PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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Pakistan Journal of Nutrition 13 (12): 710-715, 2014
ISSN 1680-5194

Bacillus subtilis Strain VITNJ1 Potential Probiotic Bacteria in the Gut of Tilapia (*Oreochromis niloticus*) are Cultured in Floating Net, Maninjau Lake, West Sumatra

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Abstract: Tilapia (*Oreochromis niloticus*) is one kind of fish are cultured in floating net (KJA) Maninjau lake, West Sumatra. This study was aimed to know about proteolytic bacteria found in the gut of fish Nila can be used as candidate probiotics. Materials used in this study were derived from Nila fish farmers in the vicinity of Maninjau lake. The research method used is the isolation, identification and characterization of bacteria. Identification was conducted on the morphological and biochemical characteristics of bacteria. It was found that 55 isolates of bacteria that are morphological and biochemical grouped into three namely genus are *Bacillus*, *Achromobacter* and *Enterobacter*. Based on the production of extracellular proteolytic enzymes known that isolates B.3.2 had the highest proteolitic index. Based on characteristics examination and PCR analysis the isolate B.3.2 was primarily identified as *Bacillus subtilis* strain VITNJ1 bacteria can be used as a candidate probiotic.

Key words: *Bacillus subtilis*, probiotic, bacteria, tilapia, west sumatra

INTRODUCTION

Maninjau is one of public waters located in the district of Tanjung Raya, Agam regency, West Sumatra. Fishing activities that take place consist of fish culture in floating net and capture fisheries. Syandri (2013) fish farming activities in floating net start since 1992 with a total of 12 units of floating net, in 1997 increased to 2854 units, up to now has grown to 13,000 units.

Intensive aquaculture operations, if not properly monitored, can result in excessive phosphorus loading, which can negatively impact the water body. Quantitative estimations of nutrients in relation to ecosystem changes are therefore essential to ensure that environmental conditions and fisheries remain sustainable in lakes (Guo and Li, 2003).

Similarly, the residual waste feed and fish feces that accumulate in the bottom waters of the lake. The environmental impact of waste (fish faeces, uneaten food, bacterial biomass) from the fish culture industry, notably from cage fish farms, is an increasing issue of concern around the world. The intensive fish culture in cages can lead to the eutrophication of water bodies and to the emergence of deleterious effects on the water quality, that are harmful for fish and humans. There are several works on the effect of fish culture in cages on water quality (Ntengwe and Edema, 2008; Azevedo *et al*., 2011). Tangko *et al*. (2007), one effort that can be done to anticipate these problems is to add probiotics in fish feed. The basic principle of the work is to utilize the ability of probiotic microbes to facilitate absorption by the digestive tract of fish (Feliatra and Suryadi, 2004).

Probiotics are products which improve intestinal microflora and support good health for host. In general, probiotics protect against infections, alleviate lactose intolerance, reduce blood cholesterol levels, improve weight gain and feed conversion ratio and also stimulate the immune system (Salminen *et al*., 2004; Agrawal, 2005). Other benefits of probiotic in aquaculture are competitor for nutrient, source of nutrients and enzymatic contribution to digestion and improve water quality (Abraham *et al*., 2008). The present experiment aimed to isolate and identify bacteria as new probiotic from intestine of Tilapia (*Oreochromis niloticus*) are culture in floating net.

MATERIALS AND METHODS

Materials: Materials used in this study is the fish Tilapia (*Oreochromis niloticus)* were obtained from fish farmers located around Maninjau lake and was transferred to the Microbiology laboratory, Faculty of Fisheries and Marine Sciences Bung Hatta University.

Culture medium: The culture medium in this research: GTA (*glucose trypton agar*)+$CaCO_3$, TSA (*trypticase soy agar*), trypton broth, sulfite agar, nitrate broth, TSIA (*triple sugar iron agar*), *Baird Parker Agar* (BPA), *brain heart infusion* (BHI), *Simmons citrate* and SMA (*skim milk agar*).

Isolation of bacteria: Isolation of bacteria from the digestive tract done by using pour plate on media *glucose trypton agar* (GTA)+$CaCO_3$ medium. One gram

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of the gut Nila homogenates suspended in 9 mL of sterile distilled water and then made up to a dilution series 10^8. Each dilution series grown on nutrient agar medium and then incubated for 48 h at a temperature 37°C (Djide *et al*., 2008).

Identification of bacteria: The identified of the isolates were determined by the standard procedure of gram staining, catalase test, motility and spore former test (Hadioetomo, 1985; Fardiaz, 1989; Lay, 1994). Characterization bacterial strains determination the using standard methods "Manual for the identification of medical bacteria" (Cowan and Steel's, 1975).

Production of proteolytic enzyme activity assay: This test is done with the procedure Bairagi *et al.* (2002). Isolates obtained from the isolation inoculated by means of streak on an agar medium enriched with skim milk (4%), incubation at 37°C for 24 h. Proteolytic enzyme production activity is shown by the formation of clear zone.

Molecular identification: The Genomic DNA was extracted from pure culture using genomic DNA extraction kit following instructions of the Pitcher *et al.* (1989, modified) and White *et al.* (1989). The 1.5 kb 16S rDNA gene were amplified by PCR using a pair of universal bacterial 16S rDNA gene primers 27 F: 5-AGA GTT TGA TCC TGG CTC AG-3 and primers 1492 R: 5-GGT TAC CTT GTT ACG ACT T-3. The PCR was carried out according to (Hiraishi *et al*., 1995). To amplified by PCR using specific primers 27 F: 5- AGA GTT TGA TCC TGG CTC AG-3 and 520 R: 5-GTA TTA CCG CGG CTG CTG-3' from genomic DNA (200 ng) on Ready-To-GO PCR Beads (Pharmacia-Biotech, Uppsala, Sweden). Phenol-chloroform-isoamyl alcohol (25:24:1) treatment, ethanol precipitation and agarose gel electrophoresis were used to purify the genomic DNA. Total volume of the PCR reaction (25 uL) consisted of 1.5 U Tag DNA Polymerase, lOmM Tris-HCl (pH 9 at room temperature), 50 mM KC1, 1.5 mM $MgCl_2$, 200 uM of each dNTPs and stabilizer including BSA. The reaction was incubated in a Gene Amp PCR System 2400 Thermocycler (Perkin-Elmer Cetus, Norwalk, Conn). The sequencing reactions were done by using the Big Dye Ready Reaction Dye Deoxy Terminator kit, purified by ethanol-sodium acetate precipitation. The reactions were run on an ABI PRISM 3130 Genetic Analyzer (PE Applied Biosystems, Foster City, CA.). The raw data results in the subsequent sequencing and assembling and trimming using Bioedit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequence data that has been further in the BLAST assembling the genomic data that has been registered in DDBJ/DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp/) or by NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS AND DISCUSSION

Isolation of bacteria: The isolation of bacteria from of the gut Tilapia (*Oreochromis niloticus*) was performed GTA+$CaCO_3$ medium through multilevel dilution method of 10^{-1} until 10^{-8} to reduce the number of microbial populations present in the media. Diluting solution used in this study are sterile distilled water. Isolates that have clear zone is thought to be acid bacteria. A total of 55 isolates suspected acid-producing bacteria based on the clear zone around the bacterial colonies grown on medium GTA+$CaCO_3$. Furthermore, isolates were grown to medium GTA repeated up to 3 times in order to obtain a single cell. Sightings colony bacterial isolates isolated from the gut of tilapia (*Oreochromis niloticus*) fish (Fig. 1).

Research on the isolation bacteria from the gut of tilapia was also performed by Gangasuresh and Bharathi (2014) from tilapia fish are healthy and the sick, Flores *et al.* (2013); Zapata (2013); Thillaimaharani *et al.* (2012) and Perdana (2011). After purification of bacterial isolates and followed by morphological and biochemical observations, then obtained several isolates of probiotic bacteria found in the gut of tilapia. Identification of isolates refer to Holt *et al.* (1994) as shown in Table 2.

Bacterial isolates of group A and B (genus *Bacillus*): Bacteria are approaching this genus has morphological characteristics as follows: colony color milky white or slightly creamy, colony shape with rounded edges wrinkles. Cells are rod shape and straight, measuring 0.5-2.5 x1.2-10 µm and often arranged in the form of a pair or chain, with round or rectangular tip. Gram+cell staining, motile, catalase and oxidase positive, methyl red negative, the optimum temperature 30-37°C and grows well on NaCl 1-3%. According to Holt *et al.* (1994), *Bacillus* sp. Gram+and usually motile by peritrichous flagella. Endospores oval, sometimes round or cylindrical and highly resistant to unfavorable conditions. They are not more than one spore per cell and sporulation can not stand on the open air. Bakteri this is aerobic or facultative anaerobic. Diverse physiological capabilities, very sensitive to heat, pH and salinity; chemoorganotroph with fermentation metabolism or respiration. Usually catalase and oxidase positive. Is widespread in a variety of habitats; few species are pathogenic to vertebrates or invertebrates. The same type of bacteria found by Musefiu *et al.* (2011) the isolation and identification of flora study of aerobic bacteria found in the digestive tract surface and catfish and tilapia fish in Ibadan, North Nigeria. The same study was also conducted by Thillaimaharani *et al.* (2012) yang examine the intestinal bacterial flora of tilapia (*Oreochromis mosambicus*, Peter, 1852) fish and found the bacteria *Virgibacillus pantothenticus*, *Bacillus cereus*, *Bacillus licheniformis*, *Enterococcus faecalis* and *Virgibacillus alginolyticus*.

Table 1: Characteristics of bacterial colonies isolated from gut tilapia based biochemical tests

	Bacterial Isolates group			
Characteristics	A	B	C	D
Gram	+	+	-	-
Shape	bacil	bacil	bacil	bacil
Endospores	+	+	-	-
Motility	+	+	+	-
Oxidase	-	-	-	-
Aerob/anaerob	A	A	A	A
Indol	-	-	-	-
Reduction of nitrate	+	+	-	-
TSIA	M/K	K/K	K/K	M/M
Glucose	-	-	+	+
Lactose	-	-	+	-
Sucrose	+	-	+	-
Gas	-	-	+	-
Citrat	-	-	+	-
Blood agar	+	+	-	-
Pigmentation	Grey	Grey	Grey	Grey
Hemolysis	-	-	-	-
Urea	-	-	+	-
Mannitol	-	-	+	-
MR	+	+	+	-
VP	+	-	-	+
OF	-	-	+	-
Gelatine	+	+	-	-
KCN			+	-
Arginine			+	-
Lysine			+	-
Malonat broth			-	-

Table 2: Proteolytic index value of 8 selected isolate

Isolate code	Proteolytic index (IP)
B2.2	23.0
B3.2	26.0
C2	26.0
C10	16.5
M3	13.0
M10	4.6
S1	8.0
S3	11.5

Bacterial isolates of group C (genus *Achromobacter*): Bacteria are approaching this genus has morphological characteristics as follows: rod-shaped member of Enterobacteriaceae and usually 1-5 µm long. As with other bacteria, Gram-negative enterobacteria have and they are facultative anaerobes, fermenting sugars to produce lactic acid and various other end products. Most also reduce nitrate to nitrite, although there are some who do not like most bacteria, Achromobacter generally less cytochrome C oxidase, although there are exceptions (eg *Plesiomonas shigelloides*). Most flagella used to move, but few are nonmotile genera. They do not form spores. There is a positive catalase reaction varies, but sometimes there are also negative, not produce gas, H_2S reaction is negative, negative oxidase test, negative indole test, negative urea test, citrate test was also negative, test against KCN medium negative, arginine test sometimes there are positive and negative lysine test. Many members of this family are the normal part of the intestinal flora found in the intestines of humans and other animals, while others are found in water or soil, or parasites on a variety of different animals and plants. According to Moeljanto (1992) the types of bacteria that are usually found in fresh fish is usually included in the class *Achromobacter*, *Flavobacte-rium*, *Pseudomonas* and *Clostridium*.

Lewis (1973) and Austin (2002) stated that the bacteria *Achromobacter* and *Enterobacter* is a bacterium commonly found in fish and shrimp in addition to *Acinetobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Serratia*, *Aeromonas*, *Alcaligenes*, *Eikenella*, *Bacteroides*, *Citrobacter freundii*, *Hafnia alvei*, *Cyt-phaga/Flexibacter*, *Bacillus*, *Listeria*, *Propionibacterium*, *Staphylococcus*, *Moraxella*, dan *Pseudomonas*.

Bacterial isolates of group D (genus *Enterobacter*): *Enterobacter* is a gram-negative bacillus shaped, with size 0.6-1.0 µm x 1.2-3.0 µm, motile, does not form spores, encapsulated, aerobic, producing gas and have flagellal. These bacteria are often found together *Escherichia coli* live freely in nature such as water, soil and also in the digestive tract of humans and animals. Oxidase negative, catalase positive and sometimes there are negative, motile, citrate test positive, indole negative, urease test positive, citrate positive, test sugar (lactose, glucose and sucrose) positive, mannitol test against positive, negative VP test and test positive OF.

The nature of growth of Enterobacter that can grow well in almost all artificial media in the microbiology laboratory. Colony shape *Enterobacter* (*Aerobacter aerogenes*) large, white-red, turbid, convex, rounded and smooth. In addition *A. aerogenes* also break down carbohydrates such as glucose and lactose to acid and gas as case *Escherichia coli*. *Enterobacter aerogenes* can live as a saprobe in the digestive tract of animals and humans. *Enterobacter aerogenes* is one type of coliform bacteria, which is a group of bacteria used as an indicator of sanitary conditions are not good for food and beverages.

These bacteria are also found in the study Uddin *et al.* (2012) which examined the bacteria found in the digestive tract of fish Mas (*Cyprinus carpio*) that in the freeze, Trakroo and Agarwal (2011) the isolation of bacteria from fish Rohu (*Labeo rohita*) at India.

Detection of extracellular proteolytic enzyme production: The ability to produce extracellular proteolytic enzymes detected using the test medium is a medium that is enriched with enzyme substrate (*skim milk*). The detection is based on the formation of hydrolysis zone around the colonies bacteria tested. The test results showed the production of extracellular proteolytic enzymes that all test isolates produce extracellular proteolytic enzymes.

This study focused on one of them on the types of proteolytic enzymes as a major component of fish feed is protein. Based on the results obtained by all of the

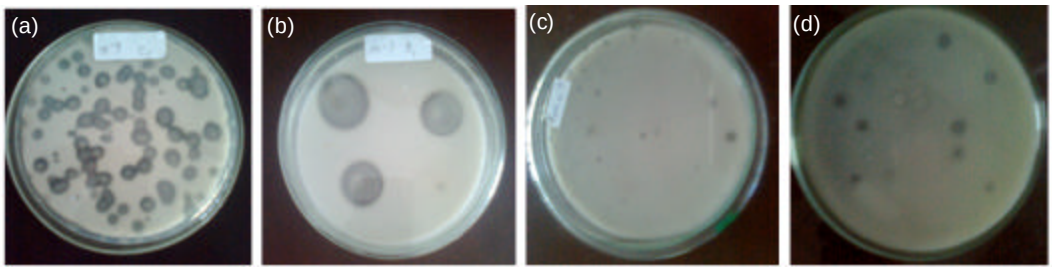

Fig. 1(a-d): Shape of intestinal bacteria colony morphology Tilapia (*Oreochromis niloticus*) based on the type of feed

isolates have the ability to produce extracellular proteolytic enzymes. The ability of probiotic bacteria to produce extracellular proteolytic enzymes have an important role in participating digest compounds that are protein. 8 selected isolates that have the highest value of the index isolates the code B2.2, B3.2, C2, C10, M3, M10, S1 and S3 (Table 2). The highest proteolytic indices are selected isolates B3.2 with proteolitic index value (IP) amount to 26 (Fig. 2). If associated with the identification based on morphological and biochemical properties of the bacteria, it is known that the selected bacterial isolates that have the highest index of proteolytic isolates B3.2 is a bacterium *Bacillus subtilis*. Geovanny and Shen (2008) showed a marked improvement in the activity of proteolytic enzymes in shrimp fed probiotic treatment compared to the control. The same study on the effect of probiotics on digestive enzyme activity was also carried out by Ziaei-Nejad *et al.*(2006); Zhou *et al*. (2009) and Musikasang *et al*. (2009) establish the ability to digest protein as one of the criteria for the selection of probiotic. The presence of these proteolytic enzymes will further increase the number of compounds that are the digestible protein that decreases the amount of waste that contains nitrogen originating from the digestive process. This is advantageous because it will reduce the amount of ammonia derived from organic N mineralization process that is expected to solve the problem of mass mortality of fish that often occur in Maninjau. Research on proteolytic enzyme activity test contained in the digestive tract of fish as a candidate probiotic was also performed by Subagiyo and Djunaedi (2011) find all of the isolates have the ability to produce proteolytic enzymes protease. Results of the study Mubarik (2001) showed that isolates NU-2 a proteolytic bacteria with proteolytic index value (IP) amounted to 1.89 and has the potential to be used as probiotics because it can produce both protease, α-amylase and glucoamylase extracellular. Geethanjali and Subash (2011) found the *Bacillus subtilis* bacteria isolated from the gastrointestinal freshwater gut labeo rohita fish originating from India, has the highest proteolytic activity and canbe used as probiotic candidates.

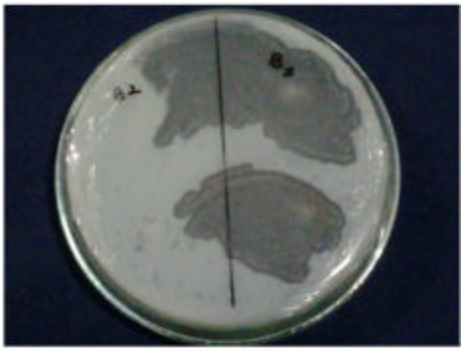

Fig. 2: Proteolytic activity of bacterial isolates selected B3.2 on *skim milk* agar (SMA) medium

Molecular identification: Methods for the detection and identification of *B. subtilis* are e.g., serotyping, pyrolytic gas chromatography, pyrolytic mass spectrometry, ribotyping, phage typing, plasmid profiles, electrophoresis in pulse electric field and polymerase chain reaction (PCR) using genera-specific and species-specific primers. Isolate B3.2 was DNA extraction for GES methods (Pitcher *et al*., 1989; Modified), amplification PCR of its 16S rDNA with specific primers 27 F: 5-AGA GTT TGA TCC TGG CTC AG-3 and primers 1492 R: 5-GGT TAC CTT GTT ACG ACT T-3 (White *et al*., 1990), purification PCR product by PEG precipitation methods (Hiraishi *et al*., 1995) and ethanol purification methods. Result of purification to analysis against by automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer, Applied Biosystems), thus for trimming and assembling by Bioedit program at BLAST program at the NCBI database, showed that the new isolate was taxonomically very close to *Bacillus subtilis* strain VITNJ1 (Fig. 3). The same study was also conducted by Al-Faragi and Alsaphar (2012) isolated *Bacillus subtilis* bacteria from intestinal of common carp (*Cyprinus carpio* L.) and might be applied as good probiotic. Ibrahim *et al*. (2014) examine the intestinal bacterial flora of Clarias gariepinus in Minna metropolis, Niger State and found the bacteria, *Bacillus subtilis* from fresh and dried fish.

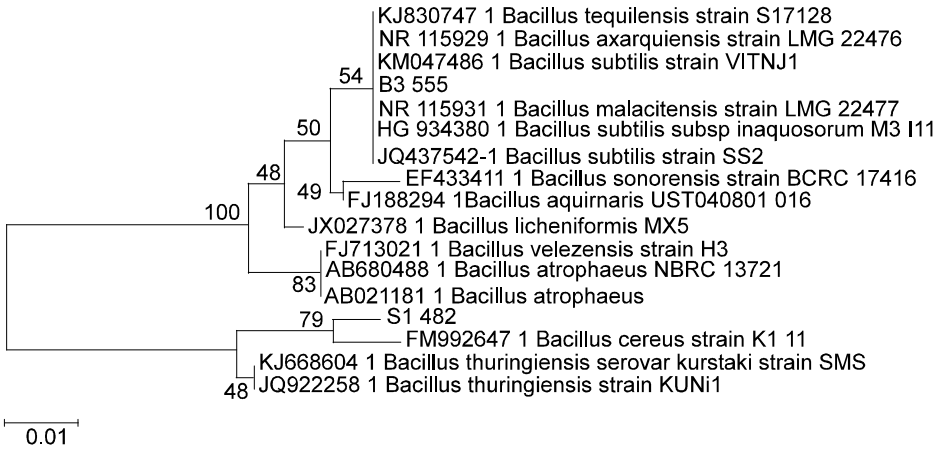

Fig. 3: The phylogenetic tree based on 16S rDNA gene sequences of bacterial isolates B.3.2

Conclusion: Based on the selection result obtained 55 isolates of bacteria from the digestive tract of tilapia (*Oreochromis niloticus*) fish that the morphological and biochemical grouped into three namely genus Bacillus, Achromobacter and Enterobacter. Based on the production of extracellular proteolytic enzymes known that isolate bacteria B3.2, have highest proteolitic index value (IP) is *Bacillus subtilis* strain VITNJ1 can be used as probiotic candidates.

ACKNOWLEDGEMENTS

This research was funded by the Directorate General of Higher Education, Ministry of Education Republic Indonesia, in accordance with the Agreement on Implementation of Fundamental Research 2014.

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