Cloning, Secretory Expression, Partial Characterization, and Structural Modeling of an Alkaline Protease from *Bacillus subtilis D-2*

Yayuan Xu,[a,b] Fazhi Xu,[c] Xiaoling Ding,[c] Kun Qian,[b] and Lvmu Li [a,c,*]

To develop a large-scale production of the protease of *Bacillus subtilis* strain D-2, the full-length gene *apr-D2* (1,149 bp) encoding the alkaline protease was cloned into plasmid pET-32a and expressed as a secretory protein in *Escherichia coli*. Sequence analysis of the deduced amino acid sequence revealed high homology with the catalytic domains of the subtilisin serine proteases. From SDS-PAGE analysis, the recombinant protein had a molecular mass of 60.4 kDa. The expressed protease was secreted into the culture medium in a functional active form. The purified recombinant protease showed a pH optimum of 10.5 and temperature optimum of 55 °C, and it was stable in the pH range from 5.0 to 13.0. The enzyme activity was slightly enhanced by Ca^{2+}, Mg^{2+}, Ba^{2+}, and SBT1. However, it was highly inhibited by Ag^{+} and PMSF. A theoretical structural model of mature protein was constructed by comparative modeling, which showed a putative catalytic triad (Asp-32, His-64 and Ser-221) with high similarity to the template. The structural characteristics that confer enzymatic specificity of the protease were analyzed. Taken together, the data suggested that the secretory expression system with pET-32a in *E. coli* was successfully constructed. Additionally, enzymatic specificity analysis of the alkaline protease indicated that it was suitable for various processing industries.

Keywords: Bacillus subtilis strain D-2; *Secretory expression*; *Escherichia coli*; *Alkaline protease; Structural model*

*Contact information: a: School of Life Science, Anhui Agricultural University, Hefei, Anhui, 230036, China; b: Institute of Agricultural Food, Anhui Academy of Agricultural Sciences, Hefei, Anhui, 230031, China; c: School of Animal Science and Technology, Anhui Agricultural University, Hefei, Anhui, 230036, China; *Corresponding author: lilvmu@126.com*

INTRODUCTION

Alkaline proteases have wide applications in different industries, such as detergent, feed processing, protein processing, and peptide synthesis (Chandrasekharaiah *et al*. 2012; Adrio and Demain 2014). Many alkaline protease genes have been cloned, and their encoded proteases have been expressed, purified, and characterized (Deng *et al*. 2010; Deng *et al*. 2011). Compared with plants, animal, and fungal proteases, bacterial proteases are the most significant due to their extracellular nature, high yield of production, limited space and short period of time required for their cultivation, and their ease of genetic manipulation. *Escherichia coli* is the most commonly used host for the expression of recombinant proteins; it has been the "work horse" for expression of protease from different microbial resources (Fu *et al*. 2000; Deng *et al*. 2014). However, one challenge is inefficient secretion and the formation of inclusion bodies. Hence, various attempts have been made to facilitate the extracellular secretion of recombinant proteins in *E. coli*, including the use of different promoters to regulate the level of expression, the use of

different host strains, co-expression of chaperones, and optimization of induced conditions (Guex and Peitsch 1997; Fu *et al*. 2003).

Bacteriocin release proteins can be used for the release of heterologous proteins from the *E. coli* cytoplasm into the culture medium (Gupta *et al*. 2002; Han *et al*. 2013; Heo *et al*. 2013). Secretory expression exogenous proteins in *E. coli* may be more desirable in large-scale industrial production. Fu *et al*. (2003) showed that the recombinant F1 protease was efficiently excreted into the culture medium using *E. coli* XL1-Blue, and the purified enzyme was stable at 70 °C for 24 h from pH 8.0 to 10.0. The enzyme exhibited a high degree of thermostability with a half-life of 4 h at 85 °C, 25 min at 90 °C, and it was inhibited by the serine protease inhibitor (Hu *et al*. 2013).

Protein function is determined by its structure (Johansson-Åkhe *et al*. 2019). The alkaline serine protease of *Bacillus pumilus* TMS55 has high alkaline protease activity and dehairing efficacy. Structural analysis shows that this protease is composed of nine α-helices and nine β-strands. It has three catalytic residues and 14 metal binding residues, which allows the rational design of its derivatives for structure-function studies and for further improvement of the enzyme (Ibrahim *et al*. 2011). Protein localization sites should be selected depending upon biochemical properties of the respective proteins.

In this study, *Bacillus subtilis* strain D-2 isolated from topsoil surface of a meat powder plant showed higher alkaline protease activity than other isolates. It effectively decomposed protein in soybean meal and blood powder and improved dietary performance of protein feedstuff (Xu *et al*. 2011). *B. subtilis* strain D-2 alkaline protease has not yet been characterized. To determine the protease of *B. subtilis* strain D-2, the alkaline protease gene was cloned for secretory expression in *E. coli* BL21(DE3) using pET-32a vectors with a signal sequence and propeptide sequence. The localization of recombinant protease on the subcellular fraction was performed on the optimal induced expression condition. Finally, the purified recombinant enzyme was fully researched by enzymatic characterization, protein sequence analysis, and structure model prediction.

EXPERMENTAL

Materials

Bacillus subtilis D-2 (HQ325006) from a laboratory stock was cultivated at 37 °C for 24 h in nutrient broth (NB). *E. coli* DH5α and *E. coli* BL21(DE3) were grown in Luria-Bertani (LB) medium at 37 °C. Ampicillin (50 mg/mL) was added to the medium when needed. The pGEMT-Easy TA cloning vector and the expression vector pET-32a used to construct recombinant plasmids were obtained from TaKaRa (Dalian, China). The plasmid mini kit used for extracting Plasmids from *E. coli* was purchased from Novagen (Madison, WI, USA). The TaKaRa mini BEST agarose gel DNA extraction kit was used for recovering DNA fragments in agarose gels. All restriction enzymes and modification enzymes were obtained from TaKaRa. All other chemicals were of the highest reagent grade that was commercially available.

Methods

Construction of target gene expression vector

A DNA fragment was isolated using PCR with two primers, PK1 (5'-CG-GGATCC-ATGAGAGGCAAAAAGGTATG-3'; 27 bp) and PK2 (5'-CC-AAGCTT-TTACTGAGCTG CCGCCTGTAC-3'; 30 bp) with the *BamHI* and *HindIII* sites at the 5'

ends, respectively. Both primers were designed according to the nucleotide sequence of the alkaline protease gene and were synthesized by Shanghai Biotechnology. The total DNA of *Bacillus subtilis* D-2 was used as a template in the PCR amplification (Jaouadi *et al*. 2012). The amplification of the target gene was performed under the following conditions. Standard PCR was carried out in 15 μL containing 1.5 μL *B. subtilis* DNA, 1.5 μL of each primer, and 12.5 μL 2× Taq mixture. The PCR cycling conditions comprised an initial step of 5 min at 94 ºC, a second step of 30 cycles including 60 s at 94 °C, 50 s at 63 °C and 60 s at 72 °C, and a third step of 10 min at 72 °C. The PCR product was inserted into the pGEMT-Easy TA simple vector and transformed in *E. coli* DH5α. The recombinant vector was named pGEMT-*apr-D2* and sequenced by TaKaRa Biotechnology. Sequence analysis was carried out using the DNAstar 7.1 software (DNAStar company, Madison, America), and homology analysis was performed using the BLAST program available from the National Center for Bio-technology Information (NCBI) (https://www.ncbi.nlm.nih.gov/)

The recombinant plasmid pGEMT-*apr-D2* was digested by the *BamHI* and *HindIII* endonucleases, and the produced fragment was inserted into pET-*32a* digested with the same enzymes resulting in pET32a-*apr-D2*. The vectors thus obtained fused thioredoxin to the amino-terminal region of the expressed protein, and they were transformed into *E. coli* BL21(DE3) for fusion protein expression with the empty plasmids pET-32a for control. *E. coli* DH5α and *E. coli* BL21(DE3) competent cells were prepared and transformed by electroporation as described previously (Joo and Choi 2012).

Expression and detection of extracellular proteolytic activity

The transformed *E. coli* harboring the appropriate recombinant plasmid pET32a-*aprD2* was inoculated into 10 mL of fresh LB containing ampicillin (50 ng/mL) and grown at 37 °C with shaking overnight. A 500 μL culture sample was then inoculated into 10 mL LB liquid medium and grown at 37 °C under constant agitation until the cell density of OD_{600} reached 0.5 (approximately 4 h). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture media at 1 mM. After another 4.5 h of incubation, the cultures were centrifuged at 10,000 × g for 10 min at 4 °C, and the pellet was resuspended in the TE buffer. The cells were lysed by ultrasonication of 5 cycles (2 s pulse, 1 s interval, and 2 min duration) on ice in the buffer. The cell lysate was centrifuged at 15,000 × g for 10 min at 4 °C to separate soluble (supernatant) and precipitated (pellet) fractions. The culture supernatant, intracellular soluble content, and inclusion bodies were used to detect the expressed product with SDS-PAGE. The extracellular protease activity was detected by detected as zones of hydrolysis on skim milk medium agar plates of the culture supernatant of *E. coli* BL21(DE3) with plasmids pET-*aprD2* and empty plasmids pET-32a for control (Jeong *et al*. 2015b).

Optimization of secretory expression condition

To optimize the secretory expression condition, different temperatures (12, 17, 22, 27, 32, and 37 °C), inducer concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mmol l^{-1} IPTG), and inducing times (0, 2, 4, 6, 8 and 10 h), were set to induce expression, followed by testing of the A_{660} and protease activity of the culture (Kim *et al*. 2010).

Production and partially purification of recombinant protease

The transformed *E. coli* BL21(DE3) colony containing the construct expression vector was inoculated in 25 mL of LB medium containing 30 μg/mL ampicillin and incubated overnight in shaker at 37 °C as seed culture. The seed culture was inoculated

into 500 mL of LB medium containing ampicillin (50 μg/mL) and then cultured at 37 °C until the optical density at 660 nm reached 0.8. IPTG was added at a final concentration of 0.4 mM to induce expression of the fusion protein. The cell-free supernatant was collected by centrifugation, after the culture was incubated at 27 °C for an additional 4 h period. The proteins were precipitated using 30 to 60% ammonium sulfate and then collected by centrifugation after incubation overnight at 4 °C. They were dissolved in buffer of pH 6.0 (containing 0.01 M NaH_2PO_4 and 0.01 M Na_2HPO_4) and dialyzed with the same buffer overnight to remove any residual ammonium sulfate. The crude extract was purified using Ni^{2+}-chelating chromatography according to the manufacturers' instructions (Beijing AOBOX Biotechnology Corporation, Beijing, China). The purified recombinant protease was stored at -70 °C before analysis. The protein concentration was measured with a BCA protein assay kit (Pierce, Rockford, America) using bovine serum albumin (BSA) as the standard.

Determination of alkaline protease activity

Protease activity was determined by Folin's phenol using 1% casein as substrate. First, 0.2 mL of enzyme solution was added to 0.8 mL of substrate solution (1% V/V, casein with 50 mM glycine-NaOH buffer, pH 10.0) and incubated at 50 °C for 20 min with controls. The reaction was terminated by adding 1 mL of 10% trichloroacetic acid (TCA), followed by holding for 10 min at room temperature and centrifugation at 10000 × g for 10 min at 4 °C. Next, 1 mL of supernatant was mixed with 3 mL of 0.4 M Na_2CO_3 and 0.5 mL of Folin's phenol. The absorbance of each sample was measured at 660 nm equivalent to 1 μmol of tyrosine, in the presence of the Folin Ciocalteau reagent by using a tyrosine standard curve (it has been revised). A single unit of protease activity was defined as the amount of enzyme that released 1 μg of tyrosine per minute under optimum assay conditions. Enzyme units were measured using tyrosine (0 to 100 mg) as standard (Deng *et al*. 2011).

Determination of proteolytic properties

The optimal temperature was determined by analyzing the enzyme activity in 50 mM glycine–NaOH (pH 10.0) in the temperature range of 30 to 80 °C. The optimal pH was determined at 55 °C using buffers with different pH (Lee and Liu 2000). The relative activity was calculated as a percentage of the maximal activity. The thermal and pH stabilities of the enzyme were assayed by determining the residual activity after incubating the purified enzyme at 45 to 65 °C for 0 to 120 min or at a pH of 4.0 to 13.0 for 12 h. The initial activity was taken as 100% (Sareen and Mishra 2008).

The effects of various metal ions on the enzyme activity were determined by incubating the reaction in mixtures containing 10 mM $AgNO_3$, $MgCl_2$, $MnCl_2$, $PbSO_4$, $ZnSO_4$, $CaCl_2$, $BaCl_2$, $FeSO_4$, or $MnSO_4$ (Deng *et al*. 2011). To evaluate the effects of inhibitors, a soybean trypsin inhibitor (SBTI), β-mercaptoethanol (ME), urea, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), guanidinium chloride (Gn-HCl), and phenylmethylsulfonyl fluoride (PMSF) were used (Li and Yu 2012). The purified enzyme was pre-incubated with each inhibitor for 30 min at room temperature before the residual activity was measured. The enzyme activity incubated under identical conditions, but in the absence of a metal ion or inhibitor, was defined as 100%. The effects of surfactants and oxidants on the *apr-D2* stability were evaluated by pre-incubating the enzyme for 1 h in solutions of dodecyl sulfonic acid

sodium (SDS), Tween 80 or H_2O_2 at room temperature before the residual activity measured. The activity of the enzyme without any additive was construed as 100%.

The amino acid sequence of *apr-D2* gene was compared with other known proteins obtained from the EMBL/GenBank/DDBJ databases using available BLAST methods (http://www.ncbi.nlm.nih.gov/blast/). A conserved sequence alignment of *apr-D2* and other related proteases was created using CLC Main Workbench software. Based on the crystal structures of the subtilisin BPN´ (PDB ID: 2st1) from *B. subtilis*, the structures of the *apr-D2* protein were modeled using Swiss-Model (http://swissmodel.expasy.org/) and the quality of the final models was analyzed using QMEANclust (http://swissmodel.expasy.org/qmean/cgi/index.cgi). The predicted structure was compared using the PyMOL Molecular Graphics System (ver. 1.5.0.4, Schrödinger, LLC, New York, USA) and exported as images.

RESULTS AND DISCUSSION

Amplification of *apr-D2* gene

The *apr-D2* gene was amplified from the total DNA of B. subtilus D-2 by PCR. The DNA sequencing results *apr-D2* contained a 1149 bp open reading frame encoding a putative 382 amino acid (aa) residues (Fig. 1). The nucleotide sequence of *apr-D2* was deposited in GenBank (GenBank accession No. JQ730856). The results of DNA sequence comparisons showed a 99% similarity to the sequences of fibrinolytic genes of *B. amyloliquefaciens* CH51 (EU414203). Therefore, the *aprD2* is an alkaline protease that is most closely related to the alkaline proteases belonging to the Subtilisin family. The gene encoding subtilisin Carlsberg from *B. licheniformis* was cloned in pBR322 vector by Jacob *et al*. (1985), and it contained an open reading frame (ORF) with 1137 bp encoding 379 aa. The alkaline protease genes of *B. lentus* and *B. alkalophilus* were cloned and sequenced, which both encoded 380 aa (Jorgensen *et al*. 2000). Almost all of them have total length of about 380 aa, which is similar to the target gene *apr-D2*. The results of signal peptide analysis are shown in Fig. 2. C_{max} values (the cleavage site score), Y_{max} values (the combination score), S_{max} values (the signal peptide score), S mean values (the mean values of the signal peptide score), D values (the classification task) and their sites are obtained. The C_{max} value was greater than 0.49, the S_{mean} value was greater 0.5, and the possible cleavage site in *apr-D2* signaling peptide region was between the 23 and 24 amino acid groups. The first 24 amino acids in the *apr-D2* gene are signaling peptide regions. But the signal sequence of *Apr-D2* gene does not have the characteristics of typical signal peptides. Rather, it is only a signal motif. When comparing the sequence to that of other homologous proteins, this signal peptide is as long as that of *Apr51* and is longer than that of *AprE2* from *B. subtilis* CH3-5 by one amino acid (Kim *et al*. 2009).

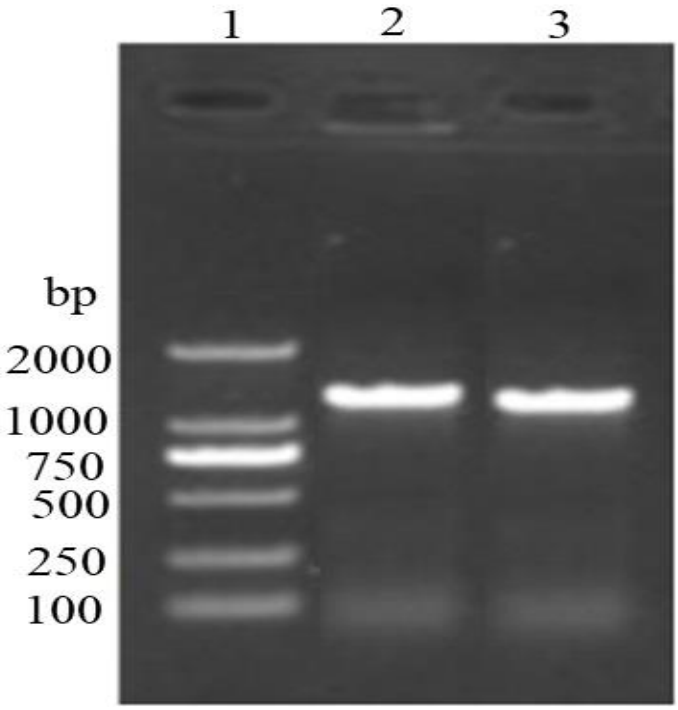

Fig. 1. The amplified product of *apr-D2* gene. Lane 1, DL2000 Markers; lane 2 and lane 3, PCR product of *apr-D2* gene

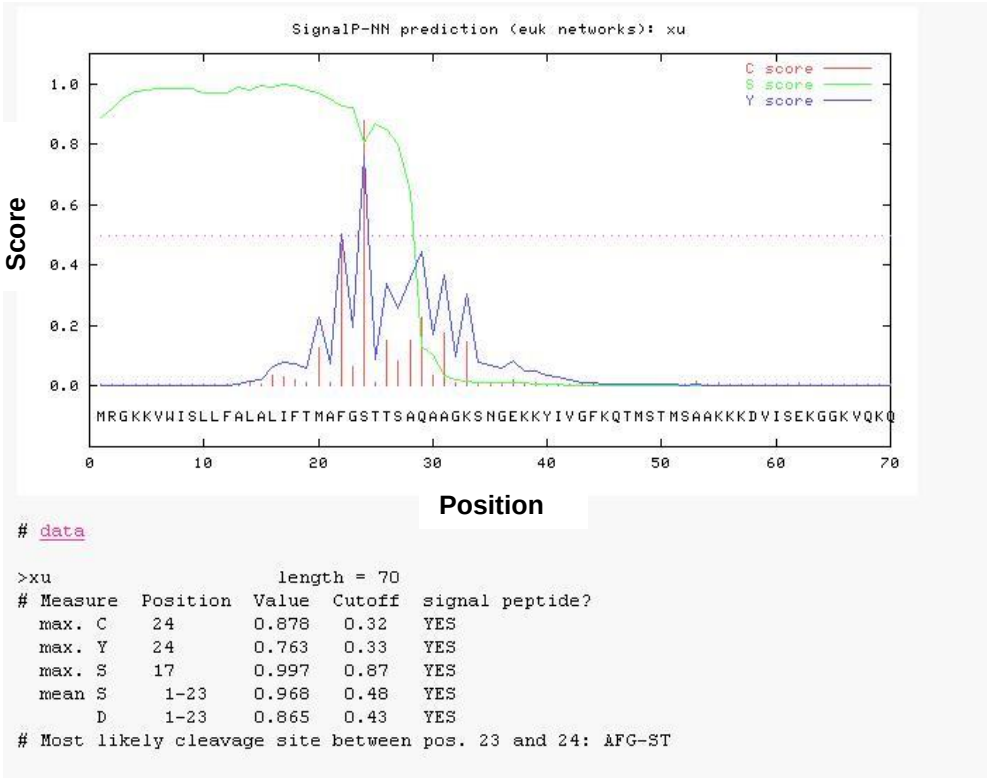

Fig. 2. Deduced signal peptide by signalp 4.0

Expression and Detection of *aprD2* Extracellular Proteolytic Activity

The expressed protein was analyzed by SDS-PAGE. The culture supernatant, intracellular soluble content, and inclusion body extracts from *E. coli* BL21(DE3) harboring plasmids pET32a-*apr-D2* all presented a strong band with MW of 60.4 kDa (Fig. 3), due to the presence of the N-terminal fusion peptide (Hu *et al*. 2013). In contrast, the cell extracts from the uninduced strain and empty expression vector strain did not show the corresponding bands. Furthermore, the culture supernatant from *E. coli* BL21(DE3) harboring plasmids pET32a-*apr-D2* displayed a hydrolyzed zone on a milk plate (skim milk 1%), while the control cannot generate the hydrolyzed zone (Fig. 4). Thus, the *aprD2* protease was successfully expressed in *E. coli* using expression vector pET-32a with signal peptide and secreted into the culture medium in a functional active form.

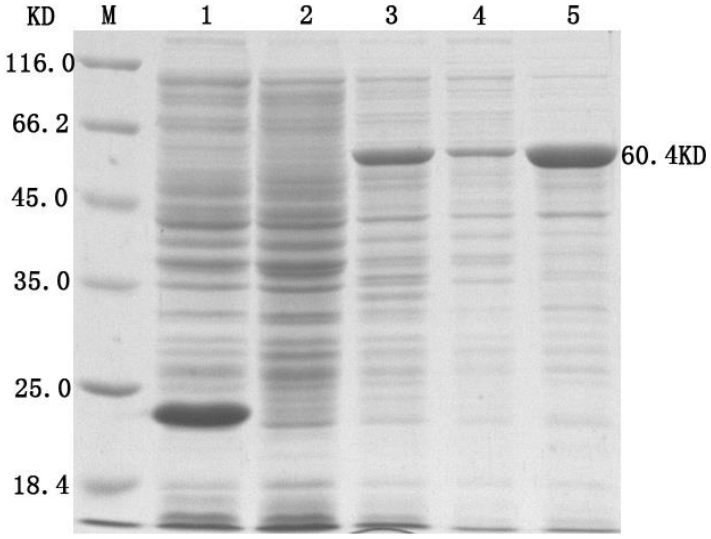

Fig. 3. SDS-PAGE analysis of expression plasmid. Lane M protein MW markers, lane 1 IPTG induced *E. coli* pET-32a, lane 2 uninduced *E. coli* harboring pET32a-*aprD2*, lane 3 intracellular soluble content of IPTG induced *E. coli* harboring pET32a-*aprD2*, lane 4 inclusion body of IPTG induced *E. coli* harboring pET32a-*aprD2*, lane 5 culture supernatant of IPTG induced *E. coli* harboring pET32a-*aprD2*.

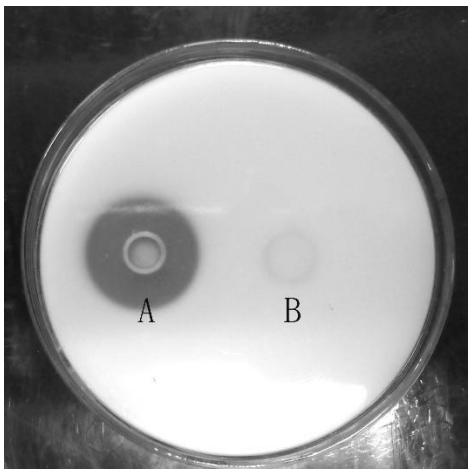

Fig. 4. The hydrolyzed zone of *aprD2* protease on a milk plate. (A) The culture supernatant from *E. coli* BL21(DE3) harboring plasmids pET32a-*aprD2*. (B) The culture supernatant from *E. coli* BL21(DE3) harboring plasmid pET-32a.

The *apr-D2* signal peptide could assist the secretion of the recombinant enzyme in *E. coli*. The signal peptide from *Bacillus* was also successfully used for expression and secretion of a-amylase and nattokinase in *E. coli* (Niu *et al*. 2006), which agrees with the present result that the signal peptide from *B. subtilis* has the function of helping secretion of heterologous protein in *E. coli*. There are several advantages to a system that releases proteins into the culture medium in a regulated manner (Jeong *et al*. 2000). Purification of the protein of interest was simplified and expressed protein was high activity because of a larger space for accumulation of the protein (Bindal *et al*. 2018).

Optimization of the Secretory Expression Condition of the *aprD2* Protease

Considering the effects of temperature on the growth of bacteria and secretory expression level, 27 °C was selected as the induction temperature after 37 °C culture until the cell density of OD_{600} reached 0.8 (Fig. 5A).

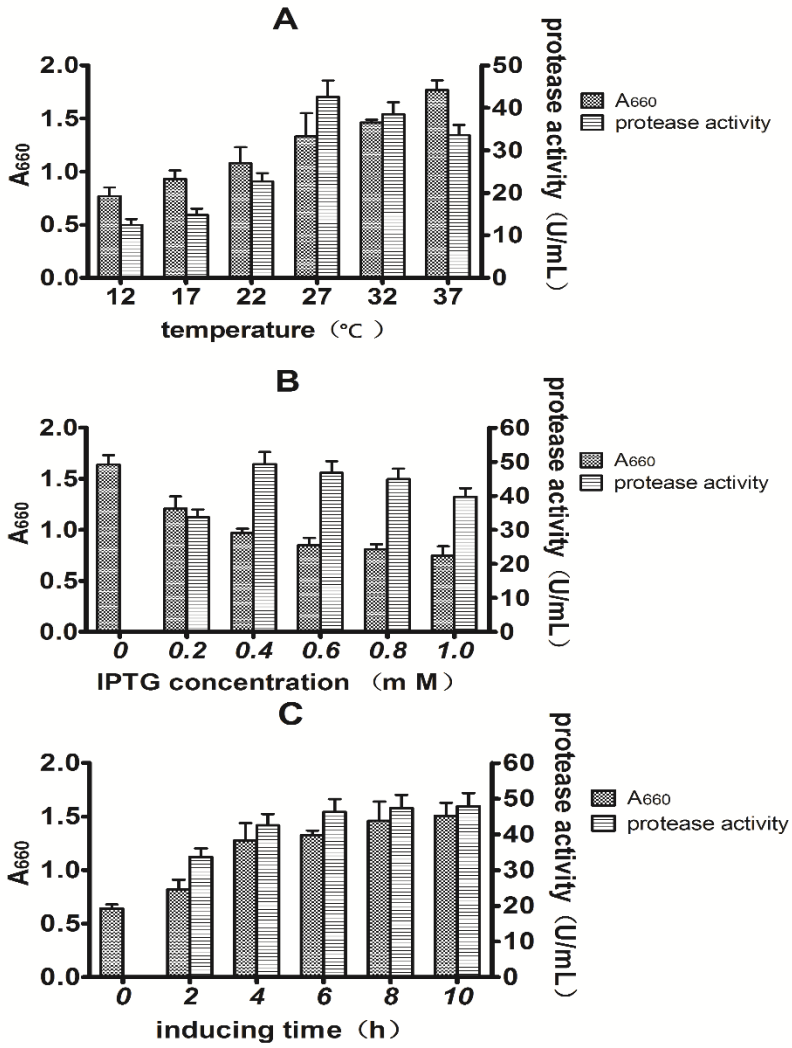

Fig. 5. Effects of inducing conditions on the cell growth and the secretory expression of active recombinant pET32a-*aprD2* in *E. coli* BL21(DE3). (A) Inducing temperature. (B) IPTG concentration. (C) Inducing time. The experiment was repeated three times; Y-error bars show SEM of triplicate experiments.

The yield of the recombinant active protease in the culture of *E. coli* is related to the temperature. With increasing temperature, the cell growth increased as well, whereas the protease activity in the supernatant culture decreased. However, at a relatively low temperature, a higher yield of active protease was detected. A moderate decrease of culture temperature could improve the secretory expression of recombinant proteins in *E. coli*, as lower temperature could help propeptides to fold properly (Ni *et al*. 2009). Secretion efficiency depends on the structures of fusion proteins, which change with temperature. High secretion efficiency of nattokinase was also obtained in *E. coli* under low temperature (Cai *et al*. 2016). Furthermore, the concentration of IPTG had no significant effect on the recombinant protein expression (Fig. 5B). Due to the toxic effects of high concentrations of IPTG on bacteria, 0.4 mmol/L was selected as the concentration of IPTG. The screening results of the expression conditions revealed that the induction time had no obvious effect on the expression levels of the recombinant fusion protein, pET32a-*aprD2*. However, the secretory expression level of pET32a-*aprD2* was relatively high after 4 h of induction (Fig. 5C). The optimal induce time of pET32a-*aprD2* in *E. coli* BL21(DE3) is much shorter than that of the recombinant F1 protease expressed by pTrcHis-TOPO in *E. coli* XL1-Blue with 24h induction (Fu *et al*. 2003). The results indicated that the strategy in this work was a potential way to produce alkaline protease of *B. subtilis* D2 for its simple cultural medium, short growth cycle, and the easy purification technique.

Determination of Proteolytic Properties

Effects of temperature and pH on protease activity and stability of aprD2

The *aprD2* protease was active over a broad range of temperatures from 30 °C to 80 °C with the optimal activity at 50 °C (Fig. 6A). It was stable up to 60 °C, retaining above 80% and 50% activity after incubation for 40 and 120 min, respectively (Fig.6B). The enzyme was active in the pH range of 4 to 13, with optimum activity at pH 10.5 (Fig. 6C), which was quite different from that of *AprE51* (pH 6.0) (Kim *et al*. 2009), although *apr-D2* shows a high homology. The enzyme activity was stable over a broad range of pH from 6 to 13, and it retained more than 50% activity after 12 h of incubation (Fig. 6D). These results suggested that the *aprD2* was a thermostable alkaline protease and could be used in various industries. The *aprD2* protease displayed superior performance at high temperatures and across a wide range of pH. Therefore, these properties made *aprD2* potentially useful for industrial bioremediation processes, including the forage industry (Peng *et al*. 2004).

Effects of Metal Ions, Inhibitors, Surfactant, and Oxidant on Enzyme Activity

The effects of various metal ions on the *aprD2* protease activity are summarized in Table 1. Ca^{2+}, Mg^{2+}, and Ba^{2+} increased the enzyme activities to 147.4%, 113.4%, and 121.3% of control, respectively. The enzyme retained over 90% of its activity in the presence of Mn^{2+} and Pb^{2+} at a concentration of 10 mM. Fe^{2+} did not significantly affect the activity of the enzyme, whereas Ag+ had a strong inhibitory effect. It has been reported that Ca^{2+} can protect stability for many enzymes, especially for protecting alkali-thermostable protease, because it can further promote the tertiary structure of the enzyme (Pan *et al*. 2004). The subtilisin family has two calcium-binding sites of Ca A and Ca B sites. Thus, Ca^{2+} was an important factor for the activity and thermal resistance of the protease. In this study, Ca^{2+} had certain activation effect on protease activity of pET32a-*aprD2*, which was in accordance with the structural features of the subtilisin family (Jeong *et al*. 2015a).

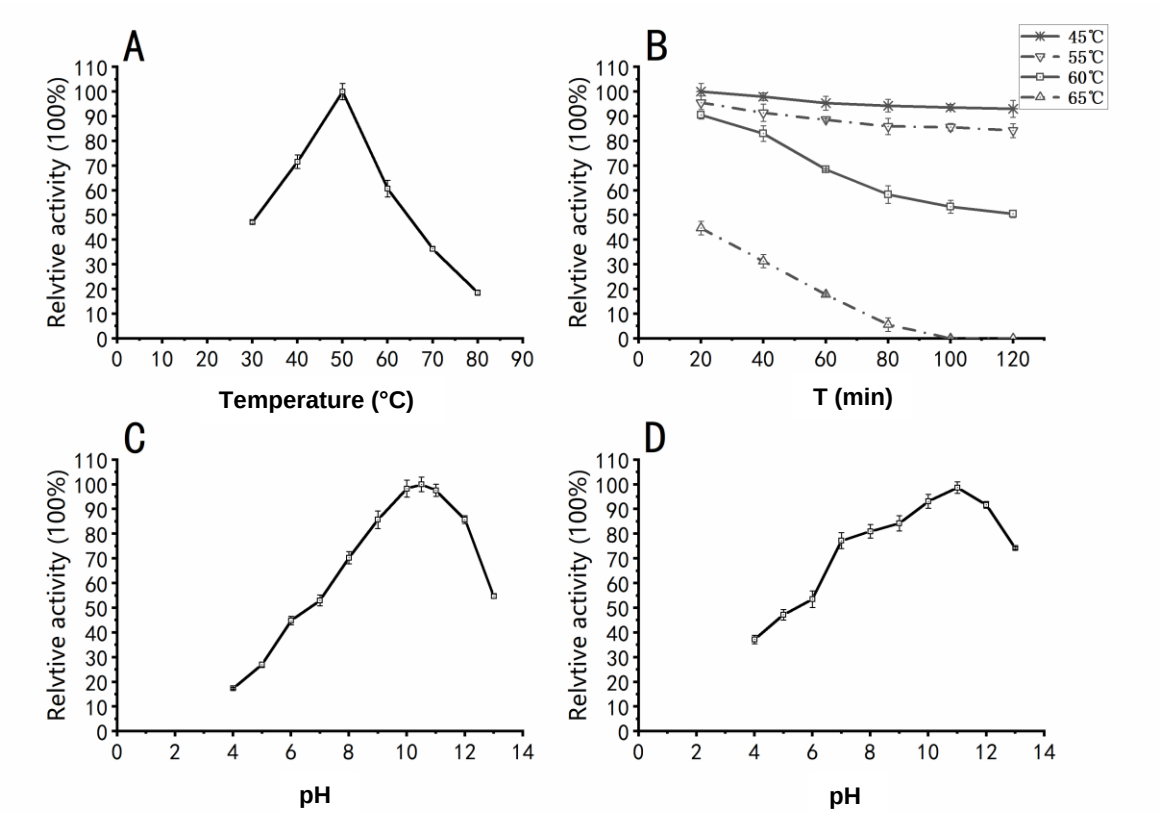

Fig. 6. Effects of temperature, pH, and NaCl on the activity and stability of the *aprD2*. (A) Effects of temperature on enzyme activity. (B) Stability of enzymes after incubation at 45 °C, 55 °C, 60 °C, and 65 °C for 0 to 120 min. (C) Effects of pH on enzyme activity. (D) pH stability of enzymes after incubation at pH 4-13 for 12 h. The experiment was repeated three times. Y-error bars show SEM of triplicate.

Table 1. Effect of Metal Salts on Enzyme Activity

Metal Salts	Concentration (mM)	Relative Activity (%)
Control	0	100.0± 4.42
Ag^{+}	10	38.23± 3.21
Mg^{2+}	10	113.45± 2.70
Mn^{2+}	10	94.56± 3.81
Pb^{2+}	10	90.23± 4.05
Zn^{2+}	10	84.78± 2.63
Ba^{2+}	10	121.29± 3.53
Cu^{2+}	10	72.46± 2.94
Fe^{2+}	10	103.74± 3.22
Ca^{2+}	10	147.45± 4.12

All values are the mean ± SD (n = 3).

The effects of various inhibitors, surfactants, and oxidants on enzyme stability of pET32a-*aprD2* are shown in Table 2. The purified enzyme was not affected by 10 mM EDTA and EGTA, which suggested that metal ions are not essential for the enzyme activity. SBTI, ME, and urea showed slight effect on the protease, while GnHCL and DTT inhibited protease activity by 47.4% and 58.4%, respectively. The enzymatic activity was very sensitive to PMSF, one of the typical serine protease inhibitors, which inhibited 92.7%

of its activity at a concentration of 5 mM, suggesting that *apr-D2* may belong to the serine proteases group (Pene *et al*. 2004). Furthermore, the *aprD2* was remarkably stable in the presence of ionic surfactants (SDS), nonionic detergents (Tween 80), and oxidants (H_2O_2), as it retained 85.6%, 87.0%, and 85.6% of its starting activity upon exposure to these chemical reagents, respectively. These characteristics of the recombinant protease showed that it could potentially be used in the feed processing industry and leather industry (Li and Yu 2012; Xin *et al*. 2015).

Table 2. Effect of Inhibitors, Surfactant, and Oxidant on Enzyme Activity

Chemical Reagents	Concentration	Relative Activity (%)
Control	0	100.00 ± 2.78
PMSF	5 mM	7.31 ± 1.36
GnHCl	1 M	52.56 ± 2.98
DTT	5 mM	41.63 ± 2.77
SBT1	3%[a]	103. 32 ± 3.47
ME	10 mM	98.66 ± 2.89
Urea	1 M	94.34 ± 3.36
EDTA	10 mM	105.22 ± 2.05
EGTA	10 mM	96.54 ± 2.90
H_2O_2	1%[b]	85.65 ± 2.35
Tween80	1%[a]	91.52 ± 3.73
SDS	0.1%[a]	87.00 ± 4.11

a: w/v; b: v/v, all values are the mean ± SD (n = 3).

Sequence Analysis and Molecular Modeling

As can be seen in Fig. 7, three catalytic residues (D32, H64, and S221) are conserved in two proteases.

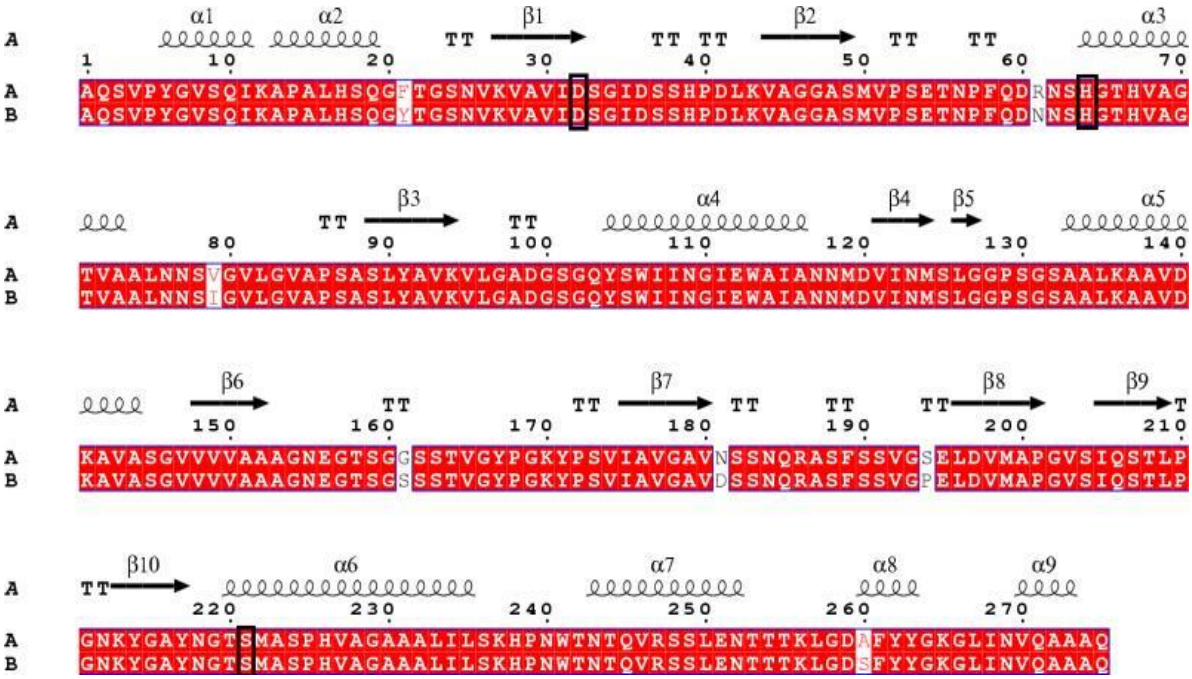

Fig. 7. Multiple sequence alignment by ESPript of A: *apr-D2* and the template used for the construction of the 3D model B: a SUBTILISIN BPN' (PDB_ID: 2st1)

In addition, these catalytic residues have one or more conserved glycine residues as neighbors. It has been previously suggested that glycine residues provide flexibility necessary for enzyme active sites (Yan *et al.* 1997).

The three-dimensional structure of *apr-D2* was displayed by comparative modeling using the defined structure of the subtilisin NOVO BPN´ of *B.* subtilis (PDB ID: 2st1) as respective template (Fig. 8). 3D domain swapping is a protein structural phenomenon that evolved as a mechanism for oligomeric assembly. Protein 3D structure prediction based on sequence alignment (Chou 2014) can provide useful information and insights for analyzing various properties of protein structure and function.

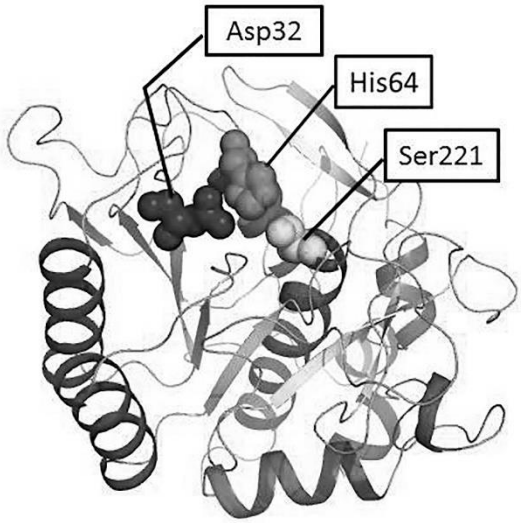

Fig. 8. Three-dimensional model of *apr-D2* and the respective active site visualized in the PyMOL software tool

CONCLUSIONS

1. The *apr-D2* gene was amplified, and the sequence comparisons showed a 99% similarity to the sequences of other alkaline protease genes.
2. The *aprD2* protease was induced to express and secreted into the culture medium in a functional active form. The optimum pH and temperature for partially purified *aprD2* activity were 10.5 and 55 degrees, respectively. It was stable in the pH range from 5.0 to 13.0 and had the ability to degrade milk.
3. The *aprD2* protease activity were increased by Ca^{2+}, Mg^{2+}, and Ba^{2+} The enzyme retained over 90% of its activity in the presence of Mn^{2+} and Pb^{2+} at a concentration of 10 mM. Fe^{2+} did not significantly affect the activity of the enzyme, whereas Ag^{+} had a strong inhibitory effect. It was not affected by 10 mM EDTA and EGTA, while GnHCL and DTT inhibited protease activity by 47.4% and 58.4%, respectively. PMSF inhibited 92.7% of its activity at a concentration of 5 mM. But it was remarkably stable under ionic (SDS), nonionic detergents (Tween 80), and oxidants (H_2O_2).
4. The three-dimensional structure of *apr-D2* was displayed and the active site residues of mature protein are Asp-32, His-64, and Ser-221.

ACKNOWLEDGEMENTS

This study was supported by a grant from the key project of the National Spark Program (No. 2014GA710002) and the Innovative Project of Anhui Academy of Agricultural Sciences (No. 18c1226).

REFERENCES CITED

Adrio, J. L., and Demain, A. L. (2014). "Microbial enzymes: Tools for biotechnological processes," *Biomolecules* 4, 117-139. DOI: 10.3390/biom4010117

Bindal, S., Dagar, V. K., Saini, M., Khasa, Y. P., and Gupta, R. (2018). "High level extracellular production of recombinant γ-glutamyl transpeptidase from *Bacillus licheniformis* in *Escherichia coli* fed-batch culture," *Enzyme Microb. Technol.* 116, 23-32. DOI: 10.1016/j.enzmictec.2018.05.004

Cai, D., Wei, X., Qiu, Y., Chen, Y., Chen, J., Wen, Z., and Chen, S. (2016). "High-level expression of nattokinase in *Bacillus licheniformis* by manipulating signal peptide and signal peptidase," *J. Appl. Microbiol.* 121(3), 704-712. DOI:10.1111/jam.13175

Chandrasekharaiah, M., Thulasi, A., Vijayarani, K., Kumar, D. P., Santosh, S. S., Palanivel, C., Jose, V. L., and Sampath, K. T. (2012). "Expression and biochemical characterization of two novel feruloyl esterases derived from fecal samples of *Rusa unicolor* and *Equus burchelli*," *Gene* 500, 134-139. DOI: 10.1016/j.gene.2012.03.027

Chou, K. C. (2004). "Structural bioinformatics and its impact to biomedical science," *Curr. Med. Chem.* 11(16), 2105-2134.

Deng, A., Wu, J., Zhang, G., and Wen, T. (2011). "Molecular and structural characterization of a surfactant-stable high-alkaline protease AprB with a novel structural feature unique to subtilisin family," *Biochimie* 93, 783-791. DOI: 10.1016/j.biochi.2011.01.011

Deng, A., Wu, J., Zhang, Y., Zhang, G., and Wen, T. (2010). "Purification and characterization of a surfactant-stable high alkaline protease from *Bacillus* sp. B001," *Bioresource Technol.* 101, 7100-7106. DOI: 10.1016/j.biortech.2010.03.130

Deng, A., Zhang, G., Shi, N., Wu, J., Lu, F., and Wen, T. (2014). "Secretory expression, functional characterization, and molecular genetic analysis of novel halo-solvent-tolerant protease from *Bacillus gibsonii*," *J. Microbiol. Biotechnol.* 24, 197-208.

Fu, X., Inouye, M., and Shinde, U. (2000). "Folding pathway mediated by an intramolecular chaperone. The inhibitory and chaperone functions of the subtilisin propeptide are not obligatorily linked," *J. Biol. Chem.* 275, 16871-16878.

Fu, Z., Hamid, S. B., Razak, C. N., Basri, M., Salleh, A. B., and Rahman, R. N. (2003). "Secretory expression in *Escherichia coli* and single-step purificationof a heat-stable alkaline protease," *Protein Expr. Purif.* 28, 63-68.

Guex, N., and Peitsch, M, C. (1997). "Swiss-Model and the Swiss-Pdb Viewer: An environment for comparative protein modeling," *Electrophoresis* 18, 2714-2723. DOI: 10.1002/elps.1150181505

Gupta, R., Beg, Q. K., Khan, S., and Chauhan, B. (2002). "An overview on fermentation, downstream processing and properties of microbial alkaline proteases," *Appl. Microbiol. Biotechnol.* 60, 381-395. DOI: 10.1007/s00253-002-1142-1

Han, X., Shiwa, Y., Itoh, M., Suzuki, T., Yoshikawa, H., Nakagawa, T., and Nagano, H., (2013). "Molecular cloning and sequence analysis of an extracellular protease from

four *Bacillus subtilis* strains," *Biosci. Biotech. Biochem.* 77, 870-873. DOI: 10.1271/bbb.120920

Heo, K., Cho, K. M., Lee, C. K., Kim, G. M., Shin, J. H., Kim, J. S., and Kim, J. H., (2013). "Characterization of a fibrinolytic enzyme secreted by *Bacillus amyloliquefaciens* CB1 and its gene cloning," *J. Microbiol. Biotechnol*. 23, 974-983.

Hu, H., He, J., Yu, B., Zheng, P., Huang, Z., Mao, X., Yu, J., Han, G., and Chen, D., (2013). "Expression of a *keratinase (kerA)* gene from *Bacillus licheniformis* in *Escherichia coli* and characterization of the recombinant enzymes," *Biotechnol. Lett.* 35, 239-244. DOI: 10.1007/s10529-012-1064-7.

Ibrahim, K. S., Muniyandi, J., and Pandian, S. K. (2011). "Molecular cloning, sequence and structural analysis of dehairing Mn^{2+} dependent alkaline serine protease (MASPT) of *Bacillus pumilus* TMS55," *Protein Pept Lett*. 18(10), 1035-1041.

Jacobs, M., Eliasson, M., Uhlén, M., and Flock, J. I. (1985). "Cloning, sequencing and expression of *subtilisin Carlsberg* from *Bacillus licheniformis*," *Nucleic Acids Res.* 13: 8913-8926.

Jaouadi, N. Z., Jaouadi, B., Aghajari, N., and Bejar, S. (2012). "The overexpression of the SAPB of *Bacillus pumilus* CBS and mutated sapB-L31I/T33S/N99Y alkaline proteases in *Bacillus subtilis* DB430: New attractive properties for the mutant enzyme," *Bioresour. Technol*. 105,142-151. DOI:10.1016/j.biortech.2011.11.115

Jeong, S. J., Park, J. Y., Lee, J. Y., Lee, K. W., Cho, K. M., Kim, G. M., Shin, J. H., Kim, J. S., and Kim, J. H. (2015a). "Improvement of fibrinolytic activity of *Bacillus subtilis* 168 by integration of a fibrinolytic gene into the chromosome," *J. Microbiol. Biotechnol*. 25(11), 1863-1870. DOI: 10.4014/jmb.1505.05062

Jeong, S. J., Heo, K., Park, J. Y., Lee, K. W., Park, J. Y., Joo, S. H., and Kim, J. H. (2015b). "Characterization of AprE176, a fibrinolytic enzyme from *Bacillus subtilis* HK176," *J. Microbiol*. *Biotechnol*. 25, 89-97.

Johansson-Åkhe, I., Mirabello, C., and Wallner, B. (2019). "Predicting protein-peptide interaction sites using distant protein complexes as structural templates," *Sci. Rep.* 9(1), 4267. DOI: 10.1038/s41598-019-38498-7

Joo, H. S., and Choi, J. W. (2012). "Purification and characterization of a novel alkaline protease from *Bacillus horikoshii*," *J. Microbiol. Biotechnol*. 22, 58-68.

Jorgensen, P. L., Tangney, M., Pedersen, P. E., Hastrup, S., Diderichsen, B., and Jorgensen, S. T. (2000). "Cloning and sequencing of an alkaline protease gene from *Bacillus lentus* and amplification of the gene on the *B. lentus chromosome* by an improved technique," *Appl. Environ. Microbiol.* 66, 825-827.

Kim, G. M., Lee, A. R., Lee, K. W., Park, J. Y., Chun, J., Cha, J., Song, Y. S., and Kim, J. H. (2009). "Characterization of a 27 kDa fibrinolytic enzyme from *Bacillus amyloliquefaciens* CH51 isolated from Cheonggukjang," *J. Microbiol. Biotechnol*. 19, 997-1004.

Kim, M. R., Choeng, Y. H., Chi, W. J., Kang, D. K., and Hong, S. K. (2010). "Heterologous production of *Streptokinase* in secretory form in *Streptomyces lividans* and in nonsecretory form in *Escherichia coli*," *J. Microbiol. Biotechnol.* 20, 132-137.

Lee, M. A., and Liu, Y. (2000). "Sequencing and characterization of a novel serine metalloprotease from *Burkholderia pseudomallei*," *FEMS Microbiol. Lett.* 192, 67-72. DOI: 10.1111/j.1574-6968.2000.tb09360.x

Li, X., and Yu, H.Y. (2012). "Purification and characterization of novel organic-solvent-tolerant beta-amylase and serine protease from a newly isolated *Salimicrobium*

halophilum strain LY20," *FEMS Microbiol. Lett.* 329(2), 204-211. DOI: 10.1111/j.1574-6968.2012.02522.x

Ni, Y., and Chen, R. (2009). "Extracellular recombinant protein production from *Escherichia coli*," *Biotechnol. Lett*. 31(11), 1661-1670. DOI: 10.1007/s10529-009-0077-3

Niu, D. D., Xu, M., Ma, J. S., and Wang, Z. X. (2006). "Cloning of the gene encoding a thermostable alpha-amylase from *Bacillus licheniformis* CICIM B0204 and functional identification of its promoter," *Wei Sheng Wu Xue Bao* 46(4), 576-80.

Pan, J., Huang, Q., and Zhang, Y. (2004). "Gene cloning and expression of an alkaline serine protease with dehairing function from *Bacillus pumilus*," *Curr. Microbiol*. 49(3), 165-9. DOI: 10.1007/s00284-004-4305-8

Peng, Y., Yang, X. J., Xiao, L., and Zhang, Y. Z. (2004). "Cloning and expression of a fibrinolytic enzyme (*subtilisin DFE*) gene from *Bacillus amyloliquefaciens DC-4* in *Bacillus subtilis*," *Res. Microbiol.* 155, 167-173. DOI:10.1016/j.resmic.2003.10.004

Sareen, R., and Mishra, P. (2008). "Purification and characterization of organic solvent stable protease from *Bacillus licheniformis* RSP-09-37," *Appl. Microbiol. Biotechnol.* 79, 399-405. DOI: 10.1007/s00253-008-1429-y

Xin, Y., Sun, Z., Chen, Q., Wang, J., Wang, Y., Luogong, L., Li, S., Dong, W., Cui, Z., and Huang, Y. (2015). "Purification and characterization of a novel extracellular thermostable alkaline protease from *Streptomyces* sp. M30," *J. Microbiol. Biotechnol.* 25(11), 1944-1953. DOI: 10.4014/jmb.1507.07017

Xu, Y. Y., Li, L. M., Xu, F. z., Ding, X. L., and Qian, K. (2011). "Screening, identification and application of a new *Bacillus* producing alkaline protease," *Acta Laser Biology Sinca*. 20(6), 830-838. DOI: 10.3969/j.issn.1007-7146.2011.06.023

Yan, B. X., and Sun, Y. Q. (1997). "Glycine residues provide flexibility for enzyme active sites," *J. Biol. Chem.* 272, 3190-3194. DOI:10.1074/jbc.272.6.3190

Article submitted: March 27, 2019; Peer review completed: May 4, 2019; Revised version received: May 11, 2019; Accepted: May 15, 2019; Published: May 21, 2019.
DOI: 10.15376/biores.14.3.5301-5315