Indian Journal of Biochemistry & Biophysics
Vol. 58, October 2021, pp. 444-450

Purification and characterization of an extracellular alkaline cold-adapted serine metalo-protease from the cold tolerant bacterium, *Stenotrophomonas* sp. BTR88

Reihaneh Nikbakhti[1], Bahar Shahnavaz[1, 2]* & Ahmad Asoodeh[3]

[1]Department of Biology, Faculty of Science; [2]Institute of Applied Zoology; &
[3]Department of Chemistry, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran

Received 20 July 2019; *revised 02 December 2019*

Proteases are the most economical enzymes in biotechnology and industry. Nowadays, a lot of attention is being paid to extremophiles microorganisms owing to the diversity of their enzymes. One hundred and Six proteolytic bacteria were isolated from Binaloud Mountain; one of them (strain BTR88) was selected as the best producer of extracellular protease and was used for further studies. This bacterium belongs to *Stenotrophomonas* sp., which were identified by the 16S rDNA sequence. Maximal protease production was detected at the beginning of exponential growth phase in the presence of starch and skim milk at 20°C and pH 9. This protease was purified to electrophoretic homogeneity with a fold: 27.5, yield: 33% and specific enzyme: 12.6 U/mg. SDS-PAGE and zymography analyses revealed a protein band of 22 kDa. The maximum activity was at pH 9 and in the range of 20-30°C; while, the enzyme exhibited a broad range of activity from 20-80°C as well as the pH of 5-10. Enzyme inhibition in the presence of phenylmethane sulfonyl fluoride (PMSF) and Ethylenediaminetetraacetic acid (EDTA) showed that the purified enzyme belongs to serine metallo-enzymes. Kinetic parameters of k_m, V_{max} and k_{cat} for the cold tolerant enzyme were determined to be 7.2 mg/mL, 1.45 mM/min and 33.2 sec^{-1}, respectively. The characteristics of activity in cold and alkaline conditions and the broad range of pH and temperature suggest that serine metalloprotease has potential use in the detergent industry.

Keywords: Binaloud mountain, Cold tolerant, Extremozyme, Protease, *Stenotrophomonas* sp.

Proteases are large groups of hydrolyzing enzymes producing polypeptides and amino acids. They have tremendous applications in various industries such as food, pharmacy, laundry and leathery[1,2]. One of the problems that industry is encountered with is Lack of stability in using enzymes. Because of the harsh conditions that are considered in these industries, the use of extreme proteases has great importance[3]. Extremophiles are the organisms that can grow and function under difficult environmental condition such as high salts, acidic or alkaline pH, high or low temperatures[4]. This adaptation is due to the presence of extremozymes in these microorganisms, which give the ability to tolerate a range of difficult environmental conditions[5].

Psychrophiles and psychrotrophs live under extreme conditions. They have an extensive distribution and diversity of biomass production. Psychrophiles are microorganisms having minimum, optimum and maximum temperatures of 0°C, ≤ 15°C and ≤ 25°C; while psychrotrophs or cold tolerating microorganisms are those they have minimum, optimum and maximum temperatures of 0°C, ≤25°C and ≤35°C[6]. From a structural and functional point of view, psychrophilic bacteria have evolved by living at low temperatures and exhibit a wide range of metabolic activities at subzero temperatures in cold ecosystems[7]. Due to the less destructive effect of low temperatures on cellular or enzyme structures, these microorganisms possess a further frequency in nature[8]. An increased flexibility in psychrophilic enzymes leads to a higher level of activity at low temperatures while mesophilic enzymes have a lower level of activity in these conditions. By use of psychrophilic enzyme, industrial processes are conducted in a short time leading to decrease cost and energy consumption. In general, addition of cold tolerant enzymes in temperature-sensitive reactions brings about an increasing yield of reaction and preventing undesirable chemical modifications and volatile compounds from the reaction[7]. Many cold tolerant enzymes from bacteria in various ecosystems have been so far reported[8].

Researchers are quite highly interested in alkaline proteases that are produced by microorganisms which are very important in biotechnology[9]. In the presence

*Correspondence:
Phone: +98-51-38805506;
Fax: +98-51-38762227
E-mail: shahnavaz@um.ac.ir

of oxidizing agents or surfactants, which are commonly used in the new formulation of detergents, alkalin proteases that are able to maintain activity and stability in alkaline pH are particularly important[10].

A great majority of cold-adapted proteases are derived from the bacterial strains[8] which vary in their thermostability and alkali stability. High proteolytic activity at lower temperatures by these proteases is important in the commercial industries, but their low thermal stability is a common drawback that limits their applications. Despite so many advances that have been made in the study of psychrophilic enzymes, reports are limited on the psychrophilic enzyme that is active at low temperature and capable of maintaining high levels of activity at high temperatures[4].

To the best of our knowledge, there is no study regarding cold-tolerant protease from psychrophilic bacteria in Binaloud Mountain in the Khorasan Razavi province.

In the first step of the present research study, the optimal growth conditions of *Stenotrophomonas* sp. BTR88 were investigated and at the second step, purification and characterization of cold tolerant alkalophilic protease from the strain was carried out.

Material and Methods

Sampling, substrate and chemicals

Soil samples were collected under snow from two locations of the Binaloud Mountains at 1000 M (58°51′9″E, 36°25′38″N) and 2000 M (58°50′57″E, 36°25′35″N) altitude in December 2011. All culture mediums used for growth of bacteria and glucose, starch, sucrose, lactose and Bovine serum albumin (BSA) were obtained from Merck (Darmstadt, Germany). Q-Sepharose was supplied by Pharmacia (GE Healthcare Life Sciences, Uppsala, Sweden). DNA extraction kit was purchased from MP Biomedicals, Qbiogene. PCR reagents were purchased from DenaZist (Mashhad, Iran). All other chemicals used were of analytical grade.

Screening and isolation protease producing bacteria

To achieve maximum efficiency in the isolation process, 100 µL of each dilution soil samples were cultured on diluted Tryptic Soy Broth (TSB) containing agar (TSA medium) and incubated at low temperature. For early verification of protease activity, 10 µL of selected isolates in defined concentration (1.5×10^8 CFU/mL) were inoculated on a mineral salt agar medium (MSM) containing (g/L): Na_2HPO_4, 5.35: NH_4Cl, 2.67: $CaCl_2.H_2O$, 0.006: $MgSO_4$, 0.006: $FeSO_4.H_2O$, 0.0024: $MnSO_4.H_2O$, 0.0006: and agar, 15 supplemented with 1% skim milk and kept at 20°C for 7–10 days. Observation of a clear halo around the colonies is indicative of protease activity with decomposition of skim milk.

Protease production

Extracellular protease was produced in the broth MSM medium supplemented with 1% (*w/v*) skim milk. The culture medium was incubated at 20°C and 150 rpm for 48 h. The medium was centrifuged at 10000 × g for 20 min at 4°C, and the supernatant (crude enzyme solution) was used as protease assay.

Protease assay

The crude enzyme solution (0.1 mL) was added to the reaction mixture (0.1 mL) containing 0.5% (*w/v*) suspension of casein in 50 of mM Tris-HCl buffer (pH 7.5). The mixture was incubated at 30°C for 10 min. The reaction was stopped by the addition of 200 µL of 10% trichloroacetic acid (TCA). After centrifugation, optical density was measured at 280 nm with tyrosine as a reference compound. Enzyme activity of protease is a measure of the quantity of adapted enzyme that can release one µmol tyrosine per min under the assay conditions.

Molecular and biochemical characterization

The genomic DNA was extracted using the FastDNA® SPIN Kit (MP Biomedicals, Qbiogene) according to the manufacturer's instructions. The bacterial species were identified based on amplification and sequencing of 16S rRNA using universal primers, namely, 27F and 1492R. Gene amplification of 16S rRNA was performed using 1.5 mM $MgCl_2$, 30 mM KCl, 10 mM Tris-HCl, 2.5 mM of each dNTP, 5–10 pmol of each primer, and 1U of Taq polymerase. Polymerase chain reaction (PCR) was carried out using the following program: initial denaturation at 95°C for 2 min, 30 cycles of denaturation at 95°C for 30s, annealing at 57°C for 30s, extension at 72°C for 30s, and final elongation at 72°C for 7 min. The PCR products were sequenced at Macrogen, South Korea.

Optimization of the fermentation medium

Optimization of conditions of the culture is effective on the growth and production of enzymes. In this study, the effects of different variables were investigated by the use of 'one factor at a time' procedure. The MSM medium was applied as a basal medium in all of the experiments. In order to determine the best time of incubation for protease production, the proteolytic activity was measured after 24, 48, 72 and

96 h. The different conditions of culture at various temperatures (15, 20, 25, 30, and 37°C); pH levels (6, 7, 9, and 11); incubation time for enzyme-substrate (5, 10, 15 and 20 min); inoculation rates (0.5, 1.5, 2.5 and 3.5% *v/v*) from seeding medium; diverse carbon sources (1% *w/v*); glucose, lactose, starch, and sucrose; various nitrogen sources (0.2% *w/v*): yeast extract, milk skim, ammonium sulfate and ammonium chloride were also investigated.

Purification of the protease

To purify the enzyme, supernatants of optimized culture were centrifuged at 10000 × g for 20 min at 4°C, and precipitated using ammonium sulphate (85%, *w/v*). The precipitates were incubated at 4°C for 24 h, collected by centrifugation at 8000 × g at 4°C for 15 min. The precipitates were dissolved in a minimum volume of 20 mM Tris-HCl buffer (pH 8.0), and dialyzed overnight against the same buffer. In order to carry out ion-exchange chromatography, the proteins were applied to a Q-Sepharose equilibrated with buffer 20 mM Tris-HCl buffer (pH 8.0). The protease activity of the collected fractions was measured at 280 nm. Protein concentration was evaluated based on the Bradford's method utilizing bovine serum albumin (BSA) as standard protein. SDS-PAGE was carried out to determine the molecular weight of the protease using a (5% *w/v*) stacking gel and a (15% *w/v*) separating gel. After electrophoresis, the gel was stained by silver nitrate (20% *w/v*). Zymogram analysis was performed in order to determine the number and size of the proteases in the sample.

Gelatin zymography was performed in polyacrylamide gels containing SDS and gelatin (0.1%). After SDS-PAGE, the gel was washed with 2.5% Triton X-100 (25% *v/v*) and placed in buffer (20 mM Tris-HCl, 0.2 M NaCl and 5 Mm $CaCl_2$) for 30 min, and incubated overnight at 37°C. The gel was stained in (0.1 % *w/v*) Coomassie Brilliant Blue at 30 min and distained in 45% methanol/45% water, and 1% acetic acid for 30 min. The protease activity was observed as a clear colorless area against the blue background.

The effect of pH and temperature on protease

Protease activity was measured at 30°C and different pH using various buffers (20 mM sodium acetate, pH 3-5; 20 mM sodium phosphate, pH 6-7; and 20 mM Tris-HCl, pH 8-10). To determine pH stability, the purified enzyme was incubated in different buffers at 4°C for 60 min and the residual protease activity was measured at 30°C as aforementioned before.

To determine thermal stability, the enzyme was pre-incubated in sodium phosphate buffer for 60 min and the residual protease activity was measured at 20-80°C.

The effects of inhibitors and surfactants on protease

The effects of inhibitors/detergents on enzyme activity were investigated by first incubating with 2 and 5 mM of different inhibitors (PMSF, β-mercapto-ethanol, EDTA) and 0.5% and 2.5% of chemical surfactants (SDS, cetyltrimethylammonium bromide: CTAB, X-100 Triton and H_2O_2) at 20°C for 60 min, and then determining protease activity.

Substrate specificity

A substrate solution (0.9 mL) containing 0.5% (*w/v*) for each of casein, BSA and gelatin in 50 mM Tris-HCl buffer (pH 9) was mixed with 100 µL of the enzyme solution during 60 min of incubation, and enzyme assay was performed under the standard conditions. The value for hydrolyzing casein 1.0% (*w/v*) was considered as 100% activity.

Determination of K_m and V_{max}

In order to determine the Michaelis–Menten constant (K_m), maximum velocity (V_{max}) and catalytic rate constant (k_{cat}), 450 µL of different casein concentration as substrate (2-14 mg/mL) was incubated with 50 µL of purified enzyme at pH 9 in Tris-HCl buffer over 60 min. K_m and V_{max} were calculated from the Lineweaver-Burke plot using GraphPad Prism 7. In addition, turnover number or k_{cat} was calculated with the formula $k_{cat} = V_{max}/[E]$.

Statistical analysis

All experiments were replicated three times. One-way analysis of variance (ANOVA) at the 95% confidence level was performed using the R software package.

Results

Screening of protease-producing strains

A total of 145 cold tolerant isolates were obtained from two sites. Among them, 106 isolates (73%) were protease positive. Table 1 shows a summary of the characteristic of nine isolates based on the highest diameter of clear zone.

These selected isolates could grow at 4°C and 20°C, but not at 37°C. The strain BTR88 with enzyme production was selected for further studies.

Molecular identification of BTR88 was conducted based on 16S rDNA. The results showed that the BTR88 strain belonged to subphylum of *γ-proteobacteria* and *Stenotrophomonas* genus. The gene sequence of *Stenotrophomonas rhizophila* (AJ293463) had a 99.86% similarity to the BTR88 strain (Fig. 1). The result of

16S rDNA sequencing was deposited as gene accession number of KM459539.

In order to identify the effective factors for protease production with *Stenotrophomonas* sp. BTR88, the one factor-at-a-time method was carried out and the results are shown in (Table 2). The enzyme production was measured during 96 h with 24 h intervals at 20°C. The amount of the enzyme produced varied for different time incubation. The protease production was 0.13, 0.31, 0.12 and 0.1 U/mL over 24, 48, 72 and 96 h. Although enzyme production was observed in various temperatures, but protease production increased at 20°C. Interestingly, protease activity relatively decreased 60% when the temperature was increased to 20 to 30°C. The growth and enzyme production continued in relative acidic and alkaline pH. However, optimum protease production was observed at pH: 9 (0.6 U/mL). The strain could grow in various carbon sources. However, the highest enzyme activity was obtained in the presence of (0.5% *w/v* starch and 1% *w/v* skim milk as carbon and nitrogen source.

Table 1 — The selected strains based on halo zone and maximum enzyme production

Strains	Altitude (m)	Clear Zone (mM)	Maximum enzyme Production U/mL (Time)
BTR88	2000	24	0.13 (48 h)
BTR821	2000	12	0.08 (48 h)
BTR45	2000	12	0.05 (48 h)
BTR48	2000	22	0.06 (48 h)
BTR814	2000	24	0.09 (72 h)
ATR201	1000	14	0.08 (48 h)
ATR2067	1000	14	0.02 (72 h)
ATR429	1000	18	0.02 (48 h)
ATR89	1000	16	0.06 (48 h)

*****Bolded parameters were selected for the growth of *Stenotrophomonas* sp. BTR88. Error bars indicate ± SD. Significant differences between the parameters are indicated by small letters.

Enzyme purification

Results of purification of the enzyme using Ammonium sulfate precipitation and ion exchange chromatography of Q-Sepharose are presented in (Table 3). Based on our results, specific activity was increased from 0.46 to 12.6 U/mg in the ion exchange step. These results revealed 27.5-fold purification.

As shown in Figure 2, the purified enzyme demonstrated a single band on the SDS-PAGE. The molecular weight of the enzyme was 22 kDa compared to the molecular weight marker.

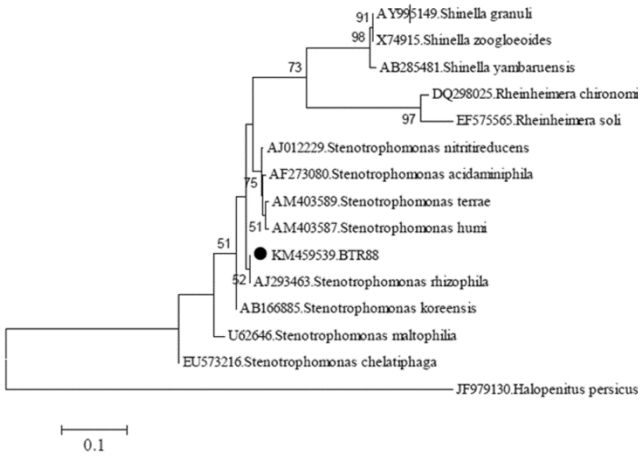

Fig. 1 — Neighbour-joining phylogenetic relationship between the 16S rRNA sequence of *Stenotrophomonas* sp. BTR88 and the 16S rRNA sequences from the data base. The sequences of the *Halopenitus persicus* were used as outgroup. Bootstrap values (%) are based on 1000 replicates

Table 2 — Effect of different parameters on protease production

Parameters	Various levels/Enzyme activity				
Incubation time (h)		960.1 ± 0.005^{b}	720.17 ±0.006^{b}	480.31±0.001^{a}	240.13±0.001^{b}
Temperature (°C)	370.12±0.03^{ab}	300.13 ±0.001^{ab}	250.16 ± 0.04^{ab}	200.31 ±0.001^{a}	150.06± 0.03^{b}
pH		110.4 ± 0.04^{b}	90.6 ± 0.004^{a}	70.55 ± 0.004^{ab}	60.48± 0.004^{b}
Inoculum size		3.50.47 ± 0.009^{ab}	2.50.4 ± 0.01^{ab}	1.50.44 ± 0.01^{ab}	0.50.55± 0.01^{a}
Carbon Source (%1 *w/v*)		Glucose 0.2 ±0.02^{b}	Lactose 0.52 ± 0.04^{a}	Sucrose 0.54 ± 0.001^{a}	Starch 0.64± 0.01^{a}
Starch (% *w/v*)		20.68 ± 0.009^{ab}	1.50.77 ± 0.03^{b}	10.54 ± 0.02^{ab}	0.50.84 ± 0.05^{b}
Nitrogen sources (%1 *w/v*)		$(NH_4)_2SO_4$ 0.0± 0.0^{b}	$(NH_4)_2Cl$ 0.0± 0.0^{b}	Yeast extract 0.4± 0.11^{a}	Skim milk 0.83 ± 0.03^{a}

Table 3 — Summary of results of purification of protease from *Stenotrophomonas* sp. BTR88

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	0.62	1.35	0.46	1.0	100
$(NH_4)_2SO_4$ precipitation	0.46	0.071	0.65	1.43	75
Q-sepharose	0.202	0.016	12.6	27.5	33

The effects of temperature and pH

The effects of pH on the stability and activity of protease from *Stenotrophomonas* sp. BTR88 were measured and showed in (Fig. 3). The maximum enzyme activity was observed at pH: 9, while it could be active in a wide range of pH from 3 to 10. The enzyme had retained 37% of its stability in the highest level of activity. Interestingly, the protease enzyme had 60% activity at pH 5 and 10 with about 40% stability residual activity after 60 min of incubation at these pHs.

The Effect of temperature on protease activity and stability is presented in (Fig. 4). The optimum temperature for the enzymatic activity was found to be 30°C. Interestingly, it was the most stable under its optimum temperature. However, protease was more stable in lower temperatures (20, 30 and 40°C). The enzyme was active at higher temperatures and retained about 60% activity at 60°C. It is interesting to note that protease was active even at 80°C.

Substrate specificity and kinetic parameters

The enzyme was able to hydrolyze casein, gelatin and BSA (5 mg/mL) as substrates. The level of activity of the enzyme towards each substrate was as follows casein: 0.125 ± 0.001; gelatin: 0.095 ± 0.002; and BSA: 0.0015 ± 0.005 U/mL. However, the greatest activity was observed towards casein. Based on Michaelis–Menten equation and non-linear regression analysis, the K_m and V_{max} values were determined to be 7.2 mg/mL and 1.45 mM/min (Fig. 5). k_{cat} was calculated by the division V_{max} to enzyme molar concentration which was 33.16 S^{-1}.

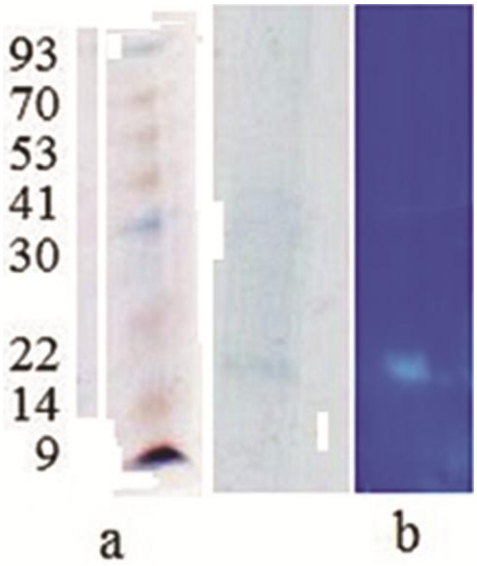

Fig. 2 — (A) SDS-PAGE of the purified protease. Lane 1: protein molecular marker weight. Lane 2: purified alkaline protease from Q-Sepharose; and (B) Zymogram: gelatin degradation is shown by a clear colorless zone against the blue background

The effects of inhibitors and detergent agent on enzyme activity

The effect of different inhibitors and detergent were investigated on protease activity (Table 4). In the presence of 2 mM of PMSF, β-mercapto-ethanol and EDTA, the enzyme activity was reduced to 19.2, 33.8 and 14.3%, respectively. The Protease lost 81 and 88% of activity in the presence of 2 and 5 mM of the PMSF, respectively. The presence of 5 mM EDTA reduced activity to 26 % of the control.

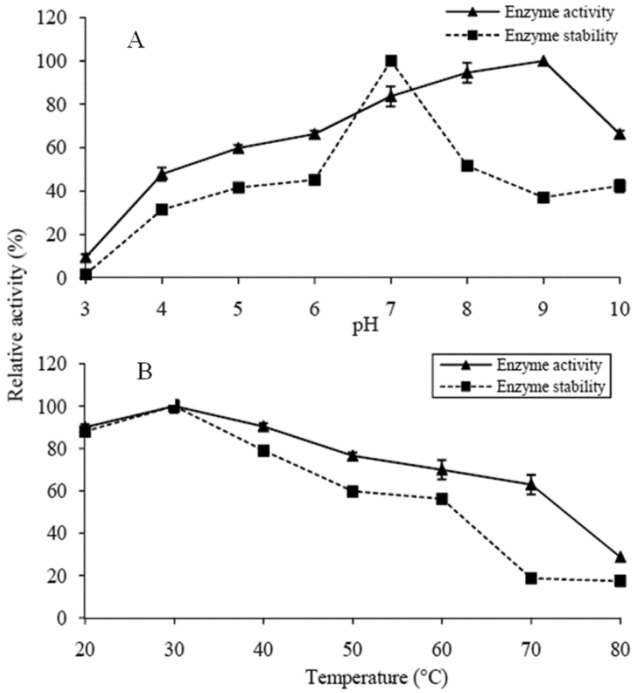

Fig. 3 — Effect of (A) pH; and (B) temperature on activity and stability of protease enzyme from *Stenotrophomonas* sp. BTR88. Relative activity is expressed as the percentage of the maximum activity (100%) under standard assay condition

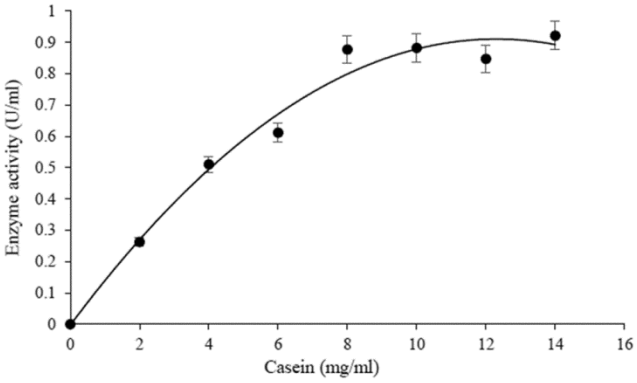

Fig. 4— Effect of casein concentration on the reaction velocity of protease. The tests were performed at 30°C and pH 9. Each value represents the mean of three replicates

Table 4 — Effect of inhibitors and detergent agent on purified protease from *Stenotrophomonas* sp. BTR88. Each value represents the mean of three replicates

	Residual activity Final concentration	
Inhibitor	2 mM	5 mM
PMSF	19.2 ± 0.39	12.08± 0.15
EDTA	33.8± 0.11	26.7± 0.11
β-Mercaptoethanol	14.3± 0.14	13.4± 0.38
Detergent	0.50%	2.50%
SDS	36.5± 0.11	34± 0.01
CTAB	36± 0.19	7± 0.2
Triton-X100	16.2± 0.12	6.3± 0.3
H_2O_2	31.8± 0.01	26.8± 0.04

However, the protease was active in the presence of detergents and bleaching agents. The residual activity of the enzyme was 34%, 7%, 6.3% and 26.8% in the presence of 2.5% (*v/v*) of SDS, CTAB, Triton X-100 and H_2O_2, respectively.

Discussion

More than 70% of isolated bacteria from Binaloud Mountains had proteolytic activity. In this study sampling was carried out from the soil under the snow, and results showed that bacterial flora could grow at around zero temperatures and were capable to hydrolyze various proteins sources. The strain of BTR88 revealed the highest proteolytic activity among the obtained strains. This strain was Gram-negative, rod shape and yellow pigment bacterium, positive catalase and oxidase, negative amylase as well as positive gelatinase and urease. Furthermore, 16s rRNA analysis demonstrated that the strain belonged to *Stenotrophomonas.* There are numerous reports concerning cold-active enzyme from bacteria[11-13]. Regarding 30% to 40% of enzyme production cost in different industries is related to culture medium cost, the importance of optimization of enzyme production is clarified[14]. Some studied have shown that simple carbon sources such as glucose, sucrose and lactose lead to complete termination of protease production[15]. In present study, the use of such carbon sources led to decrease enzyme production but did not reach to full stop production. After the final purification using exchange chromatography, the enzyme had obtained a 27.5-fold purification and a specific activity of 12.6 U/mg.

Cold tolerant protease revealed a molecular mass of 22 kDa and zymography confirmed the proteolytic activity of the obtained enzyme. Molecular mass of cold-tolerant protease was proximally similar to those reported from Gram-negative bacteria of *Planococcus* sp. CGMCC 8088 (43 kDa)[6].

A lot of cold- active enzymes obtained from psychrophilic bacteria need to low temperatures for their activity and are inactivated at high temperatures[5]. These enzymes presenting high catalytic yield at low temperatures and structural flexibility to confront with low temperature have lower thermal stability.

Purified enzyme in current study was thermostable and showed the maximum activity at 30°C. More than 80% and 20% of original activity was retained at 40°C and 80°C, respectively.

Our results revealed that the enzyme obtained from *Stenotrophomonas* sp. BTR88 is cold tolerant rather than psychrophilic enzyme. This adaptation could be related to the characteristics of the sampling area. Many researchers believe that truly psychrophilic microbes are living in marine ecosystems mostly have a temperature less than 5°C; whereas, cold-tolerant microorganisms mainly exist in terrestrial ecosystems to tolerate more temperature variations depending on change of seasons[7]. Therefore, producing cold adapted enzymes at low temperatures and stable at higher temperatures is a more beneficial strategy of psychrophilic enzyme. In these environments, temperature variation could lead to produce cold-adaptive extracellular enzymes during the long period of low temperatures in winter. In contrast, temperature increases in summer; the strains secrete thermostable enzymes to uptake organic matter available from the root of plans.

The obtained enzyme was active in wide range of pH. Sensitivity in the presence of PMSF and EDTA indicate the enzyme is metalloprotease. The purified enzyme was stable against a wide range of detergents, inhibitors and bleaching agents. The fact that the protease enzyme exhibits high stability in the presence of EDTA, SDS and H_2O_2 is of great significance in the detergent industry, because these compounds are the chelating agent that is utilized in many detergents as water-softener[16].

In application point of view, there is need to examine enzymatic properties of proteases. Kinetic parameters of some psychrophiles enzymes have been studied and compared with thermophiles and mesophiles counterparts[8]. Low temperatures result in a reduced rate of chemical reactions. Enzyme compression is increased in these conditions and the space available for enzymatic catalysis is reduced[17]. The current serine metalloprotease showed K_m and V_{max} values of 7.2 mg/mL and 1.45 mM/min, respectively. Other parameters including k_{cat} and k_{cat}/K_m were 33.16 s^{-1} and 4.62 mL/mg. Although it is not possible to compare the kinetic parameters of the present serine metalloprotease with the parameters of the serine metalloprotease from

Stenotrophomonas sp., we shall investigate the values obtained from some of the types studied. For the purpose of comparison, Serine protease from *Bacillus iranensis* (K_{cat} 3.28×10^{-2} s^{-1}, k_{cat}/K_m 1.041×10^{-2} mL/mg)[18]. k_{cat}/K_{max} ratio represents the efficiency of enzyme-catalyzed reaction. In other words, the enzyme acts more on the desirable substrate[19]. On the other hand, in comparison of two substrates, one has more k_{cat}/K_{max} value, possesses more enzyme affinity toward its own.

Protease enzymes account for 60-65% of the global sales of enzymes[10]. Although the use of microbial proteases has problems and challenges in the industry to further improve them. In spite of the systematic application of protein engineering, in order to modify and enhance the properties of enzymes, the achievement of ideal microbial protease is not possible. Therefore, protease derived from extremophiles bacteria may have unusual properties of natural types, which are more favorable in terms of application in industry.

Conclusion

Current study described purification and characterization analkaliphilic cold-adapted serine metalloprotease from *Stenotrophomonas* sp. BTR88. The purified enzyme had optimal activity at 30°C and was able to conserve its activity up to 80°C. Considering the activity of the enzyme at low temperatures and stability at higher temperature, stability against surfactants, detergents and bleaching agents suggest that the protease from bacteria *Stenotrophomonas* sp. BTR88 make it a good candidate for use in various industries.

Acknowledgement

The authors appreciate the support provided by the Ferdowsi University of Mashhad Research and Technology council (Grant number: 3/41706).

Conflict of interest

All authors declare no conflict of interest.

References

1 Dos Santos Aguilar JG & Sato HH, Microbial proteases: Production and application in obtaining protein hydrolysates. *Food Res Int*, 2018 (103) 253.

2 Karthik L, Manohar R, Kanal E, Krishnasamy G. Purification, characterization and functional analysis of a serine protease inhibitor from the pulps of *Cicer arietinum* L. (Chick Pea). *Indian J Biochem Biophys*, 56 (2019) 2.

3 Ali N, Ullah N, Qasi M, Rahman H, Khan SN, Sadiq A & Adnan M, Molecular characterization and growth optimization of halo-tolerant protease producing *Bacillus Subtilis* Strain BLK-1.5 isolated from salt mines of Karak, Pakistan. *Extremophiles*, 20 (2016) 395.

4 Dumorné K, Córdova DC, Astorga-Eló M & Renganathan P, Extremozymes: A Potential Source for Industrial Applications. *J Microbiol Biotechnol*, 27 (2017) 649.

5 Sarmiento F, Peralta R & Blamey JM, Cold and hot extremozymes: industrial relevance and current trends. *Front Bioeng Biotechnol*, 3 (2015) 148.

6 Chen K, Mo Q, Liu H, Yuan F, Chai H, Lu F & Zhang H, Identification and characterization of a novel cold-tolerant extracellular protease from *Planococcus* sp. CGMCC 8088. *Extremophiles*, 22 (2018) 473.

7 Mocali S, Chiellini C, Fabiani A, Decuzzi S, Pascale DD, Parrilli E, Tutino ML, Perrin E, Bosi E, Fondi M, Giudice AL & Fani R, Ecology of cold environments: new insights of bacterial metabolic adaptation through an integrated genomic-phenomic approach. *Sci Rep*, 7 (2017) 839.

8 Santiago M, Ramírez-Sarmiento CA, Zamora RA & Parra LP, Discovery, molecular mechanisms, and industrial applications of cold-Active enzymes. *Front Microbiol*, 7 (2016) 1408.

9 Zhou C, Zhou H, Zhang H & Lu F, Optimization of alkaline protease production by rational deletion of sporulation related genes in *Bacillus licheniformis*. *Microb Cell Fact*, 18 (2019) 127.

10 Yildirim V, Baltaci MO, Ozgencli I, Sisecioglu M, Adiguzel A & Adiguzel G, Purification and biochemical characterization of a novel thermostable serine alkaline protease from *Aeribacillus pallidus* C10: a potential additive for detergents. *J Enzyme Inhib Med Chem*, 32 (2017) 468.

11 Ko JK, Ko H, Kim KH & Choi IG, Characterization of the biochemical properties of recombinant Xyn10C from a marine bacterium, *Saccharophagus degradans* 2-40. *Bioprocess Biosyst Eng*, 39 (2016) 677.

12 Li M, Yang LR, Xu G & Wu JP, Cloning and characterization of a novel lipase from *Stenotrophomonas maltophilia* GS11: the first member of a new bacterial lipase family XVI. *J Biotechnol*, 228 (2016) 30.

13 Wang G, Wang Q, Lin X, Ng TB, Yan R, Lin J & Ye X, A novel cold-adapted and highly salt-tolerant esterase from *Alkalibacterium* sp. SL_3 from the sediment of a soda lake. *Sci Rep*, 6 (2016) 19494.

14 Ferreira RDG, Azzoni AR & Freitas S, Techno-economic analysis of the industrial production of a low-cost enzyme using *E. coli*: the case of recombinant β-glucosidase. *Biotechnol Biofuels*, 11 (2018) 81.

15 Sharma KM, Kumar R, Panwar S & Kumar A, Microbial alkaline proteases: Optimization ofproduction parameters and their properties. *J Genet Eng Biotechnol*, 15 (2017) 115.

16 Patil U, Mokashe N & Chaudhari A, Detergent-compatible, organic solvent-tolerant alkaline protease from *Bacillus circulans* MTCC 7942: purification and characterization. *Prep Biochem Biotechnol*, 46 (2016) 56.

17 Khan M & Sathya TA, Extremozymes from metagenome: Potential applications in food processing. *Crit Rev Food Sci Nutr*, 58 (2017) 1.

18 Ghafoori H, Askari M & Sarikhan S, Purification and characterization of an extracellular haloalkaline serine protease from the moderately halophilic bacterium, *Bacillus iranensis* (X5B). *Extremophiles*, 2 (2016) 115.

19 Sari E, Logoglu E & Oktemer A, Purification and characterization of organic solvent stable serine alkaline protease from newly isolated *Bacillus circulans* M34. *Biomed Chromatogr*, 29 (2015) 1356.