Characteristics of Tuna Viscera (Thunnus sp.) Hydrolysate Protein Fermented by Bacillus licheniformis

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Abstract: This study aims to analyze the nutritional composition of the degree of hydrolysis and amino acids in the internal organ waste of tuna and the protein hydrolyzate of tuna’s internal organs after fermentation using the Bacillus licheniformis bacteria. The analysis showed that the protein content of tuna offal was 53.52%, and after fermentation by Bacillus licheniformis, bacteria were able to increase protein levels from 56.04. The degree of hydrolysis of protein (DH) showed an increase of 13.24% in tuna offal to 22.28% of protein hydrolyzates are fermented tuna inards. The total essential amino acids and non-essential amino acids in the fermented tuna inards’ protein hydrolyzates increased during the fermentation process. The highest levels of essential amino acids were found in arginine as high as 3.632965 at the 96th hour, and the lowest histidine was 1.082602. In contrast, for the highest non-essential amino acids, there was glycine at 8.52223, and the lowest for tyrosine was 1.272592.

Introduction
Tuna is one of the most economically significant groups of fish species. The main types of tuna are skipjack, yellowfin, bigeye, albacore, and bluefin. Skipjack is the largest species in terms of the number of arrests, while yellowfin is the largest type of tuna in international trade (Nguyen et al., 2011). With the increasing number of tuna processing industries, the larger organs in fish and by-products will be more extraordinary. The number of by-products of fish offal in some types of fish ranges from 5-10% of the total weight of fish, where the percentage of fish offal tends to increase with fish weight.

Tuna fisheries waste consists of 17% head, 8% skin, 5% offal, 4% bone, and 2% fin (Sayana and Sirajuddeen, 2017). Jereon tuna fish still has a protein content of 18.49%, which can be used as a fish protein hydrolyzate material (Parvathy et al., 2016). Prasertsan and Prachumratana (2008) state that tuna species captured from different regions have different viscera. The viscera of tuna range from 5.18% to 7.05%. The viscera of tuna contain various enzyme activities. Spleen from yellowfin tuna contains the highest protease enzyme activity (53.38 U/mL with a specific 2.56 U/mg). In comparison, the pancreas has the highest lipase enzyme activity (0.72 U/mL with a specific activity of 0.03 U/mg).

The tuna viscera contain several types of protease enzymes that are most important such as aspartic protease (pepsin) and serine proteases (trypsin, chymotrypsin, collagenase, and elastase) (Klomklao and Bejagul, 2016). Trypsin (EC 3.4.21.4) is one of the enzymes serine proteinase found in tuna viscera, especially in the pyloric caeca and intestine parts, which function in the target’s hydrolysis proteins in amino acid types such as arginine and lysine (Unajak et al., 2012). Hydrolysate of fish protein is produced from the decomposition of fish proteins into simple peptides and amino acids through hydrolysis by enzymes, acids, bases, and fermentation (Kristinsson and Rasco 2000; Jemil et al., 2014).

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Fermentation has been considered one of the most environmentally friendly, safe, technologically, flexible, and economical ways of making protein hydrolysates. The most important type of microbes used in the fermentation process to produce proteases is Bacillus sp. (Rao et al., 1998).

Previous research conducted by Mao et al. (2013) showed that the bacteria B. licheniformis OPL-007 can be used as a fermentor agent in shrimp head waste fermentation, with nutrient content of protein hydrolysates such as bioactive (phenols), polysaccharides, amino acids, and high organic matter. Other studies used B. subtilis A26 bacteria to ferment sardines, goby fish, zebrafish blenny fish, and ray fish to produce fish protein hydrolysates (Jemil et al., 2014) and fermentation in the manufacture of zebrafish blenny protein hydrolysates using B. mojavensis (Jemil et al., 2016). This study aims to determine the chemical characteristics of the protein hydrolysate products of Tuna offal (Thunnus sp.) through a fermentation process using the bacteria B. licheniformis OPL-007 on nutrient content, hydrolysis degree, and amino acid.

Materials and methods

Research Methods

The research method used in this study is the experimental method. The experimental research method is a research method used to look for specific treatments' influence in controlled conditions. Therefore, the experimental procedure is used to reveal whether or not there are influences from the variables that have been chosen to be used as research (Sugiyono, 2009). This study aims to determine at the results of the hydrolysate products of Tuna offal before fermentation and after fermentation for 96 hours by Bacillus licheniformis.

Location and Time of Research

The research was conducted from August to November 2018. 10 kg of tuna offal were taken randomly from 10 fish sellers at the Sendang Biru Fish Auction Place, Malang Regency, East Java. This research is divided into 4 processes, namely: 1) Proximate Analysis carried out at the Nutrition Laboratory, Muhammadiyah University of Malang; 2) Determination of Degree of Hydrolyzate carried out at Universitas Brawijaya Chemical Laboratory; 3) Determination of Molecular Weight in Biomedical Labotarium, Faculty of Medicine, Universitas Brawijaya and 4) Determination of Percentage of Amino Acid in Saraswati Indo Genetech Laboratory, Jakarta.

Research Procedures

Hydrolyzate protein fermentation process

Preparation of bacteria conducted according to the published method (Islamy, 2019). The inoculum (pure culture) used is the bacterium B. licheniformis B1008 (collection of the Microbiology Laboratory LIPI Cibinong). The bacterial growth media used were Nutrient Agar (NA) and basal media (nutrients) in the test tube with sloping media.

The initial process begins with tuna viscera cleaned and separated into each part (stomach, intestine, liver, and spleen). The fat lining of the viscera is removed (Bhaskar et al., 2008). The viscera (stomach, intestine, liver, spleen) weighed to do proximate analysis to determine the initial nutrient composition and initial amino acid content.

Tuna (Thunnus sp.) Placed on the bottle as much as 100 gr added B. licheniformis as much as 3% v/w with a density of 108, then added 20% molasses stirred until homogeneous and put into 250 ml Erlenmeyer, and as a treatment carried out observations on fermentation time of day 0th and 96th hours. After incubation, the fermented media is heated in boiling water for 15 minutes to stop the enzymatic reaction. The sample was then centrifuged at 12,000 rpm for 10 minutes, and the supernatant was stored at -20°C until it was used.
Proximate Analysis

Proximate analysis of tuna hydrolyzate consists of Protein (%), Fat (%), Ash (%), Crude (%) crude fiber (%) test using the SNI 1992-01-2891, AOAC (2005) and Pratama et al. (2019) methods tested in the Nutrition laboratory Malang Muhammadiyah University.

Hydrolyzate Protein Making Process

Degree of Hydrolyzate

The degree of hydrolysis is calculated based on the percentage ratio of trichloroacetic acid (TCA). A total of 20 mL of protein hydrolyzate added 20% (b/v) TCA as much as 20 mL. The mixture is then allowed to stand for 30 minutes for precipitation, centrifuged (speed of 7,800 x g, for 15 minutes). The supernatant then analyzed its nitrogen level using the Kjeldahl method. The following formula can calculate the degree of hydrolysis:

\[
\text{% Degree Hydrolysis} = \frac{\text{Disolved Nitrogen in TCA 20\% (h/v)}}{\text{Total Nitrogen Sample}}
\]

Amino Acid Analysis (UPLC)

Testing amino acids using the Ultra Performance Liquid Chromatography (UPLC) method. Analysis of amino acids using the UPLC consists of several stages. The sample was weighed as much as 0.1 g was crushed and put into a closed test tube. The sample solution was added with 6 N HCl as much as 5-10 mL, hydrolyzed in an oven at 110 °C for 22 hours, then cooled at room temperature and transferred to a 500 mL measuring flask. Then, add distilled water to the boundary and filter with a 0.45 μL filter and piped 10 μL, adding 70 μL AccQ Fluoric Borate and divorce. Then 20 μL of the Flour Adan reagent was added to be cooked and left to stand for 1 minute and added for 10 minutes at 55 °C. then injected into the UPLC as much as 1 μL with chromatographic conditions using ACCQ-Tag Ultra C18 column, temperature 49 °C, phase of system motion PDA composition gradient detectors, flow rate 0.7 μL / minute and wavelength 260 nm.

Results and Discussion

Table 1. Proximate Composition of Fermented Tuna Viscera Protein Hydrolysate

| Proximate analysis (g kg−1 dry wt.) | Before Fermentation | After fermentation |
|-----------------------------------|---------------------|--------------------|
| Dry mater                         | 9.38                | 8.93               |
| Protein                           | 53.52               | 56.04              |
| Fat                               | 5.67                | 3.67               |
| Crude Fiber                       | 1.38                | 1.25               |
| Ash                               | 6.15                | 6.79               |

The proximate composition of protein hydrolyzates from tuna offal at the 0th to 96th hours in Table 1 above shows that the fermentation of tuna offal using Bacillus licheniformis can increase protein levels 53.52 to 56.04. The proximate analysis of dry, fat, crude fiber and ash content decreased after fermentation using Bacillus licheniformis.

Bacillus licheniformis is a species of bacteria that can grow at a temperature of 45-50°C and produce high amounts of proteases. Proteases catalyze the breakdown of peptide bonds in peptides, polypeptides, and proteins into simpler molecules such as short-chain peptides and amino
acids. The fermentation duration is related to the phase of microbial growth, which will continue to change from time to time during the fermentation process. The longer time used in the fermentation process can provide an opportunity for microbes to overhaul the components inside the substrate into components that are simpler and easier to digest (Soeka et al., 2011).

According to Aisjah (1995), a longer incubation time means more opportunities for microbes to grow and reproduce so that metabolic concentrations get higher until they become limited, which can then cause a declining growth rate. In contrast, a short incubation time results in limited microbial growth opportunities and multiply. The number of substrate components that can be converted into cell mass is also small.

Table 2. Degree of Protein Hydrolysis

| Parameters     | Before Fermentation | After Fermentation |
|----------------|---------------------|--------------------|
| Degree Hydrolysis (%) | 13.24 %             | 22.28 %            |

The data in table 2 above shows the results of the degree of hydrolysis of tuna innards before fermentation by (DH = 13.24), after fermentation using Bacillus licheniformis bacteria hydrolysis of tuna innards by (DH = 22.28%). In the study (Jemil et al., 2017), hydrolyzate protein fermentation of Sardinella fish using B. subtilis A26 produced hydrolysis degrees of (DH = 21.56%) and B. amyloliquefaciens An6 showed a higher degree of hydrolysis of (DH = 24.3%). The difference in the hydrolysis degree (DH) value is basically due to the difference in the enzyme’s specificity produced by proteolytic bacteria used. The high DH obtained can be caused by the synergistic effect of several proteases produced by bacteria during fermentation. According to Nilsang et al. (2005), the degree of hydrolysis increases with increasing hydrolysis time. This occurs because of the breakdown of the peptide bonds carried out by the enzyme. However, this DH is lower than fermented redfish protein hydrolyzate (DH = 40.9%) (Khiari and Mason 2018) and fermented anchovy waste (50%) (Yu et al., 2014; Rai et al., 2011).

Table 3. Amino Acids Composition Fermented Tuna Viscera Protein Hydrolysate

| Amino Acids (mg/100gr) | Before Fermentation | After Fermentation |
|------------------------|---------------------|--------------------|
| Arginine               | 2.876764            | 3.632965           |
| Histidine              | 0.987115            | 1.082602           |
| Isoleusin              | 1.982352            | 2.139795           |
| Leusin                 | 3.191251            | 3.196746           |
| Lisin                  | 2.067150            | 2.248013           |
| Valin                  | 2.498434            | 2.543618           |
| Fenilalanin            | 2.311895            | 2.340651           |
| Threonin               | 2.228858            | 2.867939           |
| Glutamic Acid          | 4.638858            | 5.390570           |
| Aspartic Acid          | 2.965296            | 3.088997           |
| Serine                 | 2.157002            | 2.192696           |
| Proline                | 3.053876            | 3.539084           |
The amino acid profile of fermented tuna viscera before and after fermentation is presented in Table 3. The total essential amino acids and non-essential amino acids in tuna viscera’s fermentation increased during the fermentation process. The highest levels of essential amino acids were found in arginine as high as 3.632965 at the 96th hour, and the lowest histidine was 1.082602. In contrast, for the highest non-essential amino acids, there was glycine at 8.52223, and the lowest for tyrosine was 1.272592.

Amino acids analyzed using UPLC include 15 types of amino acids. Amino acids that are not analyzed include tryptophan, proline, cysteine, asparagine, and glutamine. Hydrolysis that runs perfectly will produce hydrolysates consisting of a mixture of 18-20 kinds of amino acids. All hydrolyzed proteins will produce amino acids, but several proteins produce amino acids and produce protein molecules that are still bound (Annisa et al., 2017).

Conclusions and Suggestion
Based on research on protein hydrolyzates, tuna viscera are fermented by Bacillus licheniformis. The conclusion is that the fermentation of tuna offal using Bacillus licheniformis bacteria can increase protein levels from 53.52 to 56.04. The proximate analysis of the dry matter, fat, crude fiber, and ash content decreased after fermentation. The results of protein hydrolysis levels showed an increase from 13.24% to 22.28%. The total essential amino acids and non-essential amino acids in tuna viscera’s fermentation increased during the fermentation process. The highest levels of essential amino acids were found in arginine as high as 3.632965 at the 96th hour, and the lowest histidine was 1.082602. In contrast, for the highest non-essential amino acids, there was glycine at 8.52223, and the lowest for tyrosine was 1.272592. We suggest using Bacillus licheniformis in the fermentation process to get good tuna protein hydrolyzate.

References
Aisjah, T. (1995). Biokonversi Limbah Umbi Singkong menjadi Bahan Pakan Sumber Protein oleh Jamur Rhizophus serta pengaruhnya terhadap Pertumbuhan Ayam Pedaging. Disertasi. Universitas Padjadjaran. Bandung.
Annisa, S., Darmanto, Y. S., & Amalia, U. (2017). Pengaruh perbedaan spesies ikan terhadap hidrolisat protein ikan dengan penambahan enzim papain (the effect of various fish species on fish protein hydrolysate with the addition of papain enzyme). Saintek perikanan: Indonesian Journal of Fisheries Science and Technology, 13(1), 24. https://doi.org/10.14710/ijfst.13.1.24-30
Ariyani F., Heruwati E.S., Murdina., Wibowo., & Susetyo. (2001). Pemanfaatan kepala ikan tuna dan isi perut ikan pari sebagai sumber pepton kasar bagi media pertumbuhan mikroorganisme. Jurnal Penelitian Perikanan Indonesia, 7, 75-84.
Association of Official Analytical Chemistry (AOAC). (2000). Official Methods of Analysis. Canada: Mc Graw Hill Press.
Belkaaloul A., A. Checroun., A. I. AitAbdesalam., D. Saidi, and O. Kherouoa. (2010). Growth, acidification and proteolysis performance of two co-cultures (Lactobacillus plantarum Bifidobacterium longum and Streptococcus Thermophilus bifidobacterium longum). African Journal of Biotechnology, 9(10), 1463-1469.

Islamy, R. A. (2019). Antibacterial Activity of Cuttlefish Sepia sp. (Cephalopoda,) Ink Extract Against Aeromonas hydrophila. Majalah Obat Tradisional, 24(3), 184. https://doi.org/10.22146/mot.45315

Jemil, I., Jridi, M., Nasri, R., Ktari, N., Ben Slama-Ben Salem, R., Mehiri, M., Hajji, M., & Nasri, M. (2014). Functional, antioxidant and antibacterial properties of protein hydrolysates prepared from fish meat fermented by Bacillus subtilis A26. Process Biochemistry, 49(6), 963–972. https://doi.org/10.1016/j.procbio.2014.03.004

Jemil, I., Mora, L., Nasri, R., Abdelhedi, O., Aristoy, M.-C., Hajji, M., Nasri, M., & Toldrá, F. (2016). A peptidomic approach for the identification of antioxidant and ACE-inhibitory peptides in sardinelle protein hydrolysates fermented by Bacillus subtilis A26 and Bacillus amyloliquefaciens An6. Food Research International, 89, 347–358. https://doi.org/10.1016/j.foodres.2016.08.020

Jemil, I., Nasri, R., Abdelhedi, O., Aristoy, M.-C., Salem, R. B. S.-B., Kallel, C., Marrekchi, R., Jamoussi, K., Elfeki, A., Hajji, M., Toldrá, F., & Nasri, M. (2017). Beneficial effects of fermented sardinelle protein hydrolysates on hypercaloric diet induced hyperglycemia, oxidative stress and deterioration of kidney function in wistar rats. Journal of Food Science and Technology, 54(2), 313–325. https://doi.org/10.1007/s13197-016-2464-9

Khiari, Z., & Mason, B. (2018). Comparative dynamics of fish by-catch hydrolysis through chemical and microbial methods. Lwt-Food Science and Technology, 97, 135-143.

Klomklao, S., & Benjakul, S. (2016). Utilization of Tuna Processing Byproducts: Protein Hydrolysate from Skipjack Tuna (Katsuwonous pelamis) Viscera. Journal of Food Processing and Preservation, 41(3), e12970. https://doi.org/10.1111/jfpp.12970

Kristinsson, H. G., & Rasco, B. A. (2000). Biochemical and functional properties of Atlantic salmon (Salmo salar) muscle proteins hydrolyzed with various alkaline proteases. Journal of agricultural and food chemistry, 48(3), 657–666. https://doi.org/10.1021/jf990447v

Mao, X., Liu, P., He, S., Xie, J., Kan, F., Yu, C., Li, Z., Xue, C., & Lin, H. (2013). Antioxidant Properties of Bio-active Substances from Shrimp Head Fermented by Bacillus licheniformis OPL-007. Applied Biochemistry and Biotechnology, 171(5), 1240–1252. https://doi.org/10.1007/s12010-013-0217-z

Nguyen, H. T. M., Pérez-Gálvez, R., & Bergé, J. P. (2012). Effect of diets containing tuna head hydrolysates on the survival and growth of shrimp Penaeus vannamei. Aquaculture, 324–325, 127–134. https://doi.org/10.1016/j.aquaculture.2011.11.014
Nilsang, S., Lertsiri, S., Suphantharika, M., & Assavanig, A. (2005). Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. Journal of Food Engineering, 70(4), 571–578. https://doi.org/10.1016/j.jfoodeng.2004.10.011

Parvathy, U., Zynudheen, A.A., Panda, S.K., Jeyakumari, A., & Anandan, R. (2016). Extraction of Protein from Yellowfin Tuna (Thunnus albacares) Waste by Enzymatic Hydrolysis and its Characterization. Fishery technology, 53, 115-124.

Poonsuk, P. & Thiraratana. (2008). Comparison and selection of protease and lipase sources from visceral organs of three tuna species. Songklanakarin Journal of Science and Technology, 30(Suppl.1).

Pratama, W. W., Nursyam, H., Hariati, A. M., Islamy, R. A., & Hasan, V. (2020). Short Communication: Proximate analysis, amino acid profile and albumin concentration of various weights of Giant Snakehead (Channa micropeltes) from Kapuas Hulu, West Kalimantan, Indonesia. Biodiversitas Journal of Biological Diversity, 21(3), 1196–1200. https://doi.org/10.13057/biodiv/d210346

Rai, A. K., Jini, R., Swapna, H. C., Sachindra, N. M., Bhaskar, N., & Baskaran, V. (2011). Application of Native Lactic Acid Bacteria (LAB) for Fermentative Recovery of Lipids and Proteins from Fish Processing Wastes: Bioactivities of Fermentation Products. Journal of Aquatic Food Product Technology, 20(1), 32–44. https://doi.org/10.1080/10498850.2010.528174

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. Microbiology and molecular biology reviews: MMBR, 62(3), 597–635.

Sayana, K. S., & Sirajudheen, T. K., (2017). By-products from Tuna processing wastes an economic approach to coastal waste management. Proceedings of the International Seminar on Coastal Biodiversity Assessment, 411-420.

Soeka, Y.S., Rahayu, S. H., Setianingrum, N. & Naiola. E. (2011). Kemampuan Bacillus licheniformis dalam Memproduksi Enzim Protease yang Bersifat Alkali dan Termofilik. Media Litbang Kesehatan, 21(2), 89-94.

Unajak, S., Meesawat, P., Paemanee, A., Areechon, N., Engkgul, A., Kovitvadhi, U., Kovitvadhi, S., Rungruangsk-Torrissen, K., & Choowongkomon, K. (2012). Characterisation of thermostable trypsin and determination of trypsin isozymes from intestine of Nile tilapia (Oreochromis niloticus L.). Food Chemistry, 134(3), 1533–1541. https://doi.org/10.1016/j.foodchem.2012.03.074

Yu, X., Mao, X., He, S., Liu, P., Wang, Y., & Xue, C. (2014). Biochemical properties of fish sauce prepared using low salt, solid state fermentation with anchovy by-products. Food Science and Biotechnology, 23(5), 1497–1506. https://doi.org/10.1007/s10068-014-0205-2.