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IOP Conference Series: Earth and Environmental Science

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To cite this article: Alfabetian Harjuno Condro Haditomo *et al* 2018 *IOP Conf. Ser.: Earth Environ. Sci.* **116** 012018

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 inhibitor 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.

IOP Conf. Series: Earth and Environmental Science **116** (2018) 012018 doi:10.1088/1755-1315/116/1/012018

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.

Table 1. Antimicrobial activity test againts *A hydrophila* (mm).

No	Isolate Code	1	2	3
1	CB A_{14}	19	18	16
2	CB A_{16}	13	10	8
3	CKl A_{14}	11	12	11
4	CKl A_{18}	13	12	13
5	CKl A_{20}	17	14	14
6	CKl A_{21}	21	23	15
7	CKl A_{22}	15	15	12
8	CKl A_{23}	10	10	12
9	CKl A_{27}	13	15	15
10	CKl A_{28}	20	20	13
11	CBj A_{14}	16	11	10
12	CBj A_{18}	11	13	16
13	CBj A_{19}	15	13	9
14	CBj A_{21}	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 A_{14}	17,67	White yellowish, entire, L shaped, wet, raised	Positive, short rod
2	CB A_{16}	10,33	White pale, round, wavy, and spreading, dry, convex	Positive, long rod
3	CKl A_{14}	11,33	White milk, round, wavy, wet, convex	Positive, long rod
4	CKl A_{18}	12,67	White pale, filiform, wavy, dry, convex	Positive, long rod
5	CKl A_{20}	15	White pale, filiform, irregular, dry, raised	Positive, long rod
6	CKl A_{21}	19,67	White pale, irregular, wavy, dry, raised	Positive, short rod
7	CKl A_{22}	14	White pale, filiform, irregular, dry, raised	Positive, long rod
8	CKl A_{23}	10,67	White pale, round, entire, wet, convex	Positive, long rod
9	CKl A_{27}	14,33	White pale, rhizoid, wavy, dry, convex	Positive, long rod
10	CKl A_{28}	17,67	White pale, filiform, irregular, dry, raised	Positive, short rod
11	CBj A_{14}	12,33	White pale, round, wavy, wet, convex	Positive, short rod
12	CBj A_{18}	13,33	White pale, round, wavy, dry, convex	Positive, short rod
13	CBj A_{19}	12,33	White pale, round, entire wet convex	Positive, short rod
14	CBj A_{21}	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, CKlA21 and CKlA28 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	CKlA21	*Bacillus sp.* 13843	100	JN874760.1
3	CKlA28	*Bacillus subtilis* strain H13	99	KT907045.1

Results showed that CKlA28 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, CKlA21

5. Acknowledgement

The authors would like to thank the Dean of the Faculty of Fisheries and Marine Sciences, Diponegoro University for his permission to begin this research that Funded by Others APBN DPA SUKPA Faculty of Fisheries and Marine Sciences University of Diponegoro Fiscal Year 2017. Thanks also to Pak Marsudi, Hendra, Hanifah, Yosef, Tiara, Falah, Vava, Putri, Femy, Ilham, Fina, Thesa, Sofa, Sarah, and Mada who have helped this research. We are also grateful to the Head of Diponegoro University Integrated Laboratory, Aquaculture Laboratory Faculty of Fisheries and Marine Sciences, Diponegoro University which provides an opportunity to conduct this research.

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