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Molecular and Biochemical Analysis of Bacillus altitudinis from Freshwater Sources Implications for Water Quality and Ecosystem Health

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ABSTRACT

There are major concerns about water quality and ecosystem health as a result of the growing microbial pollution of freshwater environments. Freshwater sources are among the several environmental niches where Bacillus altitudinis, a hardy and versatile bacterium, has been found. Its metabolic properties, ecological ramifications, and prevalence in aquatic systems are still little understood, though. The purpose of this study is to examine the molecular and biochemical characteristics of B. altitudinis that has been isolated from freshwater habitats in order to evaluate its possible significance for ecosystem dynamics and water quality. Following the collection of freshwater samples from various sources, such as lakes, reservoirs, and rivers, B. altitudinis was isolated and identified using 16S rRNA gene sequencing and culture-dependent methods. The isolates' functional characteristics were assessed using biochemical profiling, which included metabolic fingerprinting, antibiotic resistance tests, and enzyme activity assays. In order to determine virulence factors, stress response genes, and biodegradation capacity, molecular characterization required genomic study. According to preliminary research, B. altitudinis produces a lot of lipase, amylase, and protease, indicating that it is involved in the breakdown of organic materials and the cycling of nutrients. Different patterns of susceptibility were found by antibiotic resistance profiling, indicating possible threats to public health should these bacteria infiltrate drinking water systems. The bacterium's capacity to adapt to polluted settings was highlighted by the discovery of genes associated with biofilm formation, heavy metal resistance, and pollutant degradation by genomic studies. Since B. altitudinis is sensitive to environmental stresses, its presence in freshwater sources may act as a bio indicator for water quality. However, further research is required to evaluate the dangers to human and environmental health due to its possible pathogenicity and resistance features. In order to enhance microbiological monitoring techniques and water management procedures, this work offers vital insights into the ecological role of B. altitudinis in freshwater systems. Developing mitigation strategies to maintain water quality and guarantee sustainable ecosystem functioning will be made easier with an understanding of the interactions between this bacterium and its surroundings.

INTRODUCTION

Freshwater ecosystems are critical for biodiversity, life support, and the provision of resources for industry, agriculture, and human consumption. But as anthropogenic activities like urban garbage, agricultural runoff, and industrial discharge have increased, these water bodies have become contaminated, endangering both the natural balance and public health (Boehm *et al.*, 2018). In freshwater environments, microbial populations are essential for contaminant breakdown, nutrient cycling, and water quality maintenance. Because Bacillus species can adapt to a broad range of environmental circumstances, such as severe temperatures, pH fluctuations, and nutritional constraints, they are extensively spread among these microbes. Although the spore-forming, Gram-positive bacteria *Bacillus altitudinis* has been isolated from a variety of environments, such as soil, air, and aquatic systems, little is known about its ecological function in freshwater ecosystems (Bae *et al.*, 2017).

Concerns regarding *B. altitudinis* possible effects on ecosystem health and water quality are raised by the growing number of cases found in water sources. While certain Bacillus species are well-known for their advantageous qualities, such their capacity for bioremediation and their ability to stimulate plant growth, others may have genes that make them resistant to antibiotics or display harmful characteristics (Ongena and Jacques, 2008). Determining whether *B. altitudinis* presence in freshwater systems presents dangers or advantages requires an understanding of its molecular and biochemical properties. Prior research has mostly examined *B. altitudinis* in soil and atmospheric settings, with little attention paid to its frequency and functional importance in aquatic habitats (Rothballer *et al.*, 2006). Examining the bacterium's activity in freshwater is essential for microbial ecology and water safety evaluations due to its adaptability and metabolic diversity.

Water quality monitoring traditionally relies on physicochemical parameters and indicator bacteria such as *Escherichia coli* and *Enterococcus* spp. Other microbial species, such as strains of Bacillus, may, nonetheless, also function as bio indicators of pollution or ecological stress, according to new research (Koler *et al.*, 2016). It has been asserted that *B. altitudinis* strong stress response mechanisms allow it to thrive in contaminated settings, such as waterways tainted by hydrocarbons and heavy metals (Bhatt *et al.*, 2019). Its capacity to generate extracellular enzymes including lipases, amylases, and proteases further suggests its function in the breakdown of organic materials and possible application in wastewater

treatment. Nevertheless, biofilm growth in water distribution systems may also be facilitated by the same enzymatic activity, posing infrastructural problems (Koler *et al.*, 2016).

The possibility of antibiotic resistance genes (ARGs) being carried by *B. altitudinis* in freshwater is one of the main concerns. There is evidence linking horizontal gene transfer between pathogenic and non-pathogenic species to the increase of antimicrobial resistance (AMR) in environmental bacteria (Bengtsson-Palme *et al.*, 2018). The presence of *B. altitudinis* in drinking water sources may be dangerous to the public's health if it carries resistance determinants. Research has revealed that certain strains of Bacillus are resistant to conventional antibiotics, such as tetracyclines and beta-lactams, which may be obtained via polluted settings. Therefore, in order to assess the potential health consequences of freshwater *B. altitudinis* isolates, it is required to characterize their antibiotic resistance profile (D'Costa *et al.*, 2006).

In aquatic environments, *B. altitudinis* interactions with other microorganisms are a crucial component of its ecology. The biogeochemical cycles in freshwater environments are influenced by microbial consortia, and the dynamics of communities may change if hardy species like *B. altitudinis* are introduced (Fuhrman, 2009). For example, its advantage over native microbial communities in nutrient-poor environments may reduce them, thereby upsetting the equilibrium of the ecosystem. The total microbial diversity in water bodies may also be impacted by the antimicrobial chemicals produced by some Bacillus species, which hinder competing bacteria (Nagakubo *et al.*, 2020). To accurately predict the effects of *B. altitudinis* colonization on freshwater microbiomes, it is crucial to comprehend these interactions. Comprehensive research on *B. altitudinis* in freshwater settings is limited, despite the species' ecological and perhaps health relevance. Rather than its functional importance in aquatic ecosystems, the majority of current study is on its taxonomy and genetic characteristics (Roman-Ponce *et al.*, 2016). Additionally, little is known about the effects of environmental variables on *B. altitudinis* activity and survival in water, including temperature, pH, and nutrient availability (Lund, 2009). Filling up these information gaps is essential to creating efficient water management plans and determining if this bacterium belongs in regular water quality monitoring programs.

This work aims to fill these gaps by performing a thorough molecular and biochemical examination of freshwater isolates of *B. altitudinis*. Specific objectives include: (1) using culture-

dependent and molecular methods to isolate and identify *B. altitudinis* from various freshwater habitats; (2) assessing its ecological role by characterizing its enzymatic and metabolic profiles; (3) assessing its antibiotic resistance patterns to identify potential health risks; and (4) examining its genomic features related to stress response and pollutant degradation. By clarifying *B. altitudinis* behavior in freshwater systems, this study will advance knowledge of microbial water quality markers and guide the development of regulations that protect aquatic ecosystems and public health.

MATERIALS AND METHOD

STUDY AREA AND ISOLATION OF SAMPLES

Three water samples were taken from different freshwater locations in the Abbottabad district, such as rivers and reservoirs. Sterile 500 mL glass bottles were used to collect water samples aseptically from several sampling locations by submerging the bottles straight into the water's surface. After being appropriately labeled, the samples were brought to the lab for examination. To isolate the bacterial strain *B. altitudinis*, aliquots of the samples were used.

ISOLATION BY MEMBRANE FILTRATION

Three 100 mL volumes of each sample were filtered using a water pump through a 0.45 µm pore-sized filter (cellulose nitrate membranes, Whatman Laboratory Division, Maidstone, England). To ensure that no air bubbles were caught, these membranes were aseptically positioned on plates with the proper selective medium. One milliliter of the treated water samples was spread out onto the nutrient agar plates in order to isolate the bacteria (Lund, 2009).

ISOLATION AND GROWTH OF BACTERIA

Following streaking onto selective medium, the samples were cultured for 24 to 48 hours at 37°C. Examine the bacterial colonies' growth characteristics and appearance during a 24-hour incubation period at 37°C. The presence of the necessary microorganisms in the growth was assessed using a number of techniques, including biochemical characterization and Gram staining (Fernandes Queiroga Moraes *et al.*, 2021).

GROWTH MEDIA SELECTIVELY USED FOR *B. ALTITUDINIS*

TABLE 3.1 TYPES OF MEDIA USED IN DIFFERENT QUANTITY/L

S. No	Media	Quantity/L
1.	Mannitol salt agar	111/ g
2.	Triptycase soy agar	tsa/l
3.	Blood agar	40g/l
4.	Baired prker agar	63g/l

MORPHOLOGY BASED CHARACTERIZATION OF ISOLATION OF PATHOGENIC BACTERIA

GRAM STAINING

A small amount of distilled water was put to a clear slide in order to carry out the Gram staining. A small quantity of pure culture was applied to the slide using a sterile needle. The needle was used to distribute the culture uniformly on the slide. A drop of crystal violet was applied to the slide smear using sterile water. After achieving a consistent dispersion, the crystal violet was swirled in and allowed to dry for around 30 seconds. After applying crystal violet stain, the slide was gently washed with distilled water that had been sterilized. To get rid of any remaining crystal violet color, a droplet of Lugol's iodine was added to the smear after the slide had been thoroughly cleaned with pure distilled water. Crystal violet and Lugol's iodine work together to hold the stain in place. Acetone was used to clean the slide following the application of Lugol's iodine. Acetone is a decolorizer that helps get rid of extra stains on the slide. A drop of safranin was applied to the slide to hide the smudge. Gram-negative bacteria acquire their characteristic hue from the counterstained safranin stain. The slide was eroded clean after being properly cleaned with water to remove any remaining safranin. The extra liquid was carefully removed from the slide using blotting paper. To preserve the discolored smear, a drop of mounting agent Canada balsam was applied to the slide. A 100X magnification microscope was used to view the slide with the plated smear (Greenwood *et al.*, 2012).

BIOCHEMICAL CHARACTERIZATION

Biochemical assays, including catalase, oxidase test, indole test, urease test, carbohydrate fermentation test and motility test were carried out. Briefly described as follow:

CATALASE TEST

The catalase enzyme, which causes hydrogen peroxide (H_2O_2) to release more oxygen, is detected by the test. It is used to differentiate between various bacteria that generate the catalase enzyme. On a sterile surface, one colony was gently mixed with hydrogen peroxide to perform the *B. altitudinis* strain catalase test. The test was successful, as evidenced by the formation of gas bubbles on the culture material's surface (Reiner, 2010).

OXIDASE TEST

The oxidase test is a method for determining if cytochrome C oxidase, also known as cytochrome a3, an enzyme involved in aerobic respiration, is present. A little piece of filter paper was treated with 1% Kovac's oxidase reagent and left to air dry. A well-isolated colony of *B. altitudinis* strains was moved from a freshly cultivated (18–24 hours) bacterial plate onto filter paper using a sterile loop. Color differences were analyzed for each test colony. After an oxidase-positive test, the color turns dark purple in ten to fifteen seconds. The color either remains the same or reacts more slowly than two minutes when oxidase-negative organisms are present (Shields and Cathcart, 2010).

INDOLE TEST

Finding out if an organism can convert tryptophan into indole is the purpose of the indole test. The Kovac's reagent, which combines concentrated hydrochloric acid, isoamyl alcohol, and paradimethylaminobenzaldehyde in an acidic environment, was used to identify indole. To perform the Indole test, a culture of *B. altitudinis* was injected into a tube containing tryptophan broth and kept at 37 °C for 24 to 48 hours. Gently stir in 0.5 ml (5 drops) of Kovac's reagent. Examine the liquid's uppermost layer; if purple or red rings show up there, a positive outcome is displayed; if yellow rings show up, a negative consequence is displayed (KOMAL, 2019).

UREASE TEST

When amino acids were decarboxylated, urea was produced. Ammonia and CO_2 were created as urea broke down. Phenol red shifted from a pale orange color at a pH of 6.8 to a magenta (pink) tint at a pH of 8.1, indicating the presence of ammonia, which made the solution more alkaline. In order to identify pH changes in solutions, phenol red was used. After a day, urease positive bacteria turned the medium pink. Negative organisms either created a yellow color change or no color change at all as a result of the acid that was generated. One or two drops of an overnight brain-heart infusion broth culture can be added to urea agar media, or a piece of a

well-isolated colony can be placed on the surface. Furthermore, incubate the tube for 48 hours to 7 days at 35 to 37 degrees. Following that, observe for at least seven days for the appearance of a pink color (Brink, 2010).

CARBOHYDRATE FERMENTATION TEST

Tests of carbohydrate utilization reveal if bacteria can ferment or oxidize a specific carbohydrate to produce acid. The way an organism consumes carbohydrates varies depending on its complement of enzymes. The fermentation pattern of a particular species, genus, or group of organisms can be used to identify it. Different carbon sources were used in this experiment. The first step was making a litter of nutrient broth and adding a little sprinkle of phenol red indicator, which changes the broth color as bacteria grow in it. Each test tube was filled with nutrient broth, and the tubes were autoclaved. Following autoclaving, a variety of carbon sources were added to the test tubes, including sucrose, arabinose, sorbitol, mannitol, galactose, fructose, maltose, and mannose. Test tubes were filled with 48-hour-old cultures of *B. altitudinis* strains, and the test tubes were monitored for any changes in the culture (Reddy *et al.*, 2022).

MOTILITY TEST

To determine if an organism can move by utilizing its flagella, this test is conducted. The kind of bacteria determines where the flagella are located. After preparing the semisolid agar, put it into test tubes to conduct the motility test for *B. altitudinis* strains. After a culture has developed on nutrient agar medium for 18 to 24 hours, affix a straight needle to the colony. Only puncture 1/3 to ½ inch deep once you are at the center of the tube. As the needle enters the medium, make sure it does so in the same direction. To determine whether a diffuse growth zone has emerged from the inoculation line, incubate for up to seven days at 35° to 37°C (Shields and Cathcart, 2011).

DISK DIFFUSION SUSCEPTIBILITY TESTING

Mueller-Hinton agar coated with different antibacterial filter paper disks is used to cultivate facultative anaerobic and pathogenic aerobic bacteria. By figuring out how sensitive or resistant these bacteria are to various antibiotic drugs, the disk diffusion susceptibility test assists clinicians in selecting alternatives to therapy for their patients. The ability of that drug to inhibit that organism can be inferred indirectly from the proliferation surrounding the disks (Hudzicki, 2009). To generate bacterial suspensions, the 0.5 McFarland standard was used.

Following the application of the suspension, Mueller-Hinton agar plates were covered with antibiotic disks. To ascertain the plates' susceptibility to antibiotics, they were incubated for 16–18 hours at 37°C. The inhibitory zones were then measured in millimeters (Hudzicki, 2009).

DNA EXTRACTION

The Qiagen RTU kit was used to extract the whole genomic DNA of the tested bacterial culture. To determine the spore concentration needed for extraction, 1 mL of the culture containing 10^8 cfu/mL was centrifuged. The extraction tube (2.5 mL) was filled with 250 µL of proteinase K to remove any potential proteins and lysis buffer AL. After centrifuging the suspension, the supernatant was disposed of. To get rid of the particles, 95% ethanol was added to the lysate. After passing the cleaned lysate through a purification micro spin column, AE buffer was used to elute the column. AW1 and AW2 were the washing buffers that were utilized. Quantification of the isolated DNA was done with the Nanodrop spectrophotometer NS1020. For subsequent downstream analysis, the isolated DNA was kept at -20 °C. 1% agarose gel electrophoresis was used to evaluate the isolated DNA's purity (Wang *et al.*, 2011).

PCR AMPLIFICATION AND SANGER SEQUENCING

The extracted DNA was amplified using 16S specific F/R primers. The sequence of 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'. The estimated PCR product was 1.4 kb to 1.6 kb. For sequencing of the PCR product, enzymatic digestion was carried out using exonuclease I and SAP enzymes and, the PCR product from agarose gel electrophoresis was passed through purification column followed by elution buffer. The cleaned PCR product was sent for Sanger sequencing using 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' primers (Wang *et al.*, 2011).

Stage	PCR Protocol	Temperature (°C)	Time (min.)
1st	Initial Denaturation	94	5.0
2nd (35 Cycles)	Denaturing	94	0.5
	Annealing	52.7	0.5
	Extension	72	2.0
3rd	Final Extension	72	5.0
4th	Hold	4	∞

BIOINFORMATICS ANALYSIS

Chromas and BioEdit tools were used to evaluate the sequence in order to determine the bacterial strain's evolutionary connection. The sequence was edited for low-quality and superfluous amplifications, and the peaks were adjusted. The highly matched sequences from the databank were obtained using the NCBI's basic local alignment search tool (BLASTn). The Clustal Omega bioinformatics program was used to perform multiple sequence alignment of the chosen BLASTn resulting sequences prior to the creation of the phylogenetic tree. The evolutionary link between *Bacillus altitudinis* and other bacterial species was determined by building and analyzing the tree after the MSA. Using the sequenced bacterial strain and MEGAX software, the evolutionary connection with other species was analyzed to create a phylogenetic tree. For the creation of phylogenetic trees, the Fast Minimum Evolution Method and Max Sequence Difference 0.75 were employed.

RESULTS

WATER SAMPLE COLLECTION

The water samples were aseptically collected and prior to microbiological examination. Water samples were subsequently moved to Laminar airflow was used throughout the process to preserve sterility and avoid cross-contamination.

FIGURE 1: WATER SAMPLE COLLECTION

WATER SAMPLE PROCESSING

A 0.45 µm pore-sized filter (cellulose nitrate membranes, Whatman Laboratory Division, Maidstone, England) was used to filter the water samples. One milliliter of the filtered water sample was then spread out onto selective medium.

FIGURE 2: WATER SAMPLE PROCESSING

MORPHOLOGICAL CHARACTERIZATION

Bacterial isolates were then characterized by morphology, by using Mannitol salt agar, blood agar. On Mannitol salt agar all *Bacillus altitudinis*, strains produce white to pale color colonies.

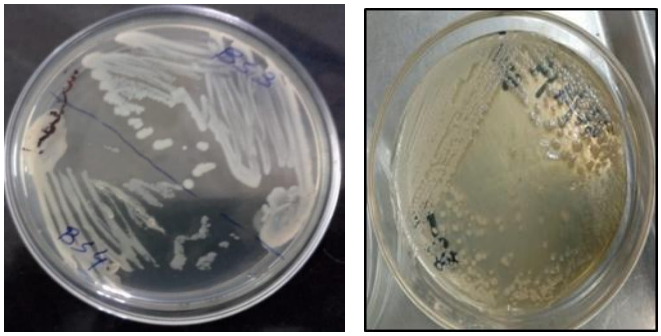

FIGURE 3: MORPHOLOGICAL CHARACTERIZATION OF BACTERIAL ISOLATES

GRAM STAINING RESULTS

Gram staining revealed that an isolated strain of *Bacillus altitudinis*, which had been grown for the overnight, was a rod-shaped, Gram-positive bacterium. The Gram response and cellular arrangement offered the initial evidence that these isolates were *Bacillus altitudinis* before doing confirmatory biochemical tests.

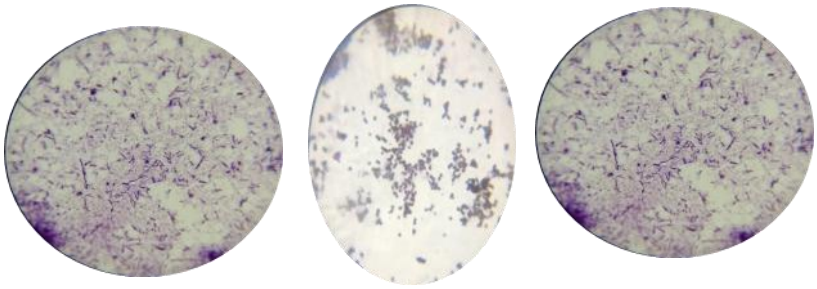

FIGURE 4: GRAM STAINING RESULTS OF BACTERIAL ISOLATES

CATALASE TEST RESULTS

The isolated bacteria generated gas bubbles on a glass slide after being treated with a few drops of 3% H2O2, indicating that the catalase test was positive. The catalase test result showed that all the *Bacillus altitudinis* bacterial strain were positive.

FIGURE 5: CATALASE TEST RESULTS OF BACTERIAL ISOLATE

OXIDASE TEST

For this, Kovac's oxidase reagent was employed. The oxidase test was used to evaluate the organism's capacity to produce cytochrome c oxidase. If the purple color developed within 30 to 60 seconds, the bacteria tested positive. All of the *Bacillus altitudinis* isolates from our investigation were oxidase positive.

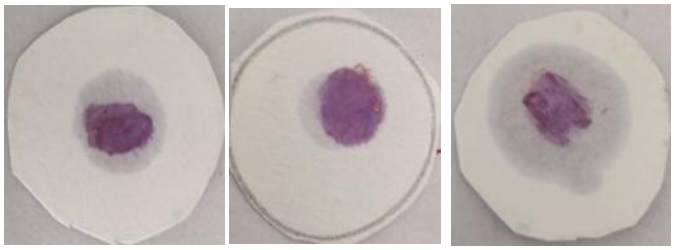

FIGURE 6: OXIDASE TEST RESULTS OF BACTERIAL ISOLATES

INDOLE TEST

A reddish-colored ring appeared on the glass tube surface as soon as the kovac's reaction was injected, indicating a successful indole test. When indole negativity is present, it is yellow or absent. Upon adding five to six drops of Kovac's reagent, every bacterial isolate had a negative indole test.

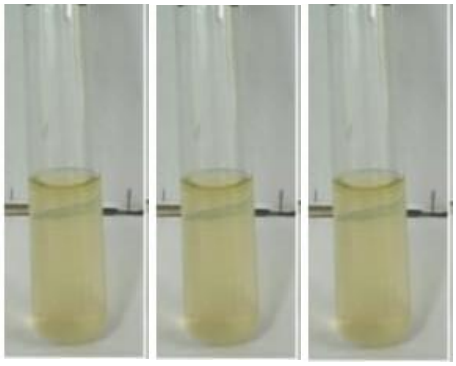

FIGURE 7: INDOLE TEST RESULTS FOR BACTERIAL ISOLATES

UREASE TEST

All the bacterial isolates are urease negative, which mean that does not break down urea into ammonia and carbon dioxide.

FIGURE 8: UREASE TEST RESULTS FOR BACTERIAL ISOLATES

CARBOHYDRATE FERMENTATION TEST

The ability of bacteria to ferment or oxidize a particular carbohydrate in order to create acid is determined by carbohydrate fermentation tests. All strains of *Bacillus altitudinis* gave a positive result to carbohydrate utilization test by utilizing mannose as a carbon source results in change in color of medium from red to yellow.

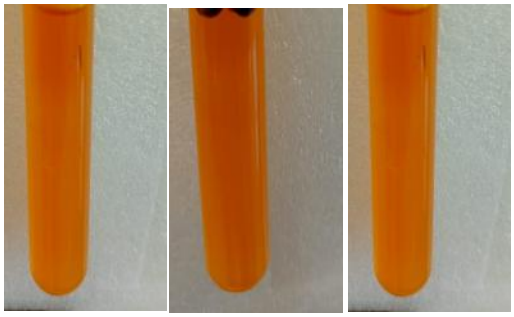

FIGURE 9: CARBOHYDRATE FERMENTATION TEST RESULTS FOR ALL BACTERIAL ISOLATES

MOTILITY TEST

Bacterial motility was assessed in this assay using a semisolid agar substrate. Bacterial motility is shown by a diffusive zone of growth from the inoculation line. *Bacillus altitudinis* strains all exhibit motility by spreading from the inoculation line.

FIGURE 10: MOTILITY TEST RESULTS FOR ALL BACTERIAL ISOLATES

ANTIMICROBIAL SUSCEPTIBILITY TESTING

To determine the antibiotic sensitivity pattern of isolated strains, sensitivity testing was performed. The highest antimicrobial resistance was substantial, with tetracycline (50%), erythromycin (75%).

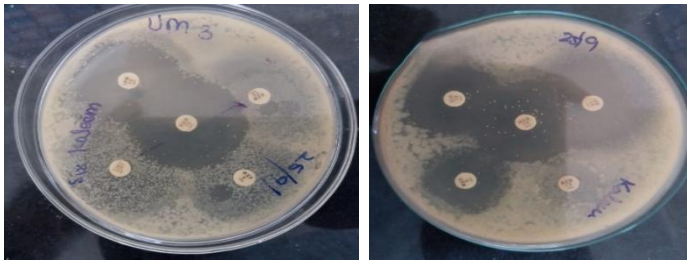

FIGURE 11: ANTIBIOTIC SUSCEPTIBILITY RESULTS FOR ALL BACTERIAL ISOLATES

The 16S rRNA gene sequence's BLASTn analysis revealed 98.37% match to the Bacillus altitudinis strain (Accession No. PQ781331.1). A phylogenetic tree created with MEGA X software demonstrated the isolate's evolutionary link to other Bacilli species. The BLAST analysis results were confirmed by the tree, which showed that the isolate grouped with B. altitudinis strains. A different phylogenetic position was shown by the distance-based tree, which clearly separated the isolate from other Bacilli species.

Table:1 Microbial information extracted from BLASTn results.

Subject	Accession No.	PQ781331.1
	Description	*Bacillus altitudinis*
	Length (b)	1266
	Start	1
	End	1266
	Coverage	100
Score	Bit	2567
	E-value	0.0
Identities	Match/Total	1239/1266
	Percentage (%)	98

TAXONOMIC HIERARCHY

TABLE:2 TAXONOMIC HIERARCHY OF THE IDENTIFIED STRAIN

Taxon	Description
Domain	*Bacteria*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillales*
Family	*Bacillaceae*
Genus	*Bacillus*
Species	*B. altitudinis*

TABLE: 3 TOP 10 BLASTN RESULTS

Scientific Name	Max Score	Total Score	Query Cover (%)	E-value	Per. Ident (%)	Acc. Len (b)	NCBI Accession No.
Bacillus sp. (in: *firmicutes*)	2567	2567	99	0	98.83	1450	MH518214.1
Bacillus altitudinis	2567	2567	99	0	98.83	1441	PQ781331.1
Bacillus	2567	2567	99	0	98.83	1447	PQ781556.1

altitudinis							
Bacillus sp. (in: *firmicutes*)	2567	2567	99	0	98.83	1450	OR392978.1
Bacillus altitudinis	2567	2567	99	0	98.83	1447	PQ813871.1
Bacillus altitudinis	2567	2567	99	0	98.83	1441	PQ782775.1
Bacillus sp. BG2-9	2567	2567	99	0	98.83	1441	KP992115.1
Bacillus pumilus	2567	2567	99	0	98.83	1453	KC764989.1
Bacillus altitudinis	2567	2567	99	0	98.83	1449	PQ813717.1
Bacillus sp. (in: *firmicutes*)	2567	2567	99	0	98.83	1452	OL955468.1

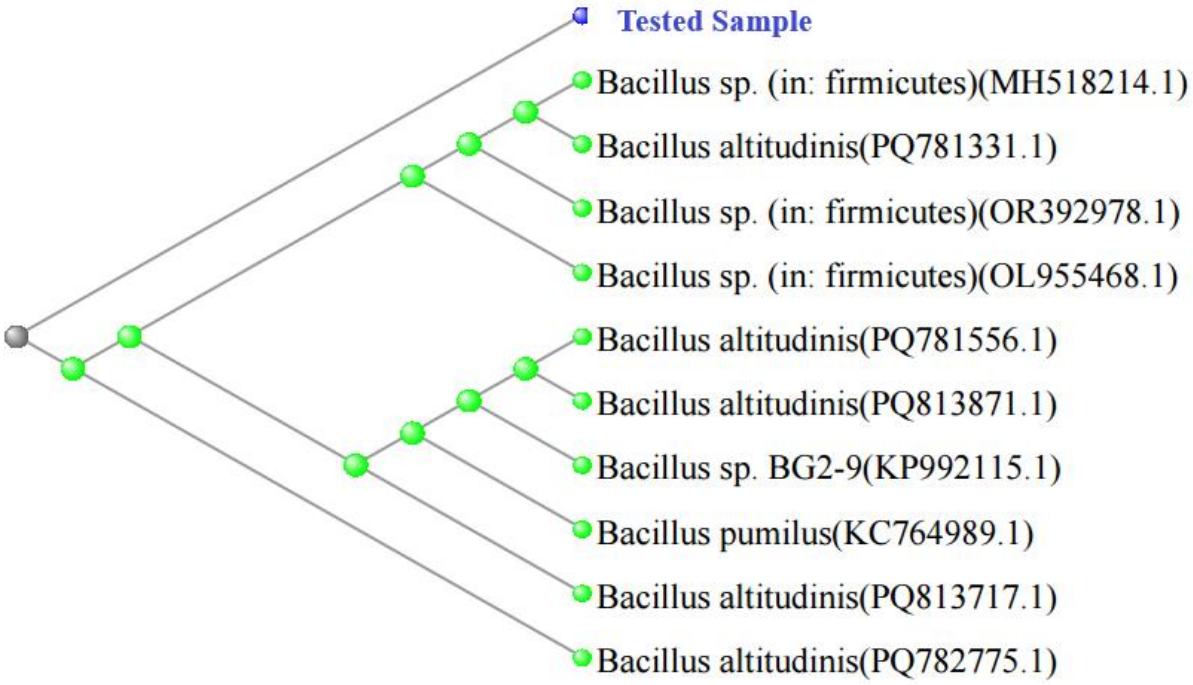

FIGURE: 12 PHYLOGENETIC TREE OF *BACILLUS ALTITUDINIS*

The isolate was determined to be a strain of *Bacillus altitudinis* based on the study of the 16S

rRNA gene sequence and the creation of a phylogenetic tree. The isolate and other B. altitudinis strains appear to have a tight evolutionary connection, according to the data. These results offer important new information on the phylogenetic linkages and genetic diversity of Bacilli species. The rod-shaped bacteria *Bacillus altitudinis* is Gram-positive. Its capacity to create endospores, which enables it to tolerate hard temperatures, and its extremophilic nature, which allows it to thrive in situations with high radiation or altitude, are its defining characteristics. *B. altitudinis* has prospective benefits in biotechnology, including bioremediation and biocontrol, and knowledge of its survival processes can be gained via research. It is an important study topic with potential applications in a variety of biotechnological sectors due to its capacity to adapt to harsh settings.

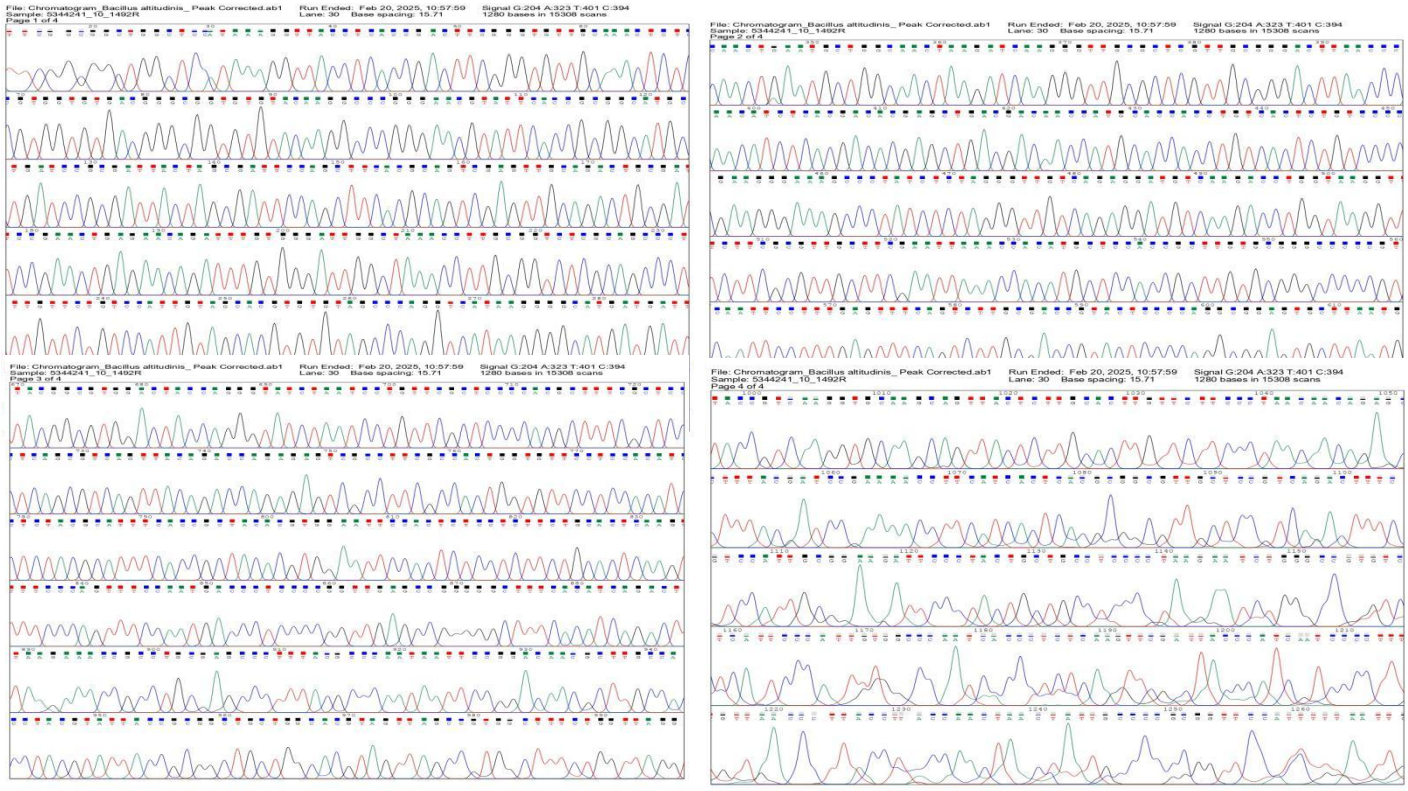

FIGURE: 13 CHROMATOGRAPHY OF *BACILLUS ALTITUDINIS*

DISCUSSION

The bacterium strain *Bacillus altitudinis* is a member of the Bacillus genus. The scientific community is interested in this strain because of its special qualities and its uses in environmental research and biotechnology. The strain was initially isolated from a high-altitude area, as implied by its name, suggesting that it may flourish in environments with low

oxygen levels and perhaps other severe factors like low temperatures and intense UV radiation. The current study focus on Molecular and Biochemical Analysis of *Bacillus altitudinis* from Freshwater Sources. During the current study bacterial strains were isolated from water samples collected from district Abbottabad region. The current investigation was designed at Abbottabad University of Science and Technology, where *Bacillus altitudinis* was isolated, purified, and examined. Bacterial isolates were then characterized by morphology, by using Mannitol salt media, on MSM all *Bacillus altitudinis* strains produce white to pale colonies. Similar study was carried out by Greenwood *et al*., 2017 which shown *Bacillus altitudinis* isolated from water source show white, pale color colonies on the surface of Mannitol salt agar. After applying the Gram-staining method, microscopic examination demonstrated that every *Bacillus altitudinis* isolate was gram positive rod A similar study was conducted by Ali *et al*., (2013) which shows *Bacillus altitudinis* was gram positive rod shaped bacteria. The biochemical analysis of the *Bacillus altitudinis* isolates was another aspect of this work. Biochemical test results show that isolated *Bacillus altitudinis* was positive towards catalase, oxidase, motility, carbohydrate fermentation test while negative towards indole and urease test. Similar study was carried out by (Price *et al*., 2012) which shows *Bacillus altitudinis* was positive results towards catalase, oxidase, motility, carbohydrate fermentation test while negative results towards indole and urease test.

Bacillus altitudinis isolates from freshwater sources are characterized molecularly and biochemically, which offers important information about their ecological function and possible effects on water quality. The study's conclusions show that *B. altitudinis* is not only common in a variety of freshwater habitats but also demonstrates metabolic diversity through its enzymatic activity. This bacterium's synthesis of extracellular enzymes including lipases, amylases, and proteases indicates that it contributes significantly to the breakdown of organic materials, which is consistent with earlier research on Bacillus species in aquatic settings (Roman-Ponce *et al*., 2016). These enzymatic properties could boost microbial-mediated degradation of contaminants and aid in the cycling of nutrients in freshwater environments. But the same characteristics could also make it easier for biofilms to grow in water distribution systems, which would interfere with infrastructure upkeep and the quality of drinkable water (Donlan, 2002). Given that *B. altitudinis* is both a potential bioremediator and a biofilm-forming agent, context-specific assessments of its prevalence in water systems are necessary.

The antibiotic resistance profile observed in *B. altitudinis* isolates is among the study's most alarming results. Similar to patterns seen in ambient Bacillus species, a number of isolates demonstrated resistance to widely used antibiotics, such as tetracyclines and beta-lactams. Since these features might be spread to harmful microbes by horizontal gene transfer, the existence of antibiotic resistance genes (ARGs) in freshwater bacteria is a major public health issue (Bengtsson-Palme *et al.*, 2018). The identification of ARGs in *B. altitudinis* raises the possibility that freshwater environments might operate as antimicrobial resistance reservoirs, requiring more stringent surveillance of bacterial populations in drinking and recreational water sources. This is especially important in areas where human-caused contamination, such pharmaceutical waste or runoff from farms, makes resistant strains more likely to spread (Boehm *et al.*, 2018). Future investigations ought to examine the genetic processes that underlie this resistance and evaluate the possibility of gene transfer to infections that are clinically significant.

The bacterium's capacity to adapt to polluted settings was highlighted by the genomic study of *B. altitudinis* isolates, which also identified genes linked to hydrocarbon breakdown and heavy metal tolerance. These results support research on Bacillus species in contaminated environments, where their adaptability in metabolism allows them to endure stress. For example, the existence of detoxifying enzymes and metal efflux pumps indicates that B. altitudinis may be used for bioremediation, especially in water systems that include heavy metals (Bhatt *et al.*, 2019). However, it is important to carefully assess the ecological trade-offs of such uses. Although *B. altitudinis* may help break down pollutants, local microbial populations may be upset if it is introduced or enriched in natural environments. According to studies, non-native strains of Bacillus can outcompete native bacteria, changing the dynamics of ecosystems and lowering biodiversity (Bhatt *et al.*, 2019). Thus, any potential use of *B. altitudinis* in bioremediation must be preceded by rigorous risk assessments to evaluate its long-term ecological impact.

The potential of *B. altitudinis* as a water quality bio indicator is another important topic of discussion. Although fecal indicator bacteria, such as *Escherichia coli*, are a major part of traditional monitoring, new research indicates that Bacillus species may be used as supplementary markers of environmental stress (Koler *et al.*, 2016). *B. altitudinis* is a viable

option for evaluating the health of ecosystems in contaminated or disturbed water bodies due to its capacity to withstand changes in pH, temperature, and nutrient availability. Its abundance may, for instance, be correlated with certain contaminants, including organic pollutants or heavy metals, offering a microbiological signature for pollution levels. However, more validation across a range of geographic and climatic settings is necessary before such biomarkers may be standardized. Furthermore, the virulence variables found in this study indicate that certain strains of *B. altitudinis* have the potential to be harmful, which warns against using them carelessly as a safe indication. A well-rounded strategy that incorporates both conventional and molecular monitoring instruments would improve the precision of water quality evaluations.

It is also necessary to have discussions about the ecological relationships that *B. altitudinis* has with other microbes in freshwater environments. The bacterium may affect the structure of microbial communities by preventing rivals from growing, as evidenced by its capacity to create antimicrobial chemicals, which is also seen in similar Bacillus species. Ecosystem processes like organic matter turnover and nitrogen cycling may be impacted in a cascade. For example, *B. altitudinis* inhibition of natural decomposers may slow down rates of degradation, unintentionally influencing oxygen levels and water clarity. Its antagonistic action against viruses or hazardous algae, on the other hand, may be advantageous for ecosystem health (Koler *et al.*, 2016). The necessity for microbiome-level research to determine the overall effect of *B. altitudinis* on freshwater environments is highlighted by these intricate relationships. Metagenomics and network analysis are two cutting-edge methods that might clarify its function in microbial consortia and pinpoint keystone species that alter its effects (Bhatt *et al.*, 2019).

CONCLUSION

This study concludes by showing that *B. altitudinis* is a functionally diverse bacterium that has important ramifications for freshwater ecosystems. While its antibiotic resistance and biofilm-forming abilities provide problems for infrastructure and public health, its enzymatic and bioremediation capacity presents chances to enhance the quality of the water. The dualistic character of *B. altitudinis* emphasizes how crucial context-specific management techniques are. Longitudinal studies to monitor its persistence and gene transfer potential in natural water systems, ecotoxicological evaluations to gauge its effect on native biodiversity, and the creation

of focused monitoring protocols to differentiate between beneficial and harmful strains should be the main areas of future research. Scientists and politicians can better utilize *B. altitudinis* potential while reducing its hazards by filling in these gaps, which will eventually help manage water resources sustainably.

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