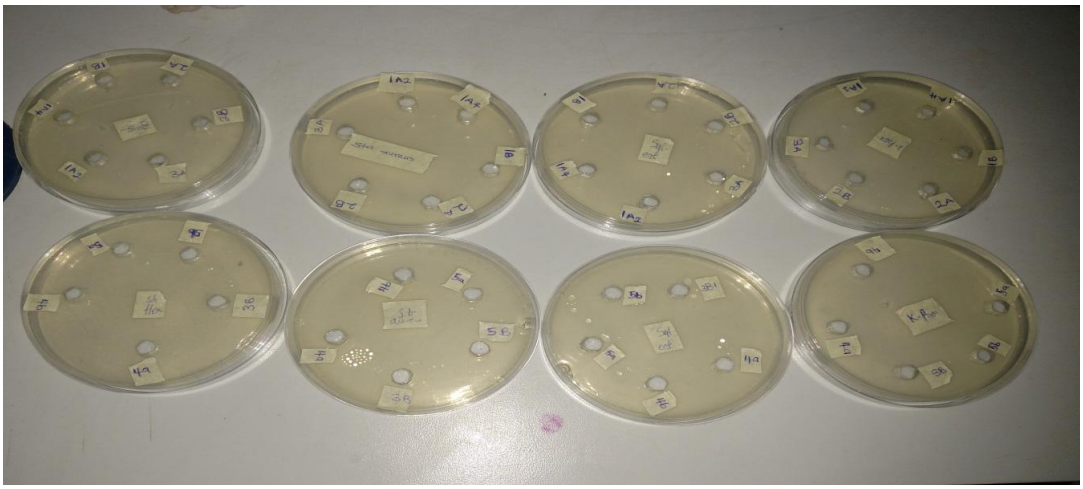

Plate 3: Testing antimicrobial effects of *Bacillus* isolates against target organisms using the Agar-well Diffusion Method. Lawns of different target organisms are growing on the plates. Each well in a plate contains 100 µL of a different TSB-cultured *Bacillus* isolate.

***Streptomyces* species and molds isolated from the soil samples on modified Streptomyces isolation agar (MSIA)**

Table 2 shows the *Streptomyces* and molds isolated in the study. Three *Streptomyces* strains were isolated on modified Streptomyces isolation agar (MSIA). *Streptomyces coelicolor* strains RzsS4 and PgsS1 were isolated from rhizosphere soil and stockyard soil respectively. *S. coelicolor* colonies started growing as tiny (punctiform) white colonies. After 14 days incubation at 35^{o}C, *S. coelicolor* showed large colonies in concentric formation with aerial mycelium having the appearance of sawdust (Plate 4 and Plate 6). *Streptomyces coelicolor* produces actinorhodin, a polyketide antibiotic. Production of actinorhodin takes place when the cultures of *S. coelicolor* enter the stationary phase of growth (Gramajo *et al*., 1993). Another *Streptomyces* species (strain CpsS3) appearing as a cream-colored and leathery colony was isolated from compost soil (Plate 5).

Four strains of *Penicillium* species were isolated on MSIA. They included CpsF1 and CpsF3 from compost soil (Plate 5), PtsF1 from poultry and *P. notatum* strain RzsF1 from rhizosphere soil (Plate 7). Fungal growth was found on MSIA despite 250,000 nystatin antifungal incorporated on the medium. *Penicillium* species were the predominant fungi isolated on MSIA.

Table 2: *Streptomycetes* and molds isolated on MSIA

Isolate code	Colony morphology and appearance of aerial mycelium.	Species isolated
Streptomyces		
PgsS1	White tiny (punctiform) colonies growing into large colonies in concentric formation with aerial mycelium having the appearance of sawdust after 14 days incubation at 35^{o}C.	*S. coelicolor*
RzsS4	White tiny (punctiform) colonies growing into large colonies in concentric formation with aerial mycelium having the appearance of sawdust after 14 days incubation at 35^{o}C.	*S. coelicolor*
CpsS3	Cream leathery smooth round colony with entire margin.	Unidentified
Molds		
CpsF3	Ash-green colonies	*Penicillium* species
CpsF3	Ash-green colonies	*Penicillium* species
PtsF1	Ash-green colonies	*Penicillium* species
RzsF1	Green colony, slightly raised, with white margin	*Penicillium notatum*

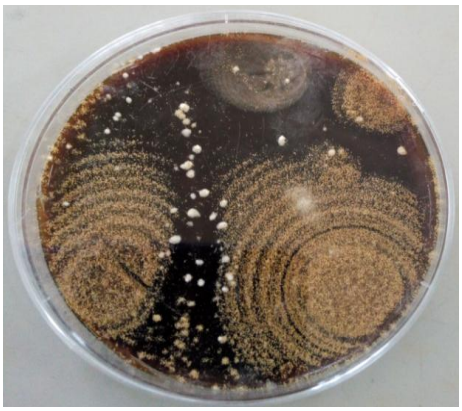

Plate 4: *Streptomyces coelicolor* PgsS1 colonies growing on MSIA.

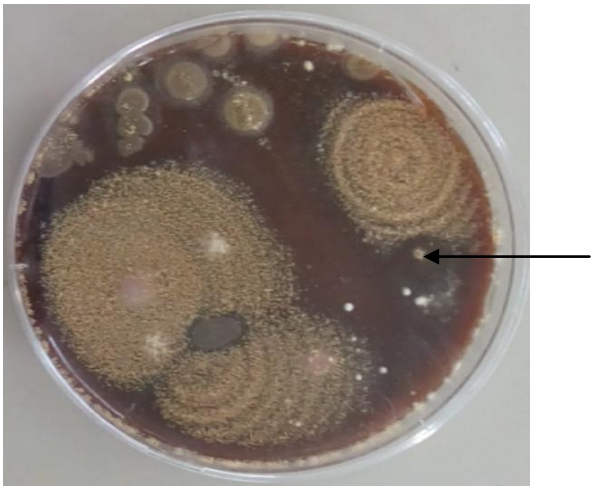

Plate 6: *Streptomyces coelicolor* RzsS4 growing on MSIA. Inhibition zones are visible around some colonies (arrow).

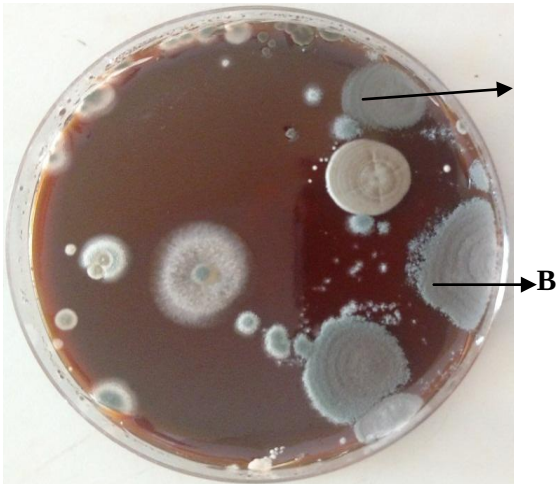

Plate 5: *Streptomyces* sp. CpsS3 (A) and *Penicillium* sp CpsF3 (B) isolated on MSIA from compost soil

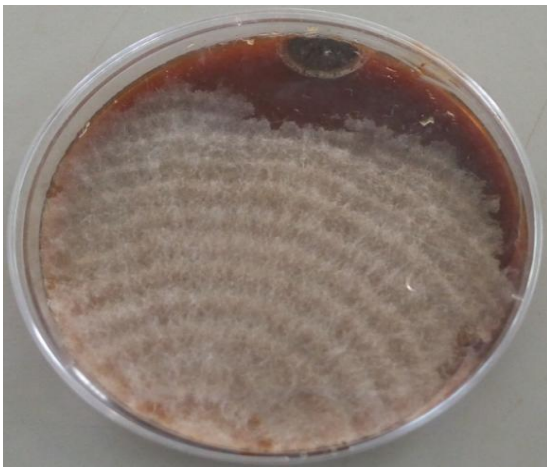

Plate 7: *Penicillium notatum* RzsF1 (top) growing as green colony with white margin

DISCUSSION

Soil samples from each of the five biotopes yielded growth of *Bacillus* species. All the soil samples yielded monocultures of *Bacillus* species on Nutrient agar. This agrees with the already established concept that antibiotic-producing microorganisms in their specific niches produce antibiotics in order to inhibit other microbial populations (Cherif et al 2003). This results in

only one population of microbe in such niches as found in the present study.

The *Bacillus* species isolated in this study all produced spores after 24 hours of growth. Sporulation is a survival strategy that enables microbes to survive adverse conditions as spores in their environment. *Bacillus* species produce antibiotics during the stationary phase of growth (postlog phase) when the genes for antibiotic production are induced (Nakano and Zuber, 1990). Coincidentally, sporulation occurs at this phase of growth as the activity of dozens of genes involved in sporulation process is induced.

Bacillus isolates in this study did not inhibit the growth of any of the target organisms. This result might be interpreted that *Bacillus* spp. do not produce antibiotics after 24 hours of growth under laboratory conditions.

Streptomyces species are notable for their ability to produce antibiotics (Chaudhary *et al*., 2013), but studies show that they hardly do so under laboratory conditions. In a study carried out in Ethiopia (Kibret *et al*., 2018), 416 actinomycete cultures were tested for bioactivity. Only six isolates (1.44%) showed pronounced antimicrobial activity. This seems to be in agreement with the report that most of the *Streptomycetes* gene clusters dedicated to the biosynthesis of metabolites remain "silent" in laboratory conditions (Kealey *et al*., 2017).

Penicillium notatum and other *Penicillium* strains were isolated on MSIA. *P. notatum* is known to produce the antibiotic penicillin which is active against Gram-positive organisms.

Modified Streptomyces Isolation agar (MSIA) seems to be a good medium for the isolation of *Streptomycetes coelicolor* and *Penicillium* species as 250,000 units of nystatin incorporated into MSIA failed to inhibit the growth of *Penicillium* species. However, 2500 mg of rifampicin added to the medium as an antibacterial completely inhibited all bacterial growth.

CONCLUSION

Microorganisms produce antimicrobial substances important in medicine. They are the source of lifesaving treatments for bacterial and fungal infections. Our study focused on soil microbiota and the findings reveal that the soil in Okitipupa and its environs is still an untapped habitat for antimicrobial producing microbes. In our laboratory, we isolated a Streptomyces strain, namely, *Streptomyces coelicolor*, which produces actinorhodin, a polyketide antibiotic that exhibits significant activity against Gram-positive pathogens including *Staphylococcus aureus* and enterococci. The present findings highlight the need for further studies towards obtaining novel antimicrobial agents out of the Streptomycetes, *Bacillus* species and molds dwelling in soils from this locality.

REFERENCES

Baltz, R. (2007). Antimicrobials from actinomycetes: Back to the future. Microbe, **2**(3): 125-131.

Berdy, J. (2012). Thoughts and facts about antibiotics: where we are now and where we are heading. Journal of Antibiotics, **65**:385-395. DOI: 10.1038/ja.2012.27

Chaudhary, H. S., Yadav, J., Shrivastava, A. R., Singh, S., Singh, A. K. and Gopalan, N. (2013). Antitibacterial activity of actinomycetes isolated from different soil samples of Sheopur (A city of central India). Journal of Advanced Pharmaceutical Technology & Research, **4**(2):118-123.

Cherif, A., Chehimi, S. and Limem, F. (2003). Detection and characterization of the novel bacteriocin entomocin 9, and safety evaluation of its producer, *Bacillus thuringiensis* spp. *Entomocidus* HD9. Applied Microbiology, **95**:990-1000

Demain, A. L. (2009). Antibiotics: natural products essential to human health. Medicinal Research Reviews, **29**:821-842. DOI: 10.1002/med.20154.

Goodfellow, M. and Fiedler, H. P. (2010). A guide to successful bioprospecting: informed by actinobacterial systematic. Antonie van Leeuwenhoek, **98**: 119-142. DOI: 10.1007/s10482-010-9460-2

Gramajo, H. C., Takano, E. and Bibb, M. J. (1993). Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. Molecular Microbiology, **7**(6): 83.

Kealey, C., Creaven, C. A., Murphy, C. D. and Brady, C. B. (2017). New approaches to antibiotic discovery. Biotechnology Letters, **39**:805-817. DOI:10.1007/s10529-017-2311-8

Kibret, M., Guerrero-Garzon, J. F., Urban, E., Zehl, M., Wronski, V-K., Ruckert, C., Busche, T., Kalinowski, J., Rollinger. J. M., Abate, D. and Zotchev, S. B. (2018). Streptomyces spp. from Ethiopia producing antimicrobial compounds: Characterization via bioassays, genome analysis, and mass spectrometry. Frontiers in Microbiology, **9**:1270. http://doi: 10.3389/fmicb.2018.01270.

Kumari, M., Myagmarjav, B. E., Prasad, B. and Choudhary, M. (2013). Identification and characterization of antibiotic-producing actinomycetes isolates. Current Research in Microbiology, **4**(1):24-31.

Kuta, F. A., Nimzing, L. and Orka'a, P. Y. (2009). Screening *Bacillus* species with potentials of antibiotics production. Applied Medical Informatics, **24**.

Lobanovska, M. and Pilla, G. (2017). Penicillin's discovery and antibiotic resistance: Lessons for the future? Yale Journal of Biology and Medicine, **90**(1):135-145.

Milner, J. L., Silo-Suh, L., Lee, J. C., He, H., Clardy, J. and Handelsman, J. (1996). Production of kanosamine by *Bacillus cereus* UW85. Applied and Environmental Microbiology, **62**(8):3061-5.

Morikawa, M., Ito, M. and Imanaka, T. (1992). Isolation of a new surfactin producer *Bacillus pumilus A-1*, and cloning and nucleotide sequence of the regulator gene, psf-1. Journal of Fermentation and Bioengineering, **74**(5):255-261.

Nakano, M. M. and Zuber, P. (1990). Molecular biology of antibiotic production in *Bacillus*. Critical Reviews in Biotechnology, **10**(3):223-40. DOI: 10.3109/0788559009038209.

Nett, M., Ikeda, H. and Moore, B. S. (2009). Genomic basis for natural product biosynthetic diversity in the actinomycetes. Natural Products Reports, **26**: 1362-1384. DOI: 10.1039/b817069j

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, synthesis and specific functions. Molecular Biology, **56**(4): 845-857.

Stein, T., Entian, K. and Heinzmann, S. (2006). Engineering *Bacillus subtilis* ATCC 6633 for improved production of the lantibiotic subtilin. Applied Microbiology and Biotechnology, **69**(5):532-6. DOI: 10.1007/s00253-005-0023-9. PubMed

van der Meij, A., Worsley, S. F., Hutchings, M. I. and van Wezel, G. P. (2017). Chemical ecology of antibiotic production by actinomycetes. FEMS Microbiology Reviews, **41:**392-416. DOI: 10.1093/femsre/fux005.

Zotchev, S. B. (2012). Marine actinomycetes as an emerging resource for the drug development pipelines. Journal of Biotechnology, **158**: 168-175. DOI: 10.1016/j.jbiotec.2011.06.002