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Antimicrobial Activities of *Bacillus subtilis* strain VITNJ1 Isolated from Tilapia(*Oreochromis niloticus*) Intestine

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Abstract : The research methodisexperimental method. Materials usedin this study wereselectedbacterial isolatesof *Bacillussubtilis*strainVITNJ1isolated fromthe digestive tractNila Tilapia(*Oreochromis niloticus*). The purpose ofthis study was power test antagonistic activity against pathogenic bacteria, test resistance toacids and bases, studiedthe form of bacterial growth curve and test candidate probiotic bacterial attachment. Of an timicrobial test against *Aeromonas hydrophila*is known that the bacterium *Bacillussubtilis* has the ability toinhibit bacterial growthinhibition zonetestwith a diameter of13mm. cangrowon a variety of pH ranges(2, 3, 4, 5, 6, 7, 8, 9, 10and11), sigmoid shaped growth curve, can beattached to thestainless steelto10^9dilution.

Keywords: *Bacillus subtilis*, probiotic, bacteria, Tilapia,West Sumatra

Introduction

Public water that has big potential to do Nila fish farming is; reservoir, lake, dam controller, and other puddles. Maninjau Lake is one of public water in Tanjung Raya District, Agam Regency of West Sumatera. Fisheries activities in Maninjau Lake such as; aquaculture in floating net and capture fisheries.

The increasing of intensification and commercialization in aquaculture production has one problem. Disease becomes the main problem in fish farming industry[1].Based on the research, we know that the using of antibiotics causes microorganisms that are resistant with drug that has antibiotic residues remain in fish meat and in environment. Moreover, an antibiotic can affect normal micro flora in digestive tract[2].In this case, the using of probiotic bacteria is a new approach, which is expected to be used in cultivation activities to control pathogens bacteria[2,3,4].

Back to nature is an ecological policy that inspires aquaculture activities such as; the using of fitofarmaka or biocontrol agents application either to control the disease or improve the water quality. Various types ofbiocontrol agents, single or mix, have been commercialized in various product; liquid or powder. Exploration and selection of biological control agents toward many microorganisms are still being done, especially in utilization of local isolates[5].

Biocontrol agent in aquatic organism is living microorganism, linked benefits with the host, and also has many positive sides such as guarantee the improvement of feed utilization, increase nutrient in feed, increase the immune response of the host toward disease, and increase the quality of water environment[6].Examples of biocontrol agent are: non-pathogenic Vibrio, *Pseudomonas*, *Plesiomonas*, *Lactobacillus*, *Bacillus,Actynomyces*, *Nitrification* and *denitrification* bacteria, *photosynthetic* bacteria, several special of yeast, and *bifidobacterium.*

Based on isolation and identification of bacteria from digestive tract of Nila fish(*Oreochromis niloticus*) that cultivated in floating net, we found one isolate of potential bacterium which has highest potential activity called *Bacillussubtilis*strainVITNJ1and it was expected to be probiotic candidate[7]. That's why this research is necessary to be done in order to characterize and examine antimicrobial activity of *Bacillussubtilis*strain VITNJ1. We expect it to able to anticipate the buildup feed problem in bottom water.

Materials and Methods

Bacterial strain and culture conditions

A bacterium that was use in this research was *Bacillus subtilis*strain VITNJ1 that is isolated from intestines of Tilapia fish (*Oreochromis niloticus)* which was obtained from fish farmer in Maninjau lake[7].Pathogenic bacteria such as; *Aeromonashydrophyla*, *Escherichia coli*, *Staphylococcus aureus*and*Bacillus subtilis*were stored in glycerol liquid and were growth in Nutrient Agar (NA) in 37^{o}C for 24 hours. Then the colonies of NA were growth in an aerobic atmosphere, and stored in incubator for 24 hours in 37^{o}C.

Probiotics Characterization

Antagonistic Bacteria Screening

Antimicrobial activity was examined by disc method by using free cell supernatant liquid from the isolate of potential bacteria[8]. Bacterium*Aeromonas hydrophila*as a pathogenic bacterium was cultured in nutrient broth, incubated in 37°C for 24 hours, then grow it in NA. at the same time, the isolate was cultured in MRS for 24 hours in 37°C; bacterial cells were centrifuged in 8000 rpm and 4°C for five minutes; supernatant was used for testing. Drop sterile disc into supernatant, dry it and placed it on MHI that contained *Aeromonashydrophila*. The petridish was incubated in 37°C for 24 until 48 hours to see the inhibition zone[2].

Effect ofpH onAntimicrobialActivity

Optimalization of antimicrobial compound formation was examined by in various pH (2, 3, 4, 5, 6, 7, 8, 9, 10, 11and 12).One ose of bacterial culture was growth in 10 ml pH adjusted MRS broth. Homogeneous culture used vortex, and it was incubated for 24 hours in 37°C. The culture that formed was centrifuged in 6000 rpm for 10 minutes and antimicrobial test was conducted using four experiment bacteria in disc method[9].

Resistance Test toward Gastric Acidity

1 ml of bacteria that was inoculated turned into series of tube contain 9 ml sterile medium with pH 2.5 (controlled by adding HCl) and pH 7.5 (controlled by adding NaOH), and they were incubated in 37°C. The amount of bacterial cells was counted every 2 hours for 8 hours long. The resistance toward acid and stomach bile salts was determined by measure the density value or *optical density* (OD) using a specphototometer at 620 nm wavelength.

The Growth of Experiment Bacteria

1 ml of isolate of bacteria was put into 9 ml liquid MRSB and was incubated for 24 hours in 37°C. Take 1% of culture and inoculate it into 90 ml of sterile MRSB and incubate it in 37°C again. The growth of the bacterium was observed every 2 hours for 8 by measuring the density value or *optical density* (OD) using a spectrophotometer at 620 nm wavelength[10].

Adhesion Test

The first steel plate was sterilized by soaking it in a detergent solution, then heat it in 40-45°C for 24 hours, rinse it with hot water (40-50°C), and autoclave it in 120°C for 20 minutes. Place the steel plate in standing erlenmeyer 1000 ml. Before that, fill the erlenmeyer with TSB 20 ml, sterilize and inoculate 1 ml of fresh bacterial culture. Cover erlenmeyer with aluminium foil and place it in a shaker for 24 hours in 29°C. Then rinse the steel plate with buffer phosfat (BF). Wipe the surface of the plate with a swab, put the swab into test tube which contain 10 ml of BF and vortex it for 1 minute. And then do serial dilution and count the population of bacteria using count plate method[11].

Result and Discussion

Antagonistic Activity Test toward Pathogenic Bacteria

Based on antagonistic activity toward pathogenic bacteria *Aeromonas hydrophyla*, noted that inhibitory of *Bacillus subtilis*strain VITNJ1 is 13 mm, it was marked by the wide of inhibition zone arising. Antimicrobial activity test in this research used a paper disc. The result of antimicrobial activity test from *Bacillus subtilis*strain VITNJ1 toward pathogenic bacterium*Aeromonas hydrophyla*can be seen in Fig. 1. The criteria of the bacteria that have to be considered to be pathogenic bacteria are its ability to inhibit the growth of pathogenic bacteria so it can compete to maintain the balance of normal microflora in the intestines. The more pathogenic bacterium in the fish intestines, the more it can harm fish cultivation. It happens because bacterium causes disease and death of the farmed fish. Also run the same research with the isolate of *Leuconostoc mesenteroides*[12]. In their research about the influence of probiotic in pathogenic bacterium that found in *Oreochromis mossambicus*has positive impact to *Aeromonas hydrophila*, *Vibrio* sp and*Escherichia coli*[13]. Bacterium *Bacillus subtilis*was isolated from the digestive tract of gold fish (*Cyprinus carpio* L) and this bacterium can inhibit the growth of *Aeromonas hydrophila*after being incubated for 24, 48, and 62 hours[14].

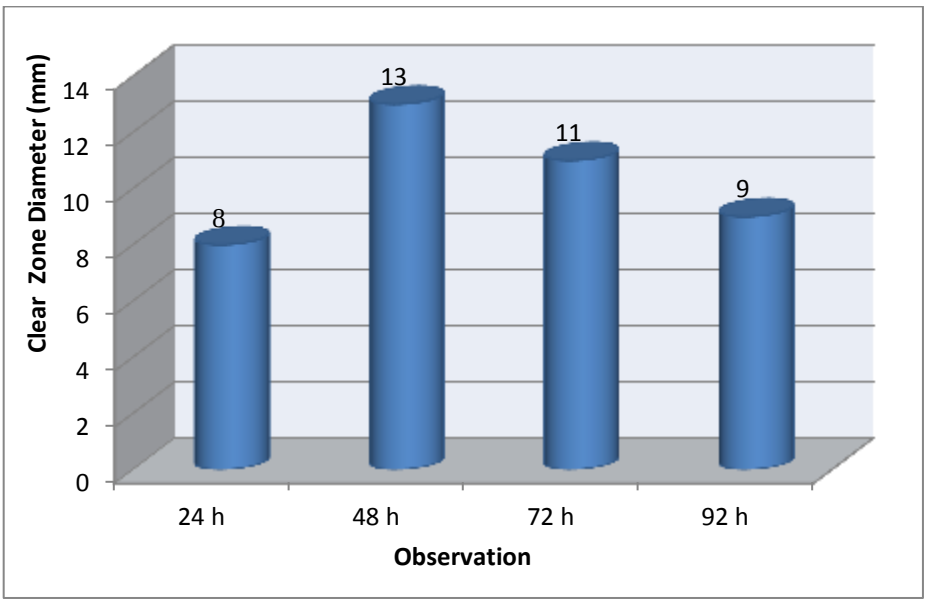

Figure 1. Antimicrobe activity test from bacterium *Bacillus subtilis*strain VITNJ1 toward pathogen bacterium*Aeromonas hydrophyla*

Figure 2.Cultivation of isolate of *Bacillussubtilis*strain VITNJ1in pH2-12range.

Resistance Test toward Gastric Acidity

Probiotic committee form FAO/WHO set a guide to examine probiotic, where probiotic bacterium has to fit the qualification which are; must be an isolate that come from human or animal; show the beneficial effect to the host; not pathogenic and poisonous; contain a lot of microbe in corpuscles; able to live and be metabolic in digestive tract; stay alive during storage periode and use; antagonistic toward pathogen. The result of the study also showed that bacterium *Bacillus subtilis*strain VITNJ1that was isolated from digestive tract of Nila Fish(*Oreochromis niloticus*) has ability to grow both in acidic and basic media. It can be seen from isolate cultivation result of *Bacillus subtilis* strain VITNJ1in various pH (fig 2).Bacterium *Bacillus subtilis* strain VITNJ1 that was growth in various pH on MRSB showed that the isolate of *Bacillus subtilis* strain VITNJ1can grow in pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11and12. This result marked with muddy culture that happened in 24 hours observation. From the cultivation result of isolate of bacterium *Bacillus subtilis*strain VITNJ1 in various pH, then we tried to see their antimicrobial activity toward four pathogenic bacteria:*Escherichia coli*, *Staphylococcus aureus*, *Salmonella thypi*and*Aeromonas hydrophyla*;the result can be seen in Fig 3.

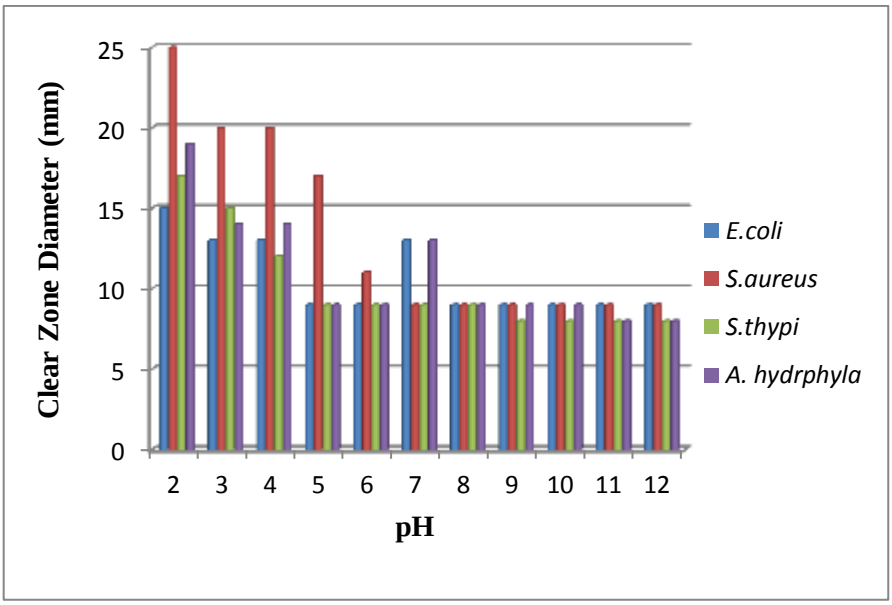

Figure 3.The effect of pH toward antimicrobial compound activity from isolate of *Bacillus subtilis*strain VITNJ1 to inhibit the growth of pathogenic bacteria

The highest antimicrobial activity of *Bacillus subtilis*strain VITNJ1 happened in pH 2 toward *Staphylococcus aureus*with 25 mm clear zone. And the lowest happened toward *Escherichia coli* with 15 mm inhibition zone.Inhibition mechanism in low ph is caused by the effort of the cells to maintain the consistency of the pH in cell. If pH is dropped, proton in the cell will step into cytoplasm of the cell. Proton (ion H^+) from acid step into cell through the trans membrane proton gradient. It will cause pH of cytoplasm decrease and it will push enzymes to work to restore the normal pH[15]. A research result antimicrobial compound that is produced by *Bacillus subtilis*that is isolated from *Labeo rohita*has highest activity in pH 5.0 – 10.0 and the highest enzyme activity happened in pH 9.0[16].

The Growth of Bacteria

The curve of the growth of bacterium*Bacillussubtilis*strain VITNJ1for 24 hours incubation periode using MRSB, measured by spectrophototometer in 620 nm wavelengths can be seen in Fig 4. Lag phase of the isolate of bacterium *Bacillus subtilis*strain VITNJ1 occurred in the first hour until the second hour. The second phase is exponential phase where the growth of the bacterium is happening very fast. In the isolate of bacterium *Bacillus subtilis*strain VITNJ1, exponential phase happens in the third until 18^{th} hour. The next phase is stationary phase that happens in 19^{th} hour until 24^{th} hour. In this phase, no additional amount of bacterial cell because the amount of the growth and death celss are the same. And the last phase is death phase, where the amount of bacterial cell drops because the nutrient and the energi reserves become less and less.

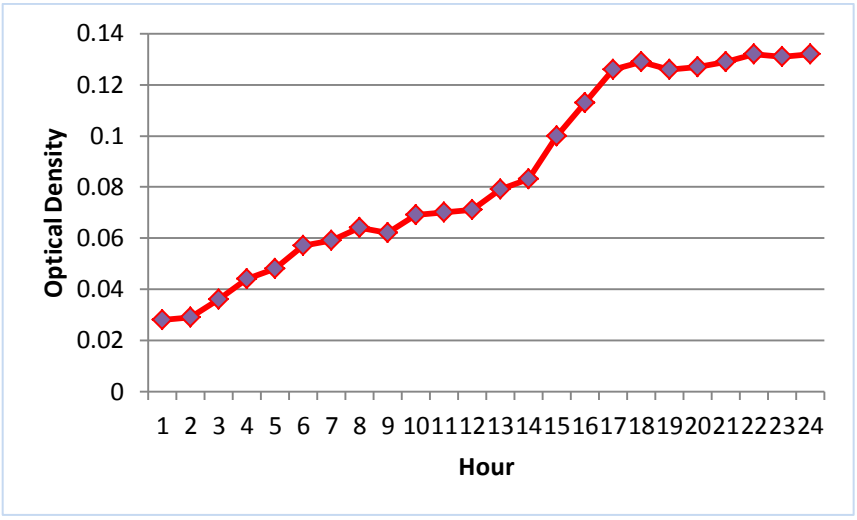

Figure 4.The curve of isolate growth of *Bacillus subtilis*strain VITNJ1 for 24 hours incubation period

The growth pattern of isolate *Bacillus subtilis*strain VITNJ1is almost the same with the a research about characterization of antimicrobial compound that was produced by bacterium*Bacillus cereus*which came from dairy product[17]. They found that bacteriocin antimicrobial compound production gets into the stationary phase after 10-16 hours incubation period. The same research was also found that thuricin 7 was produced by *Bacillus thuringiensis* BMG1.7[18]

Adhesion Test

Adhesion test on bacterium that will be probiotic candidate is intended to see how far the bacterium can patch into digestive tract of the fish. The result of adhesive test on isolate*Bacillus subtilis*strain VITNJ1 in stainless steel stab can be seen in Table 1. The result showed that until dillution 10^9, there were still bacteria that patch into the digestive tract. It means that bacterium *Bacillussubtilis*strain VITNJ1that was isolated from digestive tract of the Tilapia fish(*Oreochromis niloticus*) can be used as probiotic.Variousmodels ofattachmentin vitrocarried out includeattachmenttointestinal epithelium[19,20].Thetestattachment of*Lactobacillus casei*in vitrointestinal cellsintomiceandmodelingattachmenttostainless steelandwhichusesthe modelof attachmentLABas probioticsin vitro[21]. One of the criteria of bacterium that can be used as probiotic is able to patch to intestines[22]. Bacterium can interact with intestines epithelium cells, extracellular matrix, and mucus layer. Mucus layer that cover intestinal epithelium cells is the first contact of microorganism to patch and colonize in intestines. If the mucous is broken, bacterium will patch on intestinal epithelium cells[23]. This result is in line with the research that did a selection of probiotics amylolytic in digestive tract of Gourami fish (*Osphronemus gauramy*) using swab and planktonic method[24].

Table1.Adherence test result of isolate of *Bacillus subtilis*VITNJ1 in stainless steel stab.

No	Dilution	Populationbacteria(CFU/ml)
1	10^3	2
	10^3	2
2	10^5	1
	10^5	4
3	10^7	2
	10^7	1
4	10^9	4
	10^9	2

Conclusion

After antimicrobial test toward bacterium*Aeromonas hydrophyla*was done, we knew that bacterium *Bacillus subtilis*strain VITNJ1 had an ability to inhibit the growth of bacteria with 13 mm inhibition zone.

Based on the cultivation of isolate of *Bacillus subtilis*strain VITNJ1 in various pH on MRSB, we could see that the isolate was able to live in a medium that had pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11and 12; able to live in a medium that had pH 2.5 (acid) and pH 7.5 (basic) and the shape of the growth curve was sigmoid. The peak of the bacterium *Bacillus subtilis*strain VITNJ1 had growth occurred in 18th hour .

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