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Selection of Proteolytic Lactic Acid Bacteria with Probiotic Properties for Fish Protein Hydrolyzate Production

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ABSTRACT

This study aimed to select a proteolytic LAB with probiotic properties that can be applied to manufacture protein hydrolysate from fish heads. Tests on 20 isolates of LAB showed that nine isolates were proteolytic and non-pathogenic. A total of 5 isolates could grow well at 0.5% bile salt stress, and 3 of them could grow at pH 3. These three isolates had antagonistic ability against Salmonella bacteria, and one isolate was sensitive to the antibiotic tested. Molecular identification of the selected LAB isolates showed a 100% sequence similarity with *Pediococcus pentosaceus* with accession number MT515895.1. The LAB isolate has high proteolytic activity since it can increase the soluble fraction of fish meal powder from 32.10% to 88.38% in 48 hr. Production of protein hydrolysate using tuna waste was carried out for 30 days. Tuna waste protein hydrolysate had a medium antioxidant activity of 25.57 ± 0.93%. The hydrolyzed protein comprised 17 amino acids, including nine non-essential and eight essential amino acids, and is dominated by glutamic acid. Selected LAB isolate is potentially used in protein hydrolysate production, especially for flavor enhancers.

Keywords: antagonistic, amino acid, *Pediococcus pentosaceus*

INTRODUCTION

The fishery product processing industry produces waste of around 30-40% of the total weight of fish, consisting of the head (12.0%), bones (11.7%), fins (3.4%), skin (4.0%), spines (2.0%), and entrails (4.8%), which is more than 3.6 million tons per year (KKP 2020). If not handled properly, the waste generated can cause environmental, health, and economic problems. One way to overcome the processing waste of the fishery industry is to convert waste into products that have added value (Utomo *et al*. 2014). It has been reported that the protein content in sturgeon fish waste (*Acipenser persicus*) is 15.48%, catla *(Catla catla*) is 8.52%, and cod fish is 16.72% (Nurhayati *et al*. 2014). Thus, fish waste is a potential source of protein that can be used as an ingredient to produce protein hydrolysate.

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Fish protein hydrolysate is a product produced from breaking fish protein into simple peptides and amino acids through hydrolysis by enzymes, acids, bases, and fermentation (Kristinsson and Rasco 2000; Jemil *et al*. 2014). Protein hydrolysate is a protein derivative product that has undergone the breaking of peptide bonds in protein structures into simpler bonds through chemical processes or fermentation processes. Fermentation is one of the simplest and inexpensive techniques and can support the hydrolysis of proteins. In addition, fermentation produces proteins that are easier to digest, as they are hydrolyzed and degraded into shorter peptides and amino acids (Ramírez *et al*. 2013), so it becomes a valuable technique to improve the quality of fish products used as a source of protein (Özyurt *et al.* 2019).

Various process techniques are currently used to convert fish waste into fish meal, fish silage, fish oil, fish collagen, and fish hydrolysate. However, this is rarely used for food and additives in the food industry. The use of enzymes and microorganisms in manufacturing fish protein hydrolysate will produce compounds rich in nutrients and bioactive components. This product can be applied to the food industry, such as milk replacers, flavor enhancers, stabilizers, and protein supplements, as well as to the feed industry, microbial media, and the pharmaceutical industry (Wangkheirakpam *et al*. 2019).

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Enzymatic hydrolysis of fish protein has been carried out with catfish meat (Baehaki *et al*. 2015) and white snapper innards (Nurhayati *et al*. 2014) using papain enzyme. Generally, protein hydrolysate is used as a food additive because it has high nutritional value, such as amino acids, as well as antioxidant and antibacterial compounds (Aditia *et al*. 2018).

Lactic acid bacteria (LAB) *Pediococcus acidilactici* and *Lactobacillus plantarum* have been widely applied in the fermentation of fish silage and can eliminate fish pathogenic bacteria such as *Vibrio anguillarum* and *Aeromonas salmonicida* (Lindgren and Pleje 1983). Yin *et al*. (2006) reported LAB's ability to suppress the growth of major microflora and increase the sensory receptivity of pasta products from mackerel hydrolysate.

The protease activity of the bacteria that play a role during fermentation determines the bioactive peptides produced (Aditia *et al.* 2018). Melliawati *et al*. (2015) reported that *Lactobacillus plantarum* is a LAB isolate with proteolytic activity. In addition to proteolytics, LAB with probiotic properties has important benefits for body health. Groups of bacteria that have probiotic properties are generally found in LAB, such as *Lactobacillus* and *Bifidobacterium* (Saad *et al*. 2013). Probiotics must meet specific criteria so that they can have a positive influence on their host (Tallapragada *et al*. 2018). The probiotic characteristics required by LAB are not pathogenic, antagonistic to enteric bacteria, can adapt to the environment of the digestive tract, and can maintain the number of cells in the digestive tract (Saad *et al*. 2013). LAB can still be found in processed foodstuffs; as Jonesti *et al.* (2023) reported in tempeh flour, there are 9 logs of CFU/g. In this study, LAB with probiotic properties was used to produce hydrolysate, which has two benefits: protein hydrolysate containing probiotics.

Microbes in the fermentation process greatly influence the characteristics of the final product produced. This study aimed to select proteolytic lactic acid bacteria with probiotic properties to produce fish protein hydrolysate and evaluate the antioxidant activity and amino acid composition of the hydrolysate.

METHODS

Characterization of Lactic Acid Bacteria Isolate

LAB characterization included lactic acid production capability, catalase test, and Gram staining. The LAB isolate was grown on MRS broth medium and incubated at 37°C, for 24 hours, then inoculated on MRS Agar media with $CaCO_3$ added, then incubated at 37°C for 48 hours. The catalase test was carried out according to Sari *et al*. (2012) by adding 1–2 drops of hydrogen peroxide (H_2O_2) 3% to the prepared glass that has been applied with LAB isolate. The air bubbles formed showed a positive LAB against the catalase test. Selected LAB isolates could produce lactic acid, catalase-negative, and Gram-positive (Nikita and Hemangi 2012).

Proteolytic Activity of LAB

The protease index was tested using the well diffusion method on skim milk agar media (Nespolo *et al*. 2010). Proteolytic activity could be seen by forming clear zones surrounding the well.

Characterization of LAB as a Probiotics

Characterization as a probiotic began with a pathogenicity test (Tallapragada *et al*. 2018). Selected isolates are those that do not have the ability to hemolyze red blood cells or γ-hemolycin. Characterization continued with a tolerance test for bile salts based (Panjaitan *et al*. (2018). The number of colonies was calculated for the isolates with the highest growth rate after 5 hours of incubation. Evaluation of LAB's resistance in acid/low pH conditions was also carried out (Panjaitan *et al*. 2018). They tested the ability of LAB to fight the pathogenic (antagonistic) *bacteria Salmonella thypimurium* according to the Hamida *et al*. method. (2015), and sensitivity test to antibiotics *Amoxicillin* and *Chloramphenicol* (*Riani et al*. 2020).

Identification of Selected LAB Isolates

After the selection of LAB based on parameters as probiotics, one selected isolate was identified based on the 16S rRNA gene. DNA was extracted from bacterial cells using the PrestoTM Mini gDNA Bacterial Kit. The 16s rRNA gene was amplified by PCR using the 27F primer pair (3'-GAGTTTGATCCTGGCTCAG-5') and the 1492R primer (3'- TACCTTGTTACGACTT-5'). The DNA product was sent to 1st Base, and the DNA sequence obtained was analyzed by EzTaxon Biocloud performance. Phylogenetic trees were analyzed using MEGA 7.0.

Selected LAB Applications in Fish Protein Hydrolysate

The fish protein hydrolysate was prepared based on Torino *et al*. (2012). First, the head of the cod fish was cut into small pieces and blended. The blended fish head was mixed with 1:2 sterile aqueous (b/v) and 1% sterile glucose (b/v), then mixed using a stirring rod until everything was evenly mixed. The sample was then put into a sterile glass jar, and 10% isolated starter bacteria (v/v) with a cell count of 10^7 cfu/mL. Fermentation was carried out for 30 days at room temperature (28–30°C).

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The sample was heated for 15 minutes at 75°C to stop the fermentation. Afterward, the samples were centrifuged for 20 minutes at 10.000 g rate. The filtrates were used for amino acid testing. For antioxidant activity testing, the filtrates were freeze dryed.

RESULTS AND DISCUSSION

Characteristics of LAB

LAB is Gram-positive, round or rod-shaped, non-spore; during fermentation, lactic acid is the final product produced as the primary metabolite (Ramesh and Ray 2015). A total of 20 LAB isolates produced clear zones on MRSA media plus 1% $CaCO_3$. $CaCO_3$ reacted with lactic acid produced by LAB to form water-soluble calcium lactate.

Proteolytic Activity of LAB

Nine isolates produced proteases with various abilities (Table 1). Wikandari *et al*. (2011) explained that the proteolytic ability of LAB isolates is very diverse, from the genus level to the species. High proteolytic indices were found in IL11, IL13, and S7 isolates. The proteolytic index was determined by forming a clear zone around the LAB colony. Alemu (2015) explained the formation of clear zones around bacterial colonies, indicating that the bacteria can hydrolyze the substrate-containing proteins.

LAB Pathogenicity Test

The pathogenicity of LAB was seen from the ability to hemolyze or lyse red blood cells so that a clear zone formed in the blood gelatin. Of the nine LAB isolates tested, none of them sized red blood cells or had gamma (γ)-hemolysis, so they continued in the following probiotic LAB selection process (Table 2). The main approach in selecting potential probiotic isolates was their non-pathogenic character. All 9 LAB isolates tested did not form a lysis zone, so they were safe and not pathogenic. Hemolysis can occur because bacteria produce enzymes that have toxic properties, can classify red blood cells, play a role in increasing cell permeability, and cause cells to be susceptible to infection (Khusnan *et al*. 2002).

Tolerance to Bile Salts and Low pH

All isolates grew on MRSA media supplemented with 0.5% bile salts (*Himedia*). Nine isolates were selected as bile salt-tolerant isolates. Five survived, and one isolate

Table 1 Proteolytic Index of Lactic Acid Bacteria

Isolate Code	Proteolytic Index	Isolate Code	Proteolytic Index	Isolate Code	Proteolytic Index	Isolate Code	Proteolytic Index
IL1	0	IL6	0	IL11	28 ± 0.00	S4	0
IL2	0	IL7	0	IL12	0	S5	0
IL3	0	IL8	10± 0.00	IL13	30 ± 0.00	S6	17± 0.07
IL4	19 ± 0.07	IL9	0	S1	15 ± 0.07	S7	27 ± 0.07
IL5	20 ± 0.14	IL10	17 ± 0,07	S2	0	S8	0

Table 2 Pathogenicity of Lactic Acid Bacteria

Isolate Code	Types of hemolysis ability	
	α-haemolysis	β-haemolysis
IL4	-	-
IL5	-	-
IL8	-	-
IL10	-	-
IL11	-	-
IL13	-	-
S1	-	-
S6	-	-
S7	-	-

Table 3 Number of logs CFU/mL Lactic Acid Bacteria in the test of resistance to bile salts on De Man Rogosa Sharpe agar medium + 0.5% bile salts

Isolate Code	Hour 0 Log CFU/mL	5th Hour Log CFU/mL
IL5	5.900	5.816
IL8	5.903	5.447
IL11	5.141	6.365
IL13	5.932	6.109
S7	5.881	6.146

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(IL11) experienced an increase in the number of cells by 1 log after incubation for 5 hours under bile salt stress (Table 3). Bile salt molecules secreted in the duodenum are one of the stresses in the human digestive tract. Bile salts themselves are protective compounds in the duodenum that play a role in emulsifying fat so that they can dissolve lipids that are the constituents of microbial cell membranes (Urdaneta and Casadesús 2017). Nine non-pathogenic isolates grew in 0.5% bile salts, which were toxic to other microbes. In addition to destroying the phospholipid membrane of microbial bilayers, bile salts are also capable of denaturing proteins and chelating iron and calcium, thus causing microbial DNA damage (Urdaneta and Casadesús 2017). This study used a concentration of bile salts of 0.5%. This research aligns with Helmy *et al*. (2019), explaining that the optimum concentration of bile salts in the human intestinal tract ranges from 0.3% to 0.6%.

Exposure to bile salts in each microbe in the gut has different effects depending on the physiological resistance of the microbes (Yan *et al*. 2013). *L. acidophilus* and *L. fermentum* isolated from *dangke* can also survive and grow at a concentration of 0.5% bile salts (Adawiyah *et al*. 2015). Thamacharoensuk *et al*. (2017) tested LAB isolates on media with bile salt content at different concentrations, showing that the death of bacterial cells and the increase in bile salt concentration will increase. Five of the nine LAB isolates tested in 0.5% bile salt capsules still had high growth. The results of TPC LAB on 0.5% bile salt stress in five isolates were successfully grown in MRSA media.

As a probiotic, LAB is resistant to bile salt stress and must also be resistant to stomach acid. Boland (2016) explained that stomach acid has a pH of around 2.0. The results showed that the three LAB isolates tolerated pH 2 (Table 4). Riani *et al.* (2020) reported that LAB isolates from pineapple juice have different tolerances at pH 2 after being incubated for 2 hours. The time it takes for food to pass through the stomach was 2 hours. The stomach itself is the initial barrier, which passes through before the bacteria reach the small intestine (bile salts). Three of the five LAB isolates were selected as the best isolates based on bile salt stress and resistance to low pH. The three isolates were IL11, IL13 and S7. This condition is essential so the three isolates can pass through the digestive tract. The criteria for resistance to acid and alkaline conditions are criteria in the probiotic's selection as performe by Garcia *et al.* (2016), Phong *et al.* (2017), and Riani *et al*. (2020).

Antagonistic and Antibiotic Sensitivity

The three LAB isolates were followed by testing of antibacterial activity against *Salmonella* bacteria and their sensitivity to antibiotics. Liquid culture supernatants neutralized to pH 7 using 18-hour HCl 1N from the three isolates had antibacterial activity against the pathogenic bacteria tested. In sensitivity tests to antibiotics Amoxicillin (100 µg) and Chloramphenicol *(*50 µg), there were two isolates (IL 11 and S1) that were resistant to antibiotics, while one isolate (IL 13) was sensitive (Table 5).

Testing the activity of probiotic LAB in fighting pathogenic bacteria is very important to be understood. The antibacterial activity resulting from the three LAB isolates can be seen in Table 5. These three isolates had good inhibition according to the criteria conveyed by Jarriyawattanachaikul *et al.* (2016). Clear zones with a value of ≤ 8 mm indicate poor antimicrobial activity; clear zones between 9–14 mm indicate strong activity; clear zones of 15–19 mm indicate very strong activity; and clear zones above 19.14 mm indicate extremely strong activity. LAB is a group of bacteria that can be probiotics, but not all LABs can produce antimicrobial compounds. According to Khoiriyah *et al*. (2014), LABs were widely known in the food industry and commonly used as a fermentation starter and food preservative. Papuangan &

Table 4 Number of logs CFU Lactic Acid Bacteria /mL in the resistance test to acidic conditions grown on De Man Rogosa Sharpe agar media

Isolate Code	Hour 0 Log CFU/mL	3rd Hour Log CFU/mL	% survival rate
IL11	5.778	4.959	85
IL13	5.716	4.623	81
S1	5.813	4.580	79

Table 5 Antagonistic activity of Lactic Acid Bacteria supernatants against pathogenic bacteria and sensitivity of isolates to antibiotics

Isolate Code	Inhibition zone against *Salmonella thypimurium* (mm)	Amoxicillin	Chloramphenicol
IL11	38 ± 0.00	Resistance	Resistance
IL13	24 ± 0.14	Sensitive	Sensitive
S7	17 ± 0.07	Resistance	Resistance

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Nurhasan (2014), explained that LABs produce antimicrobial components that inhibit the growth of Gram-negative and Gram-positive bacteria. The formation of a clear zone around the LAB isolate indicates an antibacterial compound produced by three probiotic LAB isolates. These three probiotic LAB isolates can produce antibacterial compounds to fight *Salmonella* bacteria. *Salmonella* bacteria are the main cause of foodborne diseases. Generally, the Salmonella serotype is the cause of diseases in the digestive tract.

One of the essential things in selecting the safety of bacteria as a probiotic is sensitivity to antibiotics. The test results of three LAB isolates found that two isolates were resistant to antibiotics, namely IL11 and S7, and one isolate, which was sensitive to antibiotics, namely IL13. Based on Mukherjee (1988) criteria, the diameter of the sensitive category zone in *Amoxicillin* is >18 mm, the medium category is 5-12 mm, and the resistance category is <4 mm. Chloramphenicol, based on Bauer's criteria (1966), is a sensitive category with an inhibition zone diameter of 18 mm, an intermediate category of 13–17 mm, and a resistance category of <13 mm. The results of the third test of LAB isolates against the type of antibiotic Amoxicillin showed that LAB IL11 and S1 isolates had resistance properties to Amoxicillin and Chloramphenicol. Antibiotic resistance is a potential risk of probiotic application because resistance genes are transferred horizontally to other bacteria. Bacteria are resistant to certain antibiotics because they have intrinsic properties, namely by producing enzymes that inactivate antibiotic compounds (Ministry of Health of the Republic of Indonesia 2011). LAB testing toward Chloramphenicol showed that one isolate (IL13) of the three LAB isolates were resistant to Chloramphenicol. The LAB can defend against Chloramphenicol because it has the CATs gene. The gene activates the chloramphenicol acetyltransferase in LAB to activate antibiotic compounds that enter the LAB, preventing protein synthesis in LAB cells (Schwarz 2014). IL13 isolate can be used as a probiotic candidate because it is also sensitive to antibiotics and resistant to acidic and alkaline conditions.

The Identified LAB

Molecular identification using the 16S rRNA gene to the three isolates can be seen in Table 6. The Basic Local Alignment Search Tool (BLAST) program was used to analyze three LAB isolates from the NCBI website to determine the sequence's similarity to the NCBI gene bank sequence. Based on the homological analysis of the three selected LAB isolates, the sequence similarity level was 100% with *Enterococcus faecalis* with accession number MT356185.1 and *Pediococcus pentosaceus* with accession number MT515895.1.

Growth of LAB During Fermentation

The application of *P. pentosaceus* IL13 isolate, which has proteolytic activity and probiotic properties, aimed to make protein hydrolysate using fermentation techniques. Table 7 shows the growth results of LAB during fermentation. Total LAB in hydrolysate displays the amount of LAB in the fermentation liquid, total LAB at 0 hours shows the number of LAB inoculated, while total LAB at 30 hours shows the amount of LAB in the remaining fermentation pulp. The LABs could grow during fermentation. The amount of LAB at day 0 was 6.47±0.70 log cfu/mL, while on day 30, the LAB can be found in the pulp with a relatively similar amount as when inoculated, and there was also the amount of LAB in the hydrolysate. This indicates the LAB growth during the fermentation process.

Antioxidant Activity of Fish Protein Hydrolysate

The method used to determine antioxidant activity was DPPH. Samples in which antioxidants were present cause the disappearance of DPPH radical chromogens detected using a spectrophotometer (Baliyan *et al*. 2022). The antioxidant activity of cod head protein hydrolysate was 25.57 ± 0.93 ppm. Antioxidant values below 50 ppm are considered very strong (Yati *et al*. 2018). Protein

Table 6 Similarity percentage of 16S rRNA gene sequence in Lactic Acid Bacteria isolated

Isolate Code	Accession	Query cover (%)	E-value	Identity (%)
IL11	*E. faecalis* /MT356185.1	100	0.00	100
IL13	*P. pentosaceus* /MT515895.1	100	0.00	100
S7	*E. faecalis* /MT356185.1	100	0.00	100

Table 7 Number of logs CFU Lactic Acid Bacteria/mL during fermentation of cod fish heads

Fermentation time (day)	Total LAB (log CFU/mL)	Total LAB in Protein Hydrolysate (log CFU/mL)
0	6.47 ± 0.70	
30	6.60 ± 0.54	5.54 ± 0.54

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Table 8 Amino Acids content in Hydrolysate of Cod Fish Head

Types of essential amino acids	Cod fish head protein hydrolysate (%)	Non-essential amino acids	Cod fish head protein hydrolysate
Histidine	1.96	Aspartic acid	11.25
Arginine	4.59	Glutamic acid	13.93
Threonine	4.59	Serine	1.55
Valine	4.82	Glycine	3.76
Methionine	0.94	Alanine	4.63
Isoleusine	2.79	Proline	3.90
Phenylalanine	0.68	Tyrosine	2.75
Lysine	5.06	Sisteine	0.59
		Leusine	8.04

hydrolysate can contain peptides that exhibit a variety of biological activities, such as antiviral, antimicrobial, antifungal, antiproliferative, antioxidant, anticoagulant, antihypertensive, anticancer, antidiabetic, antiobesity, and calcium-binding properties (Cheung *et al*. *2015;* Gogineni and Hamann 2018*).* Most fermented bioactive peptides have a length of 2–20 amino acids. Fish fermented products of *Labeo rohita* uses *P. pentosaceus* FSBP4-40 are antagonistic to some human pathogenic bacteria (Siddegowda *et al.* 2017). The results of this study are in line with Ejuama *et al*. (2021) work, which stated that there was an increase in DPPH free radical capture activities through fermentation. Fermentation that produces amino acids strongly affects the antioxidant activity of hydrolysate (Wu *et al.* 2003). In addition, Torino *et al*. (2012) also explained that the type of peptide formed during fermentation greatly affects antioxidant activity. These peptides are formed from the activity and specificity of bacteria that play a role in fermentation.

Amino Acid Content in Protein Hydrolysate

The purpose of determining amino acids was to determine the amino acids in the hydrolysate of cod fish heads that are fermented for 30 days using *Pediococcus pentosaceus* IL13 isolate (Table 8). There were 17 amino acids constituting the hydrolysate which were fermented using *P. pentosaceus* IL13 bacteria for 30 days. The glutamic acid was the highest amino acid in the hydrolysate, in addition to hydrophobic amino acids (alanine, valine, methionine, isoleucine, leucine) and aromatics (phenylalanine, tyrosine).

Protein hydrolysate with fermentation technique has great potential to be used as a flavor enhancer. The use of *P. pentosaceus* in the fermentation process of Zhayu (a traditional fish fermentation product in China) showed the strongest umami flavor (An *et al.* 2022). Similar research by Li *et al.* (2021) using *P. pentosaceus* 30-15 in the fermentation process of Tilapia fish sausages, showed an increase in umami content and free amino acids, as well as a significant decrease in the content of biogenic amines. Ovissipour *et al*. (2011) explained that glutamic acid, aspartic acids, glycine, and alanine are amino acids that act as flavor enhancers in food products.

CONCLUSION

Selection has been carried out for 20 LAB isolates of probiotic candidates. IL13 isolate is a proteolytic LAB isolate, which has the character of being resistant to acids and bile salts, sensitive to the two antibiotics tested, and has antibacterial properties against *Salmonella thypimulium bacteria*. IL13 isolate similar to *Pediococcus pentosaceus* with its character so that the isolate can be a probiotic candidate. Cod head protein hydrolysate has antioxidant activity of 25.57 ± 0.93%, comprised of 17 amino acids. For further research, characterization of other fermentation products such as active peptides and their utilization can be performed.

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