Purification, characterization and application of thermoalkaliphilic proteases from Bacillus filamentosus, Lysinibacillus cresolivorans, and Bacillus subtilis

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Abstract

Thermophilic proteases are important industrial enzymes because they can be used at unavoidable temperature in various bioprocessing schemes. The bacterial population of Cholistan desert was explored for thermophillic proteases and their industrial applications. 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 *Bacillus filamentosus, Lysinibacillus cresolivorans*, and *Bacillus subtilis*, respectively by phylogenetic analysis. The isolates grew best at 50^{o}C while *Bacillus filamentosus* (K1), *Lysinibacillus cresolivorans* (K5), and *Bacillus subtilis* (K7) produced larger zones of hydrolysis at 37^{o}C, 45^{o}C and 50^{o}C at pH 7, respectively. The optimum temperature for protease activity was 65^{o}C for *Bacillus filamentosus* and *Lysinibacillus cresolivorans* and 55^{o}C for *Bacillus subtilis*, and the optimum pH for activity was 9 for all the three strains. The protease produced by these isolates were found active at high temperature (37^{o}C to 85^{o}C) and high pH (5–12) which make them industrially important thermoalkaliphilic proteases. Theses proteases successfully de-haired cow's skin and de-stained blood from cotton cloth pieces, which are rarely tested applications of these proteases.

Key Points

- Deserts have microflora that inhabits diverse bacterial species of different attributes that are of great industrial importance
- These bacterial species have unique ability to tolerate high temperature and pH
- The protease enzyme from these bacterial species can be used in leather and detergent industries as additives

Introduction

The global enzyme demand is increasing by 6.4% annually and proteases represents the largest commercially exploited enzyme, thus placed at the top of the list of industrially important enzymes (Dumorné 2017). Proteases demonstrate diverse physicochemical and biological functions and hence they 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 2016). In leather industry, protease is used to remove hairs from animal's skin and used for cleaning different kinds of stains like blood, grass and beetle in detergent industries. Around 70% of total proteases are used in leather and detergent industries (Mienda 2014). Nowadays, most of the industrial reactions take place at extreme conditions where normal enzymes denature, therefore, industries demand enzymes that can withstand harsh environmental conditions. Here, extremozymes are good alternatives because they can withstand harsh environmental conditions, are specific in action and environment friendly (Singh 2011). Thermophilic bacteria are considered a good source of thermostable enzymes (TE)

which make them a suitable target for many industrial processes. These thermophilic bacterial strains reside in high temperature habitat like hot springs, deserts and deep-sea hydrothermal vents etc. (Mohammad 2017; Panosyan 2017). Proteases are very important and ubiquitous extracellular enzymes found in different sources i.e. plants, animals, and microbes. But, that of bacterial origin are preferred because of easy production, extraction, and purification with less cost and time (Niyonzima and More 2015). Thermophilic proteases (TP) produced by thermophilic bacterial strains have gained attention because the enzymes produced by thermophiles are active and stable at varied temperature and therefore are of great technological and industrial importance. Proteases isolated from these sources are not only active at higher temperature but sometimes they are also active at variable pH as well as in presence of organic salts (Akel 2009). A huge number of TE have been reported from bacterial origin that are being used in various biotechnological applications, among them, genus *Bacillus* have remained a good and dominant source (Kamran 2015; Mienda 2014). Bacterial enzymes are attractive for different reasons such as their enzymes are extracellular and regarded as safe for use. Some of the *Bacillus* species producing industrially important proteases are *Bacillus subtilis* and *Bacillus licheniformis* (Parrado 2014; Uttatree and Charoenpanich 2016).

Many microorganisms contain genes for protease enzyme but, we cannot use them for large industrial scale production. So, cloning is done in other microorganisms that can produce proteases in efficient way (Saggu and Mishra 2017). Many studies reported mesophilic proteases that are stable till 50^{o}C, however, less data is available about the thermostability of microbial proteases, that are important for industrial applications. Deserts usually have high temperature and the temperature usually changes with climate, hence inhabit diverse microbial community that have adapted to different environmental conditions (Cherif 2015). Since these microbes adapt environmental fluctuations and hence can result in isolation of robust enzymes that could be able to withstand extreme conditions. In a study, *Bacillus cereus* RS3 was isolated from desert in Riyadh, Saudi Arabia with thermophilic capabilities (Shine 2016). However, there is still need to search for polyextremophilic bacteria producing enough proteases that can stand active at different harsh conditions. Therefore, this study is designed to explore bacterial population of Cholistan desert, Pakistan for purification and characterization of TP and their industrial applications.

Materials And Methods

Sample collection and bacterial isolation

50g 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^{o}C. The samples were serially diluted and about 100µl mixture was poured onto nutrient agar (NA) plates and incubated at 50^{o}C for 48 hours (Mohammad 2017). Morphologically different colonies were further purified by repeated streaking for 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 hours at 50^{o}C. The isolates that produced clear zone of hydrolysis (CZH) around their colonies were recognized as protease producing (PP) isolates (Shine 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 preliminary 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) as per manufacturer instructions (bioMerieux sa 62980, www.biomerieux.com). 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 2013). Briefly, bacterial colonies were picked from a fresh culture using a sterile tooth pack followed by inculcation in 200µl T.E buffer and placed at 90^{o}C for 10 minutes. The gDNA was obtained after centrifugation at 12000rpm for 5 minutes. 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 2016). The PCR products were sequenced 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. A multiple alignment of the related strains was performed using MUSCLE and phylogenetic analysis was conducted by the neighbor-joining distance method to compute evolutionary distance in MEGA X software and the bootstrap values were calculated based on 1000 replications (Kumar 2018). The 16S rRNA sequences of K1, K5, and K7 were deposited in NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MN493608, MN493618, and MN493619, respectively.

Bacterial Growth and protease production

Growth of the isolates were optimized at different incubation temperature and pH. For this, the isolates were grown in 10ml LB broth media and were incubated in shaking incubator at different temperatures (37, 45, 50, 55, 60, 65 and 70^{o}C) and pH (4, 5, 6, 7, 8, 9, 10 and 11) for 48 hours at 120 rpm. After 48 hours, the bacterial growth was measured by spectrophotometer at 600nm. The isolates were dot inoculated on SMA plates and incubated for protease production at different temperatures (37, 45, 50, 55, 60, and 65^{o}C) and pH (4, 5, 6, 7, 8, 9 and 10) for 48 hours. 1.5% (w/v) skim milk was used for temperature less than 55^{o}C, while for incubation at 55^{o}C and above, 2% (w/v) skim milk was used. After 48 hours of the incubation, the CZH were measured in millimeter (mm) scale using measuring tape (Hamza 2018; Shaheen 2008).

Optimization of protease activity of the isolates

Protease activity was optimized using protease assay as described earlier (Ali 2016). Briefly, the isolates were grown in LB broth at 50^{o}C and crude extract of extracellular enzymes was obtained. Then, 100µl solution of 0.5% casein prepared in 100mM phosphate buffer was added to 100µl crude enzyme and

incubated at 37, 45, 50, 55, 60, 65, 70, 75, 80 and 85^{o}C at pH 5, 6, 7, 8, 9, 10, 11, and 12 for 30 min. The reaction was terminated by adding 100µl of 15% trichloro acetic acid (TCA) and the mixture was placed at room temperature. After 10 min, the mixture was centrifuged at 13000rpm for 10 min and supernatant was transferred to another tube. Finally, 750µl 0.4M Na_2CO_3 and 150µl Folin's phenol ciocalteus reagent (3 fold diluted) was added and incubated at 40^{o}C for 20 min before measuring absorbance at 660nm.

Protease applications of destaining and dehairing

For the destaining and dehairing potential, protease enzyme was partially purified by Ammonium sulfate precipitation method followed by SDS-PAGE by method used by Ali (Ali 2016). A white cotton cloth piece (4cm^2) was stained with a drop of human blood, dried, and treated with formaldehyde. The dried cloth piece was placed in 30ml distilled water and treated with 10ml of proteases (extracted each from isolate K1, K5, and K7) followed by incubation at 50^{o}C in shaking incubator at 120rpm for 15 min. Similarly, the cloth piece was treated with 10mg/ml of local detergent (Bonus) and distilled water as positive and negative control, respectively. The results were observed, photographed, and compared (Rehman 2017). The dehairing process was carried out using a piece of cow's skin (3cm^2) and incubated in 50ml protease extract for 12 hours, while control was incubated in distilled water. After incubation, the skin was rubbed and flooded with water. The results were photographed for comparison (Rehman 2017). All the experiments were performed in triplicate and 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 soil of Cholistan desert, Pakistan. Among them, 26 (54%) bacterial isolates showed proteolytic activity. Among them, three isolates K1, K5, and K7 produced larger zones (19mm, 24mm, and 26mm, respectively) of hydrolysis on SMA plates and were therefore selected for further studies as efficient protease producers (Fig. 1).

The selected isolates (K1, K5, and K7) were gram-positive rods, and the API20E kit biochemical tests showed that all the three isolates were positive for ONPG, ADH, CIT, TDA, GEL, GLU, MAN, INO, RHA, SAL and MEL tests and negative for ODC, URE, H_2S, IND, and AMY tests (Table 1). These preliminary results identified the isolates to be of *Bacillus* origin. After that, 16s rRNA gene sequence was BLAST at NCBI database, which revealed that the isolate K1 had 94.79% sequence identity and 94% query coverage with *Bacillus filamentosus* strain SGD-14, and 94.63% sequence identity and 97% query coverage with *Bacillus endophyticus* strain 2DT, the isolate K5 had 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, while the isolate K7 had 99.89% sequence identity and 96% query coverage with *Bacillus subtilis* strain SBMP4, and 99.68% sequence identity and 99% query coverage with *Bacillus velezensis* strain FZB42 (Table 2). A neighbor joining phylogenetic tree revealed that the strain K1 belong to the genus *Bacillus* and share a distinct phyletic line with *Bacillus*

filamentosus strain SGD-14 and *Bacillus endophyticus* strain 2DT (Fig. 2). Since, the strain K1 has higher sequence identity with the *Bacillus filamentosus* strain SGD-14 (Accession number NR_134701.1), therefore, the strain K1 was identified as *Bacillus filamentosus.* This was also supported by the phylogenetic tree obtained by the maximum-likelihood algorithms, where the isolate K1 form distinct phyletic line with *Bacillus filamentosus* strain SGD-14 (Figure S1). The neighbor joining phylogenetic tree further showed that the strain K5 is affiliated with genus *Lysinibacillus* and form distinct phyletic line with *Lysinibacillus cresolivorans* strain SC03 (Accession number NR_145635.1), and K7 strain is affiliated with genus Bacillus and form distinct phyletic line with *Bacillus subtilis* strain SBMP4 (Accession number NR_118383.1) (Fig. 2). This relationship was also found in the phylogenetic tree obtained by the maximum-likelihood and Neighborhood phylogenetic algorithms (Figure S1 and Figure S2).

Table 1. 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	-	-		+
GEL	+	+		+
GLU	+	+		+
MAN	+	+		+
INO	+	+		+
SOR	+	+		-
RHA	+	+		+
SAC	+	+		-
MEL	+	+		+
AMY	-	-		-
ARA	+	-		-

Table 2. 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_134701.1
	Bacillus endophyticus strain 2DT	94.63%	97%	NR_025122.1
K5	*Lysinibacillus cresolivorans* strain SC03	99.89%	97%	NR_145635.1
	Lysinibacillus boronitolerans strain 10a	99.68%	98%	NR_041276.1
K7	*Bacillus subtilis* strain SBMP4	99.89%	96%	NR_118383.1
	Bacillus velezensis strain FZB42	99.68%	99%	NR_075005.2

Optimization of bacterial growth

Growth of bacterial isolates (*Bacillus filamentosus* strain K1, *Lysinibacillus cresolivorans* strain K5, and *Bacillus subtilis* strain K7) were observed till 70^{o}C and the optimum temperature for growth was recorded at 50^{o}C, which indicated the thermophilic nature of the isolates (Fig. 3A). The optimum pH for 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 (Fig. 3B). The diverse and extreme culturing conditions of these isolates may be possibly due to their diverse habitat (desert) where bacteria face harsh environmental conditions for their survival.

Protease production and activity

In this study, the optimum temperature of protease production was reported as 65^{o}C which revealed the thermophilic nature of the isolates. However, the temperature for high protease production varied for each bacterium such as *Bacillus filamentosus* strain K1, *Lysinibacillus cresolivorans* strain K5, and *Bacillus subtilis* strain K7 produced maximum zones of hydrolysis at 37^{o}C (28mm), 45^{o}C (25mm), and 50^{o}C (26mm), respectively (Fig. 4A). Similarly, the highest production of protease for all isolates were seen at pH 7 (Fig. 4B).

Different temperature and pH conditions affect the stability and activity of proteases therefore, temperature and pH were optimized for these isolates. The optimum activity of proteases produced by *Bacillus filamentosus* strain K1 was observed 170 U/ml at 65^{o}C and *Lysinibacillus cresolivorans* strain K5 activity was 165 U/ml at 65^{o}C, however, maximum protease activity of 190U/ml was observed at 55^{o}C for *Bacillus subtilis* strain K7 (Fig. 5A). The proteases produced by these isolates were also active at high pH values (5 to 12). While, the maximum activity of the proteases produced by *Bacillus filamentosus* strain K1 (172.3 U/ml), *Lysinibacillus cresolivorans* strain K5 (173 U/ml), and *Bacillus subtilis* strain K7 (200.6 U/ml) was observed at pH 9 (Fig. 5B).

Industrial applications of protease enzymes

After the successful partial purification of protease enzyme, the destaining and dehairing potential have been investigated. The enzyme showed the potential to remove human blood stains from white cotton cloth (Fig. 6). The protease enzymes revealed significant destaining potential as shown in Fig. 6 and this suggested the importance of these proteases **for industrial level applications** after purification by chromatographic methods. The proteases isolated in this study were also checked for their dehairing potentials using cow's skin. These proteases revealed the dehairing potential as shown in Fig. 7. Enzyme from all the three isolates have successfully removed hairs from cow's skin. Fig 7A represents the cow skin only treated with distilled water while Fig 7B, 7C and 7D represents dehaired skin treated with the enzymes of *Bacillus filamentosus*, *Lysinibacillus cresolivorans*, and *Bacillus subtilis* respectively.

Discussion

Proteases are the chief marketing enzymes worldwide because they are extensively used in leather, detergent, textile, food and pharmaceutical industries (Sinha and Khare 2013). The initial identification of 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 other researchers for initial screening of PP bacterial species based on CZH (Ali 2016; Asha and Palaniswamy 2018; Kamran 2015).

We have used API kit for the initial identification of the bacteria. This method is also used previously by several researchers to identify bacteria based on biochemical characteristics like the identification of *Bacillus sp.* isolated from marine water (Alnahdi 2012), tropical fruit flies (Thaochan 2010) and municipal waste water (Sonune and Garode 2018). The 16s rRNA gene sequencing method is used in this study for molecular identification of bacteria, which is more authentic method for microbial identification (Janda and Abbott 2007; Kai 2019). This technique has also been used for genetic studies and diversity of bacteria such as *Streptococcus* (Sasaki 2004). *Bacillus spp.* are the diverse group among all bacterial species and our results are in accordance with other researchers who identified bacteria using NCBI BLAST of 16S rRNA gene sequences (Lele and Deshmukh 2016; Mohammad 2017). In similar fashion, (Shine 2016), also identified *Bacillus* from desert soil of Riyadh, Saudi Arabia with potential of protease production using 16s rRNA sequencing (Shine 2016). In one of our previous article, *Bacillus* was reported as protease producer from salt mines of Karak, Pakistan (Ali 2016). It can be concluded that *Bacillus* is a diverse group of bacterial species that can produce protease enzymes and usually found abundantly in harsh environmental conditions.

All of our isolates showed best growth at 50^{o}C and have tolerated temperature till 70^{o}C. A slightly higher optimum temperature for growth than our study was reported by a researcher who have isolated bacteria from hot springs (El-Gayar 2017). The higher temperatures could be due to the variable conditions of the extreme sampling sites. Growth optimization is necessary because microorganisms used in fermentation technology needs 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 temperature and pH like one previously reported by (Aanniz 2015), who had isolated bacterial isolates from Moroccan

hot springs, deserts and salt marshes and have observed and optimized bacterial growth at different high temperature and pH (Aanniz 2015).

Qualitative proteolytic optimization is the direct observation and measurement of clear zones on SMA plates (Omer and Humadi 2013; Saxena 2014). The results revealed that proteases produced by all the three isolates were active at a wide range of temperature (37^{o}C to 85^{o}C) which make them industrially important thermostable proteases. According to literature, the optimum temperature for protease activity produced by a thermostable bacteria isolated from hot springs is 50^{o}C, however, proteases activity was also observed at higher temperature such as 90^{o}C (El-Gayar 2017). In previous study, the alkaline PP *Bacillus* species 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.

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 (Bezawada 2011; Mendes 2009; SD and Tambekar 2013), however, the blood destaining potential of proteases is rarely reported. These thermoalkaliphilic proteases favors 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 leather industry, which is not an ecofriendly process (Hakim 2018; Thanikaivelan 2004). In a report dehairing ability of proteases was reported for alkaline proteases (Hakim 2018). But in this study, we have isolated and investigated the dehairing potential of proteases which can stand active at high temperature and pH. The destaining and dehairing potentials of these proteases can be exploited at extreme thermoalkaliphilic conditions for industrial applications including leather and textile industry.

The three bacterial isolates were selected from soil of Cholistan desert which revealed the bacterial growth and protease production at broad ranges of temperature (37^{o}C to 70^{o}C) and pH (7 to 11). Although, the optimum pH was 9 for protease activity for all but optimum temperature was 65^{o}C in case of *Bacillus filamentosus* strain K1, and *Lysinibacillus cresolivorans* strain K5, and 55^{o}C for *Bacillus subtilis* strain K7. However, the proteases produced by these isolates have shown proteolytic activity at high temperature (37^{o}C to 85^{o}C) and high pH (5–12) which make them industrially important thermoalkaliphilic proteases at extreme operational conditions. The proteases also successfully removed blood stain from cotton cloth and hairs from cow's skin, which revealed the importance of these enzymes as detergent and/or additive of detergent in leather and textile industry. This study also recommended that Cholistan desert represents an invaluable microbial flora which can be explored for production of other important enzymes and biomolecules of industrial importance.

Declarations

Authors' contributions. IU performed the experiments, conducted statistical analysis and wrote the draft manuscript and NA helped in experiments, designed the research project, statistical analysis and in

writing the draft manuscript, and equally contributed as first author with IU. WU, MQ, MR and NU analyzed the data and provided suggestions to improve the manuscript.

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