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Screening of Indigenous Bacteria in Tilapia (*Oreocromis* sp) as a Probiotic Candidate.

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Abstract*: Tilapia (Oreochromis sp.) are ranks the seventh largest freshwater species production in the world. Tilapia feed have the ability to digest feed protein only 75% and protein efficiency ratio of 23,5%. The use of high-protein diet will increase the production cost, therefore it will result in lower production profit. To increase protein efficiency ratio and digestibility by using indigenous bacteria originated from fish intestine can be an alternative way. The purpose this study was to isolate the indigenous bacteria in Tilapia as a potential probiotic candidate. Bacterial screening was conducted trough several tests, i.e. pathogenicity, antagonism, characteristic of extracellular enzyme, growth characteristic and biochemical test. Pure culture of fourteen (14) isolates were isolated. All isolates were safe for fish as they have γ haemolytic effects on blood agar. Three isolates (NL 004, NL 007 and NL 013) were identified to have higher antagonism activities. Isolate NL 004 had the biggest capability for hydrolyzing protein, fat, and carbohydrate, (12,48 mm, 5,75 mm, 5,37 mm) respectively. Isolate NL 004 had the value best of growth rate (0,897 generation.hours^{-1}) and generation time (0,768 hours). Taking all the screening test resulted the best probiotic candidate NL 004 was a Bacillus sp.*

Key words : Bacillus sp, Feed efficiency, Isolation.

"1. Introduction"

Feed is the primary factor causing of growth increase. Commercial feed suck up 60-70% of production costs. Tilapia (*Oreochromis* sp.) require feed protein 28-40%, yet the tilapia only have protein efficiency ratio of 20-25% and the digestibility 75%. [1-3]. To improve of protein efficiency ratio and digestibility to do by increasing the number of bacterial digestion. The addition of probiotics indigen whit extraselluler digestive enzymes such as amylase, protease and lipase can be alternative way.

Probiotics are beneficial bacteria capable of producing various digestive enzymes can improve growth, feed efficiency, digestibility, increase resistance to bacterial pathogens and improve water quality [3-6]. Probiotic bacteria could be isolated from the intestines of fish, so can be an indicator found the bacteria are able adhesive to the cell wall of fish intestines. The purpose of this study is to isolate the tilapia indigenous bacteria as a potential candidate probiotic, which should increase the digestibility of the feed.

"3. Materials and Methods"

2.1 Tilapia (*Oreocromis* sp)

Tilapia used around with an average weight of 9±0.21 g. From the pond at the central of Aquaculture Punten Malang east Java. Then the fish is cut and taken away part of the intestine using sectio set.

3.2 Isolations

Tilapia taken part of gut after it grounded, then made up a 10^{-8} dilution, dilution planted on MRSA sterile media, as many as 1 ml pour plate, and incubated in a temperature of 35^0C for 48 hours. The number of colonies counted and do calsification (color, edges, surfaces and shape of colonies). To obtain pure cultures each colony is selected and then planted using ose with quadrant method, then incubation for 48 hours at a temperature of 35^0C [7].

1.3 Pathogenicity test

The pathogenicity test is to determinate characteristic of the isolated bacteria. The test was done using *sheep blood agar* media whit by planting or scraping. The test results are pathogenic if there clear zone around the colony dan contrary [8].

1.4 Antagonism *in vitro* test

Antagonism test performed to determine the ability of bacteria isolation to inhibits the growth of pathogenic bacteria (*Aeromonas hydropila* and *Edwardsiella tarda*). The test was done in a way planting pathogens MRSA media, and then paper disc placed in the middle of the media. After that

planting as many as 20 µL of bacterial isolation with a density of 10^8cfu.ml^{-1}. Positive results demonstrated the clear zone around the paper disc [6].

1.5 Extracellular enzyme test

The test performed to determine the the type of enzymes produced by candidate bacteria. The test was done by making modified test medium. In the test of enzyme with plate agar counte (PCA) whit much as 1% starch gr.V^{-1}, the test of lipase enzyme whit nutrient agar media (NA) add 1% gr.V^{-1} fish oil, and the test of protease enzyme whit backto agar (BA) media add 10% gr. V^{-1} casein. A positive result is shown on a clear zone around the paper disc [9].

1.6 Growth characteristics test

Isolation of bacteria taken as one ose then planted in sterile TSB media. Bacterial growth was observed using a spectrophotometer (Genesys 20) at 600 nm. Isolates was observed growth every hour on the first five hours, and after this whit two hous until for twenty nine hours. The results are compared with the pathogenic bacteria A. *hydropila* and E. *tarda* [10].

1.7 Identification of bacteria

The identification test is morphological and biochemical. The morphological test is Gram stain and the biochemistry test include TSIA, GAS, H_2S, catalase, oxidase test and OF. The test derivatives of carbohydrates such as glucose, lactose, sucrose, maltose, mannitol, dulcitol, inositol, arabinose, raffinosa, trehalose, and xylose. The biochemistry synthesis test includes Gelatin test, motility, Indol, Simons citrate, Ornithine Decarboxylase, phenylalanine. The test results then matched to book Cowan And Steel's Manual For The Identification Of Medical Bacteria 3nd Edition. (Cowan, 1985).

"3. Results and Discussion"

The number of colonies that grow as much as of 117 colonies in the MRSA media overall. The number of colonies selected by color, edges, surfaces and shape of colonies then obtained 14 isolates were named (NL NL 001-014). The results of isolation and then test pathogenitcity using sheep blood agar (Table 1). Pathogenicity test (test of haemolisins) is necessary to see if the bacteria harmful to fish or host, pathogenic bacteria will produce exotoksin called haemolicins that would damage blood cells making arise in the clear zone around the colony [11].

Table 1. Test of Pathogenicity and Antgonisme

Isolate	Pathogenicity test			Antagonism (mm)	
	α	β	γ	A. *hydrophila*	E. *tarda*
NL 001	Nd	Nd	+	Nd	Nd
NL 002	Nd	Nd	+	Nd	Nd
NL 003	Nd	Nd	+	Nd	Nd
NL 004	Nd	Nd	+	15.75	16,98
NL 005	Nd	Nd	+	Nd	Nd
NL 006	Nd	Nd	+	Nd	Nd
NL 007	Nd	Nd	+	14,52	16,58
NL 008	Nd	Nd	+	Nd	Nd
NL 009	Nd	Nd	+	5,16	5,93
NL 010	Nd	Nd	+	Nd	Nd
NL 011	Nd	Nd	+	Nd	Nd
NL 012	Nd	Nd	+	Nd	Nd
NL 013	Nd	Nd	+	9,43	5,92
NL 014	Nd	Nd	+	Nd	Nd

Information : Nd = No detected
+ = Detected

The results test of antagonism bacterial candidates with pathogenic bacteria A. *hydropila* and E. *tarda* **performed** (Table 1). Of the fourteen isolates, only four isolates have inhibitory effects. Only three highest isolate which is then used for next screening step. Antagonism test is necessary in to determine the ability of bacteria inhibit the growth of pathogenic bacteria with the ability to better grow [12]. The ability of antagonism is the natural activity that bacteria to secrete bacteriocins, lysozyme, and H_2O_2 [8].

Bacteriocins are proteins synthesized by ribosomes that are antagonistic to the orgenisme are on still family. Lysozyme damage the cell walls of bacteria by breaking the connection glycosides on the membrane peptidoglycan [13]. Produces enzymes myeloperoksida very important to used utilize oxygen free radicals to produce hypochlorous to kill pathogens [13]. Power of substance antagonist occurs with microbial agents that undermine and inhibit growth. Phagocytosis activity responded by first activating imflamantori response before the production of antibodies, and mediated by fagosistosis cells such as neutrophils, monocytes, and magrofag [14].

Characterization of the ability of bacteria to produce enzymes exogenous on selective media (Table 2). The three isolates obtained that able to produce proteolytic enzymes, amylolytic and

lipolytic. The ability to produce the enzyme ektraselluler will improve feed efficiency ratio and digestibility [6].

Table 2. The exracellular enzyme test isolates probiotic candidates.

Isolate	Diameter of clear zone (mm)		
	Protease	Lipase	Amilase
NL 004	12,48±0,67	5,75±0,146	5,37±0,026
NL 007	11,08±0,04	4,303±0,05	5,27±0,020
NL 013	11,53±0,06	3,78±0,166	4,35±0,045

☒Digestive tract (gut) is rich in nutrients that is a favorite of some bacteria to grow, the abundance of intestinal bacteria will increase the production of digestive enzymes. Groups of bacteria such as *Bacillus* secretly issued a hydrolysis enzyme to degrade many substrates and to grow on a wide scale source of nutrients. Extracellular enzymes can increase the amount of protein of 20-25 $g.L^{-1}$ [15]. *Bacillus* will produce hydrolysis enzyme ektraselular (polysaccharide) [17].

Table 3. Characterization of bacterial growth for 29 hours.

Isolate	Karakter Uji		
	Specific growth rate (Generation.hour^{1})	lag Fase (h)	Generation time (h)
NL 004	0,897	3	0,768
NL 007	0,5337	1	1,317
NL 013	0,629	1	1,09
A. *hydrophila*	0,252	1	2,731
E. *tarda*	0,323	1	2,13

Characterization of the best growth rate was NL 004 0,897 Genrations.hours^{-1}. Diffent of growth characterization affected the ability to utiliza nutrients (carbon), under culture conditions. Temperatute optimum of bacteria growth at 30 ^{0}C, whit a pH of 6-7 [18]. Stability of temperature will can be affect the activity sensitively of enzymes and growth [19]. Bacteria will be dormant because of environmental stress such as low temperature, nutrients disappear suddenly, and osmotic pressure [20]. Intestinal bacteria more resistant to low pH. The existence of sugar in the culture medium significantly affect (Desai 2008; Zhou *et al.,* 2013).

Morphology identification (Table 4) whit gram staining and then observed under a microscope with a magnification of 1000X. the fuction of gram staining to determine type of bacteria by gram. The ability of baterial cells to absorb colors is different gram-positive absorbing of purple color, gram-negative while absorbing red. This reaction is caused by differences of cell wall.

In gram-negative contained lipid. whereas in gram-positive there its'n are so crystal violet can be through the cell wall and tied so purple, besides gram-positive have a layered peptidoglycan [21, 22]. Biochemical test results showed a positive reaction to the test Voges-Proskauer (VP), catalase, starch, citrate, nitrate, glucose, fructose, maltose, sucrose, ribose and trehalose [6]. Catalase test is used to determine the ability and enzymes produced by bacteria. TSIA test to determine the ability of a bacterium to ferment sugars to produce acids or gas [23]. The results were analyzed by using the book identification 3ndEdition Cowan and Steel (1985).

☒Bacteria NL 004 unidentified gram + such findings in accordance with identification keys Cowan (1985), gram + bacteria with endospores, to shape of long rods (bacil) able to decipher H_2O_3 3%, motile, reactive Aesculin, Simmons' citrate +, Glycerol +, are Aerobic and facultative anaerobic is the genus Bacillus, and Clostridium. Whereas if it is round (cocci) belong to the genus Micrococcus and Staphylococu [24]. The characteristics of the genus Bacillus are gram +, catalase +, fermentatif. *Bacillus* in action with flagella peritrichous, capable of hydrolyzing starch, gelatin, casein, to reduce nitrate and the V/P reaction is + [25,26]. From the key identification has been isolate NL 004 included in the types of bacteria *Bacillus*. Bacillus has been long used as a probiotic efficient because it can produce extracellular enzymes and anti-microbial components [6]. Some studies explain that Bacillus sp can be used as probiotics because it is more resistant to changes in the environment, can be combined with other beneficial bacteria and can be used as a biocontrol microbial pathogens in fish [13].

Table 4. Identifiation test result isolat NL 004

Num	Types of test	Isolate
		NL 004
1	Gram identification	
	Color colonies	Whit milk
	Edge of colonies	undulate
	Type of Gram	+
	Bacteria shape	Bacil
2	Biochemistry test	
	TSI Agar	A/A
	Gas	-
	H_2S (Sulfur acid)	-
	Catalase	+
	Oksidase	+
	O/F	Fermentatif

3	Fermentation of carbohydrate	
	Glucose	+
	Lactose	+
	Sucrose	+
	Maltose	+
	Mannitol	+
	Dulcitol	-
	Salicin	+
	Inositol	-
	Sorbitol	+
	Arabinose	+
	Raffinose	-
	Trehalose	+
	Xylose	+
4	Synthetic Biochemistry test	
	Gelatin	-
	Motility	+
	Indol	-
	Simmons Citrate	-
	Malonate	-
	Christensen's Urease	+
	Methil Red (MR)	+
	Voges Proskauer (VP)	+
	Arginin Dihidrolase	+
	Lysine Decarboxylase	-
	Ornithin Decarboxylase	-
	Phenylalanin Deaminase	-
	Aesculin Hydrolisis	+
5	Result of analisis	*Bacillus* sp

Information: - (no reaction) ,+ (reaction), A/A (Acid/Acid).

The suplementation probiotic of *Bacillus* for 8 weeks can improve tolal leukocytes, phagocytosis activity, lysozyme activity, and serum super oxide dismutase the function to cut and inhibit xantatine and xantantin oxidase [27]. Bacillus increasing the immune response and increase resistance to vibrio, increases resistance to stress, improve survival and growth [3,4,28].

"4. Conclusion"

Based on the screening test pathogenicity, antagonism, ektraselluler enzymes and growth characteristic was found that the best candidates are NL 004 and in morphology and biochemistry test results are the type of *Bacillus* sp.

"5. Aknowledgements"

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