Scientific Research Journal,
Vol 19, No 1 (2022) 59-73
https://doi.org/10.24191/srj.v19i1.13790

Characterization of Partially Purified and Immobilized Partially Purified Protease Extracted from Silver Catfish (*Pangasius sutchi*) Viscera

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Received: 08 June 2021
Accepted: 03 December 2021
Online First: 28 February 2022

ABSTRACT

Viscera is known as one of the richest sources of proteolytic enzymes. However, the biggest problem regarding enzyme is the rapid deterioration and lose in functional properties due to inefficient treatment and improper storage condition. Therefore, in this study, protease from silver catfish viscera have been extracted, partially purified by acetone precipitation method and immobilized in the calcium alginate beads. Concentrations of 2.99 % (w/v) sodium alginate and 0.30 M calcium chloride were used to produce the immobilized partially purified protease. Then, the partially purified and immobilized partially purified protease were characterized in different range of pH, temperature and storage period in order to establish a greater protease stability during storage and increase in its efficiency. Proteolytic activity of the partially purified and immobilized partially purified protease in the alginate beads were measured by using casein as a substrate. The highest proteolytic activity for partially purified protease was recorded at pH 4 (5709.76 CDU/mg) and 40 °C (6061.14 CDU/mg) and for immobilized partially purified protease at pH 5 (3987.85 CDU/mg) and 50 °C (4956.54 CDU/mg). For storage stability, the partially purified protease has optimum storage temperature at – 40 °C while the immobilized partially purified protease at 4 °C. of 17.44 and 24.50 %, respectively for 6th days of storage. At optimum storage condition, the partially purified protease

and immobilized partially purified protease have storage efficiency of 17.44 and 24.50 %, respectively for 6th days of storage.

Keywords: protease; partially purified; immobilization; pH; temperature; storage stability

INTRODUCTION

Catfish contribute to 36.7 % of Malaysia's total freshwater aquaculture production which is behind the Nile tilapia that contribute to 44.7 % [1]. Fish viscera contributes as the biggest waste in fishing industry; this includes the digestive tissues, such as the stomach, intestines, liver, pancreas, spleen and gonads [2]. Approximately 5 to 10 % from the entire fish weight accounts for the viscera and it tends to increase according to the body weight of the fish [3]. Viscera is known as one of the richest sources of proteolytic enzymes [2]. Previous study showed that purifications method by using cold acetone (60 % concentration) effectively purified protease from Bacillus megaterium with 0.28 U/mg specific activity and 84.88 % of enzyme activity [4]. Besides, the specific enzyme activity, purification fold, and recovery percentage from acetone precipitation were higher than ammonium sulphate precipitation [5]. Cold acetone precipitation is also appropriate for commercial process as the process is simple and convenient compared to the ammonium sulphate procedure which had tedious steps and inconvenient [5].

According to Rezakhani et al. [6] immobilization enables the use of enzymes in different conditions such as chemical solvents, pH, temperature and exceptionally high substrate concentrations and allow the reuse of the immobilized enzyme [6]. Entrapment is the easiest immobilization method which will not alter the enzyme's structure, fast, low cost and the composition of the matrix can be adjusted to control the inner condition of the beads [7]. Thus, the effect of pH, temperature and storage on proteolytic activity of partially purified protease and immobilized partially purified protease were studied to establish greater stability of protease during storage and increase its usage efficiency.

MATERIALS AND METHODS

Analytical grade chemicals, brand Emsure were used in this study and purchased from Next Gene Scientific Sdn Bhd, Malaysia. The chemicals used were calcium chloride, sodium alginate, acetone, bovine serum albumin (BSA), casein, trichloroacetic acid (TCA), distilled water, sodium acetate (pH 4.0 to 5.0), sodium phosphate (pH 6.0 to 7.0), Tris-HCL (pH 8.0), L-tyrosine, hydrochloric acid, sodium hydroxide.

Silver catfish (Pangasius sutchi) viscera were obtained from silver catfish river cage, Paloh Hinai, Pahang. The viscera were rinsed, weighed, placed in airtight glass bottle, freeze dried and ground using mortar. For the crude protease extraction, the freeze-dried viscera were homogenized with 25 mM Tris–HCl (4 °C, pH 8.0) at the ratio of 1:1 [8]. Then, centrifuged at 10, 000 rpm (15 minutes, 4 °C) by using a microcentrifuge instrument (Eppendorf, Centrifuge 5418) to collect the supernatant (crude protease) [9]. Next, in the purification steps, the crude protease (0 °C) and acetone (-20 °C) were used at ratio 1:1. The cold acetone was slowly added and agitated gently to allow precipitation. Then, the solution was centrifuge at 10, 000 rpm (10 minutes, 4 °C) using a microcentrifuge. The supernatants were discarded and excess liquid was removed by using a filter paper. The pellets were dissolved in minimum amount of 0.1 M Tri-HCl buffer (pH 8.0) and centrifuged at 10, 000 rpm (20 minutes) then the supernatant (partially purified protease) was collected [10].

2.99 % (w/v) sodium alginate and 0.30 M calcium chloride was used to produce the immobilized partially purified protease. Immobilization was performed according to Geethanjali & Subash [2]. Proteolytic activity of partially purified and immobilized partially purified protease was determined according to Dapeau [11] and was calculated according to the following Equation 1 [8]:

$$Casein\ Digestion\ Unit\ \left(\frac{CDU}{mg}\right) = \frac{Et-Eb}{Es}\ x\ 50\ x\ \frac{11}{10}\ x\ DF \qquad (1)$$

Et = Absorbance of sample
Eb = Absorbance of blank
Es = Absorbance of standard (Tyrosine)
DF = Dilution factor of enzyme solution in mg

Determination of the effect of pH, temperature and storage stability study on partially purified and immobilized partially purified protease

For pH, partially purified protease and immobilized partially purified protease were incubated in different buffers for 30 minutes at 37 °C, with pH ranging from 4 to 8 and proteolytic activity determination was done [11] [8]. The buffer solutions used were sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0) and Tris-HCL (pH 8.0) each at concentration of 0.05 M [12]. For analysis of the effect of temperature, the protease was incubated at 40 to 65 °C for 15 minutes in optimum pH buffer solution [12]. After the incubation period, the sample was cooled in ice bath before proteolytic activity determination was carried out [11] [8]. The sample was analyzed for proteolytic activity at the interval of 5 °C [12]. The storage stability of partially purified and immobilized partially purified protease was determined by incubating both liquid at 4 °C and -40 °C in optimum pH obtained. The sample was stored in a glass bottle and assayed at every 3 days interval for a period of 6 days [11] [8]. The storage percentage efficiency and decrement were calculated as the following Equation 2 and 3 [13]:

$$Storage\ efficiency\ (\%) = \frac{Enzyme\ activity\ after\ storage}{Initial\ enzyme\ activity} x\ 100 \qquad (2)$$

$$Storage\ decrement\ (\%) = 100 - Storage\ efficiency \qquad (3)$$

Statistical Analysis

The data obtained were analysed by using the Analysis of Variance (ANOVA) to determine the significant difference at 5 % level and all measurements were carried out in triplicate. Duncan Multiple Range Test (DMRT) was used to identify the differences between means. Statistical program that was used is statistical software IBM Statistical Package for the Social Sciences (SPSS) Statistic 21.0 version.

RESULTS AND DISCUSSION

Effect of pH

The highest proteolytic activity for partially purified protease was recorded at pH 4 (5709.76 CDU/mg) and for immobilized partially purified protease at pH 5 (3987.85 CDU/mg) which was significantly higher ($p<0.05$) than the other pH. Based on the optimum pH, it was observed that the protease extracted from silver catfish viscera is an acidic protease with optimum activity at pH 4 and pH 5 for the partially purified protease and immobilized partially purified protease, respectively. According to Khaled et al. [14], acidic protease usually had high proteolytic activity at pH between 2 to 4 while alkaline protease at pH 8 to 10. Bougatef et al. [9], reported that fish species of Smooth hound have an optimum pH value of protease between 2.0 and 4.0. According to Normah & Nurnajwa [8], silver catfish (Pangasius sutchi) viscera contained protease that was slightly acidic and the enzyme activity increases from pH 4 until pH 6 with specific activity between 50 to 55 U/mg and decreases as the pH approaching pH 11. In addition, different molecular properties and enzyme conformation amongst different species and location of the fish will influenced the pH of the fish internal body [8]. Therefore, the highest proteolytic activity obtained at pH 4 (partially purified protease) and at pH 5 (immobilized partially purified protease) is aligned [8].

The change in optimum pH and proteolytic activity for the partially purified protease (pH 4) and immobilized partially purified protease (pH 5) may be affected by the calcium alginate matrix. Anwar et al. [13] stated that the surface of the beads and entrapped enzyme have their own individual charge thus when the two different charges interact together, a new charged microenvironment was produced. Therefore, affecting the nature of the active enzyme protein and alters the behaviour of the entrapped enzyme towards changes in pH. Besides, the loss of enzyme activity at pH values outside the optimum pH is probably due to protein conformational changes caused by repulsion of charges thus enzymes could not bind to the substrate properly [15]. Thus, a sharp decline or increase in activity before and after the optimum pH was due to the charge acquired by the support (calcium alginate).

Based on Figure 1, the sharp decline in activity showed by the immobilized partially purified protease from the optimum pH 5 to pH 8 make it easier for a person to notice if the pH value of the solution used for the incubation process was wrong or not exactly accurate compared to constant trend of decrement of activity in partially purified protease. Lastly, the overall proteolytic activity of immobilized partially purified protease was lower than partially purified protease is due to lower affinity of the enzyme to the substrates caused by diffusional limitations, decreased in protein flexibility and enzyme denaturation or leakage during immobilization process [16].

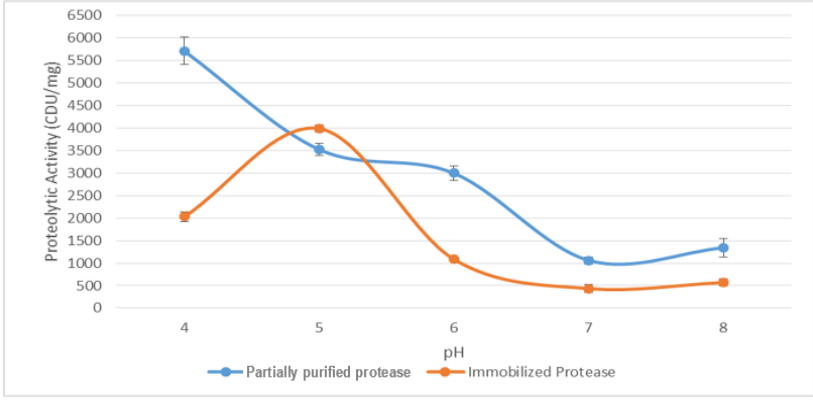

Figure 1 : Effect of pH on Proteolytic Actvity of Partially Purified Protease and Immobilised Partially Purified Protease Obtained Under Optimum Condition

Effect of temperature

The partially purified protease and immobilized partially purified proteases were assayed at different temperatures ranging from 40 to 65 °C at an optimum pH of 4 and 5, respectively. Based on Figure 2, the optimum temperature for the partially purified protease and immobilized partially purified protease were at 40 °C (6061.14 CDU/mg) and 50 °C (4956.54 CDU/mg), respectively. The optimum temperature obtained was in line with acidic protease (pH 3) from viscera of farmed giant catfish that was observed to be 40 °C and also similar with other acidic proteases extracted from sardinelle, smooth hound and european eel [17]. Anwar et al. [13] reported that immobilized proteases from newly isolated strain

of Bacillus subtilis, had an optimum temperature of entrapped enzyme at 50 °C. Furthermore, Geethanjali & Subash [2] observed that the optimum temperature for the isolated enzyme was 40 °C for both the entrapped and soluble forms. However, even at 50 °C, high activity of 98 % was observed for the immobilized enzyme.

Based on the result obtained, a shift to a higher optimum temperature that was observed from the free form of partially purified protease (40 °C) to immobilize partially purified protease (50 °C) shows that immobilization of protease plays a significant role in enzyme thermal stability. There may be several reasons responsible for these changes, which include, three-dimensional structure and activation energy of the immobilized enzyme [2]. Anwar et al. suggested that an increase in optimum temperature may result from a lower temperature in the gel microenvironment compared to the free form of enzyme solution [13]. Thus, it can be concluded that the immobilized conditioned can help in controlling the enzyme from being active and loss at lower temperature during the sample preparation process, since Malaysia temperature can reach up to 35 °C which is close to 40 °C.

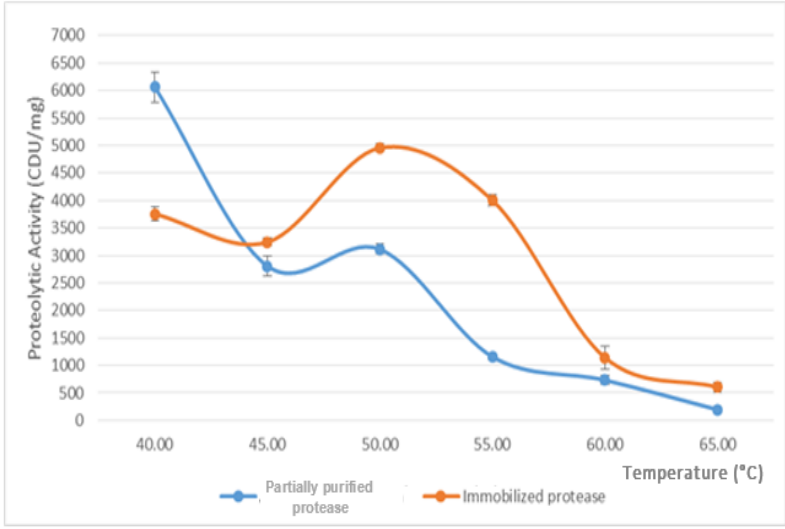

Figure 2: Effect of Temperature on Proteolytic Activity of Partially Purified Protease and Immobilised Partially Purified Protease Obtained under Optimum Condition

Storage Stability

The storage stability of partially purified protease and immobilized partially purified protease were conducted at storage temperature of 4 °C and -40 °C at an optimum pH for 6 days at 3 days interval. 4 °C was selected as it is the common temperature used to store enzyme solution and -40 °C was selected in order to have rapid freezing process and to avoid bigger size of ice crystallization.

Based on Figure 4 and 6, it was observed that the partially purified protease that was in the form of solution are slightly more stable when stored at frozen temperature (-40 °C) with only 33.50 % decrement at day 3 compared to chilled temperature (4 °C) that causes lost in proteolytic activity significantly ($P<0.05$) about 78.90 %. However, no significant difference ($P>0.05$) in percentage decrement of partially purified protease were observed for both storage temperatures on day 6. At 4 °C, the partially purified protease only retained about 11.39 % of its proteolytic activity on day 6 while at -40 °C, 17.44 %. Ali et al. [18] observed that microbial alkaline protease, from novel Bacillus licheniformis MZK03, was stable at 4 °C for 5 days with 100 % of its activity was retained and after 10 days only 33 % of its activity remained.

Meanwhile, on day 3 of the immobilized partially purified protease stored in frozen storage had already lost most of its proteolytic activity by 82.53 % and 93.81 % on day 6. In this study, a drastic decrease in the proteolytic activity at -40ºC was likely due to structural damages of the calcium alginate beads which occurred as a result of ice crystals formation within and around the beads during storage. In addition, the beads also shrink and lost its spherical shape when exposed and stored at an extreme temperature. Thus, when the sample was thawed, the enzyme which is supposedly entrapped within the beads had easily escaped into the surrounding solution.

Immobilized enzyme stored at 4 °C was likely to be more stable than those stored at -40 °C. Since, at day 3 only about 22.12 % of decrement was recorded and up to day 6 the beads still retained 24.50 % of its proteolytic activity. Anwar et al. [13] observed that the immobilized protease extracted from Bacillus subtilis stored at 4 °C showed 35 % loss of activity after

2nd days and after 10th day only about 11 % of its enzyme activity was retained. Keerti et al. [19] reported that immobilized *B*-glucosidase in alginate retained about 17.74 % of its original activity at 4 °C after 25 days. In general, if an enzyme is in solution form, it will not be stable during storage, and the activity will gradually reduce [13]. Thus, different storage condition of 4 °C and -40 °C for immobilized partially purified protease showed significant difference ($P<0.05$) in percentage decrement with 75.50 % and 93.81 %, respectively.

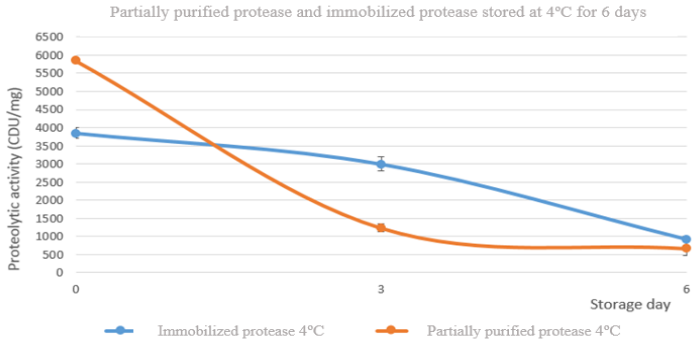

Figure 3 : Proteolytic Activity of Partially Purified Protease and Immobilized Partially Purified Protease Obtained under Optimum Condition Stored at 4 °C Storage Temperature

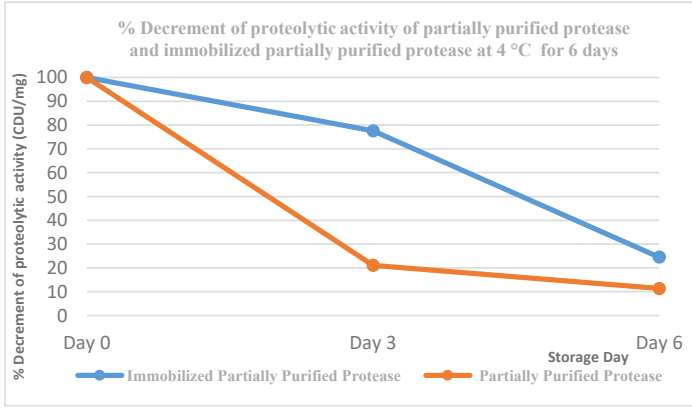

Figure 4 : % Decrement of Proteolytic Activity of Partially Purified Protease and Immobilized Partially Purified Protease Obtained under Optimum Condition Stored at 4 °C Storage Temperature

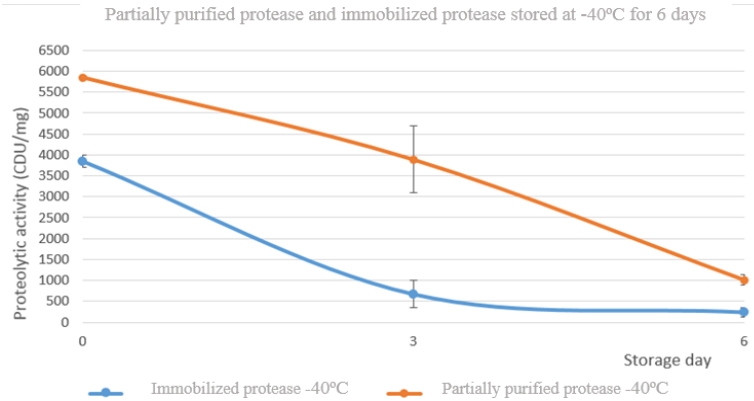

Figure 5 : Proteolytic Activity of Partially Purified Protease and Immobilized Partially Purified Protease Obtained under Optimum Condition Stored at -40 °C Storage Temperature

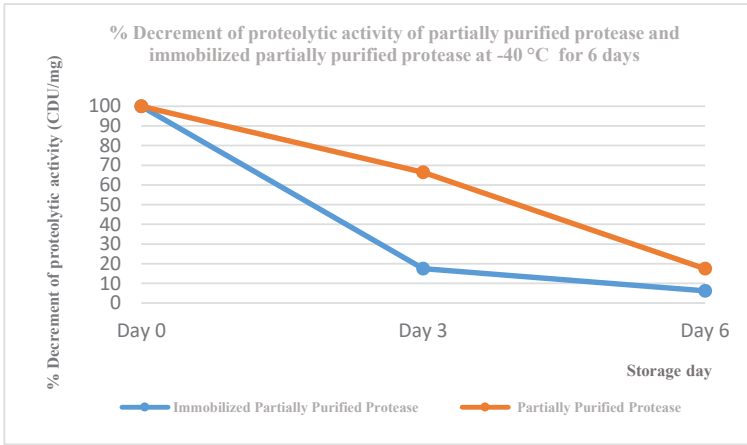

Figure 6 : % Decrement of Proteolytic Activity of Partially Purified Protease and Immobilized Partially Purified Protease Obtained under Optimum Condition Stored at -40 °C Storage Temperature

CONCLUSION

The effect of pH, temperature and storage stability on the proteolytic activity of partially purified protease and immobilized partially purified protease were successfully studied. The highest proteolytic activity for partially purified protease was recorded at pH 4 (5709.76 CDU/mg), 40 °C (6061.14 CDU/mg) and at – 40 °C (17.44 % storage efficiency, day 6). While, for immobilized partially purified protease at pH 5 (3987.85 CDU/mg), 50 °C (4956.54 CDU/mg) and at 4 °C (24.5 % storage efficiency, day 6). Therefore, it can be concluded that the immobilized partially purified protease can be easily being handle and use since the activation energy is higher thus can prevent the loss of enzyme due to room temperature condition. Next, the immobilized partially purified protease can be easily stored in the chiller and doesn't need any freezing process thus can cut the cost of electricity. Lastly, based on several studies it was mention that the immobilized enzyme beads can be recycle and reuse for a few cycles until there is no presence of proteolytic activity or the activity reach its minimum.

ACKNOWLEDGEMENT

The authors would like to express their gratitude to the Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM) Shah Alam, for the use of the laboratory facilities.

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APPENDIX A

Table 1: Effect of pH on Proteolytic Activity of Partially Purified Protease and Immobilised Protease Obtained under Optimum Condition

pH	Proteolytic activity (CDU/mg	
	Partially Purified Protease	Immobilised Protease
4	5709.76±302.95^{Aa}	2029.84±102.44^{Bb}
5	3522.65±127.01^{Bb}	3987.85±80.69^{Aa}
6	2995.95±156.05^{Ac}	1084.34±33.17^{Bc}
7	1060.04±82.96^{Ad}	434.25±93.31^{Bd}
8	1341.81±199.83^{Ad}	570.53±78.95^{Bd}

Average means ± standard deviation (n=5)
Different capital letters in a rows show significantly different between sample (P<0.05)
Different small letters in a columns show significantly different between pH (P<0.05)

APPENDIX B

Table 2: Effect of Temperature on Proteolytic Activity of Partially Purified Protease and Immobilised Protease Obtained under Optimum Condition

Temperature (ºC)	Proteolytic activity (CDU/mg)	
	Partially Purified Protease	Immobilized partially purified
40	6061.14±272.90^{Aa}	3755.07±126.11^{Bc}
45	2807.37±179.31^{Bc}	3242.36±68.52^{Ad}
50	3112.34±90.43^{Bb}	4956.54±72.16^{Aa}
55	1157.65±45.52^{Bd}	4002.58±100.89^{Ab}
60	731.86±80.33^{Be}	1141.43±205.86^{Ae}
65	195.58±62.23^{Bf}	604.05±86.79^{Af}

Average means ± standard deviation (n=6)
Different capital letters in a rows show significantly different between sample (P<0.05)
Different small letters in a columns show significantly different between temperature (P<0.05)

APPENDIX C

Table 3: Protease Activity at Different Storage Temperatures (Partially Purified Protease)

Condition	Day 0	Day 3	%decrement	Day 6	%decrement	%Storage efficiency
Frozen (-4°C) (CDU/ml)	5847.88 ± 45.8^{5aA}	3888.77 ± 795.03^{bA}	33.50	1019.89 ± 120.82^{cA}	82.56	17.44
Chilled (4°C) (CDU/ml)	5847.88 ± 45.85^{aA}	1233.89 ± 103.48^{bB}	78.90	665.93 ± 203.95^{cA}	88.61	11.39

Means within each row and column with different superscript are significantly different at $p < 0.05$.
Small letters indicate the effect of storage day on the protease activity.
Capital letters indicate the effect of storage condition on the protease activity.

APPENDIX D

Table 4: Protease Activity at Different Storage Temperatures (Immobilized partially purified Protease Obtained under Optimum Condition)

Condition	Day 0	Day 3	%decrement	Day 6	%decrement	%Storage efficiency
Frozen (-40°C) (CDU/mg)	3849.73 ± 143.29^{aA}	672.56 ± 320.40^{bB}	82.53	238.31 ± 111.65^{cB}	93.81	6.19
Chilled (4°C) (CDU/mg)	3849.73 ± 143.29^{aA}	2998.16 ± 191.82^{bA}	22.12	943.29 ± 81.91^{cA}	75.50	24.5

Means within each row and column with different superscript are significantly different at $p < 0.05$.
Small letters indicate the effect of storage day on the protease activity.
Capital letters indicate the effect of storage condition on the protease activity.