FTB Food Technology & Biotechnology

original scientific paper
ISSN 1330-9862
doi: 10.17113/ftb.56.04.18.5553

Alkaline and Halophilic Protease Production by *Bacillus luteus* H11 and Its Potential Industrial Applications

Agnieszka Kalwasińska[1]*, Urszula Jankiewicz[2], Tamás Felföldi[3], Aleksandra Burkowska-But[1] and Maria Swiontek Brzezinska[1]

[1]Department of Environmental Microbiology and Biotechnology, Nicolaus Copernicus University, Lwowska 1, PL-87100 Toruń, Poland
[2]Department of Biochemistry, Warsaw University of Life Sciences, Nowoursynowska 159, PL-02787 Warsaw, Poland
[3]Department of Microbiology, Eötvös Loránd University, Pázmány Péter sétány 1/c, HU-1117 Budapest, Hungary

Received: 4 October 2017
Accepted: 4 September 2018

***Corresponding author:**
Phone: +48566112521;
Fax: +486114443;
E-mail: kala@umk.pl

ORCID IDs: 0000-0002-2435-567X (Kalwasińska), 0000-0003-2240-6629 (Jankiewicz), 0000-0003-2009-2478 (Felföldi), 0000-0002-7449-4678 (Burkowska-But), 0000-0002-4552--3951 (Swiontek Brzezinska)

SUMMARY

This paper presents the results of the study on the production of protease by *Bacillus luteus* H11 isolated from an alkaline soda lime. *B. luteus* H11 was identified as an alkalohalophilic bacterium, and its extracellular serine endoprotease also showed an extreme alkali- and halotolerance. It was remarkably stable in the presence of NaCl up to 5 M. The enzyme was active in a broad range of pH values and temperatures, with an optimum pH of 10.5 and a temperature of 45 °C. It had a molecular mass of about 37 kDa and showed activity against azocasein and a synthetic substrate for the subtilisin-like protease, N-succinyl-L-phenylalanine-*p*-nitroanilide. The halo-alkaline protease produced by *B. luteus* H11 seems to be significant from an industrial perspective because of its tolerance towards high salinity and alkalinity as well as its stability against some organic solvents, surfactants and oxidants. These properties make the protease suitable for applications in food, detergent and pharmaceutical industries, and also in environmental bioremediation.

Key words: *Bacillus*, proteolytic bacteria, alkalohalophiles, serine endoprotease, subtilisins

INTRODUCTION

A majority of the biocatalysts obtained from microorganisms isolated from soil, water, food, *etc.* cannot be applied in harsh industrial processes owing to their low osmotic, pH, or temperature tolerance. As a consequence, new environments are explored in the search for new enzymes, and there is increasing interest in enzymes from extremophiles, as they are naturally adapted to extreme environmental conditions (*1,2*).

Proteases (EC 3.4.21) represent over 65 % of the total industrial enzyme market, of which the vast majority are alkaline proteases (*3*). These enzymes, showing exceptional activity and stability in the highly alkaline pH, are applied mainly in detergent formulations (*4,5*). Furthermore, they have various other industrial applications, such as meat tenderization, leather dehairing and tanning, silk degumming, organic synthesis, medical diagnoses, and silver recovery from X-ray films (*6,7*).

Currently available commercial alkaline proteases, *e.g.* Alcalase, Savinase, Esperase (Novozymes, Bagsværd, Denmark) or FoodPro subtilisin (Du Pont, Wilmington, NC, USA) used in organic transformations are active at wide ranges of pH (7–11.5) and temperature (30–80 °C). However, the information about their halotolerance is not easily accessible, although it would be extremely useful for the application of the enzyme in a solution with low water activity. Stable by nature and active at high salt concentrations, halophilic and moderately halophilic proteases offer important opportunities in biotechnological applications, such as food processing, biosynthetic processes, soil bioremediation and sewage treatment. Therefore, finding of novel enzymes showing optimal activities at extreme ranges of pH, salt concentration, and temperature is of great importance (*8–11*). In addition, halophilic microbes can serve as an important source of salt-tolerant genes for the salt-resistant transgenic plants (*12,13*).

Bacillus species and *Bacillus*-related genera are often defined as important sources of extracellular proteases (*14,15*). Most of these enzymes were obtained from moderately halophilic microorganisms, *e.g. Bacillus clausii* I-52 (*16*), *Bacillus subtilis* FP-133 (*17*), *Paenibacillus*

peoriae NRRL BD-62, *Paenibacillus polymyxa* SCE2 (*18*), *Halobacillus* sp. SR5-3 (*19*), *Halobacterium* sp. SP1 (1) (*20*), *Virgibacillus* sp. SK37 (*21*), *Geomicrobium* sp. EMB2 (*22*), and *Halobacillus blutaparonensis* M9 (*23*). However, reports from halophilic proteases are very limited in the literature (*24*).

An extremely artificial environment of alkaline and saline distillery lime located at the premises of a soda production facility in Janikowo, Poland (East-Central Europe) was investigated for novel strains showing interesting physiological properties, important from a biotechnological perspective (*25*). The aim of the present research is to develop conditions for the synthesis of proteolytic enzyme produced by the bacterial strain *Bacillus luteus* H11 isolated previously. In addition, the purified, halo-alkaline protease from *B. luteus* H11 was characterized.

MATERIALS AND METHODS

Medium components, chemicals and equipment

Medium components: sodium caseinate, glucose, yeast extract, peptone, agar, beef extract, tryptone, fructose, maltose, sucrose and starch were purchased from Biocorp (Warsaw, Poland). Shrimp waste originated from the Krymar (Iłowo, Poland) processing facility. Chemicals: $MgSO_4 \cdot 7H_2O$, NaCl, Na_2CO_3, K_2HPO_4, $HgCl_2$, HCl, $(NH_4)_2SO_4$, 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), HCl, NaOH, H_3BO_3, H_3PO_4, CH_3COOH, metal ions, acetone, butanol, ethanol, methanol, ethylenediaminetetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) were purchased from Alchem Grupa Sp. z o.o. (Toruń, Poland). Other chemicals: aprotinin, bestatin, diisopropyl phosphorofluoridate (DFP), ethylene-bis(oxyethylenenitrilo)tetraacetic acid tetrasodium (EGTA), E-64, Iodoacetamide, Pefabloc®, pepstatin, phenylmethylsulfonyl fluoride (PMSF), 1,10-phenantrolin, Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), cetyl trimethylammonium bromide (CTAB), sodium dodecyl sulphate (SDS), Triton X-100, Tween 80, urea, H_2O_2, N-succinyl-Gly-Gly-Phe-*p*-nitroanilide, N-succinyl-L-phenylalanine-*p*-nitroanilide, Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride, and Z-Gly-Gly-Leu-*p*-nitroanilide, Superdex 200, Bradford reagent, bovine serum albumin, polyacrylamide and amide black solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rotiphorese® NF-Acrylamide/Bis-solution and buffer components were purchased from Carl Roth GmbH+Co. KG (Karlsruhe, Germany). Britton-Robinson buffer was prepared according to Reynolds *et al.* (*26*).

Bacterial cultures were incubated in the Heraeus incubator (ThermoFisher Scientific, Waltham, MA, USA) and shaken in the IKA® KS 260 basic orbital shaker (Ika-Werke, Staufen, Germany). Enzyme reaction mixtures were incubated using the TS-100C thermo-shaker (Biosan, Riga, Latvia). Bacterial cultures/enzyme reaction mixtures were centrifuged at 6000/10 000×*g* in the Rotina centrifuge (Hettich, Tuttlingen, Germany). Absorbance measurements of cultures/reaction mixtures were performed using the Hitachi U-1900 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 600/420 nm. Vertical gel electrophoresis was performed using the Mini-protean Tetra Cell apparatus (Bio-Rad, Hercules, CA, USA).

Screening for proteolytic strains

The bacterial strain H11 used in this study was isolated as described in Kalwasińska *et al.* (*25*) from the top layer of the highly saline soda lime (6.4 % Cl^-), a byproduct of soda production, deposited at the repository ponds in Janikowo, Kuyavia, central Poland. For the evaluation of the proteolytic properties of the isolate, a nutrient medium containing (in g/L): sodium caseinate 15, glucose 5, yeast extract 1, peptone 7.5, NaCl 30, $MgSO_4 \cdot 7H_2O$, 0.02, Na_2CO_3 2, K_2HPO_4 0.2 and agar 15; pH=9, was used. The solution containing glucose and Na_2CO_3 was autoclaved separately and cooled down before its addition to the medium. The plates were incubated at 26 °C for 7 days. Protein hydrolysis was detected by Frazier's reagent (12 g $HgCl_2$, 80 mL distilled water, 20 mL concentrated HCl). Among all the proteolytic bacteria, one particular strain, H11, showing the largest bright arc, was chosen for further research aiming at the production and characterization of protease.

Strain identification

Identification of the isolated strain was based on the 16S rRNA gene sequence described previously (*25*). The obtained nucleotide sequence was deposited in GenBank, NCBI, Bethesda, MD, USA, under the accession number KJ870251. The strain was preserved with glycerol stock (20 %) and stored at –80 °C.

Growth conditions

The study included the determination of the optimal pH, salinity and incubation temperature. The strain H11 identified as *Bacillus luteus* was grown in a medium described above, except for the addition of sodium caseinate. The pH was investigated in the range of 7–11. The amount of NaCl was 0–9 % and the temperature was 10–40 °C. All experiments were performed in triplicate with shaking at 150 rpm. Bacterial growth was monitored by the absorbance measurements of cultures at 600 nm.

Alkaline protease production

A volume of 50 mL of the alkaline and saline medium containing (in g/L): peptone 7.5, glucose 5, yeast extract 1, NaCl 50, Na_2CO_3 2, K_2HPO_4 0.2 and $MgSO_4 \cdot 7H_2O$ 0.02, pH=9, was inoculated with 1 mL *Bacillus luteus* H11 suspension ($A_{600\ nm}$=1.0) and incubated at the optimal strain growth conditions of 30 °C for 72 h with shaking at 150 rpm. The culture was centrifuged at 6000×*g* and 4 °C for 10 min, and the alkaline protease activity was measured. In order to investigate the effect of different nitrogen sources on the alkaline protease production, beef extract, tryptone and shrimp shell powder were used instead of peptone and yeast extract. The shrimp shell powder was obtained from dried shrimp waste (105 °C) after grinding in the Zelmer blender ZHB1220B (Głogów Małopolski, Poland) and sterilization at 117 °C for 20 min. The effects of different

amounts of the nitrogen sources (0.5–2.5 %) on the enzyme production were also investigated. To assess the influence of different carbon sources on the bacterial growth and protease production, fructose, maltose, sucrose, and starch were used (0.5 % *m/V*) instead of glucose. In order to prevent acidification of the medium through fermentation of sugars, 100 mM Tris-HCl (approx. 50 mL for each culture), pH=8.8, was applied. Such buffered conditions enabled to maintain the alkaline pH of cultures during the three days of the experiment. Different amounts of NaCl (1–9 %) along with the best nitrogen and carbon sources were applied to investigate the optimal salt concentration for the protease production**.** All experiments were performed in three parallel repetitions, and the mean values were reported.

Protease activity assay

Protease activity was measured according to Jankiewicz *et al.* (*27*) using azocasein as the substrate. Briefly, 300 µL of the enzyme were mixed with 300 µL of 0.5 % (*m/V*) azocasein in 100 mM Tris-HCl buffer (pH=8.8). After incubation at 40 °C for 30 min, the reaction was stopped by adding 600 µL of 10 % trichloroacetic acid (TCA). In the control sample, 300 µL of the enzyme were mixed with 600 µL of 10 % TCA and 300 µL of azocasein were added after incubation. The solution was centrifuged at 10 000×*g* and the absorbance was measured at 420 nm and compared to the control sample.

Calibration curve which enables to calculate the mass correlation between the used substrate and the measured absorbance in the post-digestion sample was made according to Coêlho *et al*. (*28*). In order to do this, digestion of azocasein at the concentration of 0.3–1.5 mg/mL at 180 min by purified enzyme at the optimal conditions (40 °C, pH=10.5 and 3 M NaCl) was carried out (data not shown) and the calibration curve was obtained by plotting the azocasein concentration against its correspondent absorbance at 180 min (**Fig. 1**).

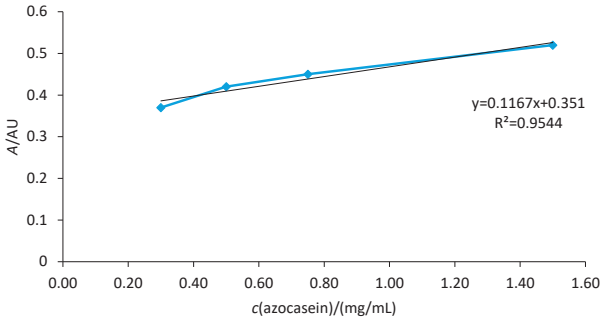

Fig. 1. Calibration curve for azocasein concentration 0.3–1.5 mg/mL after 180 min of digestion under the optimal conditions: 40 °C, pH=10.5 and 3 M NaCl

Azocasein concentration was calculated from the following equation:

$$c(\text{azocasein}) = \frac{A_{420\ \text{nm}} - 0.351}{0.1167} \qquad /1/$$

where $A_{420\ \text{nm}}$ is the absorbance of azocasein. Therefore, one unit (U) of the protease activity was defined as the amount of enzyme capable of digesting 1 mg substrate per minute, as given in the following equation:

$$\text{Activity} = \frac{c(\text{azocasein}) \cdot V^2_{\text{total}}}{t \cdot V_{\text{enzyme}}} \qquad /2/$$

where V_{total} is the sum of volumes of substrate, enzyme and TCA, V_{enzyme} is the enzyme volume used in the digestion, and t is the digestion time (min).

Enzyme purification

To obtain a clear supernatant, on the third day of growth at 30 °C, the bacterial culture was filtered and centrifuged at 9000×*g* for 10 min. The production medium contained (%, *m/V*) shrimp shell powder 2.5 and fructose 0.5, as the optimum nitrogen and carbon source, respectively, and NaCl 4 (pH=9). The protease was purified in a two-step procedure consisting of ammonium sulphate precipitation (35–85 % saturation) and molecular sieve chromatography. All protein purification stages were performed at 4 °C. Fractionation with ammonium sulphate was performed by salting out the enzyme preparation to 35 % of saturation in the first, and to 85 % of saturation in the second step. To dissolve the pellet, 50 mM Tris-HCl buffer, pH=8.8, with 2 M NaCl was used and the solution was dialyzed overnight against the same buffer. Prior to the enzyme purification, a Superdex 200 column (Sigma-Aldrich) was equilibrated with 50 mM Tris-HCl, pH=8.8, containing 2 M NaCl. Then, the 10-fold concentrated preparation was applied on the column. The active fractions were used to characterize the enzyme.

Determination of protein content

The protein concentration at all purification stages was determined with bovine serum albumin as a standard according to Bradford (*29*).

SDS-PAGE electrophoresis and zymograms

The electrophoretic separation of the samples using 10 % sodium dodecyl sulfate (SDS) (*m/V*) was carried out according to Laemmli (*30*). Zymograms were obtained after the electrophoretic separation of the samples under semi-denaturing conditions (without thermal denaturation). The polyacrylamide gel contained 0.1 % azocasein. Upon completion of the electrophoresis, the gels were placed in 0.5 % (*m/V*) Triton X-100 solution for 1 h, then transferred into 100 mM Tris-HCl buffer, pH=8.8, and stained with 0.1 % (*m/V*) amide black solution. The bright bands against the dark blue background of the gel indicated the proteolytic activity.

Effect of pH, salinity, temperature, and various chemical agents on protease activity and substrate specificity

Protease activity was studied over a pH range from 4 to 13 using 50 mM Britton-Robinson buffer. The effect of NaCl on

enzyme activity was tested at various NaCl concentrations (1, 2, 3, 4 and 5 M). The effect of temperature was studied at 30, 40, 50 and 60 °C. The thermal stability was determined at 45, 55 and 65 °C after 30, 60, 90 min of pre-incubation of the enzyme.

In order to assess the effect of specific protease inhibitors, metal ions, and other chemicals, the purified enzyme was pre-incubated in the presence of a given compound at 4 °C for 30 min, and then the residual protease activity was measured as described in the protease activity assay.

The inhibitors included: aprotinin (final concentrations 100 nM and 1 μM), Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) (final concentrations 1.0 and 10.0 μM), diisopropyl phosphorofluoridate (DFP), ethylene-bis(oxyethylenenitrilo) tetraacetic acid tetrasodium (EGTA), ethylenediaminetetraacetic acid (EDTA), Pefabloc®, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, 1,10-phenanthroline, iodoacetamide, E-64, and 10-phenantroline (final concentrations 0.1 and 1.0 mM). The metal ions included: Mg^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Cd^{+} (final concentrations 1 and 5mM). Other chemicals were: cetyl trimethylammonium bromide (CTAB), dimethyl sulfoxide (DMSO), ethanol, 2 M, H_2O_2, sodium dodecyl sulphate (SDS), Triton X-100, Tween 80, and urea (final concentrations 1.0 and 5.0 mM).

Solvents used in this study were: acetone, butanol, ethanol, and methanol. The solutions were prepared at the concentrations of 50 and 75 % by volume. Prior to the 1-hour incubation of the enzyme with the solvent, the substrate for protease was added and the residual activity was measured.

Substrate specificity was determined using N-succinyl-Gly-Gly-Phe-*p*-nitroanilide, N-succinyl-L-phenylalanine-*p*-nitroanilide, Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride, and Z-Gly-Gly-Leu-*p*-nitroanilide. The activity of protease was measured according to Bezerra *et al.* (*31*) and defined as μmol nitrophenol per 1 h. Briefly, 50 μL of the purified enzyme were mixed with 150 μL 50 mM Tris-HCl (pH=9) containing 1 M NaCl. After 5 min of preincubation, 50 μL of the appropriate substrate (5 mM in DMSO) were added, and the mixture was incubated at 40 °C for 15 min. Then, the activity of protease was measured at 420 nm.

RESULTS AND DISCUSSION

Proteolytic bacterium

The strain H11 isolated from the highly alkaline and saline soda lime was a rod-shaped Gram-positive bacterium that grew at pH range from 7.0 to 11.0 and at temperatures from 10 to 40 °C. The optimal conditions for the growth were: pH=9, 5–6 % NaCl concentration and a temperature of 30 °C. The isolate was found to be alkaliphilic and moderately halophilic. The strain H11 was assigned to the genus *Bacillus*, and it was closely related to *Bacillus luteus* (*25*).

Production of protease

Shrimp shell powder and fructose/glucose (2.5 and 0.5 % *m/V*, respectively) were the optimal nitrogen and carbon sources for the production of protease by *Bacillus luteus* H11 (**Figs. 2a** and **2b**). One of the most important challenges in enzyme production is reduction of production costs related to high expenses of substrates and media used. To achieve these goals, efforts have been made to reduce the protease production costs through both improving the enzyme yield, and the use of low-cost agricultural byproducts, wastes, or feedstocks as substrates for protease production (*32,33*). Similarly to the results obtained in this study, the shrimp shell powder was identified as the best substrate/inducer for the production of proteases by *Chryseobacterium taeanense* (*34*), *Bacillus cereus* TKU006 (*35*), *Bacillus cereus* SV_1 (*36*) and *Bacillus halodurans* CAS6 (*37*). The optimal conditions for growth and protease production of *Bacillus luteus* H11 were pH=9.0, NaCl mass per volume ratio of 4 %, and a temperature of 30 °C. Comparable results were obtained for *B. alcalophilus*, isolated from a saline soda lake, Lonar Lake, India (*38*). In our study, protease production increased with increasing salinity (1–9 % NaCl, *m/V*) and reached its maximum at 4–5 % NaCl (**Fig. 2c**). Similar to the results of the present study, *Bacillus subtilis* AP-MSU6 produced protease that had the optimal salinity of 5 % NaCl (*39*). However, moderate Gram-positive halophiles often reduce the enzyme production at a high salt concentration (*2,40*).

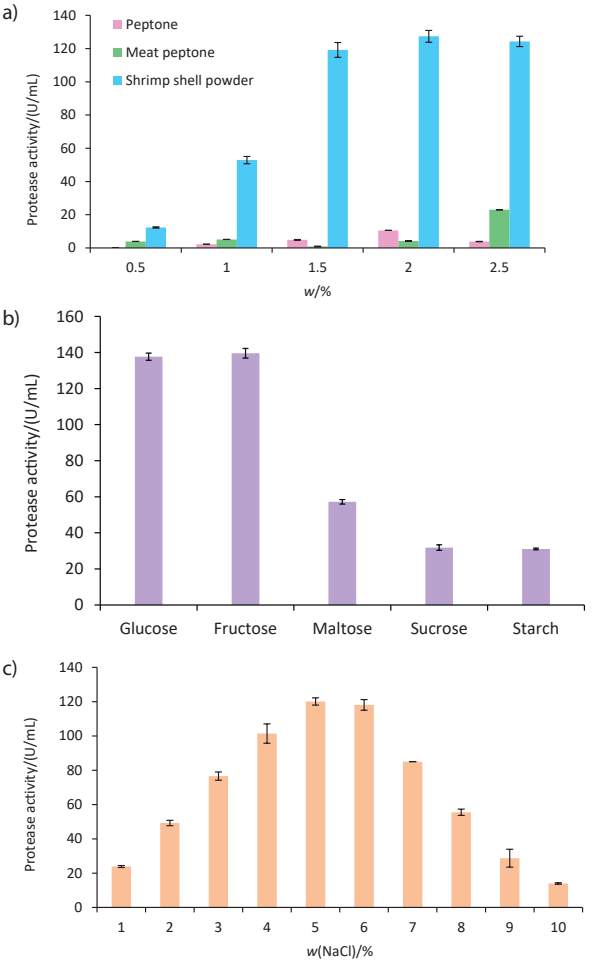

Fig. 2. Effect of: a) nitrogen and b) carbon (0.5 % *m/V*) sources, and c) amounts of NaCl on the activity of protease from *Bacillus luteus* H11. The study was evaluated by incubating bacterial culture for 72 h under optimal growth conditions: 30 °C, pH=9, and 5 % NaCl (a and b), 30 °C and pH=9 (c). Each value represents mean±S.D., *N*=3

Protease purification and molecular mass determination

The enzyme was purified 1.2 times (75.9 % recovery) in the first and 8.3 times (16.5 %) in the second step of the process. The specific activity of the protease was 115.2 U/mg. The molecular mass of the purified enzyme was approx. 37 kDa (**Fig. 3**), which is similar to the protease from halotolerant *Virgibacillus dokdonensis* VITP14 (36 kDa) (*24*), and different from *Bacillus subtilis* AP-MSU 6 (18.5 kDa) (*39*), *B. halodurans* CAS6 (28 kDa) (*37*) and *Chromohalobacter* TVSP101 (66 kDa) (*40*).

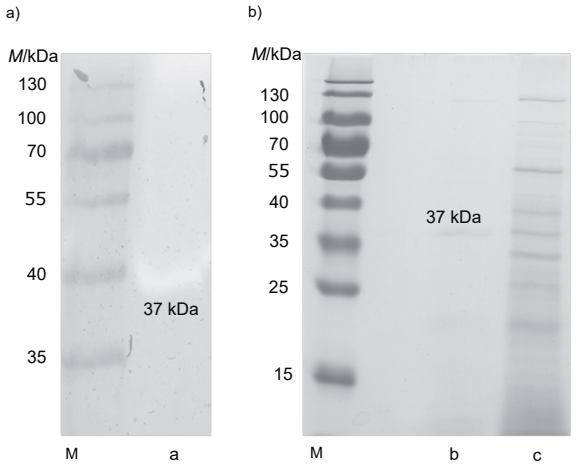

Fig. 3. The analyses of: a) zymograms and b) SDS-PAGE of protease from *Bacillus luteus* H11; M=standard molecular mass marker, a and b=purified protease, c=crude protein extract

Effect of pH, salinity, and temperature on protease activity

The purified enzyme showed optimum activity at pH=10.5 and in 3 M NaCl (**Fig. 4**). The high activity was maintained in the broad pH and salinity range, from 9 to 11, and from 1 to 5 M NaCl, respectively. The remarkable ability of the enzyme to perform its functions in a highly saline environment is similar to halophilic archeal strains such as *Natronolimnobius innermongolicus* WN18 (optimal protease activity at 2.5 M NaCl) and *Haloferax lucentensis* VKMM 007 (4.3 M NaCl) and also to an extreme halophile *Chromohalobacter* TVSP101 (4.5 M NaCl). These microorganisms were isolated from saline environments with NaCl concentration close to saturation such as soda lake, brine, and solar salterns (*14,40–43*).

In terms of pH range, the serine protease produced by *Bacillus luteus* H11 is similar to Esperase® 8.0 L (Novozymes, Bagsværd, Denmark) intended for robust cleaning at high alkalinity and elevated temperatures (*44*).

The protease secreted by *Bacillus luteus* H11 worked best in a temperature interval from 40 to 50 °C with the optimum activity at 45 °C (**Fig. 5**). The thermostability of the enzyme was measured at 45, 55 and 65 °C. The results showed that at 45 and 55 °C, after 60 min, the enzyme activity was maintained at 85 and 70 % respectively, while at 65 °C the enzyme maintained only approx. 30 % of its activity (**Fig. 6**). For most of the halophilic proteases, the reported temperature optimum was in the range from 37 to 75 °C (*14,40–43*). The thermostability of the protease from *Bacillus luteus* H11 at 30 and 40 °C, along with its tolerance

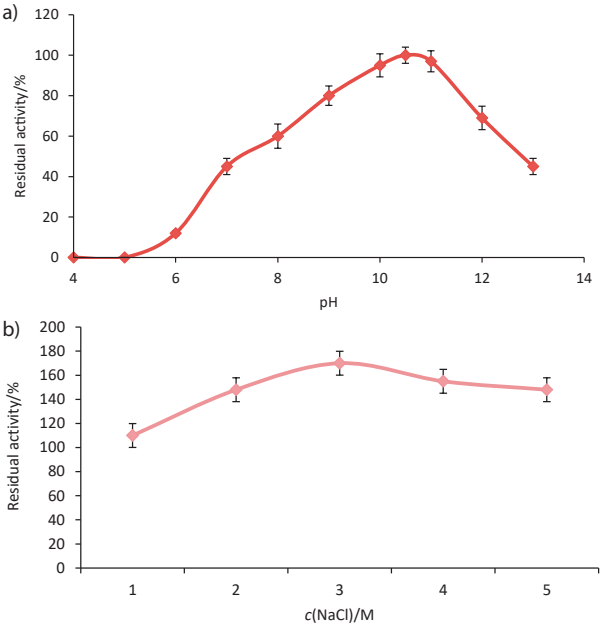

Fig. 4. Effect of pH and NaCl concentration on the activity of protease from *Bacillus luteus* H11. The study was evaluated by incubating purified enzyme substrate for 30 min under its optimal growth conditions: a) 45 °C and 3 M NaCl and b) pH=10.5 and 45 °C. Each value represents mean±S.D., *N*=3

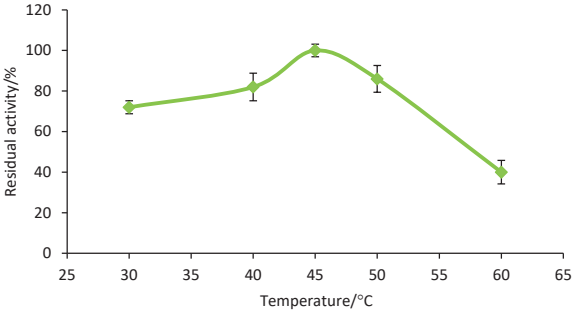

Fig. 5. Effect of temperature on the activity of protease from *Bacillus luteus* H11. The study was evaluated by incubating purified enzyme substrate in 3 M NaCl at pH=10.5 for 30 min. Each value represents mean±S.D., *N*=3

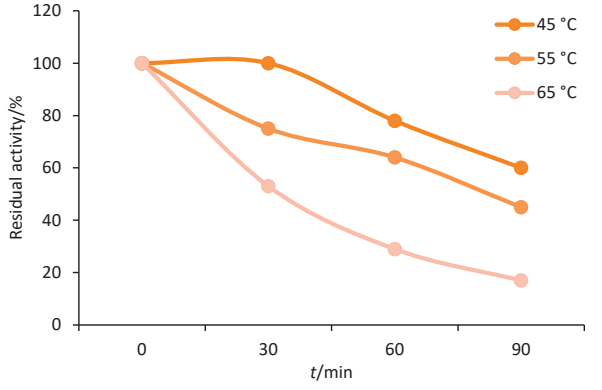

Fig. 6. Thermal stability of protease from *Bacillus luteus* H11. The study was evaluated by incubating purified enzyme in 3 M NaCl at pH=10.5 for 90 min at different temperatures (45, 55 and 65 °C). Each value represents mean±S.D., *N*=3

to alkalinity makes it useful for its potential application in detergents designed for washing at low temperatures. Rising energy costs and increased use of artificial fibres of low tolerance towards high temperatures force the enzyme manufacturers to search for novel biocatalysts, active at low temperatures (*45*).

Effect of specific inhibitors on enzyme activity and substrate specificity

In this study, specific serine protease inhibitors, Pefabloc and DFP inhibited the activity of the enzyme at the 82–95 % level and phenylmethylsulfonyl fluoride (PMSF) at the 58–75 % level (**Table 1**). Other investigated protease inhibitors such as bestatin, E-64, iodoacetamide, and pepstatin A did not affect the enzyme activity. It can be concluded that extracellular protease from *Bacillus luteus* H11 is a serine protease since the classical serine protease inhibitors, Pefabloc and PMSF, reduce its catalytic ability. In addition, the relatively high enzyme activity in the presence of TLCK and aprotinin suggests the lack of a mechanism specific to trypsin-like endoprotease. On the other hand, no effect of bestatin on the activity of protease precludes its belonging to aminopeptidase, particularly to leucine aminopeptidase. The protease activity produced by alkaline and halophilic marine *Bacillus subtilis* AP-MSU6 studied by Maruthiah *et al.* (*39*) was strongly inhibited in the presence of PMSF and thus it was confirmed as a serine protease. Similar serine protease was also observed in *B. mojavensis* A21 (*46*) and *Bacillus* sp. (*47*). The studied endoprotease was active only in the presence of N-succinyl-L-phenylalanine-*p*-nitroanilide as a substrate (data not shown). The obtained results allow classifying the enzyme among the subtilisin-like proteases, having a serine group in their active site. Such substrate specificity of extracellular endoproteases is typical for *Bacillus* species. Many alkaline, serine proteases have found practical application in biotechnology (*48*), *e.g.* Alcalase, Savinase, Esperase, Neutrase (Novozymes).

Table 1. Effects of specific inhibitors on the protease activity

	Residual activity/%	
c(inhibitor)/mM	0.1	1
none	100	100
DFP	10	5
Pefabloc	18	9
PMSF	40	25
EDTA	99	95
EGTA	90	90
1,10-Phenanthroline	100	95
Iodoacetamide	100	100
E-64	100	100
Pepstatin A	100	100
	10^{-3}	10^{-2}
Bestatin	100	100
TLCK	100	100
	10^{-4}	10^{-3}
Aprotinin	100	100

DFP=diisopropyl fluorophosphates, PMSF=phenylmethylsulfonyl fluoride, EDTA=ethylenediaminetetraacetic acid, EGTA=ethylene-bis(oxyethylenenitrilo)tetraacetic acid tetrasodium, TLCK= Nα-tosyl--L-lysine chloromethyl ketone hydrochloride

Effect of other chemical reagents including metal ions on enzyme activity

The protease activity was strongly inhibited by Zn^{2+} and stabilized by Ca^{2+} and Mg^{2+} (**Table 2**). A similar effect of a strong protease inhibition by Zn^{2+} was observed by Vidyasagar *et al.* (*40*), while simulatory effects of Ca^{2+} and Mg^{2+} were detected by Deng *et al.* (*49*) and Maruthiah *et al.* (*39*) when using alkaline protease from *Bacillus* sp. B001 and *Bacillus subtilis* AP-MSU6, respectively.

In the present study organic solvents inhibited the proteolytic activity of *Bacillus luteus* H11; however, the enzyme retained more than half of the initial activity (**Table 2**). Mesbah and Wiegel (*50*) showed that the protease from *Alkalibacillus* sp. NM-Fa4 was even more active in the presence of ethanol, showing 123 % of its initial activity, and retained 74 % of activity in the presence of methanol (50 %, by volume). According to Annamalai *et al.* (*51*), the enzyme from alkaline *Bacillus firmus* CAS 7 was enhanced with isopropanol (115.6 %) and acetonitrile (105.4 %), whereas methanol (90.3 %), ethanol (86.2 %), hexane (85.7 %) and ethyl ether (81.3 %) (25 % final, by volume) did not have significant effect on its activity. In the present study, other chemicals such as DMSO, 2 M ethanol, urea and H_2O_2, Tween 80 and Triton X-100 (1 mM) did not inhibit the protease activity (**Table 2**). Study made by Raval *et al.* (*52*) on purified protease from haloalkaliphilic

Table 2. Effect of metal ions, solvents and other chemical reagents on the protease activity

	Residual activity/%	
c(inhibitor)/mM	1	5
none	100	100
Mg^{2+}	120	119
Ca^{2+}	128	120
Ba^{2+}	100	95
Co^{2+}	100	90
Fe^{2+}	75	70
Cu^{2+}	74	60
Zn^{2+}	15	5
Cd^{+}	90	85
φ(solvent)/%	50	75
Butanol	55	45
Methanol	70	51
Ethanol	67	60
Acetone	70	50
c(substance)/mM	1	5
SDS	87	75
Triton X-100	100	85
Tween 80	106	87
CTAB	95	80
DMSO	100	105
2 M ethanol	105	110
Urea	100	100
H_2O_2	100	100

SDS=sodium dodecyl sulphate, CTAB=cetyl trimethylammonium bromide, DMSO=dimethyl sulfoxide

Bacillus pseudofirmus revealed that the enzyme showed a relatively high activity in non-ionic surfactants (Tween 80 and Triton X-100, 1 % by volume), commercial detergents, oxidizing and reducing agents, while it was not stable in anionic surfactant (1 % SDS, *m/V*). Mesbah and Wiegel (*50*) reported that an haloalkaline, thermostable protease produced by *Alkalibacillus* sp. NM-Fa4 was resistant to SDS, H_2O_2 and urea, and retained most of its activity in the presence of Tween 80 (1 %, *m/V*).

CONCLUSIONS

The serine, subtilisin-like endoprotease, produced by *Bacillus luteus* H11 isolated from alkaline and saline soda lime was purified and characterized. The alkaline protease secreted by *B. luteus* H11 may be important from industrial perspective because of its tolerance towards high alkalinity and salinity, as well as its stability against surfactants, some organic solvents, and oxidants. These characteristics make the protease useful for applications in the food, detergent, and pharmaceutical industries, as well as in environmental bioremediation. To the best of our knowledge, there is currently a lack of commercial alkaline protease on the market showing such a particular halotolerance. The possibility of using wastes from shrimp processing for the haloalkaline protease production is promising, both in lowering the overall cost of the biocatalyst production and waste utilization.

FUNDING

This research was supported by funds provided by Nicolaus Copernicus University (Toruń Poland) and partly by Warsaw University of Life Sciences to maintain research potential.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Eichler J. Biotechnological uses of archaeal extremozymes. Biotechnol Adv. 2001;19:261–78. https://doi.org/10.1016/S0734-9750(01)00061-1
2. van den Burg B. Extremophiles as a source for novel enzymes. Curr Opin Microbiol. 2003; 6:213–8. https://doi.org/10.1016/S0734-9750(01)00061-1
3. Annamalai N, Rajeswari MV, Balasubramanian T. Extraction, purification and application of thermostable and halostable alkaline protease from Bacillus alveayuensis CAS 5 using marine wastes. Proteins: Struct Funct Bioinf. 2014;92:335–42. https://doi.org/10.1016/j.fbp.2013.08.009
4. Joshi S, Satyanarayana T. Characteristics and applications of a recombinant alkaline serine protease from a novel bacterium Bacillus lehensis. Bioresour Technol. 2013;131:76–85. https://doi.org/10.1016/j.biortech.2012.12.124
5. Abdelnasser SSI, Salamah AAA, Elbadawi YB, Tayeb MAE, Ibrahim SS. Production of extracellular alkaline protease by new halotolerant alkaliphilic Bacillus sp. NPST-AK15 isolated from hyper saline soda lakes. Electron J Biotechnol. 2015;18:236–43. https://doi.org/10.1016/j.ejbt.2015.04.001
6. Shah K, Mody K, Keshri J, Jha B. Purification and characterization of a solvent, detergent and oxidizing agent tolerant protease from Bacillus cereus isolated from the Gulf of Khambhat Kunal. J Mol Catal B: Enzym. 2010;67:85–91. https://doi.org/10.1016/j.molcatb.2010.07.010
7. Kumar RS, Ananthan G, Prabhu AS. Optimization of medium composition for alkaline protease production by Marinobacter sp. GA CAS9 using response surface methodology – A statistical approach. Biocatal Agri Biotechnol. 2014;3:191–7. https://doi.org/10.1016/j.bcab.2013.11.005
8. Gómez J, Steiner W. The biocatalytic potential of extremophiles and extremozymes. Food Technol Biotechnol. 2004; 42:223–35.
9. Oren A. Industrial and environmental applications of halophilic microorganisms. Environ Technol. 2010;31:825–34. https://doi.org/10.1080/09593330903370026
10. Li X, Yu HY. Characterization of an organic solvent-tolerant α-amylase from a halophilic isolate, Thalassobacillus sp. LY18. Folia Microbiol. 2012;57:447–53. https://doi.org/10.1007/s12223-012-0160-3
11. Gupta S, Sharma P, Dev K, Srivastava M, Sourirajan A. A diverse group of halophilic bacteria exist in Lunsu, a natural salt water body of Himachal Pradesh, India. SpringerPlus 2015;4(1):274–83. https://doi.org/10.1186/s40064-015-1028-1
12. Klähn S, Marquardt DM, Rollwitz I, Hagemann M. Expression of the ggpPS gene for glucosylglycerol biosynthesis from Azotobacter vinelandii improves the salt tolerance of Arabidopsis thaliana. J Exp Bot. 2009;60:1679–89. https://doi.org/10.1093/jxb/erp030
13. Arora S, Patel P, Vanza M, Rao GG. Isolation and characterization of endophytic bacteria colonizing halophyte and other salt-tolerant plant species from coastal Gujarat. Afr J Microbiol Res. 2014;8:1779–88. https://doi.org/10.5897/AJMR2013.5557
14. Selim S, Hagagy N, Aziz MA, El-Meleigy S, Pessione E. Thermostable alkaline halophilic-protease production by Natronolimnobius innermongolicus WN18. Nat Prod Res. 2014;28:1476–9. https://doi.org/10.1080/14786419.2014.907288
15. Fujinami S, Fujisawa M. Industrial applications of alkaliphiles and their enzymes – Past, present and future. Environ Technol. 2010;31:845–56. https://doi.org/10.1080/09593331003762807
16. Joo HS, Chang CS. Oxidant and SDS-stable alkaline protease from a halo-tolerant Bacillus clausii I-52: Enhanced production

and simple purification. J Appl Microbiol. 2005;98:491–7. https://doi.org/10.1111/j.1365-2672.2004.02464.x

17. Setyorini E, Takenaka S, Murakami S, Aoki K. Purification and characterization of two novel halotolerant extracellular proteases from Bacillus subtilis strain FP-133. Biosci Biotech Biochem. 2006;70:433–40. https://doi.org/10.1271/bbb.70.433
18. Alvarez VM, von der Weid I, Seldin L, Santos ALS. Influence of growth conditions on the production of extracellular proteolytic enzymes in Paenibacillus peoriae NRRL BD-62 and Paenibacillus polymyxa SCE2. Lett Appl Microbiol. 2006;43: 625–30. https://doi.org/ 10.1111/j.1472-765X.2006.02015.x
19. Namwong S, Hiraga K, Takada K, Tsunemi M, Tanasupawat S, Oda K. A halophilic serine proteinase from Halobacillus sp. SR5-3 isolated from fish sauce: purification and characterization. Biosci Biotechnol Biochem. 2006;70:1395–401. https://doi.org/10.1271/bbb.50658
20. Akolkar AV, Durai D, Desai AJ. Halobacterium sp. SP1(1) as a starter culture for accelerating fish sauce fermentation. J Appl Microbiol. 2010;109:44–53. https://doi.org/10.1111/j.1365-2672.2009.04626.x
21. Phrommao E, Rodtong S, Yongsawatdigul J. Identification of novel halotolerant bacillopeptidase F-like proteinases from a moderately halophilic bacterium, Virgibacillus sp. SK37. J Appl Microbiol. 2011;110:191–201. https://doi.org/10.1111/j.1365-2672.2010.04871.x
22. Karan R, Khare SK. Stability of haloalkaliphilic Geomicrobium sp. protease modulated by salt. Biochemistry (Moscow) 2011;76:686–93. https://doi.org/10.1134/S0006297911060095
23. Santos AF, Valle RS, Pacheco CA, Alvarez VM, Seldini L, Santos ALS. Extracellular proteases of Halobacillus blutaparonensis strain M9, a new moderately halophilic bacterium. Braz J Microbiol. 2013;44:1299–304. https://doi.org/10.1590/S1517-83822014005000015
24. Rajeswari VD, Jayarman G, Sridharn TB. Purification and characterization of extracellular protease from halotolerant bacterium Virgibacillus dokdonensis VITP14. Asian J Biochem. 2012;7:123–32. https://doi.org/10.3923/ajb.2012.123.132
25. Kalwasińska A, Felföldi T, Walczak M, Kosobucki P. Physiology and molecular phylogeny of bacteria isolated from alkaline distillery lime. Pol J Microbiol. 2015;64: 69–77.
26. Reynolds III JE, Josowicz M, Tyler F, Vegh RB, Solntsev KM. Spectral and redox properties of the GFP synthetic chromophores as a function of pH in buffered media. Chem Commun. 2013;49(71):7788–90. https://doi.org/10.1039/C3CC44113J
27. Jankiewicz U, Larkowska E, Swiontek Brzezinska M. Production, characterization, gene cloning, and nematocidal activity of the extracellular protease from Stenotrophomonas maltophilia N4. J Biosci Bioeng. 2016;121:614–8. https://doi.org/10.1016/j.jbiosc.2015.11.011
28. Coêlho DF, Saturnino TP, Fernandes FF, Mazzola PG, Silveira E, Tambourgi EB. Azocasein substrate for determination of proteolytic activity: Reexamining a traditional method using bromelain samples. BioMed Res Int. 2016;2016:Article ID 8409183. https://doi.org/10.1155/2016/8409183
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
30. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227: 680–5.
31. Bezerra RS, Lins EJF, Alencar RB, Paiva PMG, Chaves MEC, Coelho LCBB, Carvalho Jr LB. Alkaline proteinase from intestine of Nile tilapia (Oreochromis niloticus). Process Biochem. 2005;40(5):1829–34. https://doi.org/10.1016/j.procbio.2004.06.066
32. Mukherjee AK, Adhikari H, Rai SK. Production of alkaline protease by a thermophilic Bacillus subtilis under solid-state fermentation (SSF) condition using Imperata cylindrical grass and potato peel as a low-cost medium: Characterization and application in detergent formulation. Biochem Eng J. 2008;39:353–61. https://doi.org/10.1016/j.bej.2007.09.017
33. Dutta M, Chamendra N, Pai SG, Pramod T, Siddalingeshwara KG. Isolation and screening of agro-waste substrates for protease production through solid state fermentation. Int J Curr Microbiol Appl Sci. 2014;3:774–81.
34. Wang SL, Yang CH, Liang TW, Yen YH. Optimization of conditions for protease production by Chryseobacterium taeanense TKU001. Bioresour Technol. 2008;99:3700–7. https://doi.org/10.1016/j.biortech.2007.07.036
35. Wang SL, Chao CH, Liang TW, Chen CC. Purification and characterization of protease and chitinase from Bacillus cereus TKU006 and conversion of marine wastes by these enzymes. Mar Biotechnol. 2009;11:334–44. https://doi.org/10.1007/s10126-008-9149-y
36. Ghorbel-Bellaaj O, Manni L, Jellouli K, Hmidet N, Nasri M. Optimization of protease and chitinase production by Bacillus cereus SV1 on shrimp shell waste using statistical experimental design. Biochemical and molecular characterization of the chitinase. Ann Microbiol. 2012;62:1255–68. https://doi.org/10.1007/s13213-011-0371-x
37. Annamalai N, Rajeswari MV, Thavasi R, Vijayalakshmi S, Balasubramanian T. Optimization, purification and characterization of novel thermostable, haloalkaline, solvent stable protease from Bacillus halodurans CAS6 using marine shellfish wastes: A potential additive for detergent and antioxidant synthesis. Bioprocess Biosyst Eng. 2013;36:873–83. https://doi.org/10.1007/s00449-012-0820-3

38. Kanekar PP, Nilegaonkar SS, Sarnaik SS, Kelkar AS. Optimization of protease activity of alkaliphilic bacteria isolated from an alkaline lake in India. Bioresour Technol. 2002;85:87–93.

39. Maruthiah T, Esakkiraj P, Prabakaran G, Palavesam A. Purification and characterization of moderately halophilic alkaline serine protease from marine Bacillus subtilis AP-MSU 6. Biocatal Agri Biotechnol. 2013;2:116–9. https://doi.org/10.1016/j.bcab.2013.03.001

40. Vidyasagar M, Prakash S, Mahajan V, Shouche YS, Sreeramulu K. Purification and characterization of an extreme halothermophilic protease from a halophilic bacterium Chromohalobacter sp. TVSP101. Braz J Microbiol. 2009;40:12–9. https://doi.org/10.1590/S1517-83822009000100002

41. Izotova LS, Strongin AY, Chekulaeva LW, Sterkin VE, Ostoslavskaya VI, Lyublinskaya EA, et al. Purification and properties of serine protease from Halobacterium halobium. J Bacteriol. 1983;155:826–30.

42. Kanlayakrit W, Bovornreungroj P, Oka T, Goto M. Production and characterization of a protease from an extremely halophilic Halobacterium sp. PB407. Kasetsart J (Nat Sci). 2004; 38:15–20.

43. Manikandan M, Pas L, Kannan V. Purification and biological characterization of a halophilic thermostable protease from Haloferax lucentensis VKMM 007. World J Microbiol Biotechnol. 2009;25:2247–56. https://doi.org/10.1007/s11274-009-0132-1

44. Esperase® 8.0L. Inc. Catalog # 06-3115. Strem Chemicals, Newburyport, MA, USA. Available from: https://www.strem.com/uploads/technical_notes/06-3115tech.pdf.

45. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol. 2002;59:15–32. https://doi.org/10.1007/s00253-002-0975-y

46. Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M. Two detergent stable alkaline serine-proteases from Bacillus mojavensis A21: Purification, characterization and potential application as a laundry detergent additive. Bioresour Technol. 2009;100:3366–73. https://doi.org/10.1016/j.biortech.2009.01.061

47. Zambare V. Purification and characterization of neutral serine protease from Bacillus sp. Asiat J Biotechnol Resour. 2010;3:183–92. https://doi.org/12.2010/AJOBR-2010/03/183-192

48. Parrado J, Rodriguez-Morgado B, Tejada M, Hernandez T, Garcia C. Proteomic analysis of enzyme production by Bacillus licheniformis using different feather wastes as the sole fermentation media. Enzyme Microb Technol. 2014;57:1–7. https://doi.org/10.1016/j.enzmictec.2014.01.001

49. Deng A, Wua J, Zhang Y, Zhang G, Wen T. Purification and characterization of a surfactant-stable high-alkaline protease from Bacillus sp. B001. Bioresour Technol. 2010;101: 7100–6. https://doi.org/10.1016/j.biortech.2010.03.130

50. Mesbah NM, Wiegel J. Purification and biochemical characterization of halophilic, alkalithermophilic protease AbCP from Alkalibacillus sp. NM-Fa4. J Mol Catal B: Enzym. 2014; 105:74–81. https://doi.org/10.1016/j.molcatb.2014.03.023

51. Annamalai N, Rajeswari MV, Sahu SK, Balasubramanian T. Purification and characterization of solvent stable, alkaline protease from Bacillus firmus CAS 7 by microbial conversion of marine wastes and molecular mechanism underlying solvent stability. Process Biochem. 2014;49:1012–9. https://doi.org/10.1016/j.procbio.2014.03.007

52. Raval VH, Pillai S, Rawal CM, Singh SP. Biochemical and structural characterization of a detergent-stable serine alkaline protease from seawater haloalkaliphilic bacteria. Process Biochem. 2014;49(6):955-62. https://doi.org/10.1016/j.procbio.2014.03.014