DETACHMENT BIOFILM OF Mycobacterium fortuitum ON FISH SCALES AND PVC PLASTICS SURFACES WITH ANTIBACTERIAL COMPOUNDS FROM Lactobacillus agillis

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Abstract

The activity of consumption fish or ornamental fish is an activity that has a high risk because the fish are directly exposed to the environment that is not easy to control so that the fish are susceptible to disease and even experience death. Diseases in fish are generally the result of complex or unbalanced interactions between three components in aquatic ecosystems namely weak hosts (fish), malignant pathogens and deteriorating environmental quality. M. fortuitum is a cause of. This study aims to determine the ability of the antibacterial compounds of crude BAL extract in preventing M. fortuitum biofilm cell formation and control of M. fortuitum biofilm cells at a concentration of 60% (v / v), 80% (v / v), and 100% (v / v). Isolation of LAB from pond sediments using MRSA, selection of LAB had using paper disk method was identified as Lactobacillus agillis. Detachment of biofilm cell on fish scales and PVC plastic using 0.5 g micro-glass bead is cortexed for 2 minutes. To calculate the number of biofilm cells used the TPC method. The results of this study showed the highest number of biofilm cells on day 3, namely 7 CFU logs / fish scales and 5 CFU logs / PVC plastic plates. A crude 100% antibacterial extract extract reduced the number of biofilm cells by 3 CFU logs / fish scales and 2 CFU logs / PVC plastic plates after 1 hour of contact. The 100% concentration completely prevents biofilm formation. However, at a concentration of 80% there was a decrease of 2 CFU logs / PVC plastic plates. While the concentration of 80% and 60% did not experience a decrease in the number of cells on both plates.

Keywords*:* *biofilm, Mycobacteriosis, M. Fortuitum, Lactobacillus agillis*

A. Introduction

Mycobacteriosis is the most significant disease as the main killer of fish in freshwater and infected fish allows it to spread to humans (Irianto, 2005). Mycobacteriosis is one of the diseases caused by *Mycobacterium fortuitum. M. fortuitum* is an opportunistic pathogen, saprophyte, resistant to antibiotics and disinfectants (Brooks et al. 1984) and grows in a very fast time.

Biofilm is the final product of bacterial attachment which Charaklis and Marshall (1990) define as cells of microorganisms immobilized on the substrate and trapped in extracellular polymers produced by these microorganisms. ZoBell (1943) in his report mentions that there are two types of interactions between bacteria and the surface they are attached, namely reversible interactions and irreversible interactions. It was also concluded by ZoBell (1943) that environmental conditions with limited nutrient caused nutrient to be more concentrated on the surface so that it modified the surface properties which ultimately affected the microorganism attachment process. Biofilm structure can change depending on the nutritional conditions of the environment (Hall-Stoodley, 1998). Microcolony is a small collection of several bacterial cells that will form biofilms. Polysaccharides from each cell attach to the entire surface and produce communication signals between each cell to expand the attachment field. The components of polysaccharide makes the surface polar. And this polarity makes biofilms difficult to release (Barnes and Caskey, 2002)

Based on the research of Hall-Stoodley (1998) at an incubation period of 2 hours, *M. fortuitum* was able to form biofilms on solid surfaces with cell counts of 10^6 CFU / ml and decreased the amount of density at 48 hours. Senjani (2002) states that the growth of the biofilm will increase in the same direction as the incubation time increases, but if the maximum amount has been reached then the number of cells will decrease. Characteristics of *M. fortuitum* Based on Bergey's Manual of Systematic Bacteriology (1986) which is gram positive, did not have pigments, did not have photochromogenic colonies, did not grow at 45 ^{0}C, reduces nitrate, was tolerant of NaCl (28 ^{0}C), positive for malachite green 0.01 %, positive

for Pyronin B 0.01%, positive for pospatase acid, positive for picrate 0.2%. *M. fortuitum*was resistant to acids, metals, disinfectants, and some antibiotics (Falkinham, 2002).

Charaklis (1990) states that the stages of biofilm formation include: (1) adsorption of organic molecules which are stages of conditioning on solid surfaces (Loef and Neifof, 1975), (2) Transport of bacteria to the surface (Stanley, 1983), (3) Bacterial adsorption to the surface (Oliveira, 1992), (4) Release of attached cells to the liquid phase (Marshall et al., 1971) (5) Growth of adsorbed bacteria (Dawson et al, 1981), (6) Biofilm accumulation . (7) Release of biofilm cells (detachment).Factors that influence biofilm formation include the origin of isolates, growth media, incubation time, nutritional conditions, the nature of hydrophobicity, the content of extracellular polysaccharides and others. The factors used in this study include incubation time, nutritional conditions, and hydrophobicity properties of *M. fortuitum* biofilm formation on the surface of scales and PVC plastics.The type of scales in the fish used is the stenoid type which has a rough edge surface. The shape of fish scales with surface area + 1 cm2. Scales and plastic are grown separately for control, day 1, day 3, and day 5 in liquid media NB.

M. fortuitum has the ability to stick higher on the surface of the scales than PVC plastic surfaces. During the 1st day incubation period of 5 CFU logs / plates on the surface of fish scales and PVC plastic increased up to day 3 by 7 log CFU / plates on fish scales and 5 CFU logs / plates on PVC plastic. The addition of the number of biofilm cells is thought to result from the bond between bacteria and the surface of the plate. Increasing incubation time also affects the number of cells that have reproduced and attaches and produces EPS in the surface layer of the plate.

Table 2. Total colonies on fish scales and PVC plastic surfaces

Solid Surface	Average number of biofilm cells (CFU / plate)			
	Control	**Day 1**	**Day 3**	**Day 5**
Fish Scale	0	$2,47 \times 10^5$	$2,16 \times 10^7$	$2,04 \times 10^6$
PVC	0	$2,77 \times 10^5$	$7,1 \times 10^5$	$5,9 \times 10^5$

The number of colonies on the surface of the scales is higher compared to the plastic. This is related to the hydrophobicity of the plate surface type. The surface of the scales is more rough and has a different shape on each side which results in an accumulation of cell attachments in the surface area. The posterior part of fish scales has many circular and jagged curves. The type of fish scales is stenoids. In contrast to PVC plastic, the surface shape is finer than fish scales, so the attachment area does not accumulate much

Lactobacillus agilis was the result of identification of EK2 having stem cell form, gram positive, negative catalase cell size with a width of 0.7 - 1.0 μm and a length of 4.0 - 6.0 μm. The acid production of various carbohydrates to EK2 is positive for cellobinose, fructose, galactose, glucosa, lactose, maltose, manitol, mannosa, melezitosa, melibiosa, raffinosa, ribose, salicin, sucrosa, and trehalose, while negative for arabinose, gluconate, rhamnosa , sorbitol, xylosa, and starch. EK2 isolates can grow at temperatures of 15 0C, 20 0C, 25 0C, 30 0C, 37 0C, and 40 0C. PH testing showed that EK2 isolates were able to grow at pH 5, 6, 7.5, 8, 8.5, 9, and 9.5. The use of 6.5% NaCl and 18% inhibits the growth of EK2 isolates.

Gram staining tests showed that LAB isolates from pond sediments had purple coccus and basil cell forms which were gram positive (+) bacteria. Gram-positive (+) bacteria are bacteria that have a very thick layer of peptidoglycan on the cell wall, so when testing the gram stain this bacterium will remain blue like the color of crystal violet when given safranin liquid (Fardiaz, 1992).

B. Method

LAB isolation. Isolation of LAB by growing pure isolates on MRS agar media and incubating for 24 hours at 28 0C. Pure LAB isolates were then characterized by Gram staining, catalase test, motility, and presence or absence of spores. BAL identification refers to the Mannual Laboratory (Capuccino and Sherman, 2001). Storage of isolates in 24-hour-old culture in MRS agar media and 20% glycerol at 4 0C.

Production of Potential Antibacterial Compounds. The culture of LAB isolate was inoculated at 10 mL NB then incubated at 28 0C for 15 hours. To separate cells by filtrate, centrifugation was carried out for 15 minutes at 4 ^{0}C with a speed of 10000 x g (Suranto & Ratna 2005).

Antibacterial Test on *M. fortuitum*. This test was conducted to select potential LAB. A total of 25 µL *M. fortuitum* was inoculated on MHA media rubbed using a cotton bud evenly. A total of 30 µL of BAL antibacterial compounds were dispensed on disc paper placed on the surface of the cup containing test cultures, then incubated at 28 ^{0}C for 24 hours.

Identification of LAB. Identification of potential LAB is carried out by referring to the Bergey's Manual of Determinative of Bacteria 9th edition (Holt et al., 2000). LAB was tested for its growth ability at 10 ^{0}C, 20 ^{0}C, 37 ^{0}C, and 45 0C and at pH 3, 4, 5, 6, 8, and 9.2 as well as testing 17 types of fermented sugar as the key differentiator for species.

Prevention of Biofilm *M. fortuitum*with *Lactobacillus agilis*. Fish scales and PVC plastic plates were inserted into each sterile tube by adding 1 ml of *M. fortuitum* isolate cell concentration of 10^{8} CFU / mL and then adding BAL crude antibacterial compounds with concentrations of 60%, 80%, and 100% to the media. Then aerated every 2 minutes in 15 minutes at 28 ^{0}C. The contact time was carried out for 1 hour and calculated by the total plate count method by pouring 1 mL of culture on aerobic plate count agar media, incubated for 24 hours at room temperature 28 ^{0}C (Jamilah

&Priyani, 2012). Control in the form of distilled water containing each plate with 1 ml of the pathogen of *M. fortuitum* bacteria.

Control of Biofilm *M. fortuitum*Cells with *Lactobacillus agilis*. Biofilm of *M. fortuitum* isolates was prepared on various solid surfaces with the highest number of cells based on the treatment of biofilm formation. Biofilm of Fish scales and PVC plastic that have been calculated before each are inserted into a sterile tube and then added each potential BAL crude antibacterial compound is added. Then aerated every 2 minutes in 15 minutes at 28 ^{o}C. The contact time is carried out for 1 hour and calculated by the total plate count method by pouring 1 mL of culture on aerobic plate plate agar (PCA) media, incubated for 24 hours at a temperature room 28 ^{o}C. Control is fish scales and PVC plastic which have formed biofilm biofilms with the highest number of cells soaked with sterile aquades, then treated the same as treatment (Jamilah & Priyani, 2012).

C. Research Finding

Prevention of Biofilm *M. fortuitum* Cells on fish scales and PVC plastic surfaces

Prevention of biofilm formation on the surface of fish scales and PVC plastic is carried out simultaneously, namely by adding 1 ml of pathogenic *M. fortuitum* liquid culture, fish scales and PVC plastic, and the concentration of EK2 crude antibacterial compounds on NB media contacted for 1 hour. Biofilm formation was carried out to determine the number of colonies of *M. fortuitum* bacteria during 1 hour incubation. Prevention of formation was carried out with 9 ml of NB media on each plate without the addition of *M. fortuitum* bacteria.

The formation of *M. fortuitum* biofilm with a 1-hour incubation period is about 6 CFU logs / plates on each surface of the scales and plastic. Based on the calculation the number of colonies from *M. fortuitum* biofilm on fish scales surfaces amounting to about 2.86×10^6 CFU / plate and on the surface of PVC plastic there are 2.07×10^6 CFU / plates. Variations used in controlling *M. fortuitum* biofilm using EK2 with

concentrations of 60%, 80%, and 100%.The number of biofilm cell controls during 1 hour contact was 2 CFU logs / plates for each fish scales and PVC plastic plates. Cell growth occurs allegedly *M. fortuitum* to form biofilms on fish scales and PVC plastics because distilled water is a polar solution, thus helping the formation of *M. fortuitum* to stick on the scales and plastic surfaces. Nutrients can also affect the growth of biofilm cells. Dewanti and Hariyandi (1997) state that biofilm cells are able to form biofilms in very extreme nutritional conditions (without nutrition).

From various concentrations of EK2, 100% concentration has the ability to kill 100% to prevent *M. fortuitum* biofilm formation both on the surface of fish scales and PVC plastic. Antibacterial compounds of EK2 at a concentration of 60% are unable to prevent biofilm formation. The antibacterial compound EK2 at a concentration of 60% was thought to be diluted when mixing with NB media. Diluted EK2 may affect metabolite activity in *M. fortuitum* inhibitors. Jenie et al (200) stated that yeast and peptone extract is a composition of NB (g / L) as a source of amino acids or nitrogen which functions to stimulate bacterial growth. Whereas at 80% concentration it can prevent biofilm formation with a decrease of 2 CFU logs / PVC plastic plates but on the surface of fish scales there is no decrease in the number of biofilm cells at all. This may be due to the nature of the scales as biotic surfaces that are easily attached to biofilms because they have a more diverse and rugged surface structure compared to the slippery plastic surface.

Control of *M. fortuitum* Biofilm cells on fish scales and PVC plastic surfaces

Biofilm cells with the highest count on fish scales and PVC plastic surfaces was controlled using 100% antibacterial compound EK2 which was contacted for 1 hour having a decrease in cell count by 3 log CFU / plate on the surface of fish scales and 2 log CFU / plate on the surface of PVC plastic.Control of *M. fortuitum* biofilm using 9 ml of distilled water and 1 ml of M. fortuitum on each plate did not experience a decrease in the number of biofilm cells. This is because there is no inhibition done by aquades to reduce the number of biofilm cells.

Attachment of *M. fortuitum*cells with the highest number was obtained on the third day on the surface of fish scales by 75% and PVC plastic by 66%.

Controlling the biofilm cells using the formation of the 3rd day, according to Jamilah and Priyani (1999) the longer the incubation period of biofilms, the higher number of biofilm cells formed will be difficult to control. Barnes and Caskey (2002) state that biofilm formation will be increasingly complex with the production of EPS from bacteria attached to the substrate. The release of biofilm cells from fish scales and PVC plastic is more difficult than prevention of biofilm formation which is only contacted for 1 hour. The ability to prevent *M. fortuitum* biofilm formation by up to 100% is estimated that during 1 hour contact period is the stage of adaptation and transport of bacteria to the surface which can still be influenced by its release by gravity and movement of water flow (Stanley, 1983).

D. Discussion

It is necessary to purify crude extract antibacterial compounds so that the resulting activity value is higher. The characterization of antibacterial compounds that have been carried out *on M. fortuitum* bacteria needs to be supported by other information by determining activators, inhibitors, the influence of organic solvents, and molecular weight. Further research is needed regarding variations in contact time in the prevention of biofilm formation and control of *M. fortuitum* biofilm to determine the release of biofilm cells on the surface of fish scales and PVC plastic.

E. Conclusion

EK2 was identified as *Lactobacillus agilis*. The antibacterial compound of EK2 was more effective in preventing *M. fortuitum* biofilm formation for 1 hour compared to the control of biofilms that had been formed for 3 days.

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