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Partial purification, characterization and application of thermoalkaliphilic proteases from *Priestia endophytica*, *Lysinibacillus cresolivorans* and *Bacillus subtilis* isolated from desert soil

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ABSTRACT

Aims: Thermophilic proteases are important industrial enzymes because they can be used at high temperatures in various bioprocessing schemes. The bacterial population of the Cholistan desert was explored for thermophilic proteases and their industrial applications.
Methodology and results: Three bacterial isolates K1, K5 and K7 were found promising protease producers. These isolates were preliminary identified as *Bacillus* based on morphological characteristics and biochemical tests (positive for catalase, oxidase and citrate tests, and negative for indole and urease tests). The isolates K1, K5 and K7 were further identified as *Priestia endophytica*, *Lysinibacillus cresolivorans* and *Bacillus subtilis*, respectively by phylogenetic analysis. The isolates grew best at 50 °C and *P. endophytica* (K1), *L. cresolivorans* (K5) and *B. subtilis* (K7) produced larger zones of hydrolysis at 37 °C, 45 °C and 50 °C at pH 7, respectively. The optimum temperature where protease activity was maximum was 65 °C for *P. endophytica* and *L. cresolivorans* and 55 °C for *B. subtilis*, and the optimum pH was 9.
Conclusion, significance and impact of study: The proteases produced by these isolates were found active at high temperatures (45 °C to 85 °C) and high pH (9-12), which make them industrially important thermoalkaliphilic proteases. These proteases successfully de-haired cow's skin and de-stained blood from cotton cloth pieces, which are rarely tested applications of these proteases.

Keywords: Desert, proteases, purification, thermophilic bacteria

INTRODUCTION

The global enzyme demand is increasing by 6.4% annually and proteases represent the largest commercially exploited enzyme, thus placed at the top of the list of industrially important enzymes (Naveed *et al.*, 2021). Proteases demonstrate diverse physicochemical and biological functions and hence are used in food, detergent, silk degumming, pharmaceutical, leather, film and waste processing industries. Therefore, protease accounts for more than 60% of the total enzyme sales worldwide (Singh *et al.*, 2016). In the leather industry, protease is used to remove hairs from animals' skin and used for cleaning of different kinds of stains like blood, grass and beetle in detergent industries. Around 70% of total proteases are used in leather and detergent industries (Razzaq *et al.*, 2019).

Nowadays, most of the industrial reactions take place in extreme conditions where normal enzymes denature. Therefore, industries demand enzymes that can withstand harsh environmental conditions like high pH, temperature and salt concentrations. Here, extremozymes are good alternatives because they can withstand harsh environmental conditions, are specific in action and environmentally friendly (Singh *et al.*, 2011). Thermophilic bacteria are considered one of the best sources of thermostable enzymes (TE), which make them a suitable target for many industrial processes. These thermophilic bacterial strains reside in high-temperature habitats like hot springs, deserts and deep-sea hydrothermal vents etc (Mohammad *et al.*, 2017; Panosyan, 2017).

Proteases are very important and ubiquitous extracellular enzymes found in different sources i.e., plants, animals and microbes. But the enzymes obtained

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from bacteria are preferred because of their easy production, extraction and purification with less cost and time (Niyonzima and More, 2015). Thermophilic proteases (TP) produced by thermophilic bacteria have gained attention because of their activeness and stability at varying temperatures and hence are of great technological and industrial importance. These TP are not only active at higher temperatures (85 °C), but sometimes they are also active at variable pH (3-13) and in the presence of organic salts (Santra and Banerjee, 2021). A huge number of TE have been reported from bacterial origin and are being used in various biotechnological applications. Among them, genus *Bacillus* is a good and dominant source for TE (Razzaq *et al*., 2019; Gimenes *et al*., 2021). Bacterial enzymes are attractive for different reasons, such as their extracellular nature, easy and cheap extraction and most enzymes are regarded as safe for use. Some of the *Bacillus* species producing industrially important proteases are *B. subtilis* and *B. licheniformis* (Parrado *et al*., 2014; Uttatree and Charoenpanich, 2016).

Many microorganisms contain genes for protease enzyme, but we cannot use them for industrial-scale production. So, cloning is done in other microorganisms in order to achieve large-scale production (Saggu and Mishra, 2017). Many studies reported mesophilic proteases that are stable till 50 °C. However, less data is available about the thermostability of microbial proteases. Deserts usually have high temperatures and the temperature usually changes with climate, hence inhabit diverse microbial communities that have adapted to different environmental conditions (Cherif *et al*., 2015).

Since these microbes adapt to environmental fluctuations and hence can result in the isolation of robust enzymes that could be able to withstand extreme conditions. In a study, *Bacillus cereus* RS3 was isolated from the desert in Riyadh, Saudi Arabia, with thermophilic capabilities (Shine *et al*., 2016). However, there is still needed to search for polyextremophilic bacteria producing enough proteases that can stand active at different harsh conditions. Therefore, this study is designed to explore the bacterial population from the Cholistan desert, Pakistan, for purification and characterization of TP and their industrial applications.

MATERIALS AND METHODS

Sample collection and bacterial isolation

A total of 50 g of soil samples (n=10) were collected from the desert of Cholistan, Pakistan (28.5°N 71.5°E) in sterile bottles and were stored until analysis at 4 °C. The samples were serially diluted using distilled water and about 100 µL mixture was poured onto nutrient agar (NA) plates and incubated at 50 °C for 48 h (Mohammad *et al*., 2017). Morphologically different colonies were further purified by repeated streaking several times.

Screening for proteolytic activity

Purified isolates (n=48) were dot inoculated on 1.5% (w/v) skim milk agar (SMA) plates and were incubated for 48 h at 50 °C. The isolates that produced a clear zone of hydrolysis (CZH) around their colonies were recognized as protease-producing (PP) isolates (Shine *et al*., 2016). Among them, three efficient protease-producing isolates (K1, K5 and K7) were selected for further studies such as identification, optimization of growth conditions, protease production and activity.

Identification of bacterial isolates

The selected isolates were preliminarily identified by Gram staining and biochemical characteristics. Gram staining was performed as per the method described previously and biochemical tests were performed by API kit (20E CHB) (bioMerieux, www.biomerieux.com) as per manufacturer instructions. After preliminary identification, the isolates were further identified by 16S rRNA gene-based phylogenetic analysis. The genomic DNA (gDNA) was extracted as described previously with little modifications (Packeiser *et al*., 2013). Briefly, bacterial colonies were picked from a fresh culture using a sterile tooth pack followed by inculcation in 200 µL TE buffer and placed at 90 °C for 10 min in a water bath. The gDNA was obtained after centrifugation at 12000 rpm for 5 min. The 16S rRNA gene was amplified in thermocycler using 16S rRNA gene primers, 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1510R (5′-GGCTACCTTGTTACGA-3′) using conditions described by Ali *et al.* (2016). The PCR products were sequenced (MACROGEN, Korea http://dna.macrogen.com/eng/) and BLASTed in the server of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for the related strains. Multiple alignments of the related strains was performed using ClustalW and phylogenetic analysis was inferred by using the Maximum Likelihood method and Tamura-Nei model in MEGA-X software and the bootstrap values were calculated based on 1000 replications (Kumar *et al*., 2018). The 16S rRNA sequences of K1, K5 and K7 were deposited in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MN493608, MN493618 and MN493619, respectively.

Bacterial growth and protease production

The growth of the isolates was optimized at different incubation temperatures and pH. For this, the isolates were grown in 10 mL Luria-Bertani (LB) broth media and were incubated in a shaking incubator at different temperatures (37, 45, 50, 55, 60, 65 and 70 °C) and pH (4, 5, 6, 7, 8, 9, 10 and 11) for 48 h at 120 rpm. After 48 h, the bacterial growth was measured by a spectrophotometer at 600 nm. The isolates were dot inoculated on SMA plates and incubated for protease production at different temperatures (37, 45, 50, 55, 60

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and 65 °C) and pH (4, 5, 6, 7, 8, 9 and 10) for 48 h. 1.5% (w/v) skim milk was used for temperatures less than 55 °C, while for incubation at 55 °C and above, 2% (w/v) skim milk was used. After 48 h of the incubation, the CZH were measured in a millimeter (mm) scale using a measuring tape (Hamza, 2018; Shaheen *et al*., 2008).

Optimization of protease activity of the isolates

Protease activity was optimized using protease assay as described by (Ali *et al*., 2016). Briefly, the isolates were grown in LB broth at 50 °C and a crude extract of extracellular enzymes was obtained by centrifugation at 10000 rpm for 2 min. Then, 100 µL solution of 0.5% (w/v) casein prepared in 100 mM phosphate buffer was added to 100 µL crude enzyme and incubated at 37, 45, 50, 55, 60, 65, 70, 75, 80 and 85 °C for 30 min and for pH optimization phosphate buffer for pH 5-8, and glycine buffer for pH 9-12 were used and incubated at 37 °C for 30 min. The reaction was terminated by adding 100 µL of 15% (w/v) trichloro acetic acid (TCA) and the mixture was placed at room temperature. After 10 min, the mixture was centrifuged at 13000 rpm for 10 min and the supernatant was transferred to another tube. Finally, 750 µL 0.4 M Na_2CO_3 and 150 µL Folin-Ciocalteu's phenol reagent (3 fold diluted) were added and incubated at 40 °C for 20 min before measuring absorbance at 660 nm.

Partial purification and SDS-PAGE

The crude enzyme extract was purified from all three strains by ammonium sulfate precipitation and the cultures harvested were centrifuged at 9300 rpm for 30 min. Ammonium sulfate at 70% saturation was added to the cell-free culture and shacked continuously to precipitate the protease. After centrifugation at 9300 rpm for 30 min, pellets were obtained and the resultant pellets were dissolved in 5.0×10^{-7} mM (0.5 nmol/L) phosphate buffer pH 7 (Ali *et al*., 2016).

The protease was partially purified and its molecular weight was determined using 12% SDS-PAGE under reducing conditions as described by Laemmli (1970) with slight modifications. The partially purified enzyme was mixed with loading dye and heated for 5 min in a water bath and then loaded with Thermo Scientific PageRuler Prestained Protein Ladder (14-170 kDa) for 2-2.5 h at 120 V. The gel was stained with Coomassie Brilliant Blue for 30 min and then destained for about 2 h and results was captured using camera and observed.

Protease applications of destaining and dehairing

For the destaining and dehairing potential, partially purified proteases were used. A white cotton cloth piece (4 cm^2) was stained with a drop of human blood, dried and treated with formaldehyde. The dried cloth piece was placed in 30 mL distilled water and treated with 10 mL of proteases (200 U/mL) (extracted each from isolate K1, K5 and K7) followed by incubation at 50 °C in a shaking incubator at 120 rpm for 15 min. Similarly, the cloth piece was treated with 10 mg/mL of local detergent (Bonus) and distilled water as a positive and negative control, respectively. The results were observed, photographed, and compared (Rehman *et al*., 2017). The dehairing process was carried out using a piece of cow's skin (3 cm^2) and incubated in 50 mL protease extract (200 U/mL) for 12 h, while control was incubated in distilled water. After incubation, the skin was rubbed and flooded with water. The results were photographed for comparison (Rehman *et al*., 2017).

Statistical analysis

All the experiments were performed in triplicate and the standard deviation of the data was analyzed in GraphPad Prism version 8.0.

RESULTS

Isolation and identification of protease producing bacteria

A total of 48 bacterial isolates of different colony morphology were isolated from the soil of the Cholistan desert, Pakistan. Among them, 26 (54%) bacterial isolates showed proteolytic activity and three isolates K1, K5 and K7 produced larger zones (19 mm, 24 mm and 26 mm, respectively) of hydrolysis on SMA plates and were therefore selected for further studies as efficient protease producers (Figure S1).

These isolates were preliminary identified *Bacillus* based on morphological characteristics and biochemical tests (positive for ONPG, ADH, CIT, TDA, GEL, GLU, MAN, INO, RHA, SAC and MEL tests and negative for ODC, URE, H_2S, IND and AMY tests) (Table S1). The isolates K1, K5 and K7 were further identified as *Priestia endophytica*, *Lysinibacillus cresolivorans* and *Bacillus subtilis*, respectively by 16S rRNA phylogenetic analysis. The 16S rRNA gene sequence was BLASTed at NCBI database, which revealed that the isolate K1 has 94.63% sequence identity and 97% query coverage with *Bacillus endophyticus* (now *Priestia endophytica*) strain 2DT and 94.79% sequence identity and 94% query coverage with *Bacillus filamentosus* (now *Priestia filamentosa*) strain SGD-14 (Oren and Garrity, 2020; Schoch *et al*., 2020). The isolate K5 has 99.89% sequence identity and 97% query coverage with *Lysinibacillus cresolivorans* strain SC03 and 99.68% sequence identity and 98% query coverage with *Lysinibacillus boronitolerans* strain 10a. The isolate K7 has 99.89% sequence identity and 96% query coverage with *Bacillus subtilis* strain 168, and 99.68% sequence identity and 99% query coverage with *Bacillus velezensis* strain FZB42 (Table 1). The Maximum Likelihood phylogenetic tree revealed that strain K1 belongs to the genus *Priestia* and make a separate clade with *Priestia endophytica* strain 2DT (NR_025122.1) (Figure 1). It is further revealed that strain K5 is affiliated with the genus *Lysinibacillus* and forms a distinct phyletic line with *Lysinibacillus cresolivorans* strain SC03 (NR_145635.1) and K7 strain is affiliated with the genus

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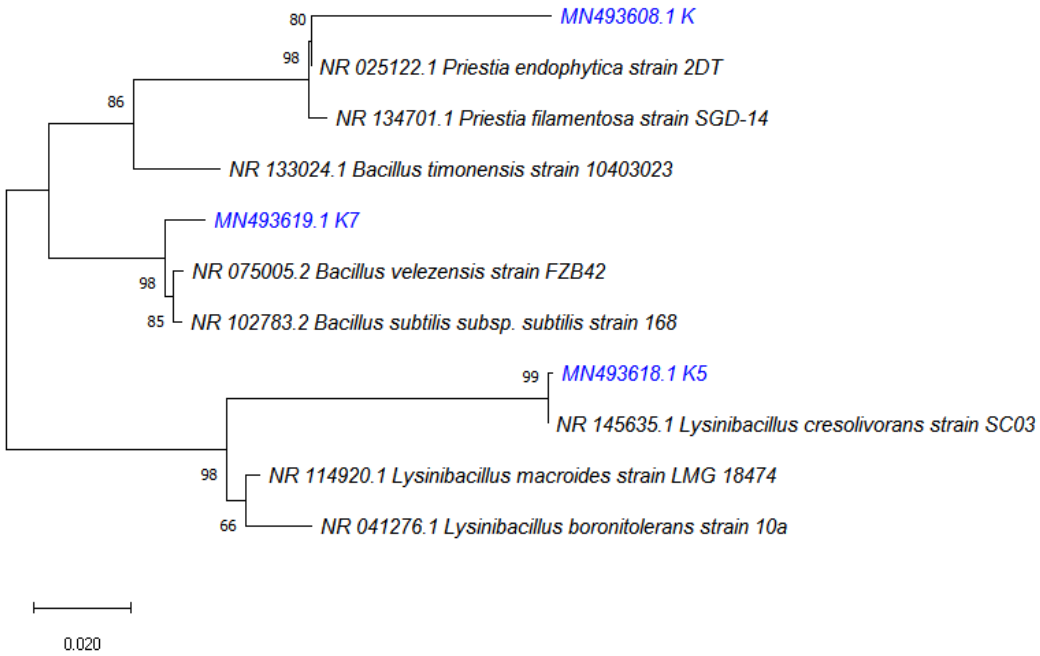

Figure 1: A maximum likelihood phylogenetic tree based on variation in 16S rRNA gene sequence.

Table 1: 16S rRNA gene sequence NCBI BLAST results of K1, K5 and K7.

Isolate	Description	Identity	Query coverage	Accession number
K1	*Bacillus filamentosus* strain SGD-14	94.79%	94%	NR_134701
	Bacillus endophyticus strain 2DT	94.63%	97%	NR_025122
K5	*Lysinibacillus cresolivorans* strain SC03	99.89%	97%	NR_145635
	Lysinibacillus boronitolerans strain 10a	99.68%	98%	NR_041276
K7	*Bacillus subtilis* strain SBMP4	99.89%	96%	NR_118383
	Bacillus velezensis strain FZB42	99.68%	99%	NR_075005

Bacillus and share clade with *Bacillus subtilis* strain 168 (NR_025122.1) and *Bacillus velezensis* strain FZB42 (NR_075005.2) (Figure 1).

Optimization of bacterial growth

Growth of bacterial isolates (*Priestia endophytica* strain K1, *Lysinibacillus cresolivorans* strain K5 and *Bacillus subtilis* strain K7) were observed till 70 °C and the optimum temperature for growth was recorded at 50 °C, which indicated the thermophilic nature of the isolates (Figure S2A). The optimum pH for the growth of three isolates were recorded as pH 7. However, growth was also observed till pH 11, which revealed the alkaliphilic nature of these bacteria (Figure S2B). The diverse and extreme culturing conditions of these isolates may be possibly due to their varied habitat (desert), where bacteria face harsh environmental conditions for their survival.

Protease production and activity

In this study, the optimum temperature where maximum protease production and activity were observed is 65 °C. However, the temperature for high protease production varied for each bacterium such as *P. endophytica* strain K1, *L. cresolivorans* strain K5 and *B. subtilis* strain K7 produced maximum zones of hydrolysis at 37 °C (28 mm), 45 °C (25 mm) and 50 °C (26 mm), respectively (Figure 2A). Similarly, the highest production and proteolytic activity for all isolates were seen at pH 7, however production was observed till pH 10 (Figure 2B).

Different temperature and pH conditions affect the stability and activity of proteases; therefore, temperature and pH were optimized. The optimum activity of proteases produced by *P. endophytica* strain K1 was observed 170 U/mL at 65 °C and *L. cresolivorans* strain K5 activity was 165 U/mL at 65 °C. However, maximum protease activity of 190 U/mL was observed at 55 °C for *B. subtilis* strain K7 (Figure 3A). Here optimum activity refers to the highest activity or production of proteases at specific temperature or pH. The proteases produced by these isolates were also active at high pH 5 to 12. While, the maximum activity of the proteases produced by *P. endophytica* strain K1 (172.3 U/mL), *L. cresolivorans*

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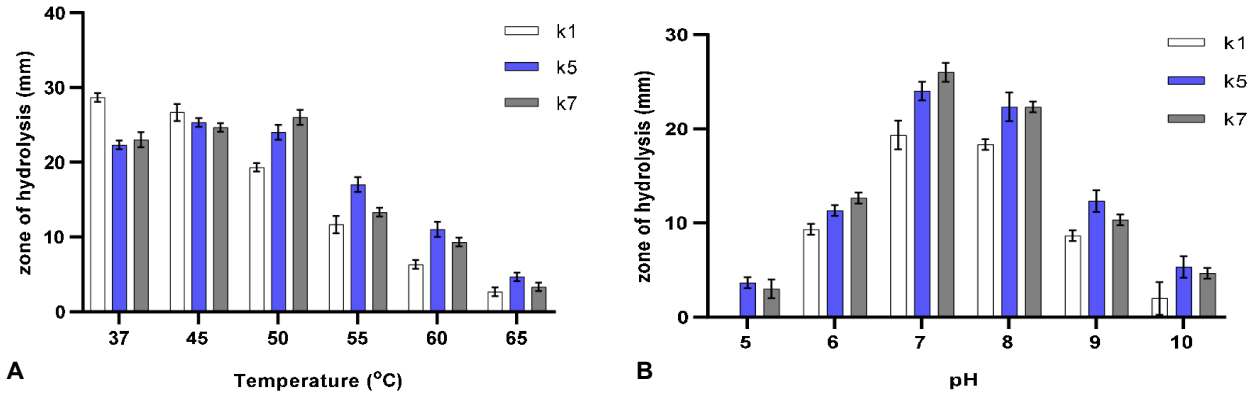

Figure 2: Protease production from *Priestia endophytica* strain K1, *Lysinibacillus cresolivorans* strain K5 and *Bacillus subtilis* strain K7 at different (A) temperature and (B) pH.

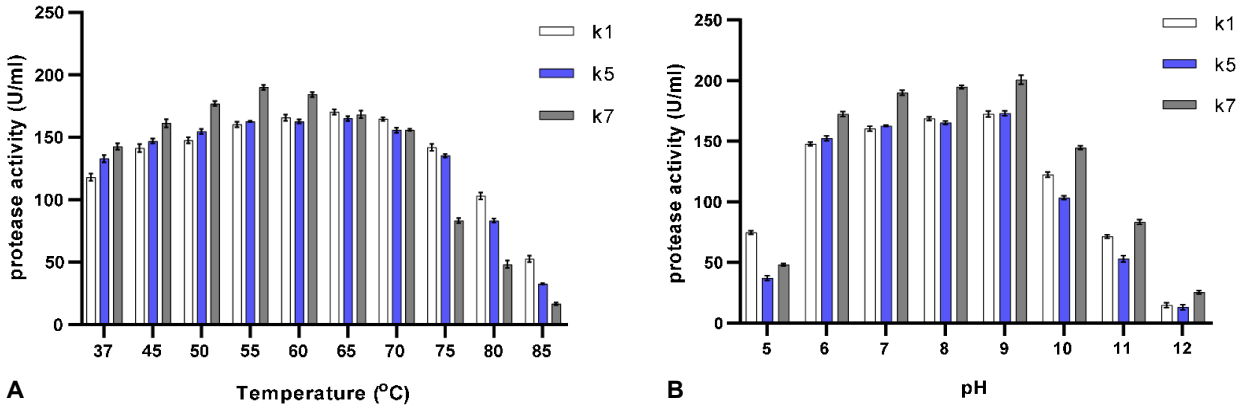

Figure 3: Protease activity optimization isolated from *Priestia endophytica* strain K1, *Lysinibacillus cresolivorans* strain K5 and *Bacillus subtilis* strain K7 at different (A) temperature and (B) pH.

strain K5 (173 U/mL) and *Bacillus subtilis* strain K7 (200.6 U/mL) was observed at pH 9 (Figure 3B).

Partial purification of protease

After the partial purification of protease, SDS-PAGE was run to determine the molecular weight of protease from *P. endophytica* strain K1, *L. cresolivorans* strain K5 and *B. subtilis* strain K7. Cell-free proteases in supernatant broth produced by the strains were partially purified by ammonium sulfate. SDS-PAGE results showed that the partially purified protease from all 3 isolates were aligned with marker protein having size of approximately 40 kDa by comparing with the ladder (Figure 4).

Industrial applications of protease enzymes

After the successful partial purification, the destaining and dehairing potential were investigated. The enzyme showed the potential to remove human blood stains from white cotton cloth (Figure 5). The protease enzymes revealed significant destaining potential as shown in Figure 5 and this suggested the importance of these proteases for industrial level applications after purification by chromatographic methods. The proteases isolated in

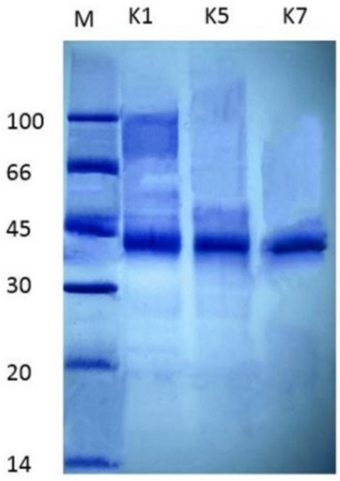

Figure 4: Partially purified enzyme on SDS-PAGE form isolate *Priestia endophytica* strain K1, *Lysinibacillus cresolivorans* strain K5 and *Bacillus subtilis* strain K7 having size of 40 kDa.

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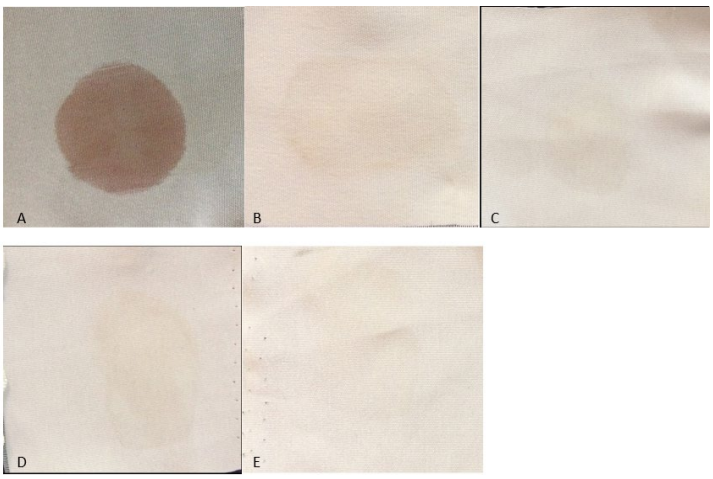

Figure 5: Blood de-staining with crude protease and local detergent. (A) Treated with distilled water, (B) Crude protease from *Priestia endophytica* strain K1, (C) *Lysinibacillus cresolivorans* strain K5, (D) *Bacillus subtilis* strain K7 and (E) With local detergent.

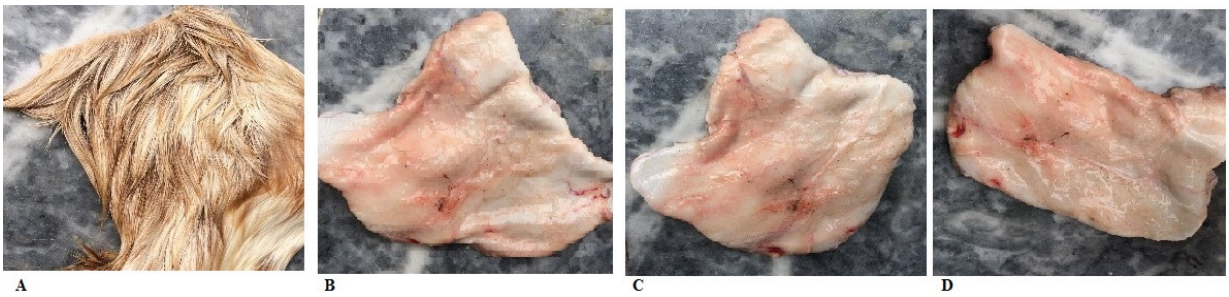

Figure 6: Dehairing of cow's skin with distilled water and crude proteases. (A) Treated with distilled water, (B) Crude protease from *Priestia endophytica* strain K1, (C) *Lysinibacillus cresolivorans* strain K5, (D) *Bacillus subtilis* strain K7.

this study were also checked for their dehairing potentials using cow's skin. These proteases revealed the dehairing potential as shown in Figure 6. Enzyme from all the three isolates have successfully removed hairs from cow's skin.

DISCUSSION

Proteases are the chief marketing enzymes worldwide because of their usage in leather, detergent, textile, food and pharmaceutical industries (Gimenes *et al*., 2021). The initial identification of protease producing (PP) bacteria was observed by direct observation of clear zones produced by PP bacteria on SMA plates. This technique has also been used by many researchers for initial screening of PP bacterial species based on CZH (Ali *et al*., 2016; Asha and Palaniswamy, 2018; Sridhara *et al*., 2021).

The initial identification of bacteria was done using API kit. This method has been used previously by several researchers to identify bacteria based on biochemical characteristics e.g., the identification of *Bacillus* sp. isolated from marine water (Alnahdi, 2012), form tropical fruit flies (Thaochan *et al*., 2010) and from municipal wastewater (Sonune and Garode, 2018) were done using same method. The 16S rRNA gene sequencing method used in this study for the molecular identification of bacteria, that is more authentic method for bacterial identification (Janda and Abbott, 2007, Kai *et al*., 2019). The 16S rRNA is a robust technique that is commonly used for the identification, genetic studies and diversity of bacteria (Gulmus and Gormez, 2020). We have identified our bacterial isolates belong to *Bacillus* sp. that is the diverse group among all bacterial species. Our results are in accordance with other researchers who used 16S rRNA gene sequences NCBI BLAST for the identification of bacteria (Mohammad *et al*., 2017; Fachrial *et al*., 2021). In similar fashion, Shine *et al.* (2016) also identified *Bacillus* from desert soil of Riyadh, Saudi Arabia with potential of protease production using 16S rRNA sequencing. In one of our previous article, *Bacillus* was reported as protease producer from the salt mines of Karak, Pakistan (Ali *et al*., 2016). It can be concluded that *Bacillus* is a diverse group of bacterial species that can produce protease enzymes and are usually found abundantly in harsh environmental habitats.

The best growth for all of our isolates were observed at 50 °C and have tolerated temperature till 70 °C. A slightly higher (75 °C) optimum temperature for growth

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than our study was reported by researchers who have isolated bacteria from hot springs (El-Gayar *et al.*, 2017). The higher temperature tolerance could be due to the variable conditions of the extreme sampling sites. Growth optimization is necessary because microorganisms used in fermentation technology need to be optimized at different parameters like pH and temperature, which is a prime step in fermentation technology. Therefore, we optimized the growth conditions both at different temperatures and pH like one previously reported by Aanniz *et al.* (2015), who had isolated bacterial isolates from Moroccan hot springs, deserts and salt marshes and have optimized bacterial growth at different high temperatures (50-75 °C) (Aanniz *et al.*, 2015).

Qualitative proteolytic optimization is the direct observation and measurement of clear zones on SMA plates (Omer and Humadi, 2013; Saxena *et al*., 2014). Our results revealed that proteases produced by all the three isolates were active at a wide range of temperature (37 °C to 85 °C), which make them industrially important thermostable proteases. Based on the literature, the optimum temperature for protease activity for a thermostable bacteria isolated from hot springs is 50 °C; however, proteases activity was also observed at a higher temperature such as 90 °C (El-Gayar *et al*., 2017). In a previous study, an alkaline PP *Bacillus* specie have been reported from salt mines (Sehar and Hameed, 2011). However, the thermoalkaliphilic proteases are least reported from desert soil, especially from deserts located in Pakistan which can be used for bio-industrial applications at extreme operational conditions.

We have partially purified thermoalkaliphilic protease from *P. endophytica* strain K1, *L. cresolivorans* strain K5 and *B. subtilis* strain K7 having the molecular weight of 40 kDa. The size range 17 to 50 kDa is a commonly reported molecular weight for different proteases produced by *Bacillus* species (Tang *et al.*, 2004; Satheeskumar *et al.*, 2013). The enzymes from the isolates were tested for its potential to remove blood clots and the results obtained were promising. The potential of proteases as detergent additives is previously reported by few researchers (Mendes *et al*., 2009; Bezawada *et al*., 2011; Emran *et al*., 2020). However, the blood destaining potential of proteases is rarely reported. These thermoalkaliphilic proteases favour the stability and compatibility of these enzymes as detergent additives. The dehairing potential of thermoalkaliphilic proteases is under studied. Usually, chemical detergents are used for dehairing processes in the leather industry which is not an eco-friendly process (Thanikaivelan *et al*., 2004; Hakim *et al*., 2018). There is a report for the dehairing ability of proteases was reported for alkaline proteases by Hakim *et al.* (2018). But in this study, we have isolated and investigated the dehairing potential of proteases that can stand active at high temperatures and pH. The destaining and dehairing potentials of these proteases can be exploited at extreme thermoalkaliphilic conditions for industrial applications, including the leather and textile industry.

CONCLUSION

The three bacterial isolates were selected from soil of the Cholistan desert which revealed the bacterial growth and protease production at broad ranges of temperature (37 °C to 70 °C) and pH (7 to 11). Although the optimum pH where protease activity (U/mL) was observed maximum was at pH 9 for all three isolates and the optimum temperature was 65 °C in case of *P. endophytica* strain K1 and *L. cresolivorans* strain K5, while 55 °C for *B. subtilis* strain K7. However, the proteases produced by these isolates have shown proteolytic activity at high temperatures (37 °C to 85 °C and high pH 9-12), which make them industrially important thermoalkaliphilic proteases at extreme operational conditions. The proteases also successfully removed blood stains from cotton cloth and hairs from cow's skin, which revealed the importance of these enzymes as detergent and/or additive of detergent in the leather and textile industry. This study also recommended that the Cholistan desert represents an invaluable microbial flora that can be explored for the production of other important enzymes and biomolecules of industrial importance.

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AUTHORS' CONTRIBUTIONS

IU performed the experiments, conducted the statistical analysis and wrote the draft manuscript and NA helped IU, and equally contributed as the first author with IU. WU, MQ, MN and NU analyzed the data and provided suggestions to improve the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY INFORMATION

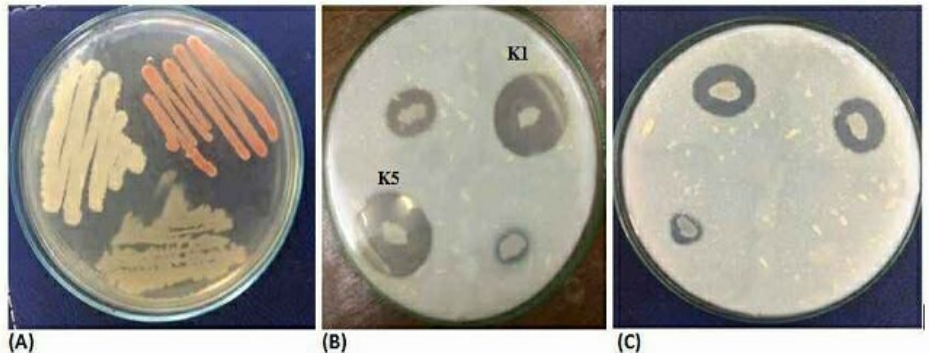

Figure S1: Growth of desert soil bacteria K1, K5 and K7 on NA and SMA plates. (A) Orange colonies of K1 and yellow colonies of K5 and K7 on NA plates, (B) clear zone of hydrolysis around K1, and K5 and (C) K7 colonies on SMA plates indicating proteolytic activity**.**

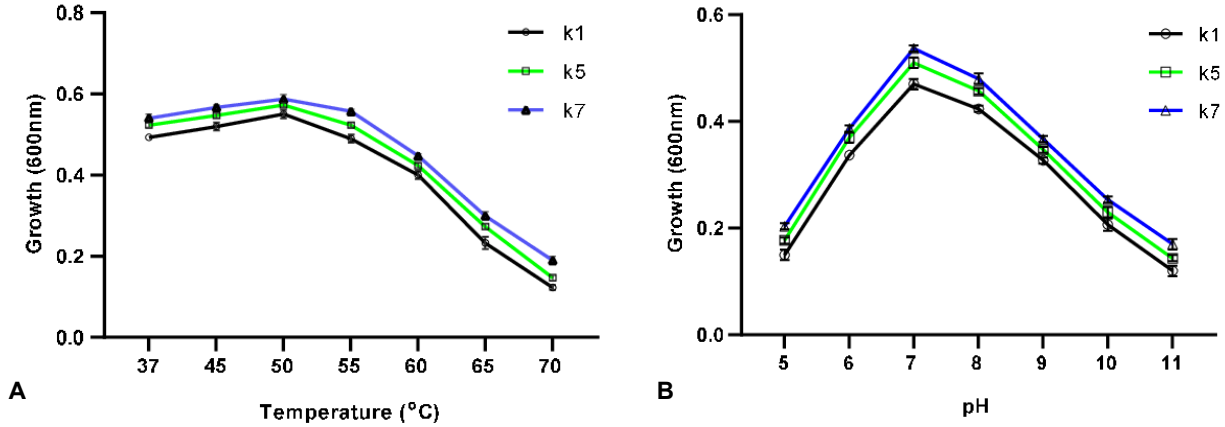

Figure S2: Growth of *B. filamentosus* strain K1, *L. cresolivorans* strain K5 and *Bacillus subtilis* strain K7 at different (A) temperature and (B) pH.

Table S1: Biochemical characteristics of the selected isolates K1, K5 and K7.

Biochemical tests	K1	K5	K7
ONPG	+	+	+
ADH	+	+	+
LDC	+	-	-
ODC	-	-	-
CIT	+	+	+
H_2S	-	-	-
URE	-	-	-
TDA	+	+	+
IND	-	-	-
VP	-	-	+

Biochemical tests	K1	K5	K7
GEL	+	+	+
GLU	+	+	+
MAN	+	+	+
INO	+	+	+
SOR	+	+	-
RHA	+	+	+
SAC	+	+	-
MEL	+	+	+
AMY	-	-	-
ARA	+	-	-

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