Screening of Potential Isolate Candidates Probiotic Against Aeromonas hydrophila from Boyolali, Indonesia

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Screening of potential isolate candidates probiotic against *Aeromonas hydrophila* from Boyolali, Indonesia

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Abstract. Mass mortality in catfish culture frequently occurs in Boyolali as a central production due to the outbreak of bacterial diseases. The main causative agent of bacterial disease is *Aeromonas hydrophila*. This research aimed to find out the bacteria isolates were potential against *A. hydrophila*. The exploratory method was commenced. Thirty-four isolates were gained from water (SBA01–SBA14) and mud (SBL01 – SBAL20) that were collected from the fish pond of Boyolali Regency, Indonesia with TSA medium. Screening the potential bacteria candidates against *A.hydrophila* using the sensitivity test that was conducted with in vitro method. Based on the screening results showed that three isolates (SBA14, SBL11, and SBL20) were potential candidates against *A. hydrophila*. On the basis of sequence 16S rDNA analysis, the result showed that SBA 14, SBL 11 and SBL 20 were closely related to *Bacillus flexus*, *Bacillus subtillis*, and *Bacillus velezensis* respectively.

1. Introduction

One type of fish disease that is often found in freshwater fish in general around the world is a bacterial disease caused by *Aeromonas hydrophila* [1]. *A. hydrophila* still becomes the cause of mass mortalities in several species including catfish in Boyolali Regency. *A. hydrophila* is unicellular, Gram-negative, motile, and opportunistic heterotrophic bacteria that can cause death in fish in a short time. This is possible because of the presence of extracellular toxin products such as hemolysin, aerolysin, cytolysin, enterotoxin, amylase, and other toxins released. *A. hydrophila* is one of the agents that cause Motile Aeromonas Septicemia (MAS) which has signs of fish with stomach edema, inflammation around the wound, bleeding in the fish's body, rotting gills, ulcers, weakness and prominent eyes (exophthalmia) [2].

Various efforts have been made to overcome the infection of *A. hydrophila*. One of the ways was by using antibiotics which have deficiencies that can make pathogens resistant and residues for fish that will be harmful if consumed by humans. Another safer solution was the use of various plant extracts and the use of probiotic bacteria that still need further research [3]. The use of probiotics as disease prevention agents is one alternative strategy. There are several probiotic bacteria that have shown to inhibit the growth of pathogenic *A. hydrophila*, namely *Bacillus subtilis*, *L. plantarum* and *B. megaterium*, *Bacillus* sp., *Lactobacillus* sp. and *Arthrobacter* sp., *Tetraselmis suecica* [4], with treatment via the feed or to the cultivation water in aquaculture [5].

There are many studies concerning the using of probiotic bacteria to prevent bacterial disease in aquaculture [6,7,8]. The present study was commenced to get potential bacteria as candidate probiotic for inhibiting *A. hydrophila* in catfish culture in Boyolali Regency.

2. Materials and Methods

2.1. Experimental materials and animals

An isolate of *A. hydrophila* was obtained from the bacterial collection of Aquaculture Laboratory, Fisheries and Marine Science Faculty of Diponegoro University. The agar media used was Tryptic Soy Agar (TSA) for the in vitro test [13] and pure isolate culture. Selective media of Glutamate Starch Phenol (GSP) as agar medium for *A. hydrophila* and increasing virulence of *A. hydrophila* test. TSB media (Tryptic Soy Broth) was used as a liquid culture medium.

2.2. Isolation of potential bacteria candidates against A. hidrophyla in pond water.

Water samples were taken using a volume of 300 ml of sample bottles in a healthy and sick catfish pond. After that, the water sample was diluted to 10^{-3} using sterile aquadest. The sample was diluted from 10^{-1} to 10^{-3}, every dilution was taken 1 ml and placed in TSA media and leveled using the spread method. Then incubated at room temperature for 24 hours. Colonies that grow and range from 30 to 300 colonies were counted according to the appearance (type) of different colonies. Each different type of colony was then purified as a candidate probiotic bacteria.

2.3. Isolation of potential bacteria candidates against A. hidrophyla in mud of fish farming ponds.

The mud of the fish culture pond taken about 1 gram, then crushed with mortar then put into 9 ml of sterile aquadest. The next step was the same as the water sample.

2.4. Identification of bacteria

Bacterial identification was carried out through bacterial morphology observations and sequence analysis through 16s rDNA. From thirty-four bacterial isolates, three isolates were characterized with the molecular approach based on methods previously used by [12]. Bacterial isolates were extracted from agar plate then suspended in sterile water (Sigma, Germany). The Polymerase Chain Reaction (PCR) was run using Eppendorf Mastercycler (Eppendorf Inc. Germany) with five freezing cycles (-80ºC) and thaw (95ºC). The universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and primers 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify nearly complete 16S rDNA gene. Determination of the sequence performed by Genetika Science, PT. (Jakarta, Indonesia). DNA sequences of the bacteria forward were compared to the BLAST *(Basic Local Alignment Search Tool*) on *National Center for Biotechnology Information, National Institute for Health* database USA to gain the homology [9,10]. Whereas the phylogenetic was constructed with Mega 6 program [11].

2.5. Sensitivity and Pathogenicity Test

The sensitivity test was carried out to see the ability of the bacteria candidates for the growth of pathogenic bacteria *A. hydrophila*. The method used was paper disc. This test performed three replicates to obtain exact clear zone. The culture of pathogenic bacteria was grown in a liquid medium (TSB) and incubated for 24 hours at room temperature. Then spread on the surface of the agar media (TSA) used L glass. The inhibiting candidate bacteria culture was grown in a liquid medium and incubated for 24 hours at room temperature. Then 0.05 ml of liquid culture was dropped on the paper disc and placed on the surface of the media that have been spread. Positive results (+) were indicated by the clear zone (inhibitory area). Negative results (-) are indicated by the absence of a clear zone.

The pathogenicity test was used to confirm whether to isolate potential candidates against bacteria *A. hidrophyla* that have been isolated from water and mud were pathogens for fish or not. This test was carried out on a laboratory scale using aquarium containers with a stocking density of 1 fish/L.

3. Result and Discussion

3.1. Result

3.1.1. Characteristic of the Bacterial Isolates

Based on the morphological characteristic, there were 34 bacteria isolates consisted of 14 isolates were gained from water (SBA01–SBA14) and mud (SBL01 – SBAL20). The characteristic of 34 isolates was presented in Table 1.

Table 1. Morphological Characteristic of 34 Isolates.

No.	Isolate Code	Source	Color in NA medium	Shape	Elevation
1.	S B A1	Water	White	Circulair	Convex
2.	S B A2	Water	White	Circulair	Convex
3.	S B A3	Water	White	Circulair	Convex
4.	S B A4	Water	White	Circulair	Convex
5.	S B A5	Water	White	Circulair	Convex
6.	S B A6	Water	White	Circulair	Convex
7.	S B A7	Water	White	Circulair	Convex
8.	S B A8	Water	White	Circulair	Convex
9.	S B A9	Water	White	Circulair	Convex
10.	S B A10	Water	White	Circulair	Convex
11.	S B A11	Water	White	Circulair	Convex
12.	S B A12	Water	White	Circulair	Convex
13.	S B A13	Water	White	Circulair	Convex
14.	S B A14	Water	White	Circulair	Convex
15.	S B L1	Mud	White	Circulair	Convex
16	S B L2	Mud	White	Circulair	Convex
17	S B L3	Mud	White	Circulair	Convex
18	S B L4	Mud	White	Circulair	Convex
19	S B L5	Mud	White	Circulair	Convex
20	S B L6	Mud	White	Circulair	Convex
21	S B L7	Mud	White	Circulair	Convex
22	S B L8	Mud	White	Circulair	Convex
23	S B L9	Mud	White	Circulair	Convex
24	S B L10	Mud	White	Circulair	Convex
25	S B L11	Mud	White	Circulair	Convex
26	S B L12	Mud	White	Circulair	Convex
27	S B L13	Mud	White	Circulair	Convex
28	S B L14	Mud	White	Circulair	Convex
29	S B L15	Mud	White	Circulair	Convex
30	S B L16	Mud	White	Circulair	Convex
31	S B L17	Mud	White	Circulair	Convex
32	S B L18	Mud	White	Circulair	Convex
33	S B L19	Mud	White	Circulair	Convex
34	S B L20	Mud	White	Circulair	Convex

The results showed that 34 isolates had white color in NA medium, circulair shape, and convex elevation.

3.1.2. Sensitivity test

Sensitivity test of 34 isolates showed inhibitory activity from 4 candidate isolates against *A. hydrophila* with a clear zone of >10 mm. The results were presented in Table 2.

Table 2. The Result of Sensitivity Test of 4 Indicated Anti-Aeromonas Isolates

No.	Isolate Code	The diameter of Clear Zone (mm)			
		X1	X2	X3	Average

1	S B A14	19	18	16	17,7
2	S B L11	13	12	15	13,3
3	S B L15	12	13	13	12,7
4	S B L20	13	23	21	19,0

The most powerful isolate in inhibiting *A. hydrophila* was SBL20 with clear zone average of 19 mm. This value was categorized as a strong inhibitory activity, followed by SBA14, SBL11, SBL15 with a clear zone of 17,7 mm; 13,3 mm; 12,7 mm, respectively and also categorized as a strong inhibitory activity. The clear zone image was presented in Figure 1.

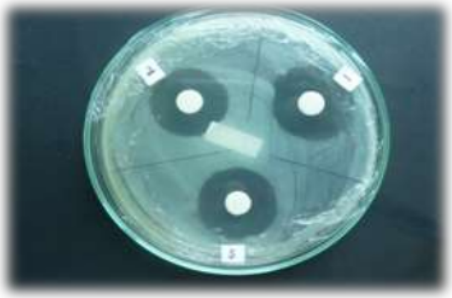

Figure 1. Clear Zone of SBL20 against *A. hydrophila*

3.1.3. Pathogenicity and Sensitivity test among four Isolates
The results of sensitivity and pathogenicity test of four isolates were shown in Table 3.

Table 3. Sensitivity among four Isolates and Pathogenicity test.

Isolate Code	S B A14	S B L11	S B L15	S B L20
S B A14		+	+	-
S B L11	+		+	-
S B L15	+	+		-
S B L20	-	+	-	
Mortality of Catfish	10 %	20 %	95 %	10 %

Based on the sensitivity test between isolates found that three isolates (SBA 14, SBL 11 and SBL 20) were potentially against *A. Hidrophyla.* Whereas, pathogenicity test showed that three isolates were a low pathogenic in catfish culture and it referred that three isolates fulfill the requirement as a potential candidate against *A. hidrophyla.* The isolates were SBA14, SBL11, and SBL20. While one isolate was known as pathogen bacteria since causing the death of catfish, and it was SBL15.

3.1.4. Molecular identification.
On the basis of sequence 16S rDNA analysis, the result showed that SBA 14, SBL 11 and SBL 20 were closely related to *Bacillus flexus, Bacillus subtillis,* and *Bacillus velezensis* respectively (Table 4).

Table 4. Molecular Characterization of Three potential bacteria against to *A. hydrophyla* in Catfish

No	Isolates	Close relative	Homology (%)	Acc. Number

1	SBA 14	*Bacillus flexus*	99	KX242400.1
2	SBL 11	*Bacillus subtillis*	99	EF528267.1
3	SBL 20	*Bacillus velezensis*	100	KY0840550.1

The phylogenetic of three potential bacteria against *A. hydrophyla* in catfish were seen in figure 2.

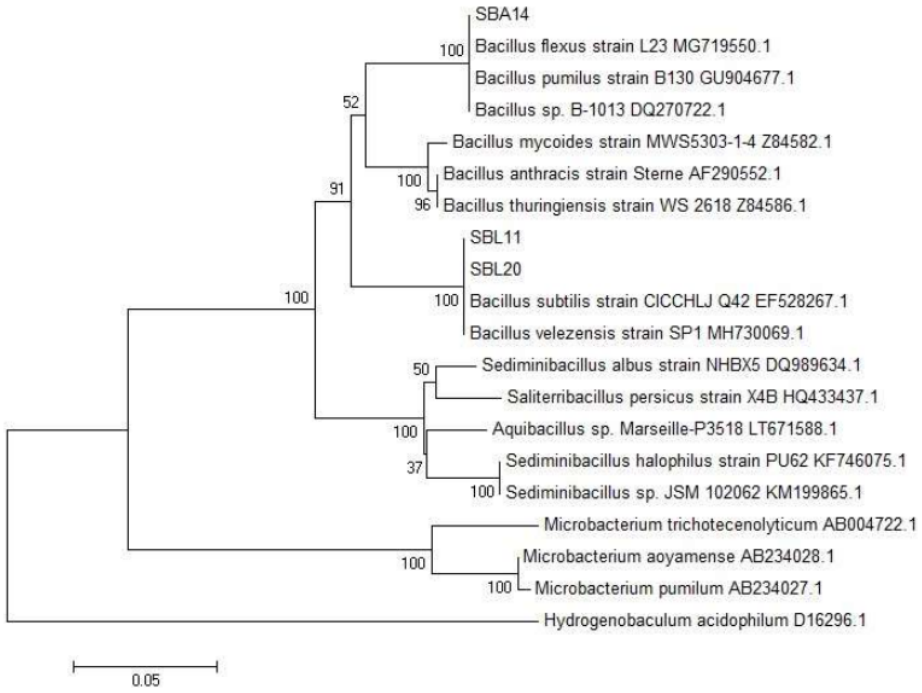

Figure 2. Phylogenetic of The Three Potential Bacteria Against *A. hydrophyla* on Catfish from Boyolali Regency

3.2. Discussion

Bacteria isolates from water and mud of catfish pond in Boyolali Regency were purified and identified. Thirty-four isolates showed morphological characteristics, such as circular shape, white color, and convex elevation. These bacteria were purified in TSA medium for In vitro screening of antagonistic bacterial activity. This test was used to screen the potential bacteria against *A. hidrophyla* as a probiotic candidate. According to Banerjee and Ray [4], the antagonistic or inhibition ability of this candidate against a variety of pathogens is the most important property. The bacteria candidate produced a wide range of bacteriocins (small peptide to larger protein) or anti-microbial compounds to inhibit pathogens or the competitors.

Sensitivity test of 34 isolates showed that the inhibitory activity of 4 isolates against *A. hydrophila* with a clear zone of >10 mm. The most powerful isolate in inhibiting *A. hydrophila* was SBL20 with clear zone average of 19 mm. This value was categorized as a strong inhibitory activity, followed by SBA14, SBL11, SBL15 with a clear zone of 17,7 mm; 13,3 mm; 12,7 mm, respectively and also categorized as a strong inhibitory activity. This interaction can be related because of the

nutritional competition of bacterial growth media and the presence of antimicrobial or bacteriocin compounds produced by SBA14, SBL11, SBL15, and SBL20. It was compatible with [12], that competition in nutrient absorption was a common phenomenon in natural habitats. Some bacterial species used antagonistic activity or inhibiting devices or organs as weapons against competitors. Desriac *et al.* [13] also stated that generally, bacteria produced several types of anti-microbial compounds or bacteriocins to inhibit or kill other competing bacterial species.

The next characteristic of probiotic candidate bacteria was non-pathogenic [4]. Four isolates were tested through pathogenicity test. The result showed that three isolates were low pathogenic in catfish culture and it referred that three isolates fulfill the requirement as a potential candidate against *A. hidrophyla* and as a probiotic candidate. The isolates were SBA14, SBL11, and SBL20. While one isolate was known as pathogen bacteria since causing the death of catfish and it was SBL15. The degree of pathogenicity depends on toxin producing capability, and it varies from one strain to another strain. For example, *A. hydrophila* is considered to be a deadly pathogen in fish [14], however few strains of this bacteria are used as probiotic candidates in fish [15].

Based on the sequence 16S rDNA analysis, the result showed that SBA 14, SBL 11 and SBL 20 were closely related to *B. flexus, B. subtillis* and *B. velezensis* respectively with a homology of 99 – 100%. These kinds of Bacillus were commonly found as probiotic in fish culture [16,17,18]. Bacillus was able to inhibit *A. hydrophila* because it contained a wide range of cyclic lipopeptides. Polypeptides or lipopeptides are polymers composed of several peptides which are bound to carboxyl (COOH) groups with amino groups. One or more polypeptides can form proteins, for example, enzymes. Polypeptides are formed through the process of gene expression that occurs in cells. According to Huang *et al*. [19], Iturin consists of seven amino acid residues that are hydrophilic and hydrocarbon tails with 10 – 13 carbons in length which are hydrophobic. Iturin and surfactin are examples of antibiotics that can inhibit bacteria growth [19]. Lang and Wagner [20] mentioned that surfactin which is a cyclic lipopeptide in addition to lowering the surface tension of a liquid also damages spheroplast and other bacterial protoplasts near the producing bacteria. The function of surfactin as an antimicrobial is related to its ability to bind hydrophobic molecules to bacterial membranes. It was further stated by Hommel & Ratledge [21] that the effectiveness of surfactin as an antimicrobial compound depends on the surfactin concentration and the resistance of the surrounding microorganism membrane which can be inhibited.

Conclusion

On the basis of 16S DNA sequence analysis, the result shows that the probiotic bacteria candidates from water and mud of catfish pond in Boyolali Regency were closely related to *B. flexus* (SBA14), *B. subtillis* (SBL11) and *B. velezensis* (SBL20). The research result found that SBA14 and SBL20 were effectively inhibited catfish mortality against *A. hydrophila* as indicated by the lowest mortality of 20%.

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