Screening of potential probiotic bacteria from catfish (*Clarias batracus*) and its antibacterial activity

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Abstract

Probiotic bacteria have an essential role in increasing fish and shrimp production in aquaculture. Probiotics also provide many benefits including increasing Food Conversion Ratio (FCR), enhancing the immune system, increasing the digestibility of feed and against pathogenic bacteria that cause disease. Many studies have been conducted to explore the fish probiotic candidate bacteria source. The purpose of this study was to obtain candidate for probiotic bacteria isolated from catfish intestines. Potential probiotic is determined on the basis of proteolytic and antibacterial activity against pathogenic bacteria.

Probiotic candidate bacteria were isolated using de Man Rogosa and Sharpe Agar (MRSA) + 1% $CaCO_3$ and then incubated at 30^oC for 3-6 days. Twelve growth colonies produced acid. Before 16s rDNA analysis, gram staining and catalase assay were performed. The results of the analysis showed that the probiotic candidate was identified as Bacillus velezensis UBL2 with 99.8% identical to Bacillus valezensis R1.16. It has proteolytic activity and antibacterial activity on gram positive and gram-negative pathogenic bacteria. Hence, B. velezensis UBL2 is a potential candidate for probiotic.

Keywords: Probiotic, proteolytic activity, antibacterial, *Bacillus velezensis.*

Introduction

The need for protein is increasing every year. Fish is one food that can satisfy the needs of human animal protein[1]. In aquaculture, there are problems such as low Feed Conversion Ratio (FCR), an increased disease caused by pathogenic bacteria and poor aquaculture water quality. Pathogenic bacteria that attack fish have a significant impact on the failure of fish farming. The method that is often used today to treat pathogenic bacteria is antibiotics. Unfortunately, antibiotic treatment will leave a residue in fish. Hence, it is not safe for human consumption[18]. It is necessary to find other methods that can fight against pathogenic bacteria, without drawbacks issues. One potential method to be developed is probiotics. Probiotics in aquaculture are bio agents that are safe for fish and food security. Probiotics have significant benefits in aquaculture by increasing fish growth, providing nutrition, increasing the immune system, increasing the digestibility of feed, improving water quality and controlling the pathogenic bacteria that cause fish disease[10]. Probiotics can control pathogenic bacteria by excreting antibacterial metabolites[12]. Many types of lactic acid bacteria have been proven to be able to act as probiotics.

Not all lactic acid bacteria can be used for probiotics. Bacteria that can act as probiotics must be non-pathogenic bacteria, non-toxic, surviving through the digestion of fish, increasing the immune system and fish nutrition[19]. Many abilities must be possessed by probiotic bacteria. Hence, it requires bacterial exploration from various sources that can consider potential probiotic candidates. This study aims to explore the probiotic candidate acid bacteria from catfish intestines.

Material and Methods

Catfishes *(Clarias batracus)* were obtained from the freshwater station at the Faculty of Fisheries and Marine Sciences, Brawijaya University. Catfish used in this study weighed about 268-300 g.

Screening of Acid Producing Bacteria: Isolation of lactic acid bacteria was carried out by taking the intestines of catfish aseptically. Samples 1 g of intestine was dissolved in 9 mL sterile Na-Physiological and then homogenized using a vortex mixer. The stock solution was then diluted and spread on de Man Rogosa and Sharpe Agar (MRSA) + 1% $CaCO_3$ and incubated at 30^{o}C for 3-6 days. Lactic acid bacteria colonies that formed clear zones on the media was pure cultured. Bacteria show a clear zone around the colony[13].

Gram and catalase analysis: The procedure refers to the standard for the identification and staining of gram bacteria. Isolate repeatedly stained using crystal violet and ethanol. The result was observed under a microscope with a magnification of 1000x. The catalase test was carried out referring to research by dripping 3% hydrogen peroxide by three drops on a 24-hour-old bacterial culture in a glass object. The positive reaction of the catalase test is shown by forming significant bubbles in the formation of oxygen gas as a result of the breakdown of H_2O_2 by the catalase enzyme produced by the lactic acid bacteria[5]. Absent of air bubbles means no oxygen gas is formed and is considered as negative catalase[7].

Qualitative proteolysis assay: The bacteria isolated from the intestines of catfish were tested for their ability to produce protease enzymes in skim milk agar media (0.5% casein, 0.25% yeast extract, 0.1% dextrose, 2.8% skim milk powder and 1.5% agar). Isolated bacteria were cultured on liquid MRS media for 24 hours at 37°C.

The well was prepared with a cork borer (7 mm) on skim milk agar media. 50 µL of cell-free medium were transferred in the well. The plate was incubated for 24 hours at 37 ° C. A clear zone around the well indicated the proteolysis activity[14].

16S rDNA molecular analysis: DNA extraction, PCR, sequencing and identification of species were carried out for isolate bacteria. Bacterial DNA was extracted using manual methods of Wizard Genomic DNA Purification Kit (Promega, Madison, Wis.). The 16S rDNA analysis was then performed using 2 20F primers (52-GTAATCGTCGGCCAGTA GAGTTTGATCCTGGCTC-32) and 1510R (52-CAGGAAACAGCTATGACC GGCTACCTTGTTACGACT-32). The PCR program was run for 30 cycles of 94°C for 2 minutes, 98°C for 10 s, 61°C for 30 s and 68°C for 1 min.

The amplified product was then sequenced using ABI 3130 xl DNA sequencer[15]. After obtaining a sequence of constituent nitrogen bases, it was then analyzed using BLAST[17]. Furthermore, species relationship analysis was carried out using the Multiple Sequence Alignment Program (MAFT) website with 13 other lactic acid bacteria[4].

Antibacterial assay: Antibacterial activity assay was conducted to determine the ability of isolated acid-producing bacteria to inhibit the growth of pathogenic bacteria. This study used four pathogenic bacteria that often cause disease in fish including *Edwardsiella tarda, Aeromonas hydrophila, Escherichia coli and Bacillus cereus*. Antibacterial analysis used the disk diffusion method. The isolated lactic acid bacteria were grown on MRS broth media and incubated at 35 °C for 24 hours. Tested-pathogenic bacteria were spread on Mueller Hinton Agar (MHA). 6 mm diameter paper disc which was impregnated with cell-free extract placed onto spread-MHA. The plates were incubated at 35 ° C for 24 hours. The clear zone around paper disc indicated the inhibitory activity of acid-producing bacteria[21].

Results and Discussion

Acid Producing Bacteria: Preliminary screening of lactic acid bacteria from the stomach of catfish was carried out using MRSA media containing 1% $CaCO_3$. MRS media are selective media against bacteria that can produce lactic acid so that the colonies that grow, accurately produce lactic acid. Twelve colonies were identified growing on the media and forming clear zones (Fig. 1). Calcium carbonate is used as an indicator of lactic acid production because $CaCO_3$ will dissolve when reacting with lactic acid so that clear zones will be formed around the growing colony[6].

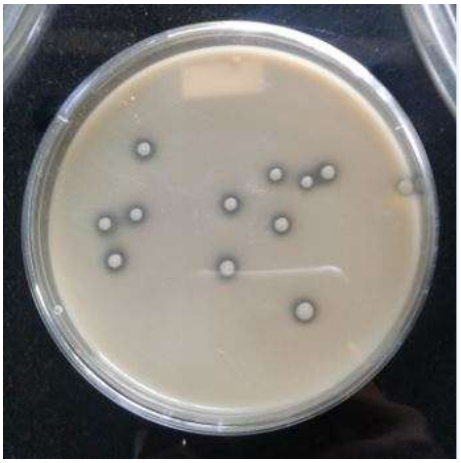

Fig. 1: Clear zone of bacteria

Lactic acid bacteria grew at 37 ° C. Lactic acid bacteria are of usually shiny white colony. Twelve colonies were pure isolated. Catalase test was applied only for the colony which showed gram-positive bacteria. Only one colony was gram-positive (code UBL2) (data not shown). Most lactic acid bacteria are gram-positive and catalase-negative[16]. Hence, UBL2 is gram-positive, catalase-negative and coccus form. Probiotic bacteria are also able to maintain the balance of microbial populations in the intestine and increase digestibility[2].

Isolate Identification: Molecular identification of isolate under the code of UBL2 was analyzed using the 16s rDNA molecular method. The results of the amplification of 16s rDNA bacteria produced an amplicon of about 1400 bp (Fig. 2). The results of the nucleotides were analyzed using BLAST on the National Center Biotechnology Information (NCBI). The results of the BLAST analysis showed that the sequences of bacterial isolates from the intestines of catfish code A51 99.8% were *Bacillus velezensis* bacteria that we identified, hence we referred to as *Bacillus velezensis* UBL2 identical to *Bacillus valezensis* R1.16.

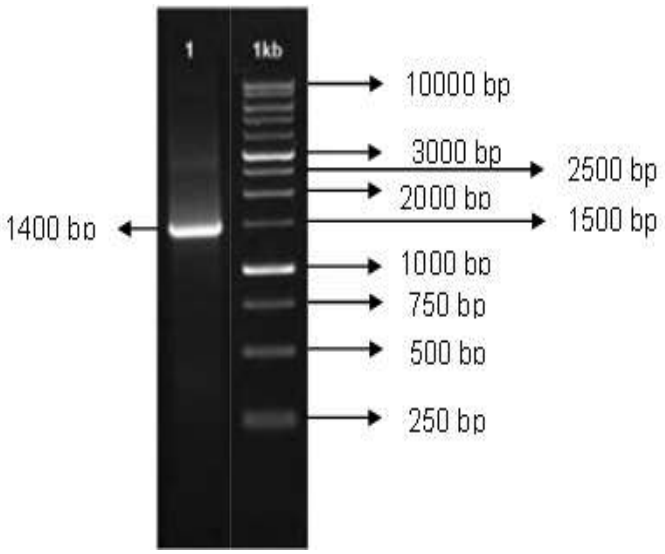

Fig. 2: 16s rDNA amplification result (1=sample; 1kb=marker)

Fig. 3: UPGMA phylogenetic tree

Identification of the kinship of 16s rDNA bacterial isolates from catfish intestines was carried out using the UPGMA method. The results of the kinship analysis show that the findings of the *Bacillus valezensis* strain UBL2 have a close relationship with other *Bacillus* species. *Escherichia coli* serves as an external comparative bacterium. *Bacillus* species are uncommon lactic acid bacteria.

Bacillus velezensis is a type of non-Lactobacillus bacteria. *Bacillus* bacteria often can produce organic acids[8]. *Bacillus velezensis* AP193 isolated from catfish *(Ictalurus punctatus)* was potential as a probiotic candidate in fish. The study was conducted by adding *Bacillus velezensis* AP193 to the feed so that it can increase the growth of catfish and can improve the quality of pond water[18].

Proteolytic Activity: *B. velezensis* UBL2 showed proteolytic activity (Fig. 4). The clear zone around the growing colony is the result of the hydrolysis activity of the extracellular protease enzyme activity produced by bacteria. Protease enzymes are enzymes that hydrolyze proteins into amino acids and simple peptides. The presence of protease enzymes in the digestive tract increases the efficiency of the absorption of nutrients especially protein. It leads to better energy production to grow. Hence, *Bacillus valezensis* strain UBL2 has the potential to become a candidate for probiotics in fish. *Bacillus* spp. is isolated from the digestive tract of freshwater fish *Labeo calbasu*. Bacillus spp. with the code of FS1, FC3 and FC6 showed proteolytic, amylolytic and lipolytic activities[9].

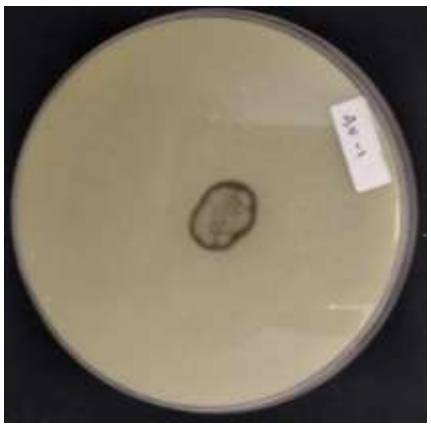

Fig. 4: The result of proteolytic activity assay

Antibacterial Activity: The isolate was tested for its antibacterial activity using four pathogenic bacteria including *Aeromonas hydrophila, Escherichia coli, Bacillus cereus* and *Edwardsiella tarda.* The clear zone in the area around the disc paper shows an antibacterial capability (Fig. 5).

Table 1
Diameter of clear zone inhibition

Bacteria	Diameter of Inhibition Zone (mm)
Aeromonas hydrophyla	11.1
Bacillus cereus	9.2
Escherichia coli	13
Edwardsiella tarda	13.7

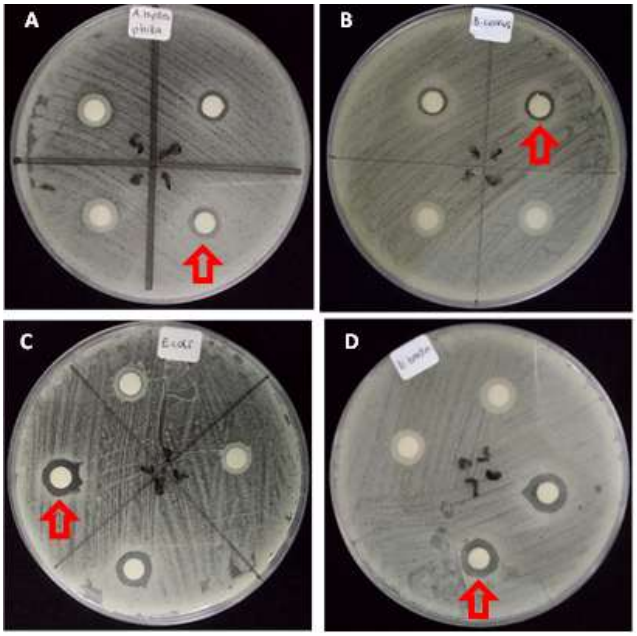

Fig. 5: Antibacterial activity of *Bacillus velezensis* UBL2 (A) *Aeromonas hydrophyla*, (B) *Bacillus cereus*, (C) *Escherichia coli*, (D) *Edwardsiella tarda*

Inhibitory zones for bacteria isolated from catfish intestine against pathogenic bacteria ranged from 9.2 mm to 13.7 mm. All pathogenic bacteria tested can be inhibited by lactic acid bacteria isolated from the intestines of catfish. The largest inhibitory zone was found in the pathogenic *Edwardsiella tarda* (13.7 mm). Inhibition zone data for each bacterium is presented in table 1. Inhibition zone diameters in the range of 0-10 mm are weak, 10-15 mm moderate and above 15 mm show good inhibitory ability[3].

Bacillus velezensis JW inhibited gram-negative (*Aeromonas hydophila, Aeromonas salmonicida, Vibrio parahemolyticus*) and gram-positive pathogenic bacteria (*L. garvieaend and S. agalactiae*). *Bacillus velezensis* strain JW produced several bacteriocins such as difficidin, bacillaene, macrolatin, bacilysin, bacillactin, bacillactin, bacillactin, bacillactin, bacillactin, bacillactin fengycin and surfactin. These antimicrobials may play a role in the growth inhibition of pathogenic bacteria[20].

Bacillus velezensis LS69 strain showed inhibition on *Escherichia coli* strain DH5α with a diameter of 10mm - 15mm. These bacteria are also reported to be able to inhibit pathogenic *Bacillus cereus* UW85 strain with an apparent diameter zone less than 10mm[11].

Conclusion

Bacillus velezensis UBL2 has proteolytic enzyme and inhibition against both gram-positive and gram-negative pathogenic bacteria. Hence, *Bacillus velezensis* UBL2 may be a potential candidate for probiotic bacteria.

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