Isolation and Characterization of Thermophilic Microbial Enzymes Protease and Amylase from Hot Spring Madkot, Pithoragarh in Uttarakhand

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ABSTRACT

Thermophilic environments such as hot springs are invaluable natural reservoirs of extremophilic microorganisms that produce thermostable enzymes with significant industrial utility. This study explored the thermophilic microbial community of the Madkot geothermal hotspot (Pithoragarh), Uttarakhand. Samples collected from high-temperature niches were subjected to enrichment and isolation under thermophilic conditions, followed by comprehensive biochemical profiling including indole, methyl red, Voges–Proskauer, phosphate solubilization, catalase, oxidase, urease and nitrate reduction tests to elucidate metabolic capabilities relevant to industrial processes. Selected thermophilic strains were molecularly identified by 16S rRNA gene sequencing and affiliated with the genera *Bacillus* and *Pseudomonas*. Both taxa demonstrated the capacity to produce industrially relevant enzymes under elevated temperature regimes: a *Bacillus* sp. isolate exhibited protease activity with a maximum specific activity of 23.05 U/ml, while a *Pseudomonas* sp. isolate synthesized amylase with a peak specific activity of 31.02 U/ml. The thermostability and activity profiles of these enzymes suggest suitability for applications that demand resilience to heat and harsh chemical conditions, including biofuel generation, high-temperature food processing, pharmaceutical enzyme formulations and bioremediation of thermally stressed or contaminated environments. Overall, the results underscore the rich, yet underexplored, thermophilic microbial diversity of Uttarakhand hot springs and emphasize their importance as a source of sustainable, heat-tolerant biocatalysts for industrial biotechnology. Future work will focus on enzyme purification, detailed thermostability profiling and cost effective pilot-scale production in relevant process conditions.

Keywords- Extremophilic, extremozymes, thermophiles, protease, amylase

INTRODUCTION

Extremophiles are organisms that can live and grow in conditions that are too harsh for most other life forms, such as very high or low temperatures, extreme pH levels, high salt content or strong pressure. Much of the Earth's surface actually experiences these extreme conditions - for instance, temperatures can drop to −89°C in Antarctic regions or rise to about 400°C on the deep seafloor. Because of this, extremophiles have evolved special strategies and mechanisms that allow them to survive and function in such challenging environments (Singh *et al.,* 2019). Extremophiles could be described as thermophiles (optimally grow on high temperature), psychrophiles (thrive well at low temperature), halophiles (optimally thrives in high concentration of salt), acidophiles (optimally thrive at low pH), alkaliphiles (optimally thrive at high pH) etc (Zhu *et al.,* 2020).

Extremophiles have recently attracted a lot of attention because they can carry out chemical reactions and have potential industrial uses even in very harsh conditions. Although, extremozymes were discovered years ago, scientists are still working to improve these enzymes through genetic engineering and to find new ones from different sources. Their goal is to get enzymes with the right properties for industrial and biotechnological applications. A report stated that the global market for extremozymes reached $4.0 billion in 2018. This growing

demand creates new opportunities for researchers and extremozymes are strong candidates to help meet these needs (Dumorne *et al.,* 2017).

Thermophiles have evolved a range of strategies that enable them to survive in harsh environments. Elevated temperatures can easily cause protein unfolding and denaturation by disrupting essential intracellular bonds, which is detrimental to cellular function. However, thermophilic microorganisms counteract this damage by producing specialized chaperones or thermosomes that help refold proteins and preserve their function under extreme heat (Annamalai *et al.,* 2016). To further prevent heat-induced protein unfolding, thermophilic bacteria utilize unique stabilizing features, such as specialized hydrogen bonds associated with hydrophobic interactions. Their enzymes also contain abundant salt bridges and additional disulfide bonds, which enhance structural stability. Moreover, other thermal-resistance mechanisms—such as increased structural compactness, oligomerization, glycosylation and strong hydrophobic interactions between subunits—play important roles in maintaining protein stability at high temperatures (Chakravorty *et al.,* 2017).

Thermophilic environments such as hot springs are reservoirs of microorganisms adapted to high temperatures and often extreme physicochemical conditions. These thermophiles produce thermostable enzymes that retain catalytic activity and structural integrity at elevated temperatures, making them valuable for industrial processes where high-temperature operation improves reaction rates, reduces contamination and enhances substrate solubility. Among industrially important enzymes, amylases and proteases occupy leading roles: amylases catalyze the hydrolysis of starch into sugars and are widely used in food, textile, paper and biofuel industries; proteases catalyze protein degradation and are essential in detergent formulations, leather processing, pharmaceuticals and waste management (Punnarath *et al.,* 2025).

Uttarakhand's geothermal sites remain underexplored sources of novel thermostable biocatalysts. The Madkot hot spring in Pithoragarh (Uttarakhand), presents a unique ecosystem influenced by Himalayan geology, variable mineral composition and elevated temperatures conditions conducive to selection of thermotolerant and thermophilic microbes with distinctive enzymatic properties. Systematic isolation and characterization of enzymes from this site can expand the catalogue of robust biocatalysts and reveal properties (e.g., temperature and pH optima, kinetic parameters, substrate specificity and thermal stability) relevant to both basic science and applied microbiology (Roy *et al.,* 2019).

Most enzymes currently available are derived from mesophiles. However, these mesophilic enzymes have limited industrial use because of their narrow stability range. In contrast, enzymes intended for industrial applications should possess broad stability, minimal product inhibition, appropriate substrate specificity and the ability to generate maximum product within a reasonable time without producing unwanted byproducts (Moni Kumar *et al.,* 2024). Nevertheless, over the past three decades, numerous thermophilic species have been identified, most of which are classified within the domains Bacteria or Archaea (Nshimiyimana *et al.,* 2018). Recognizing the limited availability of thermos stable enzymes, this paper focuses on the isolation and characterization of thermophilic microbial enzymes from the hot water spring at Madkot, Pithoragarh district, Uttarakhand.

METHODOLOGY

1. Isolation of thermophiles from study area: The thermophilic bacteria were isolated from hot water spring is located on the bank of Gori Ganga River (29.4^{o} N and 79.3^{o} E), Madkot, Pithoragarh, Uttarakhand by transferring 1 ml of hot spring water into 9 ml of sterile water with peptone and then mixed sample was homogenized. Through serial dilution the sample was diluted up to 10^{-5} and 0.1 ml of diluted aliquot was spread over nutrient agar (NA) plate and incubated at 45^{o}C for 24 to 48 hr. Each single colony with distinct morphology was selected from medium petri plate and purified by successive streaking on respective fresh NA plates (Guta *et al.,* 2024).

2. Screening of different extracellular hydrolytic enzymes: Screening for the presence of four hydrolytic enzyme activities (amylase, lipase, protease and cellulase) was performed for all bacterial isolates. The isolates were grown on NA medium with respective enzyme-specific substrates for amylase, lipase, protease and

cellulase activities at 45°C for 24 to 48 hr. The presence of relatively larger clear zones around the colonies was selected as suitable enzyme producers.

Amylase activity- Amylase activity was determined by growing isolates on a starch agar medium at 45°C for 24 hr. After incubation, the medium plates were flooded with 1% Lugol's iodine solution and a clear zone formed around a colony indicated starch hydrolysis and hence amylase production was confirmed.

Lipase activity- Lipase activity was detected on nutrient agar medium supplemented with 1% Tween 80. The formation of an opaque halo around the colonies was considered positive lipolytic activity.

Protease activity- For the protease activity test, skimmed milk agar plates were prepared and the plate was placed in an incubator at 45°C for 24 hr. The transparent clear zone around the well was considered an indication of protease activity.

Cellulase activity- Nutrient agar supplemented with 1% carboxymethyl cellulose was used for the detection of cellulose enzyme producing isolates. After incubation at 45°C for 24 hr, the plates were poured with an aqueous solution of Congo red (1% wt/vol) for 15 min. The Congo red solution was then discarded, followed by washing the plate with 1 M NaCl to detect the presence of clear halos around bacterial colonies, indicating positive result of cellulose degradation.

3. Morphological characterization of V1 and V2 bacterial strains: For the morphological characterization of both V1 and V2 bacterial strains, which was positive for amylase and protease activity, conventional microbiological procedures was followed. The isolates were characterized for their cell and colony morphologies and further for Gram staining procedure.

The temperature tolerance capacity of both the thermophilic bacteria was determined by incubating the isolated V1 and V2 bacteria on NA plate at different temperature range from 45^{o}C to 90^{o}C with 5^{o}C intervals and growth was observed after 24 to 48 hr.

4. Biochemical characterization of V1 and V2 bacterial strains: For the biochemical characterization the isolates were tested for casein, lipid, gelatin and urea hydrolysis and also tested for their ability to utilize phosphate, sulphate and nitrate. Maina (2021) protocol was used to test citrate as an alternate carbon source. The indole test was performed using Salkowski reagent on medium supplemented with L-tryptophan and the change in the color from light yellow to reddish brownish was considered as indole positive (Bric *et al.*, 1991). Voges–Proskauer test and oxidase & catalase activities were performed as described earlier by Kedia *et al.*, 2023.

5. Identification based on 16S rRNA sequencing: For the molecular identification of bacterial strains V1 and V2, genomic DNA was isolated from pure cultures and its quality was verified using 1% agarose gel electrophoresis. The 16S rDNA gene fragments were amplified and sequenced using an ABI 3730xl genetic analyzer. The resulting 16S rDNA sequences were subjected to BLAST analysis against the NCBI Gen Bank database.

RESULTS

The thermophilic bacteria, V1 and V2 were obtained from the hot spring, which is located at Madkot (Pithoragarh, Uttarakhand). Based on morphological characters both the bacterial strains are rod-shaped bacteria, while V1 is Gram-positive and V2 is Gram-negative bacteria (Table 1 and Figure 1). The temperature tolerance capacity of bacterial strains V1 and V2 were 90^{o}C and 75^{o}C respectively for 4 hrs. Hence, both bacterial strains are considered as extremophilic in nature.

Table 1: Morphological characterization of bacterial stains V1 and V2.

Bacterial Strain	**Gram Nature**	**Morphology**
V1	Gram-positive	Rod-shaped, sticky white colony, opaque, margin entire
V2	Gram-negative	Rod-shaped, light red colony, margin slightly wavy

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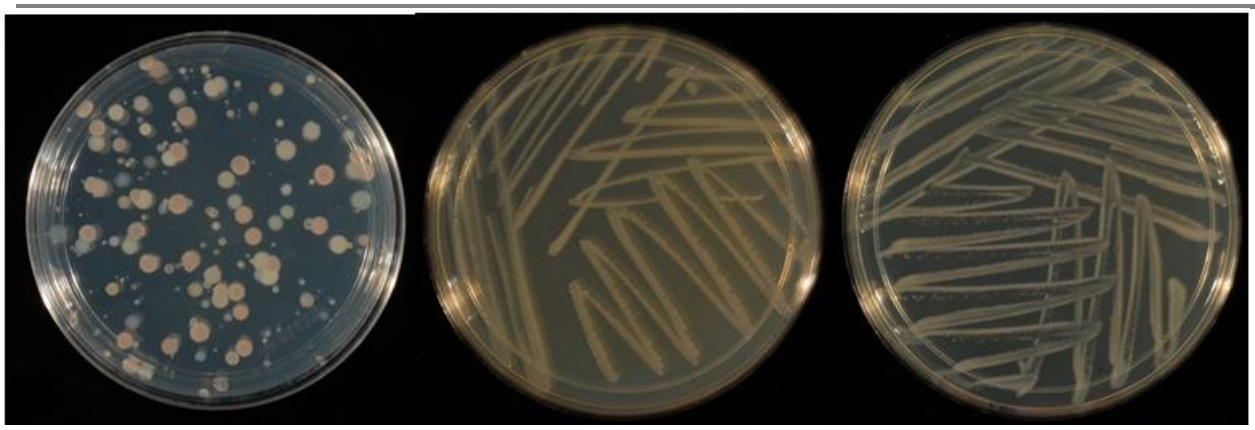

Fig. 1: Dilution plate and streaking plate culture of isolated bacteria V1 and V2.

The biochemical tests revealed that both bacterial strains were exhibited casein hydrolysis as well as phosphate solubilization. Both strains also tested positive for indole production and oxidase activity. And also, were able to utilize urea, nitrate and sulfate. Additionally, strain V1 demonstrated lipase hydrolysis and was positive for the catalase test. In contrast, both strains were not able to hydrolyze gelatin and both showed negative results for the methyl red test, Voges–Proskauer test and citrate utilization (Table 2).

Table 2: Biochemical characterization of bacterial strains V1 and V2.

Sr No.	**Name of Biochemical Test**	**Strain V1**	**Strain V2**
01	Casein Hydrolysis	+	+
02	Lipid Hydrolysis	+	-
03	Gelatin Hydrolysis	-	-
04	Urea Hydrolysis	+	+
05	Phosphate Solubilization	+	+
06	Sulphate Utilization	+	+
07	Nitrate Utilization	+	+
08	Methyl Red Test	-	-
09	Voges Proskauer Test	-	-
10	Citrate Test	-	-
11	Catalase Test	+	-
12	Oxidase Test	+	+
13	Indole production	+	+

Based on the BLAST results, the gene sequence of isolated bacterial strains V1 and V2 were showed more than 98% of homology with *Bacillus* species and *Pseudomonas* species, respectively. Thus, V1 strain was identified as a *Bacillus* sp., while strain V2 was identified as a *Pseudomonas* sp.

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Proteolytic activity of *Bacillus* sp. and *Pseudomonas* sp. was assessed on casein-containing medium by observing zones of hydrolysis. Both isolates produced clear halo zones around their colonies, indicating extracellular protease production (Figure 2).

Amylase production was evaluated on nutrient agar supplemented with 1% (w/v) soluble starch. Following incubation and iodine flooding, both *Bacillus* sp. and *Pseudomonas* sp. exhibited clear zones of starch hydrolysis surrounding the colonies, confirming amylolytic activity (Figure 2).

In contrast, neither isolate showed detectable lipase or cellulase activity under the screening conditions employed; no zones of hydrolysis were observed on lipase- or cellulase-specific media.

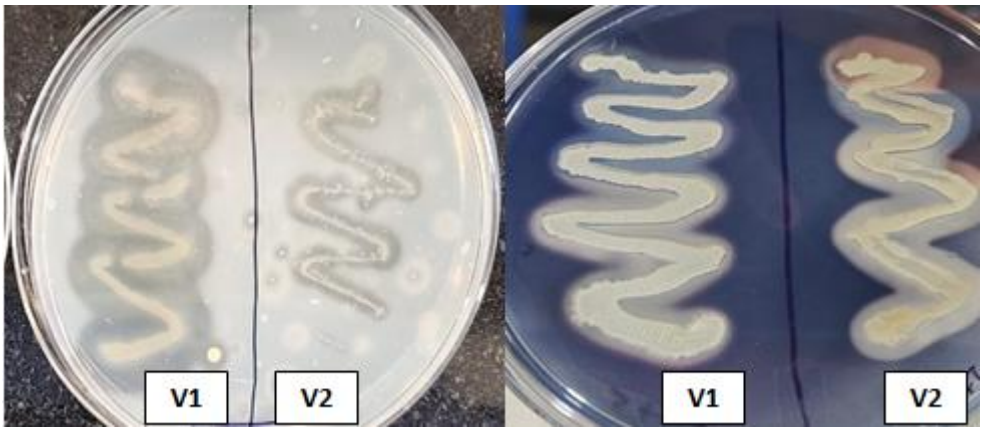

Fig. 2: Streak plate showing the hydrolysis zone of protease and amylase enzymes of isolated bacterial strains V1 and V2.

For the comparative analysis of the extracellular protease production in both the strains of bacteria, both qualitative and quantitative assays were carried out. The colony diameters and zones of hydrolysis after 24 h and 48 h were determined in *Bacillus* sp. and *Psuedomonas* sp. (Table 3).

Table 3: Colony diameters and halo-zones of protease production in *Bacillus* sp. and *Psuedomonas* sp. after 24 h and 48 h of growth.

Bacterial Strains	After 24 h		After 48 h	
	Diameter of colony (mm)	Diameter of halo-zone (mm)	Diameter of colony (mm)	Diameter of halo-zone (mm)
Bacillus sp.	10.5 ± 0.7	16.5 ± 1.2	20.5 ± 1.1	31 ± 2.7
Pseudomonas sp.	13.5 ± 1.1	19.5 ± 1.5	23 ± 0.9	27.5 ± 2.7

For the comparative analysis of the extracellular amylase production in both the strains of bacteria, both qualitative and quantitative assays were carried out. The colony diameters and zones of hydrolysis after 24 h and 48 h were determined in *Bacillus* sp. and *Psuedomonas* sp. (Table 4).

Table 4: Colony diameters and halo-zones of amylase production in *Bacillus* sp. and *Psuedomonas* sp. after 24 h and 48 h of growth.

Bacterial Strains	After 24 h		After 48 h	
	Diameter of colony (mm)	Diameter of halo-zone (mm)	Diameter of colony (mm)	Diameter of halo-zone (mm)

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Bacillus sp.	11.2 ± 1.1	14.5 ± 0.6	19.2 ± 0.8	19.5 ± 1.0
Pseudomonas sp.	12.9 ± 1.2	19.4 ± 1.3	20.5 ± 1.3	23.6 ± 0.7

The specific activities of protease of the *Bacillus* sp. and *Psuedomonas* sp. were also determined, using tyrosine as a standard. The bacterial stain *Bacillus* sp. showed a specific activity of 23.05 U/ml protein, while *Psuedomonas* sp. showed their specific activities of 21.18 U/ml protein, respectively, thus indicating that the *Bacillus* sp. exhibited more protease activity as compared to the bacterial strain *Psuedomonas* sp.

The specific activities of amylase of the only isolated *Psuedomonas* sp. was determined, using DNS reagent method and absorbance was measured at 540 nm using double beam UV spectrophotometer. The bacterial stain *Psuedomonas* sp. showed their maximum specific activity of 31.02 U/ml proteins, while *Bacillus* sp. showed a only specific activity of 19.92 U/ml protein.

DISCUSSION

The present study highlights the potential of thermophilic bacteria isolated from the Madkot hot spring as promising producers of thermostable amylase and protease enzymes. Hot springs are extreme ecological niches that support microbial communities capable of surviving high temperature, variable pH and nutrient limitation, which often leads to the production of enzymes with exceptional thermal stability and catalytic efficiency. Such properties make these enzymes attractive for industrial applications where conventional mesophilic enzymes rapidly lose activity under harsh processing conditions (Sahay *et al.,* 2017 and Alrumman *et al.,* 2018).

The results indicate that the isolated bacterial strains, *Bacillus* sp. and *Pseudomonas* sp., are thermophilic based on their ability to tolerate temperatures up to 90 °C. Among the bacterial isolates, members of the genera *Bacillus* sp. and *Pseudomonas* sp. showed notable enzyme-producing ability. This is consistent with earlier studies on hot spring microbiota, where *Bacillus* species are frequently reported as dominant hydrolytic enzyme producers because of their strong secretion systems and adaptability to stressful environments. Similarly, thermophilic or thermotolerant *Pseudomonas* species isolates have also been documented as useful producers of extracellular enzymes, including amylases and proteases, though they are generally less commonly reported than *Bacillus* in geothermal habitats (Sahay *et al.,* 2017 and Alrumman *et al.,* 2018).

A series of biochemical assays—such as indole, methyl red, Voges–Proskauer, phosphate solubilization, catalase, oxidase, urease and nitrate reduction tests—supported the initial identification of the isolate as a *Bacillus* species. Employing multiple analytical approaches provides a more complete framework for characterizing bacteria, as each method contributes unique advantages and limitations. These biochemical evaluations assess various metabolic processes, including enzyme activities and substrate utilization, thereby revealing important physiological traits of the isolates.

Thermophiles reported for the production of several extremozymes such as amylase, cellulase, chitinase, pectinase and protease which can be exploited for industrial purposes (Nayak, 2013). While, both thermophilic isolated strains were examined for the production of various extremozymes, but only protease activity was detected in *Bacillus* sp. and *Pseudomonas* sp. The culture supernatant obtained after centrifugation was used as the source of extracellular enzymes and was subjected to a protease assay. The assay showed maximum protease activity at 48 hours, a finding consistent with reports on alkaline protease from *Thermoactinomyces* sp. RM4, where enzyme activity also declined beyond this time. The reduction in activity after 48 hours was likely due to enzymatic autolysis or end-product inhibition (Verma *et al.,* 2011). In this study, *Bacillus* sp. showed specific protease activity of 23.05 U/ml which is comparatively very low in comparison to protease producing thermophilic bacteria *S_25* isolates (Shahu *et al.,* 2021). Similarly, also isolated *Bacillus* sp. showed very low amylase activity as compared to *B. subtilis* RK6 of 32.25 U/ml at alkaline pH 8.0 (Kiran *et al.,* 2018).

In the present investigation screening of the *Bacillus* sp. and *Psuedomonas* sp. were done for their ability to produce extracellular protease, using casein as a substrate and skim milk as an inducer. However, *Rojas et al.,* (2009) reported the use of skim milk as the initial substrate in *Eladia sacculum*. However, it was observed that

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this substrate masked the real activity; hence, casein was used on the plates to unmask the proteolytic activity. Thus, in their study, skim milk at initial stage might have served as an inducer like the present study. Screening of protease activity in *Bacillus licheniformis* RP1 by Sellami-Kamoun *et al.*, (2008) was done using skim milk as a substrate, but enzyme assay for its specific activity was done using casein as a substrate. Sonia *et al.*, (2014) isolated extremozymes from the soils of Uttarakhand and pointed out the protease is the economically most important biocatalyst.

The strong amylase activity observed in the isolates suggests efficient starch hydrolysis, indicating their possible role in carbohydrate turnover in the hot spring ecosystem. Thermostable amylases are especially important for industries such as starch processing, baking, brewing, textile desizing and detergent formulation because they remain active at elevated temperatures and often function without extensive cooling of the reaction mixture. Previous studies on hot spring isolates have similarly shown that thermophilic bacteria can produce amylases with high temperature tolerance and broad pH stability, supporting their biotechnological value (Alrumman *et al.*, 2018 and Sharif *et al.*, 2023).

Likewise, the production of protease by the isolates indicates their ability to degrade protein substrates under thermophilic conditions. Thermostable proteases are highly sought after in detergent, leather, food and pharmaceutical industries because they can function efficiently in hot and often alkaline environments. The recovery of protease-producing *Bacillus* and *Pseudomonas* species from Madkot hot spring aligns with reports from other geothermal sites, where proteolytic thermophiles contribute significantly to extracellular protein turnover and show strong industrial promise (Alrumman *et al.*, 2018).

The co-production of amylase and protease in the same environmental isolates is particularly important from an industrial perspective. Strains capable of producing multiple enzymes reduce downstream production costs and may be more suitable for large-scale fermentation processes. Such multifunctional isolates are especially valuable for detergent and waste-treatment applications, where mixtures of starches and proteins are often present in the substrate. Reports from other hot spring investigations in India and abroad also emphasize that thermophilic bacteria from geothermal habitats frequently possess broad hydrolytic capabilities (Sahay *et al.*, 2017 and Alrumman *et al.*, 2018).

Environmental adaptation likely explains the thermostability of the enzymes produced by these isolates. Living in a hot spring such as Madkot exposes bacteria to sustained thermal stress, which favors the evolution of proteins with more rigid tertiary structures, improved hydrophobic interactions and enhanced resistance to denaturation. These molecular features can increase enzyme half-life and preserve catalytic function at temperatures that would inactivate ordinary enzymes. This adaptive advantage makes hot spring bacteria an excellent source for discovering robust biocatalysts (Sahay *et al.*, 2017 and Ortega-Villar *et al.*, 2023).

Overall, the findings suggest that the Madkot hot spring is a rich reservoir of thermophilic enzyme-producing bacteria, particularly *Bacillus* and *Pseudomonas* species. The isolates characterized in this study may serve as useful candidates for future purification, biochemical characterization and molecular identification of thermostable amylase and protease. Further optimization of culture conditions and enzyme production parameters could enhance yield and help determine their suitability for commercial exploitation in starch, detergent, food and protein-processing industries (Sahay *et al.*, 2017 and Sharif *et al.*, 2023).

CONCLUSION

Thermophilic bacteria isolated from the Madkot hot springs (Uttarakhand) demonstrated a suite of industrially relevant traits. Both *Bacillus* sp. and *Pseudomonas* sp. isolates exhibited thermostability, phosphate-solubilizing activity and positive biochemical profiles for indole production, oxidase activity and utilization of urea, nitrate and sulfate. Extracellular protease and amylase production were confirmed by clear hydrolysis zones on casein and 1% starch agar, respectively and further supported by activity assays using crude enzyme extracts. Notably, the *Bacillus* strain showed markedly higher protease activity, whereas the *Pseudomonas* strain displayed superior amylase activity. These findings highlight the biotechnological potential of thermophiles from the Madkot hot springs, particularly for enzyme production under high-temperature conditions. However, comprehensive

biochemical characterization of the protease and evaluation of its stability and performance under industrially relevant conditions remain necessary to fully assess its application potential.

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