

<https://doi.org/10.48047/AFJBS.6.15.2024.1176-1187>



African Journal of Biological

Sciences



Research Paper

Open Access

Isolation and Characterization of Antibiotic-Producing Thermophilic *Bacillus* in Selected Hot Springs Along Lake Saiful Muluk, Pakistan

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Volume 6, Issue 15, Sep 2024

Received: 15 July 2024

Accepted: 25 Aug 2024

Published: 05 Sep 2024

doi: [10.48047/AFJBS.6.15.2024.1188-1209](https://doi.org/10.48047/AFJBS.6.15.2024.1188-1209)

Abstract:

Extreme habitats are increasingly recognized as sources of secondary metabolites, providing promising leads for the development of novel natural pharmaceuticals. Saline and hot water lakes are becoming appreciated as rich, untapped reservoirs of extremophilic microorganisms with previously uncharacterized functions. Bacilli, a large and adaptable group of bacteria, thrive in a wide range of environmental conditions due to their ability to form resistant spores, allowing them to survive in high-temperature zones where other organisms cannot. This study aimed to isolate, identify, and characterize Bacillus organisms with the potential to produce secondary metabolites with antibacterial properties from the hot springs of Lake Saiful Muluk in Pakistan. Eighty samples were collected from the surface and sediment waters of selected hot springs and inoculated directly into nutrient broth in universal bottles. Samples were initially incubated at 45°C for 48 hours, and growth was examined by checking for turbidity. Of the 80 bottles inoculated with samples, thirty-three exhibited growths. Subsequent streaking on nutrient agar and Gram staining revealed that nine of the isolated organisms were Gram-positive rods, twenty were Gram-negative rods, and four were Gram-positive cocci. Only Gram-positive rods were processed further.

Biochemical characterization showed that all nine processed isolates were catalase-positive, with no gas production upon sugar fermentation. The isolates displayed varying results for indole, motility, lactose fermentation, glucose fermentation, and H₂S production. Antibacterial profiling was performed using ATCC organisms: Staphylococcus aureus ATCC 29213, Escherichia coli ATCC 25922, Escherichia coli 35218, and Pseudomonas aeruginosa ATCC 27853. Isolates D1, D5, D8, D22, and S15 inhibited the growth of Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853, demonstrated by clear zones of inhibition around the discs. No inhibition was noted against S. aureus ATCC 29213 and E. coli 35218.

Phylogenetic analysis of amplified 16S rDNA genes revealed that D1, D5, D8, D22, and S15 formed close phylogenetic clusters with known members of Bacillus organisms, with sequence identity ranging from 88% to 99%. This study highlights the presence of thermophilic Bacillus species in the hot springs of Lake Saiful Muluk, which produce biomolecules with antibacterial properties. Characterization of these antibacterial compounds could lead to the discovery of novel antimicrobial substances effective against a wide range of pathogens.

Keywords: Thermophilic Bacillus, Antimicrobial Activity, Hot Springs, Lake Saiful Muluk, 16S rDNA Sequencing

Introduction

The rapid rise in multi-resistant bacterial strains as causative agents of infectious diseases poses a critical challenge to modern medicine, particularly in the development of effective therapies that can guarantee successful treatment outcomes (1). In 2013, there were approximately 480,000 new cases of multidrug-resistant tuberculosis (MDR-TB) worldwide, with extensively drug-resistant tuberculosis (XDR-TB) reported in over 100 countries (2). Such cases require treatment regimens that are significantly longer and less effective than those for non-resistant strains of tuberculosis.

Additionally, the emergence of resistance to last-resort antibiotics, such as third-generation cephalosporins used for treating gonorrhea, has been reported in several countries, raising concerns that gonorrhea may soon become untreatable (3). This resistance trend is not limited to tuberculosis and gonorrhea but extends to bacteria causing common infections like urinary tract infections, pneumonia, and bloodstream infections, particularly in hospital settings where highly resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant Gram-negative bacteria are prevalent (4).

In response to the growing threat of antibiotic resistance, the search for new antimicrobial agents that remain effective against resistant strains has become increasingly urgent. Extremophiles—microorganisms that thrive in extreme environmental conditions—are emerging as promising sources of novel natural products with antimicrobial properties (5). Historically, most antimicrobial compounds were isolated from terrestrial microorganisms (6). However, the discovery rate of new compounds from these sources has declined significantly over the past few decades, with soil-derived actinomycetes producing fewer clinically useful metabolites (7).

In contrast, extreme environments such as the hot springs along Lake Saiful Muluk in Pakistan offer a largely untapped reservoir of novel natural products. These environments host taxonomically diverse bacterial populations that have adapted to survive under harsh conditions, which may include extreme temperatures, salinity, and pH levels. Such adaptability suggests a high potential for the production of unique secondary metabolites not found in more temperate environments. These extremophilic bacteria, particularly those belonging to the *Bacillus* genus, have shown promise as producers of antimicrobial compounds, making them valuable candidates in the search for new antibiotics (5).

Exploring extreme habitats like the hot springs of Lake Saiful Muluk is crucial for uncovering novel sources of bioactive molecules. While antibiotics play a vital role in regulating microbial populations in natural ecosystems, only a small fraction of microbial life—less than 10%—has been explored to date. The limited studies on the diversity of extremophiles from these unique environments highlight the need for further research. By isolating and characterizing thermophilic *Bacillus* species from the hot springs of Lake Saiful Muluk, this study aims to identify new sources of antimicrobial compounds that could contribute to the development of commercial antibiotics, particularly in an era where antibiotic resistance is a growing global concern.

Materials and Methods

Study Site:

The study was conducted at the hot springs along Lake Saiful Muluk, Pakistan. This lake is a saline and alkaline body of water situated in the mountainous region of the Kaghan Valley. The hot springs in this area are known for their extreme environmental conditions, making them ideal for the study of thermophilic microorganisms.

Sample Collection:

Eighty samples were collected from the surface and sediment waters of selected hot springs around Lake Saiful Muluk. Ten sediment and ten surface water samples were collected from each of the four hot springs. The samples were collected within a temperature range of 45-60°C using sterile techniques. Each sample was inoculated directly into 15 ml of nutrient broth in universal bottles and transported to the laboratory in insulated containers.

Isolation and Culturing of Bacteria:

The inoculated samples were incubated at 45°C for 48 hours to promote bacterial growth, which was monitored by observing turbidity. Samples showing growth were streaked on nutrient agar plates and incubated at 40°C overnight to isolate single colonies (8). Gram staining was performed to categorize the bacteria as Gram-positive or Gram-negative, and only Gram-positive rods were further processed.

Biochemical Characterization:

The Gram-positive *Bacillus* isolates were subjected to a series of biochemical tests to identify their metabolic characteristics. These tests included catalase production, indole production, motility, lactose fermentation, glucose fermentation, and hydrogen sulfide (H₂S) production.

Antibacterial Activity Testing:

The antibacterial properties of the isolates were tested using the disc diffusion method against four ATCC bacterial strains: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853. The culture

supernatants from the *Bacillus* isolates were impregnated onto sterile filter paper discs and placed on Mueller-Hinton agar plates inoculated with the test bacteria. The plates were incubated at 37°C for 24 hours, and zones of inhibition were measured to assess antibacterial activity.

Molecular Characterization:

DNA was extracted from the isolates that exhibited antibacterial activity, and the 16S rDNA gene was amplified using polymerase chain reaction (PCR) with universal bacterial primers. The PCR products were purified and sequenced. The sequences were analyzed using BLAST to determine their similarity to known *Bacillus* species and to establish phylogenetic relationships.

Results

A total of 80 samples were collected from four hot springs sites along Lake Saiful Muluk and inoculated in nutrient broth. Out of these, 33 samples showed bacterial growth. Preliminary identification using morphological and biochemical methods revealed that nine of the isolates were Gram-positive rods belonging to the *Bacillus* group. The secreted compounds from these nine isolates were tested for antibacterial activity against selected bacterial strains, and five of the isolates demonstrated inhibitory effects. These five isolates were then subjected to molecular characterization through 16S rDNA analysis, revealing sequence identities ranging from 88% to 99% with known *Bacillus* species.

Isolation and Identification of *Bacillus* Colonies:

Samples were collected from four geyser sites along the shores of Lake Saiful Muluk, specifically Loburu, Losaramat, Koibobei, and Chemurkei. The isolation and identification of *Bacillus* species were critical in the initial stages to focus the study on *Bacillus* organisms exclusively. To achieve this, colony morphology and Gram staining were the primary methods used to identify and select the *Bacillus* isolates.

Location	Growth	No Growth
Loburu	8	12

Losaramat	8	12
Koibobei	9	11
Chemurkei	8	12
TOTAL	33	47

Table 1: Summary of Geyser Sampling Sites and the Number of Universal Bottles Exhibiting Growth

Morphological and Physiological Characterization

The growth obtained from the 33 universal bottles was streaked onto nutrient agar and incubated overnight at 40°C. The resulting colonies were subjected to Gram staining to determine their Gram reaction. Of the isolates, four (12.1%) were identified as Gram-positive cocci, twenty (60.6%) as Gram-negative rods, and nine (27.3%) as Gram-positive rods. For further screening, only the Gram-positive rods were considered, as *Bacillus* species are characterized by this morphology.

Upon subculturing, all Gram-positive rods exhibited robust growth on nutrient agar within 24 hours at 40°C, displaying a variety of colony morphologies. However, it was observed that these isolates tended to lose their spore-forming ability upon subculture, with spores becoming less pronounced in subsequent Gram stains. Notably, isolate D22 exhibited densely staining rods, while others displayed a branched network of rods.



D1



S15



D5

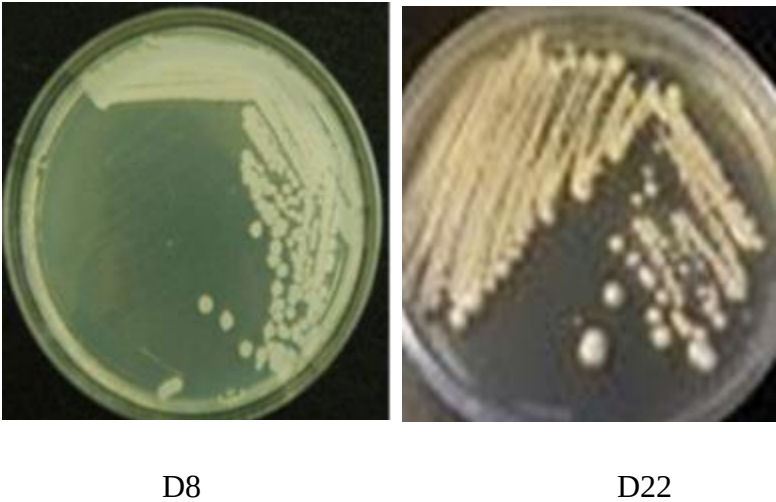


Figure 1: Colonies streaked as pure cultures from Gram positive rods exhibiting antibacterial activity as seen after 24 hours incubation at 40oC. (D1) white, raised, undulate (S15) cream colored, flat, spreading, (D5) white, flat undulate, (D8), white, flat, undulate (D22) large, white, undulate

Isolate	Colour	Form	Elevation	Margin	Cell Arrangement	Gram Reaction
S7	White	Irregular	Flat	Undulate	Large rods	Positive
D8	White	Irregular	Flat	Undulate	Small rods	Positive
S17	White	Irregular	Flat	Undulate	Large rods	Positive
D22	White	Irregular	Flat	Undulate	Large rods	Positive
D5	White	Irregular	Flat	Undulate	Large rods	Positive
D18	Cream	Irregular	Raised	Even	Large rods	Positive
D10	Cream	Spreading	Flat	Undulate	Small rods	Positive
S15	Cream	Spreading	Flat	Undulate	Small rods	Positive
D1	White	Irregular	Raised	Undulate	Large rods	Positive

Table 2: Morphological Characterization of Gram-Positive Rods Isolated from Lake Saiful Muluk

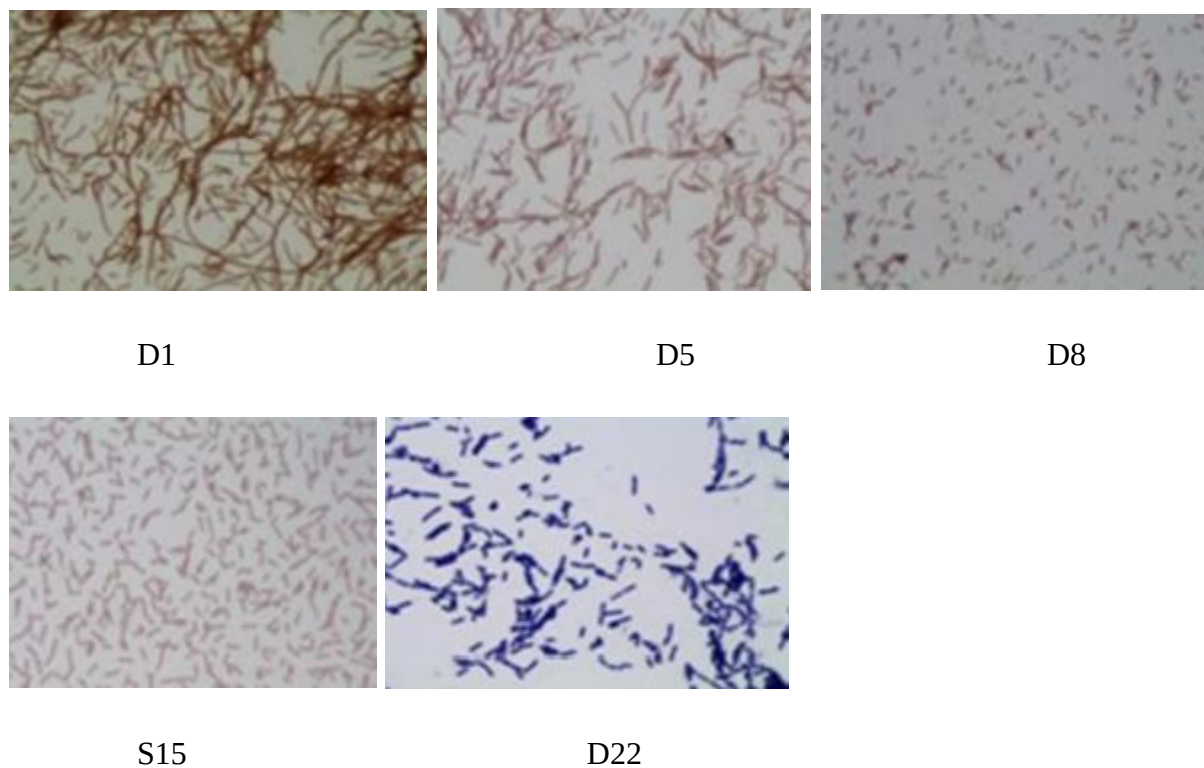


Figure 2: Gram stain images of isolates exhibiting antibacterial action against selected Gram positive and Gram negative bacteria. (D1) Gram positive filamentous rods, (D5) Gram positive filamentous rods. (D8) Small Gram positive rods, (S15) Small Gram positive rods, (D22) Large Gram positive rods.

Physiological studies showed that all the isolates were catalase positive. All but S17, D5, D18, and D1 were motile, fermented lactose/sucrose with acid production and also broke down tryptophan to produce indole. S15, D10 and D8 produced hydrogen sulfide whereas none of the isolates produced gas from carbohydrate fermentation.

Isolate	Catalase	Motility	Indole	Lactose Fermentation	Glucose Fermentation	H ₂ S Production	Gas Production
S13	+	+	+	+	+	+	-

D10	+	+	+	+	+	+	-
S7	+	+	+	+	+	-	-
D22	+	+	+	+	+	-	-
D8	+	+	+	+	+	+	-
S17	+	-	-	-	-	-	-
D5	+	-	-	-	-	-	-
D18	+	-	-	-	-	-	-
D1	+	-	-	-	-	-	-

Table 3: Biochemical results for all nine Gram positive isolates.

Susceptibility Testing

The disc diffusion assay was employed to evaluate the antimicrobial activity of the nine isolates against selected standard Gram-positive and Gram-negative bacteria. Out of the nine isolates tested, five demonstrated antibacterial activity against one or more of the standard organisms. The remaining four isolates, which showed no activity, were not pursued further. Notably, no antibacterial activity was observed against *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 35218.

As a control, the broad-spectrum antibiotic trimethoprim/sulfamethoxazole was used. Similar to the tested isolates, the control did not inhibit the growth of *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 but effectively inhibited *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922, indicated by clear zones of inhibition around the discs.

The zones of inhibition were assessed semi-quantitatively, with the smallest zones assigned a score of "+" and the largest zones assigned a score of "+++". Among the four test organisms, *P.*

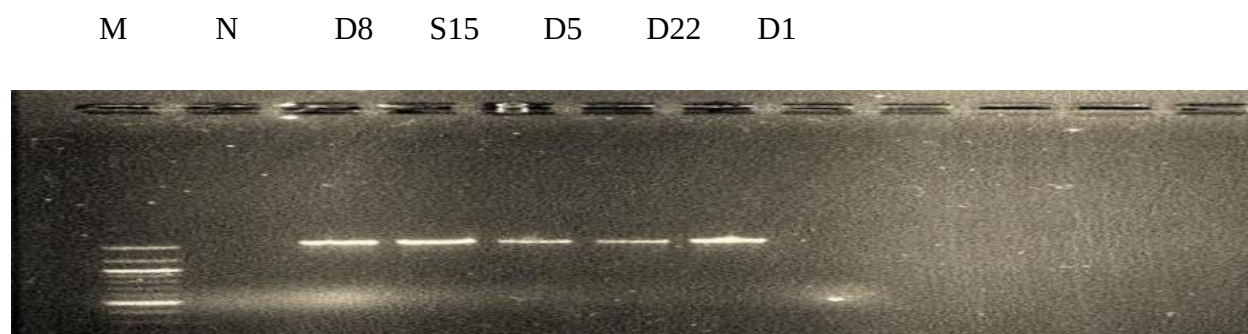
aeruginosa ATCC 27853 was the most responsive, with the largest inhibition zone observed against extracts from isolate D22. The control exhibited a similar inhibition pattern, with *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 showing no response, while *P. aeruginosa* ATCC 27853 showed the highest susceptibility.

Organism	<i>P. aeruginosa</i> ATCC 27853	<i>S. aureus</i> ATCC 29213	<i>E. coli</i> ATCC 35218	<i>E. coli</i> ATCC 25922
D10	X	X	X	X
S17	X	X	X	X
D18	X	X	X	X
S7	X	X	X	X
D22	+++	X	X	X
D1	+	X	X	X
D8	+	X	X	X
S13	+	X	X	+
D5	++	X	X	++
T	+++	R	R	++

Table 4: Semi-quantitative antimicrobial assay of extracts obtained from the Gram positive isolates against *P. aeruginosa*, *S. aureus* and two strains of *E. coli* using disc diffusion method.

DNA Extraction and Amplification

Genomic DNA was extracted from the five isolates that exhibited antibacterial activity using the phenol/chloroform method. The 16S rDNA gene was then amplified using universal primers 27F and 1525R, producing an amplification product of approximately 1500 bp. The amplicons were visualized under UV light on a 1% agarose gel stained with ethidium bromide.



Key: (N) Negative control (M) Molecular marker 1500 bp Molecular size. (D1), (D22), (D5), (S15), (D8), (D10). Represents isolate numbers.

Figure 3: Image of PCR purified DNA product

Isolate Sequences

The 16S rDNA nucleotide sequences from the five isolates that inhibited the growth of the tested bacteria were submitted to the NCBI database and assigned accession numbers KT277950-KT277954, corresponding to isolates D1, D5, D8, D22, and S15, respectively. These sequences were annotated as Seq1 for isolate D1, Seq2 for isolate D5, Seq3 for isolate D8, Seq4 for isolate D22, and Seq5 for isolate S15. The sequences for these five isolates are provided below.

```
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>Seq3. Accession number KT277952

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Phylogenetic Analysis of the Sequences

The sequence data from the five isolates were analyzed using the CLC Main Workbench version 7.0.3 software package (<http://www.clcbio.com>). Sequence alignments were carefully checked and manually corrected where necessary. BLAST analysis of the partial 16S rDNA gene sequences revealed that all five isolates with antibacterial activity were closely related to members of the genus *Bacillus*. Evolutionary analyses were performed using MEGA6 software, and a phylogenetic tree was constructed using the nearest neighbor sequences obtained from the GenBank database to determine the phylogenetic position of each isolate.

The isolates showed sequence identities ranging from 88% to 99% with known *Bacillus* species. Isolates D1 and D5 clustered together, sharing a sequence identity of 96%. These two isolates also clustered closely with *Bacillus halodurans* (Acc. NR_025446.1), with isolate D5 having a sequence identity of 99% and isolate D1 having a sequence identity of 95% with this species. Isolates D8 and S15 clustered together, sharing a sequence identity of 88%. They also clustered with *Paenibacillus thiaminolyticus* (Acc. NR_113266 and NR_040887) and *Paenibacillus dentritiformis* (Acc. NR_042861), with sequence identities of 89%, 89%, and 90% for D8, and 88%, 92%, and 94% for S15, respectively. Isolate D22 clustered with the *Bacillus cereus* group, showing a sequence identity of 93%.

Isolate	Accession	Description	Query Cover	E Value	Identity	Accession Number
D5	KT277951	<i>Bacillus halodurans</i> C-125 16S ribosomal RNA, complete sequence	100%	0	99%	NR_074984.1
		<i>Bacillus halodurans</i> strain DSM 497 16S ribosomal RNA gene, partial sequence	100%	0	99%	NR_025446.1
		<i>Bacillus halodurans</i> strain ATCC 27557 16S ribosomal RNA gene, complete sequence	100%	0	99%	NR_112056.1
		<i>Bacillus okuhidensis</i> strain GTC 854 16S ribosomal RNA gene, partial sequence	99%	0	99%	NR_024766.1
D8	KT277952	<i>Paenibacillus popilliae</i> strain ATCC 14706 16S ribosomal RNA gene, partial sequence	99%	0	97%	NR_040888.1

		Paenibacillus popilliae strain ATCC 14706 16S ribosomal RNA gene, partial sequence	99%	0	97%	NR_114456.1
		Paenibacillus thiaminolyticus strain NBRC 15656 16S ribosomal RNA gene, partial sequence	99%	0	97%	NR_113795.1
		Paenibacillus thiaminolyticus strain IFO 15656 16S ribosomal RNA gene, partial sequence	99%	0	97%	NR_040887.1
D22	KT277953	Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	100%	0	99%	NR_074540.1
		Bacillus cereus strain JCM 2152 16S ribosomal RNA gene, partial sequence	99%	0	99%	NR_113266.1
		Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence	100%	0	99%	NR_115714.1
		Bacillus cereus strain NBRC 15305 16S ribosomal RNA gene, partial sequence	100%	0	99%	NR_112630.1
S15	KT277954	Paenibacillus dendritiformis strain T168 16S ribosomal RNA gene, partial sequence	99%	0	99%	NR_042861.1
		Paenibacillus thiaminolyticus strain IFO 15656 16S ribosomal RNA gene, partial sequence	97%	0	99%	NR_040887.1
		Paenibacillus thiaminolyticus strain NBRC 15656 16S ribosomal RNA gene, partial sequence	97%	0	99%	NR_113795.1
		Paenibacillus popilliae strain ATCC 14706 16S ribosomal RNA gene, partial sequence	97%	0	99%	NR_040888.1
D1	KT277950	Bacillus hemicellulosilyticus strain C-11 16S ribosomal RNA gene, complete sequence	100%	0	97%	NR_040848.1
		Bacillus alcalophilus strain NBRC 15653 16S ribosomal RNA gene, partial sequence	99%	0	96%	NR_112722.1
		Bacillus alcalophilus strain 1 16S ribosomal RNA gene, partial sequence	100%	0	96%	NR_036889.1
		Bacillus pseudofirmus OF4 strain OF4 16S ribosomal RNA, complete sequence	100%	0	96%	NR_102774.1

		Mycobacterium smegmatis ATCC 607 16S rRNA gene	0%	0.34	100%	AM905265.1
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Table 5: BLAST Analysis Results of Isolates from Lake Saiful Muluk: Nearest Neighbors in the Database and Their Percentage Relatedness

Discussion

Bacillus species have long been utilized in various industrial applications due to their ability to produce a wide range of bioactive compounds. This study aimed to explore thermophilic Bacillus species from selected hot springs along Lake Saiful Muluk, Pakistan, and to characterize those with antibacterial activity using morphological, biochemical, and molecular methods. The results revealed the presence of diverse bacterial species in these extreme environments, with a particular focus on Gram-positive rods, as they represent the Bacillus group. Sampling was conducted on both surface (denoted as S) and sediment (denoted as D) samples, with more significant growth observed from sediment samples, potentially due to the warmer conditions below the surface. Although literature does not provide definitive support for this observation, it is plausible that Bacillus species thrive in such thermophilic environments.

Initial identification of the isolates was performed using colony morphology and Gram staining. The recognition of typical colony morphologies is crucial for bacterial identification, as different bacteria produce distinct colonies on agar media, which can provide general indications of their identity (9). The Gram staining process further narrowed down the isolates to Gram-positive rods, consistent with the characteristics of Bacillus species. Among the 33 organisms that exhibited growth on nutrient media, 27.3% were identified as Gram-positive rods.

Biochemical tests were employed to further characterize the isolates. All nine Bacillus isolates were found to be catalase-positive, consistent with the presence of the enzyme catalase, which decomposes hydrogen peroxide into water and oxygen, protecting the bacteria from oxidative damage (10). The indole test, used to determine the presence of tryptophanase, showed that some isolates were indole-positive, indicating their ability to produce indole, which has diverse biological roles, including enhancing drug resistance at higher temperatures (11).

The ability of the isolates to ferment sugars was assessed using Triple Sugar Iron (TSI) medium. Some isolates were capable of fermenting lactose and/or sucrose, while others fermented only glucose or were non-fermenters, indicating a range of metabolic capabilities among the isolates. The production of hydrogen sulfide was also noted in some isolates, indicating anaerobic fermentation of sulfate, a common process in sulfate-reducing bacteria that thrive in anaerobic environments (12).

The antimicrobial activity of the isolates was assessed using the disc diffusion method. Five of the nine isolates demonstrated antibacterial activity against standard Gram-positive and Gram-negative bacteria. Notably, isolate D22 exhibited significant inhibition against *Pseudomonas aeruginosa* ATCC 27853, aligning with previous findings that *Bacillus* species from extreme environments can produce potent antimicrobial compounds (13). The lack of activity against *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 35218 suggests specificity in the antimicrobial spectrum of the *Bacillus* isolates.

Molecular characterization through 16S rDNA sequencing confirmed that the isolates belonged to the *Bacillus* genus, with sequence identities ranging from 88% to 99% with known *Bacillus* species. Isolates D1 and D5 were closely related, clustering with *Bacillus halodurans*, known for producing lantibiotics with activity against Gram-positive bacteria (14). However, the observed activity against Gram-negative bacteria suggests that these isolates may represent distinct strains with unique antimicrobial properties.

Isolate D22, which clustered with the *Bacillus cereus* group, showed distinct morphological and biochemical characteristics compared to typical *B. cereus* strains, highlighting the potential for discovering novel *Bacillus* strains with unique bioactive compounds. Similarly, isolates D8 and S15, which clustered with *Paenibacillus* species, demonstrated significant antimicrobial activity, reinforcing the importance of *Paenibacillus* in producing bioactive secondary metabolites (15).

While 16S rDNA sequencing is a valuable tool for bacterial identification, it has limitations in resolving species-level phylogeny and may be influenced by horizontal gene transfer, as suggested by previous studies (16). Despite these challenges, the study successfully identified several *Bacillus* isolates with promising antimicrobial activity, emphasizing the potential of extreme environments as sources of novel bioactive compounds.

In conclusion, this study highlights the presence of thermophilic *Bacillus* species in the hot springs of Lake Saiful Muluk, which produce antibacterial compounds with potential therapeutic applications. Further research into the characterization and optimization of these bioactive compounds could contribute to the development of new antimicrobial agents, addressing the growing challenge of antibiotic resistance.

Conclusions

This study successfully demonstrated the antimicrobial potential of isolates from selected hot springs along Lake Saiful Muluk, Pakistan. The isolates exhibited significant antibacterial activity against selected test organisms, indicating their potential as candidates for the discovery of novel secondary metabolites with antibiotic properties. The findings underscore the importance of exploring extreme habitats, such as hot springs, as reservoirs for *Bacillus* species that may produce valuable antimicrobial compounds, especially in light of the growing concern over antibiotic resistance.

While the *in vitro* antibacterial assays provided a preliminary assessment of the isolates' antimicrobial capabilities, these results may not directly correlate with *in vivo* efficacy. Nonetheless, these assays are crucial in the initial screening process, identifying promising candidates for further investigation and development. The five isolates that demonstrated activity against all tested organisms hold particular promise for the production of novel bioactive metabolites.

Recommendations

Given the promising results obtained from the antimicrobial assays, the following recommendations are proposed:

1. **Optimization of Culture Conditions:** It is essential to develop culture conditions that closely mimic the natural habitats of the isolated organisms. This approach may enhance the production

and preservation of secondary metabolites, maximizing the potential of these organisms as sources of novel antibiotics.

2. Purification and Characterization of Metabolites: The crude secretions from the active isolates should be purified using chromatographic or other suitable methods. The purified products should then be tested against a broader range of susceptible and resistant bacteria to assess their biological activity. Additionally, the chemical structures of these metabolites should be elucidated to determine whether they represent new compounds or known structures.

3. Advanced Molecular Characterization: To complement 16S rDNA phylogenetic analysis, other molecular tools such as DNA-DNA hybridization should be employed. This would aid in the accurate taxonomic assignment of strains with ambiguous properties and in the identification of potentially new species within the *Bacillus* genus.

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