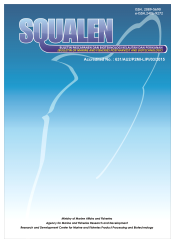

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Enzymatic Production of Fish Protein Hydrolysates in A Pilot Plant Scale

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Abstract

Protease enzyme produced from *Bacillus sp* was employed to hydrolyze fish protein hydrolysates (FPH) under controlled conditions at a batch-pilot plant scale-process. Thirty kilograms of fish meat was minced and mixed with 60 liters of water in 100 liters stainless steel vessel and 20,000 units of protease enzyme was added per kg of fish. Hydrolysis of fish was carried out at 55 ^{o}C for 6 hours. Multi stage of filtration were done to separate the FPH from unhydrolized fish residue. Mass balance were carried out to determine the rate of hydrolysis and yields. Without pH adjustment, 80% of substrate hydrolyzed could be achieved in 6 hour at 55 °C. Three kinds of products were recovered from the process, i.e solid residue, liquid FPH as filtration product, and spray dried FPH. Hydrolysis of 30 kg of fish meat substrate producing 1.7-2.0 kg of unhydrolyzed residue and 70 L of liquid FPH. After spray drying process of liquid FPH, 13 kg of FPH powder was recovered. The proximate and amino acid analysis of spray dried FPH showed that the FPH containing 20% of protein, rich in amino acids especially lysine and leucine and the residue still had 85,36 % of protein (dry basis) that could be utilized for other purpose.

Keywords: fish protein hydrolysate (FPH), enzymatic hydrolysis, protease, degree of hydrolysis

1. Introduction

The prevalence of nutritional deficiency related disease and growth retardation of children under five years old in developing countries have raised concerns and brought attentions to develop high protein content of food to overcome the problems (UNICEF, 2009). Fish is considered high protein food which provides essential nutrients for many people, especially those in developing countries (Chalamaiah, Kumar, Hemalatha & Jyotirmayi, 2012). However some people may have allergic reaction to fish protein which hinder wider acceptance of fish as food. The digestibility of unprocessed fish protein is usually lower than that of other food protein source such as egg protein. Fish protein hydrolysate which is produced through enzymatic hydrolysis can be used as an alternative protein source to reduce the possibility of allergic reaction and to increase the digestibility of fish protein.

Attempts to utilize fish wastes and underutilized fish for the production of commercially valuable food ingredients through various methods have been taken since 1980's. Fish protein hydrolysates with good nutritional composition, amino acid profile, and antioxidant activities have gained great attention of food scientists. Due to the presence of essential nutrients and bioactive components in fish protein hydrolysates, these find place in various industrial applications (Chalamaiah et al., 2012).

In Indonesia, research on FPH production have been done mainly at laboratory scale using commercial enzymes, including papain enzyme (Ariyani, Saleh, Tazwir & Hak, 2003; Salamah, Nurhayati & Widadi, 2012; Nurhayati, Nurjanah and Casti, 2013; Annisa, Darmanto & Amalia, 2017), bromelain (Wijayanti, Romadhon & Rianingsih, 2018). The use of local microbial protease from thermophilic *Bacillus sp*. to produce fish protein hydrolysate has been reported (Chasanah, Susilowati, Martosuyono, Zilda & Fawzya, 2019; Fawzya, Martosuyono & Zilda, 2017). So far, there is no publication of research on production of FPH in a pilot scale. In our recent work, FPH production was upscale by using membrane filtration technique to replace the use of centrifuse to

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separate the hidrolysate from the residue as usually done in laboratory scale. It takes much times to separate the hydrolysate by centrifugation in scaling up process. In addition, local *Bacillus* sp. protease which produced FPH without bitter taste in previous similar research (Chasanah et al., 2019) was employed to hydrolyze fish.

Any type of fish can be processed into FPH. Kuniran is a demersal fish, widely spread on the North coast of Java, commonly caught by trawlers. The maximum sustainable yield of goalband goatfish (local name: kuniran) was decreased until 2015, but currently it was recovered due to the implementation of fish resource management policies (Ma'mun, Priatna, Suwarso & Natsir, 2018). Its availability and relatively lower price was considered to be used as raw material for this research.

The enzymatic hydrolysis of protein with complex structure and containing other complex components such as fish meat cannot be independently described by simple kinetic models and laws of enzyme action. The presence of enzyme inhibitors and the complexity of protein structure may affect the effectiveness of enzyme activity (Himonides et al., 2011). Besides, the volume of reaction in large scale of hydrolysis reaction give more complexity of process compared the reaction in small laboratory scale. Variation of temperature in large scale bioreactor control may give lower efficiency of enzyme activity during the process. In this paper, hydrolysis of fish protein by protease in pilot scale bioreactor was described following the process of optimization in laboratory scale hydrolysis.

2. Material and Methods

Fish: goalband goatfish (*Upeneus moluccensis*) were collected from fish market in Cirebon. Fish were transported in container with ice and processed directly in laboratory to remove head and viscera. Fish meat was minced using silent cutter to prepare substrate for enzymatic hydrolysis. The size of fish used in this experiment was 10.8±0.7 cm length, 2.4±0.4 cm width and 13.6±3.1 gram weight.

Protease enzyme: Protease enzyme for hydrolysis was prepared by fermentation of *Bacillus sp* of RDCMFPPB collection in a minimum synthetic medium containing 0.7% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% NaCl, 0.05% yeast extract, 0.01% $MgSO_4.7H_2O$ and 0.6% skim milk. The optimum condition of bacterial fermentation and enzyme activity was taken from previous research in our laboratory (Zilda et al., 2013)

Production of fish protein hydrolysates: Hydrolysis was performed in a 100 L steel jacketed vessel heated by hot water. The unit was equipped with a temperature reader and stirrer operated with a controlled electric motor. Temperature was controlled by adjusting the gas flow in stove to maintain the temperature of water jacket. The temperature was maintained at 55±3°C. Mixture of fish mince and reverse osmosis water (1:2 w/v) heated at 55±3 °C and stirred at rate of 70 rpm. Protease enzyme was added as such that the activity was 20,000 units/kg substrate. The mixture was continuously stirred for 6 hours at a temperature 55±3 °C, after which the temperature was increased to 90 °C and held for 10 minutes to inactivate the enzyme and stop the hydrolysis process. The mixture was then left overnight to reach the room temperature before separation process. After overnight stand of, a fat layer was formed on the surface and skimmed off from the mixture.

Separation of FPH from insoluble materials (fish bone, un-hydrolyzed materials) was done using stepwise filtration. Before filtration processes, samples were taken to be separated with centrifugation as been done in laboratory scale and the results will be compared with filtration process. The first step was spinning the mixture in a spinner by filtration bag of pore size 300 and 600 mesh to separate the fish bone and rough residue. The next step were filtration using micro and ultrafiltration using membrane with pore size of 0.5 and 0.1 µm. At the end of this process yellowish clear filtrate were recovered and residual particle were collected from the filtration units. The liquid FPH was converted into powder using spray dryer with maltodextrin as filler at concentration of 20% (w/v). Condition of spray drying was determined at laboratory scale in previous experiment to determine the optimum condition. The spray drying was carried out at inlet temperature of 160 °C, outlet temperature of 90 °C, and aspirator of 90%. The production of FPH was done twice and the results were calculated to get the average score.

The yield, degree of hydrolysis and characteristics of the FPH were analyzed, including the degree of hydrolysis (DH), proximate content and amino acids compositions. Samples of hydrolysis were taken from 0-9 hours with 1 hour interval for DH measurement as described by Auwal, Zarei, Abdul-Hamid and Saari (2017). The proximate analysis in triplicate was done in RDCMFPPB chemistry laboratory. Proximate composition were determined according to SNI. Moisture content was determined by SNI 2354.2:2015 (BSN, 2015); ash content by SNI 2354.1:2010 (BSN, 2010) protein by modification of SNI 01-2354.4-2006, using Kjeltec; fat content by SNI 2354-3-2006 (BSN, 2006). Amino acids composition was analyzed in Saraswanti Indo Genetech (SIG) laboratory, Bogor using internal method developed by Saraswanti Indo Genentech laboratory (18-5-17/MU/SMM-SIG, UPLC).

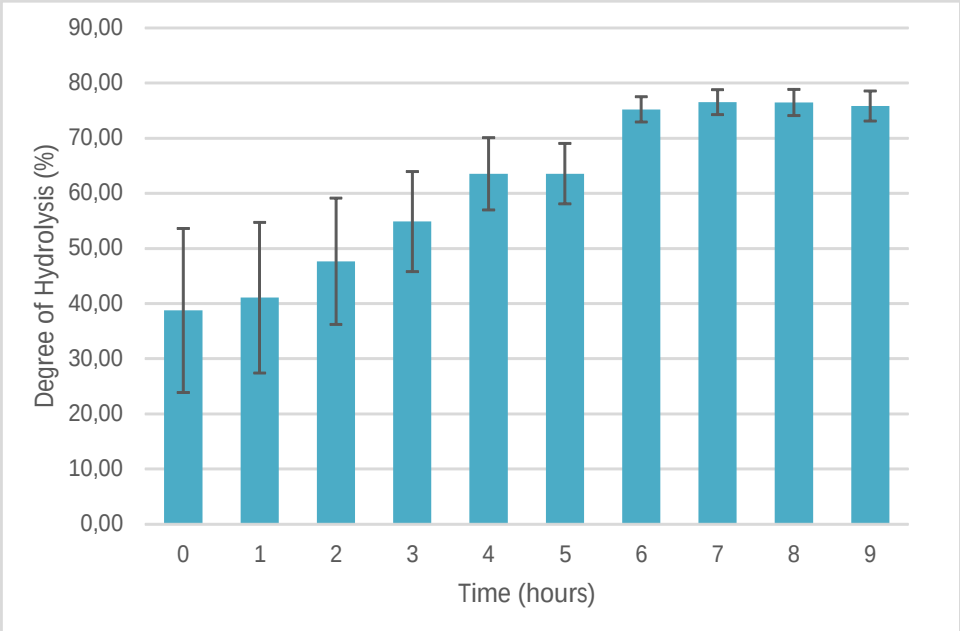

Figure 1. Degree of hydrolysis (DH) of FPH at 55 °C, for 9 hours determined by OPA method (Auwal et al., 2017)

Proximate analysis were done for raw material, liquid FPH, residual FPH, spray dried ultrafiltrated- FPH and spray dried centrifuged FPH , and amino acid composition were done for raw materials and spray dried ultrafiltrated- FPH .

3. Results and Discussions

Degree of hydrolysis (DH) was used to show the level of protein solubility after enzymatic hydrolysis. Analysis of DH was done as described by Auwal et al. (2017) using spectrophotometry technique at 340 nm. Results of DH measurement was presented in Figure 1, indicate that the degree of hydrolysis of fish protein at pilot plant scale reach the maximum after 6 hours and enter the stationary phase afterward. The maximum time for hydrolysis of this experiment was similar as reported by Fawzya et al (2017) using yellow stripe scad fish in laboratory experiment. Application of the same enzyme reach the maximum DH of 58% by method as described by Haslaniza et al. (2010) with some modifications based on ratio of TCA soluble N and total N. Several factors may affect the DH i.e, type and concentration of enzymes, as well as time of hydrolysis (Seniman, Yusop & Babji, 2014; Salwanee, Aida, Mamot, Maskat, & Ibrahim, 2013).

The entire production of FPH is outlined in figure 2 describing the step by step process of substrate preparation, hydrolysis process, and separation of products.

Mass balance calculations for the highest grade FPH powder (100% soluble spray dried powder) are shown in Table 1 which include mass of substrate, water and enzyme, volume of total mixture at the end of hydrolysis, mass of residue (insoluble matter in suspension) after removal of bones, volume of clarified hydrolysate (clear liquid containing soluble proteins) and FPH powder after spray drying.

The nutrients contained in food play an important role in health as sources of essential component for cell functions and metabolism. Fish hydrolysates can supply the protein needed as a building block of cells and tissue and metabolism process as enzymes or other functions. Table 2 shows the proximate composition on every stage of FPH production, from source materials to fish protein hydrolysate (FPH) product. The protein content of fish as raw materials is lower than the liquid filtration hydrolysate (92.24 % db vs 105.99 % db). It means that the hydrolysis process was able to improve the quality of nutrients of fish protein. However, when the hydrolysate was spray dried the protein content decreased (20.10 %db) due to the addition of maltodextrin as filler (Table 2). Protein and fat content of filtrated FPH was higher than centrifuged FPH. This finding showed that the micro and ultrafiltration technique was effectively improve the quality of FPH. However, the filtration was not able to remove the salt in FPH due to the size of salt molecules were much smaller than the pore size of the membrane.

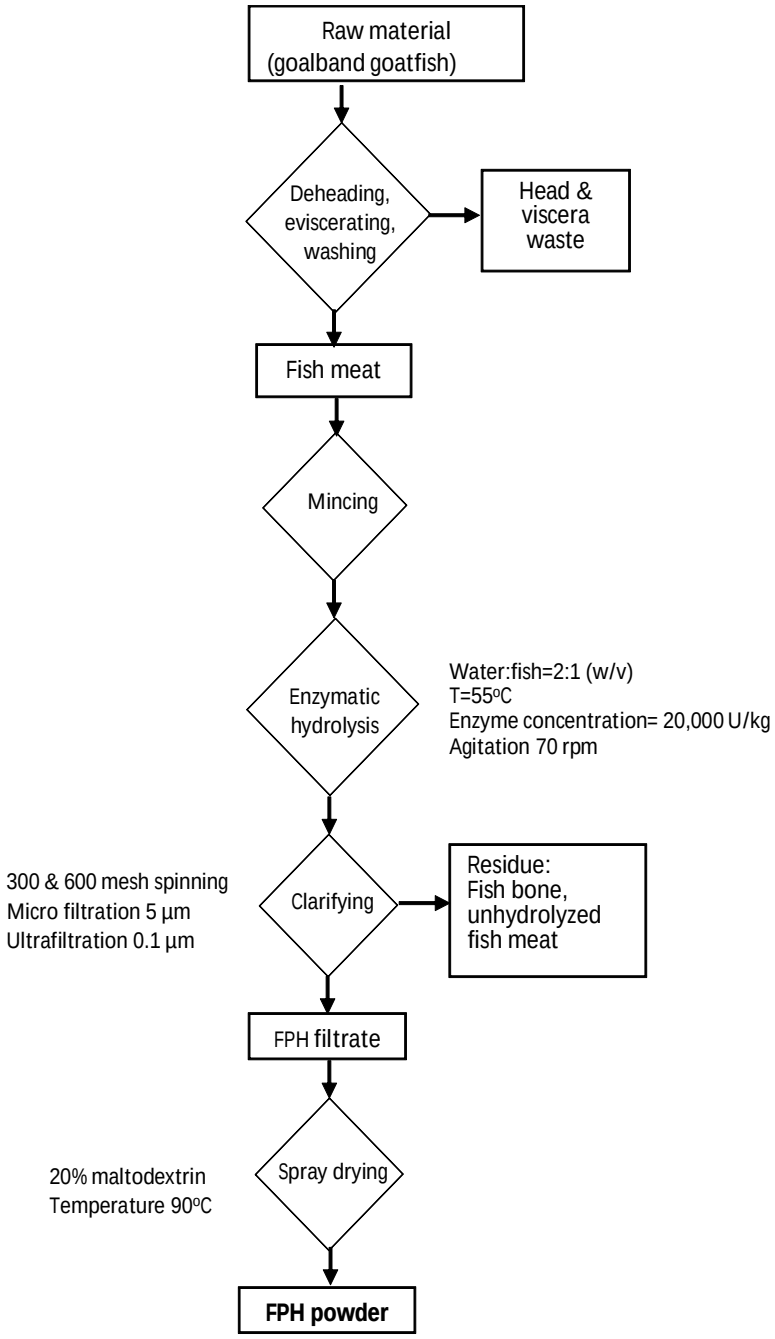

Figure 2. Production chart of fish protein hydrolysate (FPH)

Previous research on FPH stated that protein ranged between 20% to 90% of total composition depend on the source, basic of calculation (wet or dry basis) and the process of hydrolysis (type and amount of enzyme, time of hydrolysis, and drying methods) (Choi, Hur, Choi, Konno & park, 2009; Dong, Sheng, Fu & Wen, 2005; Khantaphant, Benjakul & Kishimura, 2011). The high protein content in FPH reported is mainly caused by solubilization of proteins during hydrolysis and removal of insoluble solid matter by several means of separation; centrifugation, filtration or other methods (Chalamaiah et al., 2012; Liceaga-Gesualdo & Li-Chan, 1999). High protein content of FPH offers its potential use as protein supplements for human nutrition, especially in childhood and elder. Product of enzymatic hydrolysis mainly composed of small size protein or peptides or free amino acids. These products have more advantages than unhydrolyzed fish protein in terms of digestibility and allergenic reaction. Small size peptides and free amino

Table 1. Mass balance of enzymatic FPH production

Material	Quantity
Raw fish	100 kg
Deheaded, evicerated fish	33.4 kg
Minced fish	31.8 kg
Water	63.6 L
Enzyme	2L (activity of 330 U/ml)
Liquid FPH	72.4 L
Spinner residue	1121 g
300 mesh residue	Fish bone and scales, 715 g
600 mesh residue	Unhydrolyzed fish meat 3,700 g
Microfiltration and ultrafiltration residue	43 g/L hidrolisat (= 3.1 kg)
FPH filtrate	68 L
Maltodextrin filler (20%)	13.6 kg
Spray dried FPH	13.2 kg

Table 2. Proximate analysis of materials

Sampel	Moisture (%)	Protein (%db)	Fat (%db)	Ash (%db)	Carbohydrate (%db)
goalband goatfish	77.46±0.48	92.24±2.66	1.86±0.18	7.23±0.53	
Liquid FPH (filtration)	97.83	105.99	0.43	0.14	
FPH powder (filtration *spray dried*)	3.31±0.21	20.10±7.74	0.19±0.21	2.77±1.02	70.01
Residue 600 mesh	83.06±0.03	85.36±0.18	11.16±1.77	3.78±0.06	
FPH powder (centrifuged spray dried)	3.69	18.59	1.54	0.25	

acid in FPH may easily digested and absorbed by cells in human bodies. Besides, allergic reaction in some people after consumption of fish protein is an IgE mediated immune response to specific protein in fish. These proteins are reacted with specific IgE in human bodies to create allergenic reaction (Kuehn, Swoboda, Arumugam, Hilder & Hentges, 2014). By enzymatic hydrolyisis, these proteins may lose their allergenic activity.

The fat content if FPH ranged between 0.19-11.16 % db or 0.18-1.89% wb in all samples. The results were in line with others studies that the fat content for many fish protein hydrolysates were below 5% (Abdul-Hamid, Bakar & Bee, 2002; Benjakul & Morrissey, 1997). Some kind of fish, especially fish with high fat contents may produce the fat content above 5% level for fish protein hydrolysates (Chalamaiah et al., 2012; Sathivel et al., 2003; Souissi, Bougatef, Triki-Ellouz & Nasri, 2007). The low fat content of fish protein hydrolysates is mainly caused by the fish used in the production of FPH (goalband goatfish) has low fat content and removal of lipids with insoluble protein fractions by filtration.

Moisture content of FPH powder is usually below 10% (Abdul-Hamid et al., 2002; Bhaskar, Benila, Radha, & Lalitha, 2008; Chalamaiah et al., 2012) and is mainly caused by the drying process employed in the production. The level of moisture content depends on the kind of fish processes preceeding the drying. The low moisture contents in powdered FPH have some advantages, such as high protein number per gram of consumption, lower bacterial activity and ease of handling.

Table 3. Amino acids content in kuniran fish and FPH

Amino acid	Amino acids content (μg/mg)	
	Raw material	Spray dried filtration FPH
Alanine	10.350	6.765
Glycine	10.725	6.925
Valine	8.145	4.785
Leucine	13.815	8.125
Isoleucine	7.785	4.080
Proline	7.055	4.090
Asparagine		-
Aspartic Acid	13.935	8.695
Methionine	-	-
Phenylalanine	9.025	4.230
Glutamic acid	26.185	16.290
Lysine	16.935	11.180
Histidine	4.495	3.105
Tyrosine	6.955	2.815
Tryptophan	-	-
Cysteine	-	-
Serine	5.855	5.43
Threonine	7.950	4.975
Arginine	12.060	7.160
Total	**161.27**	**98.65**

The ash content of fish protein hydrolysates was reported by many studies ranged from 0.45% to 2.7% of total composition by dry basis (Benjakul & Morrissey, 1997; Bhaskar et al., 2008; Chalamaiah *et al*., 2012). In this FPH products, the ash contents are relatively high, especially in residues. There were two factors that may contribute to these finding, i.e the salt content in fish material used in this experiments due to the presence of fish bone in the hydrolysis process and the salt presented crude enzyme used which contained salt originated from cultivation medium.

Fish protein hydrolysates are mainly composed of small size peptides and free amino acids which are have some advantages as functional foods and other functions. The amino acid composition of fish protein hydrolysates is important because of the nutritional value and the influence on the functional properties (Santos, Martins, Salas-Mellado & Prentice, 2011).

Depend on their source of raw materials, enzyme source, and hydrolysis conditions, fish protein hydrolysates have been reported to show some variation in their amino acid composition (Bhaskar *et al*., 2008; Ovissipour et al., 2009; Wasswa, Tang, Gu, & Yuan, 2007., Klompong, Benjakul, Kantachote, & Shahidi, 2009; Klompong, Benjakul, Yacha et al., 2009). The essential amino acids required for maintaining good health have been found abundantly in fish protein hydrolysates (Klompong, Benjakul, Kantachote, et al., 2009b; Sathivel et al., 2003; Yin, Pu, Wan, Xiang, Bechtel, & Sathivel, 2010). The amino acid content of FPH are lower than raw material and predominantly composed by glutamic acid, leucine, and lysine. This finding is important fact that the hydrolysis process can maintain the quality of amino acid composition. As comparison the highest amino acid content in raw fish was glutamic acid and all amino acids were presents in hyrolysisi product. Essential amino acids content in FPH are advantageous since they indicate the high quality of protein source, especially for infant where lysine is responsible mainly in human growth. Table 3 shows the amino acid composition of protein hydrolysates

prepared from fish raw material, and spraydried FPH filtration.

Other papers showed that aspartic acid and glutamic acid were found to be higher in most of the reported fish protein hydrolysates (Hou, Li, & Zhao, 2011; Klompong, Benjakul, Yacha et al., 2009a; Yin et al., 2010). These findings were in line with the results In this paper where glutamic acid was become predominant amino acid in the FPH product.

4. Conclusion

Pilot plant scale of fish protein hydrolisate (FPH) by enzymatic reaction has been done in our laboratory using controlled bioreactor and followed by series of filtration and spray drying. Mass balance of FPH production using *goalband goatfish* (*Upeneus moluccensis*) frames by protease enzyme produced by *Bacillus sp* showed that from 100 kg of raw fish produced 13.2 kg of spray dryed FPH. The protein content of spray dryed was above 25% and low fat and ash content which is rich in lysine and leucine. Both amino acids are important for growth of infant, so the FPH has a potential to be used in infant food as a protein source. Besides, the solid residue recovered from the process contains unhydrolyzed protein. This components that containing relatively high in protein (85,36% dry basis) can be used as other food ingredients such as flavor enhancer.

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