# Optimization of Enzyme Concentration for Food Protein Hydrolysate from Food Waste.

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***Abstract***

The utilization of the increasing food waste across the year must be improved for human consumption as it contained a lot of benefits to human health such as antioxidant, antihypertensive and anticancer. The effect of enzyme concentration on the enzymatic hydrolysis of fish, okara and shrimp waste was studied to get optimum hydrolysis conditions. Fish, okara and shrimp waste were hydrolysed using alcalase at different enzyme concentration which were 1, 1.5, 2 and 2.5% (v/v) at constant temperature, 60˚C, and pH, 7.5 for 4 hours. Alcalase enzyme was chosen for the hydrolysis due to its endopeptidase properties which can produce various type of peptides. Results showed that the enzyme concentration of 2.0 % was the highest degree of hydrolysis for fish waste and shrimp waste while for okara was 1.5%. FTIR results showed that the functional groups for each protein hydrolysate of fish waste, okara and shrimp waste were almost the same which were presence of hydroxyl groups and alkene groups. The results from UV-Vis Spectrophotometer shows that the highest absorbance among the protein hydrolysate in the form of supernatant was okara, 0.6963 due to the highest degree of hydrolysis which was 58.09 at the optimum condition of hydrolysis. From this study, it can be concluded that the degree of hydrolysis increases as the enzyme concentration increases until it reached maximum conversion of protein into peptides.

***Keywords*— Bioactive peptides, degree of hydrolysis, enzymatic hydrolysis, protein hydrolysate**

## 1. INTRODUCTION

In this millennium, the food waste disposal from animals and vegetables are increasing due to increase of food industries across the world. [1]. However, most of them are not properly utilised and may cause economy and environmental problems. For instance, about 2.8 million of okara produced by the tofu industry in China every year but most of them are only used as animal feeding [2]. Moreover, huge quantity of fishery waste and by- products were generated past years where they were either underutilized to produce low market value products or dumped [3]. From the recent researches, there are numerous derived- protein from the organism has been studied in order to extract the peptides effectively. The example of food waste utilization that has been studied were the enzymatic hydrolysis of *Salmo salar* (Atlantic Salmon) skin [4] and *Actinopyga lecanora* (sea cucumber) [5]. The peptide present in the food waste may contained nutrition and health-promoting functions such as antihypertensive, immunomodulatory, antioxidant, anticancer, anti-inflammatory properties and opioid-like, [6]. For instance, the bioactive peptides present in the food waste can be effective in curing diseases like the obesity and diabetes. The commercial production of peptides from food waste received a lot of interest from the community lately, however production of peptides for industrial- scale is not yet well implemented [7]. Traditionally, method of enzymatic hydrolysis is used to extract peptides. Enzymatic hydrolysis of protein involves cleavage of molecules of protein into smaller peptides and eventually become amino acid [8]. It is one of the fastest, easily controlled and safest techniques for producing peptides. Despite having low commercial value for producing peptides, it can used to enhance the functional and biological properties of the protein [9]. The size and the sequences of amino acids in the peptide chains as well as the free amino acids quantity are effected by the condition of hydrolysis conditions like pH, temperature and time which effect the hydrolysates' biological activity [10]. Previous researches from [2] and [11] found that alcalase gives the highest degree of hydrolysis (DH%) compared to papain, trypsin and other enzymes they used for the fish proteins hydrolysis. The wide range of application in food and pharmaceutical industries also have driven a high demand of peptides as people nowadays prefer natural supplements over synthetic supplements.

## 2. METHODOLOGY

### 2.1 Materials and Methods

**Samples**

Whole frozen *Leptobarbus hoevenii* (Hoven's Carp) fish, (*Glycine max)* soybeans and *Macrobrachium rosenbergii* (shrimp) were obtained from the supermarket. The waste including head, scale and tail were separated from the whole fish and shrimp. The fish and shrimp waste were then stored in the laboratory freezer at -20 ºC while the soybean stored in the room temperature, 27 ºC.

**Chemicals and enzymes**

The enzyme alcalase (≥2.4 U/g) used in this study was obtained from Sigma Company (Catalogue No. P4860). The chemicals used in this study include sodium hydroxide (NaOH), copper sulphate ($CuSO_4$), sulphuric acid ($SO_4$), Potassium Dihydrogen Phosphate ($KH_2PO_4$) and Di- Potassium Hydrogen Phosphate ($K_2HPO_4$). All these chemicals were manufactured by R & M Chemicals and were obtained from the Chemistry Laboratory.

### 2.2 Equipments

Fourier Transform Infrared Spectrometer (FTIR) model: Spectrum One, serial no. 74630 and UV- Vis Spectrometer model: Lambda 750, serial no. 750N7122801 were used in this study. Both were manufactured by PerkinElmer, Inc. FTIR was used to determine the functional groups while UV- vis Spectrometer was used to determine the peptide existense present in the food protein hydrolysate (fish, okara and shrimp). The centrifuge model: SIGMA 3-18k, manufactured by Sartorius Group was also used to separate the hydrolysate protein from other substance such fats.

### 2.3 Sample Preparation.

#### a. Fish and shrimp waste

750 g of fish waste including head, scale and tail of Leptobarbus hoevenii (Hoven's Carp) or *Macrobrachium rosenbergii* (shrimp)

was grinded in a Master (MAS- 160BL (A)-I) blender with the addition of water in a 1:1 ratio of mass of food waste and distilled water for 2 minutes to get a fine slurry mixture. Then, the waste was blended were heated to a temperature of 80˚C to make sure no microorganisms activity took place before stored in a freezer overnight.

**b. Okara**

750 g of *Glycine max (*soy beans) was soaked in 750 ml warm water at 25˚C in water overnight. Then, the soy beans is boiled with the ratio of distilled water to soybeans (3:1). After that, the boiled and soften soy beans was grinded with a Mastar (MAS-160BL (A)-I) blender for 2 minutes to get fine slurry mixture. Then, okara was dried at 50 ˚C for 4 hours in the laboratory oven to reduce the moisture. The dried okara was then mixed with distilled water with ratio of (1:1) before heated to 80 ˚C to make sure no microorganisms activity took place. The heated okara mixture then cooled down at the room temperature, 27 ºC before stored in the laboratory freezer at -20 ºC.

### 2.4 Kjeldahl Method

The Kjeldahl method is used to determine the nitrogen content in organic and inorganic samples. The formula of Kjeldahl method is as follows [12]:

$$\% \text{ N} = \frac{0.1 \text{ x Volume of titration (ml) x } 14 \text{ x } 10}{\text{Weight of sample (g) x } 1000} \quad (1)$$

The determination of the protein content is a substrate, it will be multiplied with conversion factor of 6.25 (equivalent to 0.16 g of nitrogen per gram of protein). The Kjeldahl method is divided into three stages which are digestion, neutralization and titration.

#### 2.4.1 Digestion

2 g of dried sample (fish, okara or shrimp waste) was weighted, the sample was placed in a digestion flask. The sample was mixed with copper sulphate ($CuSO_4$) and hydrochloric acid ($H_2SO_4$). The copper sulphate ($CuSO_4$) was used to catalyst the digestion process. The sample was heated at 350- 380 ˚C for about 90 minutes until it become colourless and slightly blue due to copper sulphate as well as white fumes to be seen. Then, the temperature was allowed to cool in the room temperature.

$$\text{Sample Protein (-N)} + H_2SO_4 \longrightarrow (NH_4)_2SO_4 + CO_2 + H_2O$$

#### 2.4.2 Distillation

After the sample was digested, it was taken for the distillation process. The sodium hydroxide (NaOH) in excess is added to the solution. During the process of distillation, the ammonium ion was released in the form of ammonia gas, distilled as well as received by sulphuric acid ($H_2SO_4$) in excess at the distillate.

$$(NH_4)_2SO_4 + 2NaOH \longrightarrow 2NH_3\,(gas) + Na_2SO_4 + 2H_2O$$

$$H_2SO_4\,(excess) + 2\,NH_3 \longrightarrow SO_4^{2-} + 2\,NH^{4+}$$

#### 2.4.3 Titration

The mixture of distillate and sulphuric acid ($H_2SO_4$) were titrated with a known volume of sodium hydroxide (NaOH). The excess $H_2SO_4$ which was not reacted to the $NH_3$ will react with the NaOH. Phenalphthalene was added as indicator. Once the solution was neural, the color of sample is changed, and the titration is stopped. The volume of used NaOH to neutralize the sample was calculated. By this formula, the volume reacted $H_2SO_2$ with $NH_3$ was known, mass of nitrogen can be determined, and by using protein mass (N x 6.25) [13], mass of protein can be known.

$$\text{V of (M) } H_2SO_4 = \text{V of (M) } NH_3 \quad (2)$$

V = Volume
n = number of moles

### 2.4. Enzymatic Hydrolysis

Enzymatic hydrolysis was done considering the parameter of enzyme concentration at 1, 1.5, 2 and 2.5% (v/v) for 4 hours while pH was maintained at 7.5 and temperature was fixed at 60˚C. Enzymatic hydrolysis is carried out using alcalase enzyme. After the enzymatic hydrolysis was done, the protein hydrolysate (fish waste, okara and shrimp waste) were heated to 80˚C to inactivate the alcalase enzyme. The protein hydrolysate (fish, okara and shrimp waste) were stored in a laboratory freezer at -20˚C before centrifugation process with 4100 rpm.

### 2.5 Centrifugation

The protein hydrolysate (fish waste, okara and shrimp waste) were then centrifuged with 4100 rpm for 40 minutes at 4 ˚C [22]. 2 layers were formed at the end of the centrifugation process which were solid and supernatant. The first layer which was a solid then removed using pipette and stored in a laboratory freezer at -20˚C to be analysed by FTIR and UV- Vis spectrophotometer. The second layer which was a supernatant (protein hydrolysate) was then taken out using a dropper and placed it into a clean and empty beaker to be filtered.

### 2.6 Filtration

The supernatant (protein hydrolysate) was then filtered using P5 filter paper (pore size: 4 micron) for 3 times. The filtrate was collected separately for each 1 time, 2 time and 3 times filtration. Then, they were stored in a laboratory freezer at -20 ˚C for further analysis. The purpose of filtration is to remove the impurities such as fats.

### 2.7 Fourier Transform Infrared (FTIR) Spectrometer

The protein hydrolysate from fish waste, okara and shrimp waste were separated into 6 sample, protein hydrolysate before centrifuge (1), solid first layer of protein hydrolysate after centrifuge (2), supernatant second layer after centrifuge which was not filtered (3), supernatant second layer after centrifuge which filtered one time (4), supernatant second layer after centrifuge which filtered two times (5) and supernatant second layer after centrifuge which filtered three times (6) were then analysed using FTIR in order to identify the functional groups present in the samples, in the region of 4000-400 cm -1 assisted by a computer running PerkinElmer's Spectrum software.

### 2.6 UV- Vis Spectrometer

The reading of absorbance was determined by using a spectrophotometer at wavelength of 280 nm. A 1 cm quartz cells were used for the analysis. This absorption spectroscopy usually performed with molecules dissolves in a transparent solvent like aqueous buffers. The buffers used for the absorbance measurements should not absorb light in the wavelength range of the experiment where the very low absorbance buffers include phosphate, cacodylate and borate [14]. A cuvette with phosphate buffer solution was placed in the reference beam. The protein hydrolysate from the food waste which were divided into 6 samples each (fish waste, okara and shrimp waste) which were; protein hydrolysate before centrifuge (1), supernatant second layer after centrifuge which was not filtered (2), supernatant second layer after centrifuge which filtered one time, (3) supernatant second layer after centrifuge (4) which filtered two times and supernatant second layer after centrifuge which filtered three times (5) were diluted with distilled water for a ratio of 1:50, volume of

sample to distilled water. This analysis was done in order to determine the existence of the peptides in the samples.

## 3. RESULTS AND DISCUSSION

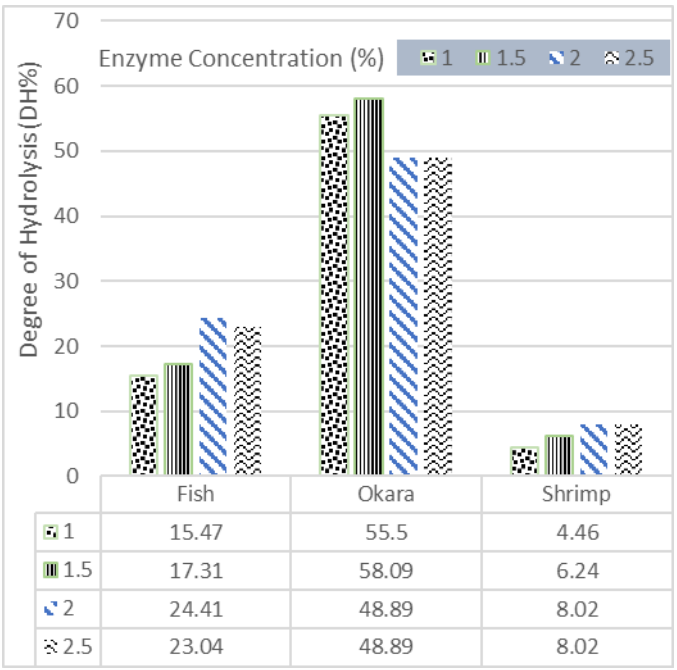
### 1) Degree of Hydrolysis

Enzymatic hydrolysis is a complex reaction that involved product inhibition as well as substrate- specific adsorption of enzymes. The enzymatic hydrolysis was done with different enzyme concentration which were 1, 1.5, 2 and 2.5% (v/v) at constant pH of 7.5 at 60 ˚C for 4 hours. The degree of hydrolysis represents the protein yield from the enzymatic hydrolysis

The degree of hydrolysis (DH%) was determined using this equation [2];

$$DH\ (\%) = BN_b / M_p \alpha h_{tot} \times 100 \qquad (3)$$

The DH represents the ratio between number of peptide bond cleaved (h) and the total number of peptide bonds in the substrate studied ($h_{tot}$) The variable B represents the amount of alkali consumed to keep the pH constant during the reaction, $N_b$ is the normality of the alkali, $M_p$ is the mass of the substrate (protein, determined as N x 6.25) in the reaction and α represents the average degree of dissociation of α- $NH_2$ groups released during hydrolysis.




| | Fish | Okara | Shrimp |
|---|---|---|---|
| 1 | 15.47 | 55.5 | 4.46 |
| 1.5 | 17.31 | 58.09 | 6.24 |
| 2 | 24.41 | 48.89 | 8.02 |
| 2.5 | 23.04 | 48.89 | 8.02 |

Figure 1 shows the result of degree of hydrolysis for protein hydrolysate (fish waste, okara and shrimp waste) against enzyme concentration.

The degree of hydrolysis (DH %) results versus enzyme concentrations (%) is shown in figure 1. The results showed that when the enzyme concentration was increased from 1 to 2 % for both fish and shrimp, the increase of DH (%) were 15.47, 17.31, 24.41% and 4.46, 6.24, 8.02 respectively. As for okara, when the enzyme concentration increased from 1 to 1.5%, the DH (%) increased from 55.5 to 58.09%. This trend showed that when the enzyme concentration increased, more enzyme molecules become associated with the food waste particles, therefore releasing more protein molecules into the system [15]. Similar results obtained by [16] where the alcalase enzyme concentration increased the overall proteolysis rate and the solubilization of protein. This results also showed the similarities with the study on hydrolysis of Salmon salar skin by [4] using alcalase where the DH increased when the concentration of enzyme increase with pH kept constant at 7.5.

[17] reported that during the initial phase of hydrolysis, there was a release of bulk soluble proteins and no increase in the release of soluble hydrolysates when additional enzyme was added to the system during the stationary phase of the hydrolysis. This may due to the product inhibition present during the hydrolysis or due to total cleavage of all the admitting peptide bonds.

From the results, the most effective hydrolysis condition for fish and shrimp waste were at the enzyme concentration is 2.0%. As for okara, the most effective hydrolysis condition for enzyme concentration is 1.5%. At this concentration, the hydrolysis may have reached the stationary phase where may reached the maximum conversion of protein to peptides. Once all the present substrate in the system were attached to the active sites of the enzyme, there will be the free enzyme which may inhibit the hydrolysis process. Thus, increasing the enzyme concentration above these effective enzyme concentrations would yield lower protein as well as not cost effective [15].

The results obtained concluded that the okara has higher degree of hydrolysis than the fish and shrimp waste. This may due to the fish and shrimp tissues are very complex substrates and contain large amount of proteinase inhibitors which makes it difficult for the hydrolysis process [18][19]. [20] also stated that the fish protein hydrolysis kinetics is complicated because of the presence of many types of peptide bonds and their enzymes specificity attack during the process. The okara also has softer structure than fish and shrimp waste that might be easier for the protein breakdown during the hydrolysis process.

### 2) Fourier Transformation Infrared Spectrometer (FTIR)

The protein hydrolysate from fish waste, okara and shrimp waste) were separated to 6 sample, protein hydrolysate before centrifuge (1), solid first layer of protein hydrolysate after centrifuge (2), supernatant second layer after centrifuge which was not filtered (3), supernatant second layer after centrifuge which filtered one time (4), supernatant second layer after centrifuge which filtered two times (5) and supernatant second layer after centrifuge which filtered three times (6) were analysed by FTIR for their characterization by determining the functional groups exist.

**a) Fish Waste**

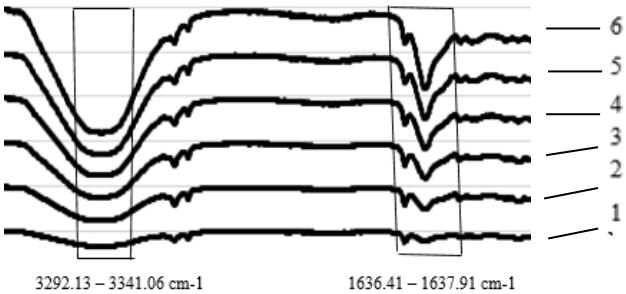



Figure 2 shows the FTIR result for fish

**b) Okara**

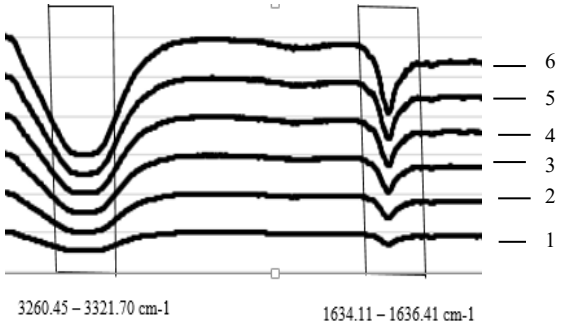



Figure 3 shows the FTIR result for okara

**c) Shrimp waste**

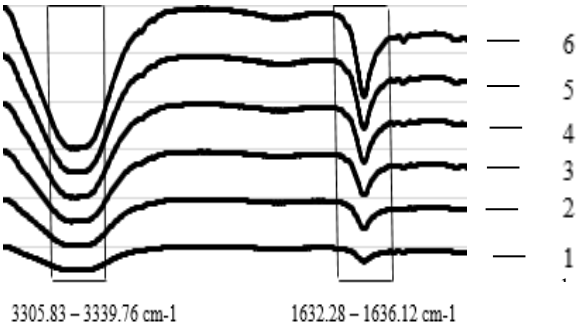



Figure 4 shows the FTIR result for shrimp waste

d) Comparison of Fish, okara and shrimp supernatant second layer after centrifuge which was not filtered (3).

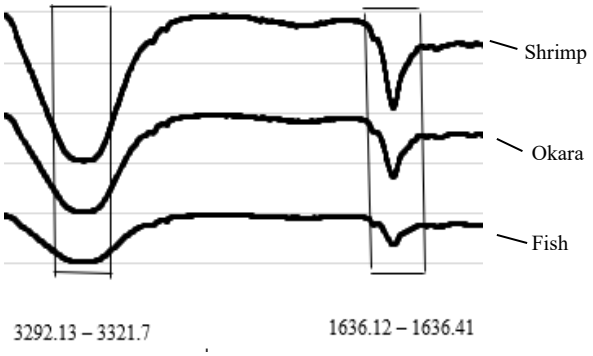



Figure 5 shows the FTIR results comparison for the supernatant (second layer after centrifuge) which was not filtered for fish okara and shrimp waste

| Sample | Wavelenght ($cm^{-1}$) | | |
|---|---|---|---|
| | From the FTIR results | Reference FTIR comparison | Reference |
| 1.Fish waste | i) 3292.13 – 3341.06<br>ii) 1636.41 – 1637.91 | i) 3345.89<br>ii) 1637.89 | [21] |
| 2.Okara | i) 3260.45 – 3321.70<br>ii) 1634.11 – 1636.41 | i) 3304<br>ii) 1539 | [22] |
| 3.Shrimp | i) 3305.83 – 3341.06<br>ii) 1632.28 – 1636.12 | i) 3287.55<br>ii) 1632.93 | [21] |

Table 1 shows the comparison of wavelength from the FTIR results obtained and FTIR results obtained by the reference for the food protein hyrolysate (Fish, okara and shrimp).

| Sample | Wavelenght ($cm^{-1}$) | | |
|---|---|---|---|
| | From the FTIR results | Reference FTIR comparison | Type of bond and vibrations |
| 1.Fish waste | i) 3292.13<br>ii) 1636.41 | i) 3345.89<br>ii) 1637.89 | i) O−H axial deformation in H2O<br>ii) N-H angular deformation in primary amine ($NH_2$ = R) |
| 2.Okara | i) 3321.7<br>ii) 1636.29 | i) 3304<br>ii) 1539 | i) O−H axial deformation in H2O<br>ii) N-H angular deformation in primary amine ($NH_2$ = R) |
| 3.Shrimp | i) 3308.89<br>ii) 1636.12 | i) 3287.55<br>ii) 1632.93 | i)O−H axial deformation in H2O<br>ii) N-H angular deformation in primary amine ($NH_2$ = R) |

Table 2 shows the comparison for the supernatant (second layer after centrifuge) which was not filtered for fish okara and shrimp waste FTIR results.

In order to determine the properties of the food protein hydrolysate, FTIR analysis was carried out for each sample. Figure 2, 3 and 4 show the results of FTIR for each 6 samples of protein hydroysate (fish waste, okara and shrimp waste). From the results, it was found that the fish waste, okara shrimp waste have the same functional group exist between the peaks 3305.83 – 3360.45.76 $cm^{-1}$ [21] [23] where it would be expected to have organic O−H axial deformation at the peaks. These may be the contributions from any bound water that may have been retained in the samples. The peaks observed at 1632.28- 1637.91 $cm^{-1}$ for fish waste and shrimp waste could be attributed to N-H angular deformation in primary amine ($NH_2$ = R) [21]. As for okara at the peaks of 1634.11 – 1636.41 $cm^{-1}$ showed that the potential existence of alkenes stretch (C=C) [23]. The protein hydrolysate before centrifuge (1) has the broadest peaks at both regions followed by (2), (3), (4), (5) and (6) which showed that it has the most presence of OH and NH bonds.

The FTIR results for the food protein hydrolysate (fish, okara shrimp waste), supernatant (second layer after centrifuge) which was not filtered from the study compromised with the results from the reference as the values are in between the range of the type of bonds and vibration for each sample. The two highest peaks for each sample were observed both at range of 3400 – 3200 $cm^{-1}$ and 1600 – 1500 $cm^{-1}$. At both peaks the fish waste showed the broadest peaks while the shrimp showed the sharpest peaks. This shows that the fish waste contained more OH and NH bonds than shrimp and okara.

From the FTIR results, at the range of 1600 – 1500 $cm^{-1}$ a significant amount of protein existed in the food protein hydrolysate (fish, okara and shrimp waste) where sequence of amino acids (amide bonds) were observed. This primary structure protein was often modeled as beads on a string, where each bead represents one amino acid unit [24].

**3) UV- Vis Spectrometer**

The protein hydrolysate from the food waste which were divided into 5 samples each (fish waste, okara and shrimp waste) which were; protein hydrolysate before centrifuge (1), supernatant second layer after centrifuge which not filtered (2), supernatant second layer after centrifuge which filtered one time (3), supernatant second layer after centrifuge which filtered two times (4) and supernatant second layer after centrifuge which filtered three times (5) were analysed using UV- Vis Spectrometer to identify protein content in them.

| Sample | Absorbance Value | | |
|---|---|---|---|
| | Fish Waste | Okara | Shrimp Waste |
| 1.Protein Hydrolysate Before centrifuge | 0.9810 | 0.8595 | 0.5899 |
| 2.Protein hydrolysate (supernatant) without filtered | 0.3315 | 0.6963 | 0.3016 |
| 3.Protein hydrolysate (supernatant) filtered 1 time | 0.3057 | 0.2193 | 0.1481 |
| 4.Protein hydrolysate (supernatant) filtered 2 times | 0.2679 | 0.1596 | 0.1617 |
| 5.Protein hydrolysate (supernatant) filtered 3 times | 0.2626 | 0.1410 | 0.1444 |

Figure 6 shows the results for food protein hydrolysate (fish okara and shrimp waste) analysed by UV- Vis spectrometer.

Protein in solution absorb ultraviolet light with absorbance maxima at 280 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280nm. Secondary, tertiary and quaternary structure all effect absorbance, thus factors like pH and ionic strength can alter the absorbance spectrum. The absorbance value determine the protein content in the sample [25].

The absorbance values in figure 6 showed the decreasing values after the protein hydrolysate was centrifuged and filtered. The highest absorbance value of supernatant was the okara which was 0.6963. This is because the okara has the highest degree of hydrolysis which was 58.89% where it hydrolysed the most protein compared to protein hydrolysate of fish waste and shrimp waste. This can be concluded that the higher the degree of hydrolysis, the higher the absorbance which results in highest peptide content.

## 4) Conclusion

Food waste that were not utilize may lead to economy, environmental and social implications problems. For instance, the poor food waste management also could lead to release of carbon footprint. Animal sources of protein, rice, heavily processed and package food are the among the major contributor to a high carbon footprint. One of the methods to reduce food waste is to extract the peptides from the food waste which can be used in the food and pharmaceutical industries. From the results obtained, the hydrolysis worked the best at enzyme concentration of 2.0% for fish waste and shrimp waste while okara at 1.5% where the DH (%) increases as the enzyme concentration increases until it reached maximum conversion of protein into peptides which also known as stationary phase in hydrolysis. Further characterization of protein hydrolysate (fish waste, okara and shrimp waste) were done by FTIR for each that showed significant amount of protein observed from the amide I band at the range of 1500 -1600 $cm^{-1}$. Analysis of protein also was done by using UV- Vis spectrometer showed that okara (supernatant) has the highest absorbance value, 0.6963 because it has the highest degree of hydrolysis which was 58.89% compared to fish and shrimp waste.

## 5) Acknowledgment

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