Probiotic Candidates from Fish Pond Water in Central Java Indonesia

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Abstract. *Aeromonas hydrophilla* is a major bacterial pathogen of intensive fresh water fish culture in Indonesia. An alternative method to control the pathogen is using probiotics. Probiotics is usually consist of live microorganisms which when administered in adequate amounts confer a health benefits on host. The aim of this research was to determine the probiotic candidates against *A. hydrophilla* which identified based on the 16S rDNA gene sequences. This research was started with field survey to obtained the probiotic candidate and continue with laboratory experiment. Probiotic candidates were isolated from fish pond water located in Boyolali, and Banjarnegara Regency, Central Java, Indonesia. A total of 133 isolates bacteria were isolated and cultured on to TSA, TSB and GSP medium. Out of 133 isolates only 30 isolates showed inhibition to *A. hydrophilla* activity. Three promising isolates were identified with PCR using primer for 16S rDNA. Based on 16S rDNA sequence analysis, all three isolates were belong to Bacillus genus. Isolate CKlA21, CKlA28, and CBA14 respectively were closely related to Bacillus sp. 13843 (GenBank accession no. JN874760.1 -- 100% homology), Bacillus subtilis strain H13 (GenBank accession no.KT907045.1 -- 99% homology), and Bacillus sp. strain 22-4 (GenBank accession no. KX816417.1 -- 97% homology).

Keywords: Probiotic candidates, Fish pond water, *Aeromonas hydrophila*, 16S rDNA.

1. Introduction
The Aquaculture has considerably grown during the last few decades as well as becoming an economically significant business [1]. Aquaculture is aimed as a solution to meet the growing demand for fish and shellfish. To meet the ongoing globalization of food shortage, improving aquaculture practices by new technological innovations is a challenging assignment for scientists and biologists [2]. There are some important aquaculture commodity in Indonesia such as walking catfish (*Clarias gariepinus*), tilapia (*Oreochromis niloticus*) and gouramy (*Osphronemus gourami*), etc. Increasing demand for domestic markets encourages the fish farmers to increase their production. With the escalation intensification of aquaculture production, diseases and declining of environmental conditions are major problems and may caused massive economic losses [3].

The negative impacts of intensification of aquaculture were bacterial disease outbreaks such as *Aeromonas hydrophila* which is still became a major problem in fresh water aquaculture. *A.*
*hydrophila* is a ubiquitous aquatic bacterium which has been implicated as a pathogen of fish [4-5] and another aquatic organisms [6-7]. Antibiotics was frequently used for prevention and treating the diseases and to keep the fish live. However this causes adverse effects on the environment and bacteria that become increasingly resistant to antibiotics administered to ailing organisms.

The need for fish protein and environmentally friendly fish cultivation requires an alternative substance to replace antibiotics and chemicals in order to support sustainable quality production of aquaculture [8-10]. An alternative solution that can be used to overcome this problem is by using probiotics. It usually contained live microorganisms that can be administered in adequate amounts in order to confer a health benefits on host. Various studies on the use of probiotics in the field of aquaculture demonstrate the ability to control potential pathogens to developed [5]. The growth performance and various immunologic parameters may be enhanced by the use of probiotics, prebiotics, immunostimulant as additional feed routine in aquatic organisms [11-12]. The types of bacteria and mechanisms by which probiotics exert their effects are still being studied, and there are still many open research opportunities on this subject.

Water as a fish culture medium is the site of various bacteria. With the bacterial screening process is expected to obtain potential isolates to increase the productivity of aquaculture in various ways. On the other hand probiotics from the same species and/or its natural environment could be best approach for better efficacy in host [13-14]. Several researches had been performed to find out potential bacteria on freshwater fish based on the 16S rDNA gene sequences. There were limited reports being documented so far describing the application of polymerase chain reaction on the diversity of potential probiotic bacteria on fresh water aquaculture derived from fish pond of central fish production in Central Java, Indonesia.

### 2. Materials and Methods

#### 2.1. Isolation of probiotics candidates bacteria from fish pond water.

Bacteria isolates were collected from fresh water fish aquaculture ponds of central production area in Boyolali Regency (from *C. gariepinus* pond), Klaten Regency (from *O. niloticus* pond), and Banjarnegara Regency (from *O. gouramy* pond). The best production of the aquaculture pond in the last harvest period became one of the requirements for sampling on selected fish ponds. Bacteria were isolated from pond water by spread plate method and cultured in to TSB (Mereck). Then, 0.1 ml of appropriate dilutions was spread on Tryptic Soybean Agar (TSA-Merck). After 24 to 72 h of incubation, the bacterial colonies were purified several times on TSA agar to get pure culture and isolated colonies. Preliminary identification of the isolates was carried out using microscopic observation.

#### 2.2. Gram staining

Morphological features of the colonies were picked and purified by a single colony to the plate. Gram stain test have done based on Gram stain protocol Animal Health Diagnostic Center, Cornell University(15).

#### 2.3. Antimicrobial activity test

Probiotic candidates were co-cultured with *A. hydrophilla* in various concentrations to evaluate their antagonistic activities using disc method. The bacterial that shows the antagonistic activity against pathogens can be classified as potential isolates. Based on the method developed by David Stout, the division of antibacterial activity can be done based on the size of the inhibitory diameter formed [16]. Based on the method, an antibacterial agent producing an inhibitory area of <5 mm is categorized as a weakly antibacterial substance; 5-10 mm are categorized as medium-strength antibacterial; 10-20 mm categorized as strong antibacterial; And > 20 mm are categorized as very powerful antibacterial. Morphological features of the colonies were picked and purified by a single colony to the plate.
2.4. Determination of pathogenicity
The potential isolates obtained were tested for their pathogenicity against 3 species of freshwater fish (C. gariepenus, T. nilotica and O. gouramy) using healthy fish that have been acclimatized for 14 days in a well-labelled glass aquarium. Fish were injected either intramuscularly (IM) with pre-prepared 0.1 ml of bacterial suspension contains $10^8$ cfu ml$^{-1}$. Control groups were injected either IM with 0.1 ml of sterile PBS. The each group (n = 10) of fish were monitored for 14 days with daily replacement of water. The parameters measured were survival rate and apparent clinical symptoms.

2.5. Molecular identification of the most potent isolate
The potential probiotic isolate was identified based on 16S rDNA sequencing. Genome DNA purification kit (Promega, Madison, USA) was used to extract the DNA for the amplification of 16S rRNA genes by the polymerase chain reaction (PCR) [24]. PCR products were resolved by electrophoresis in agarose gels and visualized by ethidium bromide staining and purified. The 16S rDNA sequence alignment was done using BLAST with the non-redundant 16S rRNA sequence database of NCBI GenBank (www.ncbi.nlm.nih.gov). Features of the colonies were picked and purified by a single colony to the plate and used for cross check.

3. Results and Discussions

3.1. Candidates Probiotic againts A. hydrophila.
A total of 133 bacterial isolates were obtained as probiotic candidates derived from cultivation of catfish, tilapia and gourami cultivation pond water. In the end there are 79 bacterial isolates left which consisted of 18 isolates from Boyolali, 31 isolates from Klaten and 30 isolates from Banjarnegara. Further, antimicrobial activity test results obtained fourteen [14] isolates probiotic candidate which showed inhibiting zone for the growth of A. hydrophila bacteria with a range of 10.33-19.67 mm. Antimicrobial activity test againts A hydrophila results are given in Table 1.

| No | Isolate Code | 1  | 2  | 3  |
|----|--------------|----|----|----|
| 1  | CB A14       | 19 | 18 | 16 |
| 2  | CB A16       | 13 | 10 | 8  |
| 3  | CKI A14      | 11 | 12 | 11 |
| 4  | CKI A18      | 13 | 12 | 13 |
| 5  | CKI A20      | 17 | 14 | 14 |
| 6  | CKI A21      | 21 | 23 | 15 |
| 7  | CKI A22      | 15 | 15 | 12 |
| 8  | CKI A23      | 10 | 10 | 12 |
| 9  | CKI A27      | 13 | 15 | 15 |
| 10 | CKI A28      | 20 | 20 | 13 |
| 11 | CBj A14      | 16 | 11 | 10 |
| 12 | CBj A18      | 11 | 13 | 16 |
| 13 | CBj A19      | 15 | 13 | 9  |
| 14 | CBj A21      | 9  | 13 | 15 |
The fourteen selected isolates were included in the strong category with a zone of > 10 mm inhibition [1]. To obtain more detailed information from selected bacteria carried out the morphological characteristics of bacterial colonies. Characteristics of Probiotics candidate isolates bacteria are given in Table 2.

Table 2. Characteristics of Probiotics candidate isolates bacteria.

| No | Isolates | Antimicrobial activity (mm) | Colony Characteristic | Gram stain and Shape |
|----|----------|-----------------------------|----------------------|----------------------|
| 1  | CB A₁₄  | 17.67                       | White yellowish, entire, L shaped, wet, raised | Positive, short rod |
| 2  | CB A₁₆  | 10.33                       | White pale, round, wavy, and spreading, dry, convex | Positive, long rod |
| 3  | CK₁ A₁₄ | 11.33                       | White milk, round, wavy, wet, convex | Positive, long rod |
| 4  | CK₁ A₁₈ | 12.67                       | White pale, filiform, wavy, dry, convex | Positive, long rod |
| 5  | CK₁ A₂₀ | 15                          | White pale, filiform, irregular, dry, raised | Positive, long rod |
| 6  | CK₁ A₂₁ | 19.67                       | White pale, irregular, wavy, dry, raised | Positive, short rod |
| 7  | CK₁ A₂₂ | 14                          | White pale, filiform, irregular, dry, raised | Positive, long rod |
| 8  | CK₁ A₂₃ | 10.67                       | White pale, round, entire, wet, convex | Positive, long rod |
| 9  | CK₁ A₂₇ | 14.33                       | White pale, rhizoid, wavy, dry, convex | Positive, long rod |
| 10 | CK₁ A₂₈ | 17.67                       | White pale, filiform, irregular, dry, raised | Positive, short rod |
| 11 | CBj A₁₄ | 12.33                       | White pale, round, wavy, wet, convex | Positive, short rod |
| 12 | CBj A₁₈ | 13.33                       | White pale, round, wavy, dry, convex | Positive, short rod |
| 13 | CBj A₁₉ | 12.33                       | White pale, round, entire wet convex | Positive, short rod |
| 14 | CBj A₂₁ | 12.33                       | White pale, irregular, dry, convex | Positive, short rod |

Gram staining test results showed that all isolates were Gram positive (blue color) with long rod shape, which means its enter into Bacillus spp genus. Some probiotics, especially of the genus Bacillus have been widely used in disease control in aquatic animals. Similar results were also reported [17], that the genus Bacillus bacteria is a Gram-positive bacteria and is included in probiotic bacterial candidates.

There is no fish mortality that caused by the all isolates as a probiotics candidate. This result is better and also in accordance with the opinion that fish mortality that caused by probiotic candidates less than 33% are considered capable of controlling pathogenic bacterial infections [18]. It has succeeded in isolating the potent probiotics of Bacillus spp. which is used to improve the immune
system of cyprinids (Labeo rohita) against the attack of A. hydrophila pathogens. The success rate obtained was very significant with the mortality range of only 20-40% compared to controls that achieved 80% mortality. The summarized sequence analysis of CBA14, CKIA21 and CKIA28 isolate are presented in Table 3.

Table 3. Genetic Diversity of Potential Probiotics Candidates Based on Gen Blast

| No. | Isolate Code | Result          | Homology (%) | Access Number   |
|-----|--------------|-----------------|--------------|----------------|
| 1   | CBA14        | Bacillus sp. strain 22-4 | 97           | KX816417.1    |
| 2   | CKIA21       | Bacillus sp. 13843 | 100          | JN874760.1    |
| 3   | CKIA28       | Bacillus subtilis strain H13 | 99          | KT907045.1    |

Results showed that CKIA28 isolate had a similarity level of 99% with Bacillus subtilis H13 strain. Bacillus subtilis H13 strain is reported on the research of cow dung as a cheap substrate for polyhydroxy butyrate production by soil bacteria. Genus Bacillus spp. commonly found from various sources. Bacterial genera Bacillus sp isolated from the healthy intestine of cyprinids (Labeo rohita) [2-3]. Bacillus spp genus is also commonly found in giant prawns [4]; fish cyprinids[5]; Bacillus spp. reported on indigestion of tilapia while another reported that Bacillus sp. in research related to natural resources of agriculture; even Bacillus spp. also found in the Mulberry plant endophytes.

Probiotic therapy offers a suitable alternative for controlling pathogens thereby overcoming the adverse consequences of antibiotics and chemotherapeutic agents. In fish culture, probiotics either in diet or bioencapsulation help in achieving natural resistance and high survivability of larvae and post larvae of fishes [6-8].

4. Conclusions

In the present study, 133 isolates bacteria were isolated from different sources and only fourteen isolates were selected on the basis of their high antimicrobial activity. Three bacteria that have potential as a probiotic candidate able to inhibit A. hydrophilla with the strong category were aligned with the 16S r-DNA sequences from the Gen Bank database (website) to identify the studied microorganism. The Results isolate CBA14 97% homology with Bacillus sp. strain 22-4, CKIA21

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