Malaysian Journal of Microbiology, Vol 20(1) 2024, pp. 85-93
DOI: http://dx.doi.org/10.21161/mjm.230307

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Production, purification and characterization of novel protease from *Bacillus amyloliquefaciens* D19 isolated in Vietnam

Tan Viet Pham[1]*, Hanh Thi Dieu Nguyen[1], Thi Luyen Bui[2] and Ngoc An Nguyen[1]

[1]Biotechnology Department, Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, No.12 Nguyen Van Bao Street, Go Vap District, Ho Chi Minh City, Vietnam.
[2]Tan Tien High School, Tan Tien Ward, Dong Phu District, Binh Phuoc Province, Vietnam.
Email: phamtanviet@iuh.edu.vn

Received 27 May 2023; Received in revised form 28 November 2023; Accepted 6 December 2023

ABSTRACT

Aims: Microorganisms play a vital role in the breakdown of natural organic compounds and are valuable objects for worldwide enzyme production. The aim of this study was to identify favorable production conditions for *Bacillus amyloliquefaciens* D19 protease, followed by the purification and chemical characterization of this novel enzyme to assess its potential applications in various fields.
Methodology and results: In this study, favorable conditions of protease production from *B. amyloliquefaciens* D19 were determined using a medium containing soluble starch (1.5%), earthworm extract (1.0%), yeast extract (0.5%), NaCl (1.0%), at pH 7.0-8.0, 37 °C for 36 h with 150 rpm shaking condition. The protease was purified and had a molecular weight of about 23 kDa. The optimum condition for casein hydrolysis was at 40 °C and pH 6.5-7.0 in the presence of 1.0 mM Na^+ or 5.0 mM Zn^{2+}. The enzymatic activity was maintained at 75-100% at 30-50 °C and in pH 6.0-10.0. The values of V_{max} and K_M were also determined as 1547 U/mg and 6.33 mg/mL, respectively.
Conclusion, significance and impact of study: The identified optimal conditions will serve as the foundation for the production of the 23 kDa *B. amyloliquefaciens* D19 protease, one of the smallest proteases within the *Bacillus* genus. Moreover, its notable heat resistance, broad pH tolerance, high substrate catalysis and moderate substrate binding affinity make this enzyme a promising candidate for various applications in the food-feed and brewing industries.

Keywords: Bacillus amyloliquefaciens, biochemical characterization, enzyme production, enzyme purification, protease

INTRODUCTION

The development of industry worldwide has led to a continuous increase in the use of industrial chemicals. As a result, environmentally friendly products have been gradually replacing harmful chemicals to minimize their negative environmental impact and improve people's quality of life (Arunachalam and Saritha, 2009; Hamza, 2017). Proteases, one of the three largest groups of industrial enzymes, account for about 60% of total commercial enzymes worldwide and are commonly used in various fields such as textiles, detergents, leather, feed, waste, and others (Gupta *et al.*, 2002; Raveendran *et al.*, 2018). The global protease market is expected to reach USD 3 billion by the end of 2024 (Sawant and Nagendran, 2014).

Protease is an enzyme that hydrolyses proteins into amino acids by breaking peptide bonds (Rao *et al.*, 1998). Extracellular proteases have strong proteolytic abilities and are used in many industrial fields (Demain and Adrio, 2008). Protease can be obtained from many sources but mainly from microorganisms. Based on chemical and structural properties, proteases can be divided into serine protease, cysteine protease, aspartic protease, metalloprotease or acidic protease, neutral protease, and alkaline protease (Hamza and Woldesenbet, 2017). Alkaline proteases are mainly obtained from bacteria and are active at alkaline pH (Gupta *et al.*, 2002).

Extracellular proteases, especially those from the genus *Bacillus*, have been widely commercialized (Gupta *et al.*, 1999). Most proteases obtained from *Bacillus* spp. are active in neutral to alkaline buffers. Their molecular mass is also quite diverse, ranging from 18-35 kDa, but can be up to 90 kDa in some cases (Yamagata *et al.*, 1995).

Surfactants and oxidizing agents strongly influence alkaline proteases. However, *Bacillus* strains can biosynthesize proteases that can work stably under the influence of many detergents, which is very important for their application in the industry (Yang *et al.*, 2000; Çalık *et al.*, 2002). Culture medium has a significant influence on the biosynthesis of extracellular enzymes. The

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 85-93
DOI: http://dx.doi.org/10.21161/mjm.230307

components in this culture medium, such as carbon, nitrogen, inorganic salts and growth factors, not only affect the growth of *Bacillus* but also affect the synthesis of their organic compounds, including enzymes (Beg *et al.*, 2002; Nascimento and Martins, 2004). In this study, favorable culture conditions for a novel protease from *B. amyloliquefaciens* D19 were determined, and characterization of this purified enzyme has provided the basis for its potential application in specific industrial fields.

MATERIALS AND METHODS

Bacterial strain and culture condition

Bacillus amyloliquefaciens D19 strain from soil in Ho Chi Minh City and stored at the Microbiological Technology Laboratory, Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City at -80 °C. The strain was originally isolated in Luria-Bertani medium at room temperature and further identified based on macro and microscopic characterization in combination with 16S rRNA sequencing using universal primer pairs: 27F-AGAGTTTGATCCTGGCTCAG and 1492R-GGTTACCTTGTTACGACTT. *Bacillus amyloliquefaciens* D19 was also previously proven to produce high-activity cellulase and a high antagonistic effect against the dragon fruit fungal pathogen *Neoscytalidium dimidiatum* (Nguyen *et al.*, 2020; 2021). Inoculum culture of *B. amyloliquefaciens* D19 was routinely prepared in Luria-Bertani broth (LB broth, tryptone 10.0 g/L, yeast extract 5.0 g/L and NaCl 10.0 g/L, pH 7.0) supplemented with 10.0 g/L casein, on a rotary shaker (150 rpm), at 37 °C overnight for further experiments.

Effect of various carbon and nitrogen sources on protease production

The effect of carbon sources on protease production was conducted in a 250 mL flask containing 100 mL of basal medium (yeast extract 5.0 g/L, NaCl 10.0 g/L) supplemented with 10.0 g/L tryptone and 1.0% of inoculum culture. Each carbon source (1.0% w/v), including rice bran, corn powder, soluble starch, maltose, lactose, and glycerol, was then added at a concentration of 10.0 g/L. The initial pH was adjusted to 7.0. The medium flasks were inoculated on a rotary shaker at 150 rpm at 37 °C for 32 h. The culture medium was centrifuged at 6000 rpm for 20 min at 4 °C and the cell-free supernatant was used for protease activity assay. The suitable carbon source concentration was subsequently screened in the 0.0-4.0% range using the same cultural conditions.

The effect of nitrogen sources on protease production was conducted in the basal medium supplemented with 1.0% inoculum culture and a suitable concentration of selected carbon source. Each of the different nitrogen sources (1.0% w/v), including tryptone, urea, casein, soya powder, gelatin, peptone, earthworm extract, KNO_3, $NaNO_3$, NH_4Cl, was then added at a concentration of 10.0 g/L. The initial pH was adjusted to 7.0. The medium flasks were inoculated on a rotary shaker at 150 rpm at 37 °C for 32 h. Cell-free supernatant was used for protease activity assay. A suitable nitrogen source concentration was subsequently screened in the 0.0-4.0% range using the same cultural conditions.

Effect of initial pH and incubation time on protease production

The effect of initial pH on protease production was determined by modifying the pH of the medium with suitable carbon and nitrogen sources in a range of pH 3.0-9.0 using 1 N NaOH and 1 N HCl. The culture was grown on a rotary shaker at 150 rpm at 37 °C for 32 h. Cell-free supernatant was used for protease activity assay.

The effect of incubation time on protease production was determined using 1.0 mL of the cell-free culture supernatant from the medium with suitable carbon-nitrogen sources and initial pH. The culture was grown on a rotary shaker at 150 rpm at 37 °C for different incubation periods at 12, 24, 36, 48, 60 and 72 h. Cell-free supernatant was used for protease activity assay.

Purification of extracellular protease from *B. amyloliquefaciens* D19

Bacillus amyloliquefaciens D19 was cultured in defined favorable medium and growth conditions. Cell-free culture supernatant was used for protein precipitation by 70% $(NH_4)_2SO_4$ solution. The precipitated protein was collected by centrifuging at 13000 rpm at 4 °C for 15 min, then immediately dissolved in buffer A (10% glycerol, 50 mM Tris-HCl pH 7.0, 2% 2-mercaptoethanol). The protein solution was subjected to overnight dialysis with the 3 kDa cut-off membrane in buffer A at 4 °C.

The dialyzed protein solution was collected after centrifuging at 13000 rpm at 4 °C for 15 min and was further purified by ion exchange chromatography using a 5 mL HiTrap Q HP column (GE Healthcare). Buffer A (50 mM Tris-HCl pH 7.0, 10% glycerol, 2% 2-mercaptoethanol) was used as the running solution and buffer B (50 mM Tris-HCl pH 7.0, 10% glycerol, 2% 2-mercaptoethanol, 1.0 M NaCl) was used as an eluting solution. The FPLC system was kept at a 2 mL/min constant flow rate. Two milliliters of eluted protein solution were collected for each fraction.

Protease activity assay

Protease activity was measured using modified Anson's method using casein as a substrate (Lanoë and Dunnigan, 1978). One unit of protease activity was defined as the amount of enzyme required to liberate 1.0 g of tyrosine/min under specified experimental conditions (37 °C, pH 7.5).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 85-93
DOI: http://dx.doi.org/10.21161/mjm.230307

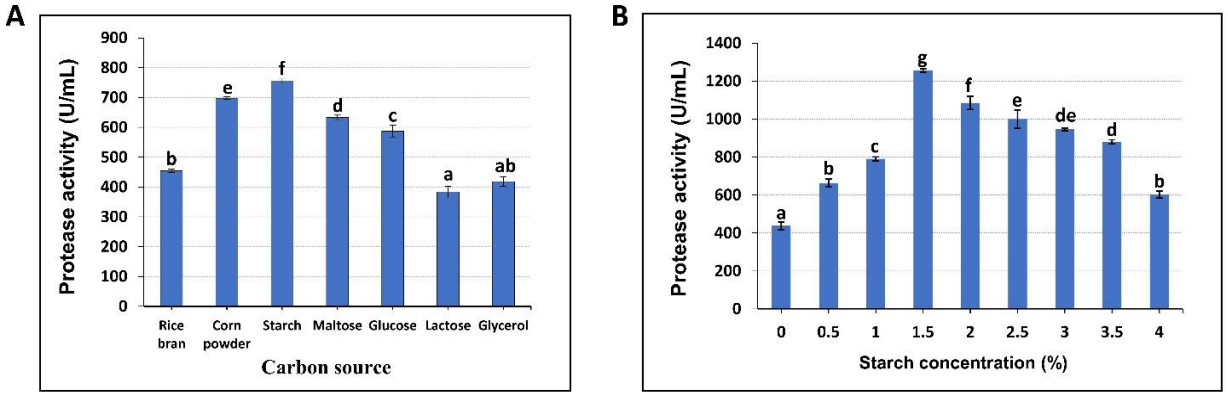

Figure 1: Effect of various carbon sources on protease production of *B. amyloliquefaciens* D19. (A) Protease activity of culture supernatant of various carbon sources; (B) Protease activity of culture supernatant of different starch concentrations.

Characterization of biochemical properties of extracellular protease from *B. amyloliquefaciens* D19

The optimum temperature for purified protease was examined from 25-70 °C and the optimum pH was examined from pH 4.0-10.0 by adjusting the temperature and pH of the reaction. Corresponding buffers used for enzymatic reaction include citrate buffer pH 3.0-6.0, phosphate buffer pH 6.0-8.0 and Tris-HCl buffer pH 8.0-10.0.

Thermal stability was determined by incubating a protease sample at 30-70 °C for 30 min and pH stability was determined by incubating a protease sample at pH 3.0-10.0 for 30 min before the enzymatic reaction. Residual activity was calculated in comparison with the non-treated protease sample (100%).

Effect of metal ion

The effect of various metal ions on the catalytic activity of purified protease was examined by adding either 10 mM of EDTA or 5 mM of different ions, including Fe^{3+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, K^{+} and Na^{+}, into the reaction. Enzymatic reaction without supplemented ion was used as a control. The reactions were conducted under optimal temperature and pH.

Enzyme kinetics

Enzyme kinetics was determined via K_M and V_{max} values using the Michalis-Menten equation to show the affinity between enzyme and substrate as well as the catalytic ability. The enzyme reaction was performed with a variation of casein concentration from 0.04-40.0 mg/mL. Enzyme activity was determined by modified Anson's method.

Statistics and data visualization

All experiments were done in triplicate and data were presented as mean of triplicate with standard deviations. Statistical analysis was done using Statgraphics Centurion 18 software (Statgraphics Technologies, Inc., The Plains, Virginia). The enzyme kinetic parameters were calculated using Prism 8.0.2 software (GraphPad Software Inc., San Diego, California, USA).

RESULTS

Effect of various carbon sources on protease production

The protease biosynthesis of *B. amyloliquefaciens* D19 in a medium containing 1.0% of different carbon sources is shown in Figure 1A.

The highest protease activity was observed in medium supplemented with soluble starch (756.17 U/mL), followed by corn powder (697.76 U/mL), maltose (633.68 U/mL), glucose (587.77 U/mL), rice bran (454.42 U/mL), glycerol (418.15 U/mL) and the lowest was with lactose (383.63 U/mL). Additionally, the medium containing 1.5% soluble starch displayed the highest protease activity (1255.39 U/mL), nearly 3-fold higher than when no starch was supplemented (437.72 U/mL) (Figure 1B). As a result, 1.5% soluble starch was selected for *B. amyloliquefaciens* D19 protease production.

Effect of various nitrogen sources on protease production

The effect of different nitrogen sources on protease biosynthesis of *B. amyloliquefaciens* D19 was examined in a culture medium containing 1.5% soluble starch and supplemented with 1.0% of various organic and inorganic nitrogen sources.

The results showed that protease activity in culture medium containing earthworm extract displayed the highest activity (1526.85 U/mL), followed by peptone (1441.16 U/mL), tryptone (1367.42 U/mL) and other nitrogen sources (Figure 2A). The amount of supplemented earthworm extract was also examined and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 85-93
DOI: http://dx.doi.org/10.21161/mjm.230307

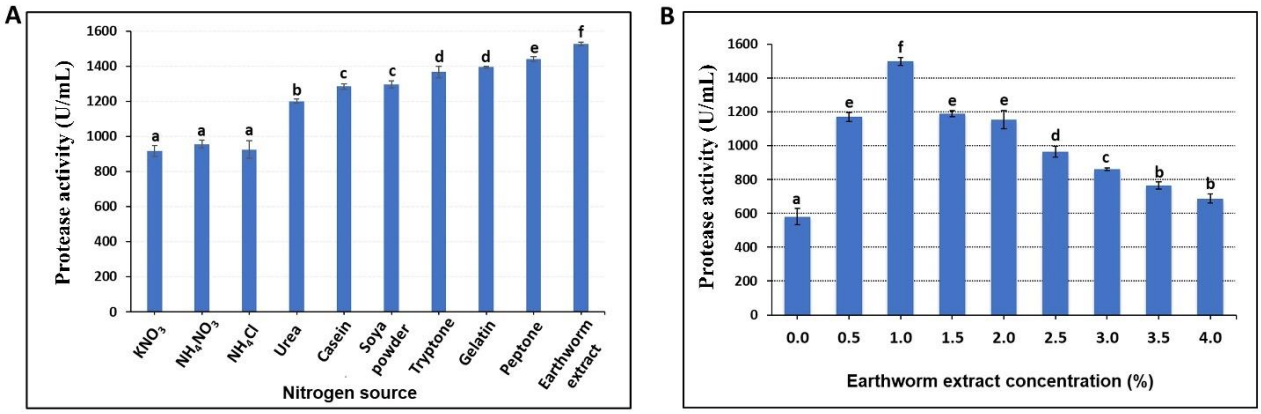

Figure 2: Effect of various nitrogen sources on protease production of *B. amyloliquefaciens* D19. (A) Protease activity of culture supernatant of various nitrogen sources; (B) Protease activity of culture supernatant of different earthworm extract concentrations.

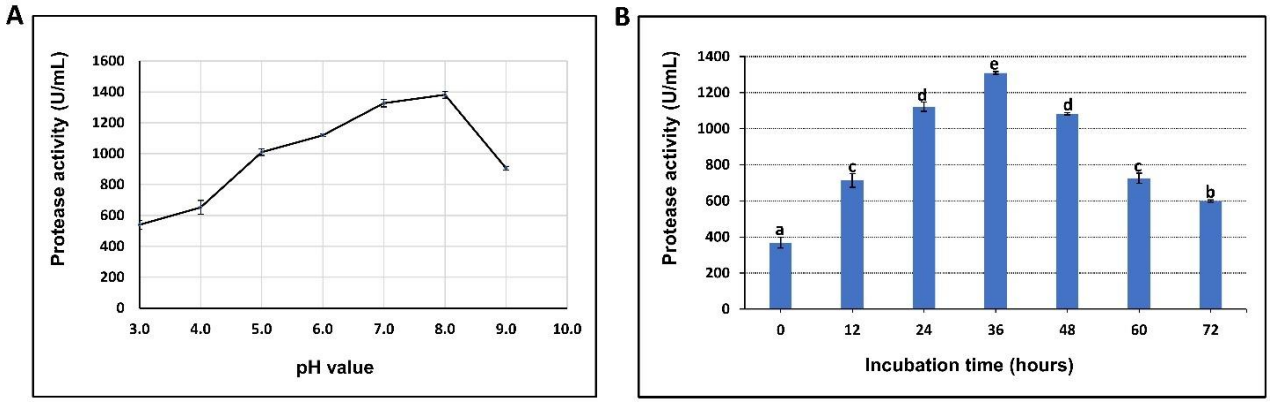

Figure 3: Effect of initial pH and incubation time on protease production of *B. amyloliquefaciens* D19**.** (A) Protease activity of culture supernatant of different initial pH; (B) Protease activity of culture supernatant after various incubation periods.

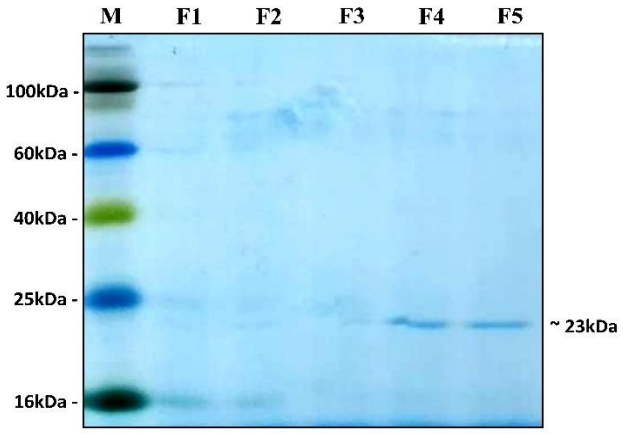

Figure 4: SDS-PAGE analysis of purified protease fractions after ion exchange chromatography. M: Protein standards; F1-F5: Protein fractions.

1.0% was the most suitable for extracellular protease production (Figure 2B).

Effect of pH and incubation time on protease production

The influence of initial pH on the protease activity of *B. amyloliquefaciens* D19 culture of seven different pH values was examined after 32 h of incubation. Protease activity reached the highest value at pH 8.0 and pH 7.0 (1381.52 U/mL and 1326.52 U/mL, respectively) while decreasing sharply at other higher and lower pH values (Figure 3A).

In addition, the protease activity of *B. amyloliquefaciens* D19 culture gradually increased and reached a maximum after 36 h of incubation (Figure 3B). Based on the obtained results, suitable culture conditions for *B. amyloliquefaciens* D19 extracellular protease biosynthesize could be defined as 36 h culture at 37 °C in

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 85-93
DOI: http://dx.doi.org/10.21161/mjm.230307

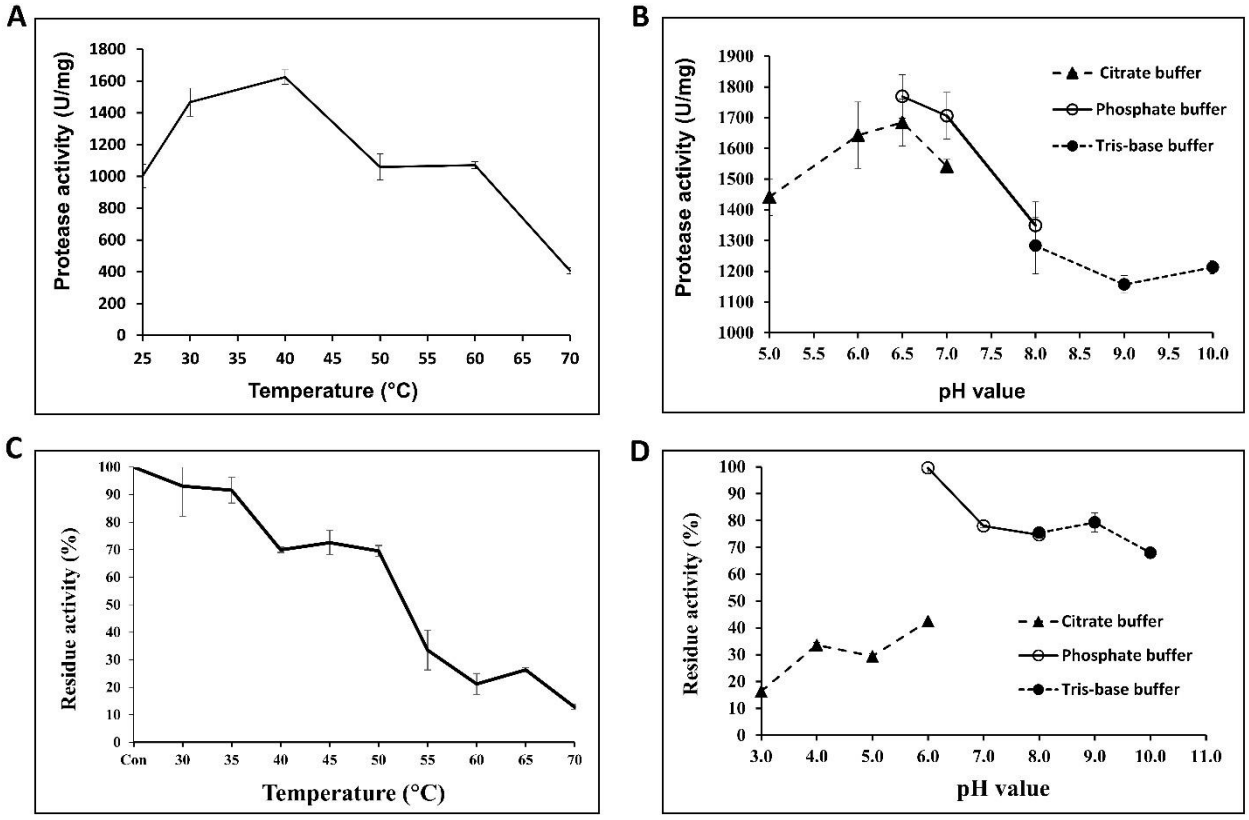

Figure 5: Effect of temperature (A) and pH value (B) on *B. amyloliquefaciens* D19 protease activity; and effect of temperature (C) and pH value (D) on enzyme stability.

basal medium containing 1.5% soluble starch, 1.0% earthworm extract, initial pH 7.0-8.0.

Purification of extracellular protease from *B. amyloliquefaciens* D19

In order to obtain pure protease fraction, *B. amyloliquefaciens* D19 was grown in defined medium and conditions and cell-free supernatant was collected for crude protein precipitation with ammonium sulphate followed by protease isolation using ion-exchange chromatography. Fractions of collected protein displaying protease activity were subsequently analyzed by SDS-PAGE (Figure 4).

After purification, the F4 and F5 enzyme fractions appeared to be pure, with one single band of approximately 23 kDa. These fractions displayed the highest protease activity compared to the others and were also used for further characterization experiments.

Effect of temperature and pH on activity and stability of *B. amyloliquefaciens* D19 protease

The effect of temperature on *B. amyloliquefaciens* D19 protease activity was studied at different reaction temperatures from 25-70 °C (Figure 5A). Protease activity was found to be highest at 30-40 °C (1438.93-1631.79 U/mg) and highly reduced at 70 °C (428.21 U/mg).

Despite the fact that it is reduced at 50-60 °C, enzymatic activity remains to be relatively high (>1000 U/mg). The optimum temperature (40 °C) was subsequently used to study the effect of different reaction buffers and pH on *B. amyloliquefaciens* D19 protease activity. The protease was shown to be active in all tested conditions (Figure 5B). The highest activity was observed at pH 6.5 in both citrate buffer and phosphate buffer, and the lowest was recorded at pH 9.0-10.0 in Tris-HCl buffer with approximately 75% remaining activity (1157.14-1213.10 U/mg).

Furthermore, the thermal stability of D19 protease at different temperatures was also investigated to provide a basis for preservation conditions and practical applications. Purified protease of *B. amyloliquefaciens* D19 was incubated at different temperatures (30-70 °C) for 30 min before being subjected to enzymatic reaction at 40 °C, pH 6.5. Relative activity was calculated in comparison with the control (without any pretreatment, 100% activity). Enzyme activity gradually decreased with elevated pretreated temperature (Figure 5C). Enzyme activity at 50 °C pretreatment was maintained at about 70%, sharply declined at 55 °C (33.52%) and dramatically reduced to only 12.8% at 70 °C.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 85-93
DOI: http://dx.doi.org/10.21161/mjm.230307

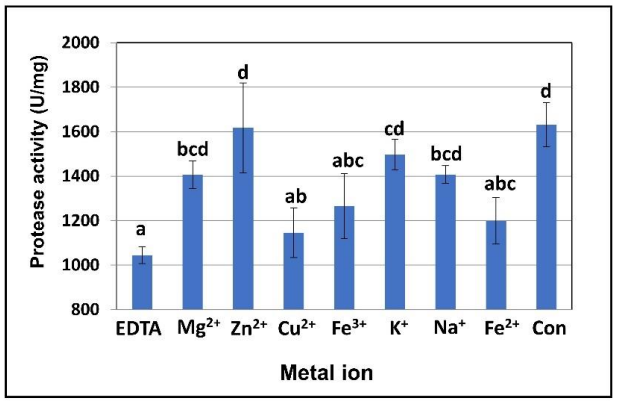

Figure 6: Effect of different metal ions on *B. amyloliquefaciens* D19 protease.

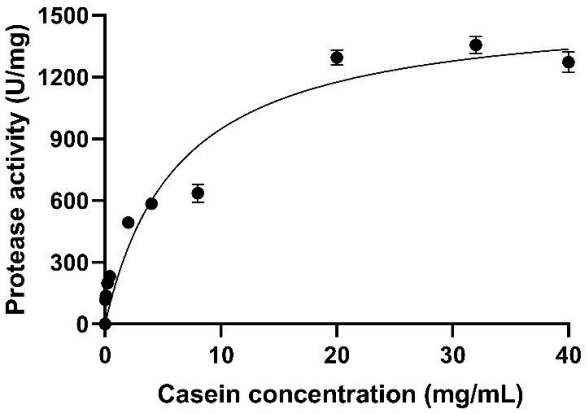

Figure 7: Extracellular protease activity from *B. amyloliquefaciens* D19 in various substrate concentrations.

Effect of metal ions on protease activity of *B. amyloliquefaciens* D19

The determination of the appropriate cofactor for D19 protease enzymatic activity will be the basis for establishing the optimum enzyme reaction conditions. In this experiment, D19 protease was assessed in different reactions containing various metal ions (5 mM) or EDTA (10 mM). The control reaction (containing ~1.0 mM residual Na^+ from the purification process) was performed without the addition of any metal ions (Figure 6).

The protease of *B. amyloliquefaciens* D19 showed the highest catalytic activity of 1630.45 U/mg in the control reaction, which contained ~1.0 mM Na^+, as well as in the reaction containing 5.0 mM Zn^{2+} (1617.78 U/mg). The reaction in which EDTA chelated any trace metal ions showed lower protease activity than those supplemented with 5.0 mM Na^+, K^+, Mg^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+} ions. The obtained results show that metal ions play a significant role in promoting the catalysis of this enzyme. Increasing the concentration of Na^+ ions to 5.0 mM reduced the enzyme activity (1407.08 U/mg).

Enzyme kinetics of *B. amyloliquefaciens* D19 protease

The catalytic activity of the enzyme was displayed by kinetic parameters. The substrate binding affinity and substrate catalysis were determined according to the Michaelis-Menten equation, and the results are shown in Figure 7. In increasing substrate concentration (0.04-40 mg/mL), enzyme catalysis increased, and V_{max} and K_M values were achieved at 1547 U/mg and 6.33 mg/mL, respectively.

DISCUSSION

In the current study, suitable media for biosynthesis and biochemical properties of extracellular protease from *B. amyloliquefaciens* D19 were identified. Starch (1.5%) and earthworm extract (1.0%), with initial pH of 7.0-8.0, 36 h of incubation at 37 °C, were found to be most suitable for D19 protease production were found to be suitable carbon and nitrogen sources for the production of this enzyme. Indeed, starch was also reported to be an appropriate carbon source for protease biosynthesis of many other strains of *Bacillus* (Chauhan and Gupta, 2004; da Silva *et al.*, 2007; Sepahy and Jabalameli, 2011; Nassar *et al.*, 2015). Earthworm extract contains mixtures of polypeptides and amino acids, which can be directly and readily available for cell metabolism. Besides, earthworm extract contains a small amount of other nutritional components such as lipids, minerals and probably growth factors, which could further favor cell growth and protease production (Hasanuzzaman *et al.*, 2010). Other suitable organic nitrogen sources were also reported for protease biosynthesis in *Bacillus*, such as soybean (Kshetri *et al.*, 2016), peptone (Puri *et al.*, 2002; Nassar *et al.*, 2015) and gelatin (Pant *et al.*, 2015). However, it is worth noting that starch and earthworm are popular, low-priced and eco-friendly compared to other purified sugars, peptone, tryptone, gelatin and beef extract derived from the meat industry. Regarding the physical requirements for protease production, the medium initial pH of 7.0-8.0 and incubation temperature of 40 °C for *B. amyloliquefaciens* D19 were observed to be similar to other Bacillus strains, including *B. subtilis* DM-04 (pH 8.0) (Mukherjee *et al.*, 2008); *B. amyloliquefaciens* MA20 and *B. subtilis* MA21 (pH 7.0, 37 °C) (Hassan *et al.*, 2013); *B. amyloliquefaciens* 35s (pH 8.0, 37 °C) (Nassar *et al.*, 2015). Additionally, the use of 1.5% soluble starch as a carbon source, 1.0% earthworm extract as a nitrogen source, an initial pH of 7.0 and an incubation temperature of 40 °C were found to be optimal for protease production, with a 36-h incubation period. Prolonged culture of more than 36 h led to continuous decline in protease activity, which can be explained by the depletion of essential nutrient content in the medium, the prolonged incubation period of more than 36 h led to continuous reduction, which can be explained by the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 20(1) 2024, pp. 85-93
DOI: http://dx.doi.org/10.21161/mjm.230307

Table 1: The enzyme activity of proteases observed from some *Bacillus* strains.

Protease source	V_{max} (U/mg)	K_M (mg/mL)	Ref.
B. amyloliquefaciens D19	1547	6.33	This study
B. siamensis CSB55	1220	0.197	Tarek *et al.* (2023)
Bacillus sp. APR-4	47.6	26.3	Kumar and Bhalla (2004)
B. cereus S8	15.0	7.5	Lakshmi *et al*. (2018)

depletion of essential nutrient content in the medium during long-term culture. Moreover, the autolysis process of many proteases could also lead to a decrease in their activity (Wang *et al.*, 2005; Sankeerthana *et al.*, 2013). Protease production in *B. amyloliquefaciens* D19 can be achieved using available low-priced nutrients with common pH and temperature and in a relatively short incubation period.

To further characterize the *B. amyloliquefaciens* D19 protease culture, the supernatant obtained from defined conditions was used for purification, resulting in a protease fraction of about 23 kDa. The molecular weight of D19 protease was similar to those obtained from *B. siamensis* CSB55 (~25 kDa) (Tarek *et al.*, 2023) or *B. amyloliquefaciens* H11 (~21 kDa) (Sai-Ut *et al.*, 2015), and was among the smallest extracellular proteases found in *Bacillus* to date. Some extracellular proteases with higher molecular weight in other *B. amyloliquefaciens* strains were also reported in previous studies including those from *B. amyloliquefaciens* FSE-68 (~27 kDa) (Cho *et al.*, 2003), *B. amyloliquefaciens* SP1 (~43 kDa) (Guleria *et al.*, 2016) and *B. amyloliquefaciens* D4 (~58,2 kDa) (He *et al.*, 2011).

Biochemical characterization results showed that enzymatic activity of the purified protease displayed in a wide range of temperatures 25-70 °C and pH 5.0-10.0, which was similar to the previously reported *Bacillus* proteases (Joshi and Satyanarayana, 2013; Sai-Ut *et al.*, 2015). In terms of thermostability and pH stability, D19 protease retained at least 75% of its residual activity at 30-50 °C and in pH 6.0-10.0. Additionally, neutral phosphate and Tris buffer can be considered proper for enzyme storage, similar to the case of protease SH21 from *B. siamensis* CSB55 (Tarek *et al.*, 2023). The fact that D19 protease showed the highest catalytic activity in the reaction containing 5.0 mM Zn^{2+} while highly inhibited by the presence of 5.0 mM of other metal ions and EDTA suggests that this could be a metalloprotease and these effects are similar to some cases of other *Bacillus* proteases (Akcan and Uyar, 2011; He *et al.*, 2011; Singh *et al.*, 2011).

Kinetic parameters of the purified D19 protease were successively determined. The V_{max} value of D19 protease was slightly higher than V_{max} of *B. siamensis* CSB55 protease SH21 (Tarek *et al.*, 2023), 32-fold and 100-fold higher than those from *Bacillus* sp. APR-4 and *B. cereus* S8, respectively (Kumar and Bhalla, 2004; Lakshmi *et al.*, 2018). The K_M value of *B. amyloliquefaciens* D19 protease was slightly lower than that of *B. cereus* S8 but more than 4-fold lower than the *Bacillus* sp. APR-4 protease (Kumar and Bhalla, 2004; Lakshmi *et al.*, 2018) suggests that it is an enzyme with very high substrate catalysis and moderate substrate binding affinity (Table 1).

CONCLUSION

In the current study, suitable media for biosynthesis and biochemical properties of extracellular protease from *B. amyloliquefaciens* D19 were identified. Starch (1.5%) and earthworm extract (1.0%) are suitable carbon and nitrogen sources for the production of this enzyme. *Bacillus amyloliquefaciens* D19 protease was purified and shown to have a molecular weight of about 23 kDa. Biochemical characterization of the enzyme showed that this enzyme is highly active in a wide range of temperatures and pH. With high V_{max} and low K_M, this protease could be an efficient enzyme for several potential applications. These results suggested that *B. amyloliquefaciens* D19 protease could be used for applications in different fields, such as food-feed and brewing industries.

ACKNOWLEDGEMENTS

We acknowledge the Industrial University of Ho Chi Minh City (IUH) for supporting this research under project number 21.2SHTP03.

REFERENCES

Akcan, N. and Uyar, F. (2011). Production of extracellular alkaline protease from *Bacillus subtilis* RSKK96 with solid state fermentation. *Eurasian Journal of BioSciences* **5, 64-72.**

Arunachalam, C. and Saritha, K. (2009). Protease enzyme: An eco-friendly alternative for leather industry. *Indian Journal of Science and Technology* **2(12), 29-32.**

Beg, Q. K., Saxena, R. and Gupta, R. (2002). De-repression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operations. *Process Biochemistry* **37(10), 1103-1109.**

Çalık, P., Bilir, E., Çalık, G. and Özdamar, T. H. (2002). Influence of pH conditions on metabolic regulations in serine alkaline protease production by *Bacillus licheniformis*. *Enzyme and Microbial Technology* **31(5), 685-697.**

Chauhan, B. and Gupta, R. (2004). Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. *Process Biochemistry* **39(12), 2115-2122.**

Cho, S. J., Oh, S. H., Pridmore, R. D., Juillerat, M. A. and Lee, C. H. (2003). Purification and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

characterization of proteases from *Bacillus amyloliquefaciens* isolated from traditional soybean fermentation starter. *Journal of Agricultural and Food Chemistry* **51(26), 7664-7670.**

da Silva, C. R. Delatorre, A. B. and Martins, M. L. L. (2007). Effect of the culture conditions on the production of an extracellular protease by thermophilic *Bacillus* sp. and some properties of the enzymatic activity. *Brazilian Journal of Microbiology* **38, 253-258.**

Demain, A. L. and Adrio, J. L. (2008). Contributions of microorganisms to industrial biology. *Molecular Biotechnology* **38, 41-55.**

Guleria, S., Walia, A., Chauhan, A. and Shirkot, C. K. (2016). Purification and characterization of detergent stable alkaline protease from *Bacillus amyloliquefaciens* SP1 isolated from apple rhizosphere. *Journal of Basic Microbiology* **56(2), 138-152.**

Gupta, R., Beg, Q. and Lorenz, P. (2002). Bacterial alkaline proteases: Molecular approaches and industrial applications. *Applied Microbiology and Biotechnology* **59, 15-32.**

Gupta, R., Gupta, K., Saxena, R. and Khan, S. (1999). Bleach-stable, alkaline protease from *Bacillus* sp. *Biotechnology Letters* **21, 135-138.**

Hamza, T. A. (2017). Bacterial protease enzyme: Safe and good alternative for industrial and commercial use. *International Journal of Chemical and Biomolecular Science* **3(1), 1-10.**

Hamza, T. A. and Woldesenbet, F. (2017). Optimization of culture growth parameters for production of protease from bacteria, isolated from soil. *Bioscience and Bioengineering* **3, 1-10.**

Hasanuzzaman, A. M., Hossian, S. Z. and Das, M. (2010). Nutritional potentiality of earthworm (*Perionyx excavatus*) for substituting fishmeal used in local feed company in Bangladesh. *Mesopotamian Journal of Marine Sciences* **25(2), 134-139.**

Hassan, M. A., Haroun, B. M., Amara, A. A. and Serour, E. A. (2013). Production and characterization of keratinolytic protease from new wool-degrading *Bacillus* species isolated from Egyptian ecosystem. *BioMed Research International* **2013, Article ID 175012.**

He, X., Ren, F., Guo, H., Zhang, W., Song, X. and Gan, B. (2011). Purification and properties of a milk-clotting enzyme produced by *Bacillus amyloliquefaciens* D4. *Korean Journal of Chemical Engineering* **28, 203-208.**

Joshi, S. and Satyanarayana, T. (2013). Characteristics and applications of a recombinant alkaline serine protease from a novel bacterium *Bacillus lehensis*. *Bioresource Technology* **131, 76-85.**

Kshetri, P., Ningombam, O. and Ningombam, D. (2016). Optimization of alkaline protease production by alkaliphilic *Bacillus* sp. KW2 in low cost medium using statistical approaches. *Applied Microbiology: Open Access* **2(2), 1000117.**

Kumar, D. and Bhalla, T. (2004). Purification and characterization of a small size protease from *Bacillus* sp. APR-4. *Indian Journal of Experimental Biology* **42, 515-521.**

Lakshmi, B., Kumar, D. M. and Hemalatha, K. (2018). Purification and characterization of alkaline protease with novel properties from *Bacillus cereus* strain S8. *Journal of Genetic Engineering and Biotechnology* **16(2), 295-304.**

Lanoë, J. and Dunnigan, J. (1978). Improvements of the Anson assay for measuring proteolytic activities in acidic pH range. *Analytical Biochemistry* **89(2), 461-471.**

Mukherjee, A. K., Adhikari, H. and Rai, S. K. (2008). Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using *Imperata cylindrica* grass and potato peel as low-cost medium: Characterization and application of enzyme in detergent formulation. *Biochemical Engineering Journal* **39(2), 353-361.**

Nascimento, W. C. A. and Martins, M. L. L. (2004). Production and properties of an extracellular protease from thermophilic *Bacillus* sp. *Brazilian Journal of Microbiology* **35, 91-96.**

Nassar, F. R., Abdelhafez, A. A., El-Tayeb, T. S. and Abu-Hussein, S. H. (2015). Proteases production by a bacterial isolate *Bacillus amyloliquefaciens* 35s obtained from soil of the Nile Delta of Egypt. *British Microbiology Research Journal* **6(6), 315-330.**

Nguyen, N. A., Hua, H. M. T., Ho, N. H. Y., Nguyen, T. D. H., Nguyen, L. H. H., Tran, T. T. T. *et al.* (2020). Isolation, identification and characterization of bacterial antagonists of the dragon fruit fungal pathogen *Neoscytalidium dimidiatum*. *Journal of Science and Technology* **44(2), 76-85.**

Nguyen, N. A., Nguyen, L. H. H., Tran, N. D. L., Bui, T. L., Nguyen, T. H., Nguyen, T. D. H. *et al.* (2021). Enhanced production of cellulose from *Bacillus amyloliquefaciens* D19. *Journal of Science and Technology* **49(1), 68-76.**

Pant, G., Prakash, A., Pavani, J., Bera, S., Deviram, G., Kumar, A. *et al.* (2015). Production, optimization and partial purification of protease from *Bacillus subtilis*. *Journal of Taibah University for Science* **9(1), 50-55.**

Puri, S., Beg, Q. K. and Gupta, R. (2002). Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. *Current Microbiology* **44, 286-290.**

Rao, M. B., Tanksale, A. M., Ghatge, M. S. and Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews* **62(3), 597-635.**

Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A. *et al.* (2018). Applications of microbial enzymes in food industry. *Food Technology and Biotechnology* **56(1), 16-30.**

Sai-Ut, S., Benjakul, S., Sumpavapol, P. and Kishimura, H. (2015). Purification and characterization of extracellular gelatinolytic protease

from *Bacillus amyloliquefaciens* H11. *Journal of Food Biochemistry* **39(1), 119-128.**

Sankeerthana, C., Pinjar, S., Jambagi, R. T., Bhavimani, S., Anupama, S., Sarovar, B. *et al.* (2013). Production and partial characterization of protease from *Aspergillus flavus* using rice mill waste as a substrate and its comparison with *Aspergillus niger* protease. *International Journal of Current Engineering and Technology* **1, 143-147.**

Sawant, R. and Nagendran, S. (2014). Protease: An enzyme with multiple industrial applications. *World Journal of Pharmacy and Pharmaceutical Sciences* **3(6), 568-579.**

Sepahy, A. A. and Jabalameli, L. (2011). Effect of culture conditions on the production of an extracellular protease by *Bacillus* sp. isolated from soil sample of Lavizan Jungle Park. *Enzyme Research* **2011, Article ID 219628.**

Singh, S. K., Singh, S. K., Tripathi, V. R. and Garg, S. K. (2011). An oxidant, detergent and salt stable alkaline protease from *Bacillus cereus* SIU1. *African Journal of Biotechnology* **10(57), 12257-12264.**

Tarek, H., Nam, K. B., Kim, Y. K., Suchi, S. A. and Yoo, J. C. (2023). Biochemical characterization and application of a detergent stable, antimicrobial and antibiofilm potential protease from *Bacillus siamensis*. *International Journal of Molecular Sciences* **24(6), 5774.**

Wang, R., Law, R. C. S. and Webb, C. (2005). Protease production and conidiation by *Aspergillus oryzae* in flour fermentation. *Process Biochemistry* **40(1), 217-227.**

Yamagata, Y., Isshiki, K. and Ichishima, E. (1995). Subtilisin Sendai from alkalophilic *Bacillus* sp.: Molecular and enzymatic properties of the enzyme and molecular cloning and characterization of the gene, *aprS*. *Enzyme and Microbial Technology* **17(7), 653-663.**

Yang, J. K., Shih, I. L., Tzeng, Y. M. and Wang, S. L. (2000). Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme and Microbial Technology* **26(5-6), 406-413.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538