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CHARACTERIZATION OF EXTRACELLULAR PROTEASE FROM *BACILLUS PUMILUS* I7

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SUMMARY

Proteases (also termed peptidase or proteinase) are the most important industrial enzymes, they occur naturally in all living organisms. A protease is an enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Therefore, these enzymes have been widely used in many industrial fields such as food processing, leather processing, weaving processing, pharmaceutical industry, waste management, washing detergent, and chemical industry. They account for approximately 40% of the total enzyme sale markets in above applications. *Bacillus pumilus* strain I7, which produces extracellular protease, was isolated from wastewater of abattoir in Hue, Vietnam. Total and specific activities of protease from *B. pumilus* I7 reached a maximum value of 64.8 unit/ml and 179 unit/mg, respectively, after 22 hours of culture in medium containing 1% soybean, 1% soluble starch, 0.1% KH_2PO_4, 0.02% $MgSO_4$ and 0.5% NaCl. Protease from *B. pumilus* I7 strongly operated at pH 8 and 50^oC. Mn^{2+} ion stimulated an increase of protease activity at 0.5 mM concentration, whereas various ions (Hg^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, Zn^{2+}, and Co^{2+}) inhibited its activity. Protease from *B. pumilus* I7 was completely prohibited by SDS (sodium dodecyl sulfate). However, PMSF (phenylmethane sulfonyl fluoride), EDTA (ethylenediaminetetraacetic acid), H_2O_2 (hydrogen peroxide) and Tween 20 partially inhibited this enzyme's activity. SDS-PAGE (SDS-polyacrylamide gel electrophoresis) with 0.5% casein showed three proteolytic bands. These bands were in the position of proteins with molecular weights of approximately 97, 65 and 48 kDa.

Keywords: *abattoir wastewater, alkaline protease, Bacillus pumilus I7, enzyme molecular weights, extracellular protease*

INTRODUCTION

Proteases are the most important industrial enzymes (Mohen *et al.,* 2005; Pawar *et al.,* 2009). They occur naturally in all living organisms. Proteases can either break specific peptide bonds, depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids. Therefore, these enzymes have been widely used in many industrial fields such as food processing, leather processing, weaving processing, pharmaceutical industry, waste management, washing detergent, and chemical industry. They account for approximately 40% of the total enzyme sale markets in above applications (Gupta *et al.,* 2002a,b).

Bacterial proteases can be classified into three different groups depending on the optimal pH of acid, neutral or alkaline. Based on the functional group presents at the active site, proteases are further classified into four prominent groups that are serine proteases (EC.3.4.21), aspartic proteases (EC.3.4.23), cysteine proteases (EC.3.4.22), and metalloproteases (EC.3.4.24) (Rao *et al.,* 1998). *Bacillus pumilus* is a Gram-positive, aerobic, rod-shaped, soil-dwelling bacterium. *B. pumilus* has been demonstrated to have the ability to secrete several extracellular enzymes such as keratinase (Rajput, Gupta, 2011), xylanase (Qu, Shao, 2011), laccase (Reiss *et al.,* 2011), lipase (Kumar *et al.,* 2011), etc. The present study aims to investigate the characterizations of extracellular protease of *B. pumilus* strain I7 which has been isolated from wastewater of abattoir in Hue, Vietnam.

MATERIALS AND METHODS

Bacteria strain and culture

B. pumilus strain I7 was isolated from wastewater of abattoir in Hue, and the sequence of 16S rRNA was deposited on NCBI with accession No. JN563928.

The biomass culture was grown in 250-ml Erlenmayer flasks containing 50 ml of LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl). The flasks with inoculum size of 100 µl from the stock were incubated at 37°C on shaker at a rotation speed of 200 rpm for overnight.

Enzyme production

Medium for extracellular protease production contains (g/l) soybean powder 10, soluble starch 10, KH_2PO_4 1, $MgSO_4$ 0.2, and NaCl 5. Fifty milliliter of medium was taken in a 250-ml Erlenmayer flask. The flask was inoculated with 5% (v/v) biomass culture and incubated at 37°C, 200 rpm overnight. The culture medium was centrifuged at 15,000 rpm/4°C for 10 min to obtain the crude extract, which served as enzyme source.

Enzyme assay

Protease activity was determined by modified procedure based on the method of Anson using casein as the substrate (Anson, 1938). One unit of protease activity is defined as the amount of enzyme required to release 1 µg of tyrosine per 1 ml per min under the standard assay conditions. Total protein concentration was determined by the method of Bradford (1976). The absorbance was measured at the 595 nm in the SmartSpec™ Plus spectrophotometer (BioRad, USA). The bovine serum albumin was used to establish the standard curve. The specific activity of enzyme (unit/mg protein) is obtained by dividing of total activity of enzyme for total proteins.

Characterization of enzyme

The effect of pH on protease was determined by assaying for the enzyme activity at 50°C with different pH levels from 6 to 11, using the following buffer systems: Sorensen buffer (pH 6-8) and glycerin-NaOH (pH 9-11). The optimal temperature was investigated in the range of 30-70°C at the optimal pH. Thermal and pH stability of protease were determined by incubating the enzyme solution for 30 min at different temperatures (30-70°C) and pH (6-11).

The effect of metal ions (Hg^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Na^{+}, Zn^{2+}, and Co^{2+}), inhibitors [phenylmethane sulfonyl fluoride (PMFS), sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), and H_2O_2] and surfactant (Tween 20) on protease activity was investigated to further characterize the enzyme. The protease was pre-incubated with the mentioned chemicals for 30 min at room temperature; afterwards the residual activity (%) was measured by standard protease assay. The final concentration of each chemical was 5 mM at the time of pre-incubation.

SDS-PAGE in the present of substrate

The supernatant was precipitated with ammonium sulfate to 60% saturation with stirrer and centrifugation was done at 15,000 rpm/4°C for 10 min. The precipitate was then dissolved in Sorensen buffer (pH 8). The solution was dialyzed against lysis buffer with Spectra/por® 4 Dialysis membrane (MW 12-14 kDa, Spectrum Laboratories Inc., USA) at 4°C for overnight.

Sodium dodecyl sulfate-(12%) polyacrylamide gel containing 0.5% casein was used to separate the secreted proteins by electrophoresis at 4°C for 3 hours. The gel was then incubated at 37°C for 2 h with gently shaking in 50 mM sodium citrate buffer (pH 8) containing 1% (v/v) Triton X-100 and stained with Coomassie Blue R-250. Protease bands were observed through the hydrolysis of casein. Molecular weight of proteases was estimated by the standard molecular weight marker (BioRad, USA) and Quanlitity-One software version 4.5 (BioRad, USA).

Statistical analysis

All the experiments were carried out in triplicates and the presented results are the mean and standard error of three values.

RESULTS AND DISCUSSION

Detection of the proteolytic activity and biomass culture

B. pumilus strain I7 showed a strong extracellular proteolytic activity on the agar plate containing 2% casein. The diameter of casein hydrolytic zone is approximately 1 cm (Fig 1). The biomass culture of *B. pumilus* I7 was incubated at 37°C for 26 h. The cell growth reached a maximum OD_{600} value of about 3 after 22 h of culture (Fig 2). Our investigations on characterization of extracellular protease from *B. pumilus* I7 were designed based on this growth profile.

Enzyme production

The results showed in figure 3 indicated that the total and specific activities of protease reached a maximum value of 64.8 unit/ml and 179 unit/mg

after 22 h of culture, respectively. The specific activity of protease from this strain is 15-fold higher than that of *B. pumilus* strain MK6-5 (Kumar, 2002). According to Huang *et al.* (2003), Kumar *et al.* (2008), and Wang *et al.* (2007), *B. pumilus* secret several extracellular enzymes, but proteases are the main enzymes due to high keratin hydrolysis.

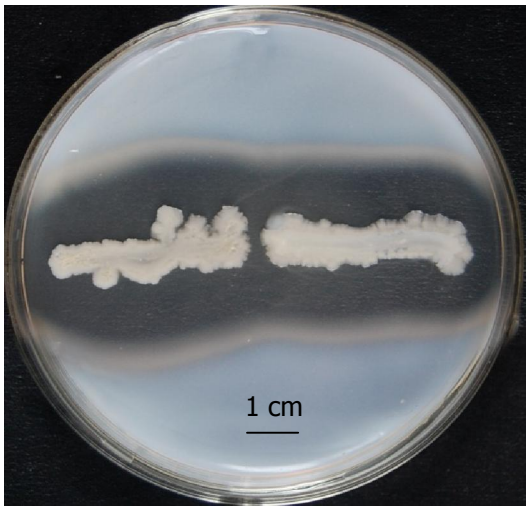

Figure 1. Extracellular proteolysis of *B. pumilus* I7 on medium containing casein

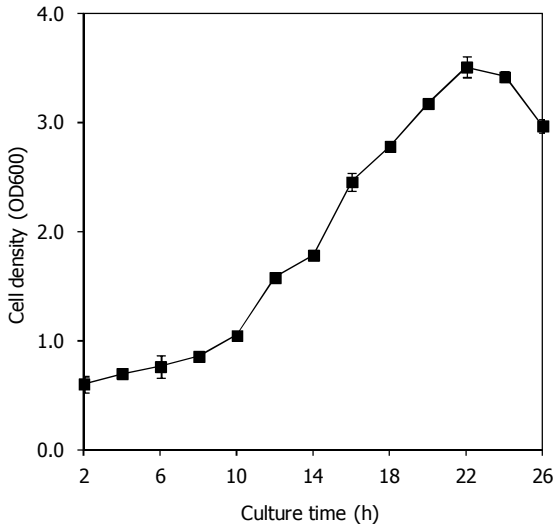

Figure 2. The growth of *B. pumilus* I7 in batch culture

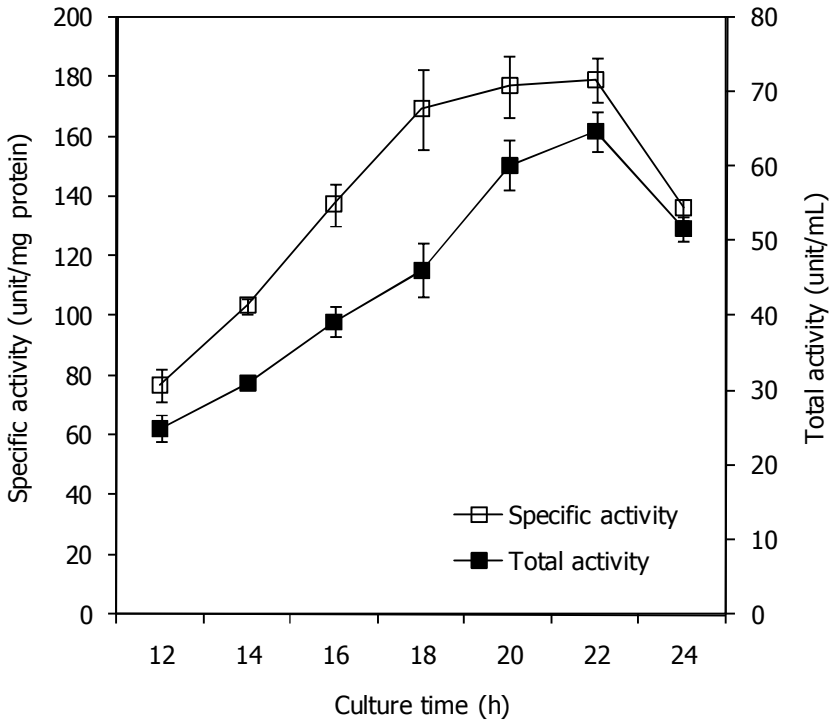

Figure 3. Protease production of *B. pumilus* I7 in batch culture

Effect of temperature on enzyme activity

Our investigation showed the optimal temperature of protease from *B. pumilus* I7 is 50°C. The enzyme exhibited lower activity when the temperature was below 40°C or over 55°C, and it was completely inactivated at 70°C (Fig 4). Proteases from *B. pumilus* TYO-67 and *B. pumilus* CBS also expressed the highest activity at 50°C (Aoyama *et al.,* 2000; Jaouadi *et al.,* 2010), while proteases from several different *B. pumilus* strains have optimal temperature of 55°C (Huang *et al.,* 2003; Kumar, 2002), 40-60°C (Jin *et al.,* 2011), 60°C (Ibrahim, 2011), or 65°C (Yasuda *et al.,* 1999). These studies indicated that *B. pumilus* strains produce many kinds of protease depending on their physiological characteristics.

Thermostability of enzyme was examined by incubating reaction mix at the various temperatures for 30 min. The figure 4 showed the residual activity of enzyme is approximately 65% when incubated at 45°C and rapidly decreases at above 50°C. Our results are in accordance with that of Huang *et al.* (2003) and Wan *et al.* (2009).

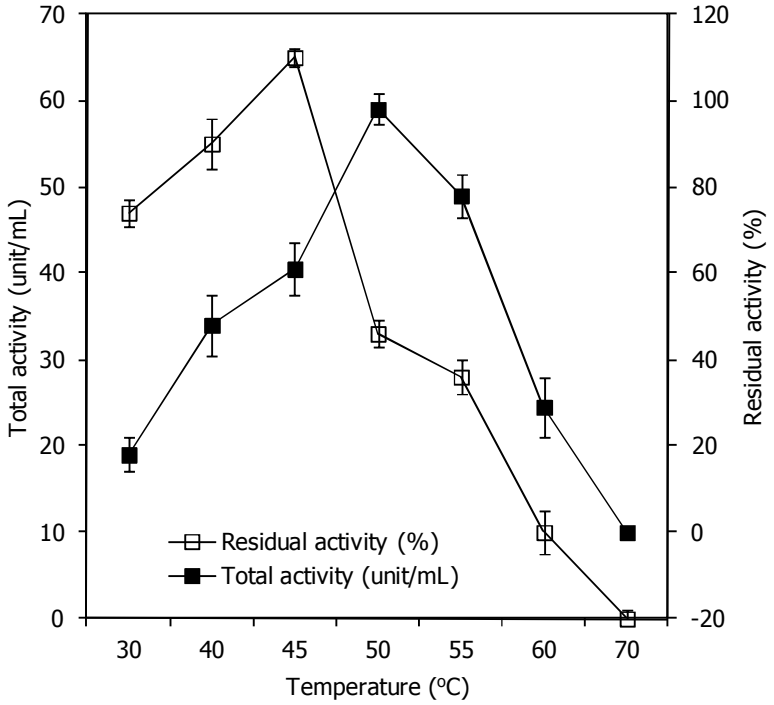

Figure 4. Thermostability of *B. pumilus* I7 protease.

Effect of pH on enzyme activity

Protease secreted by *B. pumilus* reached the optimal activity at pH 8 (Fig 5). These results indicated that secreted proteases of *B. pumilus* I7 are alkaline-like protease. Alkaline proteases of *B. pumilus* have also been reported by Kumar *et al.* (2002), Huang *et al.* (2003), Jaouadi *et al.* (2010), and Fakhfakh-Zouari *et al.* (2010). This could be suggested that alkaline proteases are dominant enzyme in the proteolytic system of *B. pumilus*. As the figure 5 showed, proteases from *B. pumilus* I7 had strong pH stability, the residual activity was above 90% in the range of pH 6-10.

Effect of chemicals on enzyme activity

Our results showed most of the metal ions tested had an inhibitory effect on protease activity. Only Mn^{2+} ion had a stimulatory effect and increased the activity by 124.57% as showed in figure 6. Ibrahim *et al.* (2011) described a manganese-dependent alkaline protease of *B. pumilus*, whereas Ca^{2+} and Mg^{2+} did not affect enzyme activity.

EDTA, H_2O_2, SDS, PMSF and Tween 20 also lowered protease activity to 52.80-81.57%. Especially, there is no residual activity of protease can be observed when SDS was added to reaction mix.

Whereas, several studies indicated that SDS partially inhibits to protease activity (Aoyama *et al.,* 2000; Huang *et al.,* 2003; Ibrahim *et al.,* 2011; Jin *et al.,* 2011). PMSF is a strong inhibitor of protease family. The studies on the influence of PMSF on *B. pumilus* protease showed the residual activity of enzyme was 0% (Aoyama *et al.,* 2000), 7.2% (Huang *et al.,* 2003) or 8% (Ibrahim *et al.,* 2011).

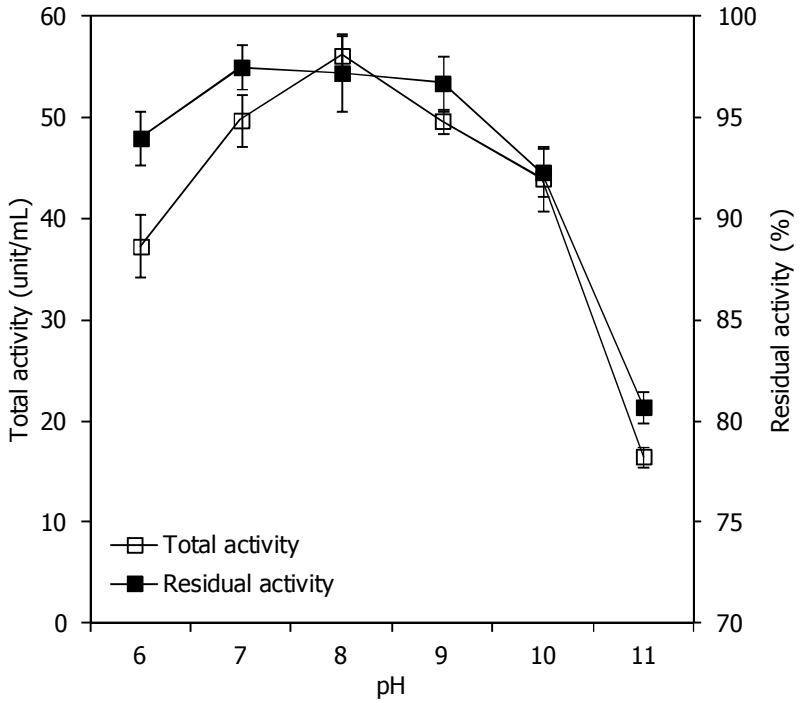

Figure 5. Effect of pH on protease activity of *B. pumilus* I7.

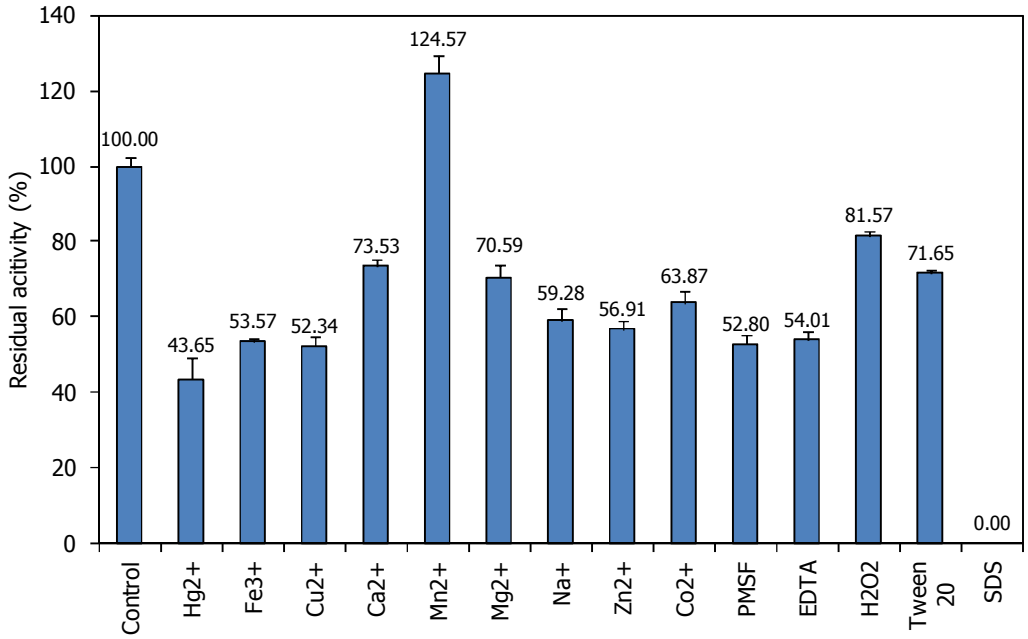

Figure 6. Effect of chemicals on protease activity of *B. pumilus* I7.

Molecular weight determination of proteases

The molecular weights of *B. pumilus* I7 proteases were determined on 0.5% casein-12% polyacrylamide gel. Proteolytic activity was expressed clearly bands at the pH 8. *B. pumilus* I7 secretes three extracellular alkaline proteases with molecular weights of approximately 97, 65, and 48 kDa (Fig 7). Among of them, a high molecular weight band (97 kDa) expressed the strongest proteolytic activity. The molecular weight of this enzyme is equivalent to alkaline protease of *B. pumilus* CN8 (Jin *et al.,* 2011). Kumar *et al.* (2008) also found a 65 kDa extracellular protease from *B. pumilus*. However, an alkaline protease with molecular weight of about 48 kDa was not detected yet. The other studies only found alkaline proteases from 30 to 35 kDa of *B. pumilus* such as Fakhfakh-Zouari *et al.* (2010), Huang *et al.* (2003), Ibrahim *et al.* (2011), Jaouadi *et al.* (2010), Wan *et al.* (2009) and Yasuda *et al.* (1999).

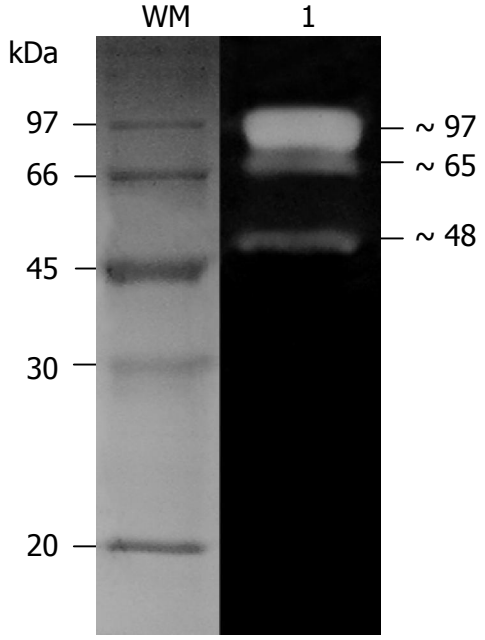

Figure 7. SDS-PAGE with 0.5% casein. WM: molecular weight maker, lane 1: extracellular proteases from *B. pumilus* I7

CONCLUSION

Total and specific activities of protease from *B. pumilus* I7 reached a maximum value of 64.8 unit/ml and 179 unit/mg, respectively, after 22 h of culture on medium containing 1% soybean, 1% soluble starch, 0.1% KH_2PO_4, 0.02% $MgSO_4$ and 0.5% NaCl. Protease from *B. pumilus* I7 strongly operated at pH 8 and 50°C. Mn^{2+} ion stimulated the increase of protease activity at 0.5 mM concentration, whereas various ions (Hg^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, Zn^{2+}, and Co^{2+}) inhibited its activity. Protease from *B. pumilus* I7 was completely prohibited by SDS. However PMSF, EDTA, H_2O_2 and Tween 20 partially inhibited enzyme activity. SDS-PAGE with 0.5% casein showed three proteolytic bands, these bands have molecular weights of approximately 97, 65 and 48 kDa.

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NGHIÊN CỨU ĐẶC ĐIỂM CỦA PROTEASE NGOẠI BÀO TỪ CHỦNG VI KHUẨN *BACILLUS PUMILUS* I7

Cù Lê Nguyên, Trần Nguyên Thảo, Nguyễn Đức Huy, Hoàng Tấn Quảng, Nghiêm Văn Tùng, Nguyễn Hoàng Lộc*

Viện Tài nguyên, Môi trường và Công nghệ sinh học, Đại học Huế

TÓM TẮT

Protease (cũng được gọi là peptidase hoặc proteinase) là các enzyme công nghiệp quan trọng nhất, chúng hiện diện trong tự nhiên ở tất cả các sinh vật sống. Protease là các enzyme thực hiện thủy phân các phân tử protein. Vì vậy, chúng được sử dụng rộng rãi trong nhiều lĩnh vực công nghiệp như chế biến thực phẩm, thuộc da, dệt, dược phẩm, quản lý chất thải, chất tẩy rửa, và công nghiệp hóa chất. Chúng chiếm khoảng 40% thị trường enzyme trên thế giới. Chủng vi khuẩn *Bacillus pumilus* I7, được chúng tôi phân lập từ nước thải lò mổ gia súc ở Huế, có khả năng sản xuất protease ngoại bào mạnh. Kết quả khảo sát đặc điểm của enzyme này cho thấy sau 22 giờ nuôi cấy trên môi trường dinh dưỡng (10 g/l bột đậu nành, 10 g/l tinh bột, 1 g/l KH_2PO_4, 0,2 g/l $MgSO_4$, và 5 g/l NaCl), hoạt độ chung và riêng của enzyme đạt cực đại là 64,83 u/ml và 179 u/mg protein. Phân tích đặc điểm enzyme cho thấy đây là protease kiềm, có nhiệt độ tối thích là 50^{o}C và pH tối thích là 8. Ion Mn^{2+} có khả năng tăng hoạt tính enzyme lên 125% trong khi các ion khác (Hg^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Na^+, Zn^{2+} và Co^{2+}) ức chế hoạt tính enzyme. SDS (sodium dodecyl sulfate) ức chế hoàn toàn trong khi PMSF (phenylmethane sulfonyl fluoride), EDTA (ethylenediaminetetraacetic acid), H_2O_2 (hydrogen peroxide) và Tween 20 chỉ ức chế một phần. Điện di trên gel có chứa cơ chất 0,5% casein cho thấy có 3 băng protein có hoạt tính protease, các băng này có khối lượng phân tử vào khoảng 97, 65 và 48 kDa.

Từ khóa: *Bacillus pumilus* I7*, khối lượng phân tử enzyme, nước thải lò mổ, protease kiềm, protease ngoại bào*

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