

A STUDY OF ANTIMICROBIAL AND CYTOTOXIC POTENTIAL OF SECONDARY METABOLITES ISOLATED FROM AGRICULTURAL SOIL BACTERIA

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Abstract

It is not a secret that *Bacillus* bacteria are used to produce secondary compounds, which are commonly used as cancer-fighting medications. These *Bacillus* species are present in various kinds of soil, such as the soil along Ring Road in Peshawar. The study aimed to investigate the soil *Bacillus* species at the Ring Road, determine the possible cytotoxic or antibacterial characteristics, and their secondary metabolic products. The *Bacillus* species were isolated, purified, and identified after standard procedures on three randomly sampled soil samples from two locations. The secondary compounds exhibited an obvious inhibition zone and had anti-bacterial activities against *Staphylococcus aureus*. They also developed a distinct inhibition zone and became antifungal to *Candida albicans*. The Brine Shrimp Lethality Test (BSLT) was also performed to determine whether the isolates were toxic to cells or not. Two strains of the soil bacillus (ASF01 and ASF02) were successfully isolated and purified using soil samples. The synthesis of secondary metabolites in bacteria is an easy, cost-effective, and eco-friendly system. The secondary metabolites were shown to have antibacterial effects against *Staphylococcus aureus*, which is a pathogenic bacterium. Secondary metabolites, on the other hand, demonstrated antifungal activity against *Candida albicans*, which is a pathogenic fungus, creating a clear zone of inhibition. The secondary metabolites also exhibited strong cytotoxicity and might be used to kill the undesired cells. (such as cancer cells)

Chapter 1

INTRODUCTION

Background of metabolites

During growth, small molecules known as metabolites are produced. Primary and secondary compounds come from two types of cell processes. Primary ones help in normal growth, making energy, and reproduction. These include

vitamins B2 and B12, amino acids, lactic acid, organic acids, and nucleotides. Secondary ones do not help the bacteria grow or reproduce, but they help the bacteria survive and work in their environment. Secondary metabolites do not take part in main life processes but often play important roles in ecological functions. Secondary metabolites are natural compounds

with many different chemical forms. They are grouped based on factors like their structure, role, and how they are made, and more than 2,140,000 types of secondary metabolites have been identified. The five (5) major secondary classes include, for example, alkaloids, fatty acid-derived chemicals and polyketides, terpenoids and steroids, nonribosomal polypeptides, and enzyme cofactors. (Thirumurugan *et al.*, 2018)

Microbial-mediated synthesis of secondary metabolites

Microorganism-produced compounds are crucial for the treatment of infectious diseases and cancer. (Osama N, *et al.*, 2022). Among microorganisms, bacteria and fungi are the best options for producing bioactive substances since they have existed on Earth for billions of years and have developed numerous biosynthetic pathways that use novel techniques to produce secondary metabolites. (Tran PN, *et al.*, 2019). The main source of bioactive secondary metabolites (SMs) is microorganisms, which can be isolated from soil microbiota, marine environments, or harsh environments. (Gakuubi, *et al.*, 2021). After development, bacteria produce complex biological compounds called microbial SMs. SMs are not involved in the growth and reproduction of the microorganisms, but are essential for certain secondary demands. (Kumar and others, 2021). Nearly 20,000 compounds have previously been found in endophytes, which are a key source of bioactive SMs among fungal species, according to recent studies. (Ancheeva *et al.*, 2020; Gakuubi *et al.*, 2021).

Microbial Secondary Metabolites as Antibiotics

Soil microbes from groups like *Bacillus*, *Cephalosporium*, *Streptomyces*, *Micromonospora*, and *Penicillium* are used to produce many kinds of medicines. Every year, around 500 new bioactive compounds are discovered. (Farag MM, *et al.*, 2020). The most well-known examples of SMs are medications with a long history and a high success rate, such as penicillin, tetracycline, and gentamicin sulphate. (Kumar *et al.*, 2021). Bacteria, especially actinomycetes like

Streptomyces, make many antibiotics like streptomycin, tetracycline, chloramphenicol, erythromycin, viomycin, lincomycin, meropenem, and daptomycin. But other kinds of bacteria can also make antibiotics. For example, the structure of the compound derived from *Chromobacterium violaceum* was used to synthesise the monobactam antibiotic aztreonam, the gramicidin A from *Bacillus*, the colistin from *Paenibacillus*, and the mupirocin from *Pseudomonas*. (Hutchings *et al.*, 2019).

Applications of microbial secondary metabolites

Potential applications of secondary metabolites in biotechnology and medicine, together with their ecological and biogeochemical impacts, have garnered significant interest. (Akbarizare M, *et al.*, 2019). One of the most significant sources of new compounds for the production of new drugs is microbial secondary metabolites. (Tedesco *et al.*, 2021). Numerous bioactive substances produced by actinobacteria have enormous potential for use in both medicine and agriculture. (Singh BP *et al.*, 2019). Furthermore, being utilized as herbicides, insecticides, plant growth regulators, and other goods like bio-pigments and surfactants in the food, pharmaceutical, cosmetics, and other industries, SMs have also been utilized recently in the agricultural field. (Kumar *et al.*, 2021). Apart from serving as the foundation for numerous pharmaceuticals (such as antibiotics and anticancer therapies), bacterial secondary metabolites are also vital to the environment, supporting microbial interactions, including competition and communication. (Zhang *et al.*, 2024) Numerous substances, including antioxidants, antimicrobials, antifungals, anticancer agents, and antivirals, are present in these metabolites and are crucial to modern medicine. (Palacios-Rodriguez *et al.*, 2024)

Bacteria producing secondary metabolites

By encoding biosynthetic gene clusters (BGCs), many bacteria produce a range of secondary metabolites, also referred to as specialized metabolites, such as antibiotics, quorum-sensing chemicals, and siderophores (Medema MH *et al.*,

2019). *Streptomyces* species can produce an average of 30 secondary metabolites due to their genetic potential. (Chevrette *et al.*, 2019). Remarkably, over 60% of these compounds are produced by soil microbes. *Bacillus* species are commonly found in soil environments and can make many helpful secondary compounds. Many significant medical conditions have been successfully treated with these medications. The well-known types of *Bacillus* are *B. licheniformis*, *B. subtilis*, *B. circulans*, *B. amyloliquefaciens*, *B. polymyxa*, *B. pumilus*, and *B. cereus*. These species frequently produce a wide range of compounds, including isocoumarins, lipopeptides, polyketides, aminoglycosides, aminopolymers, phospholipids, phosphonoligopeptides, and terpenoids, that have a range of medicinal qualities, including anticancer action. (Saxena AK, *et al.*, 2020). A recent study showed that *Streptomyces* species are no longer seen as an important source of new germ-killing drugs since no compounds from them were tested in clinical trials from 2007 to mid-2013. However, three compounds taken from rare marine Actinomycetes are now being tested in clinical research. Numerous new secondary metabolites were produced by *Streptomyces* species, according to data gathered between 2015 and 2020. From 2015 to 2020, 121 *Streptomyces* species produced 279 different natural compounds with a variety of bioactivities. (Subramani *et al.*, 2019) Microorganisms belonging to the Actinomycetales family, especially those in the *Streptomyces* genus, are well-known for their ability to create compounds with a range of antibacterial, leishmanicidal, antimalarial, and anticancer properties. (Amorim *et al.*, 2020)

Antimicrobial activity of microbial SMs

A comprehensive research has been recently carried out to determine microorganisms in different habitats to find out new and powerful compounds with diverse biological characteristics (Rammali, S. *et al.*, 2022). These works indicate that there are numerous medical applications of Actinobacteria, in particular, members of the

Streptomyces family, such as combating germs, parasites, cell damage, and cancer. These properties have been widely used in treatment therapies and have been proven to be effective in various infections (Naamane *et al.*, 2020). Several secondary metabolites displaying varying antibacterial and antifungal properties have been identified in varied sources of fungi (Masi *et al.*, 2018 and Roscetto *et al.*, 2020). Antimicrobial bacteriocins are bacterial peptides synthesized by the ribosome and are known as secondary metabolites (Darbandi *et al.*, 2022). Bacteriocins have been demonstrated in various species of bacteria associated with the genus *Klebsiella*, *Staphylococcus*, and *Bacillus* (Li and Chen *et al.*, 2023). Bacteriocin is a type of antimicrobial agents that hold potential and could be used as a new form of treatment to deal with a variety of pathogenic microorganisms (Yan *et al.*, 2024).

Chapter 3 MATERIALS AND METHODS

Study Area

This research was done in the Microbiology Lab of Sarhad Institute of Allied Health Sciences at Sarhad University of Science and IT, Peshawar.

Sample Collection

Three soil samples were taken from fields at a depth of 6 cm and kept in clean plastic bags from two areas of Peshawar. The soil heat and pH were also noted.

Bacterial Isolation

To make a mixture, one gram of soil was added to 9 ml of clean water. Then, 1 ml from the first tube was moved to the second tube, and this step was repeated up to the fourth tube. After dilution, 1 ml from each tube was placed on nutrient agar plates. After incubation, bacterial growth on all four plates was observed. To produce pure and isolated bacterial colonies, the colonies were selected and cultured again on nutrient agar plates. (Raag *et al.*, 2022).

Bacterial Identification

The isolated bacteria were then identified on the

basis of their shape, gram staining and various biochemical tests.

Phenotypic Identification

The bacteria used on the nutrient agar plates were examined based on the edges, shapes, and colors.

Gram Staining

Gram staining was used on the morphological features. A thin coating of bacterial cells were placed on a glass slide using clean water. It was dried and heated slowly. One minute later, crystal violet was added and washed. Then gram iodine was applied to a slide and after a minute washed with decolorizer. This was followed by the introduction of safranin, which was washed and dried. The slide having smear was put under a microscope and observed at 100x with oil immersion. The bacteria were categorized in terms of colour, whereby Gram-negative bacteria were considered pink in colour or red in colour, whereas Gram-positive bacteria were considered purple in colour or blue in colour (Chauhan *et al.*, 2018)

Biochemical identification

Catalase, oxidase, urease, sugar, indole, starch, spore and movement were all tests that were conducted to identify the bacteria.

Catalase Test

The catalase test was used to see which bacteria produce the catalase enzyme. This enzyme changes hydrogen peroxide into water and oxygen. A few drops of hydrogen peroxide were put on a glass slide and loopfull bacteria were mixed with it. Bacteria are classified as either catalase-positive or catalase-negative based on the formation of bubbles. (Dimri *et al.*, 2020)

Starch Hydrolysis Test

This test checked if the bacteria could break down starch. The bacteria were spread on Starch Agar plates in a line or zigzag shape and kept at 37°C for 48 hours. The plate was incubated and then 30 seconds of iodine was added, after which the additional iodine was washed off. This was

preceded by the investigation of the clear areas around the line of bacteria growth. Subsequently, the clear areas surrounding the line of bacterial growth were observed (Dimri *et al.*, 2020)

Oxidase Test

The aim of this test was to determine the bacteria that produce the cytochrome oxidase enzyme. One colony was grown on a plate and the bacteria were gently smeared on a filter paper. Once the oxidase reagent was added onto the filter paper, a change of colour was observed after 10 seconds. (Win *et al.*, 2006). The bacteria were also classified based on change in colour as oxidase-negative (pink) and oxidase-positive (purple). (Dimri *et al.*, 2020)

Indole Test

This test helps to find bacteria that make the enzyme tryptophanase, which changes the amino acid tryptophan into indole gas. (MacFaddin *et al.*, 2000). Bacterial colonies were infected, and a pure colony was cultivated on a medium plate. A few drops of Kovac chemical were applied after a 24-hour incubation period. After 24 hours, a few drops of Kovac chemical were added. A dark red ring colour development was seen in a couple of seconds. Bacteria were classified as either indole-positive or indole-negative based on the production of red rings. (Dimri *et al.*, 2020)

Urease test

The urease test is done to see which bacteria make the urease enzyme. It also helps detect urease-positive *Helicobacter pylori*. The bacterial samples were streaked on prepared urease slants and kept for 24 hours at 37°C. The colour changing red indicates whether the test is urease positive or negative. (Dimri *et al.*, 2020)

Triple Sugar Iron Test

This test helps recognize bacteria that belong to the *Enterbacteriaceae* group. Glucose makes up 0.1 percent of the test medium, while lactose and sucrose both make 1%. Phenol red and ferrous sulphate are used as markers. The medium's slant and butt were prepared. Subsequently, a slant was

used to streak the inoculum. After a day of incubation, the colour of the butt and slant changed, and gas production was observed. (Harley, 2005)

Motility Test

It is used to check if the bacteria can move. The bacterial sample was grown in nutrient broth and kept at 37°C for 24 hours. A small amount of it was then put in the middle of a cover slip. The slide was gently pressed with the cover slip and then quickly turned to avoid disturbing the drop. Under a microscope, the bacterial cell's movement on the slide was investigated. (Dimri *et al.*, 2020)

Endospore Test

Also referred to as a spore stain, this method is used to find out whether bacteria have endospores. To kill vegetative cells, the bacterial culture drop was placed on a slide and heated to 80°C for ten to fifteen minutes. Next, either safranin or malachite green was used to dye the culture. The presence of endospores was next examined under a microscope in the stained culture.

Molecular identification based on sequencing

A clean bacterial colony was picked using a sterile toothpick and mixed with 50 µL of distilled water. The mixture was then heated in a water bath at 97°C for 10 minutes. After heating, it was spun at 15,000 RPM for 10 minutes. The top liquid (supernatant) containing bacterial DNA was then collected. The DNA amount was checked by measuring absorbance at 260 nm with a UV spectrophotometer. The extracted DNA was later sent to Macrogen, Korea. Using universal primers, the 16S rRNA fragment was amplified by PCR. The results of the 16s rRNA product sequencing were provided by Macrogen. (Idris *et al.*, 2020; Martinez *et al.*, 2018).

Trimming and submission of the sequences

The purified DNA sequences were purified using FinchTV software in order to have a clear result to construct a family tree of the bacteria. They

were cleaned to find these sequences using 16s rRNA comparing them with other adjacent sequences in the database through the BLAST service of NCBI. (Idris *et al.*, 2020).

Similar sequences downloading

The NCBI blast was followed by the collection and storage of sequences with the highest level of similarity to the existing ones in the database in FASTA format (Martinez *et al.*, 2018)

Multiple sequence alignment

The evolutionary conserved segments were identified using the MEGA-7 program that was used to align some of the sequences of different species to the query sequences, or contigs in the FASTA format (Mahmoodi *et al.*, 2018).

Construction of phylogenetic tree

Molecular genetic analysis was done through MEGA-7 software to construct a phylogenetic tree using nucleotide sequences (Mahmoodi *et al.*, 2018).

Isolation of secondary metabolites

The selected bacteria had been selected using a clean loop and placed in the nutrient broth. The culture was incubated at 37°C for 24 hours after which it was centrifuged at 5000 rpm for 5 minutes. A pallet was disposed of and the supernatant that contained the bacterial secondary metabolite was collected.

Antibacterial Assay

The agar well technique was used to perform the germ-killing test. Overnight grown fresh bacteria were utilized. Muller-Hinton agar plates were made and allowed to solidify. Thereupon 100 ml of bacteria was inoculated on the plates using a clean swab and left to stand after ten minutes. The small holes (6-8 mm) were made, and 10 ml of the sample liquid were placed in each of the holes. The plates were incubated at 37°C and left at 24 hours. Once the incubation was over, the clear zone around each well was measured in millimetres by the ruler or caliper. (Pandey *et al.*, 2019)

Antifungal Assay

Antifungal activity was checked by the agar well diffusion technique. The fresh culture of the Fungus was sub cultured in Potato Dextrose Broth (PDB) and incubated for 24 hours at 27°C. Upon incubation, fungal mixture was evenly dispersed on the agar surface with the help of a clean swab. A sterile cork borer was used to make small wells (6-8 mm wide). Each of the 50-100 uL wells was added with the corresponding amount of the test sample. There were also positive and negative control that would be compared. The plates were incubated at the optimum temperature at 28 to 37d°C for 24 to 48 hours. Clear area surrounding every well was calculated in millimetres with the help of a ruler or a caliper. (Pandey *et al.*, 2019)

Cytotoxic Assay

Cytotoxic activity of secondary metabolites was assessed using the “Brine shrimp lethality assay”.

20gm of shrimp eggs were grown in sea salts at 31°C. After 48 hours, the nauplii were hatched from eggs. The nauplii were then collected in 2 vials containing 30gm nauplii each. Then the supernatant was added over nauplii. After 24 hours of incubation, live and dead nauplii were counted. (Banti *et al.*, 2021)

Chapter 4

RESULTS

. Soil Sampling:

Three Agricultural soils samples were collected from fields located at Ring road, Peshawar. The sample were obtained at a depth of 6-7 cm removing 3.0 cm of soil's upper layer. Approx. 5gm of soil was collected. The soil sample were shifted to microbiology lab at Sarhad University, Peshawar.



Figure. 1: Soil Sample Collected in beaker to make stock solution

Bacterial isolation

6-fold serial dilution was carried out on 1gm of soil sample mixed with 9 microliter (ml) of distilled water. After serial dilution, the diluent was cultured on Nutrient Agar plate and incubated for 24hours. After incubation, two

types of specific isolated colony were streaked on separate nutrient agar plates using streak plate method. After incubation, two pure cultures of bacteria were successfully isolated and named as “Bacterial Strain - 1 and Bacterial strain - 2.”

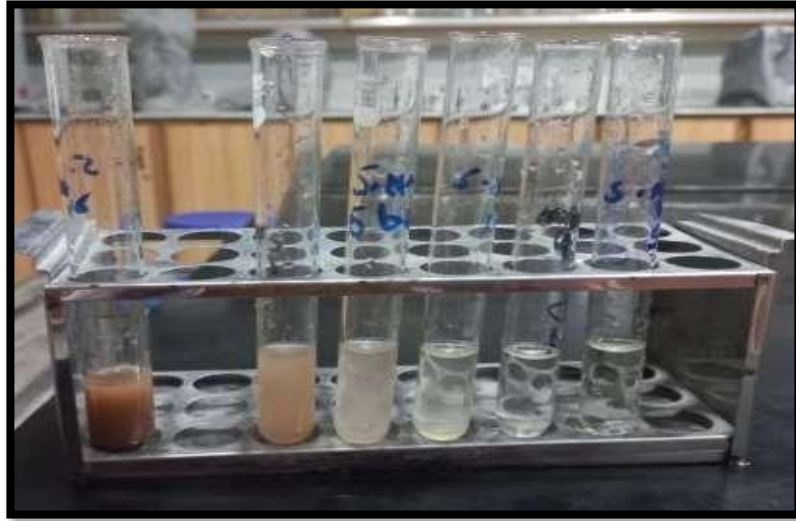


Figure. 2. Serial Dilution of soil sample

Phenotypic identification

Both the bacterial strains on nutrient agar formed purple color, smooth and circular colonies with entire margins.



Figure 3. Bacillus spp. ASF01 and ASF02 colonies

Gram Staining:

Both the bacterial strains formed purple color, rod shaped tiny colonies, which occurs individually when observed at 100x magnification under the microscope.

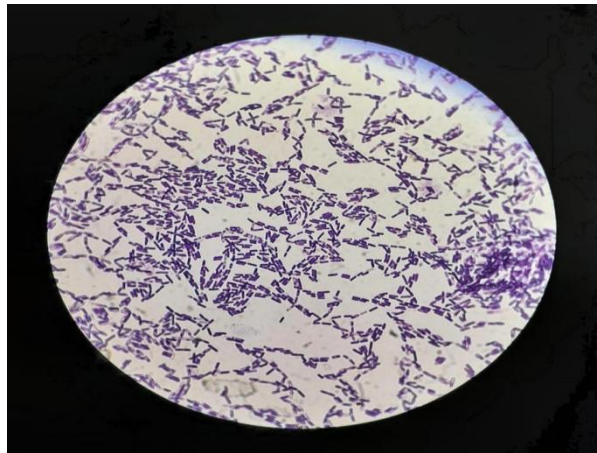


Figure. 4. *Bacillus spp. ASF01* under 100x magnification

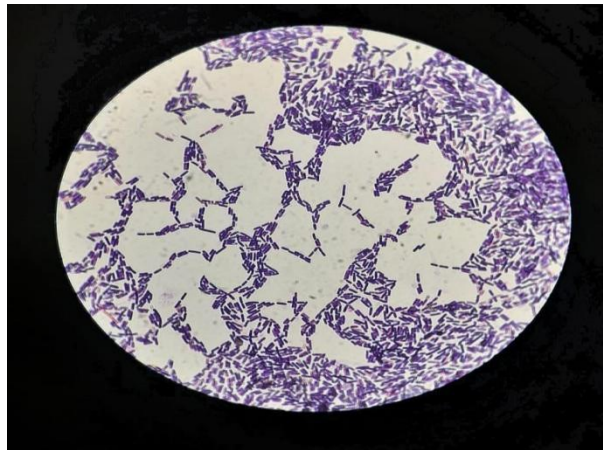


Figure. 5. *Bacillus spp. ASF02* under 100x magnification

Biochemical identification

Both the bacterial strains tested positive for catalase, oxidase, urease, motility and Starch hydrolysis whereas Indole and Endospore tested negative. A Triple sugar iron (TSI) test showed a

yellow color in butt of test tube (indicating acid formation from sugar fermentation in anaerobic environment) and red in slant color of test tube (indicating peptone utilization in aerobic environment).

Table 1. Biochemical identification of Bacterial strain -1 and Bacterial strain-2

S. No.	Biochemical test	Results
1.	Catalase	Positive
2.	Urease	Positive
3.	Oxidase	Positive
4.	Motility	Positive
5.	Starch hydrolysis	Positive
6.	Indole	Negative
7.	Endospore	Negative
8.	Triple Sugar Iron	Red/Yellow (K/A), Glucose fermentation only, peptone utilization in slant
9.	Similarity of bacteria	<i>Bacillus spp.</i>

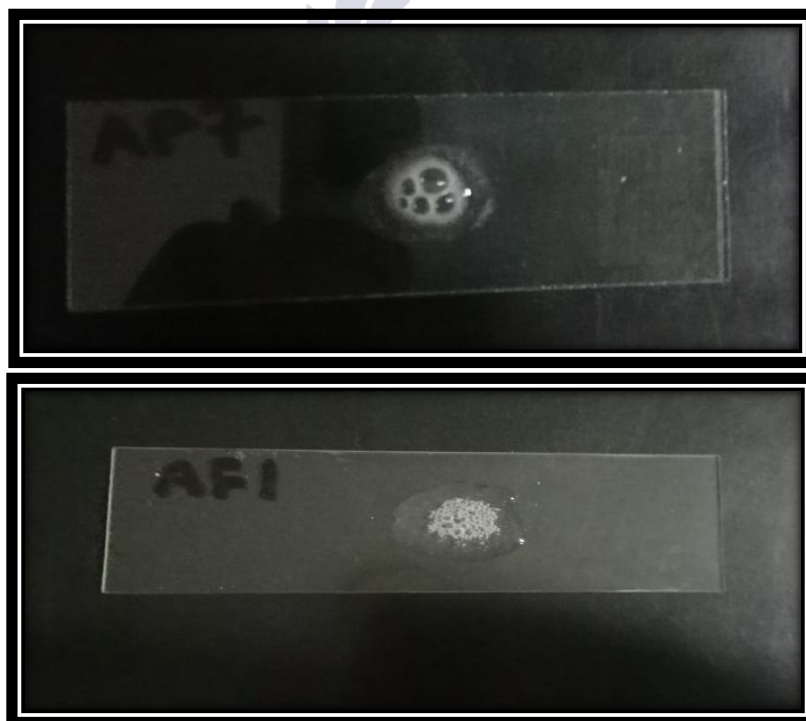


Figure. 6. Catalase Test

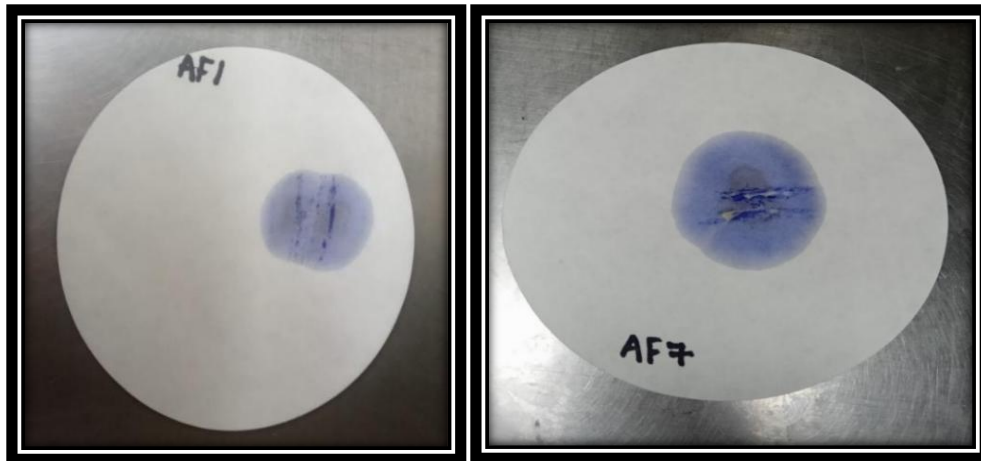


Figure. 7. Oxidase Test

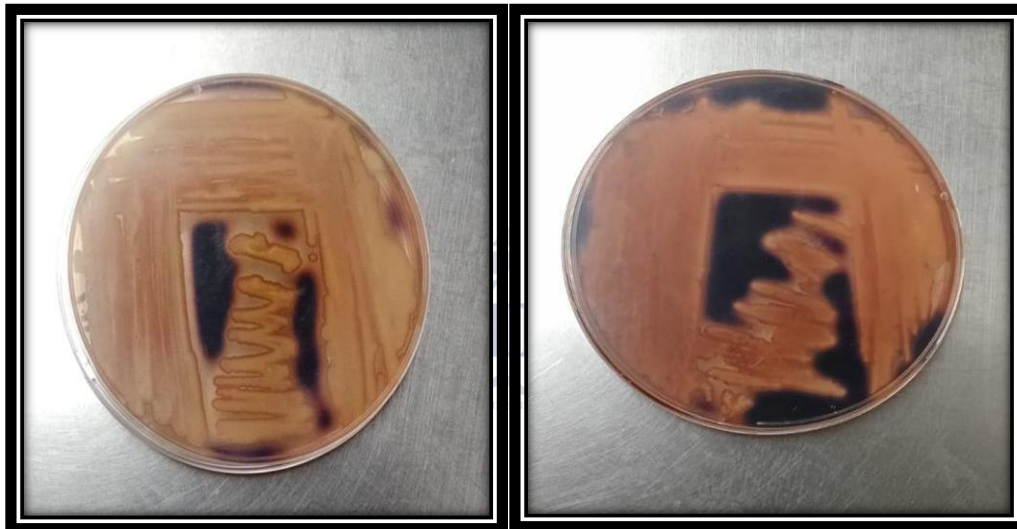


Figure. 8. Starch Hydrolysis Test

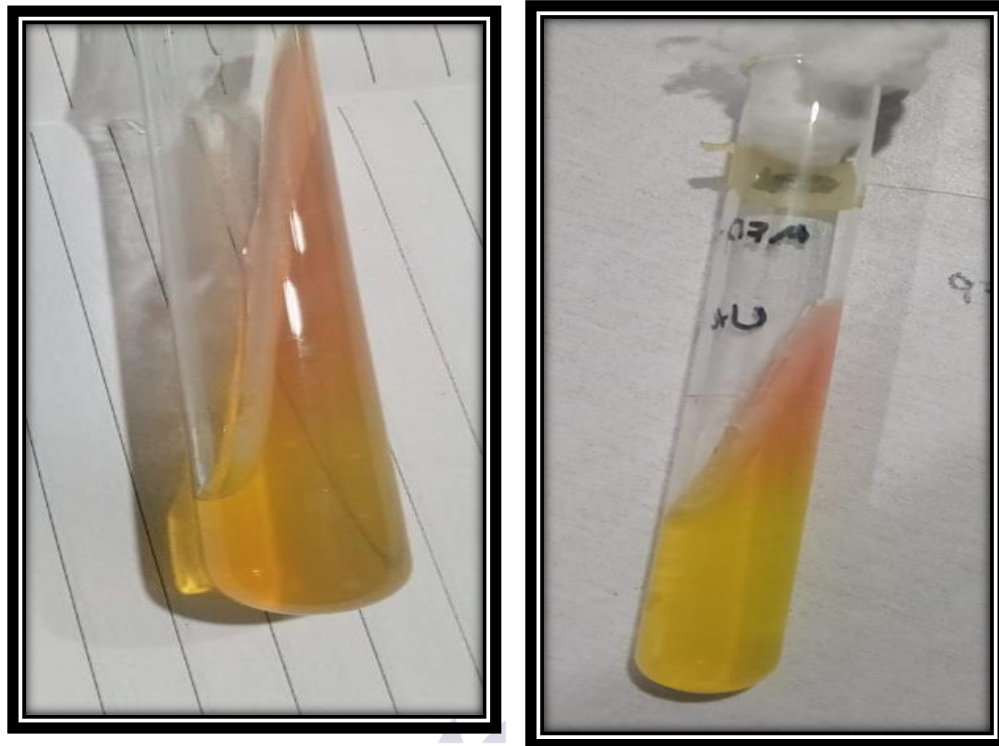


Figure. 9. Urease Test

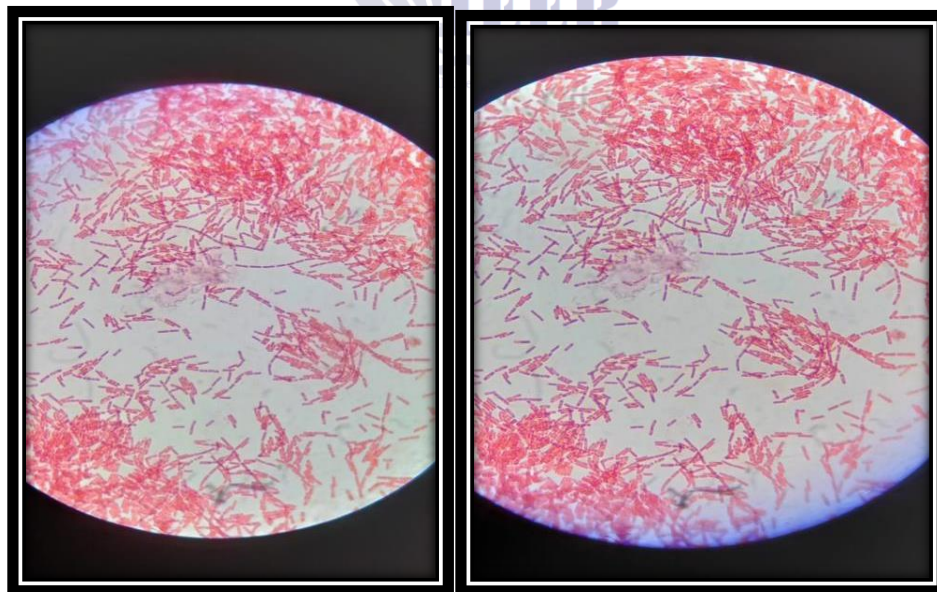


Figure.10. Endospore Test

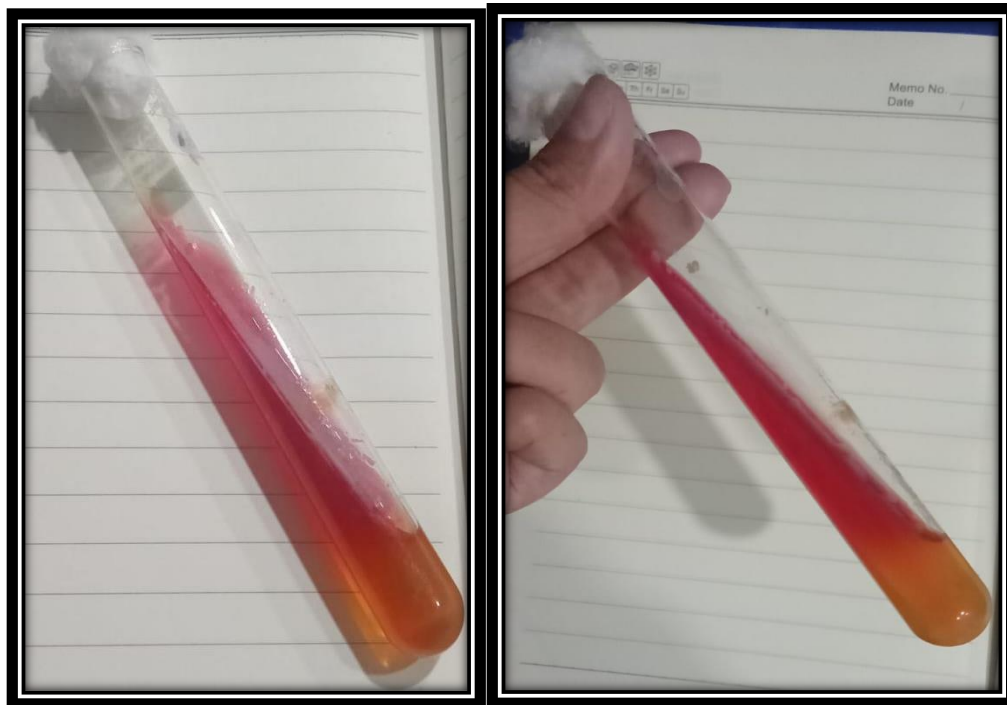


Figure. 11. TSI Test

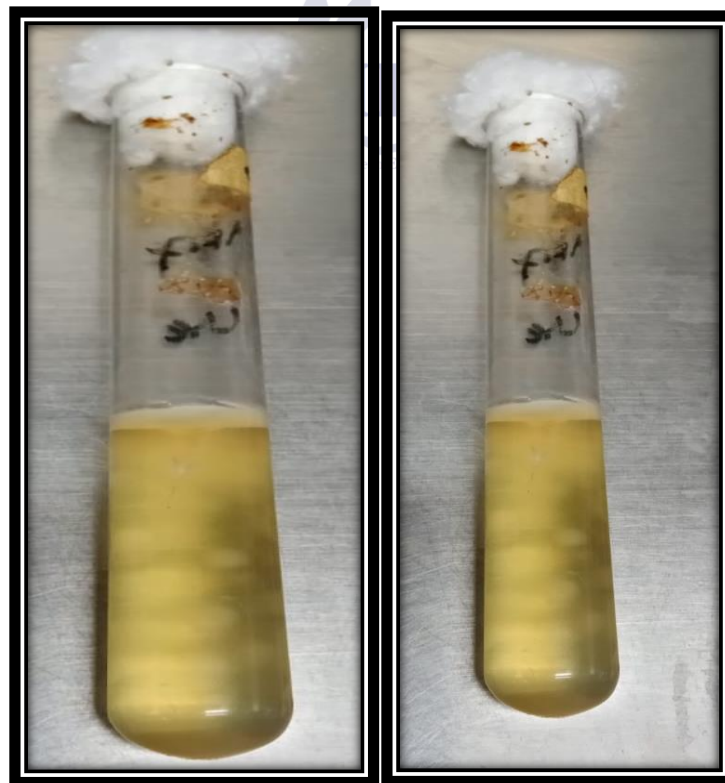


Figure.12. Motility Test

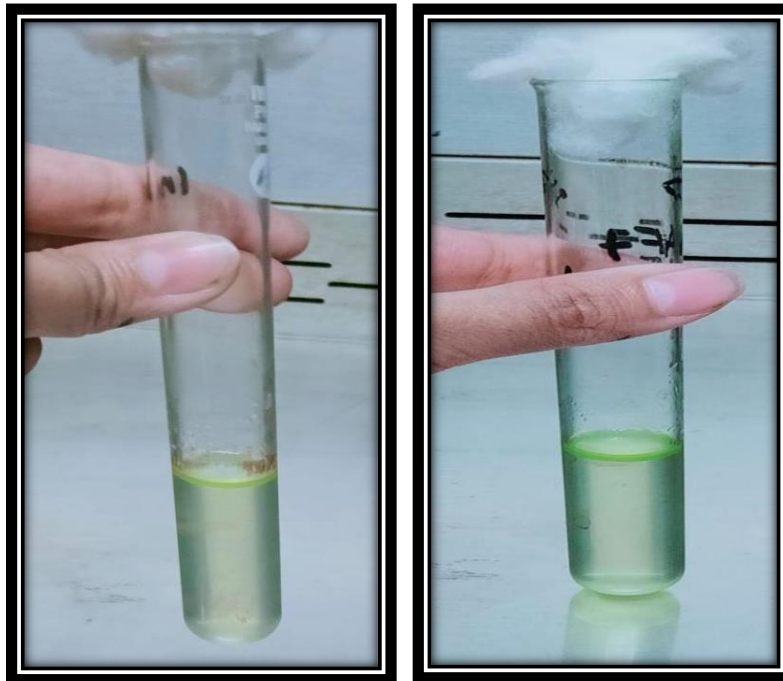
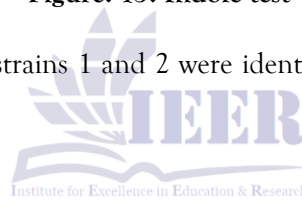


Figure. 13. Indole test

Molecular identification

Based on 16s rRNA analysis, bacterial strains 1 and 2 were identified as *Bacillus* spp. ASF01 and *Bacillus* spp. ASF02 respectively.



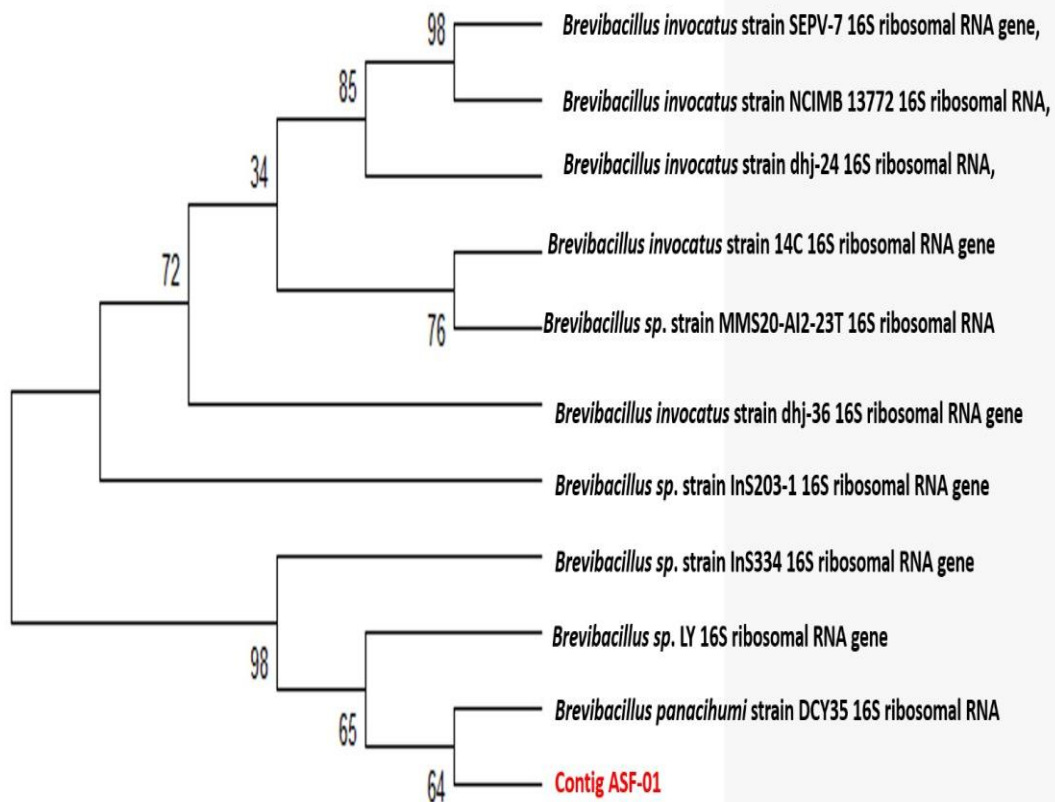


Figure: 14: Phylogenetic tree of Bacterial strain-1 with query sequence Identified as *Bacillus spp. AFS01*

Contig ASF-01

In the phylogenetic tree, Contig ASF-01 clustered within the *Brevibacillus* lineage, forming a close association with *Brevibacillus panacihumi* strain DCY35. This relationship was supported by a bootstrap value of 64%, indicating moderate confidence in this clustering. The broader clade containing *Brevibacillus* species showed high bootstrap support values (up to 98%), confirming the placement of Contig ASF-01 within the

Brevibacillus genus. However, some internal nodes exhibited low bootstrap values (e.g., 34%), suggesting uncertainty in the deeper branching order. Overall, while Contig ASF-01 is reliably assigned to the *Brevibacillus* genus, the moderate bootstrap support at the species-level node indicates that additional genetic markers or longer sequence data may be required for precise species-level identification.

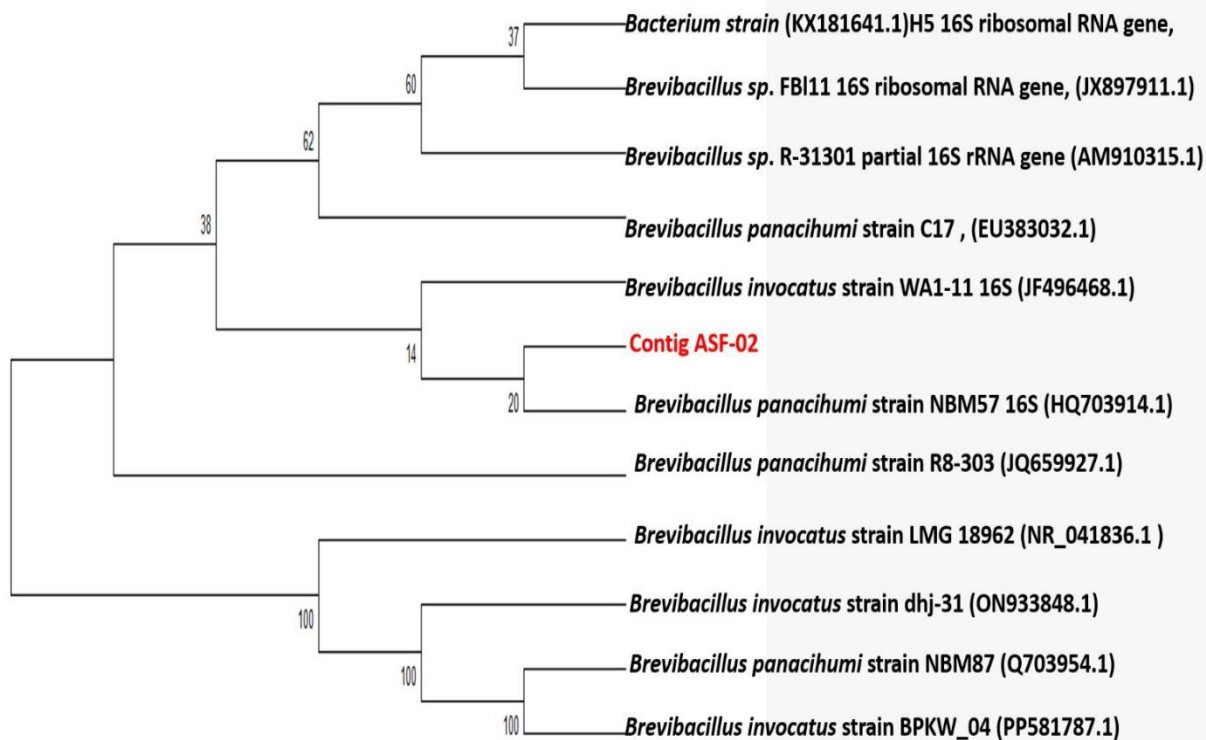


Figure: 15. Phylogenetic tree of Bacterial strain-1 with query sequence Identified as *Bacillus spp. AFS02*

Contig ASF-02

Contig ASF-02 also clustered within the *Brevibacillus* group, showing a close relationship with *Brevibacillus panacihumi* strain NBM57. However, this association was supported by a low bootstrap value of 20%, indicating weak confidence in the exact phylogenetic placement of this isolate. Several nodes within this tree displayed low bootstrap values (ranging from 14% to 38%), reflecting poor resolution among closely related taxa. In contrast, other clades within the tree exhibited very high bootstrap support values (100%), confirming the overall stability of the *Brevibacillus* genus-level clustering. The low bootstrap support associated with Contig ASF-02 suggests that its evolutionary relationship within the *Brevibacillus* lineage is uncertain and may be influenced by limited sequence length or high similarity among reference sequences.

Isolation of secondary metabolites

After incubating bacterial strain in nutrient broth for 24 hours at 37°C, the brownish turbidity was formed which was the indication of successful growth of bacterial strain. After incubation, 1 microliter of bacterial culture was shifted to Eppendorf tube and centrifuged for 5 minutes at 5000rpm. The resulting pellet was visible at the bottom of the tube and the supernatant was clear. The supernatant includes the secondary metabolites.

Antibacterial activity of secondary metabolites

Secondary metabolites synthesized by bacteria were evaluated for antibacterial efficacy against pathogenic drug resistant bacteria i.e. *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. The secondary metabolites revealed antibacterial activity against *S. aureus*, forming clear zone of inhibition. Bacterium was susceptible to secondary metabolite. Whereas the secondary metabolite does not showed any

antibacterial activity against *E.coli* and *P. aeruginosa* and no clear zone of inhibition were formed. Bacteria were highly resistant to

secondary metabolite. Ampicillin was taken as a positive control.

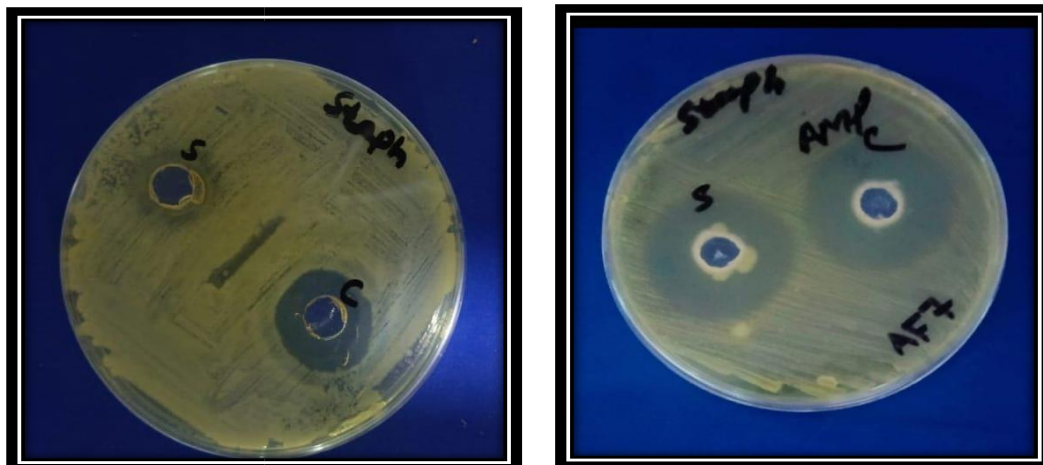


Figure 16: Antibacterial Activity of secondary metabolites against Pathogenic bacteria *Staphylococcus aureus*

Antifungal activity of secondary metabolites
Secondary metabolites synthesized by fungi were evaluated for antifungal efficacy against pathogenic fungi i.e. *Candida albicans*, *Candida auris* and *Cryptococcus neoformans*. The secondary metabolites revealed antifungal activity against *Candida albicans*, forming clear zone of

inhibition. Fungus was susceptible to secondary metabolites. Whereas the secondary metabolites do not showed any antifungal activity against *Candida auris* and *Cryptococcus neoformans* and no clear zone of inhibition were formed. Fungi were highly resistant to secondary metabolites. Clotrim was taken as a positive control.

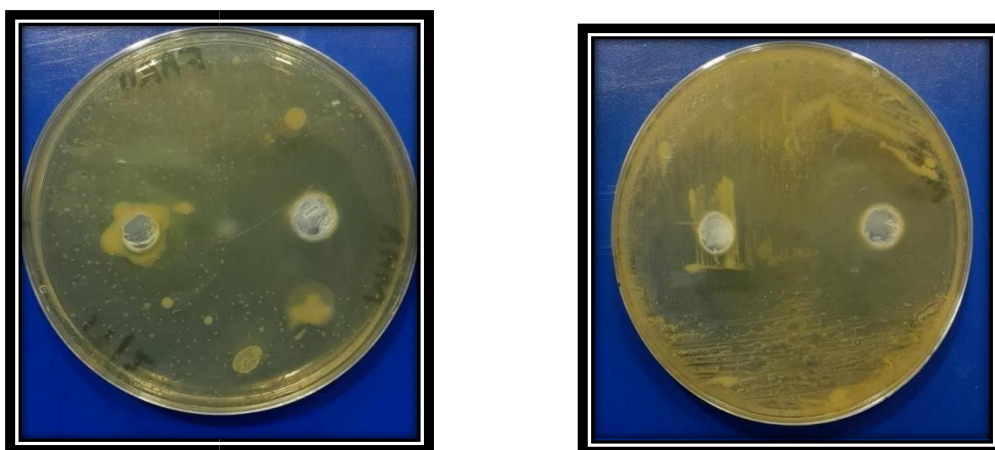


Figure 17: Antifungal Activity of secondary metabolites against Pathogenic fungi *Candida albicans*

Cytotoxic Activity of secondary metabolites

In the brine shrimp lethality assay, the negative control group (sea salt water) showed 0 mortality, confirming that test conditions were not harmful themselves. The positive control, etoposide (7.5 /ml), produced 70 mortality, validating the sensitivity of the assay. At the tested concentrations of 10 and 100g/ml, the secondary metabolites caused 16.7 lethality, while at 1000, the lethality slightly increased to 20. These findings indicate that compound induced only

mild shrimp mortality compared to the strong effect of positive control (Etoposide).

The lack of a clear dose-dependent increase suggests that the compound have relatively low cytotoxic activity in this model. Even at the highest tested concentration (1000, mortality remained far below 50%, implying that the LC50 value lies well above this range. Overall, the results demonstrate that while the test compound exerts some level of toxicity, it is weak and not comparable to the standard cytotoxic agent used as a positive control (Etoposide).

Table.2. Brine Shrimp lethality assay of secondary metabolites

Dosage (ug/ml)	No. of Shrimps	No. of survivors	Percent Lethality	Negative control (Sea salt water)	Percent Lethality of control	Positive Control (7.5 ug/ml)	Percent Lethality of positive control
10	30	25	16.67%	30	00%	Etoposide	70%
100	30	25	16.67%	30	00%	Etoposide	70%
1000	30	24	20.0%	30	00%	Etoposide	70%

**Chapter 5
DISCUSSION**

Making microbial secondary compounds through natural methods is safe, cheap, harmless, and good for the environment. These secondary compounds are chemical substances that have many different types of structures. There are known to be over 2,140,000 secondary metabolites. (Thirumurugan *et al.*, 2018). *Streptomyces* species are genetically capable of producing 30 secondary metabolites on average. (Chevrette *et al.*, 2019). Amazingly, soil bacteria are the source of around 60% of these compounds. (Saxena AK *et al.*, 2020) SMs are not involved in the growth and reproduction of the microorganisms, although being essential for certain secondary demands. (Kumar *et al.*, 2021). There are more than 500 bioactive chemicals discovered each year. (Farang MM, *et al.*, 2020)

Compounds originating from microorganisms are essential for the treatment of cancer and infectious diseases. (Osama N, *et al.*, 2022). Actinomycetes are an excellent source of SMs that have been widely utilized in the fields of medicine, agriculture, and animal husbandry due to their diverse spectrum of bioactivities, which include anti-infection, anti-cancer, anti-malarial, and anti-parasitic compounds. (Li *et al.*, 2021) Numerous secondary metabolites with different antibacterial and antifungal characteristics have been found from a variety of fungal sources. (Masi *et al.*, 2018).

In this study, bacteria taken from soil samples were used to make secondary compounds. In the same way, Amit Pandey *et al.*, (2019) aimed to find soil bacteria that make antibiotic compounds from different areas of Uttar Pradesh, India, including Lucknow. In another

study (Alqahtani *et al.*, 2022), the goal was to discover new antibiotic compounds and secondary metabolites from actinomycetes isolated from the soil of Rijal Almaa, Saudi Arabia, as we decided to use these more recent biochemical entities to eliminate the threat of the disease. (Dhaini *et al.*, 2025) Three bacterial types – *Streptomyces longisporoflavus*, *Micrococcus luteus*, and *Kocuria rosea* – were tested to find the best conditions for making antibiotic compounds that can stop *Bacillus cereus*. The findings show that actinomycetes taken from Tyre City Beach in Lebanon could be a good natural source of useful antibacterial substances. In this study, the bacteria found belonged to the *Bacillus* group. They made purple, smooth, and small rod-shaped colonies with clear edges. Tests on shape, chemical reactions, and growth features confirmed them as *Bacillus sp.* ASF01 and *Bacillus sp.* ASF02. The genetic study also proved that the isolated bacteria were *Bacillus sp.* ASF01 and *Bacillus sp.* ASF02.

The *Bacillus sp.* ASF01 and *Bacillus sp.* ASF02, in our study successfully synthesized secondary metabolites. Our results were further in consistence with the findings of Amit Pandey *et al.*, (2019), who reported the synthesis of secondary metabolites from *bacillus* specie, *Actinomycetes*.

In our investigation, a clear supernatant formed after the bacterial species had fully precipitated, indicating that bioactive secondary metabolites had been created utilizing optimized production conditions. These compounds were then further isolated using the solvent extraction method. For intracellular metabolites, methanol was utilized, while for external metabolites, chloroform. (Amit Pandey *et al.*, 2019) The antibiotic properties of bacterial isolates arise from the presence of bioactive secondary metabolites. (Singh V *et al.*, 2016)

In this research, the secondary compounds showed killing power against *S. aureus*. The bacteria *S. aureus* was sensitive to these secondary compounds. Similarly, (Sathiyaseelan *et al.*, 2011) conducted an isolation procedure on five marine actinomycetes, and one isolate demonstrated

activity against the human diseases *Vibrio cholera*, *Salmonella typhi*, *Klebsiella pneumonia* and *Escherichia coli*. In another work, Rahman *et al.* (2011) found that Actinomycetes had germ-killing effects on the tested microbes.

In this study, the secondary compounds were tested for their fungus-killing power against harmful fungi *Candida albicans*, *Candida auris*, and *Cryptococcus neoformans*. The results showed that these compounds stopped the growth of *Candida albicans*. The fungi was susceptible to secondary metabolites. In the same way, Elbendary *et al.*, (2018) found that two samples acted against *A. niger*, two against *A. flavus*, and four showed effects on *Candida albicans*. Also, Ouhdouch *et al.* (2001) found that out of 320 actinomycete types taken from different places in Morocco, 32 showed strong effects against bacteria, molds, and yeast.

Chapter 6

CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

In the recent study, *Bacillus sp.* ASF01 and *Bacillus sp.* ASF02 successfully synthesized secondary metabolites. The production of secondary metabolites by bacteria is a simple, economical, and ecologically friendly process. The secondary metabolites demonstrated antibacterial properties against pathogenic bacteria, *staphylococcus aureus*. Conversely, secondary metabolites showed antifungal action against *Candida albicans*, a pathogenic fungus, establishing a distinct zone of inhibition. Strong cytotoxicity was also demonstrated by the secondary metabolites, which may be helpful in killing harmful cells. (For example cancer cells)

6.2. Recommendations

- The chemical characterization of secondary metabolites should be explored to identify active compounds responsible for antibacterial, antifungal and cytotoxic effects.
- In vivo study should be conducted to validate the therapeutic potential and safety of these metabolites before clinical applications.

- Bacterial cultures should be optimized to enhance metabolite yield and stability for large-scale production.
- The potential of these metabolites should be investigated as lead molecules for developing novel antimicrobial and anticancer drugs

Chapter 7

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