

Characterization of Probiotics Isolated from Intestine of Mackerel Fish (*Rastrelliger sp.*) from Lembata Regency of East Nusa Tenggara

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Abstract

The research aimed to isolate, characterize, and analyze the ability of lactic acid bacteria (LAB) potential as probiotics to produce hydrolase enzyme. The LAB was isolated using MRS agar by the spread plate method. The LAB characterization includes antimicrobial activity, tolerance to low pH, bile salt, salinity, autoaggregation properties, and ability to produce hydrolytic enzymes. The isolate which has the highest ability to inhibit *Aeromonas hydrophila* is KBP 3.3, while the isolate which inhibits the highest *Streptococcus agalactiae* is KBP 1.1.1. The KBP 3.3 and KBP 1.1.1 were able to survive at pH 1 for 24 hours with a survival rate of 93.6% and 98.3%. The KBP 3.3 and KBP 1.1.1 are tolerant to 7.5% bile salt concentrations for 24 hours of 99.46% and 99.11%. The KBP 3.3 is tolerant to 0.5 % salinity for 24 hours with the highest survival rate of 113.38%, while KBP 1.1.1 is 94%. The KBP 3.3 and 1.1.1 have autoaggregation properties of 92.18% and 87.84%. The KBP 3.3 produced the highest lipase enzyme, while KBP 1.1.1 produced the protease enzyme.

Keywords: hydrolytic enzyme, lactic acid bacteria, mackerel, probiotic

INTRODUCTION

Lembata Regency is located in East Nusa Tenggara Province; one of the largest producers of marine fish in Indonesia. Fish with high economic value and widely caught in Lembata Regency are mackerel (*Rastrelliger sp.*). Mackerel is a type of fish that live in shallow sea waters and low salt salinity [1]. Mackerel production in Lembata Regency until the end of 2016 was 209.27 tons [2]. The number of mackerel catches increased until 2016 by 53.23% [3]. Mackerel is nutritious because it contains protein, omega 3, vitamin B12, vitamin D, vitamin B2, vitamin B6, phosphorus, iodine, and high selenium. Catch fish supplies such as mackerel cannot supply the increasing public demand due to natural factors such as rainfall and wind. One alternative to meet community demand for fish is aquaculture [4].

Intensive aquaculture causes the fish to experience stress, which lowers its immune system. It is a cause of increased susceptibility to disease [5,6]. Examples of infectious diseases are hemorrhagic and septicemia caused by the bacterial pathogen *Aeromonas hydrophila* [7] and *Streptococcus agalactiae* [8]. Symptoms of the disease are loss of appetite, red discoloration of the anus and base of the fins, eyes, gills, internal organs, and hemorrhagic muscles. Pathogenic bacterial infections are usually prevented by

administering antibiotics, chemotherapy, and vaccines. Specifically for antibiotic therapy, if it is carried out intensively, it will result in the emergence of microbes that are resistant to these antibiotics [9,10]. One solution to solve this problem is the application of environmentally friendly and sustainable cultivation using probiotics [7].

Probiotics are beneficial microbes that can improve the health of their host and it can be used as a pathogenic biocontrol agent in aquaculture. Previous research obtained probiotics as biocontrol agents to be applied to aquaculture. *Paenibacillus ehimensis* NPUST1 isolated from tilapia ponds that have the ability to inhibit pathogens *Aeromonas hydrophila* and *Streptococcus iniae* [7], and *Rummeliibacillus stabekisii* can inhibit the growth of *Streptococcus agalactiae* [10]. Moreover, the consortium of *Saccharomyces cerevisiae*, *Aspergillus oryzae*, and *Bacillus subtilis* can also improve the immune system of tilapia and inhibit pathogens *Aeromonas hydrophila* and *Streptococcus iniae* [11]. Probiotics from mackerel intestines from the Indonesian sea have also been reported, namely *Lactobacillus plantarum*, *Leuconostoc mesenteroides* [14], and *Bacillus megaterium* (QM B1551 strain, NBRC15308 strain, IAM 13418 strain, and ATCC strain 14581) [13]. Probiotics from mackerel intestines, especially from Indonesia, are still less explored. Therefore, the research was aimed to isolate, characterize the LAB potential as probiotics, and analyze the ability of probiotics to produce hydrolase enzyme.

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MATERIAL AND METHOD

Sample Collection and Pathogenic Bacterial Strains Used

Mackerel was obtained from a fish auction place, Lewoleba Impres Market, Lembata Regency. Morphometry of 10 fishes included weight and length of mackerel. The mackerel was then brought under cold conditions to the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University. Furthermore, the pathogen used was *Aeromonas hydrophila* obtained from the Fish Disease Laboratory, Faculty of Fisheries and Marine Sciences, Brawijaya University. Meanwhile, *Streptococcus agalactiae* was obtained from the Research Center for Freshwater Aquaculture Fisheries, Sempur, Bogor, West Java.

Isolation and Characterization of LAB

The length of the mackerel that has been used as a sample ranges from 18-19 cm. The weight of the fish is 76.5-77.2 g. Mackerel intestine contents were removed using tweezers in sterile conditions. The fish intestine of 25 g was blended using a blender in 100 mL of NaCl 0.85%. The obtained suspension was serially diluted from 10⁻¹-10⁻⁶ dilutions. Lactic acid bacteria were isolated by the spread plate method in MRS agar containing 1% CaCO₃ and incubated at 37°C for 48 hours. Lactic acid bacteria that grow are characterized by the formation of clear zones around the colony. Lactic acid bacteria were purified by the spread plate method. After that, Gram stain and catalase test were carried out to ensure that the isolates obtained were LAB [14].

Screening of Antimicrobial Activity

The antimicrobial activity test method used the well diffusion method as described by Speranza [15]. The number of cells of each lactic acid bacteria and pathogen is 10⁷ CFU.mL⁻¹. Lactic acid bacteria were cultured in MRS broth media, while *Aeromonas hydrophila* and *Streptococcus agalactiae* were cultured in Trypticase Soy Broth (TSB) media [7,16]. Pathogen isolates of 0.1 mL were spread on Trypticase Soy Agar (TSA) media. After that, 800 µL of each lactic acid bacteria was put into a well with a diameter of 5 mm, then incubated at 37°C for 48 hours. Clear zone diameters were measured and the inhibition index was calculated with formula 1 [17].

$$\text{Inhibitory index} = \frac{\text{clearzone diameter} - \text{well diameter}}{\text{well diameter}} \dots\dots\dots(1)$$

In-vitro Tests for Probiotic Properties: Tolerance to low pH, Bile salt, and Salinity

The pH variations used were pH 1, 3, and 5, while the concentration of bile salts used were 2.5%, 5%, and 7.5% [14]. The salinity variation used were 0.5%, 3%, and 6.5% [18]. Lactic acid bacteria that have antimicrobial activity and were used for further tests are KBP 3.3, KBP 4.3, KBP 4.2.1, and KBP 1.1.1. Lactic acid bacteria were each added as much as 10% with a cell density of 10⁷ CFU.mL⁻¹ into the MRS broth as measured by pH, bile salts, and salinity. Then, incubated at 37°C for 24 hours and sampling at 0, 4, and 24 hours to determine the number of cells using a hemocytometer [19]. The survival rate of lactic acid bacteria was calculated using formula 2 [20]. The N1 is the final cell amount and N0 is the initial cell amount.

$$\text{Survival rate (\%)} = \frac{\text{Log N1}}{\text{Log N0}} \times 100 \dots\dots\dots(2)$$

Auto-Aggregation Property

Lactic acid bacteria culture with 10⁷ CFU.mL⁻¹ cells was centrifuged at 6000 rpm, at room temperature for 10 minutes. The pellet was suspended in a saline buffer (0.85% NaCl) to 15 mL. The suspension was measured optical density (OD) at a wavelength of 600 nm. After that, the suspension was left for 60 minutes at room temperature, then the suspension was re-centrifuged at 3000 rpm for 2 minutes and the supernatant was measured again at the same wavelength. The percentage of auto-aggregation was calculated using formula 3 [21].

$$\% \text{ Auto-aggregation} = \frac{(\text{OD0} - \text{OD60})}{\text{OD0}} \times 100 \dots\dots\dots(3)$$

Enzymatic Activity

Hydrolase enzyme activities tested were protease, amylase, lipase, and cellulase enzymes. This test was used the paper disc diffusion method. Protease enzyme activity was tested using skim milk media and amylase test using agar strach [21]. Lipase test was used lipolytic agar [22] and cellulase test was used carboxymethyl cellulose (CMC) agar [23]. After that, incubation at 37 °C for 48 hours and clear zone was formed and also the hydrolysis index was calculated using formula 4 [22].

$$\text{Indeks hidrolisis} = \frac{\text{Diameter of clearzone}}{\text{Diameter of disc}} \dots\dots\dots(4)$$

RESULT AND DISCUSSION

The number of lactic acid bacteria obtained was eleven isolates. All of the LAB isolates were negative catalase and Gram-positive. The LAB cells are basil (KBP 1.1.1, KBP 3.3, KBP 2.1, and KBP 1.1.2.1) with cell sizes of 2-3 µm, and cocci (KBP 3.1, KBP 3.2, KBP 4.3, KBP 6.2, KBP 4.2, KBP 2.3, and KBP 4.2.1) sizes of ± 1 µm. The number of isolated lactic acid bacterial cells was 7 x 10⁶ CFU.g⁻¹. The number of LAB cells in the intestines of marine fish is usually constant during the capture and storage period of 10⁶-10⁷ CFU.g⁻¹. The fish intestine is a potential source of probiotics because LAB cell viability remains stable, compared to probiotics isolated from gills and fish skins. The amount of LAB that commonly lives in the intestines of mackerel is 2x10⁶ CFU.g⁻¹ [24]. The LABs are not dominant in the normal intestinal microbiota of fish, at variance with homeotherms, but some strains can colonize the intestine [25].

The LAB is found and symbiotic throughout the gastrointestinal tract, including the stomach and the intestines [26]. The population level of lactic acid bacteria associated with the intestine is affected by nutritional and environmental factors like chromic oxide, stress, and salinity [27]. Although some lactic acid bacteria strains can colonize the intestine of the host, living acid bacteria from food or feed preparations, are in most cases, lost from the intestine within a few days after the intake has stopped [24]. The fish intestine is prebiotic for supplementation of materials that can support the growth of LAB in the intestinal tract of hosts, while beneficial bacteria, a bacterial group of lactic acid bacteria (LAB), has elicited most interest due to their ability to release various beneficial compounds such as free fatty acids (FFA), digestive enzymes, and antimicrobial compounds [28].

Antimicrobial activity

Probiotics can inhibit pathogens by providing nutrients and enzymes to increase host growth, enhance the immune response by stimulating the immune system, and not causing environmental pollution [8]. Probiotics have inhibitory activity if the inhibitory index values range of 2.2-4.4 (moderate-very strong category) [29]. Therefore, the results show that KBP 1.1.1, KBP 3.3, KBP 4.3, and KBP 4.2.1 are the isolates that have inhibitory activity against *Aeromonas hydrophila*. The isolate with the highest inhibitory index value was the KBP 3.3 isolate of 7.16 (p <0.05). Other isolates that have high inhibitory ability are KBP 4.2.1, KBP 4.3, and KBP 1.1.1, with an inhibitory index value of 4.5, 4.1, and 2.3, respectively (Fig. 1).

Inhibitory activity by LAB against pathogens is carried out using the metabolites it produces. Probiotics *Bacillus licheniformis* KADR5 and *Bacillus pumilus* KADR6 are able to inhibit *Aeromonas hydrophila* ATCC 49140 (inhibitory index > 1.6) due to the activity of lysozyme enzymes by damaging the cell wall of pathogenic bacteria [30]. In addition, several probiotics have inhibitory activity against *Aeromonas hydrophila* Ah01, such as *Lactococcus lactis* Q-8, *L. lactis* Q-9, and *L. lactis* Z-2 [29]. This inhibitory activity is due to lactic acid compounds, which suppress lipopolysaccharide (LPS) production and damage the Gram-negative bacterial membrane [31].

Another pathogen that causes disease in fish is *Streptococcus agalactiae*. Inhibitory activity occurs if probiotics have inhibitory index values in the moderate-strong category (1 to 2.6) [9]. The results showed that KBP 1.1.1 and KBP 4.2.1 inhibit pathogens with inhibitory index values of 3.24 and 2.89, respectively (Fig. 2).

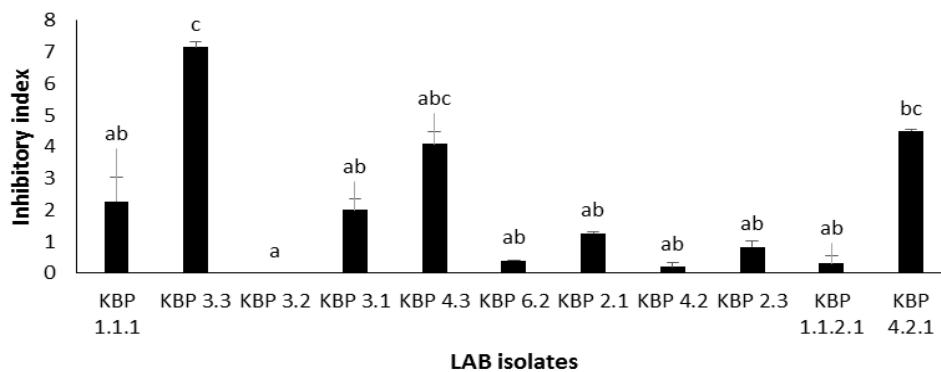


Figure 1. Antimicrobial activity of lactic acid bacteria against *Aeromonas hydrophila*

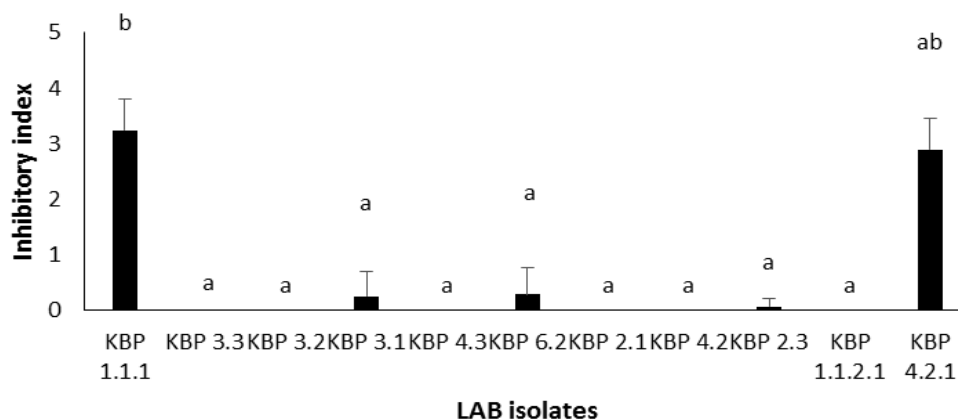


Figure 2. Antimicrobial activity of lactic acid bacteria against *Streptococcus agalactiae*

Bacteriocin type nisin A and organic acids produced by *Lactococcus lactis* subsp. *lactis* CRL 1655 is inhibiting the pathogen *Streptococcus agalactiae* [32]. Probiotics such as *Bacillus cereus* NY5 and *Bacillus subtilis* also have the ability to inhibit the pathogen *Streptococcus agalactiae* due to increased *lyzC* gene expression to produce the enzyme lysozyme [8]. Previous studies showed that the c-type lysozymes were effective in lysing both Gram-negative and Gram-positive strains. The host could recognize the bacterial challenges, generate acute stress, and enhance body immunity by secreting specific proteins such as lysozymes [33]. Moreover, some lactic acid bacteria produce bacteriocin which inhibits *Streptococcus agalactiae*, with an inhibitory index of 0.6-1.8, namely *Enterococcus faecium* BNM58 and *Weissella cibaria* BNM69 [34]. In this study, KBP 4.2.1 isolate is a potential isolate that has the ability to inhibit pathogens *Aeromonas hydrophila* and *Streptococcus agalactiae*.

Low pH Tolerance

One of the probiotic criteria is tolerance of the fish's gastrointestinal conditions [35]. The stomach pH value of tilapia ranges about 1.5 – 5.8 [14,36,37]. The intestinal pH value of tilapia ranges about 6.9 – 7.0 [14,37]. Therefore, based on the results, tolerant lactic acid bacteria isolate of gastric pH (1, 3, and 5) are KBP 3.3 and KBP 1.1.1. The survival rate of the two isolates at pH 1 was significantly different from the other two isolates for 24 hours. The survival rate of KBP 3.3 at pH 1 decreased insignificantly after incubation at 4-24 hours, from 94.7% to 93%, while the survival rate of KBP 1.1.1 increased from 95.98% to 98.3% (Fig. 3).

The isolate of KBP 3.3 had the highest survival rate at 4-24 hours, 100.86% to 98.89%. The isolate of KBP 4.2.1 had a lower survival rate than

the other three isolates at 4-24 hours, 35% to 34%. The survival rate at pH 5 did not show a significant difference (Fig. 3). Previous studies have shown that *Lactococcus lactis* CLFP 101, *Lactobacillus plantarum* CLFP 238, and *Lactobacillus fermentum* CLFP 242 were tolerant to pH 3 and 5, while at pH 1, no isolates survived [38]. Moreover, the LAB that tolerate at pH 1 are *Lactococcus lactis* (strains 8HT, 9HT, 11HT and 33HT) and *Enterococcus faecalis* 14HT [39]. Other bacteria are *Leuconostoc mesenteroides* [40] and *Pediococcus pentosaceus* tolerance to pH 3 and 5 with a survival rate of 75% [41]. Therefore, KBP 3.3 and KBP 1.1.1 have a higher tolerance at pH 1, 3, and 5 compared to previous studies.

Bile Tolerance

Tilapia and freshwater fish generally have concentrations of bile salts ranging from 1-10% [14,36]. Therefore, the results showed that KBP 3.3 and KBP 1.1.1 isolates were tolerant of the concentration of bile salt 2.5-7.5%. The survival rate of the two isolates at 4-24 was above 97%. However, KBP 3.3 isolate was one of the isolates that showed an increase in cell number during oxgal exposure of 2.5% (Fig. 4).

Along with the increase in bile salts, the survival rate of KBP 3.3 and KBP 1.1.1 did not decrease. The results of previous studies indicate that the more increased the concentration of bile salts, the value of survival rate decreases. With variations in bile salt concentrations of 2.5, 5.0, 7.5, and 10% it was reported that the survival rate of *Lactococcus lactis* CLFP 101, *Lactobacillus plantarum* CLFP 238, and *Lactobacillus fermentum* CLFP 242 was 100% [38]. Moreover, *Lactococcus lactis* (strains 8HT, 9HT, 11HT and 33HT) and *Enterococcus faecalis* 14HT also tolerance at 10% bile salt concentrations [39].

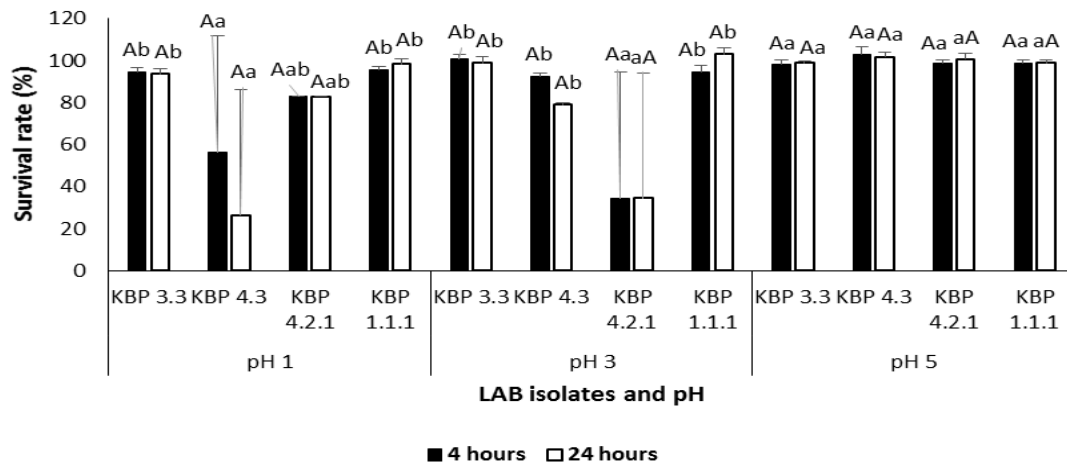


Figure 3. Lactic acid bacteria tolerance at pH 1, 3, and 5

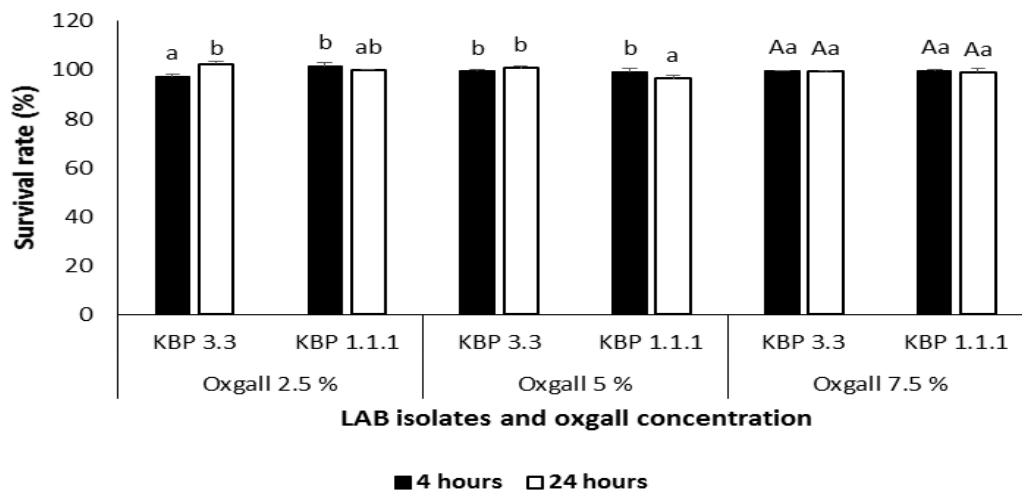


Figure 4. Lactic acid bacteria tolerance to bile salt concentration

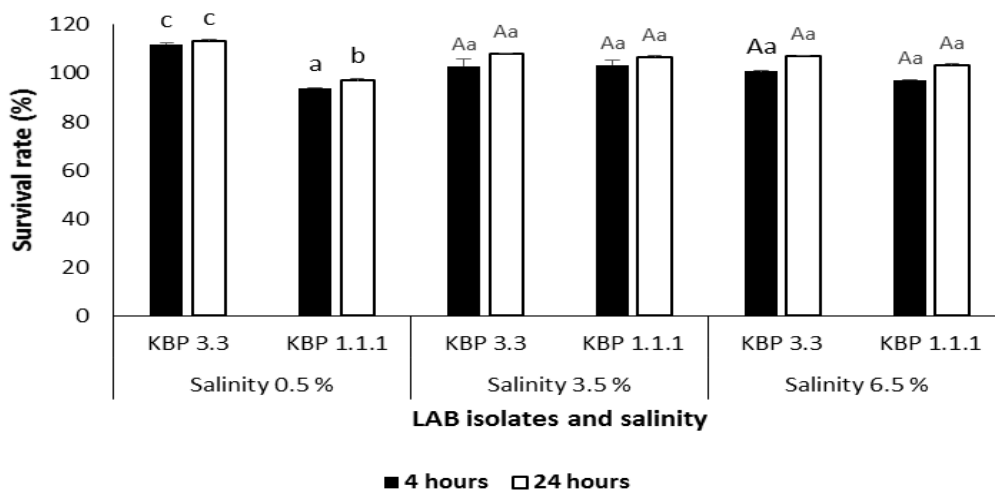


Figure 5. Tolerance of lactic acid bacteria to salinity

Salinity Tolerance

Lactic acid bacteria can be found in some seawater fish that live in tropical seas. The population of lactic acid bacteria in the aquatic environment is influenced by the temperature and salinity of freshwater [42]. Seawater salinity concentrations in Lembata regency ranged from 33.86-34.85 PSU, while water temperatures ranged from 3.5-30.01°C [43,44,45]. Moreover, freshwater salinity, in general, is 0.5-6.5% [20]. Therefore, lactic acid bacteria with probiotic potency must tolerance the host environment.

The results showed that KBP 3.3 and KBP 1.1.1 isolates were tolerant of freshwater salinity. The survival rate of KBP 3.3 and KBP 1.1.1 are above 100%. It shows that the LAB can maintain the number at 24 hours in the range of salinity 0.5-6.5% (Fig. 5). The salinity affects the ability of probiotics. It is supported by previous research that *Lactobacillus* is more commonly found in freshwater fish [46], while the *Streptococcus* species is more found in marine fish [47]. If *Lactobacillus* species are applied to marine fish, the numbers will decrease, while the number of *Streptococcus* species does not change [42]. Lactic acid bacteria have the ability to tolerate environmental conditions because of their ability to produce lizosim enzymes. Lizosim plays a role in inhibiting pathogenic microbes and surviving in environmental stressful conditions such as pH, low salinity, and temperature [48-51].

Auto-aggregation Assays

One of the probiotic criteria is to have the ability to colonize in the host intestine [52]. The ability of autoaggregation of probiotics is excellent if the value of autoaggregation is above 30-60% [53]. The results showed that KBP 3.3 and KBP 1.1.1 have the potency to colonize in the

host intestine. It is supported by the autoaggregation values of the two isolates above 50%. However, the autoaggregation value of KBP 3.3 was higher (92.18%) compared to KBP 1.1.1 (87.84 %).

The autoaggregation property depends on bacterial strains, so it varies greatly among the same bacterial species [54,55]. The good of autoaggregation property is not only seen from the results of the in vitro test but is supported by the in vivo test because many factors influence these characteristics, such as the host, defense mechanisms, native microbes in the intestine, and peristalsis [54]. Some LAB reported to have the ability to colonize are *Enterococcus faecium* strain CGMCC1.2136 (9.05%) [56], *Lactococcus cremoris* SMF110 (40.3%), *Lactococcus cremoris* SMM69 (20.1%), *Lactobacillus curvatus* BCS35 (17.9%), *Enterococcus faecium* SMF110 (40.3%), *Lactococcus cremoris* SMM69 (20.1%) [57], *Lactobacillus plantarum* (11.5%), *Lactobacillus fermentum* (14.2%), and *Lactococcus lactis* (17.2%) [38].

Enzymatic Activity

Lactic acid bacteria with probiotics potency work optimally if producing hydrolase enzymes [58]. Carnivorous and omnivorous freshwater fish need probiotics that have the activity of the enzyme hydrolase (protease, lipase, amylase, and cellulase) to help the digestive process of fish [59]. The results indicate that KBP 3.3 and KBP 1.1.1 isolates have the activity of the enzyme hydrolase, except for the amylase enzyme. The isolate of KBP 3.3 had the highest lipase enzyme activity (hydrolysis index of 2.96), while KBP 1.1.1 had the highest protease enzyme activity of 3.28 (Fig. 6).

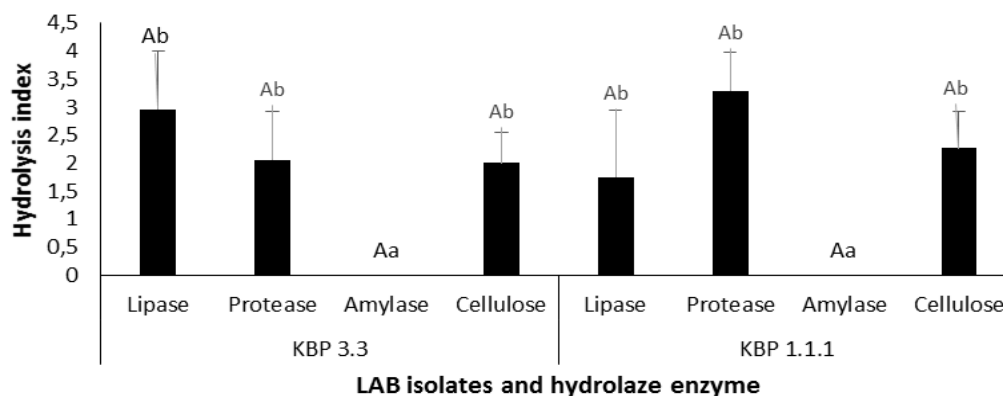


Figure 6. Hydrolase enzyme activity produced by lactic acid bacteria

Lactic acid bacteria generally have the activity of the hydrolase enzyme. It is supported by previous studies, namely *Enterococcus faecium* [60], *Lactobacillus curvatus* and *Leuconostoc mesenteroides* produce the enzyme protease, lipase, and amylase [61], *Lactococcus lactis* and *Enterococcus faecalis* produce enzymes protease and amylase [39], while *Carnobacterium sp.* produced cellulase enzymes [62]. This study together with others, confirmed that enzyme production varied according to bacterial species and food habits [63,64].

CONCLUSION

Lactic acid bacteria were successfully isolated from the intestines of mackerel as much as eleven isolates. The isolates of KBP 3.3 and KBP 4.3 are isolates that have the ability to inhibit *Aeromonas hydrophila*, KBP 1.1.1 have the ability to inhibit *Streptococcus agalactiae*, while KBP 4.2.1 have the ability to inhibit the two pathogens. The isolates of KBP 3.3 and KBP 1.1.1 tolerant to low pH, high bile salt concentrations, and low water salinity. Both of these isolates also have the ability to produce the enzymes protease, lipase, and cellulase.

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