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Screening of Amylolytic Bacteria as Candidates of Probiotics in Tilapia (*Oreochromis* sp.)

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ABSTRACT

The present study was aimed to obtain amylolytic bacteria probiotic to improve feed digestibility and growth performance in tilapia. All of the bacteria were isolated from the digestive tract of tilapia. The probiotic bacteria candidates were screened for amylolytic activity, bacterial growth rate, the resistance to acid and alkali, antagonistic activity, attachment and pathogenicity in tilapia. The selected isolates were then tested *in vivo* to observe their ability to improve feed digestibility and growth performance in tilapia. Two treatments with four replicates consisted of the control (without any probiotic) (A) and the probiotic (B) at a dose of 1% v/w feed were administered every day for 60 days. There were 41 isolates which secreted amylase and the five isolates with the largest diameter of the clear zone were NP1, NP2, NP3, NP4 and NP5. Isolate NP5 demonstrated the best results: It had amylolytic activity with a clear zone diameter of 20.3 cm, it grew rapidly with a generation time of 33.59 min, it had antagonistic properties against the pathogenic bacteria *Aeromonas hydrophila* and *Streptococcus* sp., with the inhibition zones of 12.0 and 11.0 cm, respectively. It had the ability to adhere to stainless steel plates and it was not pathogenic to tilapia. The feeding trial in tilapia showed that the probiotic treatment resulted in better feed digestibility and growth performance than those of the control.

Key words: Probiotics, amylolytic, *Oreochromis* sp.

INTRODUCTION

Tilapia (*Oreochromis* sp.) is a fish with high economic value. The intensive cultivation of tilapia faces several problems, including the relatively expensive feed price which is not followed by the increase in the selling price of the product. The ingredient which is the main source of protein is fishmeal but its availability has become more limited, thus increasing its price in the market (Lunger *et al*., 2007).

In fish metabolism, protein is a more effective source of energy than carbohydrate. The digestibility of carbohydrate in fish is relatively low because of availability and activity of amylase enzymes in their digestive systems is lower compared to those of terrestrial animals and humans. Therefore, there needs to be some efforts to increase the activity of amylase enzymes so that the utilization of protein as a source of energy could be reduced and it could be partially replaced by carbohydrate. The effort that could be done to increase the availability of digestive enzymes, especially amylase enzymes, is through the application of probiotic bacteria which produce amylase enzymes, also known as amylolytic probiotics. According to Verschuere *et al*. (2000), probiotics are

live microbes which give beneficial effects to the host by modifying the microbial community or by associating with the host, assuring that there is an improvement in feed utilization or in nutrition, improvement in the host response to disease, or improvement in the quality of the aquatic environment. In the case of the improvement of the nutritional value of feed, probiotics are able to produce several exogenous enzymes to digest feed, for instance amylase, protease, lipase and cellulase (Sahu *et al*., 2008; Wang *et al*., 2008). These exogenous enzymes would assist the host endogenous enzymes in hydrolyzing feed nutrients, for example by breaking down long-chain carbohydrates, proteins and fats in the feed. The deconstruction of these complex molecules into simpler molecules would ease digestion and absorption within the fish digestive tract. This study aimed to screen probiotic bacteria which can produce amylolytic enzymes to improve feed digestibility and the growth performance of tilapia.

MATERIALS AND METHODS

Isolation of probiotic bacteria candidates: Probiotic bacteria candidates were isolated from the intestines of 10 tilapia samples. The intestines were ground aseptically and each gram was diluted with 9 mL of physiological solution (NaCl 0.85%). An amount of 0.5 mL of inoculum source was inoculated to 10 mL of Trypticase Soy Broth (TSB) medium which was enriched with 2% starch. The culture was incubated at 29°C for 24 h. The bacterial growth was shown by the turbidity of medium. Serial dilution was done from 10^{-2} to 10^{-10} and each dilution series was spread onto a Trypticase Soy Agar (TSA) +2% starch medium. Bacterial colonies which produced amylase enzymes showed a clear zone around the bacterial colony when the medium was immersed in Potassium Iodide (KI) 1% solution. This purification method was repeated several times using the same technique and medium type until singular and uniform bacterial colonies were obtained.

Selection of probiotic bacteria candidates

Amylolytic property test and isolate characterization: The aim of this test was to measure the amylolytic property of each of the isolates. The diameter of the clear zone formed on the TSA +2% starch medium were measured and five isolates with the largest zones were selected to be used in the next step of the testing. The bacterial isolates which selected were characterized based on colony morphology, cell structure and gram staining. The physiological and biochemical characteristics were also observed consisting of oxidative/fermentative and glucose using O/F medium +1% glucose, motility and H_2S production using SIM (Sulfide Indole Motility) medium, catalase using H_2O_2 and oxidase properties using p-aminodimethy-oxalate. The data obtained were analyzed descriptively based on the methods by Cowan (1974).

Bacterial growth: Observations of bacterial growth were done to determine the bacterial generation time and the time when the exponential phase is reached. The measurement of growth phases was done by calculating the bacterial population every 2 h using the plate-count method resulting in the number of Colony Forming Units per mL (CFU mL^{-1}) (Madigan *et al*., 2003). The measurement of generation time was done to know the time that required for bacterium to divide into 2 new cells, in which it was calculated using the following equation (Fardiaz, 1992):

$$K = \frac{\text{Log } x_i - \log x_o}{0.301.t}$$

Where:

K = Growth rate constant (number of generation/time)
Xo = Number of cells at the beginning (CFU mL^{-1})
Xi = Number of cells after a certain duration of time (CFU mL^{-1})
t = Time x_i-x_o
1/k = Generation time

Resistance to acid and alkali: The resistance to acid and alkali of isolate was used to measure their ability to survive in the stomach which has a low pH and in the proximal part of the intestines which contains with bile salts. The test was done using the method explained by Ngatirah *et al.* (2000), i.e., inoculating 0.1 mL of bacterial isolate in a series of tubes containing 9 mL TSB medium at a pH of 2.5 (the pH was obtained by adding HCl) and a pH of 7.5 (the pH was obtained by adding NaOH). These test tubes were then incubated at 29°C. Observations were done at 2, 4, 6 and 8 h after inoculation and the numbers of bacteria were counted using the plate-count method (CFU mL^{-1}) (Madigan *et al.*, 2003).

Antagonistic activity test: The test for antagonistic activity was done using Kirby-Bauer method (Madigan *et al.*, 2003) using pathogenic bacteria *A. hydrophila* and *Streptococcus* sp. The pathogenic bacteria were diluted to a concentration of 10^6 CFU mL^{-1} and then 0.1 mL was spread on a TSA medium. A paper disc was put on the medium then 0.05 mL of the probiotic bacteria candidate (Np 1, Np 2, Np 3, Np 4 and Np 5) suspension at a concentration of 10^6 CFU mL^{-1} and PBS (Phosphate Buffer Saline) for control was dripped onto it. The culture was incubated at 29°C for 24 h. The antibacterial activity was obtained by measuring the inhibition zone diameter (mm).

Adhesion test: The adhesion test was done using a stainless steel plate, referring to Dewanti and Wong (1995), a sterile stainless steel plate was placed in an Erlenmeyer flask containing 25 mL of TSB medium and was inoculated with 1 mL of bacterial culture then incubated at 29°C for 24 h. After that, the stainless steel plate was swabbed thoroughly. The swab was then placed in a tube containing 10 mL of physiological solution and vortexed for 1 min. Then the number of bacteria was counted using the plate-count method (CFU cm^{-2}). Meanwhile, the number of bacteria growing in the liquid phase (planktonic) was counted by taking 1 mL of the growth medium and diluting it in 9 mL of physiological solution. The bacteria were then cultured and the number counted using the plate-count method (CFU mL^{-1}).

Pathogenicity test: The test was done by injecting 0.1 mL the probiotic bacteria candidate at a concentration of 10^6 CFU mL^{-1} into tilapia intramuscularly. The positive control tilapias were injected with *A. hydrophila,* whereas the negative controls were injected with a physiological solution. Tilapia with an average weight of 15.5±0.09 g were reared in 60×50×40 cm^3 aquarium at a density of 10 individuals per aquarium. The fish were reared there for 10 days and the fish mortality was observed daily.

Test of probiotic bacteria candidates through feeding trial on tilapia: The test-feed used in this study was low-protein (23%) pellets. The testing was done with two treatments, repeated 4 times, treatment A: Feed without any probiotic added (control) and treatment B: Feed with 1% probiotic added (Wang, 2007).

Probiotic was added to the feed by spraying it thoroughly using a syringe with an addition of 2% egg yolk (Wang, 2007). The feed was given three times a day by *ad satiation*. In order to maintain water quality, 30% of the water capacity in the aquarium was replaced every day.

Tilapia used in this study were monosexual males with average weight were 3.53±0.05 g and were reared at a density of 15 individuals per aquarium. There were 8 aquariums used sized 60×50×40 cm^3 which were placed randomly. The fish were acclimatized for 5 days were not fed for 24 h prior to the administration of the test feed. The fish were reared for 60 days for the growth performance test and for a separate 10 days for feed digestibility testing. For the feed digestibility test, feed marked with the digestibility indicator (Cr_2O_3) which was given to tilapia for 10 days and feces was collected starting day 7.

Enumeration of intestinal bacteria population: Fish intestines weighing 0.1 g were collected and were homogenized in 0.9 mL sterile Phosphate Buffer Saline (PBS). Serial dilution was performed and each series was spread onto TSA medium. The number of bacterial cells in a sample was calculated by counting the number of colonies growing on the medium multiplied by the dilution factor in CFU g^{-1} units (Madigan *et al*., 2003).

Digestive tract enzyme activity, total digestibility and carbohydrate digestibility: The activity of digestive tract enzyme that was measured was amylase enzyme (Bergmeyer, 1983). Digestibility analysis was done on the feces that had been collected by siphoning the feces at least 30 min after feeding and then the feces was dried in an oven at 110°C for 4-6 h. A nutrient content (nutrient (%)) analysis in feed and feces were then performed by proximate analysis and the Cr_2O_3 content (Cr_2O_3 (%)) in feed and feces were measured using a spectrophotometer at a wavelength of 350 nm. The total digestibility and carbohydrate digestibility were calculated at the end of the study (Watanabe, 1988) using the following equation:

$$\text{Nutrient digestibility} = 100-[1-a/a'\times b'/b]$$
$$\text{Total digestibility} = 100-[1-a/a']$$

Where:

a = Cr_2O_3 in feed (%)
a' = Cr_2O_3 in feces (%)
b = Nutrient in feed (%)
b' = Nutrient in feces (%)

Measurement of growth performance parameters: Parameters measured were Survival Rate (SR), Specific Growth Rate (SGR) that were determined according to Huisman (1987) and Feed Efficiency (FE) that were calculated according to Takeuchi (1988) using the following equations:

$$SR\ (\%) = \frac{Nt}{No}\times 100\%$$

Where:

Nt = Number of fish at the end of the maintenance period (individual)
No = Number of fish at the beginning of the maintenance period (individual)

$$SGR\ (\%) = \frac{\ln We - \ln Ws}{d} \times 100\%$$

Where:
We = Weight of fish in the end of the maintenance period (g)
Ws = Weight of fish in the beginning of the maintenance period (g)
d = Duration of the maintenance period (day)

$$FE\ (\%) = \frac{\text{Fish weight increase (g wet weight)}}{\text{Weight of the feed consumed (g dry weight)}} \times 100\%$$

Statistical analysis: This study used a complete random design with two treatments and four replications. The data obtained were analyzed using variance analysis with a 95% level of trust. To see the difference in the treatments, the test was followed by Duncan's multiple range test using the SPSS 17 program.

RESULTS

Probiotic bacteria candidate isolates: The isolation from 10 tilapia samples resulted 41 bacterial isolates which produced amylase enzymes or are amylolytic bacteria. These bacteria were then used in the probiotic bacteria candidate selection phase. From the starch hydrolysis test, 5 bacterial isolates with the largest diameter clear zones were obtained (Fig. 1). The morphological, physiological and biochemical characteristics and the bacteria species observed based on the description by Cowan (1974) are presented in Table 1. The ability to degrade or hydrolyze starch from the highest to the lowest was shown by isolate Np 1 (24.0 mm), Np 4 (20.7 mm), Np 5 (20.3 mm), Np 2 (16.0 mm) and Np 3 (14.3 mm), respectively.

Bacterial growth phase: The growth curves which were results of 24 h observations (Fig. 2) show that each bacterial isolate had a varied pattern, similar to the results of the generation time analysis results (Table 2). Based on the lag phase (I), isolate Np 4 and Np 5 had the longest maximum growth time and could reach the exponential phase quicker (12 h) than the other isolates. Based on the observation of colony numbers, the static phase of the Np 5 isolate was longer, approximately 3 h, very different from the other isolates whose static phase only lasted

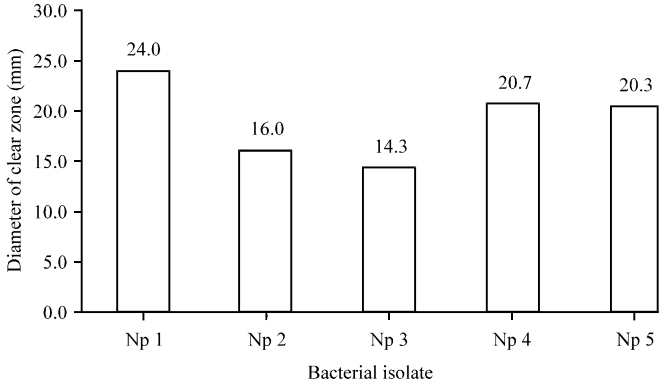

Fig. 1: Diameter of the clear zone, the result of starch hydrolysis by the amylolytic bacteria isolates

around 1-2 h. This shows that the Np 5 bacterial isolate had a longer maximum growth period compared to the other bacterial isolates. Based on the observation of the generation times of the

Table 1: Morphological, physiological and biochemical characteristics and the results of the identification of the probiotic bacteria candidates based on Cowan (1974)

			Physiological and biochemical characteristic testing						
Isolate	Shape	Gram staining	O/F test	Glucose	Oxidase	Catalase	Motility	H_2S	Assumed bacteria
Np 1	Rod	-	F	+	+	+	+	+	*Beneckea* sp., *Vibrio* sp. *Plesiomonas* sp., *Aeromonas* sp. *Enterobacteria* sp.
Np 2	Rod	-	F	+	-	+	+	-	*Haemophilus* sp.
Np 3	Rod	+	F	+	+	+	+	+	*Bacillus* sp.
Np 4	Rod	+	F	+	-	+	+	+	*Listeria* sp.
Np 5	Rod	+	F	+	+	+	+	+	*Bacillus* sp.

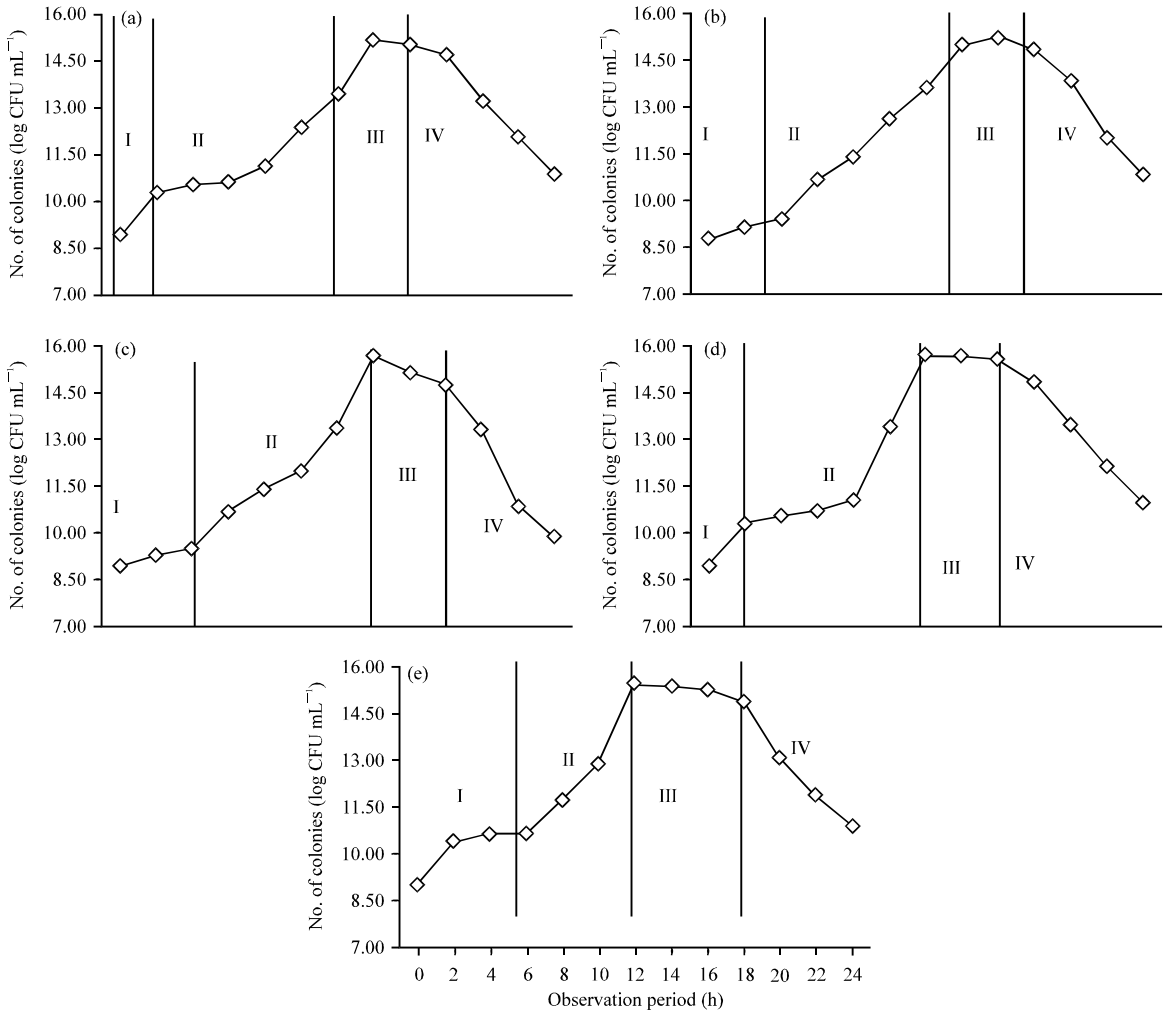

Fig. 2(a-e): Growth curves of probiotic bacteria candidate isolates, (a) Np1, (b) Np2, (c) Np3, (d) Np4 and (e) Np5

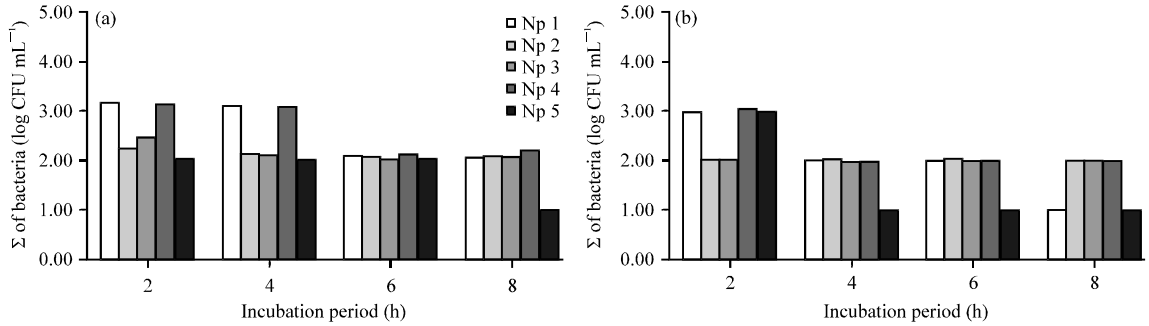

Fig. 3(a-b): Difference in the number of probiotic candidate bacteria on TSB (a) pH 2.5 and (b) pH 7.5 media and on the control media

Table 2: Generation time of the probiotic bacteria candidates

Bacterial isolates	Maximum growth time (h)	Generation time (min)
Np 1	14	40.57
Np 2	16	45.17
Np 3	14	37.59
Np 4	12	31.90
Np 5	12	33.56

five amylolytic probiotic bacteria candidate isolates (Table 2), isolate Np 4 had the shortest generation time, 31.90 min, followed by isolates Np 5, Np 3, Np 1 and Np 2.

Resistance to acid and alkali: The differences between the number of bacteria in the control media and the treatment media at each observation period are presented in Fig. 3. Based on the results obtained, the difference of bacteria on the pH 2.5 medium and on the control medium from smallest to largest were isolates Np 5, Np 2, Np 3, Np 4 and Np 1, respectively. At the observation periods of 4, 6 and 8 h, the least difference was shown by isolate Np 5. All the isolates could also survive at alkaline conditions up to the 8 h. At the 2 h observation period, the least difference between the number of bacteria on the alkaline medium and the control was shown by isolate Np 3, followed by isolate Np 2, Np 5, Np 1 and Np 4, respectively. At the observation periods of 4, 6 and 8 h, isolate Np 5 showed the least difference among all the isolates. Therefore, isolate Np 5 had the least difference at the 4-8 h observation periods both in acidic (pH 2.5) and alkaline (pH 7.5) conditions.

Antagonistic activity test: The results of the observations on the probiotic bacteria candidates antagonistic activity against the pathogenic bacteria *A. hydrophila* and *Streptococcus* sp., are presented in Table 3. The antagonistic activity against the pathogenic bacteria *A. hydrophila* was observed by measuring the inhibition zone as showed in Fig. 4a. The largest inhibition zone was demonstrated by isolate Np 5 at 12.00±0.00 mm, followed by isolate Np 3, Np 2, Np 1 and isolate Np 4, respectively. The subsequent test with the pathogenic bacteria *Streptococcus* sp., isolate Np 5 also proved to have the strongest antagonistic activity with an inhibition zone of 11.00±0.00 mm, followed by isolate Np 1, Np 3, Np 2 and isolate Np 4, respectively (Fig. 4b).

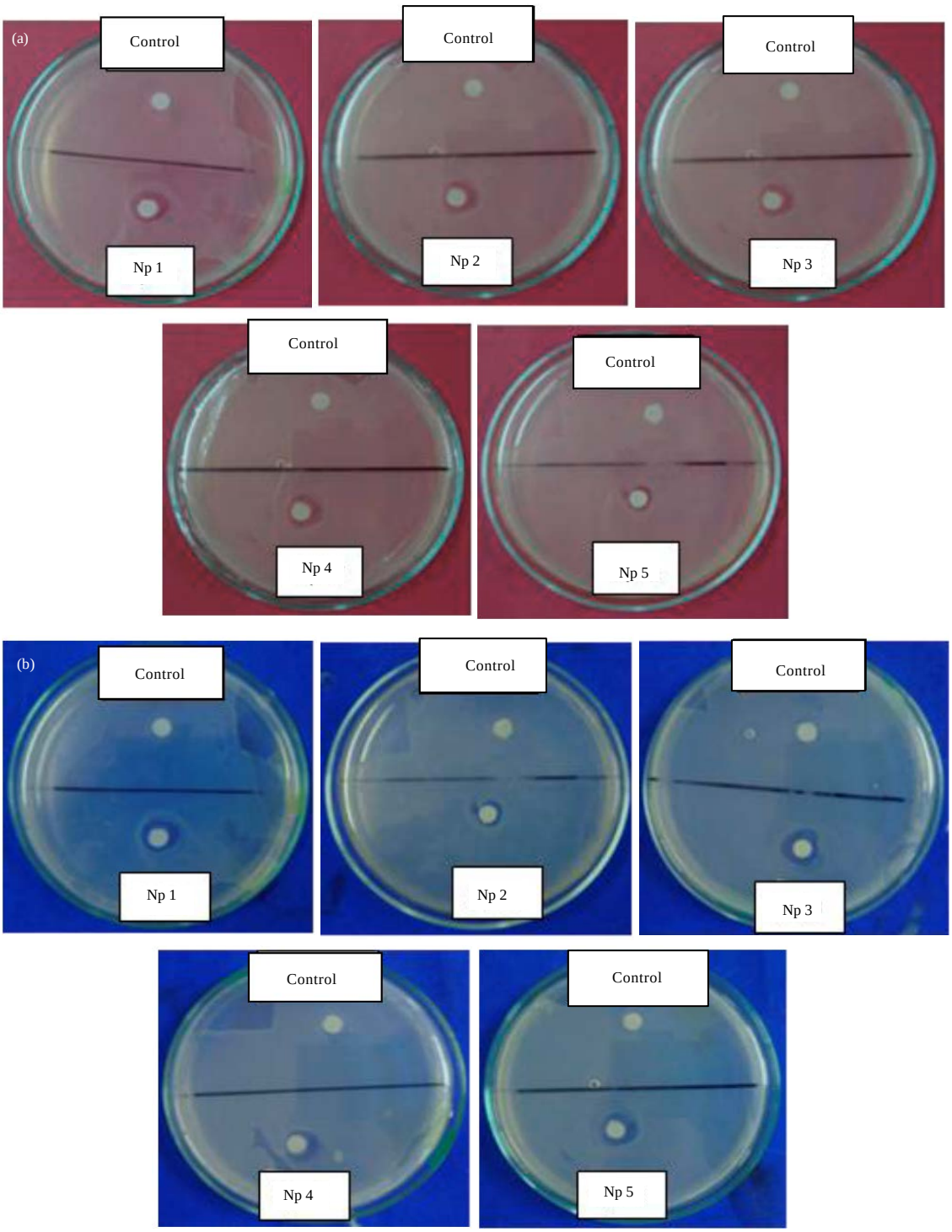

Fig. 4(a-b): Descriptive inhibition zone pictures of isolate Np 1, Np 2, Np 3, Np 4 and Np 5 in antagonistic test to (a) *A. hydrophila* and (b) *Streptococcus* sp.

Table 3: Probiotic bacteria candidates antagonistic activity against pathogenic bacteria in fish

	Diameter of inhibition zone (mm)	
Isolate	*A. hydrophila*	*Streptococcus* sp.
Np 1	10.33±0.58	10.00±1.00
Np 2	11.00±0.00	9.33±1.53
Np 3	11.67±0.58	9.67±0.58
Np 4	9.67±1.15	9.00±1.00
Np 5	12.00±0.00	11.00±0.00

Table 4: Results of the test of probiotic bacteria candidate ability to adhere to stainless steel plates

	Bacterial population	
Isolates	Swab (CFU cm^{-2})	Planktonic (CFU mL^{-1})
Np 1	$8.83\times10^{6}\pm0.31$	$5.70\times10^{11}\pm0.95$
Np 2	$7.50\times10^{5}\pm0.46$	$4.73\times10^{12}\pm0.31$
Np 3	$6.70\times10^{6}\pm0.66$	$6.37\times10^{11}\pm0.59$
Np 4	$4.23\times10^{6}\pm0.87$	$8.93\times10^{11}\pm0.40$
Np 5	$1.57\times10^{7}\pm0.40$	$8.33\times10^{10}\pm0.74$

Table 5: Results of the pathogenicity test for the probiotic bacteria candidate on tilapia

Treatments	SR (%)
Negative control	100
Positive control	0
Np 1	100
Np 2	70
Np 3	100
Np 4	50
Np 5	100

Adhesion test: The adhesion test is based on the bacteria ability to form biofilm on stainless steel plates. The results of the test of the probiotic bacteria isolates ability to adhere to stainless steel plates are presented in Table 4. The results of the observation showed that isolate Np 5 had the largest population from the swabs at $1.57\times10^{7}\pm0.40$ CFU cm^{-2} and the smallest planktonic bacterial population at $8.33\times10^{10}\pm0.74$ CFU mL^{-1}, followed by isolate Np 1, isolate Np 3 and isolate Np 4, respectively. The smallest bacterial population from the swabs was shown by isolate Np 2 at $7.50\times10^{5}\pm0.46$ CFU cm^{-2} which also had a planktonic bacterial population of $4.73\times10^{12}\pm0.31$ CFU mL^{-1}.

Pathogenicity test: The results of the test of the probiotic bacteria candidates pathogenicity are presented in Table 5. Test results after rearing for 10 days showed that the fish survival rate when tested using isolates Np 1, Np 3 and Np 5 was 100%, whereas when tested using isolates Np 2 and Np 4, it was 70 and 50%, respectively. The survival rate for the negative control was 100% while for the positive control was 0% or, in other words, none of the fish survived.

Based on the descriptive analysis, the bacterial isolate selected as the probiotic candidate for the subsequent test phases was isolate Np 5. The considerations were: (1) It had a fairly large starch-hydrolyzation diameter, 20.3 mm, (2) It grew quickly with a population reaching

Table 6: Growth parameters of tilapia

	Treatment	
Parameter	A	B
BP (log CFU g^{-1})	6.0±0.03[a]	6.5±0.06[b]
AEA (U min mL^{-1})	0.316±0.02[a]	0.466±0.002[b]
CD (%)	44.25±2.82[a]	67.32±4.67[b]
TD (%)	42.51±2.33[a]	55.43±2.03[b]
SGR (%)	3.79±0.47[a]	4.01±1.12[b]
SR (%)	100±0.00	100±0.00
FE (%)	31.2±3.04[a]	40.0±7.08[a]

Different superscript letters on the same row indicated significantly different results ($p<0.05$), BP: Bacterial population, AEA: Amylase enzyme activity, CD: Carbohydrate digestibility, TD: Total digestibility, SGR: Specific growth rate, SR: Survival rate and FE: Feed efficiency

29.8×10^{14} CFU mL^{-1}, (3) It was able to survive well in both acidic and alkaline pHs, (4) It had the strongest antagonistic activity against the pathogenic bacteria *A. hydrophila* and *Streptococcus* sp., (5) It had the ability to adhere to solid substrate and (6) It is not pathogenic.

***In vivo* assays of the probiotic:** The results of the test of whether the probiotic NP5 was effective in influencing the growth performance of tilapia are presented in Table 6. The study results showed that the bacterial population in the probiotic treatment was 6.5±0.06 log CFU g^{-1}, higher than the control which was 6.0±0.03 log CFU g^{-1}. The results of the amylase enzyme activity measurement showed that the activity in the treatment using probiotic was 0.466±0.002 U min mL^{-1}, higher than and significantly different from the activity in the control which was 0.316±0.02 U min mL^{-1}. The carbohydrate digestibility in the treatment using the probiotic was 67.32±4.67%, whereas in the control it was merely 44.25±2.82% ($p<0.05$). Similar results were found in the total digestibility rate in the probiotic treatment which was 55.43±2.03%, higher than the control which was 42.51±2.33% ($p<0.05$).

The specific growth rate in the probiotic treatment was 4.01±1.12%, higher than and significantly different from the control which was 3.79±0.47%. The feed efficiency rate for the probiotic treatment (40.00±7.08%) was higher than but not significantly different from the control (31.20±3.04%).

DISCUSSION

Probiotic bacteria candidate isolates: The presence of a clear zone shows that the carbon-source macromolecule which in this case was starch, had been hydrolyzed as a source of energy by the bacteria. Amylolytic bacteria are bacteria which are able to secrete amylase enzymes which play an important role in the fish digestive process as a catalyst for feed-nutrient hydrolysis in the fish digestive tract. Bacteria are able to adjust to their environment which is rich in complex molecules by secreting exogenous enzymes. Exogenous enzymes hydrolyze macromolecules, producing simpler molecules, for example hydrolyzing polysaccharides into sugar. These simple molecules are then transported into the cytoplasm to be utilized as a source of energy or as precursors in the synthesis of cell components.

Bacterial growth phase: The growth of the bacterial isolates was measured in order to determine the growth phases and generation time. These are useful to determine the best time to harvest the

cells to produce a product or metabolic compounds such as enzymes, antimicrobials, vitamins, organic acids, fatty acids, amino acids and peptides. As industrial microorganisms which must be mass-produced and have fast growth rates (Madigan *et al*., 2003), probiotic bacteria must reach their exponential phases quickly and have a short generation time. Generation time is the time needed by the bacterium to split itself into two.

Resistance to acid and alkali: The isolate resistance to acidic and alkaline pHs reflects the probiotic bacteria candidate ability to survive in the stomach acidic conditions and bile salts. It is presented in the difference between the number of bacteria in the control media and the number in the treatment media (acidic and alkaline pHs) during the observation period. Isolates with the smallest difference in numbers between the control media and the treatment media demonstrate their resistance to acidic and alkaline conditions. Isolate Np 5 has better survival ability in both acidic and alkaline conditions among all the isolates. This indicates that isolate NP5 would be able to survive the passage through the low pH in the stomach and the high pH of bile salts. Tolerance to stomach acids and bile salts is an important condition for probiotic candidates (Kesarcodi-Watson *et al*., 2008) because when the probiotic bacteria enter the fish body, they will pass through the stomach which is acidic and then meet with bile salts which are alkaline when passing through the intestines.

Antagonistic activity test: One of the important criteria for probiotic bacteria candidates is having antagonistic activity or having the ability to suppress the growth of pathogenic bacteria in the fish digestive tract. The presence of pathogenic bacteria in the digestive tract of fish and the cultivation environment in large numbers could cause disease in the fish. The probiotic bacteria ability in inhibiting the development of pathogenic bacteria shows their ability in maintaining the balance of micro flora in the fish digestive tract, thus reducing the probability of disease. Normal flora in the digestive tract play protective role which is important in suppressing pathogenic bacteria, stimulating local and systemic immunity and altering the intestinal metabolic activity. Normal flora also suppresses pathogenic bacteria through competition for nutrients and places to adhere on the intestinal wall (Verschuere *et al*., 2000; Irianto, 2003).

Adhesion test: The adhesion test was done as an approach to the bacteria ability to adhere to the surface of the fish intestines. The bacterial population from swabs shows the bacteria ability to colonize or to adhere. These current results were consistent with the results obtained by Wirawati (2002) and Aslamyah (2006), an increase in the population on solid substrate is followed by a decrease in the planktonic bacterial population.

Pathogenicity test: The deaths of tilapia in treatment with Np 2 and Np 4 in the pathogenicity test is assumed to be because the isolates injected were pathogenic to the fish. This assumption was based on the clinical signs demonstrated by the treatments using Np 2 and Np 4, i.e., inflammation at the injection site, anorexia and finally death.

***In vivo* assays of the probiotic:** The results showed that the supplementation of the probiotic Np 5 (B) in feed was able to produce a better growth performance than the control (A). The supplementation of probiotic in feed was meant to increase the population of probiotic bacteria in the tilapia digestive tract so that the action mechanisms of the probiotic in producing exogenous enzymes for digestion increased. The number of intestinal bacteria in probiotic treatment proved

that the probiotic given was able to survive and adhere to the fish intestinal wall and the probiotic will increase the digestive tract enzyme activity. The increased amylase enzyme activity was also observed in tilapia given the probiotic *Bacillus subtilis* (Taoka *et al*., 2007) and white shrimp given the probiotic *Bacillus* sp through feed (Wang, 2007). The increase in the activity of digestive enzymes in the tilapia digestive tract had a positive correlation to digestibility rate. The high carbohydrate digestibility in the probiotic treatment was strongly related to the high amylase enzyme activity in the probiotic treatment due to the addition of the amylolytic probiotic to the feed. Increased digestive enzyme activity could help the fish to degrade nutrients, thus increasing digestibility and improving feed efficiency (Cerezuela *et al*., 2011). This indicated that the supplementation of amylolytic probiotic could increase exogenous enzyme activity and could be utilized well to improve growth, even though it was not yet able to significantly improve the feed efficiency rate. Similar results were reported by Wang (2007) in the supplementation of the probiotic *Bacillus* sp. in white shrimp and by Aly *et al*. (2008) in the supplementation of *Bacillus pumilus* in tilapia.

The improvement of growth in the test fish due to the addition of the probiotic in the feed showed the response to carbohydrate utilization as a source of energy; this showed the presence of the protein sparing effect for growth. Bureau *et al*. (2003) explained that carbohydrate is not a superior energy source for fish; it is no better than protein or fat. Protein administered in feed is not used as an energy source but for growth and replacement of damaged tissue. The energy in feed which is non-protein source could affect the amount of protein used for growth. Therefore, probiotics in feed could improve the effectiveness of the utilization of feed carbohydrate, making the utilization of feed protein more efficient and having better responses in fish growth.

CONCLUSION

The addition of the amylolytic probiotic *Bacillus* NP5 which was screened from the digestive tract of tilapia to feed could increase digestive enzyme activity, improve nutrient digestibility and improve growth performance in tilapia.

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