Optimization, Purification, and Characterization of Haloalkaline Serine Protease from a Haloalkaliphilic Archaeon *Natrialba hulunbeirens* Strain WNHS14

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The present study addresses isolation, optimization, partial purification, and characterization of a haloalkaline serine protease from a newly isolated haloarchaeal strain isolated from Wadi El Natrun in Egypt. We expected that a two-step sequential statistical approach (one variable at a time, followed by response surface methodology) might maximize the production of the haloalkaline serine protease. The enzyme was partially purified using Hiprep 16/60 sephacryl S-100 HR gel filtration column. Molecular identification revealed the newly isolated haloarchaeon to be *Natrialba hulunbeirens* strain WNHS14. Among several tested physicochemical determinants, casamino acids, KCl, and NaCl showed the most significant effects on enzyme production as determined from results of the One-Variable-At-A-time (OVAT) study. The Box-Behnken design localized the optimal levels of the three key determinants; casamino acids, KCl, and NaCl to be 0.5% (w/v), 0.02% (w/v), and 15% (w/v), respectively, obtaining 62.9 U/ml as the maximal amount of protease produced after treatment at 40 °C, and pH 9 for 9 days with 6-fold enhancement in yield. The enzyme was partially purified after size exclusion chromatography with specific activity, purification fold, and yield of 1282.63 U/mg, 8.9, and 23%, respectively. The enzyme showed its maximal activity at pH, temperature, and NaCl concentration optima of 10, 75 °C, and 2 M, respectively. Phenylmethylsulfonyl fluoride (PMSF, 5 mM) completely inhibited enzyme activity.

Keywords: Protease, Haloalkaliphilic, archaea, *Natrialba hulunbeirens*, enzyme production

Introduction

Halophilic proteases are widely exploited in the processing of leather, detergents, and food. Since, halophilic proteases are adapted to extreme environments, they are surprisingly stable and therefore they could be of a great importance for industrial processes that are performed under high values of temperature and ionic strength and in the organic solvents [15]. The domain

Archaea was first suggested by Woese and Fox [44] as one of the three domains of life. Within this domain is a separate cluster for the halophilic archaea which grow optimally in media with high NaCl (3.5–5.2 M) [34]. In hypersaline environments, extremely halophilic archaea are often the dominant members of the microbial population due to their ability to balance osmotic pressure using a low-energy strategy [30]. Many haloarchaea produce proteolytic enzymes that function at high salt concentrations (3–4 mol l⁻¹ NaCl) at which other proteins usually precipitate [24]. Such unique properties make these enzymes fundamentally and biotechnologically

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important for application. In industrial scale the use of halophilic microorganisms is appreciated where no contamination risk. In this way, a new approach for cultivating halophilic microorganisms, such as open ponds, could be adopted instead of traditional more expensive closed bioreactors [37]. Optimization of medium components can help in increasing the yield of the enzymes, therefore this study focused on enzyme production, optimization, purification and characterization of newly produced haloalkaliphilic protease enzyme by *Natrialba hulunbeirensis* strain WNHS14 isolated from Wadi El Natrun, El Hamra Lake, Egypt.

Materials and Methods

Isolation, screening and identification

The haloalkaliphilic strains used in this study were isolated from soil samples collected from Wadi El Natrun, El Hamra Lake, Egypt. Sediment and water samples were collected during winter from El Hamra Lake (pH 10), Wadi El Natrun in Egypt, where the temperature was about 20°C. For the enrichment of haloalkaliphilic archaea, media were supplemented with 1 ml water sample or 1g sediment sample. Samples were cultivated into 250 ml Erlenmeyer flask containing 50 ml of the medium, where six different media (pH 10) were tested (M1–M6) [8, 9, 12, 16, 17, 45], respectively, then kept under shaking condition (200 rpm) at 37 and 45°C for 14 days. Subsequent dilutions of 14-days-old culture were placed out into the same medium for further purification. The purified isolate was maintained on agar plate of the same medium, subcultured at regular intervals of 1 month and stored at 4°C. All isolates were screened for production of haloalkaliphilic protease each under growth preferred medium, temperature and pH (10). WNHS14 was selected as the most potent strain under growth conditions: temperature (45°C), pH (10), salinity (15%) using M2 medium after 9-days. Subsequently, the selected isolate (WNHS14) was characterized morphologically, microscopically (scanning electron microscope (SEM)) and identified by 16S rRNA gene sequencing using specific archaeal forward 16S primer (5'-ATTCCGGTTGATCCTGCCGG-3') and reverse 16S primer (5'-TACGGYACCTTGTTACGACTT-3'). The 16S rRNA sequence was aligned with the deposited 16S rRNA sequences deposited in the international

nucleotide sequence database collaboration (INSDC) to assess its similarity through BLASTn search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), subsequently, the sequence was deposited at GenBank under the accession number KP765047.1.

Enzyme assay

Protease activity was measured by the method of Kembhavi *et al.* [20] using casein as a substrate and cell-free supernatant as a source of enzyme under reaction conditions using glycine -NaOH plus 2.5 M NaCl buffer, pH 9 at 45°C for 10 min. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min under the experimental conditions. A standard curve with tyrosine was established.

Enzyme production

Pre-culture preparation. A starting culture of *Natrialba hulunbeirensis* WNHS14 was grown in 250 ml Erlenmeyer flask containing 50 ml of the medium as described by Xu *et al.* [45] which has the following composition in g/l: casamino acids, 7.5; yeast extract, 10; trisodium citrate, 3; MgSO₄·7H₂O, 0.3; KCl, 2; FeSO₄·7H₂O, 2×10^{-5} ; MnCl₂·4H₂O, 3.6×10^{-4} ; NaCl, 200; Na₂CO₃, 8.0. The preculture cells were grown under conditions (45°C), (200 rpm) till reach OD₆₀₀ (~0.6). Then this culture was used for inoculating the production media using 2% inoculum size.

OVAT-approach. Stepwise OVAT approach was applied initially to determine the effect of different nitrogen sources, carbon sources, temperature(s) and pH(s) on the production of protease using the above mentioned medium as a basal medium at fixed time (9 days) and shaking (200 rpm).

Effect of carbon and nitrogen sources on protease production

Effect of different carbon and nitrogen sources on protease production was carried out by replacing yeast extract (10 g/l) and casamino acids (7.5 g/l) in the basal medium with a sole nitrogen source (5 g/l) either organic (peptone, yeast extract, casamino acids, skimmed milk, soybean flour and malt extract) or inorganic (NH₄)₂SO₄, NH₄Cl, urea and NaNO₃. Subsequently, the effect of different carbon sources was estimated through replacing

trisodium citrate (3 g/l) by other various carbon sources (5 g/l) and keeping the nitrogen source (casamino acids at conc. 5 g/l). Sugars such as xylose, glucose, fructose, sucrose, maltose and lactose were used. Furthermore, other carbon sources such as starch, mannitol and sodium acetate were tested as well.

Effect of temperature and pH on protease production.

Temperature was tested by incubating the cultures in a range of temperature (30–50 °C) with increments of 5 °C. In order to test the effect of pH on protease production, the optimum medium was adjusted at different pHs (7.0–10.5 with increment 0.5). Cultural conditions were set at optimum temperature for 9 days with shaking.

Response surface methodology (Box-Behnken Design)

The next step of the optimization was to determine the optimum levels of significant variables screened by OVAT approach. For this purpose, response surface methodology using Box-Behnken design was adopted.

Table 1. Box-Behnken design matrix for three independent variables with experimental and predicted levels of protease activity by *N. hulunbeirensis* strain WNHS14.

Trial	Cas.acids* (X ₁)	KCl (X ₂)	NaCl (X ₃)	Protease activity (U/ml)	
				Experimental	Predicted
1	-1 (0.5)	-1 (0.02)	0 (20)	45.60	39.78125
2	1 (1)	-1 (0.02)	0 (20)	17.60	21.43125
3	-1 (0.5)	1 (0.2)	0 (20)	41.60	37.76875
4	1 (1)	1 (0.2)	0 (20)	16.00	21.81875
5	-1 (0.5)	0 (0.11)	-1 (15)	47.20	55.34375
6	1 (1)	0 (0.11)	-1 (15)	45.60	44.09375
7	-1 (0.5)	0 (0.11)	1 (25)	15.60	17.10625
8	1 (1)	0 (0.11)	1 (25)	2.200	-5.94375
9	0 (0.75)	-1 (0.02)	-1 (15)	54.00	51.67500
10	0 (0.75)	1 (0.2)	-1 (15)	56.95	52.637500
11	0 (0.75)	-1 (0.02)	1 (25)	5.000	9.312500
12	0 (0.75)	1 (0.2)	1 (25)	4.400	6.725000
13	0 (0.75)	0 (0.11)	0 (20)	18.00	17.76000
14	0 (0.75)	0 (0.11)	0 (20)	17.50	17.76000
15	0 (0.75)	0 (0.11)	0 (20)	17.78	17.76000

*Casamino acids.

Levels of independent variables presented between brackets are expressed in terms of (w/v) %.

This optimization process involves three main steps: performing the statistically designed experiment, estimating the coefficients of the structured mathematical model and predicting the response and checking the adequacy of the model. Table 1 presents the design matrix with both coded and real values [expressed in (w/v)%] of the studied variables along with the trial response. Each variable was studied on three levels, coded -1, 0, and +1 for low, middle, and high concentrations, respectively. The combinations were performed and the haloalkaline protease activity was calculated for each trial after 9 days incubation. The results were fitted to the following second-order polynomial structured model for three variables:

$$Y = \beta_0 + \beta_1(X_1) + \beta_2(X_2) + \beta_3(X_3) + \beta_{12}(X_1X_2) + \beta_{13}(X_1X_3) + \beta_{23}(X_2X_3) + \beta_{11}(X_1)^2 + \beta_{22}(X_2)^2 + \beta_{33}(X_3)^2$$

where, Y is the predicted response (haloalkaline protease activity), β_0 is constant, β_1 , β_2 and β_3 are linear coefficients, β_{12} , β_{13} and β_{23} are cross product coefficients, and β_{11} , β_{22} and β_{33} are quadratic coefficients. The maximal predicted response of variables and coefficients were calculated using Microsoft Excel 2007.

Statistical analysis of data

Multiple non-linear regression was carried out for the enzyme activity data using Microsoft Excel 2007 to estimate the t-values, p-values, and confidence levels expressing the p-value as a percentage. The quality of fit of the second-order polynomial model equation was expressed via the multiple correlation coefficients *R* and determination coefficient *R*². The relationship between the responses and the experimental levels of each of the variables was visualized using three-dimensional graphs generated by STATISTICA 5.0 software. The optimal enzyme activity value was estimated using the *Solver* function of the Microsoft Excel tools. To determine the validity of the quadratic polynomial model, a verification experiment was carried out under the predicted optimal conditions, while monitoring the haloalkaline protease activity.

Growth curve and protease production. Incubation of *Natrialba hulunbeirensis* WNHS14 was carried out under initial and optimized conditions while monitoring

the growth and haloalkaline protease production.

Purification of the Haloalkaline protease. The cell-free culture supernatant was concentrated by ultrafiltration (Amicon ultra-50 centrifugal filter device, Merck KGaA, Germany) with 10 kDa membrane filter. The concentrated enzyme was applied onto the Hiprep 16/60 sephacryl S-100 HR gel filtration column equilibrated with 100 mM glycine-NaOH buffer, pH 9 containing 2 M NaCl. Fractions were collected at a flow rate of 0.7 ml/min and analyzed for protein content and proteolytic activity. Fractions containing high proteolytic activity were pooled together. The pooled fractions were then concentrated and desalted against 100 mM glycine-NaOH buffer, pH 9 using Amicon ultra-50 centrifugal filter device (membrane cut off of 10 kDa).

Protein methods. Protein concentration was determined by the method of Lowry *et al.* [25]. A standard curve with BSA was established. SDS-PAGE [23] was performed to monitor the purification steps. Protein gels were stained with silver staining according to the method of Mortz *et al.* [28].

Enzyme characterization

Effect of temperature. The optimum temperature for the partially purified protease was determined by assaying enzyme activity under various temperatures (40–85°C) in 2.5 M NaCl/100 mM glycine-NaOH buffer pH 9, using casein substrate (1%). In order to monitor the thermal stability, samples of the partially purified enzyme were pre-incubated at different temperatures (55–75°C). Aliquots were then taken at different time intervals, and the remaining activity was then measured.

Effect of pH. Relative protease activity was determined using buffers (100 mM) of varying pH, viz. phosphate-citrate (pH 5.8), phosphate (pH 5.8–8), tris-HCl (pH 7.8–9), glycine-NaOH (pH 9–10), carbonate-bicarbonate (pH 9.2–10.4), bicarbonate-NaOH (pH 10–11), phosphate-NaOH (pH 11–12) and KCl-NaOH (pH 12, 12.4). All buffers had NaCl concentration of 2.5 M. To study the pH stability, the enzyme was pre-incubated with the above-mentioned buffers at room temperature for 60 min and aliquots were taken at different time intervals to measure the residual protease activity.

Effect of NaCl

To determine optimum salt concentration, the amount of NaCl in assay mixture was varied from 0.5 to 4.5 M. The activity was calculated and expressed in percentage relative activity considering the maximum value as 100%. To measure the activity of NaCl-free protease, the purified enzyme solution was first eluted with 100 mM Na₂CO₃-NaHCO₃ buffer (pH 10) by ultrafiltration using Amicon ultra-50 centrifugal filter device, with 10 kDa membrane filter. Then, the NaCl-removed protease was subjected to proteolytic activity assay in the absence of NaCl.

Effect of some additives. The effects of some surfactants (Triton X-100, Tween 80) and reducing agent (SDS) on the partially purified protease activity were tested at 0.1, 0.5, 1% final concentration. The effects of protease inhibitor (PMSF); chelating agent, (EDTA) and some metals of the salts (CaCl₂, MgSO₄, ZnSO₄, HgCl₂, CuSO₄, KCl, MnCl₂, FeSO₄ & CoCl₂) on protease activity were investigated at 1 and 5 mM final concentration. The enzyme was pre-incubated with each additive in 2 M NaCl/100 mM Na₂CO₃-NaHCO₃ buffer for 30 min at room temperature.

Results and Discussion

Isolation of Haloalkaliphilic-protease producer isolates from Wadi-El-Natron and El-Hamra Lake

Most of the colonies obtained showed different shades of pink, orange and red colors while others had white, beige and colorless colonies. Total of 49 isolates were obtained, out of which 10 were from water (WNHW1-WNHW10) and 39 from sediment (WNHS1-WNHS39). Quantitative estimation of the proteolytic activity for the isolates was monitored along 12 days using isolation medium. Most of the isolates did not show any proteolytic activity during the first 3 days. However, they started to produce extracellular protease after 6 days incubation. WNHS14 had the highest proteolytic activity (10 U/ml) among the isolates under investigation, after 9 days incubation therefore, it was selected for further studies.

Phenotypic and genotypic characterization for WNHS14 strain

The physiological and morphological characteristics of

Fig. 1. Morphological characteristics for colony in agar plate (A) and cells micrograph (B & C) under SEM of the selected isolate (WNHS14).

the isolate WNHS14 showed that it is haloalkaliphilic (pH 10, NaCl up to 30%), Gram- negative, small round orange pigmented colonies, cocci-shaped cells with diameter of 1.3 μm as illustrated by the scanning electron micrograph (Fig. 1A–C). To identify this strain, the full length of 16S rRNA gene was amplified, sequenced and deposited at GenBank (Accession no. KP765047.1). Comparison of the obtained sequence with other sequences available at INSDC revealed the WHW14 is a typical Archaea, class *Halobacteria* and 99% similar to *Natrialba hulunbeirensis*.

It was pointed out that members of the class *Halobacteria* displayed extreme polymorphism, having several morphologic shapes ranging from rods to pleomorphic rods, cocci, pleomorphic, squares and triangles [4]. Colonies of halophilic archaea are various shades of red due to the presence of C50 carotenoids (bacterioruberins) [21]. Halophiles can be loosely classified as slightly, moderately or extremely halophilic, depending on their requirement of NaCl. Slight halophiles grow optimally at 0.2–0.85 M (1–5%) NaCl; moderate halophiles grow optimally at 0.85–3.4 M (5–20%) NaCl; and extreme

halophiles grow optimally at 3.4–5.1 M (20–30%) NaCl [7]. WNHS14 isolate is considered extreme halophiles, due to its ability to grow optimally at 15–20% NaCl with significant growth in presence of 30% NaCl and lack of growth below 15% NaCl.

OVAT approach for enhancing enzyme production

Haloalkaliphilic archaea provide a valuable resource of proteases which have potential for application to biotechnology. The effect of various nutritional factors on the production of protease in haloalkaliphilic archaea has been poorly investigated. Therefore, the effect of nitrogen, carbon sources, temperature and pH on the haloalkaliphilic protease production by haloarchaeon WNHS14 were highlighted and analyzed. Data showed that among the organic nitrogen sources used, casamino acids, skimmed milk and yeast extract enhanced the extracellular protease production (36.2, 33.3 and 25.7 U/ml, respectively). The nitrogen sources affecting extracellular protease production by studied strain appear to be somewhat different from those reported for the production of haloalkaliphilic extracellular protease from

Fig. 2. Effect of incubation temperatures (A) and pHs (B) on haloalkaline protease production by *N. hulunbeirensis* strain WNHS14.

the haloarchaeon *Halo geometricum* sp. TSS101 [42] and that secreted by the extremely halophilic archaeon *Halobacterium* sp. Js1 [43], which were stimulated by soybean flour and peptone. However, they both were inhibited by urea and ammonium chloride and they were activated by skim milk [42, 43]. On the other hand the added carbon sources did not improve the extracellular protease production by WNHS14, and the maximum (36.79 U/ml) was obtained in absence of any carbon source. Similarly, the presence of glucose had no significant effect on the production of CP1 protease from the moderately halophilic bacterium *Pseudoalteromonas* sp. strain CP76 [36]. Besides, glucose and starch couldn't be utilized by *Halobacterium* sp. Js1 [43], while the proteolytic production by *Haloferax lucentensis* VKMM 007 increased significantly upon using soluble starch as a carbon source [27]. Additionally, *N. hulunbeirensis* strain WNHS14 showed gradual increase in the protease production with increasing temperature and pH (Fig. 2A, B), respectively and the optima at 40°C and 9 (44.9 U/ml). For extracellular enzymes, temperature was found to influence their secretion, possibly by changing the physical properties of the cell membrane [35]. Haloalkaliphilic microorganisms require not only high salt but also alkaline pH for growth and enzyme secretion. The optimum pH range between 9 and 10 for protease production is common among alkaliphilic and haloalkaliphilic organisms [1, 32].

Response surface methodology (Box-Behnken Design)

Based on OVAT test, casamino acids (X_1), KCl (X_2) and NaCl (X_3) showed significant effect on protease production. Thus, they were further studied at three levels: -1, 0 and +1 to determine the optimum response region for haloalkaline protease activity.

Presenting the experimental results in the form of surface plots, Fig. 3 shows that lower casamino acids, KCl, and NaCl concentrations supported a high protease activity level. To predict the optimal point, a second order polynomial function was fitted to the experimental results (nonlinear optimization algorithm) of protease activity:

$$Y = 17.76 - 8.575 X_1 - 0.406 X_2 - 22.069 X_3 + 0.6 X_1 X_2 - 2.95 X_1 X_3 - 0.888 X_2 X_3 + 5.001 X_1^2 + 7.439 X_2^2 + 4.889 X_3^2$$

where X_1 , X_2 and X_3 are casamino acids, KCl and NaCl respectively. At the model level, the correlation measures used to estimate the regression equation are the multiple correlation coefficients R and determination

Fig. 3. Three-dimensional surface plots representing the effect of casamino acids, KCl and NaCl on the haloalkaline protease enzyme yield ($\text{Uml}^{-1}\text{min}^{-1}$) by *N. hulunbeirensis* strain WNHS14. (A) KCl versus casamino acids, (B) NaCl versus casamino acids, (C) KCl versus NaCl.

coefficient R^2 . The closer the value of R is to 1, the better the correlation between the measured and the predicted values. In this experiment, the value of R was 0.97 for the haloalkaline protease activity, indicating a high degree of correlation between the experimental and predicted values. Meanwhile, the value of the determination coefficient, $R^2 = 0.95$, for the haloalkaline protease activity, being a measure of fit for the model, indicated that only about 5% of the total variations were not explained by the enzyme activity.

The optimal levels of the three components, as obtained from the maximum point of the polynomial model, were then estimated using the *Solver* function of the Microsoft Excel tools and found to be casamino acids, 0.5%; KCl, 0.02%; NaCl, 15% with a predicted activity of 62.9 U/ml. As such, the value of the enzyme activity in the optimized medium was more than 6 fold the value under basal conditions, thereby emphasizing the necessity and value of the optimization process.

To determine the accuracy of the quadratic polynomial model, a verification experiment was carried out under the predicted optimal conditions. As a result, the estimated haloalkaline protease activity was 58.2 U/ml, while the predicted value from the polynomial model was 62.9 U/ml. This high degree of accuracy of (92.53%) was an evidence for the model validation under the following optimal conditions: 0.5% casamino acids, 0.02% KCl and 15% NaCl at pH 9 adjusted with sterile Na_2CO_3 .

Most microorganisms utilize organic and inorganic nitrogen sources to produce mainly amino acids, nucleic acids, proteins, and cell wall components. The availability of nitrogen sources in the media control the production of extracellular proteases. The synthesis of protease by microorganisms can be repressed by either an excess or a deficiency of nitrogen [28].

The organic nitrogen sources used in this study supported the proteolytic production from *N. hulunbeirensis* strain WNHS14, while the absence of organic nitrogen sources did not support it. The maximum effect on protease secretion (36.2 U/ml) was observed by using casamino acids as a nitrogen source. Thus, casamino acids, the best nitrogen source, was further investigated via response surface methodology to assess its optimum concentration for the protease production. Casamino acids has also been reported to be the best organic nitrogen for

protease production in the haloalkaliphilic *Bacillus* sp. RGR-14 [33]. Nevertheless, protease production was repressed in the presence of high concentrations of casamino acids. The experimental results showed that a lower casamino acids concentration (0.5%) supported a high protease production level from *N. hulunbeirensis* strain WNHS14. There are several reports on the repressive role of organic nitrogen sources in alkaline protease production in which the end products of nitrogen metabolism repress the synthesis of proteases [5, 6, 10]. The extremely halophiles are salt-loving organisms. They not only tolerate but require NaCl concentrations above 10 to 15% for survival, and optimal growth is often obtained above 20% NaCl [24]. Halophiles have evolved to live within their environmental conditions, rather than to adapt ways to circumvent it. They had to evolve a system to deal with extreme osmotic stress. Halophiles respond to osmotic pressure elevation in different ways. Some halophiles, mostly halophilic bacteria and eukaryotes, prevent the entry of inorganic salts into the cell and synthesize small organic molecules (like ectoine), known as osmolytes, to balance the osmotic pressure. The aerobic halophilic archaea, though, survive by accumulation of KCl, i.e., inorganic ions, in the cytoplasm to provide osmotic equilibrium [13, 29, 36]. Most halophilic enzymes are inactivated and denatured at concentrations below 1 M NaCl [2].

Solubility, stability and conformation of a protein can be significantly affected by salt concentrations which ultimately affect its ability to function. At environments with low salt concentration, halophilic proteins have a very low solubility [26, 36]. Therefore, the study of NaCl and KCl concentrations and their effect on the extracellular protease production by *N. hulunbeirensis* strain WNHS14, a halophilic archaeal strain, was studied. The optimum NaCl concentration for the proteolytic production from *N. hulunbeirensis* strain WNHS14 was found to be 15%. These results could be compared with that of the haloalkaliphilic bacterium S-20-9 in which protease secretion was maximum at 15% NaCl [18]. A much higher salt requirement (20%) for a serine protease secretion was reported in another halophilic archaeobacteria *Halogeometricum* sp. TSS101 and *Halobacterium* sp. Js1 [42, 43].

The statistical optimization method is widely used and has a growing acceptance in biotechnology. For our

knowledge, this study constitutes the first report of the statistical optimization of medium components for the extracellular protease production from *Natrialba* sp. However, response surface methodology has been used to optimize the protease production by the haloarchaeon *Haloferax lucentensis* VKMM 007 and that produced by the haloarchaeon *Halobacterium* sp. SP1(1) [3, 27].

Growth curve and protease production

Culturing of *N. hulunbeirensis* strain WNHS14 was carried out under basal and optimized conditions while monitoring the growth and haloalkaline protease production. As shown in Fig. 3, the basal medium showed a typical growth pattern and recorded a relatively low enzyme activity with the maximum (10 U/ml) attained after 170 h. Enzyme activity was detected in this complex medium, containing casamino acids (0.75%) and yeast extract (1%), as *N. hulunbeirensis* cells entered the stationary growth. On the other hand, using the optimized medium, containing casamino acids (0.5%), the growth rate of *N. hulunbeirensis* strain WNHS14 was significantly faster than that using the initial medium, reaching the stationary phase earlier (81 h) compared to the initial medium (120 h). Furthermore, under the optimized conditions, higher level of extracellular protease (58.2 U/ml) was produced at earlier stages of growth (early exponential phase). Protease production maximized after 57 h incubation, which is 27 h earlier compared with the proteolytic enzyme from the extremely halophilic archaeon *Haloquadratum* sp. TSS101 [42].

The growth and protease production by *N. hulunbeirensis* strain WNHS14 indicates that there is a distinct growth associated nature of protease production. High enzymatic production coincided with high growth rate of cells. On the contrary, the extracellular protease production by the haloarchaeon *Natrialba magadii* ATCC 43099 is stimulated when the cells are stressed to grow

at a slow rate [6]. Moreover, the archaeon *Natronococcus occultus* NCIBM 2192 was found to produce an extracellular protease in the late exponential and stationary growth phases and during starvation. The protease production from *Natronococcus occultus* NCIBM 2192 is thought to be induced in response to high cell density which may be indicative of quorum sensing signaling [31].

Purification of haloalkaline protease

The steps that used for the haloalkaline protease purification from *N. hulunbeirensis* strain WNHS14 were showed in Table 2. It was indicated that during purification steps the total protease activity reduced to about 428.4 U (~23%), and also the total protein content reduced to 0.334 mg (~97%) in comparison with the initial reading. However, the specific activity at the end of the purification steps was found to be 1282.63 U/mg comparing to 144.04 U/mg at the culture filtrate. It was indicated that the protease enzyme at the end of the purification steps was purified to 8.9 fold. Fig. 4 illustrates the pattern of protein profile before and after the purification. By comparing both patterns, the results revealed that there was an obvious disappearance of different protein bands from the crude enzyme after purification using gel filtration.

Haloalkaline protease characterization

The studied protease enzyme works optimally at 75°C when was examined in presence of 2.5 M NaCl and pH 9 and thus can be classified as a thermoactive protease. The enzyme possessed 100% stability after 60 min at 55°C, but it was less stable at higher temperatures. Moreover, although the optimum temperature of the enzyme activity is 75°C, it possessed only 28.47% stability after 1 h incubation at the same temperature. Rapid thermal inactivation of such enzymes might be related to the increased rate of activity at high temperatures as

Table 2. Purification table of haloalkaline protease enzyme produced by *N. hulunbeirensis* strain WNHS14.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
CFS ¹	1862.4	12.93	144.04	1.00	100
Conc. CFS ²	968.0	2.55	379.61	2.64	52
Gel filtration colum*	428.4	0.334	1282.63	8.90	23

1: cell free supernatant, 2: concentrated cell free supernatant.

*Hiprep16/60 sephacryl S-100 HR column.

Fig. 4. Growth curve and haloalkaline protease production by *N. hulunbeirensis* strain WNHS14 in initial and optimized media.

reported by Ghorbel *et al.* [11]. This is in agreement with haloalkaline protease isolated from *Natrialba magadii* [12]. Besides, the studied enzyme showed a broad pH range of activity with an optimum at pH 10, declined slightly by increasing the pH and reached to about 91% relative activity at pH 11. The enzyme was highly stable at pH ranging from 6 to 12 at room temperature throughout 1 h. These results were highly similar with the literature results for the optimal pH [42] and for pH stability [12]. By testing salt dependence of enzyme, it was found that WNHS14 protease works optimally at 2 M and retained 41% at 4.5 M when assayed in 100 mM carbonate buffer (pH 10). No activity was observed in absence of NaCl and complete irreversible loss of the enzyme activity even after elution of protein with non-saline buffer. These results revealed that the *N. hulunbeirensis* strain WNHS14 protease is a typical halophilic protease and similar to the haloalkaliphilic serine protease produced by *Natrialba magadii* [12] and by *Natronococcus occultus* NCBM 2192 [41]. In the contrary, the protease activity of the halophilic *Bacillus* sp. EMB9 was regained upon reintroducing NaCl/KCl to the preparation soon after dialysis [40]. Following up the enzyme activity in presence of some surfactants showed that a slight decrease in the enzyme activity (3.25–11%) with Tween-80, and 33.68–33.14% with Triton X-100 at 1% concentrations (Table 3). A sharp decrease in the

Table 3. Effect of some surfactants, SDS, protease inhibitors and metal ions on activity of the partially purified *N. hulunbeirensis* strain WNHS14 haloalkaline protease enzyme.

Treatments	Residual activity (%)		
	0.1%	0.5%	1%
Control (no addition)	100.0	100.0	100.0
Tween-80	96.72	94.54	89.00
Triton X-100	66.32	66.58	66.86
SDS	72.96	32.65	4.590
	1 mM		5 mM
EDTA	94.26	91.26	
PMSF	21.00	0.00	
Ca ²⁺	97.96	95.41	
Co ²⁺	99.44	59.18	
Hg ²⁺	61.24	30.26	
Mn ²⁺	96.87	89.88	
K ⁺	99.49	97.85	
Cu ²⁺	90.24	79.59	
Mg ²⁺	100.09	94.72	
Fe ²⁺	93.64	80.33	
Zn ²⁺	85.20	67.86	

proteolytic activity (95%) was resulted by incubating the enzyme with SDS at 1% concentration (Table 3). Similar results were obtained with *Natrinema* sp. strain J7 [39] and *Halobiforma* sp. strain BNMIITR [14] where SDS had a negative effect on the proteolytic activity. Moreover, as shown in Table 3 PMSF caused a complete inhibition of the partially purified enzyme activity at 5 mM, whereas 1 mM caused 79% inactivation. PMSF is known to sulphonate the essential serine residue in the protease active site, causing a total loss of enzyme activity [22]. The enzyme complete inactivation by PMSF suggests that the protease produced by *N. hulunbeirensis* strain WNHS14 belongs to serine proteases family. This is not surprising as all of the extracellular proteases isolated so far from halophilic archaea are serine proteases. The limited inhibitory effect observed by EDTA (a specific inhibitor of metallo-type proteases) indicates that the metal ions are important but not essential for the WNHS14 proteolytic activity. Similar results were reported by Giménez *et al.* [12]. Additionally, all tested metal ions at low concentrations (1 mM) did not significantly affect the protease activity except for Hg²⁺ which decreased the proteolytic activity of the partially purified enzyme by 38.76% (Table 3). Zn²⁺, Co²⁺ and Hg²⁺

Fig. 5. SDS-PAGE of haloalk 103 aline protease from *N. hulunbeirensis* strain WNHS14. Lane M shows protein molecular weight protein marker, lane 1 shows the crude extract, and lane 2 shows the partially purified enzyme after elution from HiPrep16/60 sephacryl S-100 HR gel filtration column.

inhibited protease activity by 32.14, 40.82 and 69.74% at 5 mM, respectively (Table 3). This observations are in agreement with the halophilic serine protease isolate *Natrialba asiatica* 172P1 strain and *Natronolimnobius innermongolicus* WN18 strain [19]. However, the activity of this protease enzyme was enhanced in the presence of Ca^{2+} , Mn^{2+} and Mg^{2+} ions [38]. This is in contrary of the present results at which these ions have no effect on the proteolytic activity. In conclusion this study has focused on the production, optimization, purification and characterization of the protease enzyme from a haloalkaliphilic archaeal species. Statistical optimization enhanced the production of protease by 6-fold. To our knowledge, this is the first report of the production of extracellular proteolytic enzyme by *N. hulunbeirensis* and also the first report of the statistical optimization of medium components for the extracellular protease production from *Natrialba* sp. The promising properties of the newly haloalkaline serine protease from *N. hulunbeirensis* strain WNHS14 would underpin its efficient exploitation in several industries to cope with demands of worldwide enzyme markets.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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