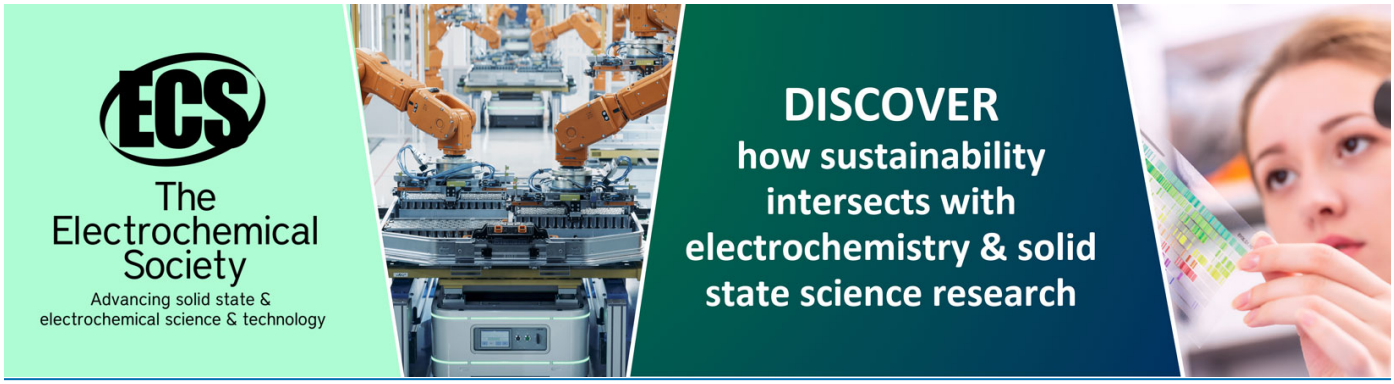
Screening of protease and lipase sources from visceral organs of *Euthynnusaffinis*



Screening of protease and lipase sources from visceral organs of *Euthynnusaffinis*

Vivi Mardina[1)*], Tisna Harmawan[2)], Fitriani[1)], Goldha Maulla Hildayani[3)], Faridah Yusof[4)]

[1)]Department of Biology, Faculty of Engineering, Samudra University, Kampus Unsam Meurandeh, Langsa 24415 Indonesia
[2)]Department of Chemistry, Faculty of Engineering, Samudra University, Kampus Unsam Meurandeh, Langsa 24415 Indonesia
[3)]Department of Chemistry, Faculty of Mathematical and Natural Science,University of Sumatera Utara, Medan Indonesia
[4)]Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, PO Box 10, Kuala Lumpur 50728

vmardina@unsam.ac.id

Abstract. *Euthynnusaffinis* is one of tuna species that distributed throughout the world. Their visceral accounts for approximate less than 20% of body weight and its value are still underutilized. This study screened four the potential individual visceral organ to produce protease and lipase enzyme viz. spleen, stomach, pancreas, and liver. Among the individual visceral organ, liver exhibited the highest source for protease activity (57.786 U/ml), while pancreas revealed as the best source for lipase activity (69.425 U/ml). Therefore, the finding of alternative sources for protease and lipase in this study might converted visceral waste by product to generate protease and lipase enzyme that may be applied in biotechnological process.

1. Introduction

Tunas are widely distributed throughout the world. Generally they are found in temperate to tropical waters between about 45° north and south of the equator and are broadly classified into coastal, neritic and oceanic species. They are grouped taxonomically in the family *Scrombridae*, which comprise of 50 species and forms the third largest product in the international seafood trade with almost 10% of the total trade in value terms (FAO 2008). One of the principal market species of tuna *is Euthynnusaffinis* [1].

Euthynnusaffinis (Figure 1), the alternative name as the 'little tuna' or 'kawakawa' is a medium sized tuna that be found in the Indian Ocean. Indonesia is considered to be among the most important tuna fishing countries in Asia besides Japan, Taiwan, and South Korea. Increasing demand for industrial tunas has been started since1940s with the global catch reached more than 4.3 milliontons in 2005 and estimated to be increase in further years [1] which Indonesia alone contributed 374,047 tons in 2015 [2].

Figure 1. *Euthynnusaffinis*

Increasing demand for industrial tuna will involve tuna in processing at both household and industrial scale. As a consequence, this may produce large amounts of fishery waste that consist of liquid and solid waste. Fishery wastes which are the waste of fishery activity, account for 25-30% of the fish processing amount or approximately 20 million tons per year [3]. These include head, tail, fins, bone, skin and fish innards that have been used as microbial growth medium, organic liquid fertilizer and collagen [4, 5]. Unlike the previously mentioned waste, the viscera waste as a by-product of fish processing has not been handled intensively, while it is abundant and inexpensive waste; so it may cause environmental and health problems [6]. In order to overcome these problems, conversion viscera waste into another product that has added value particularly protease and lipase enzymes [6, 7, 8] consider to be a promising technology [9] and able to minimize environmental pollution [6,7], besides improve people's welfare in future. Therefore, this study will focus to conduct the screening of protease and lipase sources from the individual visceral organ of *Euthynnusaffinis*as the main objective.

2. Materials and Methods

2.1. Materials

All chemicals used for analysis are analytical grade and obtained from C.V Multikreasi Medan. UV-Vis 1240 Spectrophotometer Shimadzu was used for optical absorbance measurement and VS-GoocFi Multi-tube Carrier Refrigerated Centrifuge was used routinely for centrifugation process. Experiments were conducted in triplicates.

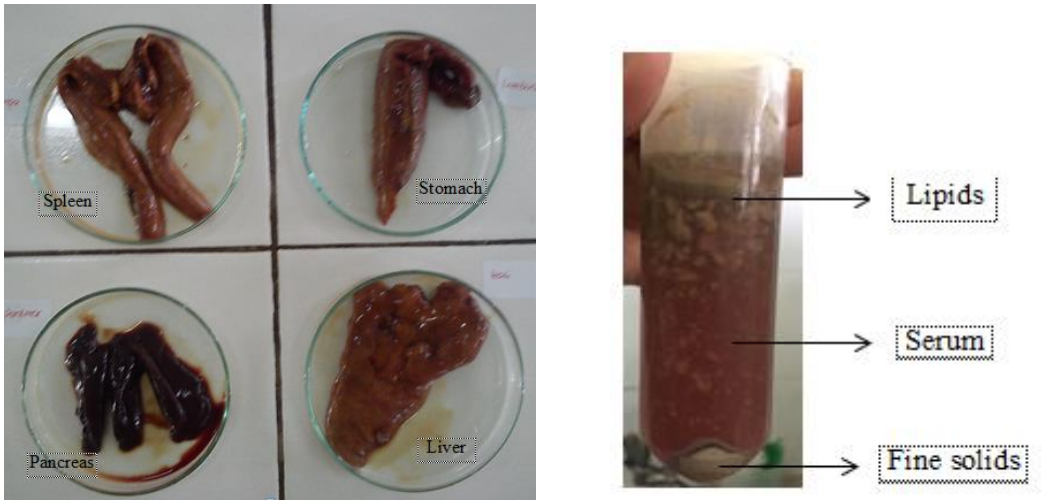
2.2. Methods

2.2.1. Sample Preparation for Source of Enzyme

Fish viscera were obtained from the local market in Langsa, Aceh, Indonesia and separated to individual viscera organ (stomach, liver, pancreas and spleen). The individual viscera organs were then cleaned and kept frozen at -20^{o} in sealed plastic bags until needed for enzyme extraction. Each viscera organ of *Euthynnusaffinis*is illustrated in figure 2(a).

2.2.2. Extraction Process

Extraction process of the samples was performed by a method that was suggested by Prasertsan and Prachumratana [8] with slight modification.The Individual visceral organ of *Euthynnusaffinus*was mixed with the phosphate buffer solution of pH 7 in the 1:2 (w/v) ratios of viscera to buffer solution. The mixture was then homogenized for 3 min before filtration through cotton cloth to remove solid residues. The fine particles left in the filtrate were removed by centrifugation at 10,000 rpm for 30 min at 4^{o}C. The supernatant (serum that was in the second layer) was used as the crude enzyme and determined for activities of protease and lipase (figure 2b).



(a) (b)

Figure 2. Individual viscera organs of *Euthynnusaffinus* (a), mixture of viscera and buffer solution after centrifugation (b)

2.2.3. Protein Assay

The protein concentration of the sample solution was measured by the method of Lowry *et al* [10] using bovine serum albumin as the reference standard. In brief, the preparing mixtures of 2% Na_2CO_3, 0.1 M NaOH, 0.5 % $CuSO_4.5H_2O$, and 1% sodium potassium tartrate was added to 0.5 ml of the sample solution prior adding the Folin reagent . The average of triplicate optical density (OD) was read at the wavelength of 600 nm.

2.2.4. Enzymatic Assay

Proteolytic activity was conducted according to the procedure described by Mardina *et al* [11]. A 2.5 ml of 1% (w/v) casein as substrate was equilibrated to 37^{o}C for 5 min prior adding 0.5 ml crude enzyme. The solution was incubated for 10 min at 37 ^{o}C. The enzyme reaction was stopped by adding 2.5 ml of 0.11 mM Tricholoaceic acid, then allow to stand at room temperature for 30 min. 1 ml supernatant was mixed with 2.5 ml of 0.5 M Na_2CO_3 and 0.5 ml of 0.1 N Folin reagent for 20 min. The OD was measured at the wavelength of 660 nm. The unit of protease activity is defined as the amount of enzyme required to liberate 1µmole of tyrosine per minute under the assay condition.

Lipolytic activity developed by Tripathi*et al* [12] is based on a spectrophotometric assay using pNPP (paranitrophenolpalmitate) as substrate. The substrate was dissolvedin 1% (w/v) Triton 100-X and 2% sodium dodecyl sulphate. The reaction mixture consisted of 0.5 ml enzyme extract, 0.5 ml of 0.2 M Tris-HCl (pH 8) and 0.5 ml pNPP stock solution. The reaction mixture was incubated for 10 min at 37 ^{o}C and 1 ml NaOH was added to stop the reaction. After centrifugation at 10.000 rpm for 10 min at 4 ^{o}C, the OD was measured at 410 nm. One unit of lipase activity was defined as the amount of enzyme which liberated 1 µm of pNPP per min from p-nitro phenol phalmitate.

Enzyme activities was calculated according the equation below

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

2.2.5. Standard Calibration Curve for Protease and Lipase Activities

The procedures were performed by following method of Mardina*et al* [11] and Yusof *et al* [13] using 1.1 mM L-tyrosine and 5 mM p-nitrophenolpalmitate. The absorbency was read for the amount of L-tyrosine solution completed to 4 ml by adding deionized water, 0.5 M Na_2CO_3, and 0.1 N folinciocalteu's phenol reagent at 660 nm. The relationship between the absorbency and the µM L-tyrosine was plotted as y/x line plot. On the other hand, calibration curve for lipase was prepared by using pNPP (5 mM), and 0.1 M phosphate buffer (pH 7). P-nitrophenol standard solution was arranged into ten individual test tubes (0.05-0.5 ml). The absorbance of each sample was measured at 410 nm and constructed by plotting A410 versus the pNPP concentration in each sample.

3. Results and Discussions

*Euthynnusaffinis*popularly known as the 'little tuna' or 'kawakawa' is a medium sized tuna that was in Indonesia's sea. *E. affinis* can be recognized by the pattern of broken diagonal lines on the upper sides and two or five dark spots above the pelvic fin (figure 1). The maximum averages and length of kawakawa are 13.6 kg and 100 cm. However, the common length is up to 60 cm[1]. In this study, the length of *Euthynnusaffinis* was 50 cm. The average was 2.1 kg, 1.8 kg, and 1.0 kg with the viscera yields of 14.7%, 16.7 %, and 8% respectively. These results were higher than the previous report that stated that the visceral yield of tuna of 7 – 8 % of the whole body [8]. These can be explained that the weight of visceral in this study include the cheek, gill and innards of fish.

Due to the requirement for calculation of specific activity of enzyme, measurement of protein concentration was necessary, hence bovine serum albumin as the reference standard was established (figure 3). The findings revealed that the highest protein content were in spleen, liver, stomach and pancreas respectively (table 1). The results of this study was supported by Chaijaroen and Thongruang [7] who extracted the digestive enzyme from Nile tilapia (*Oreochromisniloticus*) viscera waste and found protein content in the range of 4.82 – 5.47 mg/ml. Similarly, Sabtecha*et al* [9] reported the protein contents of visceral fish from species of Red snapper (*Lutjanuscampechanus*, Seer fish (*Scomberomorusguttatus*), Great barracuda (*Sphyraena barracuda*), Milk shark (*Rhizoprionodon*), Sardines (*Sadonellalongiceps*)were in the range of 3.77 – 9.44 mg/ml.

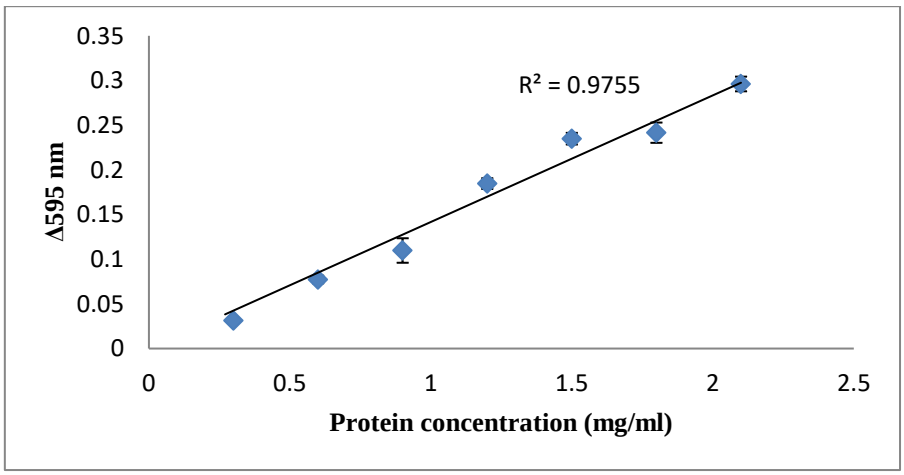
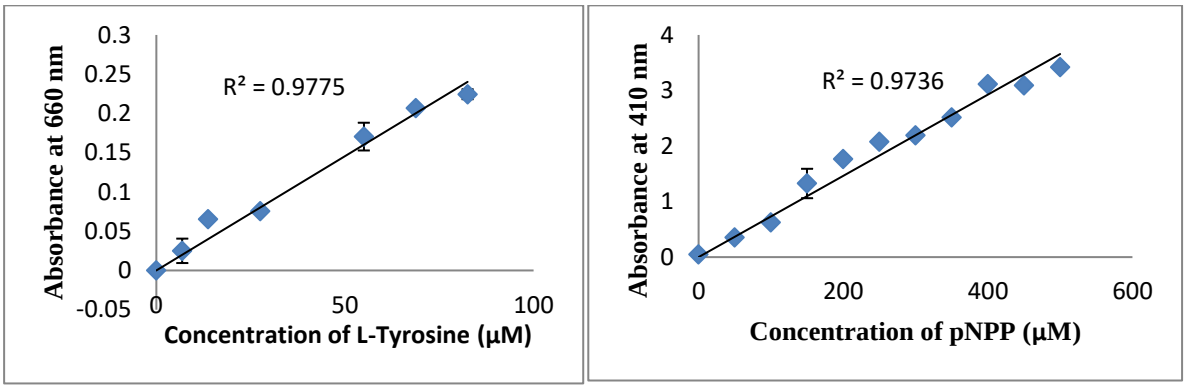


Figure 3. Standard curve for protein using bovine serum albumin

Protease assay was performed using casein as substrate that reacts through hydrolysis by protease to amino acid (tyrosine) and peptide fragments. Free tyrosine developed its colours (blue) by reacting with Folin & Ciocalteu Phenol reagent and the amount of tyrosine equivalents release was determined using L-tyrosine standard curve at absorbance of 660 nm (figure 4a), while absorbance of lipase activity was read at 410 nm and plotted against the concentration of p-nitrophenol as substrate in standard curve (Figure 4b). Standard curve in figure 4 represented the determination coefficient (R^2) of the model was goodness of fit, which means 97.75% (figure 4a) and 97.76% (figure 4b) variability in the response on variable could be explained by the linear model. The R^2 value is always between 0 and 1.0 that imply stronger model with better prediction to response.



(a) (b)

Figure 4. L-Tyrosine standard curve for measuring protease activity (a), pNPP standard curve for measuring lipase activity (b)

The study isolated and selected the viscera organs viz. spleen, stomach, pancreas and liver as sources of protease and lipase enzymes. Among the individual viscera organ, liver of *Euthynnusaffinis* exhibited the highest protease activity (57.786 U/ml), while the pancreas revealed the highest lipase activity (69.425U/ml) (Table 1).The observed results were slightly different from the results of Prasertsanand Prachumratana [8] that found the spleen and pancreas of skipjack tuna had the highest protease (25 U/ml) and lipase enzyme activities (0.28 U/ml) respectively. These differences could be explained by Dizhi*et al* [14] that concluded their study on the change of visceral properties and digestive enzyme activities was influenced by the different dietary groups of fish.

Table 1. Comparison on the highest protease and lipase activities extracted from individual viscera organ of *Euthynnusaffinis*

Individual Visceral Organ	Protein content (mg/ml)	Protease		Lipase	
		Activity (U/ml)	Spesific activity (U/mg)	Activity (U/ml)	Spesific activity (U/mg)
Spleen	9.802	47.667	4.863	62.548	6.381
Stomach	6.076	50.453	8.304	60.575	9.969
Pancreas	4.615	57.053	12.362	69.425	15.043
Liver	8.412	57.786	6.869	68.054	8.090

4. Conclusion

The study highlighted on the screening of sources of hydrolase enzymes (protease and lipase) among the individual visceral organ of *Euthynnusaffinis*. The results found that visceral of *E.affinis*. are rich of protein content and hydrolase enzyme (protease and lipase). Among the individual viscera organ (stomach, liver, pancreas and spleen), liver revealed the highest protease activity (57.786 U/ml), pancreas showed the highest lipase activity (69.425 U/ml).

Acknowledgements

The authors are grateful to the Ministry of Research Technology and Higher Education through the PDP research grant of 114/UN54.6/LT/2018 for providing financial support.

References

[1] Ahmed Q, Yousuf F, Sarfraz M, Ali Q M, Balkhour M, Safi S Z and Ashraf M A2015 *Euthynnus affinis* (little tuna): fishery, bionomcs, seasonal elemental variations, health risk assesmet and conservational management *Frontiers in life Sci*. **8** 71 – 96.

[2] Erika Fatma 2015 Development of sustainable tuna processing industry using system dynamics simulation *Procedia Manufacturing***4**(2015), 107 - 114

[3] Rustad T 2003 Utilisation of marine by-products *Electronic journal of Environmental, Agricultural and food chemistry (EJEAFChe)***2** (4): 458 – 463.

[4] Arruda L F, Borghesi R and Oetterer M 2007 Use of fish waste as silage – a review *Brazilian archives of biology and technology an international journal***50** (5): 879 – 886.

[5] Jayathilakan K, Sultana K, Radhakrishna K.and Bawa A S 2012 Utilization of byproducts and waste materials from meat,poultry and fish processing industries: a review*J. Food Sci Technol***49** (3): 278 – 293.

[6] Shobana A and Subash A 2013 Partial characterization of protease from the visceral organ waste of Cobia (*Rachycentron canadum*) *Journal of biology, agriculture and healthcare***3** (14): 5 – 9.

[7] Chaijaroen T and Thongruang C 2016 Extraction, characterization and activity of digestive enzyme from Nile tilapia (*Oreochronis niloticus*) viscera waste *International Food Research Journal***23** (4): 1432-1438

[8] Prasertsan P and Prachumratana T 2008 Comparison and selection of protease and lipase sources from visceral organs of three tuna species *Songklanakarin journal of science and technology* **30:** 73 – 76.

[9] Sabtecha B, Jayapriya J and Tamilsevi A 2014 Extraction and characterization of proteolytic enzymes from fish visceral waste: potential applications as destainer and dehairing agent *International Journal of ChemTech Research***6** (10): 4504 – 4510.

[10] Waterborg J H Lowry 2002 Method for protein quantitation, *The Protein Protocols Handbook 2nd Ed* ed Walker J M Humana press Inc., Totowa, NJ chapter 2 pp 2 – 9.

[11] Mardina V, Yusof F and Alam M Z 2015 Statistical optimization of physicochemical factors for protease production by *Bacillus licheniformis* on skim latex serum fortified media *Journal of Engineering Science and Technology***sp issue 6** (1): 42 – 52.

[12] Tripathi R, Singh J, Bharti R K and Thakur I S 2014 Isolation, purification and characterization of lipase from Mycobacterium sp and its application in biodiesel production*Energy Procedia***54** : 518 – 529.

[13] Yusof F, Khanahmadi S, Amid A and Mahmod S S 2016 Cocoa pod husk, a new source of hydrolase enzymes for preparation of cross-linked enzyme aggregate *Springer plus***5**: 1– 18.

[14] Dizhi X, Shude X, Qingyang W , Fang C , Shuqi W , Cuihong Y, Yuanyou L 2018 Changes of visceral properties and digestive enzymes in the herbivorous marine teleost Siganus canaliculatus fed on different diets*Acta oceanol sin***7**(2): 85 – 93.