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EXTREMELY THERMOSTABLE, EDTA-RESISTANT ALKALINE PROTEASE FROM A THERMOPHILIC *GEOBACILLUS SUBTERRANEUS* C2-1 ISOLATE

Ezgi Yardımcı Akkır[1], Yeliz Buruk Şahin[2], Serap Gedikli[1], Pınar Aytar Çelik[3], Ahmet Çabuk[3,4]*

Address(es):
[1] Graduate School of Natural and Applied Science, Department of Biology, Eskisehir Osmangazi University, 26480, Eskisehir, Turkey
[2] Department of Industrial Engineering, Faculty of Engineering and Architecture, Eskisehir Osmangazi University, 26480, Eskisehir, Turkey
[3] Graduate School of Natural and Applied Science, Department of Biotechnology and Biosafety, Eskisehir Osmangazi University, 26480, Eskisehir, Turkey
[4] Faculty of Art and Sciences, Department of Biology, Eskisehir Osmangazi University, 26480, Eskisehir, Turkey

*Corresponding author: pinaraytar@gmail.com

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ABSTRACT

In this study, the alkaline protease-production capacity of a *Geobacillus subterraneus* C2-1 isolate from the Çitgöl thermal spring was investigated and optimised using a Plackett-Burman experimental design. In addition to the incubation time, which was the most important parameter, other significant factors were the peptone, glucose, and $MgSO_4.7H_2O$ concentrations, the activation time and the inoculum amount.
The highest protease activity of the *Geobacillus subterraneus* C2-1 isolate was observed using 14.75 g L^{-1} glucose as the carbon source, 7.51 g L^{-1} yeast extract as the nitrogen source, an inoculum amount of 3.56%, a temperature of 56.35 °C, and a reaction time of 168 h. In the presence of 1.0 mM SDS, the protease activity did not change and even increased. Furthermore, 100 mM EDTA yielded a five-fold enhancement in specific activity. The presence of chemical denaturants, chelators, and even heavy metals did not alter the protease activity of the C2-1 isolate.

Keywords: Thermostable protease, *Geobacillus subterraneus*, Plackett-Burman experimental design, EDTA resistance

INTRODUCTION

Active proteins and enzymes can catalyse metabolic processes and biological functions under extreme conditions. Increasing the solubility of polymeric substrates and decreasing undesirable complications at high temperatures have allowed the use of thermophilic organisms in biotechnological applications **(Rao *et al.*, 1998)**. The main reason for the interest in extremophiles is the development of their ability to produce enzymes for industrial use **(Wiegel, 1998; Ravot *et al.*, 2006; Champdore *et al.*, 2007; Kumar *et al.*, 2011).** Extremozymes with high thermal stability have been reported to be resistant to detergents, chaotropic agents, organic solvents and extreme pH conditions. The enzymes produced by extreme thermophilic microorganisms are called thermozymes and function optimally at temperatures between 60 °C and 125 °C **(Niehaus *et al.*, 1999).**
Thermostable protease extremozymes that exhibit great biotechnological potential are significant **(Niehaus *et al.*, 1999; Anwar and Saleemuddin, 1998; Haki and Rakshit, 2003).** High temperatures increase the rate of the biological hydrolysis reaction and reduce the risk of contamination **(Scandurra *et al.*, 1998).** Moreover, the more thermostable enzymes have been found to be resistant to proteolysis and denaturation **(Tsai *et al.*, 2001).**
Approximately 80% of the products obtained from thermophilic organisms used in the industrial field are enzymes, particularly hydrolases, including proteases **(Rawlings et al. 2007).** Protease enzymes are one of the most important industrial enzymes, and different varieties of these enzymes have been used in the food, textile, detergent, leather and pharmaceutical industries **(Walsh 2002).**
A Plackett-Burman experimental design is based on a first-degree model and may be used in industrial research to determine the effectiveness of a designed process. In this design, quantitative variables that contribute to the results of various trials used to measure the process of interest are targeted to increase their effectiveness. In addition to the fact that this experimental design is a material cost-saving process, it has significant advantages at the industrial scale, such as power reduction and minimisation of lost time. Validation experiments performed with an acceptable confidence interval consistency and satisfactory reliability have also been tested **(Plackett and Burman 1946).**

This study reveals the ability of the extracellular protease enzyme from a *Geobacillus subterraneus* C2-1 strain isolated from the Çitgöl hot springs. Additionally, experiments investigating the stability of this enzyme in changing stress conditions were conducted.

MATERIAL AND METHODS

Microorganism

Thermal springs in the localities of Çitgöl and Naşa (Kütahya, Turkey) with a high temperature were selected as the sampling area. To isolate the microorganisms inhabiting the water in these thermal springs, nutrient broth medium was used as a growth medium and incubated at 60 °C for 120 h under static conditions. The pure cultures were stored at +4 °C and maintained on nutrient agar.

Enzyme production

The isolates obtained from thermal springs in Çitgöl and Naşa were screened for protease activity. Basal medium containing 5 g L^{-1} peptone, 10 g L^{-1} glucose, 0.5 g L^{-1} NaCl, 0.1 g L^{-1} $CaCl_2.2H_2O$, 0.3 g L^{-1} K_2HPO_4, 0.4 g L^{-1} KH_2PO_4, 0.1 g L^{-1} $MgSO_4.7H_2O$, and 5 g L^{-1} yeast extract was used for protease production. After growth optimisation for protease production, the parameters were adjusted to the optimised conditions, and the isolates were incubated. At the end of the incubation period, culture broth was collected by centrifugation at 14000 rpm and +4 °C for 15 min. Extracellular extract (supernatant after centrifugation) was used for crude enzyme.

Protease activity assay

Protease activity was measured using the Anson method **(Anson, 1938).** A reaction mixture containing 2.5 mL of 0.6% casein solution in 50 mM glycine-NaOH-NaCl buffer with a pH of 10.5 and 0.5 mL of crude enzyme was incubated at 30 °C for 20 min, and the reaction was then stopped by the addition of 2.5 mL of trichloroacetic acid. The mixture was then incubated at room temperature for

30 min and centrifuged at 9000 rpm for 5 min. Subsequently, 0.5 mL of the supernatant was mixed with 0.5 M sodium carbonate and 0.5 mL of Folin-Ciocalteu reagent, and the resulting mixture was incubated at room temperature for 30 min. The amount of tyrosine in the solution was measured by reading the absorbance at 660 nm. The protein concentrations were measured using the Bradford method **(Bradford, 1976).**
Protease activity = [(A_{660} / Slope) x Total volume] / [Enzyme volume x Incubation time]
One unit of enzyme activity was defined as the amount of enzyme yielding the release of 1 µg of tyrosine (from 0.6% casein) per minute at 30 °C **(Takami *et al.*, 1989).**

Screening of protease activities of thermophilic isolates from thermal springs

Screening experiments were performed to determine the proteolytic enzyme-production ability of 16 different isolates from thermal springs in Çitgöl and Naşa. After incubation for 120 h at 50 °C, each culture supernatant was subjected to protease activity and total protein measurements.

Effect of different carbon and nitrogen sources on protease production

The carbon and nitrogen sources used for the growth of microorganisms are important for enzyme production. For this purpose, the effect of various carbon sources (10 g L^{-1}) was studied by replacing glucose in the basal medium with sugars or sugar alcohols (e.g., lactose, maltose, sucrose, glycerol, and starch). Yeast extract was replaced by various inorganic and organic (e.g., soytone, casein, gelatine, urea, and ammonium sulphate) nitrogen sources to obtain an equivalent nitrogen concentration. These experiments were performed by incubating at 50 °C for 120 h, and the other conditions were maintained constant. After incubation, the protease activities of the culture supernatants were measured.

Plackett-Burman experimental design

The components of the medium used for the C2-1 isolate, which was found to produce protease, were designed and optimised using a Plackett-Burman experimental design, a statistical method based on a first-order model **(Plackett and Burman, 1946).** This method shows the effects of other independent parameters in the system on any dependent parameter, allowing elimination of an argument and yielding the following equation (1), where *Y* is the response (result), β_0 is the model shift, βi is the linear coefficient, and *Xi* is the level in the form of the number of arguments.

$$Y = \beta_0 + \Sigma \beta i Xi \qquad \text{Eq.1}$$

Protease production was optimised using a Plackett-Burman design with 11 (n) factors, corresponding to the most important parameters affecting the process. The parameters used in the experimental design consisted of medium components, such as peptone, glucose, KH_2PO_4, $MgSO_4.7H_2O$, yeast extract, and $CaCl_2.2H_2O$, and environmental parameters, such as pH, temperature, incubation time, activation time and inoculum amount. Each variable was tested at high (+1) and low (-1) levels, and the examined parameters and levels are given in Table 1.

Table 1 Media Components and Their Corresponding Levels in Plackett-Burman Experiments

Symbol	Variables	Experimental Value	
		Low (-1)	High (+1)
A	Peptone (g L^{-1})	3	10
B	Glucose (g L^{-1})	5	15
C	KH_2PO_4 (g L^{-1})	0.2	1
D	$MgSO_4.7H_2O$ (g L^{-1})	0.3	0.6
E	Yeast Extract (g L^{-1})	2	10
F	$CaCl_2$. $2H_2O$ (g L^{-1})	0.05	0.5
G	pH	5	11
H	Temperature (ºC)	50	80
J	Incubation time (day)	3	7
K	Activation time (hour)	3	7
L	The amount of inoculum (%)	3	10

Characterisation of the crude enzyme

Determination of optimal reaction temperature and pH

To define the optimal pH for protease activity, casein was dissolved in 50 mM glycine–NaOH buffer at pH values ranging from 8.0 to 12.5. To determine the effect of temperature on enzyme activity, varying temperatures between 20 °C and 80 °C were assessed.

Definition of thermal and pH stability of the protease

Thermal stability was investigated by pre-incubating the culture supernatant at various temperatures, such as 20, 30, 40, or 60 °C for 1 h, 80, 85, or 90 °C for 1 h, 5 h and 24 h, and 95 °C for 24, 48, 72, 96 and 120 h, before measuring the protease activity as described above. The pH stability of the protease was determined by incubation for 1 h at 60 °C and pH values ranging from 8 to 12.5. The remaining activity of the protease was calculated as the specific activity.

Impact of heavy metals and toxic compounds on protease activity

To examine the effect of heavy metals and toxic substances, such as $FeCl_3$, $FeSO_4$, $CuCl_2$, $CdCl_2$, $CuSO_4$, KCl, NaCl, $CaCl_2$, $CoCl_2$, $BaCl_2$, $MnCl_3$, KCN, and $HgCl_2$, the protease was incubated for 20 min with each of these metals at final concentrations of 5 mM and 10 mM. For KCN and $HgCl_2$, the final concentration was also adjusted to 100 mM.
The maximal activity of the raw protease was expressed as 100%. The residual activity was calculated using the crude protease enzyme. Control solution prepared without any metal ions or toxic compounds was used in the experiments for comparison purposes.

Effect of some denaturants on the protease

The protease was incubated with SDS at a final concentration of 1.0 and 5.0 mM, urea at a final concentration of 0.5, 1.0, 5.0, and 10.0 mM, and with EDTA at a final concentration of 5.0, 10.0 and 100.0 mM to determine the stability of the enzyme. Control solution prepared without any denaturant was used in each of the experiments for comparison purposes.

Characterisation of producer isolate

16S rDNA sequence analysis for molecular identification of producer isolate was carried out. DNA was extracted from the bacterium by MoBio DNA isolation kit. The amplification of DNA sample was carried out in 20 µL reaction (GoTaq, Promega, Southampton, U.K.) with 2.5 mM $MgCl_2$. The sequence of the forward and reverse primer were 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3'), respectively. PCR reaction was carried out utilizing an initial denaturation step at 95 °C for 5 min, 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1.5 min at 72 °C, followed by a final extension step at 72 °C for 10 min [11, 12]. The purified 16S rRNA gene was sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analyzed on an automated DNA sequencer (Applied Biosystems). 16S rRNA gene sequences were initially compared to reference sequences at the National Center for Biotechnology Information using BLAST (NCBI, 2009).

RESULTS AND DISCUSSION

Screening of proteolytic activity

Sixteen isolates with different morphological characteristics were obtained from the Naşa and Çitgöl hot springs in Kütahya Province. We determined the specific activities of these isolates by measuring the amount of total protein and protease activity. When the *Geobacillus subterraneus* C2-1 isolate exhibited improved growth in basal medium, it produced the protease with the highest activity (302 U mg^{-1}) among those produced by the other isolates, and this isolate was thus selected for further studies. The relative activity profiles of all of the tested strains are given in Figure 1. In this figure, the C2-1 protease was assumed to equal 100%, and the other proteases are expressed relative the activity of the C2-1 protease.

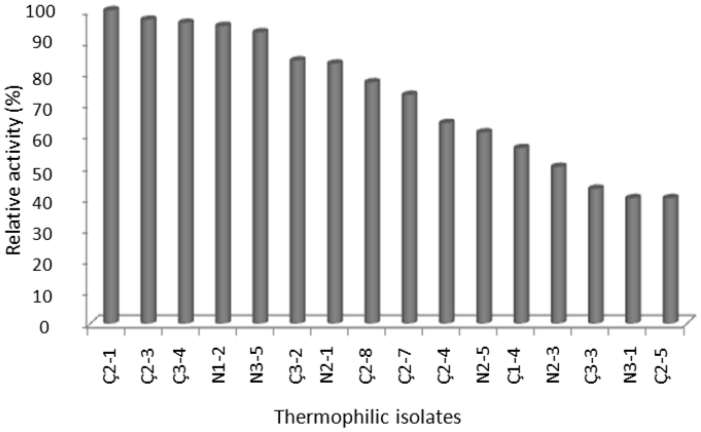

Figure 1 Screening of the abilities of various isolates to produce proteolytic enzymes (Working conditions; incubation time: 120 hours, incubation temperature: 50 ºC, inoculum amount: 10%)

Investigation of carbon and nitrogen sources for protease production of the C2-1 isolate

To increase the protease-production ability of the C2-1 isolate, the most effective carbon and nitrogen sources were determined. Of the seven carbon sources investigated, the highest enzyme activity was observed with the use of glucose, whereas the use of sodium acetate as the carbon source yielded the lowest activity (Fig. 2). Similarly, Boominadhan *et al.* screened *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus megaterium* and *Bacillus licheniformis* isolated from wastes for protease activity and found effective protease activity when glucose was utilised **(Boominadhan *et al.*, 2009)** Akel *et al*. also found that glucose can be used as a carbon source for the production of protease from the hyperthermophilic *Bacillus* strain HUTBS71 **(Akel *et al.*, 2009).** A study based on the protease derived from *Conidiobolus coronatus* showed that glucose was the best carbon source followed by fructose (85–90%) and that all other carbon sources yielded only 50–60% activity compared with that obtained using glucose **(Laxman *et al.*, 2005).** Phadatare found the highest activity with arabinose followed by sucrose **(Phadatare *et al.*, 1993),** whereas Sutar *et al.* reported that sucrose, glucose and fructose could serve as good carbon sources for protease enzyme production **(Sutar *et al.*, 1992).** In another study, lactose was found to be the best carbon source for protease production by *Bacillus brevis* **(Banarjee *et al.*, 1999).** Zhu and his team produced protease from the incubation of the *Geobacillus* sp. strain YMTC 1049 with glucose **(Zhu *et al.*, 2007),** and Bajaj and Sharma obtained the most effective protease activity with the use of lactose **(Bajaj and Sharma 2011**).

After testing various nitrogen sources for protease production by *Geobacillus subterraneus* C2-1, we found that ammonium sulphate yielded less activity and that yeast extract resulted in markedly higher activity (Fig. 3). Laxman *et al.* reported that diammonium hydrogen phosphate was the best nitrogen source and that all other inorganic nitrogen sources yielded only 80–90% protease activity compared with yeast extract **(Laxman *et al.*, 2005).** Johnvesly and Naik observed that sodium nitrate and potassium nitrate support protease production and that ammonium nitrogen completely inhibits production. Yeast extract, followed by casein, represented the best sources for protease enzyme production by the thermophilic *Bacillus* sp. JB-99, whereas beef extract and peptone were poor organic nitrogen sources **(Johnvesly and Naik 2001).** Boominadhan *et al.* studied protease production by *Bacillus* sp. and found that ammonium carbonate was the best nitrogen source **(Boominadhan *et al.*, 2009),** whereas Zhu and co-workers and Akel *et al.* identified yeast extract as the best nitrogen source for protease production by *Geobacillus* sp. YMTC 1049 and hyperthermophilic *Bacillus* HUTBS71, respectively **(Zhu *et al.*, 2007; Akel *et al.*, 2009).** Bajaj and Sharma used mustard as a nitrogen source and found that it yielded the highest protease production by *Streptomyces* sp. DP2 **(Bajaj and Sharma 2011).**

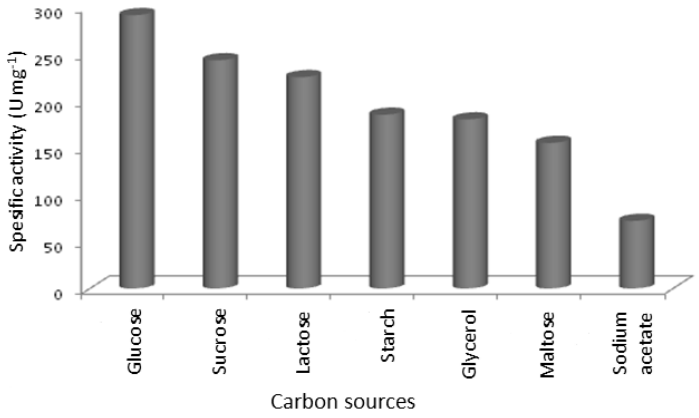

Figure 2 Effect of different carbon sources on protease enzyme production (Working conditions; incubation time: 120 hours, incubation temperature: 50 ºC, inoculum amount: 10%)

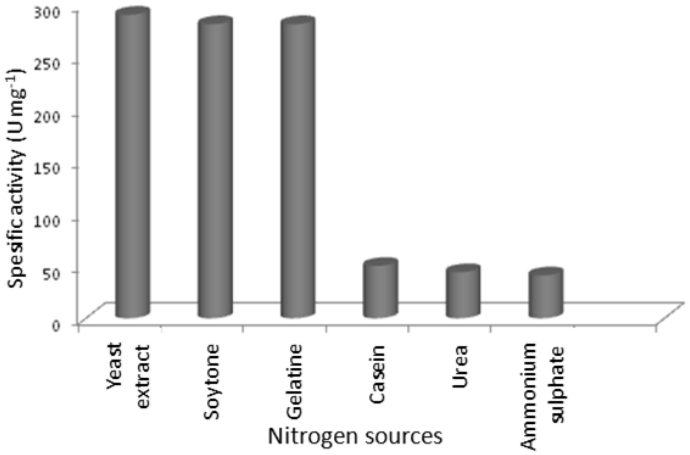

Figure 3 Effect of different nitrogen sources on protease enzyme production (Working conditions; incubation time: 120 hours, incubation temperature: 50 ºC, inoculum amount: 10%)

Determination of optimal protease production conditions

The nutritional needs (amounts of the components) and some environmental factors for protease production by *Geobacillus subterraneus* C2-1 were determined using a Plackett-Burman experimental design. This one-factor-at-a-time experiment requires many classical optimisation experiments and is thus a time-consuming process, although the experimental design allows results to be obtained rapidly using fewer assays.

The Plackett-Burman experimental designs and the response values for the activity of the thermostable protease are presented in Table 2. The results of a statistical analysis using Design Expert software are given in Table 3. Six of the 11 independent factors were found to be statistically significant. The data were analysed through analysis of variance (ANOVA). Figure 4 shows the Pareto chart of the effects with the level of significance. Among the factors, the incubation time was found to exhibit the highest level of significance, with a contribution of 54.77%. Additionally, peptone and glucose were among the most significant factors, with contributions of 14.58% and 11.33%, respectively. Other important factors affecting the process were found to be the magnesium ion amount, the activation time and the inoculum amount.

Based on the PBD results, the optimal levels of each media component were determined to be 9.99 g L^{-1} peptone, 14.75 g L^{-1} glucose, 0.45 g L^{-1} KH_2PO_4, 0.3 g L^{-1} $MgSO_4.7H_2O$, 7.51 g L^{-1} yeast extract, and 0.25 g L^{-1} $CaCl_2.2H_2O$, and the other optimised factors were a medium pH of 7.54, an incubation temperature of 56.35 °C, an incubation time of seven days, an activation time of 6.91 h and an inoculum amount of 3.56%. These optimised values produced a specific protease production activity of 1478 U mg^{-1}, whereas the predicted value for these optimal conditions was 1532 U mg^{-1}. The experimental results under the optimal conditions fell within the prediction interval (95%PI low: 1393.58 and 95%PI high: 1670.41 U mg^{-1}).

Using a Plackett-Burman experimental design, the optimal amount of inoculum (3.56%) was determined in this study. Johnvesly and Naik found that the highest protease activity of thermophilic and alkaliphilic *Bacillus* sp. JB-99 was obtained using an inoculum amount of 5% **(Johnvesly and Naik, 2001),** whereas Joo *et al.* studied the production of this enzyme by the alkaliphilic *Bacillus* sp. I-312 strain with an inoculum amount of 0.2% **(Joo *et al.*, 2005).** Nadeem *et al.* found maximal enzyme production with an inoculum amount of 2.0%, but further increases in the inoculum amount resulted in decreases in alkaline protease production (Nadeem et al. 2008). With higher inoculum concentrations, the nutrient might be rapidly consumed, which may ultimately result in less growth and decreased alkaline protease production. Similar findings were also observed by Shafee and co-workers **(Shafee *et al.*, 2005).** These results indicate that the inoculum amount has a significant impact on cell growth and protease production.

Table 2 Effect of media components and environmental parameters on the production of thermostable protease

	Experiments											
Factors	1	2	3	4	5	6	7	8	9	10	11	12
A	10	3	10	3	3	3	10	10	10	3	10	3
B	15	15	5	15	5	5	5	15	15	15	5	5
C	0.2	1	1	0.2	1	0.2	0.2	0.2	1	1	1	0.2
D	0.6	0.3	0.6	0.6	0.3	0.6	0.3	0.3	0.3	0.6	0.6	0.3
E	10	10	2	10	10	2	10	2	2	2	10	2
F	0.5	0.5	0.5	0.05	0.5	0.5	0.05	0.5	0.05	0.05	0.05	0.05
G	5	11	11	11	5	11	11	5	11	5	5	5
H	50	50	80	80	80	50	80	80	50	80	50	50
J	3	3	3	7	7	7	3	7	7	3	7	3
K	7	3	3	3	7	7	7	3	7	7	3	3
L	3	10	3	3	3	10	10	10	3	10	10	3
Enzyme Activity	948.4	614.5	655.4	995.1	1177.2	816.6	757.0	1316.5	1531.0	637.2	913.8	542.7

Table 3 Statistical parameters for the various components included in the Plackett-Burman screening study

Variable	Media Component	Effect	SS[a]	Contribution (%)	p-value[b]	Significance
A	Peptone (g L^{-1})	223.1	149368.8	14.6	0.0003	Yes
B	Glucose (g L^{-1})	196.7	116053.8	11.3	0.0005	Yes
C	KH_2PO_4 (g L^{-1})	25.5	1949.3	0.2	>0.1	No
D	$MgSO_4.7H_2O$ (g L^{-1})	-162.1	78815.7	7.7	0.0013	Yes
E	Yeast Extract (g L^{-1})	-15.6	725.9	0.1	>0.1	No
F	$CaCl_2$. $2H_2O$ (g L^{-1})	25.3	1921.7	0.2	>0.1	No
G	pH	-27.7	2304.6	0.2	>0.1	No
H	Temperature (ºC)	28.6	2446.4	0.2	>0.1	No
J	Incubation time (day)	432.5	561179.3	54.8	< 0.0001	Yes
K	Activation time (hour)	138.2	57318.2	5.6	0.0027	Yes
L	The amount of inoculum (%)	-132.4	52552.6	5.1	0.0032	Yes

[a] Sum of Squares, [b] p-values<0.05 were considered to be significant

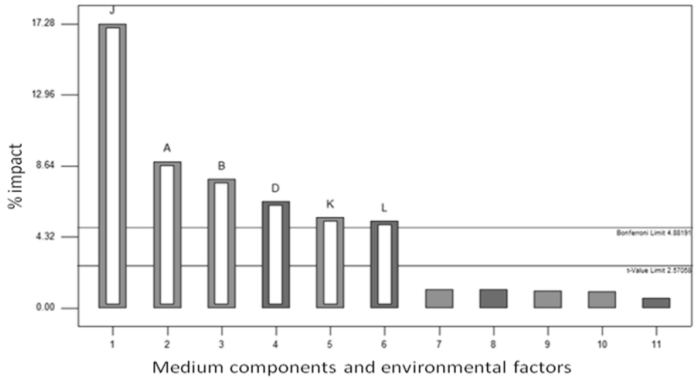

Figure 4 Experimental determination of the impact value (%) of the Pareto chart.

Our findings from optimisation studies related to the effect of the incubation temperature on growth showed that the *Geobacillus subterraneus* C2-1 isolate grown at nearly 57 °C yielded the highest enzyme activity. Annamalai *et al.* reported that the optimal temperature for protease production by *S. alveayuensis* CAS 5 was 55 °C **(Annamalai *et al.*, 2014).** In contrast, Voorhorst *et al.* showed that the hyperthermophilic archaeon *Pyrococcus furiosus* could produce alkaline protease at 95 °C **(Voorhorst *et al.*, 1995).**

The effect of incubation time on enzyme production was determined, and maximal enzyme synthesis was obtained following 168 h of incubation. Zhu *et al.* found that protease production by *Geobacillus* sp. YMTC was obtained after 8 h **(Zhu *et al.*, 2007).** According to the study conducted by Bajaj and Sharma, protease production by *Streptomyces* sp. DP2 was obtained after 72-96 h of incubation **(Bajaj and Sharma, 2011).**

Characterisation of crude enzyme

After the optimal production conditions were defined, the incubation was performed using the determined environmental parameters and medium components, and the resulting biomaterial (crude enzyme) was utilised for further characterisation experiments. The effects of temperature, pH, metal ions and denaturants on the activity of the produced enzyme were investigated. At the end of the optimisation experiments, the optimal reaction temperature (Fig. 5a) and pH (Fig. 5b) were found to be 40 °C and 10.5 (1682 U mg^{-1}), respectively. The specific activities at pH 9.0 and 10.0 were 1526 and 1552 U mg^{-1}, respectively. Additionally, a nearly constant specific activity of approximately 1500 U mg^{-1} was found at a pH range of 11.0-12.5.

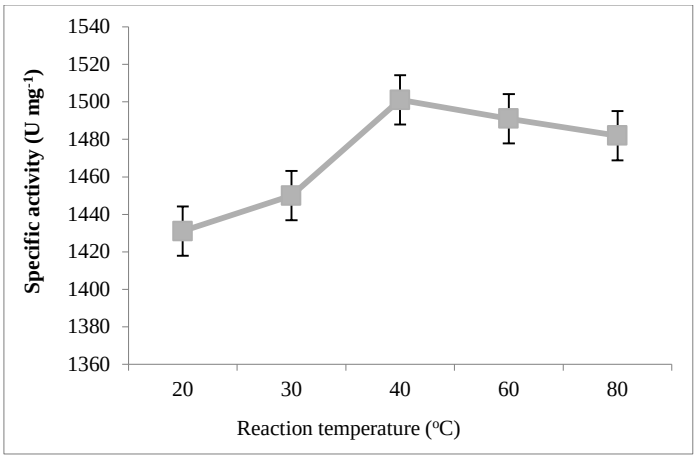

Figure 5a Optimal reaction temperature of the protease enzyme (Working conditions; reaction pH: 10.5; reaction time: 20 min.)

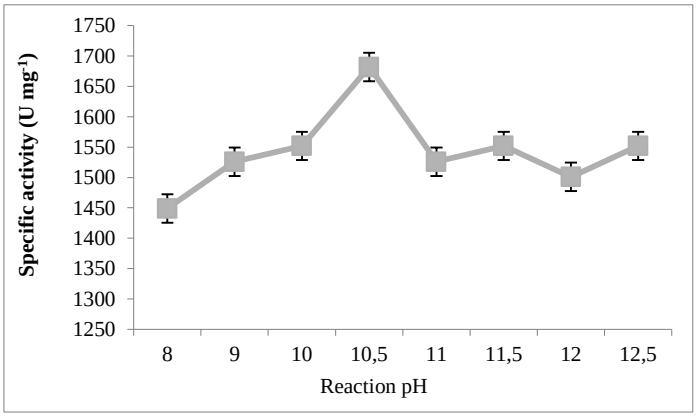

Figure 5b Optimal reaction pH of the protease enzyme (Working conditions; reaction time: 20 min., reaction emperature: 40 °C)

The specific enzyme activities at 20 and 60 °C were 1519 and 1614 U mg^{-1}, respectively, and this activity was maintained with further increases in the temperature. The pH and temperature stabilisation experiments revealed that the

activity increased with increasing temperatures. The activities of 1519 U mg^{-1} at 20 °C and 1654 U mg^{-1} at 85 °C were maintained for 24 h; therefore, the activities of the enzyme were maintained at higher temperatures. For example, an activity of 2289 U mg^{-1} was found to be maintained for 1 h at 95 °C, but this value decreased to 1166 U mg^{-1} after incubation for 120 h. The maintenance of the activity of this protease at 95 °C demonstrates that it exhibits a hyperthermophilic character. Voorhorst *et al*. found the highest activity of a protease from the hyperthermophilic archaeon *Pyrococcus furiosus* at 95 °C and pH 7.5 **(Voorhost *et al*., 1995),** whereas Annamalai *et al*. reported that the best activity of the protease from *Bacillus alveayuensis* CAS 5 strain was observed at 50 °C and pH 9.0 **(Annamalia *et al*., 2014).** In contrast, Zhu *et al*. determined that the protease obtained from *Geobacillus* sp. YMTC 1049 strain was effective at 75 °C and pH 7.5 (Zhu et al. 2007).

In this study, the thermostable character of and the maintenance of activity under both alkaline conditions and a large temperature range observed for the protease produced by the *Geobacillus subterraneus* C2-1 isolate are considerable advantages for the usability of this protease by various industries (Figs. 6 and 7), particularly the detergent industry, which requires protease that preserve their stability under increasing temperature and pH conditions **(Rao *et al*., 1998).**

The investigation of the effects of heavy metals on alkaline protease activity showed no considerable decrease in activity. In this investigation, heavy metals and toxic compounds, such as $FeCl_3$, $FeSO_4$, $CuCl_2$, $CdCl_2$, $CuSO_4$, KCl, NaCl, CaCl, $CoCl_2$, $BaCl_2$, $MnCl_3$, KCN and $HgCl_2$ (at final concentrations of 5 mM and 10 mM, KCN and $HgCl_2$ were also investigated at a final concentration of 100 mM) were added to the reaction, and the specific activities were then measured. The enzyme produced was determined to be resistant to heavy metals. A decrease of approximately 5-10% was obtained in the presence of 10 mM $HgCl_2$ or $BaCl_2$, 100 mM KCN, or 5 mM NaCl, $FeSO_4$ or $FeCl_3$. Furthermore, 100 mM Hg ion caused a 50% decline. However, an activity increase was observed in the presence of Mn^{2+} and Cu^{2+} ions (Fig. 8). Akel *et al*. found similar results related to changes in activity in the presence of manganese ion **(Akel *et al*., 2009).** According to the study conducted by Zhu *et al*., the activity of the protease RH-1 was enhanced by the addition of 10 mM EDTA and EGTA. In addition, the activity of the protease can be activated by the addition of Ca^{2+} and Mg^{2+} but partially inhibited by Ba^{2+}, Zn^{2+}, Pb^{2+}, Co^{2+}, Mn^{2+} and Cu^{2+} **(Zhu *et al*., 2007).**

To determine the effects of different denaturants on the alkaline protease activity, urea, SDS and EDTA were added to the reaction environment at varying concentrations. At the end of the incubation period, the relative activities were defined compared with the control solution without denaturant, and the results are presented in Figure 9.

The protease obtained from the *Geobacillus subterraneus* C2-1 isolate was highly stable in the presence of the denaturants used in this study. Urea (0.5, 1.0 and 10.0 mM) had no significant effect on protease activity (Fig. 9). Banarjee also reported that a maintenance of protease activity at a rate of 95% in the presence of urea **(Banarjee *et al*., 1999).** Another study showed an enhancement in enzymatic activity in the presence of high concentrations of urea (4–6 M) **(Zhu *et al*., 2007).**

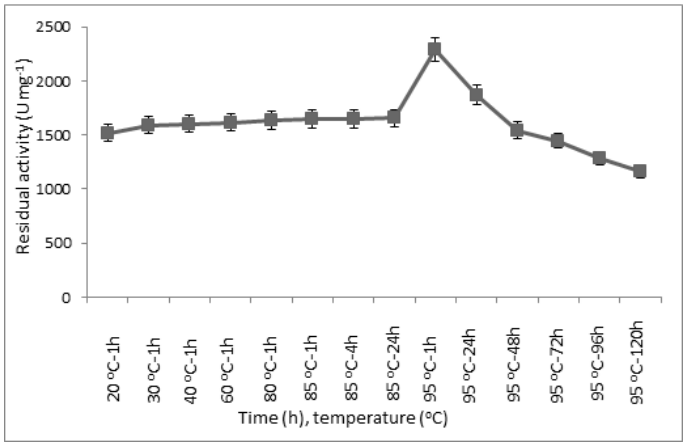

Figure 6 Effect of incubation temperature on protease stability (Working conditions: reaction pH: 10.5; reaction time: 20 min.)

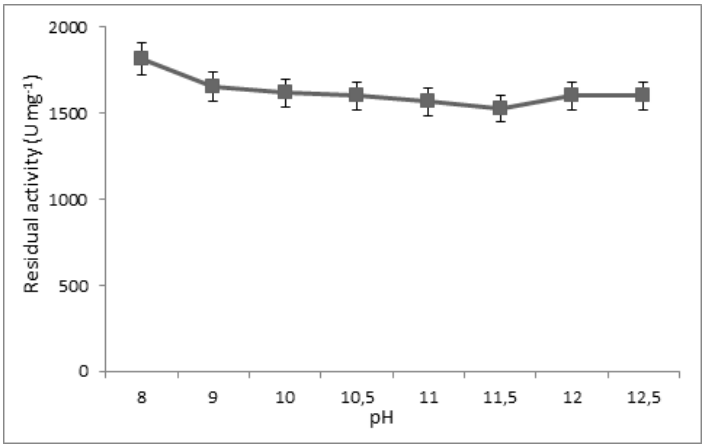

Figure 7 Effect of incubation pH on protease stability (Working conditions: reaction pH: 10.5; reaction time: 20 min.)

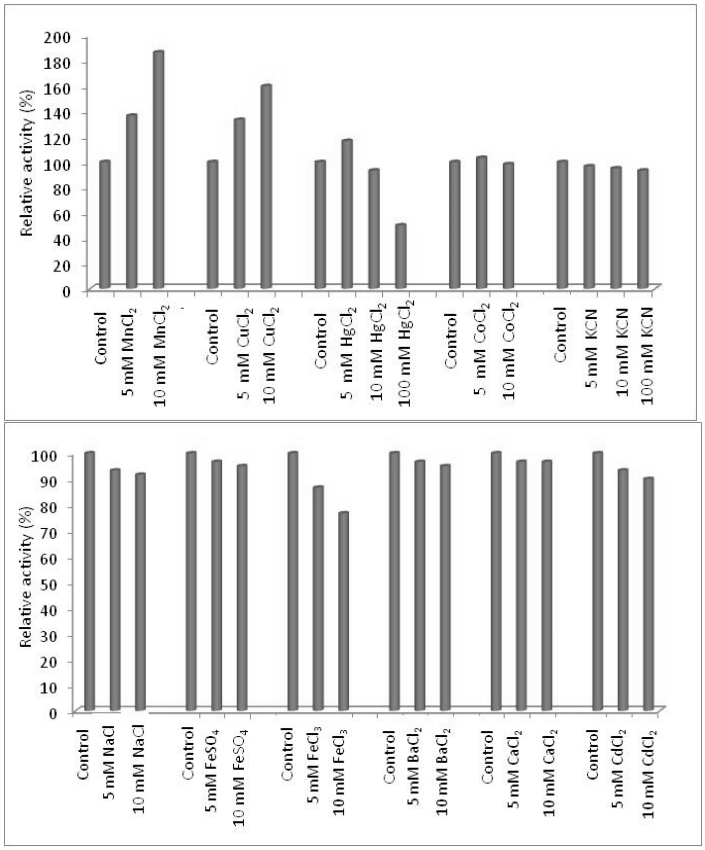

Figure 8 Effect of some metals and toxic compounds on enzyme stability (Working conditions: reaction pH: 10.5; reaction time: 20 min.)

Bacterial proteases are known to be unstable in the presence of SDS. However, the addition of 1.0 mM SDS did not result in any alterations in protease activity in this study, and the addition of 5 and 10 mM SDS decreased the enzyme activity by only 7% and 15%, respectively (Fig. 9). The SDS resistance of the protease may be an important characteristic for industrial products, such as detergents with protease additives. Similar results related to SDS resistance are detailed in the literature **(Uyar *et al*., 2011; Özçelik *et al*., 2014).**

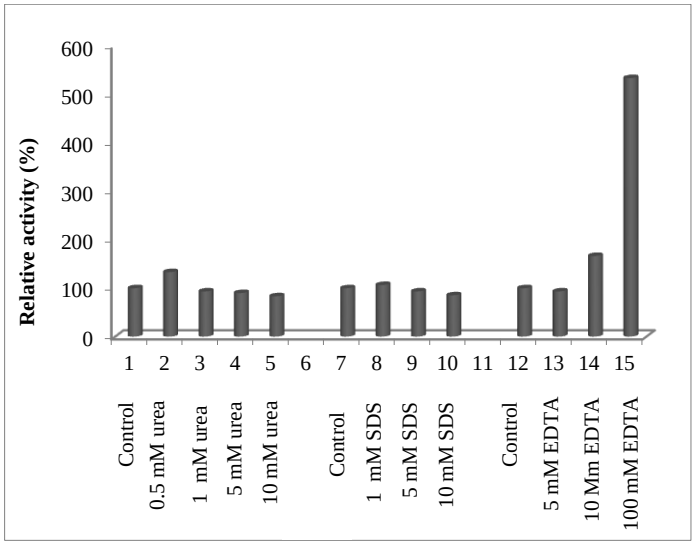

Fig. 9 Effect of some denaturants on enzyme stability (Working conditions: reaction pH: 10.5; reaction time: 20 min.)

Surprisingly, the specific activity of the protease was increased by the presence of EDTA in the reaction environment used in this study. The addition of 100 mM EDTA resulted in a five-fold enhancement in specific protease activity. Due to the rigidity of the native conformations of thermophilic proteases, chemical denaturants, chelators, and even heavy metals might not cause any notable changes in activity **(Manning and Colon, 2014).** Kujo and Oshima observed that the activity of NAD-dependent glutamate dehydrogenase extracted from the archaeon *Pyrobaculum islandicum* was increased in the presence of some denaturants, such as urea **(Kujo and Oshima, 1998).** The protease in this study was found to be resistant to chemical denaturants, including SDS, urea and EDTA, and we demonstrated that the protease exhibited structural stability, similarly to the protease RH-1 **(Zhu *et al.*, 2007)** from *Geobacillus* sp. YMTC 1049. In addition, the protease from *Geobacillus subterraneus* C2-1 obtained in this study might be a monomer, and it is also possible that its disulphide bridges might not be fractured in the presence of denaturants. This finding is supported by the fact that the protease investigated in this study preserved its activity at high temperatures and in the presence of heavy metals and denaturants. The results indicate that the conformation of the enzyme might be extremely rigid and that this protease exhibits good packing associated with thermostability. Similar results are found in the literature **(Chen and Wesley, 2004).** Besides, urea with low concentrations due to the unfolding effect of urea on substrate may enhance enzyme activity thus making it a better substrate. Similar findings were given in the literature **(Hill *et al.*, 1959; Garg and Virupaksha, 1970)**

Identification of producer isolate

The partial 16 S rRNA gene sequence of protease producer strain was identified. The sequence was aligned and compared with the bacterial sequences available in the GenBank database. According to BLAST analysis, the highest similarity level (100%) detected was from the 16S rDNA sequence of the strain of *Geobacillus subterraneus* FZWP-19. deposited in the GenBank (accession number JX914497).

CONCLUSIONS

Our results contribute to the literature due to the lack of studies on an extremely resistant protease from a strain of *Geobacillus subterraneus.* The fact that this protein maintains its specific activity in the face of increasing temperatures, varying pH levels, and the presence of different heavy metals and denaturants demonstrates its important advantages and potential for a wide range of industrial uses.

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