
# Identification of Microbial Community in The Rearing Water Red Hybrid Tilapia (*Oreochromis mossambicus* x *Oreochromis niloticus)* Supplemented with Wood Vinegar

By

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**DECLARATION**

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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I certify that the report of this final year project entitled "Identification of Microbial Community in The Rearing Water Red Hybrid Tilapia (*Oreochromis mossambicus* x *Oreochromis niloticus)* Supplemented with Wood Vinegar" by CHOW WAI CHUN, matric number F14A0047 has been examined and all the corrections recommended by examiners have been done for the degree of Applied Science (Animal Husbandry Science) with Honours, Faculty of Agro Based Industry, University Malaysia Kelantan.

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## ABSTRACT

Red hybrid tilapia (*Oreochromis mossambicus x Oreochromis niloticus*) is an important aquaculture species for many countries and also commercially produced and consumed worldwide. Development of tilapia industry is significantly increased in Malaysia. However, the disease of tilapia affects the development of tilapia aquaculture and also cause the economic losses to the tilapia industry. In order to overcome the disease of tilapia, wood vinegar is applied as antimicrobial agent. Hence, study of the microbial community in the water of tilapia culture is necessary. Therefore, the detail of important microbial community in wood vinegar that play important role to overcome fish disease were revealed. In this study, water from the tilapia culture containing wood vinegar and the control without wood vinegar were cultured in the agar plate for pure bacteria isolation. The isolated bacteria were identified through Gram staining method, catalase test and oxidase test. In the tilapia culture containing wood vinegar and the control without wood vinegar, oxidase test showed oxidase negative and catalase test show catalase positive. From the Gram staining result, the result showed that there were more bacteria Gram negative compare to bacteria Gram positive but there are no significance. Further test for molecule analysis are required to carry out to further identify the bacteria species.

Keywords: *Oreochromis mossambicus x Oreochromis niloticus,* wood vinegar, Gram staining, oxidase test, catalase test

**Pengenalpastian mikrob Komuniti dalam Ternakan Air Tilapia Hibrid Merah (*Oreochromis mossambicus x Oreochromis niloticus*) ditambah dengan cuka kayu**

## ABSTRAK

Tilapia hibrid merah (*Oreochromis mossambicus x Oreochromis niloticus*) adalah spesies akuakultur penting untuk banyak negara dan juga dihasilkan secara komersial dan digunakan di seluruh dunia. Pembangunan industri tilapia meningkat dengan ketara di Malaysia. Walau bagaimanapun, penyakit tilapia memberi kesan kepada pembangunan tilapia akuakultur dan juga menyebabkan kerugian ekonomi dalam industri tilapia. Dalam usaha untuk mengatasi penyakit tilapia, cuka kayu digunakan sebagai agen anti-mikrob. Oleh itu, kajian tentang mikrob komuniti dalam air kultur tilapia adalah diperlukan. Oleh itu, terperinci daripada komuniti mikrob penting dalam cuka kayu yang memainkan peranan yang penting untuk mengatasi penyakit ikan sudah didedahkan. Dalam kajian ini, air dari kultur tilapia yang mengandungi cuka kayu dan kawalan tanpa cuka kayu sudah kultur dalam plat agar untuk bakteria pengasingan tulen. Bakteria terpencil sudah dikenal pasti melalui kaedah pewarnaan Gram, ujian katalase dan ujian oksidase. Dalam kultur tilapia yang mengandungi cuka kayu dan tanpa cuka kayu, ujian oksidase menunjukkan oksidase negatif dan ujian katalase menunjukkan katalase positif. Dari hasil pewarnaan Gram, hasilnya menunjukkan bahawa terdapat lebih bakteria Gram negatif bandingkan dengan bakteria Gram positif. ujian selanjutnya untuk molekul analisis perlu dijalankan untuk mengenalpasti lagi spesies bakteria.

Kata kunci: *Oreochromis mossambicus x Oreochromis niloticus,* cuka kayu, pewarnaan Gram, ujian oksidase, ujian katalase

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| | |
|---|---|
| TMPD | N, N, N, N-tetra methyl-p-phenylenediamine dihydrochloride |
| TSA | Tryptone Soy Agar |
| $H_2O_2$ | Hydrogen peroxide |
| µL | Millimetre |
| ° | Degree |
| X | Magnitude |
| % | Percentage |

# CHAPTER 1

## INTRODUCTION

In the present days, red hybrid tilapia (*Oreochromis mossambicus x Oreochromis niloticus*) serve as an important aquaculture species for many countries due to the high demand of the red hybrid tilapia. This species of red hybrid tilapia is cultivated commercially in order to supply and fulfil the consumer demand in the whole world. During the year 2003, the aquaculture industry had estimated to produce and cultivate a total amount of 2.3 million metric tons of tilapia (Boyd, 2004). However, the development in tilapia aquaculture had faced an obstacle, which is the spreading of diseases among the tilapia. This causes a great loss in terms of economic benefit to the tilapia industry. Even though there are reports that mention the tilapia have a better resistance towards bacteria compare to the other types of fish, but the tilapia is also susceptible to certain diseases (Boyd, 2004). An example of disease that can increase the mortality rate of the tilapia is Edwardsiellosis. In order to overcome Edwardsiellosis, wood vinegar is recommended to add into the water surrounding of the cultivation of tilapia fish.

Wood vinegar is a natural by-product that obtained from the charcoal production (Yatagai, Nishimoto, Hori, Ohira, & Shibata, 2002). Besides that, wood vinegar also can be known as pyroligneous acid (Mas, Torija, García-Parrilla, & Troncoso, 2014). Nowadays, wood vinegar is widely used as an antimicrobial agent

for the plants and crops. The wood vinegar is able to restrain the pests and diseases that spread among the plants and crops. The enhancement of certain beneficial microbes in the plants and crops can be done with the application of wood vinegar. This will further help to increase the resistance of the plants and crops toward diseases. Besides that, wood vinegar can be used to increase the fertility rate and promote the growth of the plants and crops. The application of wood vinegar also able to improve the soil quality and enhance the quantity yield of the fruit produced. Wood vinegar is generally safe and secure to be used and utilised in the livestock production typically aquaculture and even for the plant and crops. Certainly, the over dosage of wood vinegar will become toxic to the livestock, fish, plant and crops (Burnette, 2010).

Recently, wood vinegar had been discovered to be used in enhancing the fish production of the aquaculture fish farming (Nopadon Pirarat et al., 2015). Wood vinegar is one of the sources that can help to improve the resistance of fish toward the disease like Edwardsiellosis. The disease Edwardsiellosis that infect the tilapia are caused by the bacteria called *Edwardsiella tarda* (Kebede & Habtamu, 2016). Hence, a study in the microbial community for the wood vinegar is necessary in order to develop a better cultivation and production of tilapia fish.

## **1.1** Problem Statement

Wood vinegar have been widely used in plant and crops. Nowadays, wood vinegar has been analysed and discovered to be used as an antimicrobial agent for the plant and crop. This is to restrain the pests and overcome the disease of crops by

improving the resistance against the disease. The resistance against diseases can be done by the enhancement some beneficial microbes in the plant and crops (Yang et al., 2016). Until recently, there are only a few number of beneficial microbes that have been discovered and able to be enhanced by the wood vinegar in the plant and crops. However, there are not many research done and little information about the application of wood vinegar in the aquaculture field. Therefore, the study of the microbial community in wood vinegar for aquaculture is necessary to be applied for obtaining data and information about the importance in stimulating the fish immunity.

## **1.2** Research Question

1. What is the dynamic of the microbial community in tilapia culture compared to the tilapia culture supplemented with wood vinegar?
2. What is the important microbial community in wood vinegar that play important role to overcome fish disease?

## **1.3** Objectives

1. To determine the dynamic of the microbial community in tilapia culture compared to the tilapia culture supplemented with wood vinegar.
2. To determine the important microbial community in wood vinegar that play important role to overcome fish disease.

## 1.4 Scope of Study

This research focuses on the dynamic of the microbial community in wood vinegar and the importance of microbial community in wood vinegar that plays an important role in preventing the fish disease in tilapia.

## 1.5 Significance of Study

The aim of this study is to define the dynamic of the microbial community in tilapia culture compared to the tilapia culture supplemented with wood vinegar. Besides, this study focuses on the role of wood vinegar in the prevention of fish disease. There are only a few number of beneficial microbes that have been discovered and able to be enhanced by the wood vinegar in the plant and crops. However, there are not many research done and little information about the application of wood vinegar in the aquaculture field. Therefore, the study of the microbial community in wood vinegar for aquaculture is necessary to be applied for obtaining data and information about the importance in stimulating the fish immunity. Further study of the usage of wood vinegar will help in enhance the strategies for the prevention of disease of fish in aquaculture fish farming.

## **1.6** Limitation of Study

Redundant bacterial colonies might be resulted from this study and cannot be differentiated until the molecular identification had be done. Besides, there is a possibility that some important microbes might be lost during the cultivation of the colonies. This is because the culture media does not favour the growth of this bacteria.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Red Hybrid Tilapia

The red hybrid tilapia (*Oreochromis mossambicus x O. niloticus) is* a native aquaculture species in Africa. There are many tilapia aquacultures in others country outside of Africa (Kocher, Lee, Sobolewska, Penman, & McAndrew, 1998). Hence, tilapia aquaculture is the second most fish farming in the world. Tilapia can tolerate a wide range of salinity. However, tilapia can thrive at the salinity up to 15 ppt. Besides, tilapia grows well at the water temperature between 29 $^{o}$C and 31 $^{o}$C. Definitely, tilapia will stop feeding if the water temperature below 17 $^{o}$C. and high mortality will occur if the water temperature below 20 $^{o}$C. Hence, this is very important to maintain the water temperature for the commercial tilapia aquaculture. Furthermore, tilapia can tolerate lower levels of dissolved oxygen (DO) concentrations of less than 0.3 mg/L. Indeed, Nile tilapia can thrive at the levels of dissolved oxygen (DO) concentrations of above 1 mg/L. Disease resistance and growth of tilapia will decrease if the levels of dissolved oxygen (DO) concentrations of less than 1 mg/L in a long period. Besides, tilapia grows well at the range of pH value between pH 6 to pH 9. Furthermore, tilapia consumes wide range and many type of food such as plankton, detritus and decomposing organic matter. Market size for the tilapia is around 400g to 500 grams. Tilapia will take around

five to six months to grow up to around 400 to 500 grams under optimal condition (EVANS, PASNIK, PERES, LIM, & KLESIUS, 2005).

Nowadays, tilapia serve as an important aquaculture species for many countries and also commercially produced and consumed worldwide (Njiru & Okeyo-Owuor, 2004). However, the disease of tilapia affects the development of tilapia aquaculture and also cause the economic losses to the tilapia industry. Tilapia have good resistance from bacteria compare to the other type of fish, but there are some disease cause high mortality to the tilapia such as Edwardsiellosis (Kwon, Nam, Kim, Kim, & Kim, 2005). Hence, the aquaculturists need to find some ways to overcome the disease of tilapia (Yatagai et al., 2002). One way to overcome the disease of tilapia such as Edwardsiellosis is application of wood vinegar to tilapia culture (Nopadon Pirarat et al., 2015). The microbial community in the wood vinegar can be defined by using gram staining. Hence, the important role of wood vinegar to overcome fish disease can be revealed with define the dynamic of the microbial community in wood vinegar.

## 2.2 Wood Vinegar

Wood vinegar also known as liquid oil. In Philippines, wood vinegar known as *Mokusaku.* Wood vinegar need collected in a place with cool condition. Besides that, wood vinegar need to undergo sedimentation process after collected. Duration of the sedimentation process is almost six months. From the sedimentation process, toxic substances in the wood vinegar can be separated. As a result, three layers were

formed. In the middle part are wood vinegar while above part and the bottom part are water and the tar (Burnette, 2010).

Wood vinegar can be used together with the charcoal for the soil supplement. Wood vinegar can also use for fertigation to increase agriculture yield. Soil condition and soil nutrients can be improved by fertigation. Wood vinegar can activate beneficial microorganisms and increase the plant energetic. Besides, wood vinegar can control pathogenic bacteria in the soil and can used to feed good microorganisms (Hwang, Matsushita, Sugamoto, & Matsui, 2005).

Wood vinegar is a mixture of water and organic compounds. Wood vinegar obtained by extracting the smoke rising when burning the wood. Besides that, wood vinegar contains phenolic compounds and organic acids. Wood vinegar also used to remove bad odour in the animal farm. Wood vinegar also can increase egg production. This is because by adding wood vinegar in the feed that increase the feed intake of the hens. Intestinal calcium intake in rats also was improve by wood vinegar. Wood vinegar also increase the feed intake of the pigs. As a result, fed pigs with wood vinegar is better than other organic acids (Choi, Shinde, Kwon, Song, & Chae, 2009).

Wood vinegar can improve the crops quality, control pests and prevent plant disease and soil disease. Wood vinegar can help plants to grow strong roots (Tsuyoshi, Review, Joseph, Paananen, & Gould, 2015). Wood vinegar is natural by-product produced from charcoal production. Besides that, wood vinegar also called pyroligneous acid (Oramahi & Yoshimura, 2013). Nowadays, wood vinegar is widely

used as antimicrobial agent for the plant and crop to restrain the pests and overcome the disease of crops (Baimark & Niamsa, 2009).

Indeed, wood vinegar can help the plant and crops to improve the resistance against the disease by enhance some beneficial microbes to the plant and crops (Hwang et al., 2005). Besides, wood vinegar also used to promote the growth of the plant and crop. Furthermore, wood vinegar can improve soil quality and enhance the quantity yield of the fruit produced. Wood vinegar is safe and secure to be used for the livestock, fish, plant and crops (Zhang, Wei, Yao, Chao, & Xiaojuan, 2014). Wood vinegar will become toxic to the livestock, fish, plant and crops while applied overly (Burnette, 2010).

### 2.3 Microbial Community

Communities are known as multi-species assemblages, in which organisms live together in a contiguous environment and interact with each other. Conditions in microorganisms' microenvironments influence by the microorganisms react to and in turn. Microbes strongly interacting with each other in a microenvironment comprise a local community. An obligation for the constricted interrelationship between microbes and their microscale physical and chemical environments is important for delineation of microbial communities (Konopka, 2009).

Microbial community structures and composition can be correlated with the changes in certain functions. Temporal variability of microbial biomass dynamics is complex. Microbial community structure and composition also change with time (Hussain et al., 2012). The differentiation of microbial communities determines by examine the distribution of microbial groups and to identify the patterns of microbial abundance and community level diversity. The distributions of both Gram-negative and Gram-positive bacteria were significantly (Fierer, Schimel, & Holden, 2003).

## 2.4 Bacteria Colony Morphology

Bacteria are grow at media in form of colony. Colonies can be determined by the shape, form, margin, colour, elevation and size of the colony. The selected colony was circled, marked and assign number to each colony (Phumudzo, Ronald, Khayalethu, & Fhatuwani, 2013). Observe and record the colony morphology is important in bacteria identification (Meloni, Bertoloni, Busolo, & Conventi, 1980).

Bacterial colonies are normally shiny and smooth in appearance. Growth time, plate colony density and culture medium composition can affect the colony morphogenesis (Sousa, Machado, Nicolau, & Pereira, 2013). There are four types of colony form which are circular, irregular, filamentous and rhizoid in Figure 2.1. Five types of colony elevation are raised, convex, flat, umbonate and crateriform. Five types of colony margin are entire, undulate, filiform, curried and lobate.

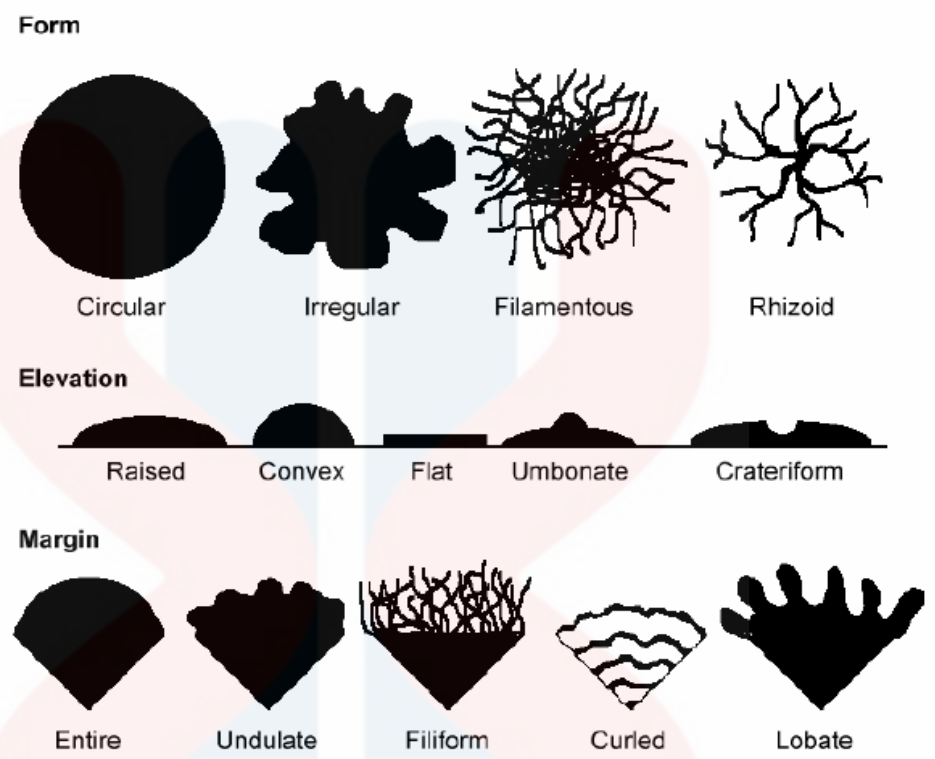


Figure 2.1: Basic colony morphology types.

**2.5 Gram Stain**

Gram-negative rods (GNR) are *Prevotella*, *Bacteroides*, *Fusobacterium*, and *Porphyromonas.* Gram-positive cocci (GPC) is *Streptococcus pyogenes*. GNR and GPC are contributing peritonsillar abscess. GNR are resistant to penicillin while GPC are sensitive to penicillin. (Takenaka, Takeda, Yoshii, Hashimoto, & Inohara, 2012).

In year 1883, gram staining procedure was introduced by Carl Friedlander. Positive gram staining material was inside the cell wall was introduced by Bartholomew and Mittwer. There are stages of gram stain in Figure A.1. An ionic bond formed

between the dye and the cell can help in primary stain. Dye has basic group while the cell has acid group. Iodine enter the cell and precipitate with dye was formed. Alcohol play important role in gram stain for the differentiation of gram negative bacteria and gram-positive bacteria. Alcohol easily pass though cell membrane and causes the gram-negative cell colourless and dissolves precipitate of iodine and dye. Alcohol difficult pass though cell membrane and causes the gram-positive cell very slow become colourless and difficult dissolves precipitate of iodine and dye. Counterstain is used to stain the colourless gram negative cell (Bartholomew & Mittwer, 1952).

Heat fixed smear can help to fix the bacteria to the slide. The bacteria are killed and smeared on the slide after the slide is heated in Figure B.1. Also, air dry was allowed then gram stain was carried out. Gram stain can help in differentiate the bacteria based on the cell morphology and gram reaction. In year 1921, gram stain was modified by Hucker. In year 1904, the final step of gram stain which is counterstain was introduced by Cart Weigert (Christian & Gram, 1980).

Fresh culture will provide a more accurate result and culture in broth form are more recommended. Refilter the crystal violet precipitate can prevent contamination. Clean the slide with the alcohol can provide a more accurate gram stain result. Gram positive cell hold the primary dye precipitate while gram negative cell releases the primary dye precipitate when rinse by ethanol or acetone. Gram positive cell contain peptidoglycan tightens cell wall and not allow the rinse enter the cell. Gram negative cell has lipid cell wall allow the rinse enter the cell and rinse out the dye precipitate. Thus, the gram-negative cell become colourless. Safranin is used in Counter stain.

From the counter stain, gram negative cell become pink in colour while gram positive cell become purple in colour (Beveridge, 2001).

Gram positive cell has thick cell wall that contain layers of peptidoglycan. The walls contain pores and the pores was closed to prevent dye and iodine complex escape when dehydrated by alcohol. Gram negative cell has thin layer of peptidoglycan that allow entering of the solvent. In gram negative cell, alcohol penetrates the thin layer of peptidoglycan that rich in lipid and remove the dye and iodine complex (Beveridge, 2001).

**2.6 Catalase Test**

Catalase test can help in determine presence of catalase enzyme in bacteria by breaking down hydrogen peroxide to the oxygen and water. Catalase can only break down hydrogen peroxide but other peroxide all cannot broken down by catalase. A false positive result may be produced while media include all red blood cells. Besides that, a false negative result may be produced while the culture is not fresh culture (Whittenbury, 1964).

Catalase test can help in differentiated *staphylococci* and *micrococci*. The growth of *alloiococci* are very slow. *Aerococci* and *alloiococci* are catalase positive but no presence of cytochrome enzymes and catalse. From porphyrin test, *stomatococci*

has presence of cytochrome although has negative catalase reactions (Facklam & Elliott, 1995).

Catalase activities of family member of *Enterobacteriaceae* has been studied. From the study, it was found that more advantage by using more amount of hydrogen peroxides. Thus, this is better use more than 3% of hydrogen peroxide ($H_2O_2$). Also, from the study it showed that, catalase test can complete by using put 3% of $H_2O_2$ on blood-containing medium. Most of the anaerobes are catalase negative. Catalase test is play an important role in differentiated aerobic and anaerobic organisms (Sharon & Slide, 2017).

Catalase test can be conduct by using blood plate, but not easy to carried out due to the colony not cultured well. This is because catalase test does not have reaction while the nutrient agar does not have colonies. In contrast, while catalase test conduct on blood agar catalase test do have some reaction such as although blood agar does not have colonies but there is some reaction occur such as a few bubbles occur (Fung & Petrishko, 1973).

From the study there are two types of method to differentiate family *Enterobacteriaceae* which are inoculated colony on the plate and add 3% of $H_2O_2$ by using dropper bottle, Pasteur pipette covered with cover slip and filter paper to the broth tube. From the result, the inoculated colony on the plate and add 3% of $H_2O_2$ by using dropper bottle and Pasteur pipette also have bubbles occur while catalase positive. It also showed that the most efficient and most sensitive catalase test method

is add 3% of $H_2O_2$ to the colony on the plate by using Pasteur pipette and covered with cover slip. This is because there are more sensitive under the cover slip (Taylor & Achanzar, 1972).

Viable cultures are available for catalase enzyme in catalase test. Part of the *Aerococcus* species and *Enterococcus* species may produce a weak catalase reaction. Hydrogen hydroxide must store in fridge and escape from light. This is very importance to let anaerobic bacteria exposed to the air around half an hour during culturing the bacteria. A false positive result may be produced while the inoculating loops react with hydrogen peroxide.

*Staphylococcus aureus* and *Streptococcus mitis* are the control organisms for the catalase test. *Staphylococcus aureus* is control organism for positive catalase test while *Streptococcus mitis* is control organism for negative catalase test. There are two types of method which are tube or bottle method and agar slant method. Tube or bottle method is adding hydrogen peroxide solution to the bottle. On the other hand, agar slant method is adding hydrogen peroxide solution to the agar slant.

Bubbles produced in the catalase test is caused by the oxygen gas break down from the hydrogen peroxide solution. Cell that need oxygen have to make catalase and also obtain oxygen by break down hydrogen peroxide. A false positive may be produced while the agar was contaminated. Also, a false positive may be produced while metal contact with hydrogen peroxide. It is suitable to put the slide with dark colour cover to observe the catalase test reaction so can easily observe the bubbles.

Catalase is enzyme that exist in almost all the bacteria that exposed to the oxygen. Catalase catalyse the breakdown of hydrogen peroxide to the oxygen and water. Catalase is very efficient and can breakdown large amount of hydrogen peroxide in a second. Catalase is made up of many amino acids and form a tetramer that contain four polypeptide chains. Iron group in the catalase can help catalase more easily attach to hydrogen peroxide. The optimum pH and temperature for catalase are different with different type of species.

Louis Jacques Thénard was recommended breakdown of hydrogen peroxide and also noticed catalase in year 1811. Oscar Loew was found catalase in plants and animal and also named catalase in year 1990. James B.Sumner and Alexander Dounce was crystallised catalase in year 1937. In year 1938, the molecular weight was found (HSI, 2014).

Hydrogen peroxide has strong oxidation capacity, it causes the hydrogen peroxide become toxic. Lactic acid bacteria utilise sugar and alcohols while presence of oxygen. This is because oxygen used as hydrogen acceptor (Whittenbury, 1964). Catalase was described as first bacterial enzymes by Gottstein in year 1893. In year 1923, first bacterial classification arrangement was established by McLeod and Gordon (HSI, 2014).

Catalase is used to neutralize hydrogen peroxide. There are two types of method which are slide method and tube method in Figure C.1. In the slide method,

hydrogen peroxide added to the colony on the slide. In the tube method, hydrogen peroxide added to the colony into the test tube (Reiner, 2013).

## 2.7 Oxidase Test

Oxidase test was published by Gordon and McLeod for classifying *Gonococci*. Oxidase test can used for classifying *Gonococci* by observe the presence of indophenol blue from the bacteria. Indophenol blue will form while bacteria undergo oxidation of dimethyl-p-phenylenediamine and α-naphthol. A better method was published by Gaby and Hadley in year 1958. In this method, N, N-dimethyl-p-phenylenediamine oxalate was used in oxidase test. From the result, it showed that all *Staphylococci* were oxidase negative (Shields & Cathcart, 2013).

Gram negative bacteria contain the enzyme cytochrome oxidase. N, N-dimethyl-p-phenylenediamine oxalate and α-naphthol will react to indophenol blue when presence of the enzyme cytochrome oxidase. Oxidase test can help in differentiate *Neisseria* from other gram-negative diplococci. Besides that, oxidase test can help in differentiate *Aeromonas hydrophila* from *Escherichia coli.* Also, oxidase test can help in differentiate *Plesiomonas shigelloids* from *Shigella sonnei* (Prior, Methods, Methods, & Level, 2011).

Bacteria can divide to oxidative positive bacteria and oxidative negative bacteria by observe the oxidise of N, N, N, N-tetra methyl-p-phenylenediamine

dihydrochloride (TMPD). Bacteria that has respiratory mechanism will get high TMPD oxidase values. Lactic acid bacteria have low TMPD oxidase values. This is because lactic acid bacteria lack of type c cytochrome and lack of cytochrome that contain electron transport system. Terminal oxidase activity in diversity of physiological bacteria can be measured by TMPD oxidase reaction. TMPD oxidation require type c cytochrome in bacterial electron transport systems. *Escherichia coli* is the oxidase negative bacteria that has the highest TMPD oxidase values (Jurtshuk & Mcquitty, 1976).

Oxidase test can help in determine presence of the cytochrome oxidase enzyme in the organism. Besides, oxidase test can help in differentiate *Moraxella*, *Neisseria*, *Pasteurella* and *Campylobacter* species. Also, oxidase test can help in differentiate *Pseudomonads* species. Transport of electrons from NADH to oxygen electron acceptors can be catalysed by cytochrome oxidase or indophenol oxidase. Normally, oxidase positive bacteria contain cytochrome oxidase or indophenol oxidase. N, N, N, N-tetra methyl-p-phenylenediamine dihydrochloride (TMPD) is the oxidase test reagent. It was used as electron acceptor for the enzyme oxidase. Indophenol blue coloured compound was form by the oxidase reagent (Hemraj, Diksha, & Avneet, 2013).

Aerobic organisms that can utilise oxygen as final hydrogen acceptor normally contain a cytochrome system. Water or hydrogen peroxide will be the end product of the metabolism. Media that contain tellurite and fermentable carbohydrates not suitable for oxidase test. This is because the reaction of the oxidase test may prevent by the media that contain tellurite and fermentable carbohydrates. The media must be fresh cultured. A false positive result may be produced while oxidation products formed

during flame sterilisation. Nichrome inoculated loops is not recommended to use in oxidase test (Shields & Cathcart, 2013).

*Pseudomonas aeruginosa* and *Acinetobacter lwoffii* are control organisms for oxidase test. *Pseudomonas aeruginosa* is oxidase positive while *Acinetobacter lwoffii* is oxidase negative. There are four types of oxidase test method which are filter paper method, direct plate method, swab method and impregnated oxidase test strip method in Figure D.1. In the filter paper method, filter paper put into the reagent solution. In direct plate method, two drops of reagent added to the colonies on the agar plate. In swab method, the swab dip into the reagent then attach to the colony. In the impregnated oxidase test strip method, scrape fresh growth with loop and stick on the filter paper. For observation, blue colour was examined within ten second.

Oxidase test can help in identifying of *N.meningitids*. Besides that, other members of genus *Neisseria* may also oxidase positive (Prior et al., 2011). Oxidase test can also help in identifying Neisseria, Aeromonas, Alcaligenes, Pseudomonas, Campylobacter and vibrio's which are oxidase positive while Enterobacteriaceae which is oxidase negative. Oxidase positive organisms have higher TMPD oxidase value while oxidase negative organisms have lower TMPD oxidase value (JURTSHUK & MCQUITTY, 1976).

*Neisseriacae* and *Pseudomonadacae* are oxidase positive while Enterobacteriaceae is oxidase negative. In year 1957, cytochrome oxidase test in a broth culture was introduced by Gaby and Hadley. In year 1960, the use of agar slope

cultures in oxidase test was introduced by Ewing and Johnson (STEEL, 1961). In year 1928, use of dimethyl-*p*-phenylenediamine dihydrochloride solution in oxidase test and oxidase systems in oxidase test was introduced by Gordon and McLeod.

Oxidase test can help in differentiate *Neisseria gonorrhoeae* from *Staphylococcus* species and *Streptococcus* species. *Neisseria gonorrhoeae* is oxidase positive while *Staphylococcus* species and *Streptococcus* species are oxidase negative. In oxidase test, the use of tetra-methyl-*p*-phenylenediamine dihydrochloride solution can increase the sensitivity of the oxidase test.

# CHAPTER 3

# METHODOLOGY

## 3.1 List of Material and Apparatus

### 3.1.1 Chemicals and Reagents

Wood vinegar, Tryptone Soy Agar (TSA), alcohol, sterile distilled water, Hydrogen peroxide ($H_2O_2$), N, N, N, N-tetra methyl-p-phenylenediamine dihydrochloride (TMPD), Gram stain reagents.

### 3.1.2 Equipment and Apparatus

Water tank, 100 µL micropipette, portable bunsen burner, glass spreader, incubator, rubber glove, inoculating loop, micropipette tips, cotton swab, dropper, glass slide, light microscope, parafilm.

## 3.2 Nile tilapia, *Oreochromis niloticus*

In this study, the species of the red hybrid tilapia is *O. mossambicus x O. niloticus*. The cultivation and growth of the fingerlings size red hybrid tilapia with a weight of three to five grams was done in the water tank that located at the aquaculture laboratory, UMK Campus Jeli. Commercial fish pellet was used to cultivate the growth of the red hybrid tilapia. For every single water, 6 fingerlings were culture and placed in the water tank. The fingerlings were separated and growth at two different types of environment. A ratio of 1:500 for the dilution of wood vinegar by water were used to create one of the water environment, while another water environment does not consist any wood vinegar. The water environment that does not have any wood vinegar were act as the control part of this research. Three biological replicates were used for each treatment. The three to five grams fingerlings size red hybrid tilapia (*O. mossambicus x O. niloticus*) were cultured with wood vinegar solution for the duration of approximately four weeks' time.

## 3.3 Sampling and analysis of culturing water

A total number of four water samples were collected throughout the whole conduction of this research. The first water sample was collected in the first week after the wood vinegar is added into the water tank. After a duration of three days reaches, the second water sample was collected before the water that originally in the water tank were replaced. The third water sample was collected during the fourth week of this research conducted by adding in the wood vinegar into the water tank. After

another time duration of three days passed, the last water sample was collected before the water that originally in the water tank were replaced.

## 3.4 Isolation Water Sample Method

### 3.4.1 Serial Dilution

The serial dilution method was used to dilute the water sample collected. One conical flask and seven test tubes were prepared beforehand and the serial dilution were marked by 100ml of the dilution blank which is $10^{-2}$, and continuously with $10^{-3}$, $10^{-4}$ and $10^{-5}$. The water sample were poured into the $10^{-2}$ dilution blank and were further shaken vigorously. After that, $10^{-2}$ blank were enabled to wait for 30 seconds. Then, 1ml of the $10^{-2}$ dilution were transferred into the $10^{-3}$ tube by using micropipette. Then, fresh and sterilized pipettes were used to continue the next step, where the 1ml of $10^{-3}$ dilution was transferred into $10^{-4}$ dilution blank, and the 1ml of $10^{-4}$ dilution was transferred into the $10^{-5}$ dilution blank.

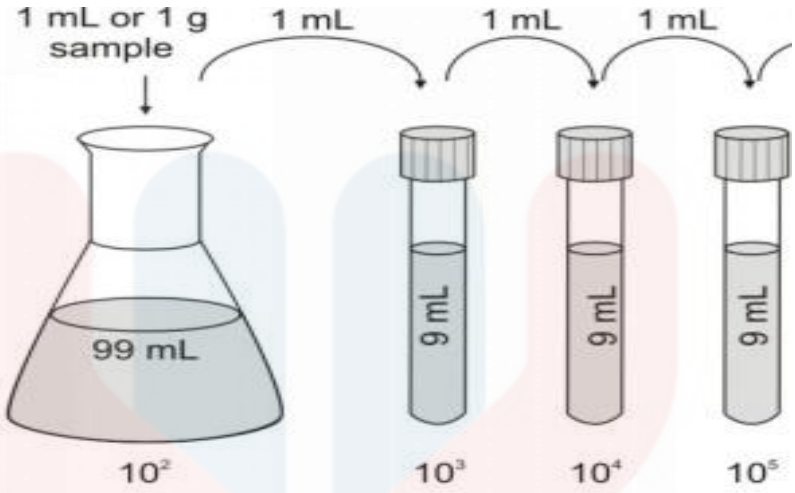


Figure 3.1: The water sample was diluted, and serial dilution was prepared. Source: (http://elte.prompt.hu/sites/default/files/tananyagok/PracticalMicrobiology/ch05s03.html)

### 3.4.2 Spread Plate Method

0.1 mL of $10^{-5}$ diluted sampling solution from the water tank was transferred into the Tryptone Soy Agar (TSA) by using the micropipette. The micropipette tips did not reuse and once the micropipette tips were used, a new micropipette tips were change and replaced. The glass spreader was sterilised with alcohol and further flamed with Bunsen burner. Firstly, the glass spreader was placed into the alcohol. After that, the glass spreader was put through the Bunsen burner to burn the glass spreader. Hence, the glass spreader that contain alcohol was burn and sterilized by the flame of Bunsen burner. After the glass spreader are enable to cool down for few seconds, the glass spreader was used to spread the diluted sampling solution that transferred onto the surface of the Tryptone Soy Agar (TSA). Then, the Tryptone Soy Agar (TSA) plate was rotated with the direction of top-to-bottom or side-to-side motion in order to spread the diluted sampling solution on the surface of the Tryptone Soy Agar (TSA). After the

Tryptone Soy Agar (TSA) plate being covered and sealed with plastic paraffin film, the Tryptone Soy Agar (TSA) plate was incubated with an inverted position in the incubator at 37°C.

### 3.4.3 Streak Plate Method

After the Tryptone Soy Agar (TSA) plate that had been spread with diluted sampling solution and incubated overnight, there are lots of colonies of cells formed or growth on surface of the agar inside the Tryptone Soy Agar (TSA) plate. Then, an inoculating loop was used for the streak plate of the bacteria colony. The inoculating loop was sterilised by flame it with the Bunsen burner before use. A single well-isolated colony was spotted and scoop up from the Tryptone Soy Agar (TSA) plate by using the sterile inoculating loop. Then, a first streak was started in the direction of backwards and forwards. After first streak has been done, the Tryptone Soy Agar (TSA) plate was turned around 90° anticlockwise direction for the process of second streak. The second streak was just the same process like the first streak by streaking in three to four parallel lines with the direction of backwards and forwards. Next, the third streak and fourth streak were repeated in the same ways like the first streak and second streak mentioned previously. After the streaking of the colony on the Tryptone Soy Agar (TSA) finished, the plate was covered and sealed with plastic paraffin film. Then, the Tryptone Soy Agar (TSA) plate was incubated with an inverted position inside the incubator.

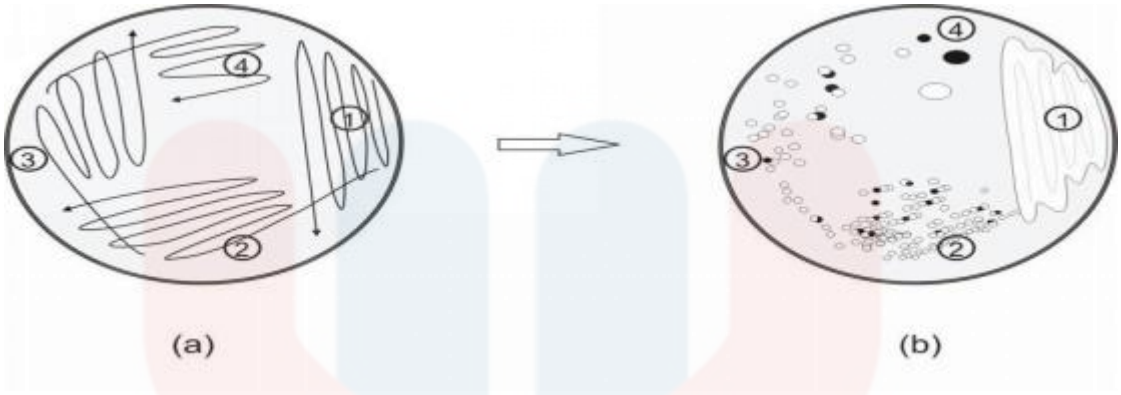


Figure 3.2: Streaking Technique. Adapted from (http://blogs.fit.edu/wp-content/uploads/2014/05/Florida-Tech-Streaking-1.jpg)

### 3.5 Gram Staining Test

The colony that inoculated on the Tryptone Soy Agar (TSA) plate was used for the Gram staining test. A single colony was picked and choose from every single streaked Tryptone Soy Agar (TSA) plate to undergo gram staining. Firstly, the bacteria smears were stained with Crystal Violet for one minute. Then, rinse with water. Next, stain the smears with Gram's Iodine for one minute. After that, rinse carefully with acetone and rinse only until the blue colour stops coming out of the smear. Then, rinse with water and counter-stain with Safranin for at least ten seconds. Next, rinse with water and allowed to be air dried. After the slide had completely dry, the slide was place to the microscope. The low power (10X) lens was used for finding a good field. A drop of immersion oil was added and immerse the 100X lens into the oil on the sample glass slide by rotating the nosepiece.

### 3.6 Oxidative Test

The targeted colony was inoculated by cotton swab. After that, 1% of N, N, N, N-tetra methyl-p-phenylenediamine dihydrochloride (TMPD) that act as Kovacs oxidase reagent was added onto the surface of the cotton swab. The reactions were observed within five to ten seconds. Purple colour that appeared within five to ten seconds indicates a positive reaction.

### 3.7 Catalase Test

A glass slide was cleaned and the targeted colony was inoculated by using inoculating loop and placed on the centre of the glass slide. After that, a drop of 3% hydrogen peroxide was placed to the targeted colony. The inoculating loop was sterilized after each inoculation that had been done. Bubbles produced or formed indicates the reaction are positive, while on the other hand, it will be a negative reaction.

# CHAPTER 4

## RESULTS AND DISCUSSION

Total four water sample were taken with four different times. The first water sample was taken during the first week after the wood vinegar was added into the water tank. After three days, the second water sample was collected before any changes to the water. The third water sample was collected during the fourth week after the wood vinegar was added into the water tank. After another three days, the last water sample was collected before the changes to the water.

The water sampled were diluted by serial dilution method. Amount of 0.1 mL of $10^{-5}$ diluted solution sampling from the water tank has been transferred into the Tryptone Soy Agar (TSA). From the spread plate and streak plate, the colony was inoculated and the morphological of bacteria was observed and recorded. The morphological of bacteria was shown and tabulated in the Table 4.1.

Based on the Figure 4.1, the growth of the bacteria on nutrient agar was observed and recorded. The colony that growth on the nutrient agar were used for gram staining test, catalase test and oxidase test. Hence, the colony morphology such as form, elevation and margin of each colony was recorded and observed.

Figure 4.1: Growth of colony bacteria on nutrient agar.

Based on the result observed, the sample mainly showed a circular form, raised elevation, entire margin and creamy white colour colonies. It was obviously that there are no contaminations throughout the whole conduction of the streak plate as the growth of the bacteria on nutrient agar along the streaking line and all the colonies do share the same colony morphology with the original colony. From the result, some of the bacteria is small, so they need a big surface-to-volume ratio to support their inside body biochemistry.

The growth of bacteria is depending on the nutrient intake of the bacteria. The bacteria in the nutrient agar have different ability in competition on nutrient. The cell shape in and of itself, and there are consist other nutritional situations may create conditions that suitable one bacterial shape over another. Besides, motility also will affect the growth of the bacteria. Motility establish heavy particular pressure on cell shape. A certain length-to-width ration are better off as rod by the fast cells. Also, shape

ratios in line with the environments must be adapted by the chemotactic cells. Besides that, cells that near surfaces or drive to viscous environments may do best if they are lightly spiral or curve. Predation also will affect the growth of the bacteria. The bacteria may have grown various type of cell shapes to prevent eaten by protozoa (Young, 2007).

Nutrient agar is a medium that contain high nutrient content and can be used as general purpose to culture various type of microorganism (Uthayasooriyan, M., Pathmanathan, S., Ravimannan, N., & Sathyaruban, 2016). Thus, it can help in the identification and classification of bacteria by observing the colony morphology. Also, it can provide very useful information and data for the bacteria cultured.

Bacterial colonies are normally shiny and smooth in appearance. Growth time, plate colony density and culture medium composition can affect the colony morphogenesis (Sousa et al., 2013). The morphological of bacteria was shown and tabulated in Table 4.1. The presumptive bacteria genus was shown and tabulated in Table 4.2.

Table 4.1: The morphological of bacteria that cultured from the water sample collected on four different times.

| Sampling | Treatment | Sample ID | Form | Elevation | Margin | Colour |
|---|---|---|---|---|---|---|
| 1 | With Wood Vinegar | A1, A3, C1, C2 | Circular | Raised | Entire | Creamy White |
| | | B1 | Circular | Flat | Entire | Creamy White |
| | | B2, B3 | Circular | Raised | Entire | Pale Yellow |
| | | A2, C3 | Circular | Raised | Undulate | Creamy White |
| | Control | D1, E2, E3, F1, F2, F3 | Circular | Raised | Entire | Creamy White |
| | | E1 | Circular | Flat | Entire | Creamy White |
| | | D2 | Circular | Raised | Entire | Pale Yellow |
| | | D3 | Circular | Raised | Undulate | Creamy White |
| 2 | | A1, A3, B1, B2, | Circular | Raised | Entire | Creamy white |

| | | | | | | |
|---|---|---|---|---|---|---|
| | With Wood Vinegar | B3, C1, C2, C3 | | | | |
| | | A2 | Circular | Convex | Entire | Creamy white |
| | Control | D1, D2, E1, E2, E3, F1, F3 | Circular | Raised | Entire | Creamy white |
| | | D3, F2 | Circular | Raise | Entire | Creamy white |
| 3 | With Wood Vinegar | A1, A3, B1, C1, C2, C3, | Circular | Raised | Entire | Creamy white |
| | | A2, B2 | Circular | Raised | Undulate | Creamy white |
| | | B3 | Circular | Flat | Entire | Creamy white |
| | Control | D1, D2, D3, E1, E2, E3, F1 | Circular | Raised | Entire | Creamy white |
| | | F2 | Circular | Raised | Undulate | Creamy white |
| | | F3 | Circular | Flat | Entire | Creamy white |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4 | With Wood Vinegar | A1, A2, B1, B2, B3, C1, C2, C3 | Circular | Raised | Entire | Creamy white |
| | | A3 | Circular | Raised | Undulate | Creamy white |
| | Control | D1, D2, D3, E1, E2, F1, F2 | Circular | Raised | Entire | Creamy white |
| | | F3 | Circular | Raised | Undulate | Creamy white |
| | | E3 | Circular | Flat | Entire | Creamy white |

Table 4.2: The presumptive bacteria genus that cultured from the water sample collected on four different times.

| Sampling | Treatment | Sample ID | Gram Stains (+/-) | Shape | Catalase (+/-) | Oxidase (+/-) | Presumptive Bacteria Genus |
|---|---|---|---|---|---|---|---|
| 1 | With Wood Vinegar | A1, A3, B3, C2, C3 | - | Coccus | + | - | Staphylococcus |
| | | A2, B1 | + | Coccus | + | - | Staphylococcus |
| | | B2 | + | Bacillus | + | - | Staphylobacillus |
| | Control | E2, E3, F1, F2, F3 | - | Coccus | + | - | Staphylococcus |
| | | D1, D2 | + | Bacillus | + | - | Staphylobacillus |
| | | D3, E1 | + | Coccus | + | - | Staphylococcus |
| 2 | With Wood Vinegar | A1, A2, B2, C1, C2, C3 | - | Coccus | + | - | Staphylococcus |
| | | A3, B1, B3 | - | Bacillus | + | - | Staphylobacillus |
| | Control | D2, D3, E1, F1 | - | Coccus | + | - | Staphylococcus |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | E2, E3, F2, F3 | + | Coccus | + | - | Staphylococcus |
| | | D1 | - | Bacillus | + | - | Staphylobacillus |
| 3 | With Wood Vinegar | A1, A3, B2, B3, C1, C2, C3 | - | Coccus | + | - | Staphylococcus |
| | | A2, B1 | + | Coccus | + | - | Staphylococcus |
| | Control | D1, D2, D3, E1 | - | Coccus | + | - | Staphylococcus |
| | | E3, F1, F2 | + | Bacillus | + | - | Staphylobacillus |
| | | E2 | - | Bacillus | + | - | Staphylobacillus |
| 4 | With Wood Vinegar | A1, A3, B1, B3, C1, C2, C3 | - | Coccus | + | - | Staphylococcus |
| | | A2 | - | Bacillus | + | - | Staphylobacillus |
| | | B2 | + and - | Bacillus | + | - | Staphylobacillus |
| | Control | D2, D3 | - | Coccus | + | - | Staphylococcus |

| | | | | | | |
|---|---|---|---|---|---|---|
| | D1, E1, E2, F1, F2, F3 | - | Bacillus | + | - | Staphylobacillus |
| | E3 | + | Bacillus | + | - | Staphylobacillus |

**4.1 Gram Staining**

Gram staining is a method that can used to differentiate microorganisms in the microbiology field. This method can differentiate the bacteria into gram positive bacteria and gram-negative bacteria. Besides that, there are various type of microorganism that can be differentiated and identified by the gram staining method.

Gram positive bacteria cell has a thick layer of peptidoglycan and low lipid content. While decolourizing the bacteria cell, the thick wall will dehydrate and enclose the pores in the cell wall. This causes the colour of the stain could not exists in the cell. Then, the acetone cannot remove the crystal violet iodine complex which is stick to the thick layer of the peptidoglycan. Hence, the purple colour of the stain was observed.

On the other hand, gram negative bacteria cell has a thin layer of peptidoglycan and high lipid content. While decolourizing the bacteria cell, the decolourizers will dissolve the lipids in the cell wall. Then, the crystal violet complex was able to be

remove by the alcohol. Hence, the red colour was observed when the bacteria smear on the glass slide was counter-stained with safranin.

However, gram staining was not able to completely provide information for the identification of the species of the bacteria that just totally depend on the shape and the colour of the bacteria. Thus, further biochemical test such as catalase test and oxidase test were carried out to further the identification of the species for the bacteria cultured. Figure 4.2 showed result of gram staining where gram positive bacteria were stained. Figure 4.3 showed result of gram staining where gram negative bacteria were stained.

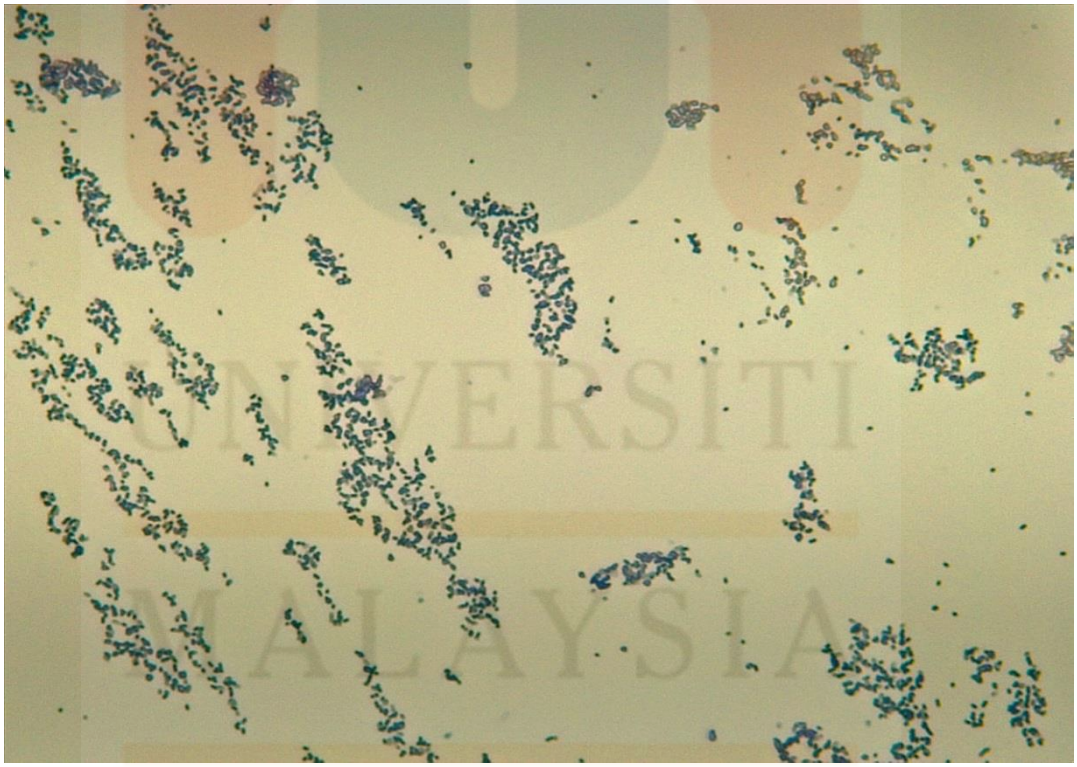

Figure 4.2: Gram positive bacteria were stained.

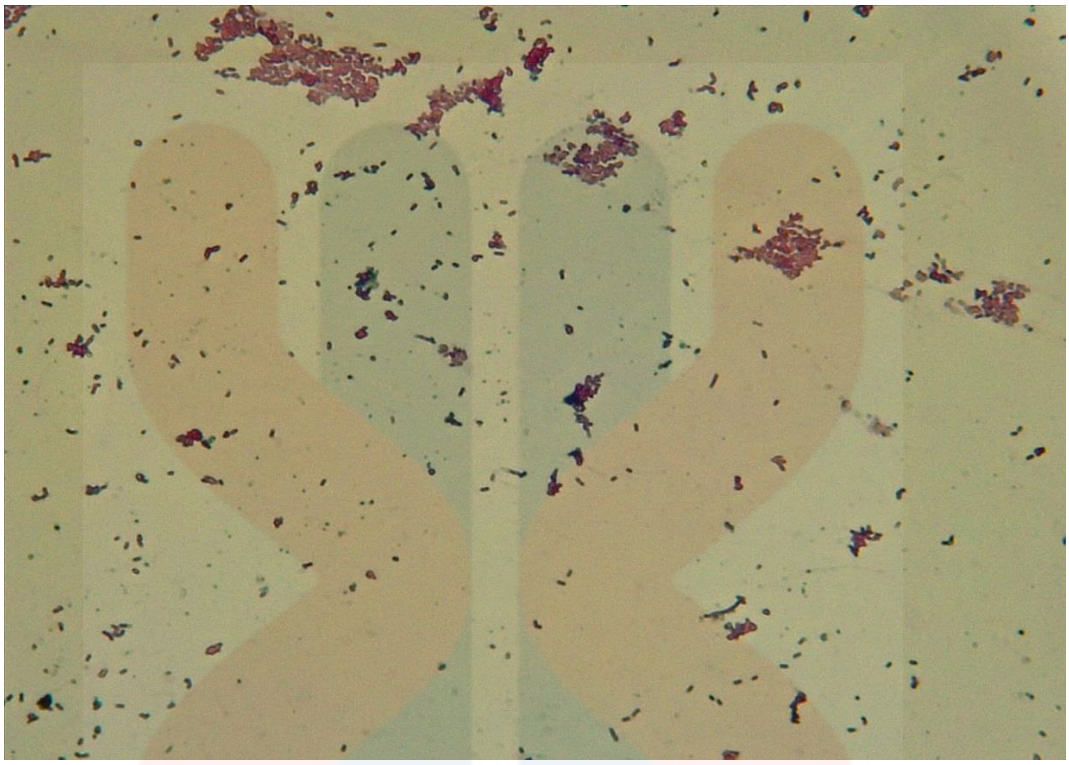

Figure 4.3: Gram negative bacteria were stained.

**4.2 Catalase Test**

Catalase test was performed by using the chemical, hydrogen peroxide as an indicator. Positive reaction was resulted from the production of bubbles, while the result will be another way round, a negative reaction. From the Figure 4.4, it was observed as catalase positive. This is because the bacteria were appeared with lots of bubbles after the chemical hydrogen peroxide was added (Facklam & Elliott, 1995).

Catalase test is a method that can be used to differentiate microbes in the microbiology field. This method is able differentiate the species *Staphylococci* bacteria

and the species *Streptococci* bacteria. The result that had been observed and showed as catalase positive, the bacteria was considered as *Staphylococci*, otherwise the bacteria will be consider as *Streptococci* when the result showed a catalase negative (Taylor & Achanzar, 1972).

Catalase is a common enzyme that produced by living organisms which respire using oxygen, such as bacteria. The enzyme catalase acts as a catalyst in the decomposition of the hydrogen peroxide into oxygen and water. The enzyme catalase can prevent the damage of the bacteria during the oxygen metabolism (Fung & Petrishko, 1973).

In this study, it was suggested that the absence and presence of wood vinegar does not affect the distribution and the presence of *Staphylococci* bacteria and *Streptococci* bacteria. This is because the result of catalase test for the treatment with wood vinegar and without wood vinegar also gives out a catalase positive result. Wood vinegar will give out catalase positive result and presence of *Staphylococci* bacteria (Ashtavinayak & Elizabeth, 2016).

Hydrogen peroxide is unstable, so it need to be refrigerated and avoid from the exposure of sunlight. Besides that, the culture that used in the catalase test must be fresh, otherwise a false catalase negative results can be produced. Also, the apparatus that used in the catalase test such as the glass slide must be sterile before used, otherwise a false catalase positive results can be produced (Whittenbury, 1964).

In this study, it was suggested that the absence and presence of wood vinegar does not affect the distribution and the presence of *Staphylococci* bacteria and *Staphylobacilli* bacteria. This is because the result of catalase test for the treatment with wood vinegar and without wood vinegar also gives out a catalase positive result.

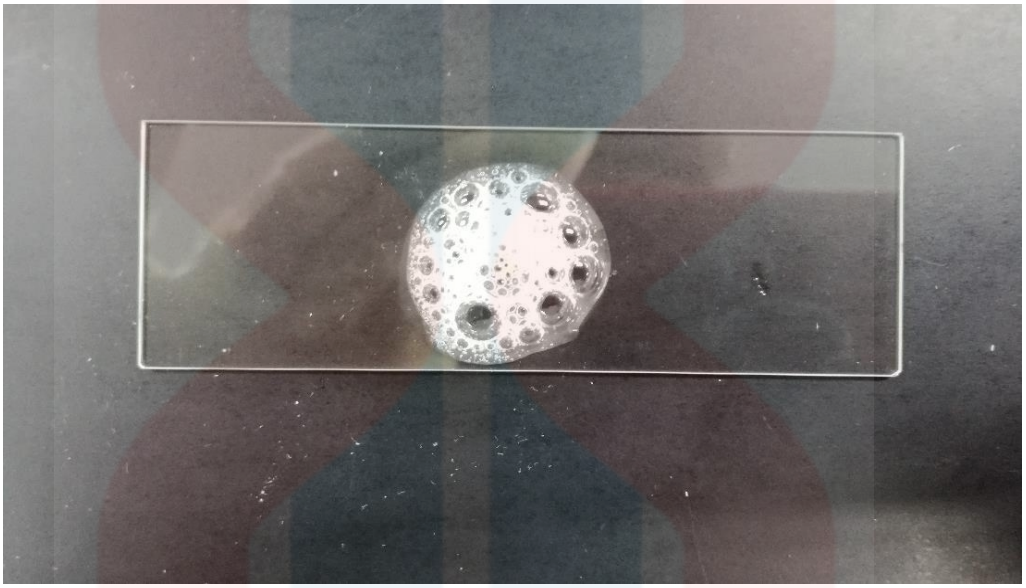

Figure 4.4: Result of catalase positive as a lot of bubbles can be observed.

**4.3 Oxidase Test**

Oxidase test is performed by using the chemical, N, N, N, N-tetra methyl-p-phenylenediamine dihydrochloride (TMPD) as an indicator. The targeted colony was inoculated by using cotton swab. After that, 1% of N, N, N, N-tetra methyl-p-phenylenediamine dihydrochloride (TMPD) that act as Kovacs oxidase reagent was added to the surface of the cotton swab. The observation for the reaction was done within five to ten seconds. Purple colour that appeared within five to ten seconds indicates a positive reaction otherwise it was a negative reaction. Figure 4.5 showed

oxidase negative as cotton swab did not have any colour changes within the time of ten seconds.

Oxidase test is a method that can be used in the differential of microbes in the microbiology. This method can differentiate *Staphylococci* bacteria and *Micrococci* bacteria. The oxidase test is used to determine the presence of cytochrome c oxidase which is an enzyme that plays an important role in the electron transport chain. Oxidase reagent such as N, N, N, N-tetra methyl-p-phenylenediamine dihydrochloride (TMPD) are able to determine the ability of the bacteria to produce cytochrome c oxidase. While the result showed oxidase positive, the bacteria are considered as aerobic bacteria otherwise the bacteria can be considered as anaerobic bacteria due to the oxidase negative result produced. In this study, it was discovered that the bacteria which was found in the rearing water of the tilapia are anaerobic due to the negative results produced from the oxidase test. This means that the bacteria do not require any oxygen in their respiratory process (Shields & Cathcart, 2013).

Gram negative bacteria contain the enzyme cytochrome oxidase. N, N-dimethyl-p-phenylenediamine oxalate and α-naphthol will react to indophenol blue when presence of the enzyme cytochrome oxidase. Bacteria that has respiratory mechanism will get high TMPD oxidase values. Lactic acid bacteria have low TMPD oxidase values. This is because lactic acid bacteria lack of type c cytochrome and lack of cytochrome that contain electron transport system. Terminal oxidase activity in diversity of physiological bacteria can be measured by TMPD oxidase reaction. TMPD oxidation require type c cytochrome in bacterial electron transport systems. (Jurtshuk & Mcquitty, 1976).

In this study, it was suggested that the absence and presence of wood vinegar does not affect the distribution and the presence of *Staphylococci* bacteria and *Staphylobacilli* bacteria. This is because the result of oxidase test for the treatment with wood vinegar and without wood vinegar also gives out an oxidase negative result (Prior et al., 2011).

Figure 4.5: Result of oxidase negative as cotton swab did not change the colour within ten seconds.

# CHAPTER 5

## CONCLUSION AND RECOMMENDATION

In conclusion**,** the objective of this study was achieved. Throughout this research, the gram staining method is successfully identified the bacteria isolated. In this research, in total there are 49 bacteria Gram negative and 20 bacteria Gram positive. In tilapia culture supplemented with wood vinegar, there are 8 Gram positive and 26 Gram negative. In tilapia culture without wood vinegar, there are 12 Gram positive and 23 Gram negative. There are more Gram negative compare to Gram positive but there are no significance.

Throughout this research, biochemical test such as oxidase test and catalase test are successfully identified the bacteria isolated. In this research, oxidase test showed oxidase negative while catalase test showed catalase positive.

In tilapia culture supplemented with wood vinegar the fish growth well and do not have fish disease but there is no significance. In tilapia culture without wood vinegar the fish also do not have fish disease. In this research, there are no evidence to determine the important role of wood vinegar to overcome fish disease, but it sure that wood vinegar does not cause negative impact to the fish.

The recommendation for this research such as gram staining difficult differentiate the bacteria species by only observe the morphological characteristic. Hence, further test such as Polymerase Chain Reaction (PCR) are required to carry out to further identify the bacteria species.

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## APPENDIX A

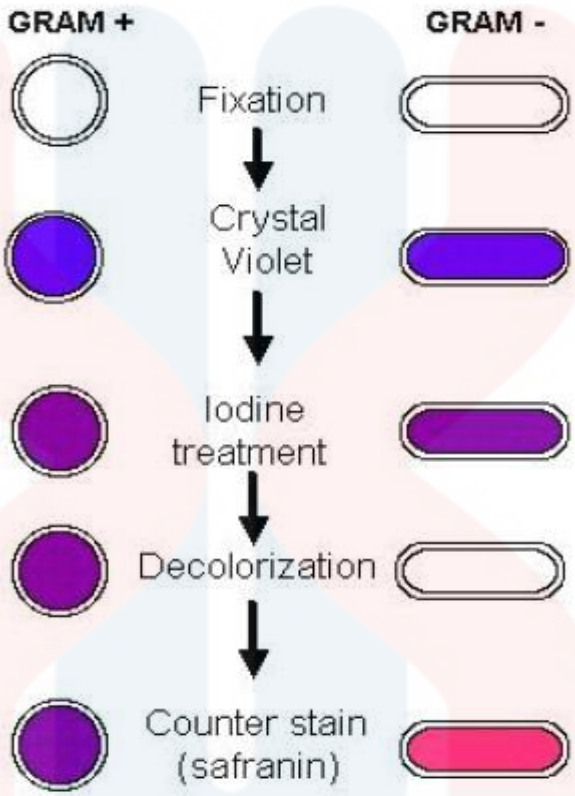


Figure A.1: Stages of Gram Stain.

## APPENDIX B

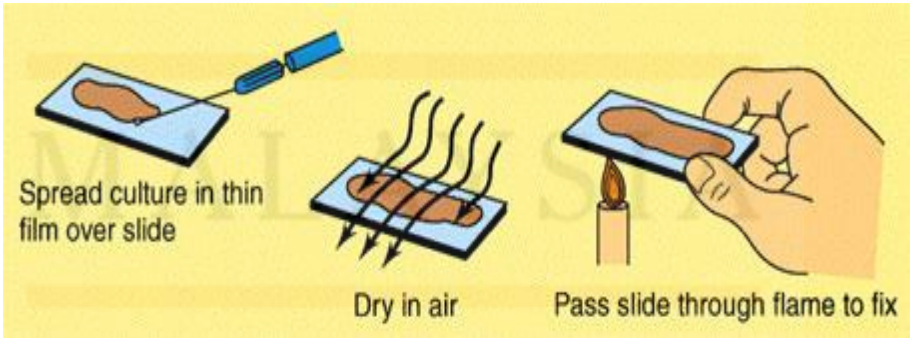


Figure B.1: Preparation of the bacteria smear.

**APPENDIX C**

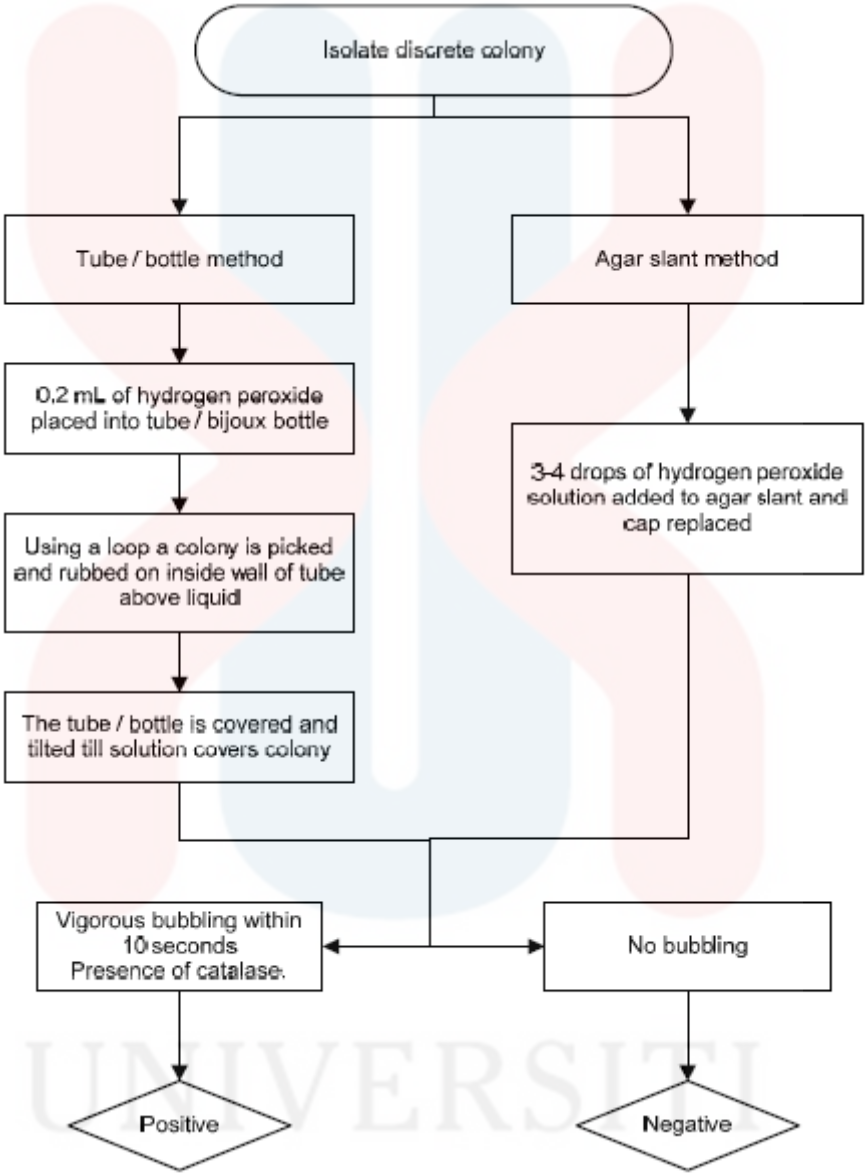


Figure C.1: Procedure of Catalase test. There are two types of method for catalase test which are tube or bottle method and agar slant method.

**APPENDIX D**

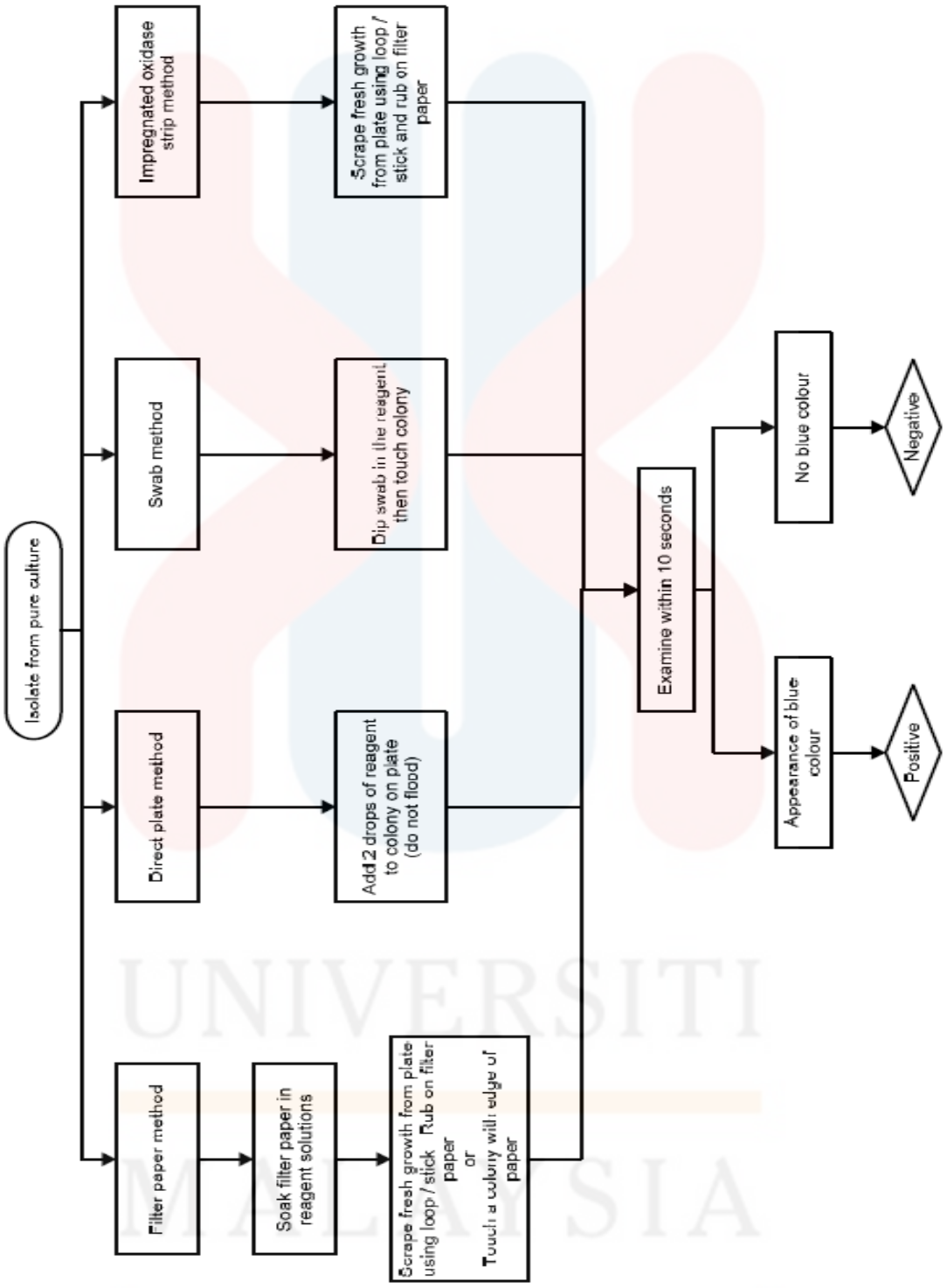


Figure D.1: Procedure of Oxidase test. There are four types of method for oxidase test which are filter paper method, direct plate method, swab method and impregnated oxidase strip method.