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ANTIBIOTIC PRODUCTION BY MICROBES ISOLATED FROM SOIL

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ABSTRACT: Antibiotic is one of the most important commercially exploited secondary metabolites produced by bacteria, fungi and *Streptomyces* and employed in a wide range. Most of the antibiotics used today are from the microbes. Bacteria are easy to isolate, culture, maintain and to improve their strain. *Bacillus* species being the predominant soil bacteria because of their resistant endospore formation and production of vital antibiotic like polymyxin, bacitracin etc. are always found inhibiting the growth of the other organisms. In the present research study, screening of bacteria, fungi and *Streptomyces* with potential antibiotic activity was carried out. Among the microbes isolated and identified, *Bacillus subtilis, Penicillium chrysogenum* and *Streptomyces* sp were selected on the basis of their anti-bacterial activity. The inhibitory activities of the isolated microorganisms were checked against *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and *Klebsiella pneumonia* (ATCC 4352). It was observed that *Penicillium chrysogenum* metabolites showed maximum antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumonia* with a zone of inhibition of 17mm, 11mm, 19.8mm and 8.2mm respectively. *Bacillus subtilis* metabolites showed activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* measured as zone of inhibition of 13.4mm and 13.8mm respectively whereas *Streptomyces* sp showed minimum activity against all the four tested organisms.

INTRODUCTION: Antibiotics, in one form or another, have been in use for centuries. The vast majority of novel antibiotics have been detected by screening of "wild isolates" obtained from soil and other natural habitats. Although a wide taxonomic range of microbes have the ability to produce antibiotics. Thus, over 55% of the antibiotics detected between 1945 and 1978 originated from the genus *Streptomyces*, representing a total of more than 5,000 compounds [1].

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With advances in organic chemistry many antibiotics are now also obtained by chemical synthesis, such as the sulfa drugs. Drugs used in the chemotherapy of infectious diseases are classified into two groups. Drugs that have been synthesized by chemical procedures in the laboratory are called synthetic drugs while those produced by bacteria and fungi are called antibiotics [2].

The antibiotics are widely distributed in the nature, where they play an important role in regulating the microbial population of soil, water, sewage, and compost.

Of the several hundred naturally produced antibiotics that have been purified, only a few have been sufficiently non-toxic to be of use in medical practice.

Those that are currently of greatest use have derived from a relatively small group of microorganisms belonging to the genera *Penicillium, Streptomyces, Cephalosporium, Micomonospora and Bacillus* [3].

Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. While many antibiotics are known to exist, efforts to discover new antibiotics still continue. Therefore, many species such as *Streptomyces, Bacillus and Penicillium* have been studied continuously for their ability to produce antibiotics [4].

In addition, due to the fact that *Bacillus* species have produced antibiotics in the soluble protein structure and that these antibiotics have been found to be cheaper and more effective in studies conducted to date, these microorganisms are preferable for commercial production.

Currently, the target is to produce antibiotics such as polymyxin and bacitracin from *Bacillus* [5, 6]. It was reported that members of the species *Bacillus* generally produced polypeptide type bacteriocines, and that these antibiotics generally affect Gram positive bacteria [7, 8]. The apparent increase of the occurrence of antibiotic resistance among bacteria during the past years and its possible implication in public health has led to an intensified surveillance of bacterial resistance in many countries.

Treatment of infectious diseases caused by pathogenic bacterial and fungal strains was one of the most traditional problems in the clinical field [9, 10]. This necessity encouraged the investigators to synthesize novel and more potent inhibitory compounds (like azoles and quinolones derivatives) [11, 12] to fight them. However, the adverse effects and also appearance of bacterial or fungal resistances persuaded the investigators to study on natural products from microorganisms or herbal extracts to discover novel and safe lead compounds [9, 10].

It was not until 1940 with the discovery of penicillin, the first, best-known and most widely used antibiotic [13, 14] in 1928 by an English Bacteriologist, late Sir Alexander Fleming that the first clinical trials of penicillin were tried on humans. This antibiotic was obtained from a blue green mould of the soil called *Penicillium notatum*.

Penicillin was discovered accidentally in 1928 by Fleming, who showed its efficacy in laboratory cultures against many disease producing bacteria. This discovery marked the beginning of the development of antibacterial compounds produced by living organisms.

Another antibiotic, streptomycin was isolated in 1944 by Waksman, a Microbiologist, from a species of soil bacteria, called *Streptomyces griseus*, particularly tubercle bacilli, and has proved to be very valuable against tuberculosis. A vigorous search for more antibiotics was on at this time and in 1947, another antibiotic, chloromycetin was discovered by Burkholder [14, 15]. It was isolated from *S. venezuelae*. It has a powerful action on a wide range of infectious bacteria both Gram positive and Gram negative.

Most of the peptide antibiotics produced by *Bacillus* are active against Gram positive bacteria [16]. However, compounds such as polymyxin, colistin, and circulin exhibit activity almost exclusively upon Gram-negative forms, whereas bacillomycin, mycobacillin, and fungistatin are effective agents against molds and yeasts [17]. As more antibiotics were discovered, designed and studied, scientists found that they had different properties. Some of these properties include their source, range of activity and their kinds. These were used to classify those [14].

The objective of the present study is production, extraction and assay of antimicrobial metabolites from bacterial, fungal and *Streptomyces* isolates using soil as source.

MATERIAL AND METHODS:

Isolation and screening of microbial isolates: In the present study, soil sprinkle technique was used to isolate antibiotic producing bacilli. For this purpose about 20-30 particles of soil were sprinkled on the surface of nutrient agar plates seeded with the test organism one Gram positive *Staphylococcus aureus (*ATCC 29213) and three Gram-negative *Escherichia coli (*ATCC 25922), *Pseudomonas aeruginosa (*ATCC 27853), *Klebsiella pneumonia (*ATCC 4352). The plates were incubated at 30°C for 24 hours. Antibiotic activity was checked by zone of inhibition, surrounding a colony.

Different colonies having zones of inhibition were picked and streaked on separate nutrient agar plates to get pure cultures. These isolates were used as the source of antibiotic producing microbes. All strains were stored at 4°C and subcultured periodically.

The soil fungi were isolated by both the Direct Soil Inoculation and the Soil Dilution Techniques using the pour plate method. The Media used for the isolation were Potato Dextrose Agar (PDA). Plates were incubated for 5 days at 28˚C. Pure cultures of fungal isolates were identified using both macroscopic (cultural) and microscopic (morphological) features with reference to Barnett and Hunter [18].

Each fungal isolate was streaked on Nutrient Agar as a straight line and incubated at 30˚C. After two days of incubation, the test organisms, one gram positive *Staphylococcus aureus (*ATCC 29213) and three gram-negative *Escherichia coli (*ATCC 25922), *Pseudomonas aeruginosa (*ATCC 27853), *Klebsiella pneumonia (*ATCC 4352) were streaked perpendicular to the streaked line of the growing fungus.

This was then incubated at 37˚C for 24 hours, after which the zone of inhibition of each test organism from the streaked line of the growing fungus was measured.

For Streptomyces, 1g of the soil were suspended in 100 ml of physiological water (NaCl 8.5 g/1) then incubated in an orbital shaker incubator at 28 ^{0} C with shaking at 200 rpm for 30 min. Mixtures were allowed to settle, and serial dilutions up to 10^{-5} were prepared using sterile physiological water and agitated with the vortex at maximum speed. An aliquot of 0.1 ml of each dilution from 10^{-2} to 10^{-5} was taken and spread evenly over the surface of starch casein agar medium.

The media are added to antibiotic to inhibit bacterial and fungal contamination, respectively. Plates were incubated at 28^o and 37^{o}C and monitored after 48, 72, and 96 h. Repeated streaking on starch casein agar plates led to purify bacterial colonies that showed an actinomycetes like appearance. The isolated strains are preserved at 4^{o}C during two months and maintained for longer period by serial subculture.

Identification of antibiotic producing soil microbes: Isolated bacterial strain was identified morphologically (shape, gram staining, spore staining, spore shape, sporangium dilatation and motility) and biochemically (sugar utilization, starch utilization, casein hydrolysis, indole production, citrate utilization, methyl red-voges proskauer (MR-VP), oxidase production, catalase production, nitrate reduction, gas production from glucose according to the Bergey's Manual of Determinative Bacteriology [19].

Pure cultures of fungal isolates were identified using both macroscopic (cultural) and microscopic (morphological) features with reference to Barnett and Hunter [18].

For Streptomyces, a medium containing 10 g of yeast extract per liter and 10 g of glucose per liter and pH 7.2 (yeast extract-glucose medium) was used for general cultivation of actinomycetes strains. The media used for morphological characterization of strain were those described by Shirling and Gottlieb [20]. Cultural characteristics of cells in various media were recorded after incubation for 14 days at 30°C and morphological observations were made with a light microscope by using the method of Shirling and Gottlieb [20].

Inoculum preparation: The inoculum of *Bacillus* species, identified as *Bacillus subtilis*, selected on the basis of maximum activity against the test organisms, was prepared in Tryptic Soy broth (pH7.3) at concentration of 10% (v/v) by incubating at 30°C for 72 hours.

The fungus was sub-cultured on PDA plates and incubated at 30 ^{0}C for 3-5 days to obtain the spores used for antibiotics production. Spores were washed into a sterile beaker using 0.1% Tween 80 in 0.1M potassium phosphate buffer at pH 7.0. The spore suspension was standardized such that 1 in 10 dilutions has an absorbance of 0.48 at 530nm.

The inoculum of *Streptomyces* specie, selected on the basis of maximum activity against the test organisms, was prepared in *Streptomyces* growth medium was used at concentration of 10% (v/v) by incubating at 28°C for 4 days.

Production medium for antimicrobial compounds:

1. **For Bacteria**: About 50 ml of the Synthetic medium (g/L); L-glutamic acid 5.0; KH_2PO_4 0.5; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; $MnSO_4.H_2O$ 0.01; NaCl 0.01; $FeSO_4.7H_2O$ 0.01; $CuSO_4.7H_2O$ 0.01; $CaCl_2.2H2O$ 0.015; Glucose 10; and pH 7), was taken in 250 ml flask as production medium. After sterilization of synthetic media concentrated glucose solution previously sterilized by 0.2 µm pore size filter paper, was added to give a final concentration of 1% in the medium.

2. **For *Penicillium***: About 50 ml of the Synthetic medium (g/L); 6.0g Ammonium acetate, 0.5g $NaSO_3$, 0.02g $ZnSO_4.7H_2O$, 0.25g $MgSO_4.7H_2O$, 6.0g KH_2PO_4, 0.02g $FeSO_4.7H_2O$, 0.5g Phenylalanine and 10.0g carbon source (lactose) (pH-6.0) was taken in 250 ml flask used as production medium. The flasks were incubated with intermittent shaking for 21 days after which the contents were sieved through cotton wool and filter to remove cells.

3. **For Streptomyces**: About 50 ml of the Synthetic medium (g/L); 40g Sucrose, 12.8g $H_3C_6H_5O_7.H_2O$, 6.0g $(NH_4)_2SO_4$, 0.25g $MgSO_4.7H_2O$, 0.15g K_2HPO_4, 11g $CaCO_3$,0.25g $MnSO_4.4H_2O$, 0.04g $ZnSO_4.7H_2O$, 0.016 g $K_2Cr_2O_7$ was taken in 250 ml flask used as production medium. After sterilization of synthetic media then add 5% mycelium inoculums. And incubate at 28^oC with intermittent shaking (200 rpm) for 6-7 days.

Antibiotic production condition and Purification: Shake flask fermentation method was used for bacterial antibiotic production. Seventy two hours old inoculum was prepared in Tryptic Soy Broth (pH 7.3) at concentration of 10% (v/v). Inoculum was added to the production medium and incubated for 24 hours in orbital shaker at 120 rpm. After incubation, culture was centrifuged to get cell free supernatant and was further used for antimicrobial activity.

The purification of penicillin from the production media began with filtration of the broth. In the first stage, large solids and microbial cells were separated by filtration, as filtration is the most versatile method for removing the insoluble from the broth.

Penicillin rich aqueous broth was treated with activated charcoal to remove pigments and impurities. After filtration and carbon treatment, penicillin recovery was done by liquid-liquid extraction (solvent extraction). Penicillin was extracted from an aqueous phase into the solvent butyl acetate. Potassium ions were added to precipitate the salt of penicillin and this was further used for antimicrobial activity.

The purification of antibiotic from the production media of *Streptomyces* began with filtration of the broth. In the first stage, large solids and microbial cells were separated by filtration, as filtration is the most versatile method for removing the in soluble from the broth and cell free filtrate was further used for antimicrobial activity.

Antimicrobial activity by Agar diffusion assay: Agar well diffusion method was used to check the cultures for the production of antimicrobial metabolites. Twenty-four hours fresh cultures of one Gram positive *Staphylococcus aureus* (ATCC 29213) and three Gram-negative *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 4352) were diluted with pre-sterilized normal saline. A sterilized cotton swab was dipped in the diluted cultures and lawns were prepared over the agar surface.

Wells were made in the inoculated plates using sterile corn borer. About 80 µl cell free supernatants were added in the wells and the plates were incubated at 37ºC for 24 hours. After 24 hours, the zones of inhibition were observed. The diameter of the zone of inhibition was measured in mm with well size of 6mm.

RESULTS AND DISCUSSION:

Isolation, Screening and Identification of microbial isolates: By using soil sprinkle technique, five different bacterial colonies having zones of inhibition were picked and streaked on separate nutrient agar plates to get pure cultures. Out of 5 bacterial isolates only one bacterial isolate showing maximum inhibition zone was selected and identified as *Bacillus subtilis* (**Table 1**).

TABLE 1: MORPHOLOGICAL AND BIOCHEMICAL TESTS FOR THE IDENTIFICATION OF *BACILLUS SUBTILIS*

Test	Morphological and Biochemical tests
Grams staining	+
Shape	*Cocco bacilli*
Spore formation	+
Motility	+
Gelatin	+
Indole production	-
Citrate utilization	+
Mannitol	+
Methyl red (MR)	-
Voges-Proskauer (VP)	+
Oxidase	+
Catalase	+
Starch hydrolysis	+
Nitrate reduction	+
Casein hydrolysis	+
Gas production from glucose	-

By using soil dilution technique three different fungal cultures having zones of inhibition were picked and streaked on separate potato dextrose agar plates to get pure cultures. Out of three fungal strains, one isolate showing maximum inhibition zone was selected and characterized as *Penicillium chrysogenum* by its shiny blue green mold surrounded by white mycelium on potato dextrose agar. Such colonies were visualized under light microscope using phenol blue to confirm the mycelium and conidia of the fungi.

By using soil sprinkle technique seventeen different *Streptomyces* colonies were obtained. But five species having zones of inhibition were picked and streaked on separate starch casein agar plates to get pure cultures. Out of five species only one *Streptomyces* species (Isolate 3) showing maximum inhibition zone was selected (**Table 2**).

TABLE 2: MORPHOLOGICAL IDENTIFICATION OF *STREPTOMYCES* BASED ON PIGMENTATION CHARACTERISTICS

Characteristic Observed Isolate	*Spore-mass colour*	*Substrate mycelium colour*	*Diffusible pigment production*
Isolate 3	*White to pink*	Pink	+

Antimicrobial activity by Agar diffusion assay: Samples drawn during batch fermentations were subjected to agar well diffusion assay, using *Staphylococcus aureus* as test organisms. Antimicrobial activity was measured in terms of zone of inhibition. The incubated samples were evaluated and optimum antimicrobial activity of inoculum of *Bacilius species* was ensured at 48hours.

It was observed that *Penicillium chrysogenum* metabolites showed maximum antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumonia* with a zone of inhibition of 17mm, 11mm, 19.8mm and 8.2mm while *Bacillus* metabolites showed activity against *Staphylococcus aureus* and *Pseudomonas florescens* measured as zone of inhibition of 13.4mm and 13.8mm and *Streptomyces* sp showed minimum activity against all the four tested organisms with inhibition zone of 7.2mm, 7.5mm, 10.6mm and no zone was observed in case of *Klebsiella pneumonia* (**Table 3**).

Schallmey [21], isolated soil bacteria which shows antibiotic activity under normal growth condition and was found inhibiting some gram positive and some gram negative organism both *Bacillus lentus* and *Bacillus alvei* also shows antibacterial activity against *Staphylococcus aureus. Bacillus pumillus* only show slight zone of inhibition on *Proteus* spp while it is inactive against others.

The culture extracts of *P. chrysogenum* exhibited significant antibacterial effects against isolates. The results show that the organism produces antibiotics that are effective against the bacterial isolates. This is consistent with the fact that most strains of *Penicillium chrysogenum* produce β-lactam antibiotics, mainly penicillins [22].

Out of 22,500 microbial bioactive metabolites, 10,100 metabolites are produced by Actinomycetes [23]. *Streptomyces* are especially prolific, producing around 80% of total antibiotic products [24].

The present study was focused on antimicrobial metabolites production from soil microbial isolates. The strains showed good inhibition against all bacteria in cross streak method were selected.

TABLE 3: ANTIMICROBIAL ACTIVITY OF MICROBIAL METABOLITES AGAINST TEST ORGANISMS

Test Organism	Control	*Bacillus* metabolite	*Penicillium* metabolite	*Streptomyces* metabolite
Staphylococcus aureus (ATCC 29213)	26 mm	13.4 mm	17mm	7.2mm
Pseudomonas aeruginosa ATCC 27853)	27 mm	13.8 mm	11.0mm	7.5mm
Escherichia coli (ATCC 25922)	25 mm	0 mm	19.8mm	10.6mm
Klebsiella pneumonia (ATCC 4352)	25 mm	0 mm	8.2mm	0mm

CONCLUSION: In search of new antibiotics, relatively simple and rapid methods have been developed for screening microorganisms for antibiotic producing ability. Soil samples are commonly employed in the isolation of antibiotic producing organism.

The detection of these antagonistic substances revealed interesting properties that justify its importance and its study on the potential application in biological control of pathogenic microorganisms and spoilage food. For these reasons, the biochemical nature and the best conditions to production of the substances studied in this work are being investigated to further purification experiments. Many microorganisms have been evaluated for the production of antimicrobial substance. However the high cost and low yields have been the main problem for its industrial production [25].

In the present study, the microbial isolates with antimicrobial activity from soil were isolated. Among all screened isolates, *Penicillium chrysogenum* metabolite showed maximum inhibition against both gram positive as well as gram negative bacteria. Production of antibiotic by microorganisms from soil is affected by many factors including nitrogen and carbon source. Therefore there is a great need to optimize with different substrates that provides maximum production of antimicrobial substance.

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