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Extraction and Screening of Various Hydrolases from Malaysian Channel Catfish (*Ictalurus punctatus*) Viscera

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Abstract:

Malaysia is a large producer of fish industry estimated to be 1.5million tonnes every year. 35-50% of the fish weight is its viscera, which is usually disposed into the landfills or sea leading to massive environmental problems. However, various hydrolases can be extracted from this highly potential by-product source. In this study, protease, lipase, α-amylase and cellulase activities were studied. Protein concentration of the whole viscera of *Ictalurus punctatus*was 0.798596 mg/mL, protease gives the highest specific activity among the hydrolases (13.57 U/mg), cellulase was (1.43 U/mg), lipase (1.425 U/mg) and the α-amylase was (0.689 U/mg).

Keywords: Hydrolase, protease, lipase, cellulase, α-amylase.

1. Introduction:

Fish is considered as one of the main dishes consumed everyday by Malaysians. In 2010, it was estimated that Malaysia produces 1.5 tonnes of fish annually and this number is able to be increased in the coming years. As for any process waste is generated, fish waste includes (guts, bones, skin, head, fins) that is usually collected and dumped in waste sites or in the seas causing health and environmental problems including contamination of the soil and water sources and sickening odors.

Catfish in general and channel catfish in specific*Ictalurus punctatus*(**Figure 1**) are bottom feeders which means they live at the bottom of the rivers or ponds and eat various kinds of feed such as: aquatic plants, other fish, decaying vegetation, fish eggs and crayfish as well as snails, aquatic bugs and minnows. Moreover, catfishes are carrion eater means that they eat dead things such as dead fish, dead bugs, or anything else that is dead (Yukozimo, 2010). Hence, depending on the fish diet, channel catfish stomach will release different hydrolases to breakdown the content of the food.

Figure 1: channel catfish *Ictalurus punctatus*

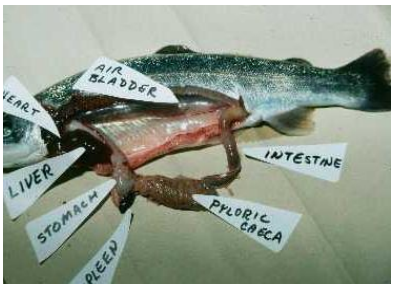

Figure 2: Fish viscera anatomy(link)

Proteases are involved in digesting long protein chains into short fragments, breaking the peptide bonds that link the residues of amino acid together to generate shorter fragments. Protease also accounts for almost quarter of the total global production of enzymes (Gupta *et al*, 2002). They have number of applications in biotechnological processes and industrial applications, i.e. food processing, pharmaceuticals, leather industry, detergent industry, etc.

Lipase on the other hand catalyzes the hydrolysis of various forms of fatty acyl esters (fats, lipids). Commonly lipases are found in nature and have been found in animals, higher plants and microorganisms (Ohnis *et al*., 1994). Lipases have wide application in pharmaceuticals and it is one of the main components of detergents.

Cellulase enzyme breaks down cellulose, Li *et al*, (2012) stated many applications of cellulase in industry, such as fuels, food, brewery, wine, animal feeds, textile, laundry, pulp/paper and agriculture. Further, cellulase has uses as chemicals.

Lastly, α-amylase is a starch degrading enzyme, it degrade starch and related polymers to yield product characteristic of individual amylatic enzymes (Aiyer, 2005).Amylases have many potential applications in several industrial processes such as in food, textiles, paper industries, detergents, fuel ethanol from starches, fruit juices, alcoholic beverages, sweeteners, etc. (Singh *et al*, 2011).

In this study, the four hydrolases are chosen to determine their activities from the most common fish in Malaysian waters channel fish *Ictalurus punctatus.*

2. Materials and Methods:

2.1 Materials

All chemicals used for analysis are obtained from Essen Haus Sdn. Bhd., Bradford reagent from Bio-rad, the substrates and reagents were of analytical grade and obtained from Sigma–Aldrich.Sartorius VivaSpec Spectrophotometer (New York, USA) was used for optical absorbance measurements andEppendorf centrifuge 5804 (Hamburg, Germany). Experiments were done in triplicates.

2.2 SamplePreparation:

60 Channel fishes(*Ictalurus punctatus*) were obtained from Aminah canteen/IIUM that was originally brought from Pasar Selayang, Batu Caves, Malaysia. Viscera was separated from the rest of fish body and kept in ice while being transported to Molecular lab/IIUM and kept in -20°C till being used. It was then cleaned and separated from the fish head and stored again in -20°C.

2.3 Extraction process:

Each fish viscera weight is 10-12.5g that is about 7% of total weight (total weight for an individual fish is around 89g). Total viscera weight of the 60 fishes was 749g. Cold 0.5M sodium phosphate buffer (pH 7.4) was used for to blend the viscera for extraction process in ratio of 1:2 (w/v) viscera to buffer solution (Prasertsan and Prachumratana, 2008). The mixture was then filtered bya muslin cloth to remove the solid residues. Followed by the removal of the fine particles form the filtrate by centrifugation at 12000rpm for 1hour at 4°C, **Figure 3** shows the mixture of viscera and buffer solution after centrifugation, the second layer which is the supernatant inform of serum was separated from the rest to be the crude enzyme and then stored at -20°C to be used for further processes.

Figure 3: Mixture of viscera and buffer solution after centrifugation, the top layer is a layer of lipids, the second layer is the serum that is separated from the rest to be the crude enzyme and bottom layer is fine solids.

2.4 ProteinConcentration

Bradford method (Bradford, 1976) is used to determine the protein content with a standard of BSA and scanning by Magellan Data Analysis Software at absorbance wavelength 595nm.

2.5 EnzymaticAssays

Protease activity assay: Using the procedure described by Hagihara *et al.* (1958) using casein as substrate to measure the activity of protease. Casein solution was prepared by dissolving 1g of casein in 100ml Tris-HCL buffer pH 8, 1 ml of enzyme solution was added to casein and incubated for 20min at 35°C. The reaction is terminated by adding 4ml of 10% trichloroacetic acid TCA. Absorbance was measured at 280nm and tyrosine standard curve was prepared. The equation below to measure the enzyme activity (Sigma-Aldrich) with modification:

$$\text{Enzyme activity } \left(\frac{\text{Units}}{\text{ml}}\right) = \frac{(umole\ \ of\ tyrosine\ \ released\ ) * Total\ \ volume\ \ of\ assay\ (mL)}{volume\ \ of\ used\ enzyme\ \ (mL) * Time\ of\ assay\ * volume\ \ in\ cuvette\ (mL)} \quad (1)$$

One unit of protease is described as the enzyme required to hydrolyze casein to produce color equivalent to 1.0 µmole of tyrosine per minute.

Lipase activity assay: (Yi *et al.*, 2009, Zhu *et al.*,2013) procedure was followed with some modifications to determine the amylase activity in the light absorbance at 420nm caused by the release of p-Nitophenol because of hydrolysis of *pNPP*, the substrate solution which was a mixture of isopropanol, 30mg pNPP, 0.5M Tris-HCL buffer, 0.4% Triton X-100 and 100mg of gum arabic was prepared and incubated at 40°C for 10min then the enzyme was added to the substrate solution, 2ml of 96% ethanol was added to stop the reaction. Lipase activity is determined by using p-nitrophenyl palmitate standard curve.

One unit of lipase activity is defined as the required enzyme to liberate 1 uM of p-nitophenol/min under the experimental conditions.

α-amylase activity assay: (Bernfeld, 1955) procedure was followed to determine the amylase activity by measuring the absorbance at 540nm,1% (w/v) starch solutionwas incubated at 20°C then 1m of sample solution was added and the mixture is incubated for 30min at 20°C.Followed by the addition of 1 ml of color reagent that was prepared by heating (not boiling) 20mL dinitrosiliclyce acid (DNS) in water bath and slowly adding sodium potassium tartrate buffer (12g sodium potassium tartrate in 8 mL 2M NaOH) and diluted with 40mL dH_2O. The whole mixture was placed in a boiling water bath for 15min then directly in ice bath to cool down to room temperature and 9mL dH_2O is added.0.2 % (w/v) maltose is used for the preparation of standard curve.

One unit of α-amylase is defined as the amount of the enzyme that liberates 1.0 µmol of reducing sugar/min with maltose as a standard (Sajedi *et al*., 2005).

Cellulase activity assay: (Lone *et al.*, 2012) method was followed with some minor modifications. Using the glucose as a substrate for cellulase and light absorbance at 540nm,0.5% Carboxymethyl cellulose (prepare in 0.01M sodium citrate buffer pH 4) was added to 50uL of sample solution and incubated at 40°C for 30 min, the reaction was terminated by 100uL of dinitrosiliclyce acid and placed in boiling water bath for 5 min, then diluted with dH_2O for the mixture to be 1mL.1unit of cellulase is equal to 1 umol of glucose per minute.

3. Results and discussion:

The environmental condition of the extraction process must be considered while conducting the extraction process. Each enzyme has its range of pH values and temperature, outside this range the enzyme will be inactivated. Hence, when pH of the medium is higher than 7.0, higher enzyme activity can be reached (Lv *et al.*, 2008).For example,the highest specific activity of enzyme from gastric mucosa of Polar cod (*Boreogadus saida*) was achieved at a pH of 7.3 (Meinke *et al.*, 1972). In addition to that, it has been stated that the lipase activity can be determined only within the range of pH 6-11 (Prasertsan and Prachumratana, 2008). Temperature is the other important factor to be considered in extraction process to maintain proteins' shape and activity. According to (Doonan, 1996; Zhao, 2011)to minimize protein denaturation as well as the autolysis of enzymes, the sample must be kept at (0-4°C) during the process.

Based on Bradford assay, the protein concentration of the crude enzyme mixture 798.596 ug/mL = 0.798596 mg/mL, it was observed that the color of the assay was stable blue and in gradient based on the concentration of BSA in preparation of standard curve (shown in **Figure 4**.), and the sample was dark blue indicating the presence of active proteins and that the reaction occurred between the protein and the dye. The high protein concentration in the viscera of channel catfish can be related to the diet of the fish itself, as mentioned earlier this type of fishes can digest a variety of food that is a high protein intake, therefor; the stomach will release digestive enzymes (hydrolases) to breakdown (hydrolysis) of food.

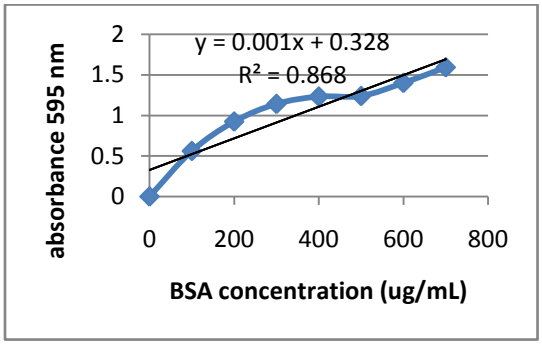

Figure 4: BSA standard curve

In protease activity assay, caseinplayed the role as a substrate that will go through hydrolysis by protease to amino acids and peptide fragments, tyrosine is one of the amino acids released, and free tyrosine will react with Folin & Ciocalteu Phenol reagent changing its color from green to blue, the mixture is incubated at 35°C to give the enzyme a best shape of active side to the attachment with the substrate for suitable time, then the reaction was terminated by trichloroacetic acid (TCA). Dark blue color formed when testing the sample that indicates the reaction of protease in the sample with the F-C reagent. The protease of the channel catfish viscera was 13.57 U/mg because hydrolysis of protein starts in the stomach and continues through the digestive system. The L-Tyrosine standard curve is shown in **Figure 5.**

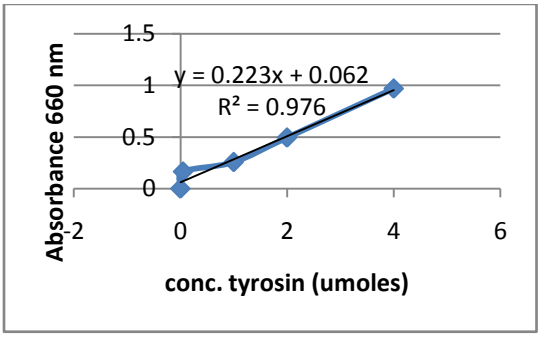

Figure 5: L-Tyrosine standard curve to measure protease activity

To determine the activity of lipase, p-nitrophenyl palmitate was used as substrate, it is worth mentioning that lipase catalyzed reaction cannot be performed at acidic medium (pH 4-6) or highly alkaline medium (pH 10-12), it is best at neutral or mildly alkaline (Kanwar *et al.*, 2005). However, based on the standard curve prepared (**Figure 6**) the lipase specific activity was 1.425 U/mg, this result is considered higher than reported results of three tuna species: Yellowfin (*Thunnus albacares*) 0.0538 U/mg, Skipjack (*Katsuwonus pelamis*) 0.0221 U/mg and Tonggol (*Thunnus tonggol*) 0.0338 U/mg (Prasertsan and Prachumratana, 2008). The reason behind that could be related to the difference in the diet of each fish species, catfish digest more oil and lipids from different sources at the bottom of the river allowing the digestive system (i.e. pancreas) to release more lipase for the hydrolysis of lipids.

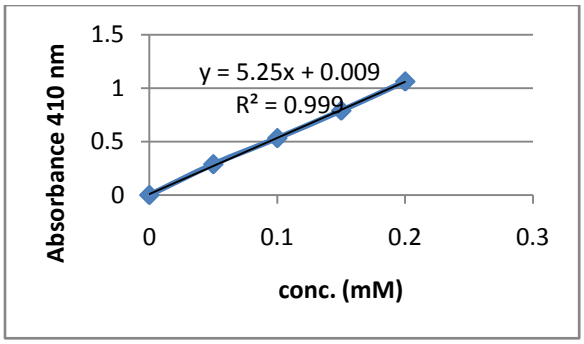

Figure 6: p-nitrophenyl palmitate standard curve to measure lipase activity

α-amylase activity on the other hand was measured under certain conditions for the substrate (Maltose) to be hydrolyzed, starch is one of the content of the fish feed that is goes through hydrolysis in the stomach by amylase enzyme to form reducing groups of maltose.in this study, it was found that the amylase activity of the sample is 0.689 U/mg. compared with other fish sources like different species of tuna fish (Prasertsan and Prachumratana, 2008), they didn't contain amylolytic enzyme activity. The pH of the medium is chosen to be pH 6.9 at which the highest alpha-amylase activity can be, based on (Tizon *et al.*, 2012) the optimal amylolytic activity was at pH 6.0-8.0 and this is in agreement with the findings in crystalline style extracts of various marine sources.

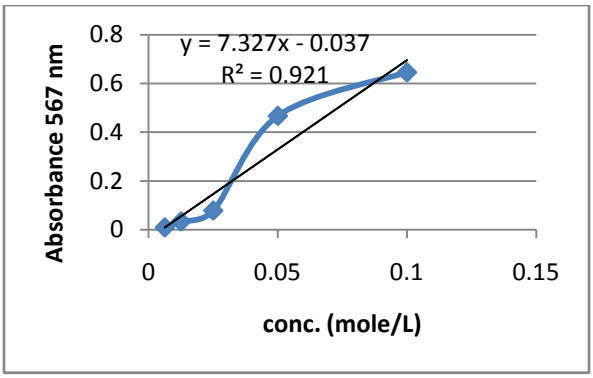

Figure 7: Maltose standard curve to measure α-amylase activity

The cellulase activity was measured using glucose as a substrate,**Figure 8** the glucose standard curve is shown. The specific activity of cellulase was 1.43 U/mg. this result shows the presence of cellulose in the fish feed and the ability of the fish to digest cellulose, since most of animals cannot digest the cellulose and they rely on marine bacteria or fungi in nutrient extracting and recycling (Tizon *et al.*, 2012).

Cellulase activity is important in dissolving the cell wall of green algae to extract the intracellular nutrients.

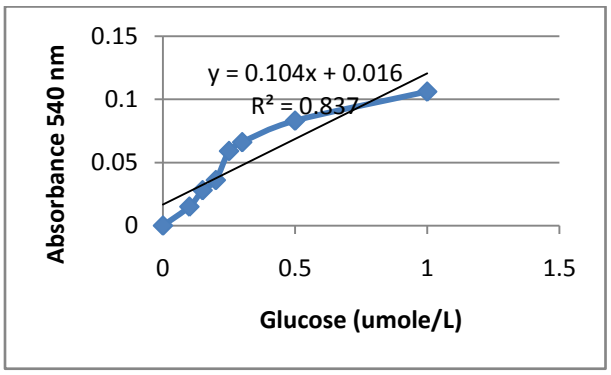

Figure 8: Glucose standard curve to measure cellulase activity

Conclusion:

The whole viscera of channel catfish (*Ictalurus punctatus*) possessed high protein content, and high protease, lipase, amylase and cellulase activities. Among the four studied hydrolases protease gave the highest specific activity. This rich source of enzymes can be used for further studies and characterization to be applied later in industry and at the same time reduce the environmental risks of the fish waste.

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