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

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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 alkalo-halophilic bacterium, and its extracellular serine endoprotease also showed an extreme alkal- 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, alkalo-halophiles, 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, Bagsvaerd, 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 halo-philic microorganisms, e.g. *Bacillus clausii*1-52 (16), *Bacillus subtilis* FP-133 (17), *Paenibacillus*
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 lutes* 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: MgSO₄·7H₂O, NaCl, Na₂CO₃, KH₂PO₄, MgCl₂·6H₂O, (NH₄)₂SO₄, 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), HCl, NaOH, H₂BO₃, H₃PO₄, CH₃COOH, 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(oxy ethylenenitril)tetraacetic acid (EGTA), E-64, lidoacetamide, Pefabloc®, pepstatin, phenylmethylsulfonyl fluoride (PMSF), 1,10-phenanthroline, Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), cetyl trimethylammonium bromide (CTAB), sodium dodecyl sulphate (SDS), Triton X-100, Tween 80, urea, H₂O₂, N-succinyl-Gly-Gly-Phe-p-nitroanilide, N-succinyl-L-phenylalanine-p-nitroanilide, Na-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 (Bio-Rad, Hercules, CA, USA).

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 lutes* 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, Na₂CO₃ 2, KH₂PO₄ 0.2 and MgSO₄·7H₂O 0.02, pH=9, was inoculated with 1 mL *Bacillus lutes* H11 suspension (A₄₅₀nm=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 2ZH1220B (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 for 10 min. The protein concentrations at all purification stages were determined with bovine serum albumin as a standard according to Bradford (29).

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](image)

Azocasein concentration was calculated from the following equation:

\[
c_{\text{azocasein}} = \frac{A_{420 \text{ nm}} - 0.351}{0.1167} \tag{1}/
\]

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:

\[
\text{Activity} = \frac{c_{\text{azocasein}} \times V_{\text{total}}}{V_{\text{enzyme}} \times t} \tag{2}/
\]

where \( V_{\text{total}} \) is the sum of volumes of substrate, enzyme and TCA, \( V_{\text{enzyme}} \) 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 9000xg 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 of 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), Na-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) (final concentrations 1.0 and 10.0 µM), disopropyl phosphorofluoridate (DFP), ethylene-bis(oxyethylenenitritol) 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: Mg2+•••Ca2+, Ba2+, Co2+, Fe2+, Cu2+, Zn2+, Cd2+ (final concentrations 1 and 5 mM). Other chemicals were: cetyl trimethylammonium bromide (CTAB), dimethyl sulfoxide (DMSO), ethanol, 2 M, H2O2, 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-Leu-p-nitroanilide, N-succinyl-L-phenylalanine-p-nitroanilide, Nα-benzoyl-Gly-Phe-nitroanilide, Nα-tosyl-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 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 lake 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, (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). Similarly 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).
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 archaean 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, Bagsvaerd, 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

| c(inhibitor)/mM | Residual activity/%  
|----------------|------------------|
|                | 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             |
| 10³             | 10²              |                  |
| Bestatin       | 100              | 100             |
| TLCK           | 100              | 100             |
| 10³             | 10¹              |                  |
| Aprotinin      | 100              | 100             |

DFP=diisopropyl fluorophosphates, PMSF=phenylmethylsulfonyl fluoride, EDTA=ethylenediaminetetraacetic acid, EGTA=ethylenbis(oxyethylenenitriilo)tetraacetic acid tetrasodium, TLCK=Na-tosyl-L-lysine chloromethyl ketone hydrochloride

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

| c(inhibitor)/mM | Residual activity/%  
|----------------|------------------|
|                | 1               | 5               |
| none           | 100             | 100             |
| Mg²⁺           | 120             | 119             |
| Ca²⁺           | 128             | 120             |
| Ba²⁺           | 100             | 95              |
| Co²⁺           | 100             | 90              |
| Fe²⁺           | 75              | 70              |
| Cu²⁺           | 74              | 60              |
| Zn²⁺           | 15              | 5               |
| Cd²⁺           | 90              | 85              |
| 0(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             |
| H₂O₂           | 100             | 100             |

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

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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