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Isolation, Purification, and Characterization of Protease from a Local *Bacillus* Strain Adapted to Extreme Temperatures in Southern Iraq

Hyder Najy Alzobaidy[1*], Ghasan M Hasan[1], Suhad K R Al-Magsoosi[2] & Jasim M Awda[3]

[1]Department of Animal Production, [2]Department of Plant protection, College of Agriculture, Wasit University, Wasit, Iraq

[3]Department of Food Science, College of Agricultural Engineering Science, University of Baghdad, Baghdad, Iraq

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Bacteria, with their amenability to genetic manipulation and cultivation, represent a favored source of protease enzymes. This study aims to isolate and purify protease from a local *Bacillus* strain in southern Iraq, known for its resilience to extreme heat. In August 2022, soil samples were collected from Wasit Province, Iraq, and plated on a casein-hydrolyzed medium. *Bacillus* strains were selected based on microscopic and colony characteristics, with the most active isolate identified as *Bacillus subtilis* through morphological assessment and 16S rRNA sequencing. Protease production occurred through submerged fermentation, yielding a crude enzyme with enzymatic and specific activities of 241.3 U/mL and 613.3 U/mg, respectively. Partial purification with chilled acetone resulted in an enzyme with enzymatic and specific activities of 935.0 U/mL and 745.0 U/mg, respectively. Further DEAE chromatographic purification showed a single peak of protease activity with increasing sodium chloride ionic strength. The enzyme's specific activity reached 2491.8 U/mg, with a recovery rate of nearly 90%. Subsequent gel column filtration via 1.5 × 60 cm Sephadex G-100 displayed a single peak of protease activity and increased specific activity (4264.10 U/mg) with a 73% recovery rate. Molecular weight determination using Sephadex G-100 column indicated a size of 29.3 kDa for the *B. subtilis* protease. Regarding thermal stability, the enzyme demonstrated initial stability at 60°C, but prolonged exposure reduced activity. In contrast, exposure to 70°C and 80°C resulted in rapid declines in enzymatic activity. This study highlights the isolation and purification of a robust protease enzyme from a local *Bacillus* strain, underscoring its potential significance in various industrial applications.

Keywords: *Bacillus subtilis*, Climate change, Enzymes production, Submerged fermentation, Thermostability

Introduction

Proteases are involved in many biological activities, including digestion of food, blood coagulation, recycling of intracellular proteins, antigen performance, and activation of other proteins, including enzymes, neurotransmitters, and hormones.[1] There are many industrial uses for proteases, whether as a mixture or purified. They are considered one of the largest groups of industrial enzymes and represent 60% of total enzyme sales in markets.[2] The search for proteases with enhanced properties, such as thermal stability and catalytic efficiency, is a topic of continuous interest.[3,4] In recent years, the industrial demand for proteases with tailored properties has surged. The ability to withstand extreme temperatures, in particular, has gained prominence due to its relevance in various biotechnological processes.[5] While proteases from mesophilic organisms have been widely explored, the hunt for proteases from extremophiles, organisms thriving in extreme environments, offers a promising avenue for discovery.[6] Microbial protease is a preferred source of this enzyme because of the rapid growth of the microbes, their simple nutritional requirements, the limited space needed for their cultivation, uncomplicated climate control, and the ease with which they can be genetically manipulated to produce new enzymes with different properties.[7] Generally, microbes secrete enzymes and other metabolites extra cellularly to the surrounding environment, so they are comparatively easier to extract than plant or animal intracellular enzymes as there is no need to smash the cell wall.[8] *Bacillus* species is commonly distributed in soil, water, air, and degraded plant residues. This type of bacteria tolerates the hard conditions of the environment, such as temperature and low water activity, by forming bacterial spores.[9] *Bacillus* species, renowned for their adaptability, have garnered attention as potential sources of robust proteases.[10] Their ubiquitous presence in soil, and the ease of genetic manipulation[11], make them an attractive target. However, the specific adaptation of *Bacillus* strains to extreme temperatures in regions like

*Author for Correspondence
E-mail: hynajy@uowasit.edu.iq

southern Iraq remains relatively underexplored in the literature.

This study's innovation lies in isolating and purifying protease from a local *Bacillus* strain in a severe heat-prone region. This choice is grounded in the organism's natural adaptation to extreme temperatures, suggesting the potential for unique enzymes with remarkable thermal stability. While previous *Bacillus* protease studies exist, research on strains from extreme hot environments is rare. By exploring protease production in *Bacillus* strains adapted to harsh conditions, this research aims to uncover enzymes with distinct thermal resilience, offering innovative possibilities across various industries. Thus, it highlights extremophilic microorganisms as sources of valuable proteases.

Materials and Methods

Materials

All materials were of reagent-grade quality in the conducted experiments: Casein-hydrolyzed medium and yeast extract powder from (HiMedia, Mumbai, India); Sephadex G-100, DEAE Sephadex, Phosphate buffer, Tris-HCl buffer, casein and protein markers from (Merck, Darmstadt, Germany); Trichloroacetic acid, acetone, HCl, NaOH and NaCl from (CDH, New Delhi, India); Presto Mini gDNA Bacteria Kit from (Geneaid, New Taipei City, Taiwan); PCR Pre-Mix. (Bioneer, Daejeon, South Korea); Agarose gel (RPI corp., Illinois, USA); and Dialysis bag (Thomas Scientific Co., PA, USA).

Isolation of Protease-Producing Microorganisms

In August 2022, soil samples were taken from various locations in Wasit Province, in the southeast of Iraq. The preparation of the samples was carried out in accordance with the procedure described by Seyfzadeh *et al*.[12] After being appropriately diluted, these samples were plated on casein-hydrolyzed medium. Plates were incubated for 24 hours at 35°C. Protease-producing organisms were indicated by the presence of a clear zone around the growth[13]. *Bacillus* strains were selected based on their microscopic and colony properties. Subsequently, the isolate with the highest activity was chosen for further study, and 16S rRNA was used to identify it.

Identifying *Bacillus Subtilis* by 16S rRNA

The DNA is extracted from the isolates using Presto Mini gDNA Bacteria Kit according to the instruction manual. The 16S rRNA gene was amplified using PCR (CFX96, BioRad, CA, USA). This involved using universal primers (Table 1) that anneal to regions of the gene and allow for its amplification. The amplification was achieved in 20 µ volumes by using PreMix PCR.[14] An agarose gel was used to examine the amplified PCR product. The PCR product is sequenced, and the BLAST bioinformatics program is used for identification. To identify the species, the resulting sequence was compared to a database of 16S rRNA sequences from the National Center of Biotechnological Information (NCBI).[15]

Table 1 — Universal 16S rRNA primers

Primer	Sequence	No. of nitrogen bases
104 F	5-CGGGTGAGTAACACGTG 3-	17
1390 R	5-CGGGTGTGTACAAGGCCC 3-	18

Protease Production and Partial Purification

Protease production was achieved according to Sharma *et al*.[16] with some modification; it took place in submerged fermentation conditions utilizing yeast extract casein medium. The medium used in production contained (g/L): glucose, 10; yeast extract, 5; casein, 5; K_2HPO_4, 2; KH_2PO_4, 2; $MgSO_4.7H_2O$, 1.0. After sterilization, the pH was adjusted to 9.0. After 48 hours, cultures were centrifuged (DM0424, DLAB Scientific Co., Ltd., Kuala Lumpur, Malaysia) at 10,000 rpm for 20 minutes, and the supernatants were exposed to cold acetone (−15°C) precipitation. The enzyme solution from step 1 was added to a volume of 2:3 (V:V) acetone while being stirred slowly. The suspension was instantly centrifuged for 10 minutes at 9,000 xg. The precipitate was placed in 0.01 M Tris-HCl buffer, pH 7.5, and dialyzed against 0.01 M Tris-HCl buffer. After dialysis, the enzyme activity and protein content were assessed.

Protease Assay

The method of Seyfzadeh *et al*.[12] was used to assess the activity of the protease. In a tube containing 1.8 mL of 1% (w/v) casein (dissolved in 0.2 M phosphate buffer, pH 7.2), a diluted enzyme (0.2 mL) was added. The tube was then incubated (100–800, Memmert, Schwabach, Germany) at 37°C for 30 min. The proteolysis was then stopped by adding 3 mL of a 5% (w/v) trichloroacetic acid solution. The blank solution was made in the same way as the working solution, minus the addition of the enzyme solution. The absorbance at 280 nm of the precipitate and the supernatant was measured using spectrophotometer (T60 U, PG Instruments ltd., Lutterworth, UK) after the mixture was filtered through filter paper (Whatman No. 1). A unit of protease is defined as the

amount of enzyme necessary to cause a 0.001 increase in absorbance at 280 nm/min. Protease activity was expressed as U/mL using the following equation:

$$\text{Enzymatic activity (U/mL)} = \frac{A280\ nm}{0.001\times30\times0.2}$$

where, A = absorbance at 280 nm; 0.001= from enzyme unit definition; 30 = reaction time (min); 0.2 = enzyme solution volume

Protease Purification

Ion Exchange Assay

Twenty grams of the DEAE Sephadex exchanger were dissolved in 500 milliliters of distilled water while stirring (Mtops MS300HS, PLT Scientific, Selangor, Malaysia) constantly, allowed to settle, heated in a water bath at 95°C for 5 hours while continuously stirring, and then filtered. The resulting slurry was placed in a vertical column and left overnight. The column was then treated with 30 mL of 0.5 N HCl solution and 30 mL of 0.5 N NaOH solutions. After that, 1 mL of 50 mM phosphate buffer (pH 7) was injected into the column with the desired dimensions (2.5 × 20 cm). After the column was properly equilibrated, a concentrated enzyme was carefully poured onto the surface of the beads. The phosphate buffer was used to wash the column to remove unbound proteins. The fraction collector (2110, BioRad, CA, USA) was configured to collect 3 mL of each fraction at a rate of 50 mL per hour. NaCl (0.1– 0.5M) dissolved in 0.05 M phosphate buffer is used to create a gradient elution that is used to dislodge other proteins bound to the column. Spectrophotometry at 280 nm and protease activity assays were used to determine each fraction's optical density and enzymatic activity, respectively. Fractions donating protease activity were pooled and kept at 4°C until the next purification step.[17]

Gel Filtration Assay

The gel filtration column was prepared using Sephadex G-100 According to manufacturer's instructions. An amount of 15 g of Sephadex powder was suspended in 500 mL of distilled water and heated with stirring using a hot plate stirrer to 90°C for 5 hours. Then, the suspension was washed twice with a phosphate buffer solution and suspended in an appropriate amount of the same solution. Next, the suspended Sephadex gel was degassed using a vacuum pump, then filled in the column and gradually on the inner wall of the column to give a gel with dimensions of 60 × 1.5 cm. The column was then equilibrated with phosphate buffer solution at 0.05 M and pH 7.0, three times the gel's volume. Following column preparation, the concentrated enzyme solution was gently added to the column surface and eluted with 0.05M phosphate buffer pH 7.0 at a flow rate of 40 mL/h (3 mL for each fraction). Each fraction's optical density (at 280 nm) and enzyme activity were determined. Protease activity fractions were pooled and stored at 4°C.

Molecular Weight Estimation

The protease's molecular weight was estimated using a 1.5 × 60 cm Sephadex G-100 gel column. The column is loaded with blue dextran solution, and the void volume (Vo) is calculated as the volume of the first eluted peak. As molecular marker proteins, glucose oxidase (80000), bovine serum albumin (67000), ovalbumin (45000), trypsin (23000), and myoglobin (17000) were utilized. The linear relationship between the ratio of the recovery volume of each protein marker to the void volume (Ve/Vo) versus its molecular weight was subsequently plotted.[18]

Thermostability Assay

The thermostability of the enzyme was measured following the method described in Ali *et al*.[19] with some modifications. The thermostability of the purified protease was evaluated at varying temperatures of 50°C, 60°C, 70°C, and 80°C for varying times (15 to 90 minutes) in the presence of 2 mM $CaCl_2$, and relative protease activities were determined under standard assay conditions. As a control, the enzyme was incubated at 50°C in the presence of calcium ions, and it was presumed to have 100% activity.

Statistical Analysis

Microsoft Excel version 2112 was used for the analysis. The standard deviation (S.D.) of replicate values was employed for rigorous statistical analysis. This allowed for the assessment of data consistency, measurement of variability, and determination of the significance of observed differences, ensuring the reliability of the results.

Results and Discussion

Identification of *Bacillus Subtilis* by 16S rRNA

Several bacterial isolates were found in soil samples from various locations in Wasit territory (Iraq). All of the bacterial cultures were evaluated using casein-hydrolyzed medium plates. Isolates showed the largest zone of hydrolysis on casein-hydrolyzed medium plates, making them the most

likely candidates to be significant protease-producing isolates. *Bacillus* strains were selected based on their microscopic and colony properties. *Bacillus subtilis* isolates are Gram-positive, aerobic, and spore-forming bacteria. Colonies that are typically off-white or cream in color and produce a distinctive wrinkled or folded appearance were selected. Isolate purity was confirmed through extensive streaking. The presence of a clearance zone indicates that *Bacillus subtilis* is producing protease. According to 16S rDNA analysis, the isolate was indeed *Bacillus subtilis*. The isolate was identified as *Bacillus subtilis* based on its morphology, biochemical profile, and 16S rRNA sequencing. The standard protocol for extracting and purifying genomic DNA was followed. Agarose gel analysis revealed a single band of DNA with a particularly high molecular weight. The 16S rRNA gene was amplified by PCR. Results in Fig. 1 show a single PCR amplicon band of about 1500 bp. The obtained PCR amplicon was subjected to additional purification and processing before being sequenced. The phylogenetic analysis and nucleotide homology of the 16S rDNA gene sequence for the isolated microbe in NCBI GenBank confirmed the isolate's close association with *Bacillus* sp. There was about 98% similarity to other strains of *Bacillus subtilis*. Based on the findings, the isolated strain of *Bacillus subtilis* will be subjected to further purification analysis. In accordance with the findings[20], a distinct band with a molecular weight of 1500 bp was observed, confirming the presence of genetic material belonging to the *Bacillus* genus.

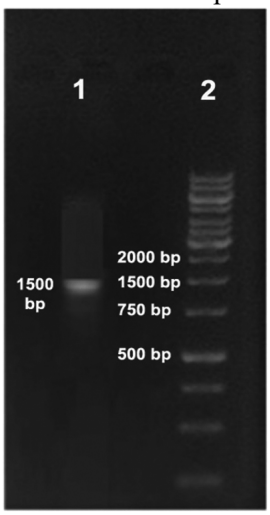

Fig. 1 — 1.2% agarose gel with a single 1500 bp and 16S rRNA amplicon band. Lane 1: 16S rRNA amplicon; Lane 2: ladder

Bacillus Subtilis Protease Purification Steps

Based on Table 2, the enzymatic activity and specific activity of the crude enzyme are 241.3 U/mL and 613.9 U/mg, respectively. After precipitation with chilled acetone, the partially purified enzyme exhibited enzymatic activity and specific activity of 935.0 U/mL and 745.0 U/mg, respectively. The increase in both enzymatic activity and specific activity after acetone precipitation indicates successful partial purification by removing impurities, resulting in a more concentrated and purified enzyme preparation. According to a previous study[21], the isolated *Bacillus subtilis* strain's crude filtrate protease had a relatively low enzymatic activity of 0.410 U/mg but a high overall protease activity of 165.9 U. Protease crude extract activity and specific activity were reported to be 5791.9 U and 2036 U/mg, respectively, in the interesting findings.[22] Previous research[23] revealed that the *Bacillus* protease pellet obtained after cold acetone precipitation had a specific activity of 4975.9 U/mg.

Ion Exchange Chromatography

The protease particle was chromatographed on a DEAE Sephadex G-100 column after partial purification of the enzyme with cold acetone. Elution was carried out in a phosphate buffer with a linearly increasing sodium chloride gradient (0.1–0.5 M). Results shown in Fig. 2 demonstrate that protease activity was eluted in a single peak when the ionic strength of the sodium chloride salt was increased. Polyethylene glycol was used to concentrate the activity-containing fractions to an 8 mL volume after they were dialyzed against phosphate buffer. After DEAE chromatographic purification, the enzyme's specific activity increased to 2491.8 U/mg, and its recovery was almost 90% (Table 1). The

Table 2 — Purification steps of *Bacillus subtilis* protease

Purification step	Volume mL	Activity U/mL	Protein mg/mL	Specific activity U/mg	Total activity U	No. of folds	Yield %
Crude enzyme	25	241.3 ± 9*	0.393 ± 0.02	613.9	6032.5	1	100
Acetone precipitation	6	935.0 ± 13	1.255 ± 0.04	745.0	5610.0	1.2	92.9
Ion exchange	8	672.8 ± 18	0.27 ± 0.04	2491.8	5382.4	4.0	89.2
Gel filtration	9	511.7 ± 17.3	0.12 ± 0.03	4264.1	4605.3	6.9	76.3

*Values are the mean of n ± standard deviation (n = 3)

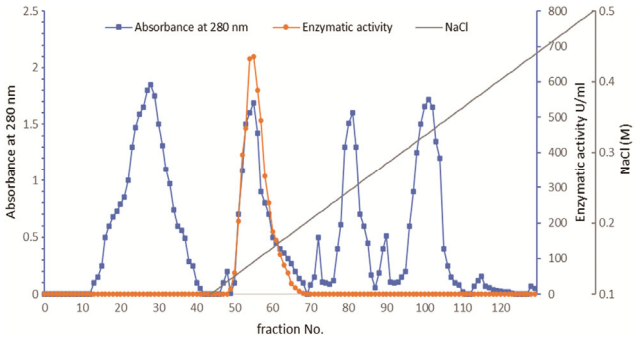

Fig. 2 — Ion exchange chromatography purification profile of protease from *Bacillus subtilis*. The partially purified enzyme solution was loaded onto a 2.5 cm × 20 cm DEAE Sephadex G-100 column. The column operated at a flow rate of 50 mL/h, and 3 mL fractions were collected using phosphate buffer for elution

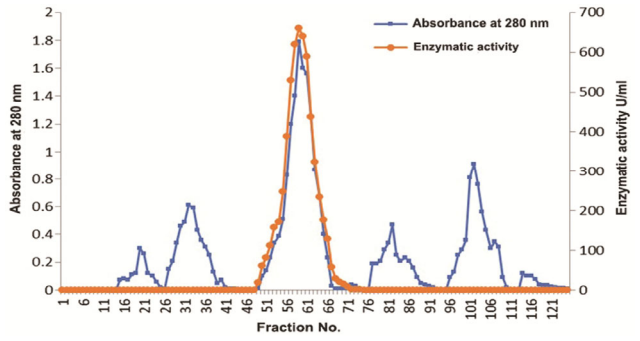

Fig. 3 — Gel filtration with 1.5 × 60 cm Sephadex G-100 equilibrated with 0.05 M phosphate buffer at pH 7.0 with a flow rate of 25 mL/h and a fraction volume of 3 mL

selective binding of the enzyme to the ion exchange resin results in increased specific activity and decreased impurities.[24] It found that the final purification of *Bacillus subtilis* protease by DEAE cellulose ion exchange chromatography led to a specific activity of 7040 U/mg and a 38.6% yield. D'Souza *et al.*[25] demonstrated that the specific activity increased to 876.36 U/mg when *Bacillus cereus* S46-protease was chromatographed on a DEAE cellulose column equilibrated with 1 M Tris.HCl buffer at pH 7.0.

Gel Filtration

Gel filtration with 1.5 × 60 cm Sephadex G-100 was used for further purification. The results in Fig. 3 displays 6 peaks of proteins and one peak representing protease activity was observed following elution. Protease activity was found in fractions 48–67. The enzyme's specific activity increased to 4264.10 U/mg, resulting in a final recovery of nearly 73% (Table 1). The use of gel filtration further purified the protease by separating it from proteins of different sizes. The specific activity increased significantly, and a high

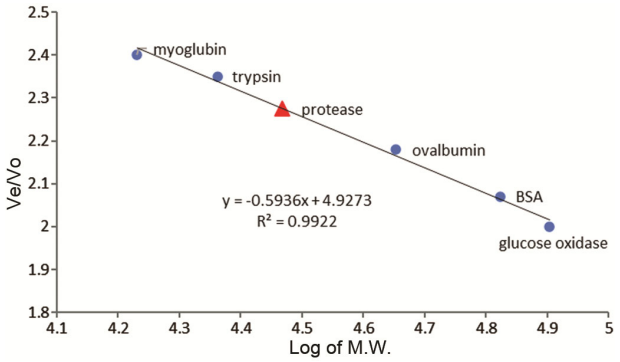

Fig. 4 — Standard curve for protease molecular weight determination using Sephadex G-100 (60 × 1.5 cm) gel filtration chromatography

recovery rate was achieved, making the enzyme preparation suitable for various applications due to its improved purity and concentration. Hu *et al.*[26] demonstrated that purifying protease from *Bacillus subtilis* with ammonium sulfate 40–70%, UNOsphere Q anion exchange, and Sephadex G–75 gel filtration led to a maximum specific activity of 10764.3 U/mg with a 66.0 fold purification and a 14.8% yield. Protease activity from *B. subtilis* was shown to increase 3.0 fold after gel filtration chromatography, with a specific activity of 80 U/mg and a recovery of 3.18%, as shown in the study.[27]

***B. Subtilis* Protease Molecular Weight**

The enzyme's molecular weight was determined using a Sephadex G-100 column. The protease's molecular weight was determined using a plot of Ve/Vo against log molecular weight (Fig. 4). The use of a Sephadex G-100 column allows for the estimation of the protease's molecular weight based on its elution behavior in comparison to known molecular weight standards. The *B. subtilis* enzyme has a molecular weight of 29.3 kDa, which falls within the molecular weight range of 27 to 71 kDa for *Bacillus*-derived proteases.[28] Mahmoud *et al.*[29] reported that SDS-PAGE analysis of *Bacillus subtilis* protease revealed the presence of a single band at approximately 48 kDa. Recently, Li *et al.*[30] discovered that after purification of *Bacillus cereus* protease, SDS–PAGE revealed a highly enriched protein band with a molecular mass of approximately 28 kDa.

***B. Subtilis* Protease Thermostability**

The findings from Fig. 5 indicate that the protease exhibited a state of initial stability when subjected to a temperature of 50°C. Nevertheless, when subjected to a temperature of 60°C, it demonstrated favorable initial stability. However, prolonged exposure to this

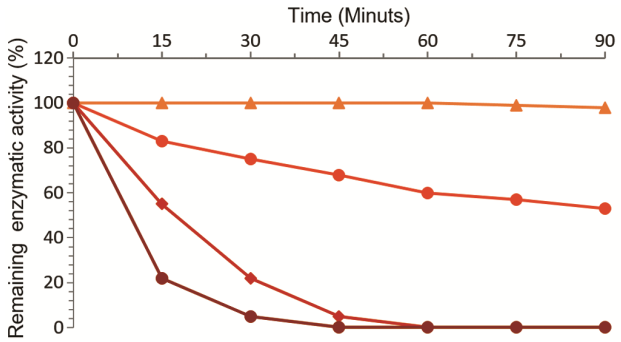

Fig. 5 — Relative thermostability of purified protease at different temperatures

temperature resulted in a decrease in its level of activity. In addition, when exposed to a temperature of 70°C, the rapid decline in activity made it inappropriate for use in high-temperature applications. The enzyme exhibited a rapid decline in activity when exposed to a temperature of 80°C. These findings are consistent with the fundamental rules regulating the behavior of enzymes when subjected to thermal stress. These principles include the denaturation of enzymes and the subsequent decline in their activity as a result of prolonged exposure to high temperatures.[31,32] The findings of Miao *et al.*[33] reveal a significant decrease in residual activity for the wild-type *Bacillus* protease as heat treatment times increase, notably reaching only 36.59% residual activity after exposure to 65°C for 30 minutes. Meena *et al.*[34] demonstrated that the optimum condition for protease production by *Pseudomonas aeruginosa* was found at temperature 45°C.

Conclusions

In conclusion, this study successfully isolated and purified a robust protease enzyme from a local *Bacillus* strain in southern Iraq. The enzyme displayed notable thermal stability, particularly at 60°C, for more than one hour, making it suitable for applications requiring elevated temperatures. However, its sensitivity to prolonged exposure to higher temperatures, such as 70°C and 80°C, indicates potential limitations. Future research avenues may explore genetic modifications and bioprocessing strategies to enhance the enzyme's thermal resilience. This study highlights the untapped potential of extremophilic microorganisms in extreme environments, promising opportunities for biotechnology and industrial sectors. The practical applications of this unique protease enzyme warrant further investigation and utilization, with potential benefits across various industries. This work represents a stepping stone in harnessing extremophilic microorganisms for biotechnological advancements, aligning with the growing demand for tailored enzymes.

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Conflict of interest

The authors declare that there is no conflict of interest among the authors of the manuscript.

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