

Production of peptone from boso fish (*Oxyeleotris marmorata*) for bacterial growth medium

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Abstract. Underutilized *Oxyeleotris marmorata* fish is abundant and widespread in Indonesia. The study aimed to use *O. marmorata* fish for peptone production using papain from dried latex of papaya fruit. The peptone was applied as nitrogen sources for bacterial growth. The resulted peptone was optimized at 50-65°C for 5-8 hr, using 0.1% of papain at pH 6.0. Characterization of peptone was based on the soluble protein content, N-amino content, % degree hydrolysis (DH), SDS PAGE profile and growth of bacteria *Escherichia coli* and *Staphylococcus aureus*. The results indicated that the optimum condition for hydrolysis was at 50°C for 7 hr ($p < 0.05$). Fish peptone soluble protein content was of 8.6 mg/mL, α -amino was 0.59%, and AN/TN 5.47%. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) profile of peptone showed a major band with molecular weight between 17-28 kDa. Fish peptone effectiveness for *E. coli* and *S. aureus* growth was similar with commercial bacterial peptone.

1. Introduction

Peptone is a water-soluble mixture of polypeptides and amino acids which are widely used in many biological and biotechnological applications. Peptone is primarily obtained from bovine or porcine origin, such as meat, internal organs, gelatin, and milk, as well as from plants and yeasts [1]. Peptone is a partially digested protein, and prepared by the enzymatic or acidic hydrolysis of proteinaceous material. The resulted hydrolysate contains secondary protein derivatives such as polypeptides, dipeptides, and amino acids. They provide a source of nitrogen which is water soluble, does not coagulate on heating, and is therefore particularly suitable for inclusion in microbiological culture media [2]. Peptone is one of the good supplements and replacement of serum for the growth of the mammalian cells and production of the biological products. The benefits of peptone supplementation in cell culture applications have been well documented for many years as it contains low molecular weight nutrients, peptides and free amino acids which are good for cell culture [3].

Fish peptone is one of the alternative approaches for protein hydrolysate production for nitrogen source in microbial growth. The use of fish is not only an eco-friendly but also as a cheaper raw materials for media formulation. The utilization of sardine and mackerel wastes from fish processing industry has been studied for peptone recovery. The recovered peptones performance in microbial growth was found to be affected by hydrolysis method [4].

Indonesian marine biodiversity provides significant biological potential and is able to be exploited for its fish, seaweed, and other biological potential. Boso fish (*Oxyeleotris marmorata*) is a widely distributed species of sleeper goby native to fresh and brackish waters in Indonesia [5]. *O. marmorata* fish was potential for peptone production because of its abundant and cheap due to it less favored for people. Indonesia still imports peptone to fulfill its demand since there is no peptone industry available in Indonesia. Imported peptone within the last two years increase by 3,296 tones with the value of USD 12,5 million in 2012 and become 5,102 tones with the value of USD 20,76 million in 2013 [6]. The study aimed to utilize *O. marmorata* fish for peptone using papain from dried latex of papaya fruit.



2. Experimental

2.1. Materials

Boso fish (*O. marmorata*) was obtained from north beach of Indramayu, Indonesia. Papaya latex was harvested directly from papaya fruit at a California papaya farm in Bandung, Indonesia. *Escherichia coli* and *Streptococcus aureus* strains were obtained from Research Unit for Clean Technology collection. Oxoid as commercial peptone was used for comparison.

2.2. Methods

2.2.1. Enzyme preparation

Papain enzyme was prepared from papaya latex by drying it at 50°C for overnight. Enzyme activity of dried papain was characterized using casein as substrates. Yield of papain enzyme was of ±30% w/w.

2.2.2. Determination of papain activity

Papain activity was determined by modification of Afaq, S. and Iqbal, J. (2001) methods [7]. One mL of 2% casein (w/v) was mixed with 2 mL of phosphate buffer 0,1 M pH 7,0 and incubated at 50°C for 5 min. This solution was added with 0.5 mL of papain sample and incubated at 50°C for 20 min. The reaction was stopped with addition 1 mL of TCA 20% (v/v) and incubated at 50°C for 20 min. Control sample solution was prepared by the addition of 50 µl L-cystein chloride with 2 mL of phosphate buffer 0.1M, pH 7.0, 0.5 mL of papain, 1 mL of TCA 20%, and incubated at 50°C for 20 min. This solution was added with 1 mL of casein 2% and incubated at 50°C for 20 min. Tyrosine (0-450 µg/mL) was used as the standard solution. Sample and its control were centrifuged at 4°C, 6.000 rpm for 10 min. Absorbance of supernatant was measured by a spectrophotometer UV-Vis at 280 nm. The unit activity of papain enzyme was calculated based on µg tyrosine per gr of enzyme.

2.2.3. Peptone preparation

Peptone preparation was carried out by using a modification of Fahrniah et al. (2009) methods [8]. The frozen of boso fish was thawed and mixed with distilled water with a ratio 1:4. Samples were blended and pH adjusted to 6.0. The optimization of peptone production was carried out in a water bath by using 0.1% of papain at 50-65°C for 5-8 hr. Enzymatic hydrolysis process was stopped at 85°C and allowed to stand for 15 min. Peptone extract was vacuum-filtrated and filtrate was stored at -20°C. For molecular weight assay, the selected peptone extract was freeze dried.

2.2.4. Analysis of soluble protein content

Soluble protein content was analyzed by using a modification of Lowry (1951) method [9]. A 0.5 mL of peptone extract was added to a reaction tube and mixed with 5 mL of solution C (50 mL of Lowry A and 1 mL of Lowry B solution), then incubated at room temperature for 30 min. The sample solution was mixed with 0.5 mL of 1N Folin reagent. Absorbance was measured by a spectrophotometer UV-Vis at 500 nm. A series concentration of bovine serum albumin (BSA) was used for standard curve of protein.

Determination of Degree Hydrolysis

Degree hydrolysis (DH) of peptone extract was calculated using relationship between α -amino nitrogen (AN) and total nitrogen (TN) according to equation [10]:

$$\%DH = \frac{\alpha - \text{amino Nitrogen (AN)}}{\text{Total Nitrogen}} \times 100$$

Total nitrogen was determined by Kjeldahl method [11]. α -Amino nitrogen was analyzed using a modification of Sudarmadji (1997) method [12]. Five mL of peptone extract was mixed with 10 mL of distilled water and 0.2 mL of saturated K-oxalate (K-oxalate : water = 1:3) and 5 drops of 1% phenolphthalein, then stirred the solution for 2 min. The mixture was titrated with 0.1 N NaOH to pink

color and added with 2 mL of formaldehyde. Titration was continued with 0.1 NaOH to its final end-point. The concentration of α -AN was calculated using the following equation:

$$\alpha - AN (\%) = \frac{V}{W \times 10} \times N_{NaOH} \times 14.008$$

V: titration volume, W: weight of sample.

2.2.5. Statistical analysis

The data of soluble protein content and % DH was evaluated by General Linear Model with univariate analysis. Duncan's multiple range tests were used to compare the means to identify which groups were significantly different from others. Significance was defined at $P < 0.05$.

2.2.6. Application of peptone produced as bacterial growth media

Bacterial growth assay was determined by Rahayuningsih and Wiranti (2014) method [6]. The strains of *E. coli* and *S. aureus* were used to test the microbial growth performance. The inoculum was prepared by inoculating one loops of strains to 100 mL of Nutrient Broth (NB) media and incubated on a shaker incubator at 30°C for 18 hr. Furthermore, 1 mL of inoculum was inoculated into 100 mL of NB media contains 5% (v/v) of peptone extract. Both strains were grown at 30°C and cultivation was performed for 24 hr on a shaker incubator. The assay was compared with a commercial peptone. The growths of both microbes were monitored every 2 hr by measuring optical density at 600 nm.

2.2.7. Molecular weight analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done on all samples and protein marker on a discontinuous buffered system according to Laemmli in Bollag et al. (1996) method [13]. Twenty μ L of each sample was added with 40 μ L of sample buffer. Samples were placed in a foam rack and placed in a beaker of boiling water for 4 min. The prepared of polyacrylamide gel was placed in an electrophoresis unit. Running buffer was filled to the upper buffer chamber of the gel until the buffer reaches halfway between the tops of the short and long glass plates. Five μ L of standard protein markers and 25 μ L of each samples were added to the polyacrylamide gel. Electrophoresis was conducted at a constant 200 V for 30 min. The gel was removed and placed in staining solution.

3. Results and discussion

Fish peptone has been produced by enzymatic hydrolysis process using papain enzyme from dried papaya latex. The proteolytic activity of papain was of 68.46 ± 4.95 unit, of which unit activity was calculated based on μ g tyrosine production per gram of enzyme. Latex obtained from unripe papaya (*Carica papaya* L.) fruits is a mixture of proteolytic enzymes including papain, chymopapain A and B (EC 3.4.22.6), endopeptidase papain III, endopeptidase papain IV and endopeptidase papain omega [14]. Catalytic activity of papain involves hydrolysis of proteins with broad specificity for peptide bonds, but preference for an amino acid bearing a large hydrophobic side chain [15]. Meanwhile, protein and fat content of boso fish were $67.32\% \pm 1.86$ and $4.02\% \pm 0.22$, respectively.

Generally, enzymatic hydrolysis process influenced by some conditions such as temperature, time, pH, substrate and enzyme concentrations. In this study, the optimization of fish peptone production was carried at 50-65°C for 5-8 hr. Other conditions, such as pH, substrate and enzyme concentrations have been determined. In this study, optimum pH of substrate was 6.0 with 25% (b/v) substrate and 0.1% of enzyme concentration. Protein soluble and ratio of α -amino (AN) to total nitrogen (TN) were analyzed to evaluate the optimum condition of fish peptone production. The effect of hydrolysis temperature on soluble protein content of fish peptone extract was monitored for 4-8 hr. The data is presented on Figure 1.

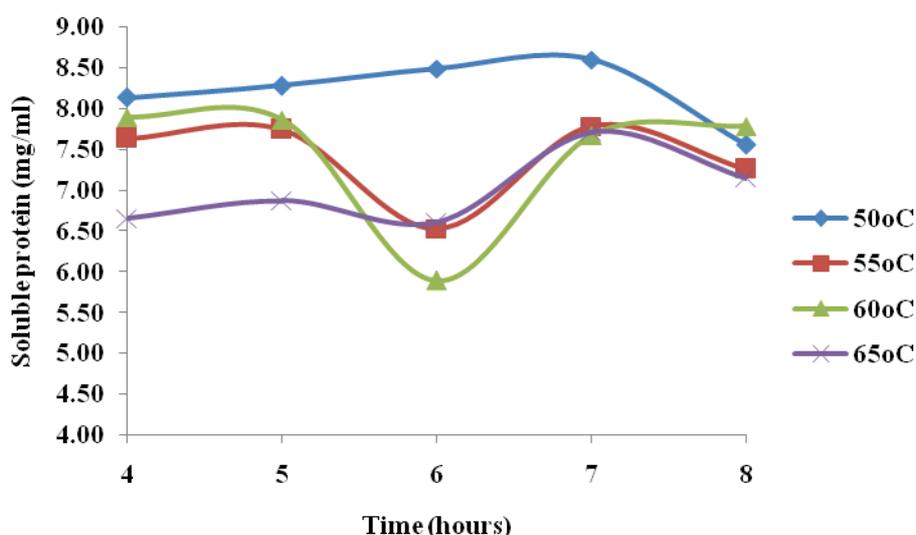


Figure 1. The effect of hydrolysis temperature on soluble protein content of peptone.

Figure 1 shows that the highest soluble protein content was obtained at 50°C after 7 hr of enzymatic hydrolysis. At this condition, fish peptone had the soluble protein content of 8.6 mg/mL. Statistical analysis followed by Duncan test ($p < 0.05$) showed that hydrolysis temperature of 50°C was significantly different with other hydrolysis temperatures. Rahayuningsih and Wiranti (2014) reported the best temperature for peanut meal peptone production using 0.4% of crude papain was 55°C for 4 hr. Moreover, the production of soybean meal peptone production was at 60°C for 5 hr [8]. Based on statistical analysis, hydrolysis time was also significant to soluble protein content. The data showed that the soluble protein content of fish peptone at hydrolysis temperature 55-65°C, after 5 hr hydrolysis was decreased and increased after 6 hr hydrolysis. Temperature correlates with the chemical reaction due to the changes of kinetic energy. The increase of enzymatic reaction will be performed on certain temperature. When hydrolysis temperature is too high, protein enzyme denatures, tertiary structure of enzyme disrupts and the catalytic activity of this enzyme decreases [6]. In this study, protein soluble of fish peptone was increased after 7 hr hydrolysis. This phenomena was occurred at hydrolysis temperature 55-65°C, but was not occurred at 50°C. This assumed that the stability of protein was disrupted at temperature more than 50°C. Degree hydrolysis was generally used for monitoring the hydrolysis process in peptone production. Peptones are partially digested protein; they are prepared by the enzymatic or acidic hydrolysis of proteinaceous material. These hydrolysates contain secondary protein derivatives such as polypeptides, dipeptides, and amino acids. Degree hydrolysis of proteins was measured by the number of peptide bonds cut, divided by the total number of peptone bonds [2]. The effect of hydrolysis temperature to AN/TN of fish peptone extract was also monitored for 4-8 hr (Figure 2).

Figure 2 shows that degree of hydrolysis (AN/TN) of fish peptone production at 50-65°C for 4-8 hr was not significantly different ($p > 0.05$). Degree of hydrolysis of protein depends on three basic principles namely the quantification of the nitrogen released by protein hydrolysis, the determination of free amino groups, and the titration of protons released [10]. Based on the data in Figure 1, the optimum condition for enzymatic hydrolysis was reached at 50°C for 7 hr. At this condition, fish peptone product had α -amino content 0.59% and AN/TN 5.47%. Study of fish peptone using enzymatic hydrolysis of silver carp by-products in *S. aureus* media showed that degree of the protein hydrolysate from alcalase and trypsin was of 4.94 and 4.6%, respectively [1].

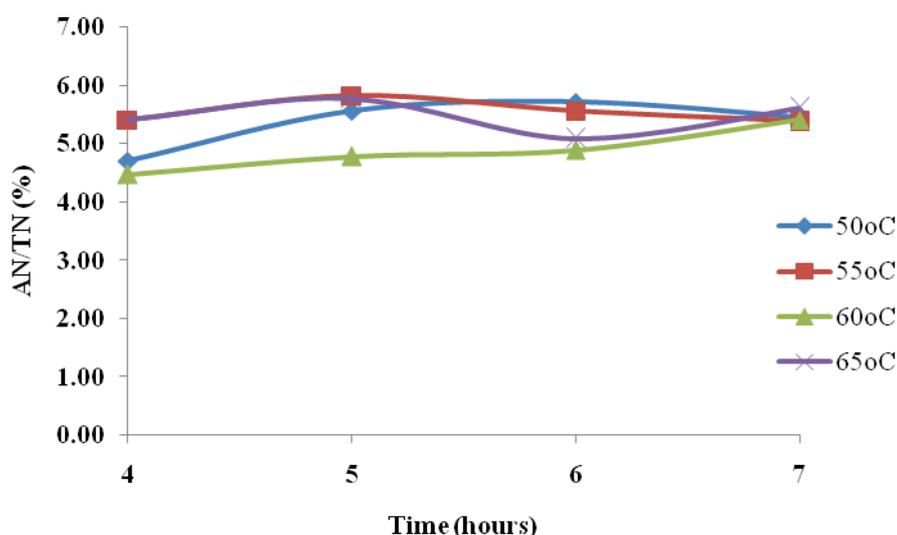


Figure 2. The effect of hydrolysis temperature on AN/TN of fish peptone extract.

SDS PAGE profile shows that the molecular weight was of between 17 -28 kDa (arrow sign) whereas commercial peptone (CP) was ≤ 6 kDa (Figure 3). Commercial peptone used in this study was produced from soya bean as raw material. Beside raw materials, parameters such as time, pH, temperature and enzyme concentration was also influences to the enzymatic activity co-operatively [16]. The molecular weight of peptone from fish waste which hydrolyzed by alcalase was < 6.5 kDa and by alkaline was < 21 kDa [4]. The hydrolysis of a protein molecule is a gradual process by which the gigantic molecule of protein is converted in to products of successively lower molecular weight such as peptones [2].

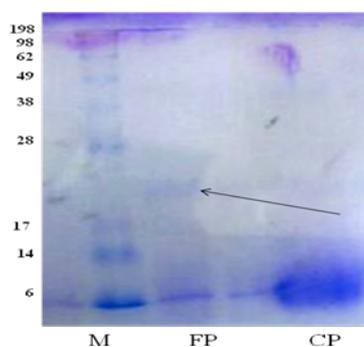


Figure 3. SDS PAGE profile of fish peptone extract from boso fish. M: protein marker, FP: fish peptone extract, CP: commercial peptone.

Fish peptone extract was tested for bacterial growth media and compared with commercial peptone. In this study, the concentration of fish peptone extract in media was calculated based on the protein content which equal with the protein content in commercial peptone. The growth of *E. coli* and *S. aureus* was monitored every two hr until 24 hr. The growth curves of *E. coli* and *S. aureus* is presented in Figure 4.

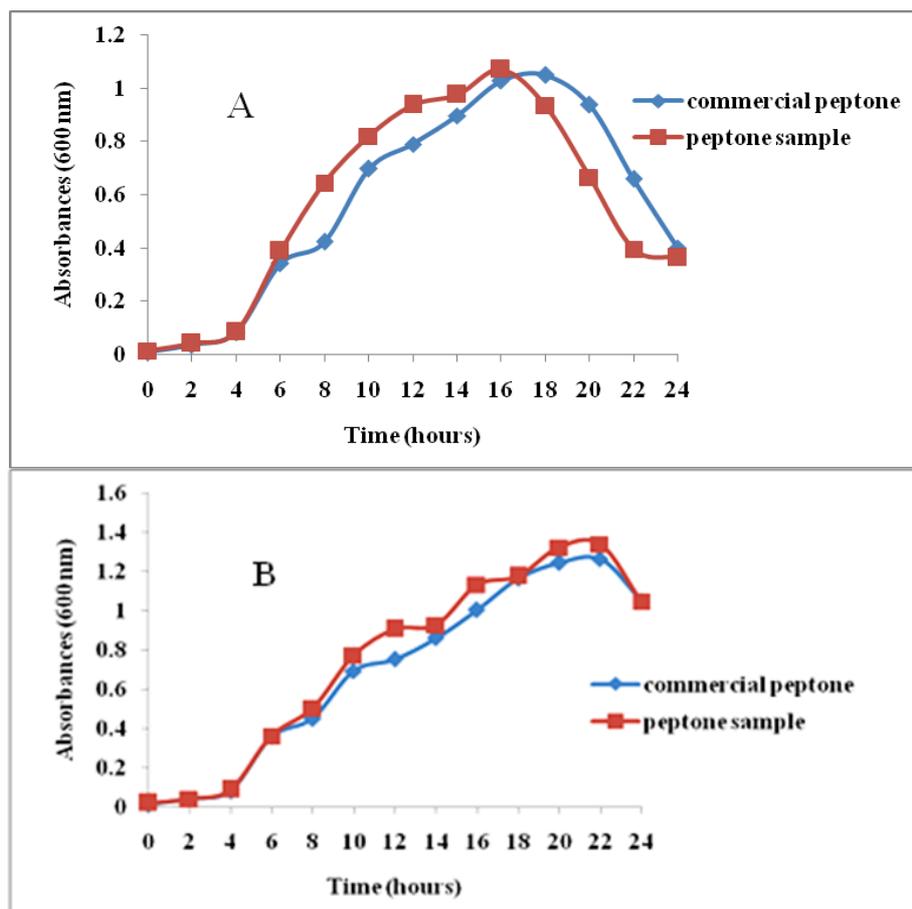


Figure 4. Growth curves of *E. coli* (A) and *S. aureus* (B).

Cell density for the peptones from boso fish showed superior performance in *E. coli* compared with commercial peptone. The data shows that lag phase of *E. coli* in peptone was about 4 hr. Log phase of *E. coli* in fish peptone sample was occurred for 12 hr with the maximum absorbance was $A_{600} = 1.0275$ whereas commercial peptone was occurred for 16 hr with the maximum absorbance was $A_{600} = 0.9350$, respectively. Furthermore, the absorbance of *E. coli* decreased, indicating that the death phase was occurred after 16 hr for peptone sample and 18 hr for commercial peptone. Vieira et al. 2005 [17] reported the performance of peptones produced from different fisheries by-product for *E. coli* compared with commercial peptone. The better performance of fish peptone in comparison with commercial media was also reported by other researches [1]. As shown on Figure 4B, the growth curves of *S. aureus* in fish peptone sample was similar with commercial peptone. *S. aureus* showed the same lag phase with *E. coli*. However, *S. aureus* has longer log phase than *E. coli*. The maximum absorbances of *S. aureus* in both fish peptone sample ($A_{600} = 1.2450$) and commercial peptone ($A_{600} = 1.323$) were reached after 22 hr incubation. Rahman et al (2004) reported peptone from waste beer industry gave the same effectiveness for *S. aureus* as that from commercial Bacto peptone and yeast extract. The maximum absorbances of *S. aureus* in peptone from waste beer industry reached after 16 hr incubation. The lag phase is defined to transition to the exponential phase after the initial population has doubled. The lag phase is thought to be due to the physiological adaptation of the cell to the culture conditions. This may involve a time requirement for induction of specific messenger RNA (mRNA) and protein synthesis to meet new culture requirements. Meanwhile, during log phase or exponential growth the rate of increase of cells in the culture is proportional to the number of cells present at any particular time and the death phase is characterized by a net loss of culturable cells [18].

4. Conclusion

Peptone has been successfully produced from Boso fish (*O. marmorata* of which the optimum condition for hydrolysis process was obtained at 50°C for 7 hr ($p < 0.05$). Soluble protein content of the resulted peptone was of 8.6 mg/mL whereas α -amino content and AN/TN was of 0.5 and 5.47%, respectively. SDS PAGE profile of fish product showed a major band with molecular weight between 17-28 kDa. The effective of fish peptone for *E. coli* and *S. aureus* growth was similar with commercial bacterial peptone.

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