Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Scholars Research Library

Annals of Biological Research, 2012, 3 (4):1747-1756
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

Phenotypic and protease purification of two different thermophilic *Bacillus* strains HUTBS71 and HUTBS62

Aqel, H.[1,2*]

[1]*Clinical Laboratory Sciences Program; King Saud Bin Abdulaziz University for Health Sciences; Riyadh, Saudi Arabia*
[2]*Department of Medical Laboratory Sciences; Hashemite University; Zarqa; Jordan*

ABSTRACT

The thermophilic proteases were purified from two different Bacillus strains HUTBS62 and HUTBS71. The two strains showed variation in ability to grow at different pH-values and temperatures, pH 5-11 and 28-73°C (HUTBS71) and pH 5-7 and 37-63°C (HUTBS62), respectively. The purified enzyme from the two different strains also showed variation in purification folds and %yields in different steps of purification methods. Ammonium sulfate fractionation was achieved at 75-80% for HUTBS71 and 55-60% concentrations for HUTBS62. The purification fold and yield were 10-fold and 67% for strain HUTBS71, and 6.5-fold and 61% for strain HUTBS62, respectively. Sephadex G-100 purification step achieved 40-fold purification and 16.7% yield from strain HUTBS71, and 32-fold purification and 12% yield of protease from strain HUTBS62. DEAE ion exchange chromatography step achieved 60-fold purification and 1.7% yield for strain HUTBS71, and 53-fold purification and 2% yield for strain HUTBS62. The molecular weight of purified proteases from HUTBS71 and HUTBS62was 49 kDa and 48 kDa, respectively.

Key words: Protease; *Bacillus*; Purification; Ammonium sulfate; Sephadex-G-100; Ion exchange.

INTRODUCTION

Proteases (EC. 3.4.21-24 & 99) are a complex group of enzymes collectively known as peptidyl-peptide hydrolases. They are responsible for the hydrolysis of peptide bonds in a protein molecule by the process of proteolysis. Bacteria belonging to *Bacillus* species are the most important source of many commercial microbial enzymes [1]. Microbes represent an excellent source of enzymes such as proteases, because of their broad biochemical characters [2]. These proteases, especially from *Bacillus* are the most widely exploited industrial enzymes with major application in detergent formulation [3], pharmaceutical, leather, laundry, food, waste processing industries, chemical synthesis, production of high fructose corn syrup and polymerase chain reaction [4, 5, 6].

The importance of enzymes as source for industrial applications has been well recognized and it was reported that proteases count for nearly 65% of the total world enzyme sales. They are classified according to their optimum pH to acidic, neutral and alkaline proteases [7].

The aim of this study is to: (a) Phenotype the isolated tested thermophilic bacteria; (b) Purify protease enzymes from thermophilic *Bacillus* strains HUTBS71 and HUTBS62 using three different methods: (1) Ammonium sulfate

fractionation, (2) Sephadex-G100 gel filtration (3) DEAE ion exchange chromatography; and (c) Determine the molecular weight of the purified enzymes.

MATERIALS AND METHODS

Organisms

Bacillus strains HUTBS62 and HUTBS71 were previously isolated in Dr. Hazem Aqel's Microbiology Lab in the Hashemite University. The water specimens were obtained from Mai'ns hot-spring, Jordan. The colony characteristics, cell morphology, Gram reaction, and biochemical tests of the two strains were determined. They were also screened for protease activity using casein agar medium [8].

Enzyme production

For maximum production of protease each bacterial strain was left to grow in a medium containing the following: 0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 0.5 g/l glucose, 0.4 g/l Na_2HPO_4, 0.085 g/l Na_2CO_3, 0.02 g/l $ZnSO_4$, 0.02 g/l $CaCl_2$, 0.02 g/l $MgSO_4$ (Huang *et al*., 2006). Two thousand five hundred of the above medium was inoculated with 1٪ of old bacterial culture and incubated in bioreactor (Electrolab 351EMC, Switzerland) at 50°C for 50 h under a continuous stir (at 150 rpm). The medium was adjusted to a pH 7.0. After completion of growth, the whole broth was centrifuged using (Universal 32, Hettich, Germany) at 14000 x g for 30 min at 25°C, and the clear supernatant was recovered and used for purification.

Protein concentration

The protein concentration was determined according to the method of Lowry *et al*. [9]; taking crystalline bovine serum albumin as the standard.

Protease assay

The proteolytic activity of the enzyme was assayed in triplicate using casein (Hi Media, India) as a substrate as described by Huang *et al.* [7] where a mixture of 400 µl casein solutions (2% (w/v) in 50 mM Tris-HCl buffer pH 7.2) and 100 µl of the sample were added to a tube. The reactions were carried out at 65°C in water bath (Memert, Germany) for 10 min and then terminated by the addition of 1 ml 10% trichloroacetic acid (w/v). The mixture was centrifuged at 14000 x g for 20 min in order to remove the precipitate formed. A 500 µl supernatant was carefully removed to measure tyrosine content using a Folin-phenol method described by Michel and Francosi [10].

One unit of protease activity (U) was defined as the amount of enzyme that hydrolyzed casein to produce 1.0 µmole of tyrosine per min at 65°C.

The number of units of protease activity per ml was calculated using the following equation: Unit ml^{-1} = (µmole of tyrosine × reaction volume)/(sample volume × reaction time ×volume assayed) = µmoles min^{-1} ml^{-1}.

Ammonium sulfate precipitation

The microorganisms were grown for 50 h as described previously. The cells were separated by centrifugation (14000 x g, 30 min) and the supernatant was fractionated by precipitation with ammonium sulfate. The precipitates between 30-90% for HUTBS71 and HUTBS62 were collected using centrifuge at (14000 x g, 20 min). All the subsequent steps were carried out at 25°C. The pellet from each strain was dissolved in 50 mM Tris-HCl buffer pH 7.2, dialyzed for 3 h against the same buffer, or concentrated by filter tube and concentration was achieved by centrifugation of filter tubes at 14000 x g for 15 min at room temperature and the active fraction retained above the filter was stored at 4°C for further study.

Sephadex G-100 gel filtration chromatography

The protein fraction obtained earlier were loaded onto a column of Sephadex G-100 (1.5 cm x 27 cm) (Fluke, Switzerland) equilibrated with Tris-HCl buffer pH 7.2. The column was eluted with the same buffer at a flow rate of 0.5 ml min^{-1}; fractions with high protease activities were pooled, concentrated using filter tubes, concentration was achieved by centrifugation at 14000 x g for 15 min at room temperature and active fraction was stored at 4°C for further study.

Ion-exchange chromatography

Two ml of the active fractions collected earlier and concentrated from gel filtration column were applied onto the DEAE Sepharose fast flow column equilibrated with 50 mM sodium acetate buffer pH 4.8 or 50 mM Tris-HCl buffer pH 7.2 for fractions from strains HUTBS71 and HUTBS62 respectively ; each column was washed with 10 column volumes of equilibration buffer; then protease and other proteins were eluted using 50 mM Tris-HCl buffer pH 7.6 or 50 mM Tris-HCl buffer pH 8.6 for fractions of strain HUTBS71 and HUTBS62, respectively, and flow rate of 0.5 ml min^{-1} was maintained at 0.5 ml min^{-1} and 3 ml fractions were collected.

Fractions active with protease were collected pooled, dialyzed, and concentrated by filter tubes (after centrifugation at 14000 x g for 15 min at room temperature) or by dialysis against sucrose crystal for 2-3 h, then stored at 4°C for further analysis**.**

PAGE or SDS-PAGE electrophoresis

Poly-acrylamide gel electrophoresis (PAGE) or Sodium Dodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) was done essentially as described by Laemmli [11]; using a mini-gel apparatus (80 mm × 60 mm × 0.75 mm; 10 lanes), with 7.5% (w/v) polyacrylamide separating gel. A 0.1 (w/v) gelatin was included in separating gel for protease activity staining only in PAGE. Protein molecular weight markers (Promega, USA) or protease samples were applied. Electrophoresis was carried out at 100 mA for approximately 1.5 h or until bromophenol blue tracking dye reached the bottom of the gel. For protease activity staining, the gel containing 1% gelatin was incubated at 37^{o}C in 50 mM Tris-HCl buffer pH 7.2 for 15 h, the protease caused hydrolysis of gelatin included in the gel. Gel was then stained for protein bands with Coomassie Brilliant Blue R-250 (Bio-Rad USA) and distained with 10% acetic acid and 10% ethanol as usual. In PAGE gel the clear bands against blue background represent the site of active protease while SDS-PAGE protein bands are blue against a clear background.

RESULTS

Phenotypic characteristics of the new isolates

The new isolates of *Bacillus* species (HUTBS62 and HUTBS71) showed a variation in colony morphology from raised, smooth, circular and entire colonies to flat, rough, erose and raised colonies. The color of colonies for both strains was white. The Gram stain showed also variation in the cell morphology and spore location from short to long thick rods, with central and sub-terminal spores. The broth growth of the isolates showed one of the following: Turbidity or layer at surface only. The optimum pH-values and temperatures for growth of each strain were pH 5-7 and 37-63^{o}C (HUTBS62), pH 5-11 and 28-73^{o}C (HUTBS71), respectively. Both strains hydrolyzed casein, starch and gelatin (Table 1).

Ammonium sulfate fractionation

Detailed description of enzyme activity and protein concentration in ammonium sulfate fractions are presented in Figure 1. *Bacillus* strain HUTBS71 showed two peaks of protease activity; the maximum enzyme activity was found in 75-80% saturation fraction, and the second was a minor protease activity peak was found at 55-60% saturation fraction. Ammonium sulfate fractionation resulted in 10 purification fold and 67% yield. Whereas, *Bacillus* strain HUTBS62 showed two peaks of protease activity. The maximum enzyme activity was found in 55-60% saturation, and a second minor protease activity peak was found at 75-80% saturation. Ammonium sulfate fractionation resulted in 6.5 purification fold with 61% yield.

Sephadex G100 gel filtration column

The elution profile of protease activity and protein concentration from strains HUTBS71 and HUTBS62 eluted from Sephadex G100 gel filtration column is shown in Figure 2. Gel filtration fractionation for *Bacillus* strain HUTBS71 resulted in a higher peak around fraction 13 and a lower peak around fraction 16; this gel filtration purification step resulted in 40-fold purification and 16.7% yield. Whereas, gel filtration fractionation for *Bacillus* strain HUTBS62 resulted in a single protease activity peak around fraction 15 and this purification step resulted in 32-fold purification and 12% yield.

Ion exchange chromatography

DEAE Sepharose ion exchange chromatography column eluted fractions for both strains HUTBS71 and HUTBS62 is presented in Figure 3. For *Bacillus* strain HUTBS71, two active protease peaks were eluted from the column: one peak eluted at fraction 15 and the second peak eluted at fraction 18. Sepharose fractionation resulted in 60-fold

purification and 1.7% yield. On the other hand, a single peak was eluted, *Bacillus* strain HUTBS62, around fraction 13 from the Sepharose column. this ion exchange chromatography fractionation step resulted in 53-fold purification and 2% yield.

Polyacrylamide gel electrophoresis (PAGE and SDS-PAGE)

PAGE (7.5%) analysis was performed to evaluate purification progress and the number of protease isoenzymes present in strain HUTBS71 and HUTBS62 and each purification step was examined by PAGE. The purified protease eluted from DEAE ion exchange chromatography column migrated as a single band in the native PAGE upon protease activity staining for both HUTBS71 and for HUTBS62. The result is presented in Figure 4.

SDS-Gel electrophoresis (7.5%) reveals that molecular weight of protease purified from HUTBS71 and HUTBS62 consists of a single subunit of 49 kDa and 48 kDa, respectively. The result of SDS-gel electrophoresis is presented in Figure 5.

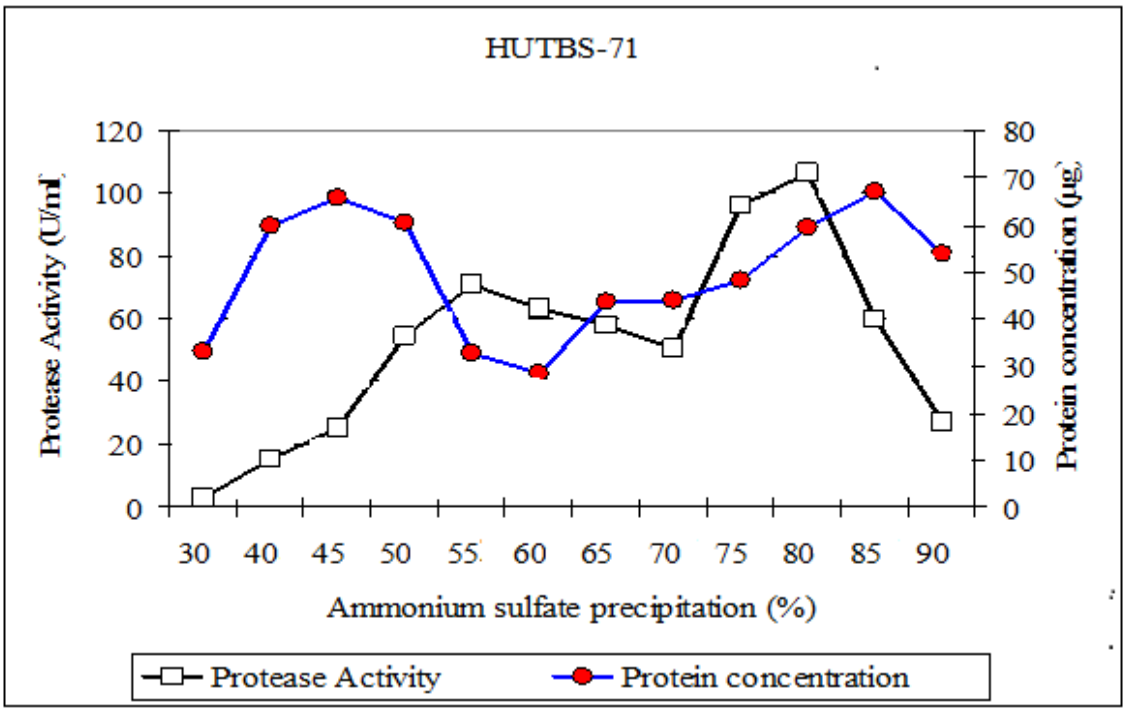
Summary of purification of protease

Protease was purified by ammonium sulfate fractionation, Sephadex G100 gel filtration and DEAE ion exchange chromatography. Protease from *Bacillus* strain HUTBS71 was purified 57-fold and 1.7% yields while, *Bacillus* strain HUTBS62 was purified 53-fold with 2% yield. Purification summary of proteases from both strains is presented in Table 2.

Table 1. Bacterial colonial characterization, cell morphology, hydrolysis enzymes and other biochemical tests. Where WRSCE: white, raised, smooth, circular and entire colonies; WFREI: white, flat, rough, erose and raised colonies; VP test: Voges-Proskauer test; TDA: Tryptophane diaminase; LDC: Lactate decarboxylase; G+ve: Gram positive; G-ve: Gram negative.

	***Bacillus* species strain**	
	HUTBS62	**HUTBS71**
Optimum temperature	37-63^{o}C	28-73^{o}C
Optimum pH-values	5-7	5-11
Culture examination:		
Colony morphology	WRSCE	WFREI
Broth growth	Turbid	Layer at surface
Microscopic examination:		
Gram stain	G+ve short, thick rods with central spore.	G+ve long, thick rods with central and subterminal spore.
Enzyme hydrolysis:	+ve	+ve
Casein	+v	+ve
Starch	+ve	+ve
Gelatin	-ve	+ve
Growth in 7% NaCl	-ve	+ve
Oxidase test	-ve	+ve
Indole test	+ve	-ve
VP test	+ve	-ve
TDA	-ve	+ve
LDC	-ve	+ve
Acid from glucose		

Figure-1. Protease activity and protein concentration in ammonium sulfate fractions for both *Bacillus* strains HUTBS71 and HUTBS62.



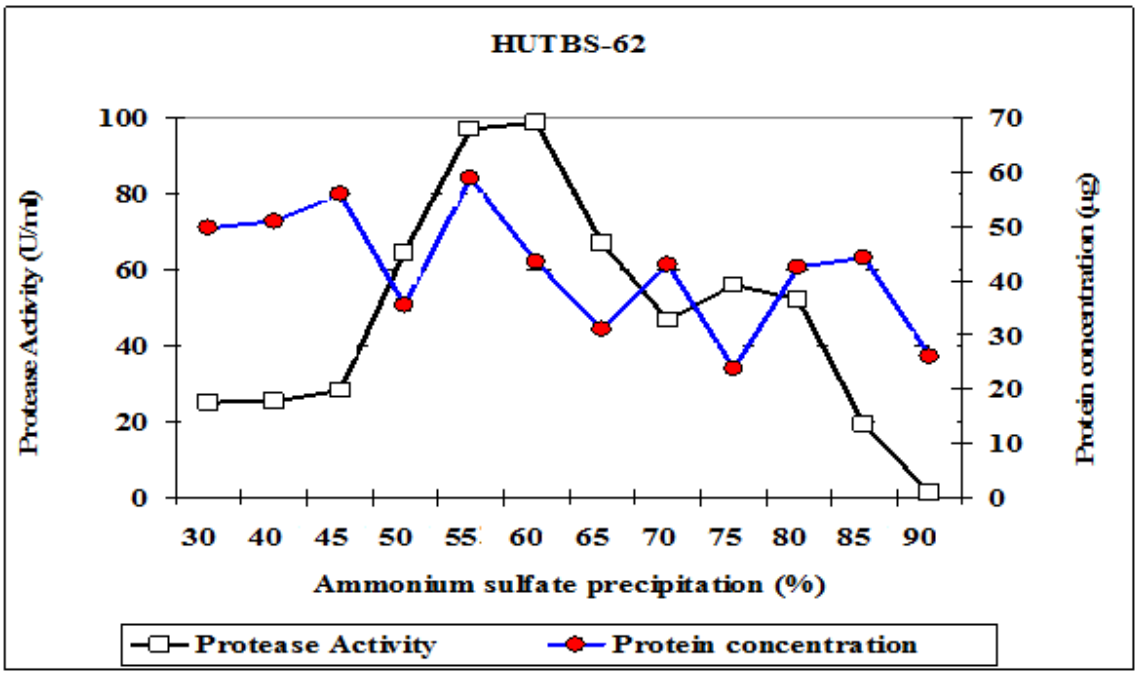
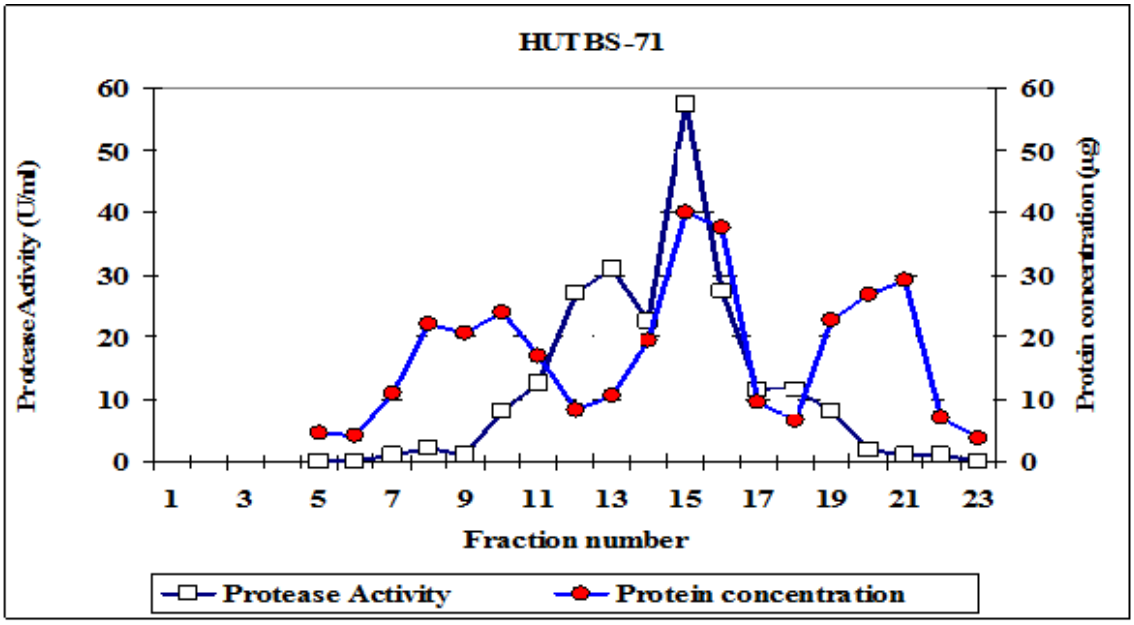


Figure 2. Protease activities and protein concentrations in each fraction for *Bacillus* strains HUTBS71 and HUTBS62 eluted from Sephadex G-100 column.



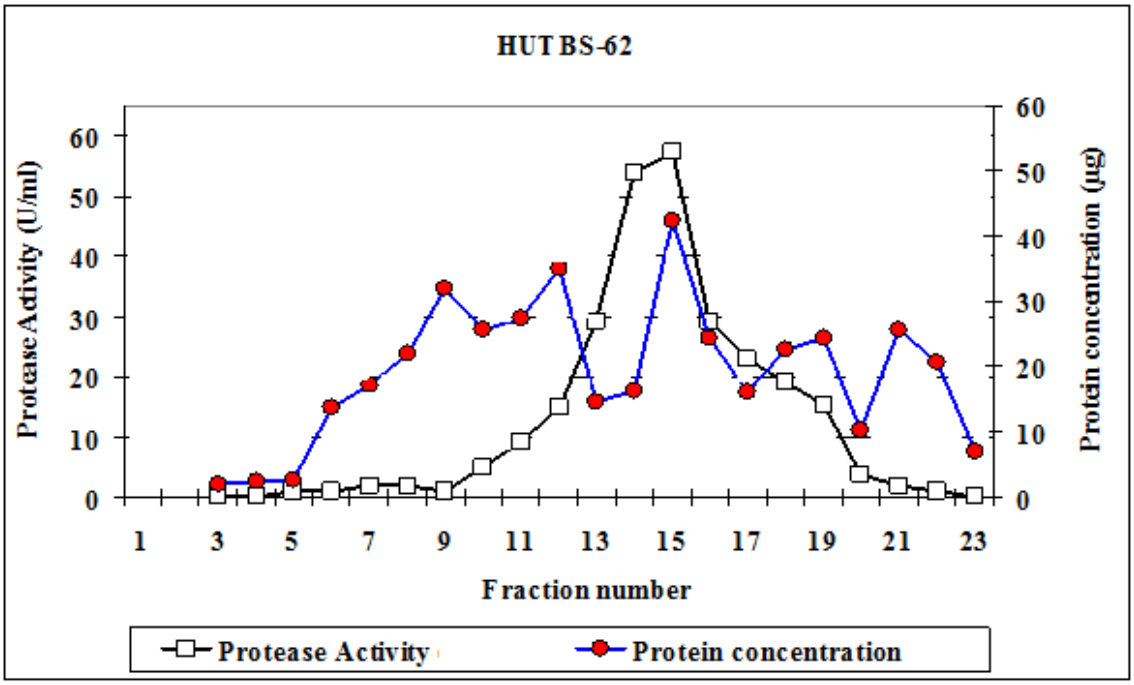
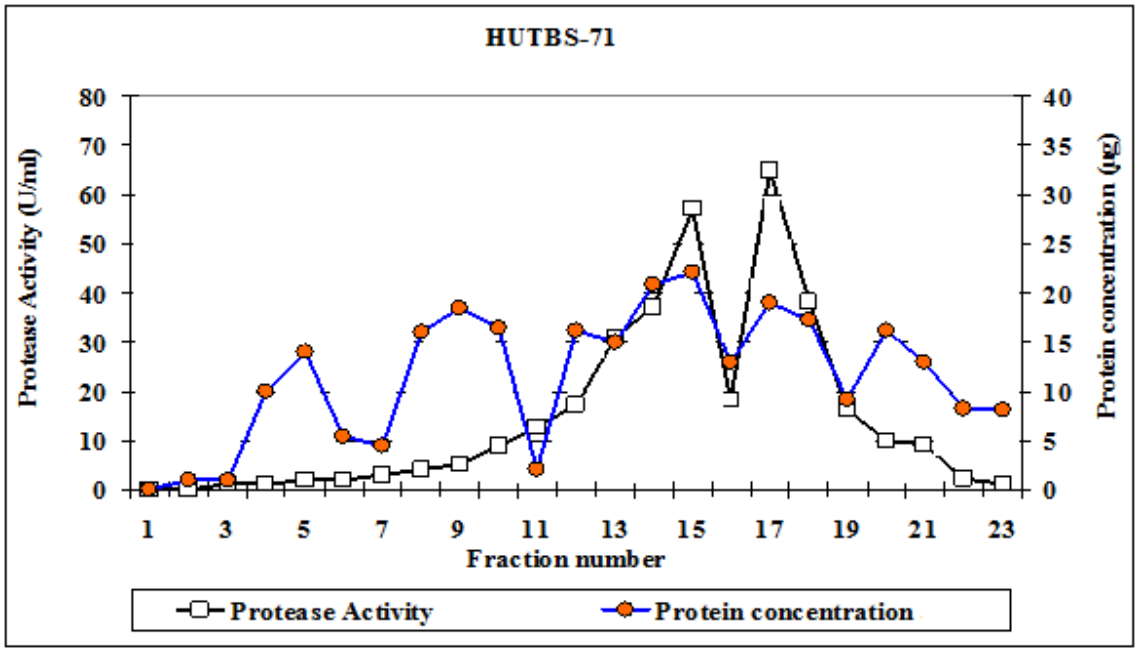


Figure 3. *Bacillus* strains HUTBS71 and HUTBS62 protease activity and protein concentration eluted from DEAE-Sepharose column



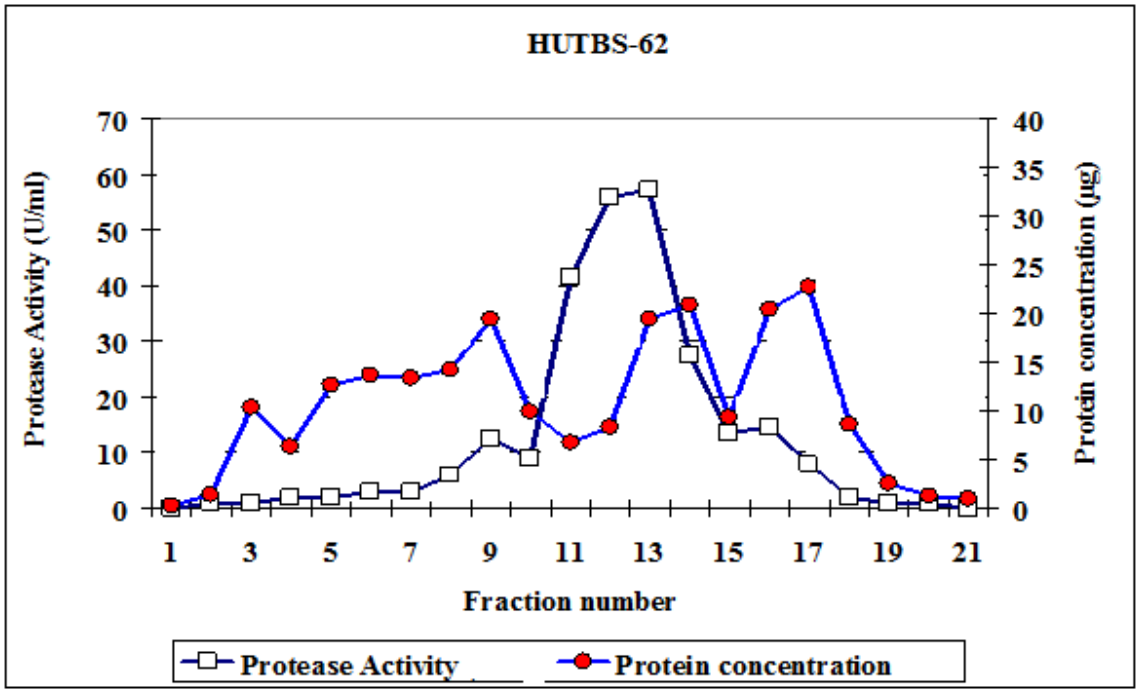


Figure 4. Polyacrylamide Gel Electrophoresis (7.5%) with patterns of proteins isolated at different purification steps from *Bacillus* HUTBS71 and HUTB62 strains stained for protease activity. 0.1% gelatin is included in the gel

HUTBS71 strain: lane A gel filtration fraction lane B supernatant media; lane D ammonium sulfate fraction and lane G ion exchange chromatography fraction.
HUTBS62 strain: lane C media supernatant; lane E ammonium sulfate fraction; lane F gel filtration fraction and lane H ion exchange chromatography fraction.

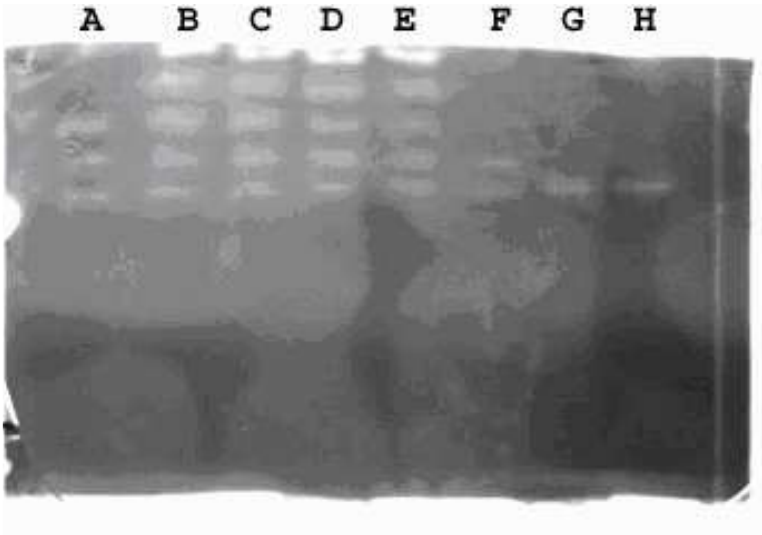


Figure 5. SDS-Polyacrylamide gel electrophoresis of protease from HUTBS71 and HUTBS62. Lane A: purified protease from HUTBS62; lane B: purified protease from HUTBS71 and Lane C : protein markers (25, 35, 50, 75, 100, 150 kDa).

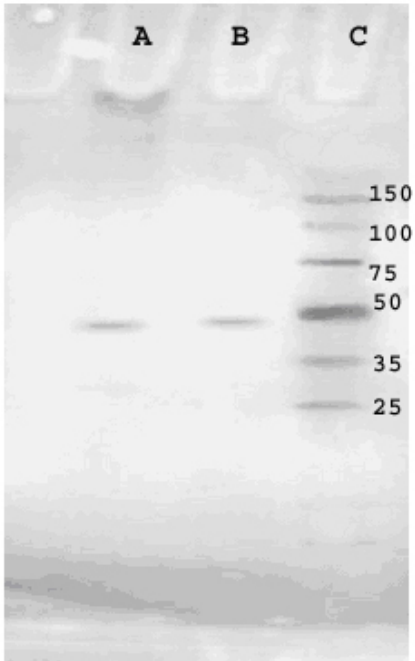


Table 2. Summary of Protease purification from *Bacillus* strains (a) HUTBS71 and (b) HUTBS62.

(a)

Purification Steps	Volume (ml)	Protein (mg ml^{-1})	Enzyme activity (U ml^{-1})	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Culture media	50	24	3.2	159	6.6	1	100
Ammonium sulfate (75%-80%)	14	11.3	51	724	64	10	67
Sephadex G-100	12	3	59	717	267	40	16
DEAE – Chromatography	11	1.4	48	533	392	59	1.7

(b)

Purification steps	Volume (ml)	Protein (U ml^{-1})	Enzyme activity (U ml^{-1})	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Culture media	50	22.5	6.4	321	14	1	100
Ammonium sulfate (55%-60%)	13	8	52	672	83	6.5	61
Sephadex G-100	12	1.5	57	679	453	32	12
DEAE-Chromatography	10	0.63	47	468	744	53	2

DISCUSSION

Thermophilic microorganisms secrete a numbers of proteolytic enzymes such as proteases with unique properties that make these enzymes of special interest for industrial application; its application is very broad and is mainly used in food and detergent industries [12]. *Bacillus species* were considered as an ideal host for the industrial production of the extracellular enzymes [13]. An extracellular protease was isolated from cells of thermophilic *Bacillus* HUTBS71 and HUTBS62 strains which were isolated from thermal water of Mai'n hot spring and purified to homogeneity by ammonium sulfate precipitation, Sephadex G-100 gel filtration chromatography followed by Sepharose ion-exchange chromatography. Charles *et al.* [14] purified extracellular alkaline protease from *Aspergillus nidulans* HA-10 in two-step procedures involving ammonium sulfate precipitation and sephadex G-100 column chromatography.

Electrophoresis studies revealed the presence of four protease bands secreted in the medium for each strain with comparable R_f values and all bands were recovered by ammonium sulfate fractionation but at different concentrations. The purified protease from the two strains was the fast moving band. The discrepancy between the numbers of protease active fractions in column chromatograms and native PAGE stained for protease activity may represents an artifact such as a partially hydrolyzed enzyme.

The purified proteases from HUTBS71 and HUTBS62 are monomeric proteins. The molecular weight was estimated to be about 49 kDa and 48 kDa for HUTBS71 and HUTBS62 strains, respectively. Begum *et al.* [15] reported a similar result for protease molecular weight from *Pseudomonas aeruginosa*. Charles *et al.* [14] showed a molecular weight of proteases from *Aspergillus nidulans* HA10 of 42 kDa.

The two new thermophilic proteases were purified from two new different *Bacillus* strains HUTBS62 and HUTBS71. Enzymes from both strains showed variation in fold purification, % yield and molecular mass and even they differ from previous purified proteases from other *Bacillus* species.

Further work on effect of temperature, pH, thermostability stability, metal ions and EDTA on activity is currently under investigations.

REFERENCES

[1] R. Camila, B. Andréa and L. Meire. *Brazil. J. Microbiol.*, **2007**, 38, 253-258.
[2] N. Bhashar, E. Sudeepa, H. Rashmi and A. Selvi. *Bioresource Technology*, **2007**, 14, 2758-2764.
[3] B. Qasim and G. Rani**.** *Process Biochemistry*, **2002**, 37, 1103-1109
[4] F. Olajuyigbe and J. Ajele. *Afri. J. of Biotech*, **2005**, 4, 776-779.
[5] S. Kumar, C. Tsai and R. Nussinov. *Oxford J Protein Engineering*. **2000**, 13, 179-191.
[6] D. Cowan, R. Daniel and H. Morgan. *Trends Biotechnology*, **1985**, 65, 4559-4567.
[7] G. Huang, Y. Tiejing, H. Po and J. Jiaxing. *Afri. J. Biotech.*, **2006**, 5, 2433-2438.
[8] H. Akel and M. Atoum, M. *Microbiologica*, **2003**, 26: 249-256.

[9] O. Lowry, N. Rosebrough, A. Farr and R. Randall. *J. Biol. Chem.*, **1951**, 193, 265-275.
[10] L. Michel and L. Francosi. *Anal. Biochem.*, **1986**, 157, 28-31.
[11] U. Laemmli. *Nature*, **1970**, 227, 680-685.
[12] T. Yandri, H. Dian, and H. Sutopo. *Europ. J. Sci Resea.*, **2008**, 23, 177-186.
[13] B. Qasim and G. Rani. *Enzyme and Microbial Technology*, **2003**, 32, 294-304.
[14] P. Charles, V. Devanathan, A. Periasamy, M. Ponnuswamy, P. Kalaichelvan and K. Byung. *J. Basic Microbiol*, **2008**, 48, 347-352.
[15] S. Begum, I. Ahmed, F. Alam, H. Samsuzzaman, N. Absar and H. Ashraf. *Pak. J. Med. Sci*., **2007**, 23, 227-232.