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Cold and Salt Tolerant Hydrolase Producing Bacteria from the High Altitude Cold Desert of Ladakh, India: Characterization and Comparative *In Silico* Analysis

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Abstract: The high-altitude cold desert of Ladakh represents a polyextreme environment where low temperature, repeated freeze-thaw cycles, desiccation, intense solar radiation, and salinity fluctuations collectively shape microbial survival. In the present study, water samples from Pangong Lake and the Indus River were used to isolate culturable bacteria across NaCl gradients (0.9-12%, w/v) at 20 and 37°C. A total of 112 bacterial isolates were recovered and screened for extracellular amylase, protease, endoglucanase, and xylanase production, revealing broad hydrolytic potential along with notable pigmentation diversity. Six representative enzyme-hyperproducing isolates were selected for phenotypic, biochemical, and 16S rRNA gene-based characterization and showed closest affiliation to *Halobacillus trueperi, Alkalibacillus flavidus, Glutamicibacter nicotianae, Brachybacterium rhamnosum,* and *Pantoea vagans*. To extend the culture-based observations, comparative *in silico* analyses were performed using publicly available reference proteomes corresponding to the closest identified taxa. These analyses revealed trends associated with adaptation to low temperature and osmotic stress, including variation in glycine-to-proline ratio, shifts in mean isoelectric point, and reduced salt-bridge networks in modeled cold shock proteins relative to the thermophilic reference *Thermus thermophilus*. Comparative gene mining also indicated the presence of UV-repair and oxidative-stress functions relevant to persistence in high-altitude environments. Together, these findings highlight the biotechnological potential of culturable Ladakh bacteria as sources of hydrolases active under combined cold and saline conditions, while providing preliminary comparative insights into molecular traits associated with survival in high-altitude cold-desert habitats.

Key words: Psychrotolerants, halotolerance, cold-active hydrolases, Ladakh, polyextreme environment, 16S rRNA characterization, *In silico* proteomics, molecular adaptation.

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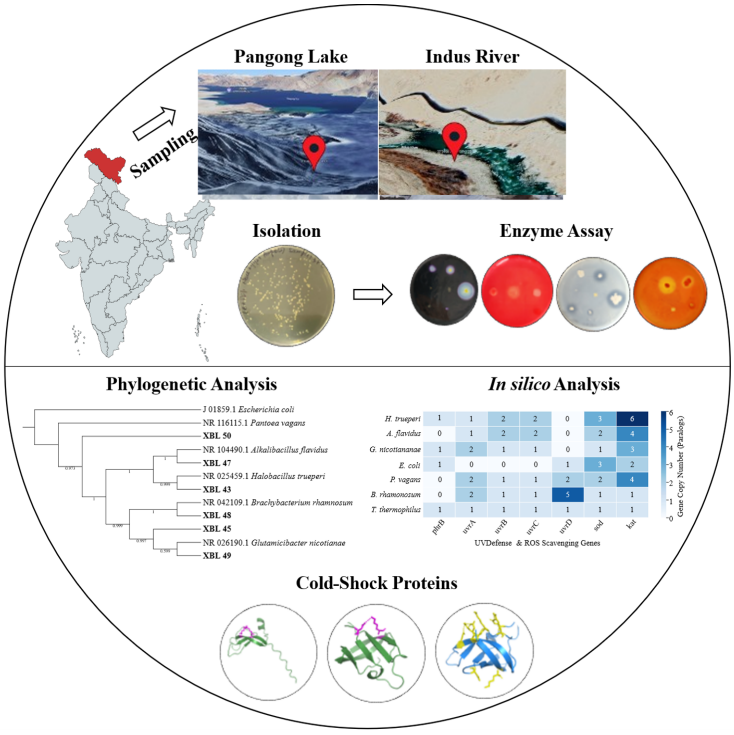
High-altitude aquatic habitats of Ladakh provide a natural setting for investigating microorganisms adapted to



Graphical abstract.

multiple environmental stresses, including low temperature, salinity, desiccation, and intense solar radiation (Karan *et al.*, 2012; Karan *et al.*, 2020; Khare *et al.*, 2025; Pandey *et al.*, 2025). In such ecosystems, microbial life is shaped not by cold alone, but by the combined effects of reduced temperature and low water availability (Pandey *et al.* 2026). Pangong Lake, for example, is a brackish high-altitude system, and together with associated riverine habitats such as the Indus River, represents an ecologically relevant niche for microorganisms capable of functioning under both cold and saline conditions (Chaudhari *et al.*, 2020; Rathour *et al.*, 2020; Yadav *et al.*, 2024).

Cold-adapted microorganisms from extreme environments are of considerable biotechnological interest because they produce biomolecules that remain functional under conditions that inhibit many conventional mesophilic systems (Mangiagalli *et al.*, 2020; Passarini *et al.*, 2020; Sajjad *et al.*, 2020). Among these, extracellular hydrolases are particularly important because of their broad industrial relevance in food processing, detergents, textiles, biomass conversion, environmental applications, and low-temperature biotransformation (Karan and Khare, 2010; Kumar *et al.*, 2012; Grötzinger *et al.*, 2018; Renn *et al.*, 2021; Pandey *et al.*, 2025). Cold-active enzymes are of high relevance because they often display increased catalytic efficiency at low or moderate temperatures, which can reduce energy input and limit unwanted side reactions (Laye *et al.*, 2017; Laye and DasSarma, 2018; Al-Maqtari *et al.*, 2019; DasSarma *et al.*, 2020; Karan *et al.*, 2020). Likewise, salt-tolerant enzymes are valuable because activity under high ionic strength is frequently associated with tolerance to reduced water activity and, in some cases, enhanced performance in harsh industrial conditions (Karan *et al.*, 2011; Karan *et al.*, 2012; Kumar *et al.*, 2012; DasSarma *et al.*, 2013; Vogler *et al.*, 2020; Sysoev *et al.*, 2021; Ghasemi *et al.*, 2025).

Hydrolase-producing microorganisms inhabiting cold and saline environments are therefore promising resources for enzyme bioprospecting. Previous studies from cold deserts and saline ecosystems have shown that extremophilic and halotolerant bacteria can serve as sources of industrially important enzymes such as amylases, proteases, endoglucanases, lipases, and xylanases (Kumar *et al.*, 2011; Karan *et al.*, 2012; DasSarma and DasSarma, 2015; Littlechild, 2015; Amoozegar *et al.*, 2017). In Himalayan cold environments, microbial diversity studies have also highlighted the

potential of cold-adapted bacteria as sources of pigments, enzymes, and other functional biomolecules with ecological and applied significance (Bisht *et al.,* 2013; Pandey *et al.,* 2023). However, despite this promise, there are limited culture-based studies investigating the extracellular hydrolase potential of microorganisms from high-altitude Ladakh under combined cold and saline conditions.

Adaptation to these habitats involves maintaining membrane function, macromolecular stability, and enzyme activity under low thermal energy and osmotic stress (Karan *et al.,* 2012; Laye *et al.,* 2017; Parvizpour *et al.,* 2017). At the protein level, cold adaptation is often associated with greater conformational flexibility, while salt adaptation commonly involves changes that help preserve hydration, solubility, and function in environments of reduced water activity (Karan *et al.,* 2012; Gunde-Cimerman *et al.,* 2018; Karan *et al.,* 2020; Yoo *et al.,* 2023; Pandey *et al.,* 2026). These adaptive trends are useful for interpreting extremophile physiology, but culture-based isolation and screening remain essential for identifying microorganisms with real biotechnological potential. In this context, sequence- and structure-level comparisons can complement experimental screening by providing preliminary insight into molecular features associated with survival and function in polyextreme habitats.

In the present study, we investigated culturable bacteria recovered from Pangong Lake and the Indus River in the high-altitude cold desert of Ladakh across a NaCl gradient and two incubation temperatures. All isolates were screened for extracellular amylase, protease, endoglucanase, and xylanase production. To identify hydrolase-producing strains with potential relevance to cold and saline environments, representative high-performing isolates were then characterized phenotypically, biochemically, and by 16S rRNA gene sequencing. To extend these observations, selected taxa were further examined through comparative *in silico* analyses based on publicly available reference proteomes. This integrated approach links culture-based enzyme bioprospecting with comparative molecular analysis to better understand the functional and adaptive potential of bacteria inhabiting the high-altitude cold desert of Ladakh.

Materials and Methods

Site Description and Sample Collection: Water samples were collected on 13 December 2023 from two high-altitude aquatic environments in Ladakh, India. Sample 1 was collected from Pangong Lake (33°41′00.6″N, 78°41′53.4″E), and appeared clear, exhibiting a temperature of 4°C, a pH of 6.77, and an electrical conductivity (EC) of 13460 µS cm^{-1}. Sample 2 was collected from the Indus River (33°34′06.7″N, 78°07′43.3″E), which appeared clear, with a temperature of 4°C, a pH of 6.68, and an EC of 260 µS cm^{-1}.

Primary Cultivation and Isolation: To recover culturable bacteria from these high-altitude aquatic habitats under different salt and temperature conditions, serially diluted water samples were plated on Luria-Bertani (LB) agar supplemented with 0.9%, 3%, 6%, 9%, and 12% (w/v) NaCl. A dual-temperature isolation strategy was adopted, with duplicate sets of plates incubated statically at 20°C and 37°C for up to one week. Incubation at 20°C was done to proliferate the growth of psychrotolerant population (Margesin and Miteva, 2011, De Maayer *et al.,* 2014). Distinct colonies were repeatedly streaked to obtain pure cultures.

Screening for Extracellular Hydrolytic Enzymes: All 112 purified isolates were screened for extracellular hydrolytic enzyme production. Amylase-producing isolates were identified on starch agar using Gram's iodine. Endoglucanase (CMCase) and xylanase activities were evaluated on carboxymethylcellulose (CMC) agar and xylan agar, respectively, followed by staining with 0.5% Congo red and destaining with 5 M NaCl. Proteolytic activity was screened on skim milk agar. To assess each isolate under physiologically relevant ionic conditions, the screening medium was supplemented with the same NaCl concentration as the isolation medium from which that strain was originally recovered (Supplementary Table S1 and S2). Each assay was performed in triplicate, with mean halo-zone diameters calculated after subtracting the colony diameter, which served as a comparative semi-quantitative descriptor to prioritize isolates for downstream characterization (Karan *et al.,* 2012).

Selection of the Representative Strains via Biochemical and Enzymatic Characterization: To reduce redundancy among the 112 isolates and select representative strains for further

study, isolates were first grouped based on colony morphology and pigmentation. Representative isolates were then compared on the basis of hydrolytic enzyme production and distinct phenotypes. Six isolates showing strong enzyme production and distinctive phenotypic characteristics were selected for further characterization and designated as the XBL (Xtremophiles Biotech Lab) strains. These isolates were examined by Gram staining, endospore staining, and capsule staining. Biochemical characterization of the bacterial isolates was performed using standard colorimetric and culture-based assays. Rapid spot tests were utilized to determine catalase activity using 5% (v/v) H_2O_2 and oxidase activity using N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (Reiner, 2010; Shields, 2010). To evaluate specific metabolic and enzymatic pathways, isolates were inoculated into respective broth and agar media and incubated at their respective temperatures for 24-48 h. Mixed-acid fermentation and acetoin production were assessed in MR-VP broth using methyl red and Barritt's reagents, respectively (McDevitt, 2009). Nitrate reduction was evaluated in nitrate broth by detecting nitrite formation with sulfanilic acid and α-naphthylamine, utilizing zinc dust to confirm true negative results (Buxton, 2011). Extracellular enzyme production was determined via gelatin hydrolysis in nutrient gelatin deeps and urease activity marked by a phenol red transition to pink (Cruz and Torres, 2012). Additionally, complex metabolic traits were studied using citrate utilization test on Simmon's citrate agar (green to blue shift), carbohydrate fermentation and gas/H_2S production on Triple Sugar Iron (TSI) agar slants, and motility, along with further H_2S and indole confirmation, in SIM (sulfide indole motility) agar deeps (Lehman, 2005; MacWilliams, 2009a; MacWilliams, 2009b).

To define the operational ranges of the major hydrolases produced by the six selected isolates, crude enzyme preparations were assayed across pH, temperature, and NaCl conditions. Each isolate was grown in starch, carboxymethyl cellulose, xylan and casein media for amylase, endoglucanase, xylanase and protease enzyme respectively, supplemented with its respective optimal NaCl concentration (Supplementary Table S1 and S2) at the respective growth temperatures with shaking (200 rpm) for 48 h. Cells were removed by centrifugation (4500 rpm, 15 min, 4°C; Model- Z446K Brand- Hermle, Germany), and the cell-free culture supernatant was used as the crude enzyme source. Hydrolytic activity was assessed against the corresponding substrates under varying pH (4.0-11.0, using sodium acetate, sodium phosphate, Tris-HCl, and glycine-NaOH buffers across the appropriate ranges), temperature (10-55°C), and NaCl concentration (0-15% w/v). Reducing sugars released from polysaccharide substrates were quantified by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) at 540 nm, and proteolytic activity was estimated using the azocasein assay at 440 nm (Frankena *et al.*, 1985). Activity at each condition was analyzed relative to the maximum observed activity for that enzyme, and the operational range was defined as the set of conditions retaining ≥ 20% of that maximum. All assays were performed in triplicate, with mean values summarized in Supplementary Table S3.

16S rRNA Sequencing and Comparative In Silico Analysis: The 16S rRNA genes of the selected isolates were sequenced and analyzed by BLAST against the NCBI database for taxonomic assignment. For comparative *in silico* analysis, annotated genome assemblies and translated proteomes of the closest related reference organisms (showing >98% similarity) were selected, along with *Escherichia coli* and *Thermus thermophilus* as mesophilic and thermophilic controls. These closely related reference taxa served as predictive models for cold adaptations in these lineages. Their whole genomes were retrieved using the NCBI Datasets tool. Proteome-wide glycine-to-proline (G/P) ratios were then calculated using a custom Python script implemented with Biopython (Cock *et al.*, 2009). Specifically, these metrics were calculated as global, proteome-wide aggregate ratios (i.e., the organism's total glycine content divided by its total proline content) rather than as per-protein averages. This macroscopic "bulk pool" approach treats the entire translated proteome as a single biochemical pool, preventing very short, low-complexity peptides from being disproportionately weighted equally with massive, multi-domain proteins. This methodology accurately reflects the organism's

Table 1. Recovery, pigmentation, and hydrolytic enzyme profiles of culturable bacteria isolated from the Indus River and Pangong Lake under different incubation temperatures and NaCl concentrations in the isolation medium

Incubation temperature (°C)	NaCl concentration in isolation medium (% w/v)	Total isolates	Prominent pigmentation profiles	Amylase (+)	Protease (+)	Endoglucanase (+)	Xylanase (+)
Indus River*							
37	0.9-9	39	Red, orange, brown, pink, yellow	18	16	5	14
20		26	Salmon orange, red, yellow, transparent	3	6	1	0
Pangong Lake**							
37	0.9-12	28	Yellow, transparent, salmon orange, light red	12	4	2	7
20		19	Cream, yellow, white, peach	9	2	4	0

*Indus River sample collected at 4°C; pH 6.68.
**Pangong Lake sample collected at 4°C; pH 6.77.

total genomically encoded metabolic demand and the macroscopic biophysical cost of thermal adaptation.

Identification of Cold Shock Proteins and Structural Modeling: Putative Cold Shock Proteins (CSPs) were identified from the reference proteomes using annotation-based searches for the terms "cold shock" and "CspA". To enable a comparable structural analysis, sequence corresponding to the canonical 69-amino-acid *E. coli* CspA template (PDB ID: 1MJC) was selected, and one representative cold shock protein (CSP) per organism was retained for analysis. Three-dimensional models of the selected CSPs were generated using AlphaFold2 (Jumper *et al.,* 2021, Yang *et al.,* 2023). Intramolecular salt bridges were then analyzed in the predicted structures using the PDBParser and NeighborSearch modules of Biopython. Structural comparisons and visualization were carried out in PyMOL (Schrödinger LLC 2015).

Comparative Analysis of UV-Repair and Oxidative Stress-Related Genes: To compare selected traits potentially relevant to survival under high-altitude conditions, annotated reference proteomes were screened for proteins associated with UV repair and oxidative stress response. A custom Python pipeline using Biopython (v1.81) and regex-based text mining was used to identify proteins related to direct DNA repair (*phrB*), nucleotide excision repair (*uvrA, uvrB, uvrC,* and *uvrD*), and oxidative stress defense (superoxide dismutase and catalase). The number of annotated hits for each category was compiled into a data matrix, and a comparative heatmap was generated using seaborn and matplotlib.

Results and Discussion

Primary Cultivation, Ecological Distribution, and Community-Wide Enzymatic Profiling: Initial isolation under different incubation temperatures and NaCl concentrations yielded 112 distinct bacterial isolates. As summarized in Table 1, culturable bacteria were recovered from both Pangong Lake and the Indus River across a broad range of saline conditions. Isolates from Pangong Lake were recovered across the fully tested NaCl range up to 12% (w/v), whereas isolates from the Indus River were recovered up to 9% (w/v). These observations indicate that both high-altitude aquatic habitats harbor diverse culturable bacteria capable of growth under combined cold and saline conditions.

Macroscopic examination revealed considerable variation in colony pigmentation, including yellow, cream, white, peach, red, orange, brown, pink, transparent, and salmon-orange phenotypes. Representative culture plates are shown in Supplementary Figure S1. The frequent occurrence of pigmented colonies is notable in the context of high-altitude environments, where microorganisms are exposed to strong solar radiation and associated oxidative stress. Pigments such as carotenoids and melanin have been linked to protection against UV and other environmental stresses in extremophilic microorganisms (Kumar *et al.,* 2012; García-López *et al.,* 2017; Pathak *et al.,* 2020; Sajjad *et al.,* 2020; Pandey *et*

Table 2. Phenotypic, enzymatic, and biochemical characteristics of the selected XBL isolates from the Indus River and Pangong Lake

Source	Indus River			Pangong Lake		
Strains	XBL43	XBL45	XBL48	XBL47	XBL49	XBL50
Morphology	Gram-positive rod	Gram-positive coccus	Gram-positive rod	Gram-positive rod	Gram-positive coccus	Gram-negative rod
Incubation temperature (°C)	37	37	37	37	37	20
Endospore	+	-	-	+	-	-
Capsule	+	-	-	-	-	-
Amylase (mm)	6	8.5	-	14	16	-
Protease (mm)	16	-	13	14	-	10
Endoglucanase (mm)	-	-	-	-	5	-
Xylanase (mm)	8	7.5	10	15.5	5	-
Indole production	-	-	-	-	-	-
Methyl Red	+	+	+	+	+	+
Voges-Proskauer	-	-	-	-	-	-
Citrate utilization	-	-	+	+	+	+
H_2S production	+	+	-	+	+	+
Nitrate reduction	+	+	-	+	+	-
Gelatin hydrolysis	-	-	-	-	+	-
Oxidase	-	-	+	+	-	+
Urease	+	+	+	+	+	+
SIM motility	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
TSI reaction	Yellow / Red	Red / Red	Yellow / Yellow	Red / Yellow	Red / Red	Yellow / Yellow

Note: Hydrolytic enzyme activities are presented as halo-zone diameters in mm; "-" indicates no detectable activity under the screening conditions.

al., 2023). Although pigment identity was not determined in the present study, the observed chromogenic diversity may reflect adaptation to these conditions.

The enzyme plate assays, conducted as a preliminary, semi-quantitative screening of all 112 isolates for extracellular amylase, protease, endoglucanase, and xylanase, revealed broad hydrolytic potential within the culturable bacterial population. Amylase-positive isolates were detected in both the samples, with comparable proportions in Pangong Lake isolates recovered at 20°C and 37°C. Protease-positive isolates were most frequent among Indus River isolates recovered at 37°C, while endoglucanase-positive isolates were detected at lower frequencies across all groups. Xylanase activity was observed only among isolates recovered at 37°C from both sites and was not detected among the 20°C recovery groups under the present screening conditions. This pattern suggests that the expression or detection of xylanolytic activity may be influenced by incubation temperature, although this will require confirmation through strain-level studies under controlled conditions (Rizzatti *et al.,* 2004). Further, upon preliminary quantitative assessment, the enzymes were found to be active at a pH range of 4.0-11.0, a temperature range of 10-55°C, and salinity up to 15% NaCl (w/v), showcasing great industrial relevance (Supplementary Table S3).

Overall, the results show that the culturable bacterial fraction recovered from these Ladakh habitats includes multiple hydrolase-producing strains with potential relevance to cold and saline environments. Detailed colony characteristics and raw halo-zone measurements for all primary isolates are provided in Supplementary Tables S1 and S2.

Phenotypic, microscopic, and biochemical characteristics of the selected XBL strains: To reduce redundancy among the primary isolates, representative strains showing strong hydrolytic activity and distinct phenotypic features were selected for further characterization. This

Table 3. 16S rRNA gene-based identification of the selected XBL isolates by NCBI BLAST analysis

Isolate	Accession No.	Closest BLAST hit	Query coverage (%)	Identity (%)	Reference accession no.
XBL43	PV241795.1	*Halobacillus trueperi*	98	98.66	NR_025459.1
XBL45	PV241801.1	*Glutamicibacter nicotianae*	100	98.72	NR_026190.1
XBL47	PV241810.1	*Alkalibacillus flavidus*	100	99.83	NR_104490.1
XBL48	PV241814.1	*Brachybacterium rhamnosum*	100	100.00	NR_042109.1
XBL49	PV241815.1	*Glutamicibacter nicotianae*	100	99.46	NR_026190.1
XBL50	PV241817.1	*Pantoea vagans*	100	99.74	NR_116115.1

process yielded six isolates, designated XBL43, XBL45, XBL47, XBL48, XBL49, and XBL50. Their phenotypic, microscopic, enzymatic, and biochemical characteristics are summarized in Table 2.

Microscopic examination showed that five of the six selected isolates were Gram-positive, while one isolate, XBL50, was Gram-negative. Most isolates were rod-shaped, whereas XBL45 and XBL49 were coccoid. Among the selected strains, only XBL43 showed positive staining for both endospore and capsule formation, while XBL47 showed positive results for endospore staining only. The presence of a capsule in XBL43 may be relevant to persistence under osmotic stress and desiccation, although this was not investigated further in the present study.

The selected isolates also differed in their extracellular hydrolytic enzyme profiles. XBL49 showed the highest amylase activity among the six isolates, with a clearance zone of 16 mm, whereas XBL43 and XBL47 exhibited activity against multiple substrates. Protease activity was observed in XBL43, XBL47, XBL48, and XBL50, while endoglucanase activity was detected only in XBL49. Xylanase activity was observed in XBL43, XBL45, XBL47, XBL48, and XBL49, with XBL47 showing the largest clearance zone. These differences indicate substantial functional variability among the selected isolates.

Biochemical profiling further highlighted diversity in substrate utilization and related metabolic traits. All six isolates were positive for methyl red, SIM motility, catalase, and urease, and negative for Voges-Proskauer and indole production. Citrate utilization was positive in XBL47, XBL48, XBL49, and XBL50, while nitrate reduction was observed in XBL43, XBL45, XBL47, and XBL49. Five of the six isolates were positive for H_2S production, with XBL48 as the only exception. Gelatin hydrolysis was detected only in XBL49. Oxidase activity was positive in XBL47, XBL48, and XBL50, whereas XBL43, XBL45 and XBL49 were oxidase negative.

The Triple Sugar Iron (TSI) profiles also varied among the selected strains. XBL45 and XBL49 showed a red/red reaction, XBL47 showed a red/yellow reaction, and XBL43, XBL48, and XBL50 showed yellow/red, yellow/yellow, and yellow/yellow reactions, respectively. Together, these results indicate that the selected XBL isolates differ not only in enzyme production but also in several phenotypic and biochemical characteristics, supporting their selection as representative strains for subsequent molecular identification and comparative *in silico* analysis.

Molecular identification and phylogenetic analysis of the selected XBL isolates: 16S rRNA gene sequencing followed by NCBI BLAST analysis was used to determine the closest taxonomic affiliations of the selected XBL isolates (Table 3). The six isolates showed high sequence similarity to recognized bacterial taxa, with query coverage values of 98-100% and sequence identities ranging from 98.66 to 100.00%. XBL48 showed 100.00% identity to *Brachybacterium rhamnosum*, while XBL50 showed 99.74% identity to *Pantoea vagans*. XBL45 and XBL49 both showed the highest similarity to *Glutamicibacter nicotianae*, whereas XBL43 and XBL47 were most closely related to *Halobacillus trueperi* and *Alkalibacillus flavidus*, respectively. These results provided the taxonomic basis for selecting related reference organisms for subsequent comparative *in silico* analyses.

Phylogenetic analysis based on 16S rRNA gene sequences further supported the placement of the selected isolates within their respective bacterial lineages (Fig. 1). The Maximum Likelihood tree grouped each isolate with its closest reference sequence, in agreement with the BLAST-based identifications. Together, the

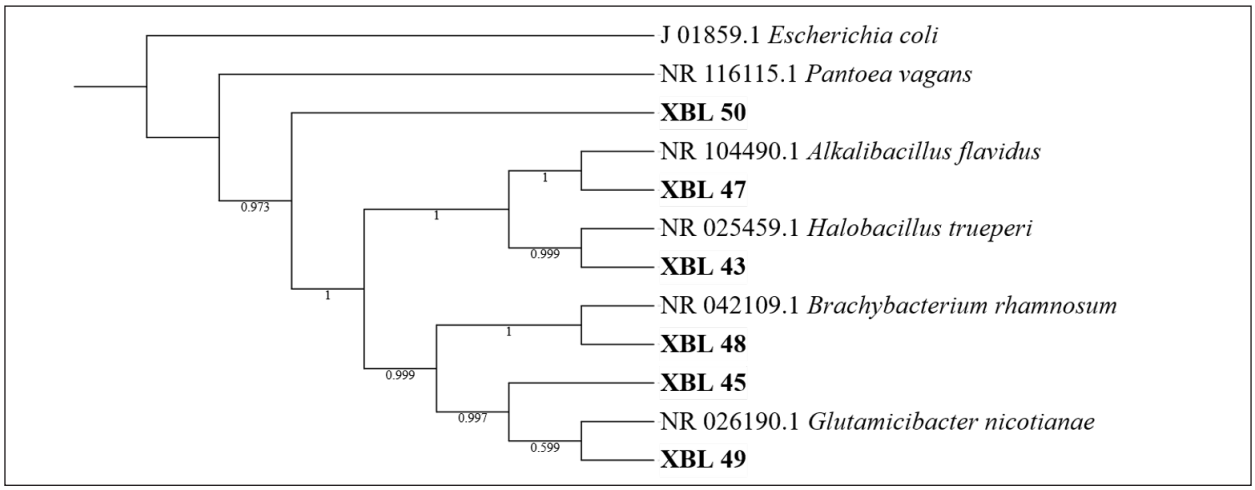


Fig. 1. Phylogenetic analysis of the selected XBL isolates based on 16S rRNA gene sequences. The tree was constructed using the Maximum Likelihood method with the Tamura-Nei model. Bootstrap values from 1,000 replicates are shown at the nodes. The selected Ladakh isolates clustered with their closest reference taxa, including Halobacillus trueperi, Alkalibacillus flavidus, Glutamicibacter nicotianae, Brachybacterium rhamnosum, and Pantoea vagans.

sequence similarity and phylogenetic results indicate that the selected XBL isolates represent taxonomically diverse bacteria recovered from the high-altitude aquatic habitats of Ladakh.

Comparative proteome-wide physicochemical features associated with cold and saline adaptation: To complement the culture-based characterization, comparative proteome-wide physicochemical features were examined using publicly available reference proteomes of *Alkalibacillus flavidus* (Ref Seq: GCF_040545925.1), *Brachybacterium rhamnosum* (Ref Seq: GCF_054957695.1),

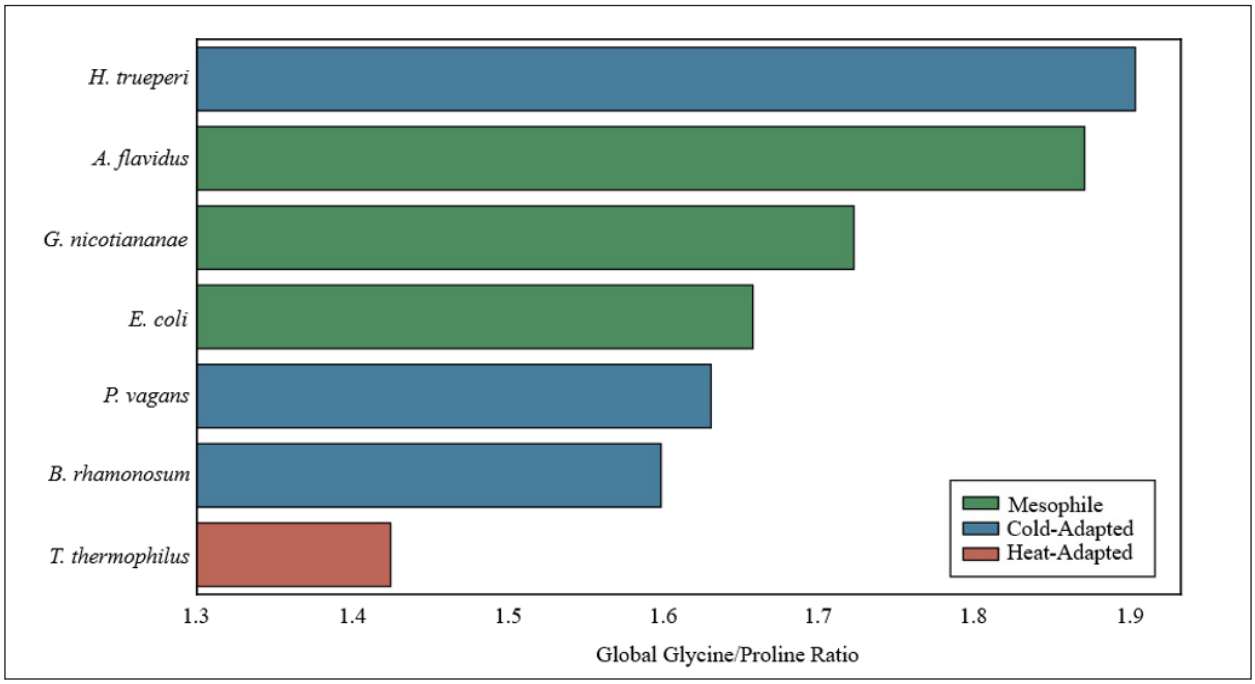


Fig. 2: Comparative glycine-to-proline (G/P) ratios of reference proteomes corresponding to the closest identified taxa and selected control organisms. Values were calculated from translated reference proteomes using Biopython. The analyzed taxa included organisms related to the selected Ladakh isolates (H. trueperi, A. flavidus, G. nicotianae, P. vagans, B. rhamnosum), together with E. coli and T. thermophilus as mesophilic and thermophilic controls. Higher G/P ratios in some taxa may be consistent with increased conformational flexibility, although this feature should be interpreted as a comparative trend rather than a universal signature of cold adaptation.

Table 4. Comparative proteome-wide physicochemical features of reference taxa related to the selected Ladakh isolates and selected control organisms

Taxa/reference organisms	G/P ratio	R/K ratio	Mean aliphatic index	Mean isoelectric point (pI)	Mean aromaticity
H. trueperi	1.90	0.68	91.96	6.27	0.093
A. flavidus	1.87	0.83	93.56	5.86	0.090
G. nicotianae	1.72	1.71	94.78	6.37	0.069
E. coli	1.66	1.26	95.50	6.93	0.082
P. vagans	1.63	1.58	95.09	6.93	0.079
B. rhamnosum	1.60	4.76	94.75	6.27	0.057
T. thermophilus	1.42	2.40	102.43	7.49	0.080

Glutamicibacter nicotianae (Ref Seq: GCF_003687415.2), *Halobacillus trueperi* (Ref Seq: GCF_003386945.1), *Pantoea vagans* (Ref Seq: GCF_004792415.1), together with *Escherichia coli* (Ref Seq: GCF_000005845.2) and *Thermus thermophilus* (Ref Seq: GCF_000091545.1) as mesophilic and thermophilic controls. The analyzed organisms differed in glycine-to-proline (G/P) ratio, arginine-to-lysine (R/K) ratio, mean aliphatic index, mean isoelectric point (pI), and aromaticity (Table 4).

Among the parameters examined, the G/P ratio showed moderate variation across the selected taxa (Fig. 2). The highest values were observed in *H. trueperi* (1.90) and *A. flavidus* (1.87), whereas the thermophilic reference *T. thermophilus* showed the lowest value (1.42). Because glycine can increase local flexibility whereas proline can impose conformational constraint, higher G/P ratios have often been discussed in relation to cold adaptation (Ho *et al.,* 2005). However, such trends should be interpreted cautiously, as no single compositional feature is universal across all cold-adapted proteins or organisms.

Additional proteome-level properties also varied among the analyzed taxa (Table 4). The mean aliphatic index was lowest in *H. trueperi* (91.96) and highest in *T. thermophilus* (102.43), consistent with the general view that thermophilic proteomes tend to retain features associated with greater structural stability. The mean isoelectric point was lower in several of the cold-adapted or salt-associated taxa than in *T. thermophilus*, which may reflect differences in protein surface properties and hydration behavior under low-temperature or saline conditions. Likewise, the R/K ratio varied across organisms, with lower values in *H. trueperi* and *A. flavidus* and a higher value in *T. thermophilus*. Since charged residues contribute to ionic interactions and surface electrostatics, such variation may be relevant to differences in protein stabilization strategies across thermal and osmotic regimes.

Overall, these comparative patterns are consistent with the view that bacteria from cold and saline environments may combine multiple compositional strategies to maintain protein function under conditions of low temperature and reduced water availability (Pandey *et al.,* 2026). At the same time, the observed trends were not uniform across all taxa, indicating that adaptation is likely shaped by lineage specific as well as environmental factors. These results, therefore, provide a comparative context for the selected Ladakh isolates rather than direct evidence of proteome-wide adaptation in the isolates themselves.

Comparative salt-bridge patterns in modeled cold shock proteins: To examine whether the comparative proteome-level trends were also reflected at the level of a functionally relevant cold-response protein, Cold Shock Proteins (CSPs) from the selected reference taxa and control organisms were modeled and analyzed for intramolecular salt-bridge patterns (Fig. 3). Cold Shock Proteins are small nucleic acid-binding proteins involved in cellular responses to low temperature and help maintain RNA and DNA function during cold acclimation (Casanueva *et al.,* 2010).

The modeled CSPs differed in the number of predicted intramolecular salt bridges. Among the proteins analyzed, the thermophilic control *Thermus thermophilus* showed the highest number of predicted salt bridges, whereas most of the other modeled CSPs showed one, and *Glutamicibacter nicotianae* showed two (Fig. 3). This pattern is consistent with the general view that thermophilic proteins often retain more stabilizing interactions, while proteins from

cold- or salt-associated organisms may rely on comparatively reduced ionic cross-linking or alternative structural solutions to preserve conformational mobility at low temperature.

At the same time, these results should be interpreted cautiously. The present analysis is based on AlphaFold2 models of selected reference proteins and provides comparative structural context rather than direct biophysical evidence for the Ladakh isolates themselves. In addition, adaptation to cold and salinity is often multifactorial, involving changes in surface charge, hydration, local flexibility, and other intramolecular interactions rather than salt-bridge number alone (DasSarma *et al.,* 2013; Karan *et al.,* 2020). Therefore, the observed differences in CSP salt-bridge patterns are best interpreted as one structural feature that may contribute to adaptation under low-temperature and osmotic stress conditions.

Comparative analysis of UV-repair and oxidative stress-related genes: To assess whether taxa related to the selected Ladakh isolates also differed in genomic traits relevant to high-altitude stress, annotated reference proteomes were screened for genes associated with UV repair and oxidative stress response (Fig. 4). The analysis included proteins related to direct photoreactivation (*phrB*), nucleotide excision repair (*uvrA, uvrB, uvrC,* and *uvrD*), and oxidative stress defense, including superoxide dismutase (*sod*) and catalase (*kat*).

The resulting heatmap showed variation among the analyzed taxa in the number of annotated proteins assigned to these categories. In particular, several taxa related to the Ladakh isolates showed higher counts for one or more DNA-repair or oxidative-stress functions than the thermophilic control *Thermus thermophilus*. Such differences may be relevant to persistence in environments where microorganisms experience both strong solar radiation and oxidative stress. However, these results should be interpreted as comparative annotation-based patterns rather than direct evidence of gene expansion in the Ladakh isolates themselves.

The observed distribution of catalase- and superoxide dismutase-related annotations is also broadly consistent with the importance of oxidative-stress defense in exposed high-altitude habitats (Dosek *et al.,* 2007; Pérez *et al.,* 2017; Kumar *et al.,* 2022). The comparative analysis with the culture-based assay suggests that taxa related to the Ladakh isolates possess multiple genomic features that may support survival under combined radiation and oxidative stress.

The generated heatmap identified extensive paralogous redundancy in the nucleotide excision repair (NER) pathway (*uvrA-D*) and

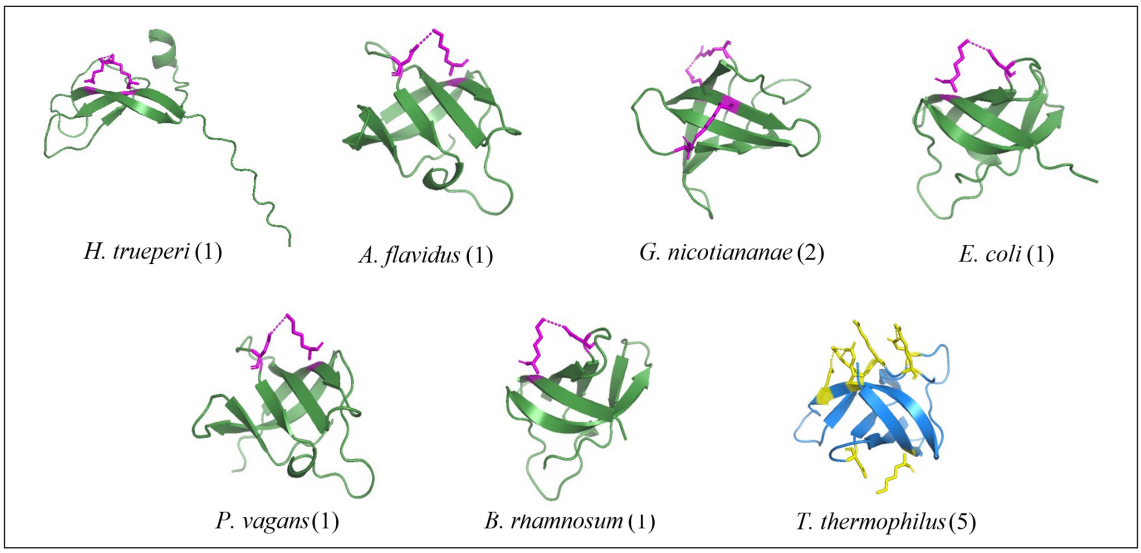


Fig. 3: Comparative intramolecular salt-bridge networks in modeled Cold Shock Proteins (CSPs) of selected reference taxa and control organisms. Monomeric CSP models were generated using AlphaFold2 from selected canonical cold shock protein sequences. Predicted intramolecular salt bridges were identified using a distance cutoff of <4.0 Å between acidic (Asp, Glu) and basic (Arg, Lys, and His) side chains. Numbers in parentheses indicate the total number of predicted intramolecular salt bridges in each model.

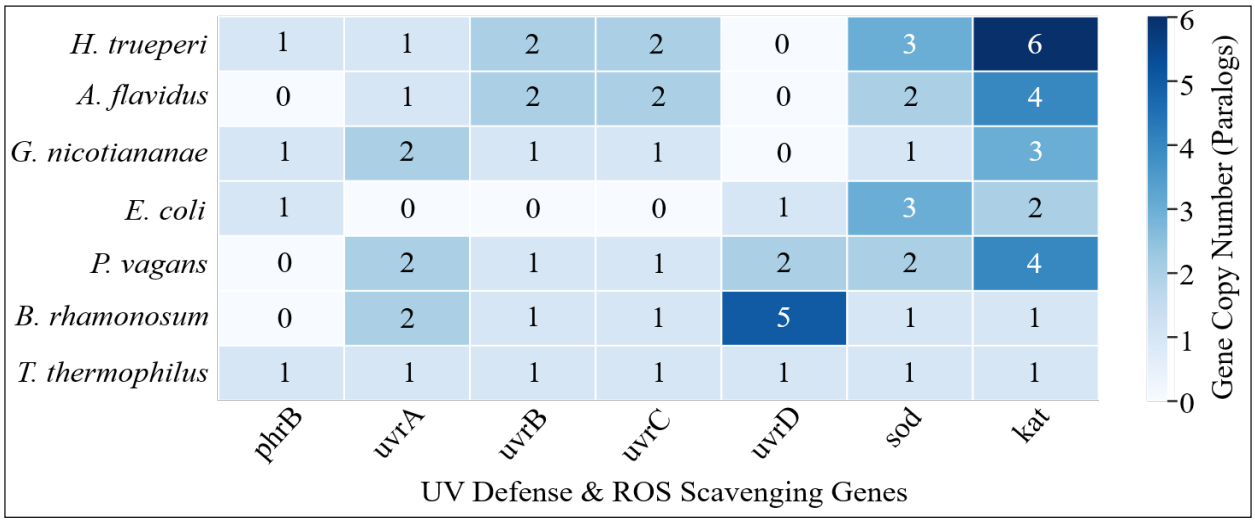


Fig. 4. Comparative distribution of annotated UV-repair and oxidative stress-related genes in reference taxa related to the selected Ladakh isolates and control organisms. The heatmap shows the number of annotated proteins associated with direct DNA repair (phrB), nucleotide excision repair (uvrA, uvrB, uvrC, and uvrD), and oxidative-stress defense (superoxide dismutase and catalase) across the analyzed reference proteomes. Values represent annotation-based counts and are presented as comparative descriptors rather than direct genomic measurements of the Ladakh isolates.

direct DNA repair machinery (*phrB*) relative to mesophilic and thermophilic references, supporting rapid excision of UV-induced lesions. Extremophilic genomes also display a high prevalence of paralogs encoding primary oxidative stress enzymes, including superoxide dismutase and catalase. This genomic redundancy aligns with the uniform *in vitro* catalase activity observed across the XBL strains (Table 2). The expanded genetic repertoire enables efficient neutralization of cytotoxic reactive oxygen species and preserves genomic integrity under intense trans-Himalayan radiation.

Conclusions

This study highlights the high-altitude cold desert of Ladakh as a promising source of culturable bacteria with extracellular hydrolytic potential under combined cold and saline conditions. A total of 112 isolates were recovered from Pangong Lake and the Indus River across different NaCl concentrations and incubation temperatures, and screening revealed broad production of amylase, protease, endoglucanase, and xylanase among the culturable bacterial population. Six representative isolates were selected for further characterization, and their phenotypic, biochemical, 16S rRNA gene-based identification and quantitative enzyme activity characterization showed that they represent taxonomically diverse bacteria with distinct hydrolytic profiles.

The comparative *in silico* analyses provided additional context for understanding traits associated with persistence in these environments. Reference taxa related to the selected isolates showed variation in proteome-wide physicochemical properties, including glycine-to-proline ratio, aliphatic index, and isoelectric point, together with differences in predicted salt-bridge patterns in modeled cold shock proteins and in the distribution of annotated UV-repair and oxidative stress-related genes. Although these observations are comparative rather than direct genomic measurements of the Ladakh microbial ecosystem, they are consistent with the view that survival in high-altitude cold-desert habitats involves a combination of compositional, structural, and stress-response strategies.

Overall, the study provides a culture-based foundation for enzyme bioprospecting from Ladakh and identifies selected bacterial isolates as promising candidates for future investigation of hydrolases functioning under low-temperature and saline conditions. These findings also contribute to a broader understanding of how microorganisms persist in polyextreme environments shaped by cold, osmotic stress, and high solar radiation.

Further, the future work will include spectrophotometric quantification and detailed kinetic characterization (Vmax, Km, kcat) of the most promising hydrolases under defined cold and saline conditions, for their utilization in relevant industrial settings.

Competing Interests

The authors declare no competing interests.

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