

Euthynnus affinis viscera-an alternative source for protease and lipase enzymes: Characteristic and potential application as destainer agent

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Abstract. *Mardina V, Harmawan T, Fitriani, Sufriadi E, Febriani, Yusof F. 2020. Euthynnus affinis viscera-an alternative source for protease and lipase enzymes: Characteristic and potential application as destainer agent. Biodiversitas 21: 5858-5864.* This study described the characterization of protease and lipase, extracted from the viscera of *Euthynnus affinis* and their applications as stains removal. Enzyme increased a few folds (3.99 folds for protease and 2.45 folds for lipase) after the submission to partial purification processes by ammonium sulfate precipitation and dialysis. Molecular mass assessment by SDS-PAGE showed that the enzyme solution contains three protein bands, estimated at 70, 42, and 24 kDa for protease and 32 kDa for lipase. Zymography confirmed the presence of protease and lipase in the sample. The protease and lipase exhibited optimum activities at pH 7.0 and pH 8.0 respectively, active at temperatures from 35 to 75°C with optimal activities at 65°C for both. The enzymes were stable at alkaline pH after 90 minutes (mins) of incubation and were also stable up to 65°C, showing the remaining activities of more than 70%. Samples containing protease and lipase as de-staining agents were tested by their ability to remove blood and palm oil stains, with results proving they were effective. Overall, the study showed that crude fish visceral extract contains protease and lipase and is useful in removing household stains, and could substitute for commercial detergents.

Keywords: *Euthynnus affinis* viscera, lipase, protease, partial purification, stain removal

Abbreviations: BSA: bovine serum albumin, CL: crude lipase, CP: crude protease, DL: dialysate fraction of lipase, DP: dialysate fraction of protease, kDa: kilodaltons, M: marker, pNPP: p-nitrophenyl palmitate, SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, TCA: trichloroacetic acid

INTRODUCTION

Enzymes play a pivotal role in biotechnological industries, facilitating the development of green, sustainable and modern industry. The global enzyme market attained \$6 billion in 2017 (Arnau et al. 2020) and is predicted to attain a value of \$6.32 billion by 2021 (Chapman et al. 2018). Of the 60% of enzymes marketed worldwide in 2018, proteases accounted for 20% (Razzaq et al. 2019) particularly in technical enzymes, coming second after carbohydrases and before lipases in the world enzyme market (Li et al. 2012). One of essential and profitable application for technical enzyme is in detergent, where the total global market size was 1.11 billion in 2016 and projected to increase to 1.33 billion in 2021 (Chapman et al. 2018) or a compound annual growth rate (CAGR) of approximately 11.5% throughout 2015-2020 (Singh et al. 2016).

The development of enzymes in detergent formulation started with crude trypsin used as additives on 'Burnus' in 1913 (Amara et al. 2009), followed by Bio-40, Biotex, Maxatase, Era plus[®], Tide[®], Dynamo[®] and others (Kumar et al. 2008). The addition of enzymes in detergent mix was motivated by energy and environmental concerns

by washing in lower temperatures and reducing pollution by non-biodegradable chemicals (detergent) as well as excellent specificity. The addition of enzymes such as protease and lipase to detergent releases proteinaceous material or insoluble triglycerides from stains by catalyzing the breakdown of chemical bonds after water is added. Enzymes are also better than conventional chemicals, as they can remove stubborn stains but degrade before entering waterways, alleviating, or preventing water pollution (Naganthran et al. 2017).

Technological development in detergent industries has allowed subtilisins, a protease enzyme from *Bacillus subtilis*, as additive. However, Ktari et al. (2014) stated that this enzyme is not ideal for detergent since it has not fulfilled specific criteria as detergent additive viz. low thermal stability and short shelf life. Thus, it is pertinent that countries-especially in Asia that have abundant, inexpensive, and alternative sources of the enzyme such as *Euthynnus affinis* viscera, investigate properties of enzyme source that is compatible with the composition of detergent. The study highlighted the extraction, partial purification, and characterization of protease and lipase enzymes from the liver and pancreas of *E. affinis* as well as their application

as bio detergent for the removal of stains.

MATERIALS AND METHODS

Materials

Euthynnus affinis was collected from Langsa Market, Aceh. Materials in this study were analytical grade that consists of phosphate buffer, Tris, TCA, Na₂CO₃, Folin reagent, pNPP, Triton X-100, SDS, NaOH, tyrosine, HCl, ammonium sulfate, Coomassie Brilliant Blue R-250, glycine, loading dye, and the protein molecular weight marker (10-175 kDa).

Procedures

Preparation for the source of enzyme

E. affinis viscera organ viz. liver and pancreas were separated and washed by aquadest for enzyme extraction. The phosphate buffer (1 M) at pH 7 was added to the individual organ in the ratio of 1:2 (w/v) for liver and pancreas to the buffer solution. The mixture was homogenized before filtered through a cotton cloth to remove solid residues and centrifuged at 10,000 rpm, 4°C, for 10 mins. The supernatant was then collected as the crude enzyme (Mardina et al. 2018).

Protease and lipase assay

The protease assay had a few procedures. The substrate was prepared by diluting the 1% (w/v) casein in 50mM Tris buffer (pH 8). The proteolytic activity was measured by incubating the enzyme with casein for 10 mins. It was followed by adding 2.5 mL of 0.11 mM TCA then left in room temperature for 30 mins before centrifuged at 10,000 xg for 10 mins in 4°C. The supernatant was mixed with 2.5 mL of 0.5 M Na₂CO₃ and 0.5 mL of 0.1 N Folin reagent for 20 mins, showing absorbance at 660 nm. The protease activity unit was defined as the amount of enzyme required to liberate 1µmole of tyrosine per minute under the assay condition (Mardina et al. 2015; Mardina and Yusof 2016).

For the lipase, the substrate was prepared by dissolving pNPP with 1% (w/v) Triton X-100 and 2% sodium dodecyl sulfate. The lipolytic activity was assayed by mixing 0.5 mL crude enzyme with 0.5 mL of 0.2 M Tris-HCl (pH 8) and 0.5 mL pNPP stock solution. The mixture was incubated at 37°C for 10 mins before adding 1 mL NaOH, centrifuged to collect the supernatant which measured at 410 nm. One unit of lipase activity was defined as the amount of enzyme which liberated 1 µm of *p*-nitrophenol per min from pNPP (Tripathi et al. 2014).

Protease and lipase activities were analyzed by the following formula (Mardina et al. 2018):

$$\frac{\text{Protease activity } \frac{\text{Units}}{\text{ml}}}{(\text{umole of tyrosine release} + \text{total volume of assay (ml)})} = \frac{(\text{volume of used enzyme (ml)} \times \text{time of assay} + \text{volume in cuvette (ml)})}{(1)} \quad (1)$$

$$\frac{\text{Lipase activity } \frac{\text{Units}}{\text{ml}}}{(\text{umole of p-nitrophenol release} + \text{total volume of assay (ml)})} = \frac{(\text{volume of used enzyme (ml)} \times \text{time of assay} + \text{volume in cuvette (ml)})}{(2)}$$

Standard calibration curves for protease and lipase enzymes

The standard curve for protease activity was analyzed by the method of Mardina and Yusof (2018). The tyrosine was prepared at the concentration of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µL. Varying concentration of the dH₂O, Na₂CO₃, and Folin reagent was mixed to the tyrosine solution to obtain the total mixture of 4mL. Absorbance was read at 660nm. The standard calibration curve for lipase was prepared by having 5 mM *p*-nitrophenol (0.05-0.5 mL in 0.1 M phosphate buffer (pH 7), following the method described in Yusof et al. (2016). The absorbance of the standard solution was read at 410 nm. The relationship between the absorbencies and the concentrations of standard solution, µM L-tyrosine and mM *p*-nitrophenol were plotted as y/x line plots for protease and lipase activities respectively.

Partial purification

Partial purifications of the crude enzymes were carried out using the suggested method by Mardina and Yusof (2016). Crude enzymes were fractionated by 100% (w/v) ammonium sulfate overnight at 4°C with constant stirring. The precipitates were recovered by centrifugation at 10,000 xg, 4°C for 15 mins. Moreover, they were resuspended in 50 mM Tris-HCl buffer, pH 8 before dialyzed against the same buffer for 24 h.

Protein assay

Protein concentration in the sample was determined by the Bradford method as suggested by Mardina and Yusof (2018) and Waterborg (2002) with BSA as the reference standard. The prepared solution consists of 0.1 mg /mL BSA, 0.1M sodium acetate, and such amount of Bio-Rad protein Dye reagent. For example, each tube (tube 1 and 2) had 0 mL and 30 mL BSA solution and mixed to 800 µL and 770 µL sodium sulfate before completed to 200 µL protein Dye reagents. The experiment was performed in triplicate and the absorbance was read at 595nm.

Molecular weight determination

SDS-PAGE was carried out using 4% (w/v) stacking and 10% (w/v) separating gel according to Laemmli (1970). 15 µL enzyme solutions were added to 5 µL loading dye and heated for 3 mins at 100°C to denature the protein. The electrophoresis was conducted at 125 volts, 60mA for 70 minutes. The protein bands were visualized through Coomassie blue (0.1%, w/v) staining for 20 mins and then de-stained in acetic acid (10%, v/v) for 60 mins. 10-175 kDa of the protein molecular weight marker was taken as the standard.

Detection of protease and lipase activities by zymography

Staining of protease and lipase activities was performed on SDS-PAGE, according to Ktari et al. (2014), emphasizing not to heat the enzymes before electrophoresis. Electrophoresis condition was set as the mention above. Gels were submerged in a renaturing solution containing 2.5% Triton X-100 and agitated for 30 mins and then immersed in incubation/developing buffer. Finally, the gels were stained using Coomassie Brilliant Blue R-250 for visualization.

Effect of pH on activity and stability of enzyme

The effects of pH on protease and lipase activities were studied over a pH range of 4.0-9.0, using casein and p-NPP as the substrates. The measurement of pH stability against enzymes was prepared by incubating crude enzyme for 60 mins at 37°C in a different buffer with residual activities measured under the standard assay conditions (According to formula of 1 and 2). The following buffer system consists of citrate buffer (pH 4-5), Tris-HCl buffer (pH 6-8), and glycine-NaOH buffer (pH 9) (Mardina and Yusof 2016).

Effect of temperature on activity and stability of enzyme

The effect of temperature on protease and lipase activities was investigated from 35°C to 75°C, using casein and p-NPP as a substrate in the phosphate buffer, pH 7.0. Thermal stability was examined by incubating the enzyme preparation for 90 min at a different temperature and aliquots were withdrawn in intervals (30 min, 60 min, and 90 min) to calculate the remaining activity. The non-heated crude enzyme was set as a control (Mardina and Yusof 2016).

Efficacy of the protease and lipase for removal of stains

Protease and lipase as destainer were tested based on the method of Mahmoud et al. (2015) with slight modification. 20 µL of chicken blood and palm oil were poured into a white 4 x 4 cm² cloth and left for 10 mins at room temperature. Afterward, the cloth was dried for 5 mins inside an oven (80 °C). The samples were incubated for 30 minutes (60°C) at different treatment condition, as follows: (i) 100 mL dH₂O in beaker + cloth as a control; (ii) 100 mL dH₂O in beaker + stained cloth; (iii) 100 mL dH₂O in beaker + stained cloth + detergent components (1% SDS, 1% tween 20, 1% Triton X-100, and 1% H₂O₂); (iv) 100 mL dH₂O in beaker + stained cloth + the crude enzymes (protease and lipase); (v) 100 mL dH₂O in beaker + stained cloth + detergent components (1% SDS, 1% tween 20, 1% Triton X-100, and 1% H₂O₂) + the crude enzymes (protease and lipase).

The de-staining activity of protease and lipase was observed for 10, 20, and 30 min incubation respectively at an optical density of 518nm. Visual examination of various pieces was done to identify the performance of enzymes in stain removal.

RESULTS AND DISCUSSION

Purification of protease and lipase

Various methods exist to recover protease and lipase enzymes from crude sample extract. For efficient protein recovery in native conditions, this study submitted the crude extract to ammonium sulfate precipitation as the initial step of enzyme concentration (Mala and Takeuchi 2009), followed by dialysis to remove excess salt. The result revealed that specific activity increased two times for the protease, and slightly improved for the lipase (Table 1).

Table 1. Partial purification for protease and lipase enzyme from *Euthynnus affinis* viscera

Purification step	Total activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Protease					
Crude enzyme	57.786	8.412	6.869	100	1
(NH ₄) ₂ SO ₄ precipitation	51.358	3.867	13.281	88.876	1.93
Dialysis	43.089	1.5702	27.410	74.566	3.99
Lipase					
Crude enzyme	69.425	4.615	15.043	100	1
(NH ₄) ₂ SO ₄ precipitation	42.345	2.116	20.012	60.994	1.330
Dialysis	37.534	1.018	36.870	54.064	2.450

Characterization of the protease and lipase

SDS-PAGE and zymography of protease and lipase

SDS-PAGE and zymography analysis estimated the molecular weight number of proteases and lipase enzymes in the crude enzymes and the dialysis samples. Zymography staining revealed the proteolytic and lipolytic activities, with casein and p-NPP as a substrate for protease and lipase, respectively. Figure 1 showed three major bands of protease activity with different molecular weights. However, only one major clear band was shown by lipolytic activity in the dialyzed sample. These findings inferred that at least three major proteinases and one lipase were present in the liver and pancreas of *E. affinis*.

The molecular weight of the protease after dialysis was found to be 70, 42, 24 kDa on SDS-PAGE and the zymography analysis confirmed that the proteolytic activity was displayed by 42 kDa (Figure 1.B). A similar result was recorded by Chaijaroen and Thongruang (2016) who found the partial and crude protease from *Nile tilapia* viscera exhibited three protein bands on SDS-PAGE assigned at 56.22, 31.33, and 26.71 kDa. Geethanjali and Subash (2018) found that protease from *Labeo rohita* viscera revealed a molecular weight of 38 kDa. Freitas-Junior et al. (2012) stated that the molecular weight of protease derived from several fish visceral falls within a range of 23-28 kDa. Sabtecha et al. (2014) observed that the purified proteases extracted from visceral waste of Red snapper, Great barracuda, Sardines, Seer fish, and Milk shark exhibited a single clear band of molecular mass of 35 kDa, 34 kDa, 62 kDa, 32 kDa-50kDa, 20 kDa-35 kDa respectively. Notably, alkaline proteases with a molecular mass between 18 and 90 kDa have also been reported.

SDS-PAGE and zymography analysis forecasted the expected molecular mass of lipase was about 32 kDa (Figures 1.A and 1.B). This finding was supported by Sharma et al. (2014) who characterized lipase from the alimentary canal and digestive gut of *Catla catla* (catla) with 30 and 67 kDa in dialysate fraction of SDS-PAGE. Similarly, Kurtovic et al. (2009) reported that a partially purified lipase from the hepatopancreas of neo flying squid (*Ommastrephes bartramii*) showed a molecular weight of 33 kDa, lower compared to the pancreatic lipase from mammalian species.

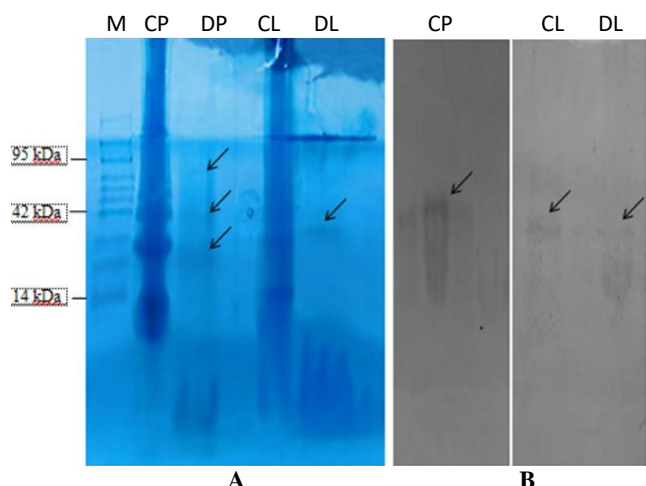


Figure 1. SDS-PAGE (A) and zymogram analysis (B). (M: marker, CP: crude protease, DP: dialysate fraction of protease, CL: crude lipase, DL: dialysate fraction of lipase)

Optimum pH and pH stability on protease and lipase activities

Optimum pH for the protease and lipase were determined through a variety of buffer ranging from pH 4 to pH 9, resulting in pH 7 for protease and pH 9 for lipase (Figures 2.A and 2.B). The optimum pH for protease was

lower than the protease from Zebra blenny viscera, which was pH 8 as the optimal pH for proteolytic activity (Ktari et al. 2014). However, the optimum pH for the lipase activity was in line with Prasertsan and Prachumratana (2007), who found pH 9 as the optimum pH for lipase from tonggol tuna viscera. Kuepethkaew et al. (2016) reported that the optimal pH for lipase activity from the pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas was 8.5. Furthermore, Gorgun and Akpinar (2012); Sae-leaw and Benjakul (2017) stated that the optimum pH for lipase from the liver of *Cyprinus carpio L* and *Lates calcarifer* was 8. These differences could be explained by the changes in digestive enzymes in fish viscera due to different diets (Dizhi et al. 2018).

The stability of protease and lipase against pH was investigated by incubating the enzyme extract in different pH buffers for 60 mins with the results in Figures 3.A and 3.B, presented as residual activities. Figure 3.A shows that the activities were maintained high from pH 4 to pH 8 (approximately 93-98%); however, the activity decreased significantly above pH 8 and only retaining approximately 66% at pH 9. As in the case of lipase enzyme in Figure 3.B, the activities were maintained high (approximately 90 to 99%) in the tested pH values range (pH 4 to 9); however, it dropped to 75% at pH 6.

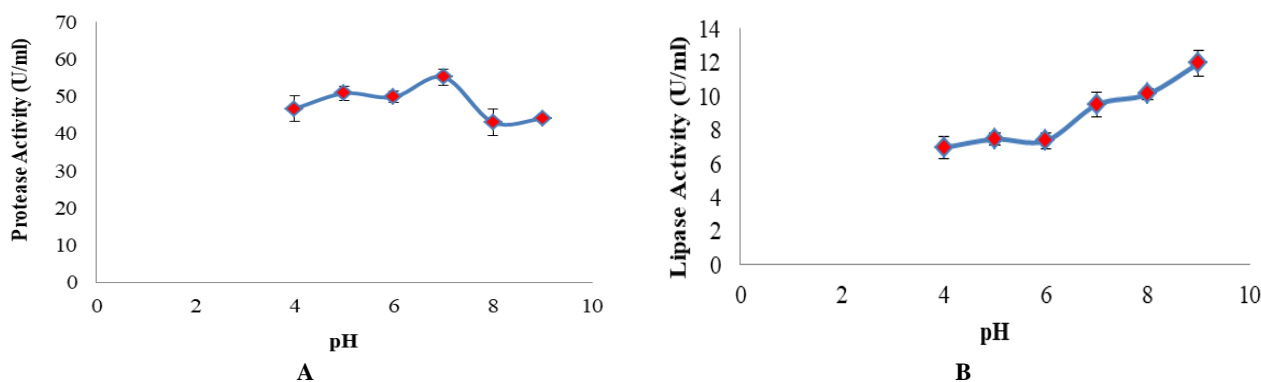


Figure 2. pH profile of the crude protease (A) and lipase (B) from viscera of the *Euthynnus affinis*. Protease and lipase activities were assayed in the pH range from 4.0 to 9.0 at 37°C

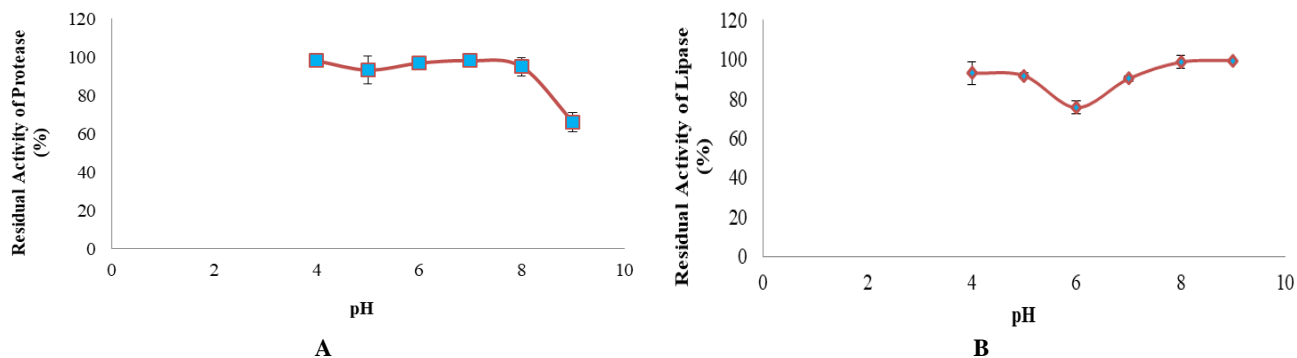


Figure 3. The pH stability of (A) the crude protease and (B) lipase from *Euthynnus affinis* viscera. The pH stability was determined by incubating the enzyme in different buffers (pH 4 to 9) for 60 min at 37 °C, and the residual activity was examined at pH 7 and 37°C using casein and pNPP as substrate. Enzyme activity before incubation was taken as control (100%)

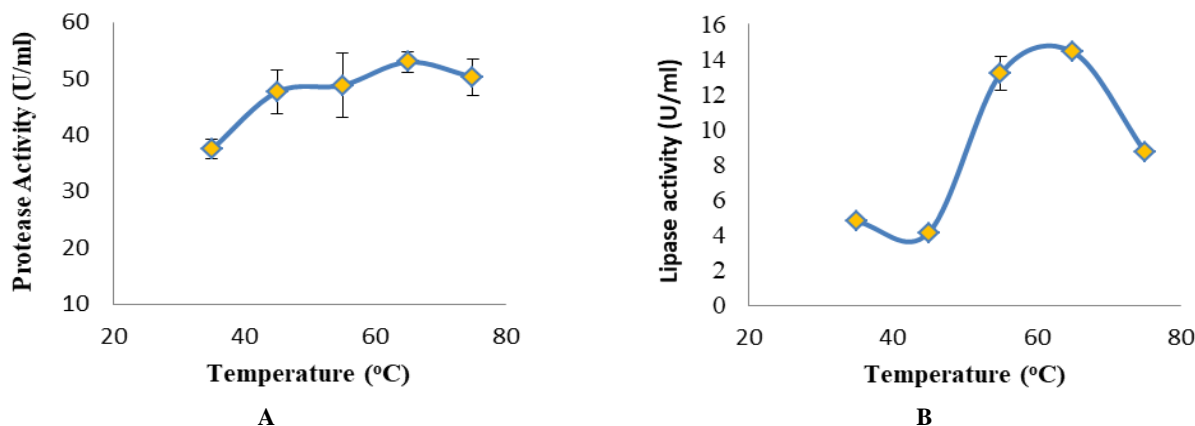


Figure 4. Temperature profile of the crude protease (A) and lipase (B) from the liver and pancreas of *Euthynnus affinis*. Enzyme activity was assayed at different temperatures ranging from 35 °C to 75 °C at pH 7 using casein and pNPP as substrates

Miyanaga and Unno (2011) stated that enzyme activity and stability were conditioned on the reaction solution pH value, where the active sites of enzyme consist of amino acids with some ionic groups. The dissociation state of amino acid residue significantly influences the structure of the active site, the affinity to the substrate, and the catalytic activity. In this study, the decrease of residual lipase activity might be related to the state of acidic ionization or basic amino acids as it is in Tris-HCl medium (pH 6). Acidic amino acids have carboxyl functional groups in their side chains. Basic amino acids have amine functional groups in their side chains. If the state of ionization of amino acids in a protein is altered, then the ionic bonds determining the protein's 3-D shape can be altered. This can lead to altered protein recognition or inactivation of an enzyme. Changes in pH may not only affect the shape of an enzyme but also change the shape or charge properties of the substrate, making the substrate unable to bind to the active site or unable to undergo catalysis. To counteract this, Liu and Cheng (2000) suggested the use of soluble additives, immobilization technique, protein engineering, and chemical modification.

Nevertheless, it is expected that the residual activity will decrease or have a total loss of activity beyond pH 9, from extremely high or low pH values. These results are aligned with Kurtovic et al. (2009), who reported most purified or partially purified fish digestive lipases have pH stability in the range of pH 5.0-pH 9.5.

Optimum temperature and temperature stability of protease and lipase activities

The effect of temperature on protease and lipase from the liver and pancreas of *E. affinis* was investigated by assaying enzyme activities at different temperatures. Figures 4.A and 4.B show that protease and lipase were active at different degrees of temperatures, from 35 to 75°C, but both enzymes work optimally at 65°C. For thermal stability, both enzymes were stable up to 65°C which showed residual activities of more than 70% (81% for protease and 72% for lipase) even after a 90 mins incubation (Figures 5.A and 5.B). In particular, protease was shown to be stable up to 75°C, while maintaining

about 78% residual activity, even after 90 mins incubation. Unlike protease, lipase revealed a significant decrease in residual activity (34%) when incubated at 75°C for 90 mins likely due to thermal denaturation (Figure 5.B).

The enzyme stability trend follows previous studies. Chaijaroen and Thongruang (2016) reported that the crude protease from Nile Tilapia had an optimum temperature of 60°C, and the enzyme activity decreased significantly after 30 mins of incubation. Additionally, the crude proteases from Zebra Blenny (Ktari et al. (2014) and Cobia (*Rachycentron canadum*) (Shobana and Subash 2013) viscera displayed maximum activity at 60°C. However, this study identified that visceral protease from *E. affinis* has a slightly higher optimum temperature (65°C) compared to protease extracted from Nile Tilapia, Zebra Blenny, and Cobia.

The optimum temperature for lipase of *E. affinis*, 60°C, is also aligned with Kuepethkaew et al. (2016), who studied laundry detergent-stable lipase from the Pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas. Kuepethkaew et al. (2016), found that pH stability was significantly affected by temperature, as shown in Figure 5.B, where the fish protease was highly stable at a temperature range between 35°C and 65°C, maintaining more than 70% of its original activity after 90 mins of incubation. In contrast, lipase activity decreased significantly when the enzyme was exposed above 75°C with the residual activity of about 34% after 90 mins of incubation.

Application of protease and lipase as destainer agent

A simple washing method was conducted to evaluate the ability of protease and lipase enzymes as a new bio-friendly detergent formula to remove stains. Figure 6 visualizes the results of the enzyme performance in the removal of stains. Furthermore, their ability was analyzed in the presence and absence of component detergent and presented in the optical density at 518 nm (Figure 7).

Figure 6 shows that the crude enzymes could effectively remove the stains after incubation/washing for 30 min, with no addition of detergent components (no. c-3). It is also compatible with detergent components such as

sodium dodecyl sulfate (SDS), Tween-20, Triton X-100, and hydrogen peroxide (H₂O₂) to de-stain the blood and palm oil as described in No. c-4. Furthermore, the reflectance of the stained cloth treated with and without supplement of the enzymes was measured at OD518nm (Figure 7). Observation at OD518nm showed that the stain removal rate on Experiment 3 was highest compared to other treatments.

Moreover, the ability of the crude enzymes to remove blood and palm oil stains was investigated further by

rinsing the treated cloth using acetone, and solution measurement was taken at O.D. 518nm (Figure 7). The reflectance of the acetone on the rinsing step revealed the low value of experiment 3; suggesting that the stained cloth in experiment 3 had cleaned (the blood and palm oil stains had been removed in previous washing step). Thus, interestingly, the direct applicability of the crude extracts without downstream processing deems it acceptable as a substitute for commercial ones.

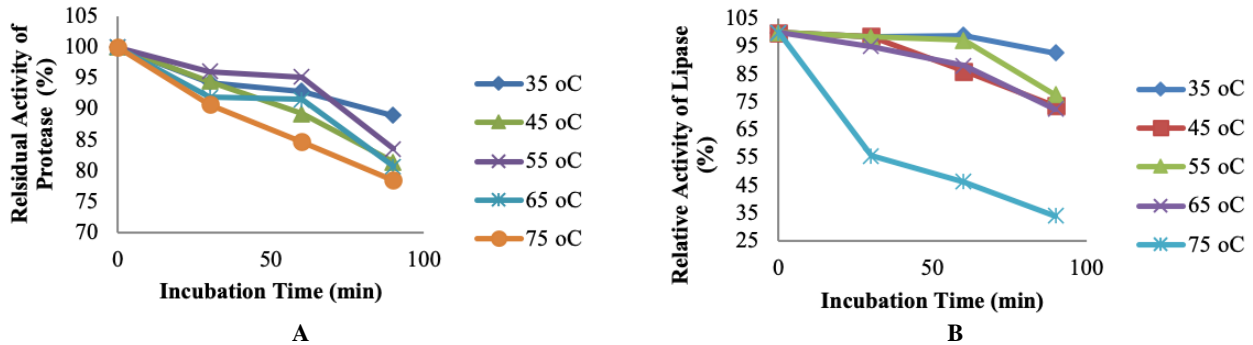


Figure 5. Thermal stability of the liver protease (A) and the pancreas lipase (B) of *Euthynnus affinis*. The enzymes were incubated at different temperatures for 30 min, 60 min, 90 min. The residual enzyme activities were assayed at pH 7.0 and 37 °C. The non-heated enzyme was considered as control (100%)

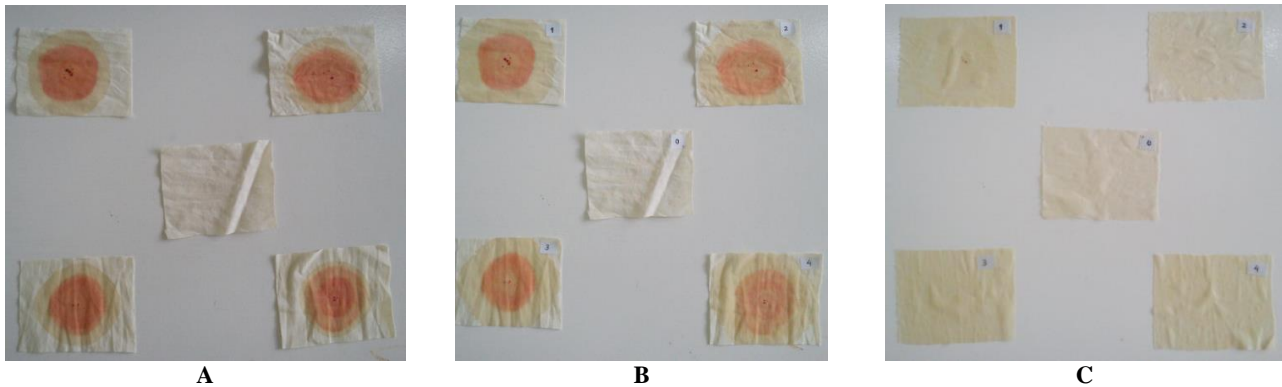


Figure 6. Application of the crude enzyme (protease and lipase) from *Euthynnus affinis viscera* on the destaining of blood and palm oil. A. The stained clothes, B. The stained clothes after drying, C. The stained cloth after incubation/ washing. (0: control, 1: the stained cloth, 2: the stained cloth with detergent only, 3: the stained cloth with the crude enzymes only), 4: the stained cloth with detergent components and crude enzymes (protease and lipase)

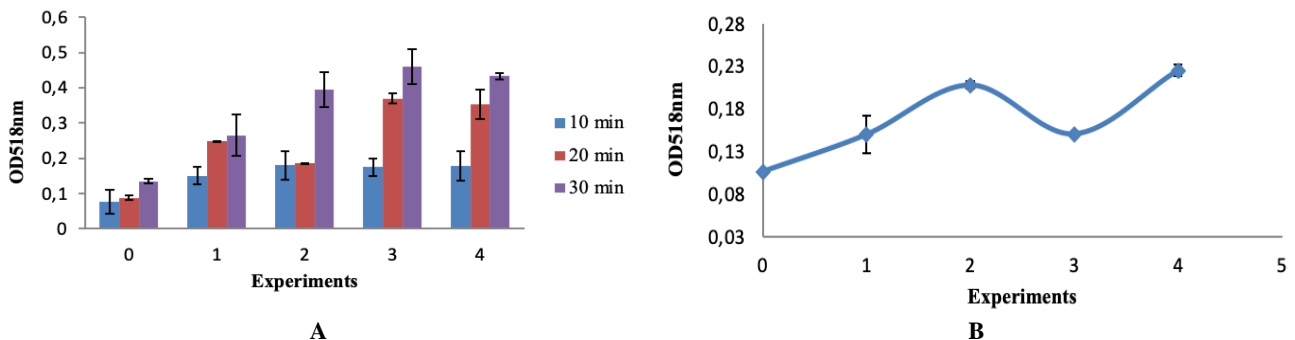


Figure 7. A. At washing step, B. At rinsing step. Reflectance of blood and palm oil-stained cloth with and without the addition of the crude protease and lipase from liver and pancreas of *Euthynnus affinis* (0) Control; (1) the stained clothes; (2) the stained clothes with the addition of detergent components; (3) the stained clothes with the supplement of the crude enzymes only (protease and lipase); (4) the stained clothes with detergent components and the crude protease and lipase

The study characterized crude and partially purified protease and lipase from *E. affinis* viscera to determine their use as additive detergent, with findings suggesting their properties as destainer agents. For instance, they were stable at alkaline pH (pH 8 for protease and pH 9 for lipase) with the residual activity more than 80% and stable at a temperature range of 35°C to 65°C by maintaining the residual activity of approximately 75%. Additionally, the ability of these partially purified crude enzymes to remove blood and palm oil stain indicate that they can be used as an additive or destainer agent in the detergent industry.

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