Isolation of *Aeromonas sobria* JC18 from milkfish (*Chanos chanos*) intestine with proteolytic and cellulolytic activities for fish probiotic

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Abstract. Attention on the probiotic application for the improvement of fish resident intestinal microbiota has risen in the last decades. It is demonstrated that probiotics may function not only by direct inhibition of pathogenic bacteria or manipulation of enzymatic digestion of feed but also by modulation of immunity in fish. In the present study, we aimed to isolate the autochthonous microbiota of marine fish intestines as probiotic candidates based on cellulolytic and proteolytic activity. Bacteria were isolated and purified on a cellulose agar with 24h of incubation. A total of 18 bacterial strains were purified and stored in -80°C. Phenotypic screening based on the antibiotic resistance, antagonistic activity against pathogenic bacteria, resistance to an acidic environment, and ability of colonization in fish intestine found a selected strain, namely JC18. Infection test, molecular and phenotypic characterizations revealed that the JC18 isolate was a non-pathogenic *Aeromonas sobria*. It is hence revealed that the milkfish intestine is a potent source of proteolytic bacteria for fish probiotic screening.

1. Introduction

The aquaculture industry has great potential to develop in the world. In 2014, aquaculture contributes about 35.3% of world fish production with an increase of 5.2% each year [1]. Along with the increase in aquaculture practice, an outbreak of disease occurs more frequently [2] that needs mitigation with various approaches, including water quality management, feeding management, vaccination, immunostimulant, and probiotic application.

Probiotics have been chosen as one of the ways to overcome problems in intensive aquaculture practice. Probiotics in aquaculture are defined as a live microbial adjunct that has a beneficial effect on the host [3]. Study shows that probiotics are beneficial in the fish digestive tract by producing extracellular enzymes such as proteases, amylases, lipases and growth factors such as vitamins, fatty acids, and amino acids [4].

Protein is one of the nutrients required by fish in high amounts. In general, fish needs 30-40% of protein content in the diet [5]. Enhancement of protein digestibility in the fish diet is important to increase feeding efficiency. Accordingly, the purpose of this study was to find potential proteolytic bacteria as a candidate of fish probiotic from the autochthonous microbiota of marine fish intestine.
2. Materials and Methods

2.1 Isolation and Purification
Milkfish (Chanos chanos) naturally live in the open estuarine area of the national mariculture center Balai Besar Pengembangan Budidaya Air Payau (BBPBAP) Jepara, Indonesia was selected as an object for bacterial isolation. A total of three fish was collected. Fish were anesthetized with ice and dissected for the intestine examination. The intestine was opened for bacterial isolation by using the loop with streak method on a Carboxyl methyl cellulose (CMC) agar plate. The inoculated plate was incubated at 28°C for 24h. Grown bacteria were purified on CMC agar and the purified bacteria were cultured in Tryptone Soya Broth (TSB) for 24h at 30°C, and stored in the medium with an addition of 50% glycerol at -80°C.

2.2 Screening for probiotic properties
2.2.1 Hydrolytic enzyme activity
The purified bacteria were screened qualitatively for the ability to produce proteolytic and cellulolytic enzymes by using skim milk agar and CMC agar, respectively. The examination was continued with semi-quantitative screening based on the hydrolytic index. Bacteria were grown in TSB for 24h and inoculated to a paper disk (Ø 0.8 mm) (10⁷ cells/disc). The bacteria-containing disk was then incubated at 28 °C for 24 hours. The proteolytic hydrolysis index (PHI) were determined based on the diameter of the clear zone and the diameter of the disk.

2.2.2 Acid tolerance
Acid tolerance tests were determined by culturing the bacteria in tryptic soy broth (TSB) medium with pH 4 (pH 7 as a positive control. Acid-resistant bacteria will grow in such medium as indicated from the turbidity after 24h of incubation as similar to those in the positive control.

2.2.3 Antagonistic activity
Antagonistic test against fish pathogenic bacterium Vibrio harveyi SB25 and Photobacterium damsela subspecies piscicida GD05 was conducted with a direct cross-inoculation method and followed by 24 h of incubation. The antagonistic activity was determined based on the inhibition zone obtained in the growth line of the pathogenic bacteria inoculation site.

2.2.4 Antibiotics resistance
The test was carried out by growing the isolates on a TSA medium containing 5 mg/ml of antibiotics. The antibiotics that were used are Oxytetracycline, Kanamycin, Ampicillin, and Rifampicin. Overnight bacterial cultures in TSB were dropped on the antibiotic agar plates in duplicate, followed by incubation at 28 ° C for 48 h.

2.2.5 Isolate survival in the fish intestine
The test was carried out by administrating 0,1 ml of 10⁸ cells/ml bacteria in PBS medium orally at Tilapia. Observations were conduct at 2 and 24 hours after administration. Bacterial sampling was carried out by taking two cm from the upper intestine. The amount of bacteria observes using the total plate count (TPC) method was incubated for 24 hours at 28 ° C.

2.3 Molecular Identification
The DNA was extracted from 24 hours culture of the selected isolate using Wizard Genomic DNA Purification Kit (Promega) according to the protocol. PCR was carried out to targeted 16S rDNA with the use of primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACTT-30). The amplification process is regulated by the process of pre-denaturation (95 ° C, 3 minutes), denaturation (95°C, 30 seconds), annealing (55°C, 30 seconds), elongation (72°C, 1 minute). Denaturation, annealing, and elongation processes are a cycle that was repeated 30 times. The next stage is the final extension (72°C, 5 minutes) and cooling (12°C, 5 minutes). The determination sequence of base nucleotide was carried out by using the services of First Base (PT Genetics Science Indonesia). The reading of the sequence of results obtained is done by depositing the primary forward sequence and the reverse primary sequence using the software BioEdit. The obtained
sequence results then aligned with the BLAST database (Basic Local Alignment Search Tools) provide by GenBank. Phylogenetic tree construction was carried out using MEGA software version 7.0 with the Maximum Likelihood statistical method based on the Tamura-Nei model, a bootstrap consensus of 100 times replication, and partial deletion used in the existing data gaps.

2.4. Biochemical characterization

Phenotypic identification was conducted by biochemical examinations, which include Gram staining, and test on catalase, oxidase, oxidative or fermentative, Motility, ornithine decarboxylase, and fermentation of glucose, sucrose, and lactose. The biochemical characters of bacterial strain were matched to the characters of *Aeromonas* spp for identification [6].

3. Results and Discussion

3.1. Proteolytic index

A total of 62 bacterial isolates with 12 additions from the previous collection were growth in cellulolitic agar, and examined for the proteolytic activity based on the protein hydrolysis index (PHI) were conducted in the present study. The results showed that 42 isolates had proteolytic activity. A total of 25 isolates among them has a relatively high protein hydrolysis index (PHI) that range between 1.07 – 1.88 (Table 1).

Table 1. Protein hydrolysis index (PHI) of cellulolytic bacterial strains.

| No | isolate | Growth in cellulolitic agar | Protein hydrolysis index | No | Isolate | Growth in cellulolitic agar | Protein hydrolysis index |
|----|---------|-----------------------------|--------------------------|----|---------|-----------------------------|--------------------------|
| 1  | JAL 10  | +                           | 1.38                     | 15 | JM 2    | +                           | 1.14                     |
| 2  | JAL 11  | +                           | 1.25                     | 16 | JM 4    | +                           | 1.41                     |
| 3  | JAL 12  | +                           | 1.56                     | 17 | JM 13   | +                           | 1.16                     |
| 4  | JAL 27  | +                           | 1.38                     | 18 | JC 9    | +                           | 1.23                     |
| 5  | JAL 28  | +                           | 1.23                     | 19 | JC 10   | +                           | 1.13                     |
| 6  | JAL 30  | +                           | 1.25                     | 20 | JC 18   | +                           | 1.70                     |
| 7  | JAL 31  | +                           | 1.42                     | 21 | JC 23   | +                           | 1.41                     |
| 8  | JAL 36  | +                           | 1.53                     | 22 | JC 24   | +                           | 1.51                     |
| 9  | JAL 37  | +                           | 1.25                     | 23 | JC 29   | +                           | 1.12                     |
| 10 | JAL 39  | +                           | 1.31                     | 24 | JC 30   | +                           | 1.17                     |
| 11 | JAL 40  | +                           | 1.07                     | 25 | JC 31   | +                           | 1.52                     |
| 12 | JAL 41  | +                           | 1.88                     | 26 | JC 32   | +                           | 1.80                     |
| 13 | JC 28   | +                           | 1.51                     | 27 | JC 33   | +                           | 1.63                     |
| 14 | JC 25   | +                           | 1.32                     |    |         |                             |                          |
| 28-42 | JAL 07, JAL 08, JAL 19, JAL 25, JAL 26, JAL 34, JAL 38, JAL 42, JT 3, JM 11, JM 20, JC 6, JC 17, JC 21, JC 22 | + | 0.00 |

3.2. Acid tolerance and antibiotic resistance properties

Acid tolerance tests were carried out with proteolytic isolates found 26 acid-tolerant strains. Furthermore, examination of the 26 strains based on the antibiotic resistance found 22 isolates sensitive to antibiotics, while 4 isolates were either resistant to some or all antibiotic tested. The antagonism test found no interruption or interference activity either with *Vibrio harveyi* or *Photobacterium damselae* subsp. *piscisida*. Five best isolates were selected based on the proteolytic activity from 24 isolates that do not carry antibiotic resistance characters, including JC18, JC31, JC32, JAL12, and JAL41 strains.
Table 2. Acid resistance of selected bacterial strain.

| No. | Isolate | Growth in pH 4 | pH 7 |
|-----|---------|----------------|------|
| 1-26| JM 4, JM 11, JM 13, JC 9, JC 10, JC 17, JC 18, JC 21, JC 23, JC 24, JC 25, JC 28, JC 33, JAL 10, JAL 11, JAL 12, JAL 27, JAL 28, JC 30, JAL 36, JAL 37, JAL 39, JAL 41, JC 31, JC 32, JAL 31 | + | + |
| 27-28 | JM 2, JAL 30 | - | + |

Table 3. Antibiotic resistance of selected bacterial strains.

| No. | Isolate | O* | K** | A*** | R**** |
|-----|---------|----|-----|------|-------|
| 1-22| JM 4, JM 11, JC 9, JC 10, JC 17, JC 18, JC 24, JC 25, JC 28, JC 30, JC 31, JC 32, JC 33, JC 24, JC 31, JAL 12, JAL 27, JAL 30, JAL 31, JAL 36, JAL 39, JAL 41 | S | S | S | S | S | S |
| 23-24| JC 21, JC 23 | S | S | S | R | R | S | S |
| 25 | JAL 37 | R | S | S | S | S | S | S |
| 26 | JM 13 | R | R | R | R | R | R | R |

*Oxytetracycline, **Kanamycin, ***Ampicillin, ****Rifampicin

3.3. Survival ability in the fish intestine

The five best isolates were tested in the endurance in the fish intestine for 24 hours post-oral inoculation. We found two strains, JAL12 and JC18, were relatively stable to live in the fish intestines. All bacterial strains did not produce disease signs or mortality of fish.

Table 4. Survival of selected bacteria in the fish intestines.

| Isolate | Number of bacteria at hours post-inoculation (10⁴ CFU/mg intestine) |
|---------|---------------------------------------------------------------|
|         | 2 h                               | 24 h                     |
| JC18    | 6.6±0.22                          | 6.5±0.14                 |
| JC31    | 6.5±0.05                          | 0.0±0.00                 |
| JC32    | 6.5±0.12                          | 0.0±0.05                 |
| JAL12   | 7.1±0.27                          | 6.0±0.01                 |
| JAL41   | 7.0±0.01                          | 6.0±0.13                 |

3.4. Phenotypic and molecular identification of the JC18 strain

The result of molecular identification for JC18 based on the 16S rRNA gene found the high similarity to four different species of Aeromonas spp., namely A. veronii, A. sobria, A. hydrophyla, and A. salmonicida (Figure 1). Furthermore, the biochemical test showed that isolate closest to Aeromonas sobria (Table 5).
Figure 1. Phylogenetic tree of JC18 strain.

Table 5. Biochemical characteristic of JC18 strain.

| Characters       | JC18 | A. veronii[6] | A. sobria[6] | A. hydrophila[6] | A. salmonicida[6] |
|------------------|------|---------------|--------------|------------------|------------------|
| Gram             | -    | -             | -            | -                | -                |
| Cell shape       | rod  | rod           | rod          | rod              | rod              |
| oxidase          | +    | +             | +            | +                | +                |
| catalase         | +    | +             | +            | +                | +                |
| H₂S production   | -    | -             | -            | +                | -                |
| Motility         | +    | +             | +            | +                | -                |
| Ornithine        | -    | +             | -            | -                | -                |
| Decarboxylase    | -    | +             | -            | -                | -                |
| D-Glucose, gas   | -    | -             | -            | -                | -                |
| Glucose, acid    | +    | +             | +            | +                | +                |
| Sucrose, acid    | -    | -             | +            | +                | -                |
| Lactose, acid    | -    | -             | -            | +                | d                |

3.5. Discussion

Enzymes produced by permanent gut endosymbionts and probiotics in digestion are crucial to increase nutrient intake in fish, because the quantity and activity of endogenous enzymes in fish is not sufficient for complete metabolism of the ingested feed [7]. The results obtained was different for all isolates with the range of PHI between 1.075 – 1.8875 that can occur do to different factor. Peptidase types in the form of threonine- and metallopeptidases have a neutral pH optimum. Aspartic, glutamic and cysteine peptidases have an acidic pH optimum, and serine peptidases have a neutral to basic pH optimum [8]. In general enzyme activity in the digestive tract are highly depend on dietary supplements (carbohydrates, proteins and lipids), physiological factors (age, body temperature, gut pH, and structure of digestive tract), and other environmental conditions [9]. Further study is needed to perform the relationship and influential parameters for production and activity protease.

One of the main conditions of the strain that can be used as a probiotic agent is to have acid resistance to ensure that organisms will survive passage into the intestines [10]. Bacteria that have acid resistance have a mechanism to prevent damage due to acidic environment. Common mechanisms for acid
Antibiotic resistance in bacteria can be obtained intrinsically or via mutations in chromosomal genes and by horizontal gene transfer [12]. Bacteria isolation from capture fish in this study is intended to minimize the possibility of antibiotic resistance in isolates due to antibiotics exposure and horizontal gene transfer. Antibiotic resistance genes may be transferred by different mechanisms such as conjugation, transformation, or transduction. Although the isolation carried out is assumed to obtain isolates without antibiotic resistance genes, it is still found some antibiotic-resistant isolates. These results can occur as in the previous study that found antibiotic resistance microbe from a cave with no human activity [13]. Based on those assumptions it is suspected that the resistance properties possessed by some isolates are likely to occur naturally due to evolution.

Antagonism in bacteria is the ability of bacteria to inhibit or kill other bacteria. The antibacterial effect of bacteria is due to the following factors, either singly or in combination: the production of antibiotics, bacteriocins, siderophores, enzymes (lysozymes, proteases), hydrogen peroxide, and intestinal pH changes by the production of organic acids [2]. The inability of isolates to inhibit opportunistic bacteria can occur due to environmental influences. The state of the bacterial environment both in vitro and in vivo can influence the production inhibitors. This is supported by a study that suggests the composition, form, and oxygen might influence bacteriocin production or the number of bacteriocins released into the medium [14] [20]. In addition, the results still need to be reviewed to see the possibility of the antagonistic ability of isolates with other mechanisms (such as immunostimulatation, digestive enzyme production, and place or nutrition competition). Further in vivo study is necessary to confirm antagonism activity, because in some cases of positive results in in vitro is not able to show antagonism in vivo, as well as the opposite [15].

The ability of microorganisms to colonize is considered as one of the main criteria in the selection of potential probiotic candidates, this ability is related to attachment efficiency in intestinal epithelium to reduce or prevent pathogen colonization [16]. This test is intended to determine the comparison of the ability of intestinal resistance between isolates. The endurance ability in the digestive tract of JC18 isolate is possible because they have a higher replication power than other isolates, as stated that bacteria capable of colonizing have a higher multiplication rate than the level of release from the digestive tract [17]. The trend of decline in isolates can still be considered reasonable because the test period is relatively short; it is still inadequate for bacteria in colonizing. Probiotic bacteria can colonize the digestive tract when applied or given for a long time [18]. A previous study showed that Bacillus sp. can only dominate up to 50% of the total bacteria against Vibrio sp. colonized in the hepatopancreas of shrimp juvenile after 20 days of application on water [19].

Molecular and biochemical identification of JC18 in the present study found the identity to Aeromonas sobria with 99% of similarity. Aeromonas sobria is a mesophilic motile Gram-negative rod ubiquitous in freshwater aquatic environments but also has been isolated from the intestinal tract of apparently healthy fish such as perch and zebrafish. A. sobria, in general, was known as pathogenic bacteria, some studies report that A. sobria can act as the pathogen of perch, carp, and catfish [21] [22] [23]. On the other hand, some studies in rainbow trout have shown positive results of using A. sobria as a probiotic by control infections of pathogenic bacteria (Aeromonas salmonicida, Lactococcus garvieae, Streptococcus iniae, Vibrio anguillarum, Vibrio ordalii and Yersinia ruckeri), stimulation of immunity by the increased number of leucocytes, enhanced phagocytic and respiratory burst activity [24] [25].

Isolation of large amounts of proteolytic and cellulolytic bacteria from the intestine of milkfish in the present study indicated that the fish species is a potential source for bacterial isolation for a fish probiotic candidate. The non-pathogenic Aeromonas sobria JC18 with enzymatic activity and ability to stand with low pH, and survive in fish intestine revealed the potential application as fish probiotic. Hence, further study on the probiotic efficacy in fish needs to be conducted for the development of the Indonesian aquaculture industry.
4. Conclusion

Potential probiotic for fish, namely *Aeromonas sobria* JC18 with proteolytic and cellulolytic activities, was isolated from the intestine of milkfish (*Chanos chanos*) naturally grows in brackish water environment of Central Java, Indonesia.

Acknowledgment

This research was funded by Program Rekognisi Tugas Akhir Universitas Gadjah Mada (number: 3143/UN1.P.III/DIT-LIT/PT/2021) to Indah Istiqomah, Ph.D

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