Physicochemical Parameters and Microbiological Quality of Water from Local Fish Farm

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Physicochemical Parameters and Microbiological Quality of Water from Local Fish Farm

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This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

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DECLARATION

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>API</td>
<td>Analytical Profile Index</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved Oxygen</td>
</tr>
<tr>
<td>PPM</td>
<td>Parts Per Million</td>
</tr>
<tr>
<td>BOD</td>
<td>Biochemical Oxygen Demand</td>
</tr>
<tr>
<td>TAN</td>
<td>Total Ammonia Nitrogen</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Sterilize Double Distilled Water</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulse-field gel electrophoresis</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>RAPD</td>
<td>Randomly amplified polymorphic DNA</td>
</tr>
<tr>
<td>Rep-PCR</td>
<td>PCR amplification of repetitive bacterial DNA element</td>
</tr>
<tr>
<td>AGE</td>
<td>Agarose Gel Electrophoresis</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
</tbody>
</table>
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ABSTRACT

The outbreak of fish disease has threatened the development of aquaculture industry in years. Enterobacteriaceae is a member of gram-negative bacilli and contain more than 30 genera. They act as the main indicator for the bacteriological water quality. The study was conducted at Malaysian Mahseer breeding farm near Asajaya to determine the physicochemical parameter and occurrence of Enterobacteriaceae family in fishes in the ponds which this could reflect the healthiness of water. Water sample were collected from the ponds and analyzed for the presence of Enterobacteriaceae. Samples were plated on MacConkey agar and colonies formed were further testes by ‘String Test’. Gram-negative colonies were analyzed with (GTG)\textsubscript{3} PCR. The phylogenetic tree was constructed based on the DNA banding pattern from the (GTG)\textsubscript{3} PCR. Suspicious species were identified using API 20 E Diagnostic Kit. Four major clusters were shown in the dendrogram indicate the diversity of the bacteria strains. Twelve strains of Escherichia coli, four strains Serratia odorifera, one strain of Serratia liquefaciens and eight strains of Aeromonas hydrophila/caviae/sobria were detected.

Key words: Enterobacteriaceae, (GTG)\textsubscript{3} PCR, API 20E Diagnostic Kit

ABSTRAK


Kata kunci: Enterobacteriaceae, (GTG)\textsubscript{3} PCR, kit API 20E.
1.0 INTRODUCTION

Freshwater aquaculture industry is highly important for the development of Malaysia. It provides tremendous and steady export revenue and increases the local security of food in the country (National Aquaculture Sector Overview, 2012). Common freshwater fish species that are normally bred for sale are tilapia, eel, catfish, tuna, rainbow trout, carp and a number of species. In order to consistently supply healthy aquatic organisms, water quality management is crucial in aquaculture industry.

Fish diseases emerged as a popular and formidable issue for most fish farmers. Threat of the fish diseases has slowed down the progress of aquaculture (National Aquaculture Sector Overview, 2012). These diseases have originated from a few agents such as parasites, fungus, viruses, bacteria or improper physical environment, which lead to the unnecessary loss of the industry. According to Chuah et al (2010), Edwardsiellosis tarda outbreak has led to a heavy financial loss of the fish farmers where the Edwardsiellosis tarda infected fishes are founded.

Bacteria are one of the causative agents that result in several significant diseases that might create a massive loss to the Malaysian aquaculture industry. Hazardous bacteria strains that are commonly found in the freshwater are mostly gram-negative such as Aeromonas hydrophila, Pseudomonas fluorescens, Vibrio vulnificus, Vibrio parahaemolyticus, Edwardsiella ictaluri and Edwardsiella tarda. The degree of existence of these strains in the source correlates with the likeliness of disease found on the fishes within the source. For instance, Genus Vibrio commonly causes necrosis and septicemia. Disease prevention and control is steps necessary to be taken in order to hamper the massive damage to the
industry. Early conformation of the presence and characterization of the hazardous strains are crucial to suppress the disease outbreak.

Diverse physicochemical factors such as pH, temperature, turbidity, dissolved oxygen (DO), Biochemical Oxygen Demand (BOD), Total Ammonia Nitrogen (TAN) and total suspended solids (TSS) have a large impact on the fish health, growth and their hostility to the disease triggered by the causative agents. Most of the massive fish death’ cases that occur around the world are mainly due to the changes of environment factor. For example, 10,000 of fishes were killed in Butterfly Lake in South Knoxville due to depletion of water’s oxygen level after the fluctuation of atmosphere temperature (Hickman, 2012). Similar case happened in Lake Taal, Philippine where 750 tons of fishes was found death in 2011 after climate changed over the weekend (Li, 2011).

The objectives of this research project were as below;

i) to detect the level of physicochemical factors in selected local fish farm

ii) to detect the potential disastrous microbiological factors that might cause the transmission of infectious diseases that may eventually lead to zoonosis.

iii) to determine the genetic diversity distance between isolates.
2.0 LITERATURE REVIEW

2.1 Tor tambroides

*Tor tambroides* and *Tor douronensis* or its common name is mahseer that are most valuable and overpriced fish in Malaysia (Litits *et al.*, 2007). Their high market price is largely due to the fine and tasty taste of the flesh. Also, the species population is decreasing sharply due to the environment degradation by human activities, for example, logging, deforestation and overfishing has endangered the *Tor* sp.. Combination of these events has exaggerated the price of the fish. According to Ingram *et al.* (2005), the price of *T. douronensis* has reached RM100/kg while the price of *T. tambroides* has reached RM400/kg in the Kapit, Sarawak. Many entrepreneurs have also started to breed this overpriced fish, therefore generating a high demand in the fresh water fish industry.

2.1 Enterobacteriaceae

Members of *Enterobacteriaceae* comprised of more than 170 recognized species today. They possess of few easily distinguishable and unique characteristics, which are Gram-negative bacteria rod, some of them are motile, facultatively anaerobic, absent of oxidase and presence of catalase. Their optimum growing temperature is at 37°C (some prefer 25-30°C). Common habitats of them are in living organisms (plants and animal), soil and water. Members that are included in the family are *Citrobacter* species, *Enterobacter* species, *Escherichia* species, *Hafnia alvei*, *Klebsiella species*, *Morganella morganii*, *Proteus* species, *Providencia* species, *Salmonella* species, *Serratia* species, *Shigella* species and *Yersinia* species (UK Standards for Microbiology Investigations, 2011). Most of the bacteria species under family *Enterobacteriaceae* are pathogenic to living organisms, therefore their presence are the main indicator of disease outbreak most of the
time. According to Kamble et al. (2012), genus *Aeromonas* causes the furunculosis and hemorrhagic Septicemia diseases to the fishes.

### 2.2 *Vibrionaceae*

The family *Vibrionaceae* is a very significant bacterial group that is found all around the world in different area that affects all types of marine and freshwater fishes (Kumiko et al., 1993; Gwedelynne et al., 2005; Safinaz et al., 2011). Vibrios are Gram-negative, motile rods, have a facultatively fermentation metabolism, and presence of oxidase. Most of the strains are pathogenic to human and animals. Moreover, Vibrio species are normally inhabited in the intestinal flora or marine and freshwater fishes (Simidu et al., 1977). Vibrio species like *V. splendidus*, *V. tubiashii*, *V. fluvialis*, *V. neonatus*, *V. ezurae*, and *V. ponticus*, *V. harveyi*, *V. splendidus*, and *V. tubiashii* are often connected with the diseases occurring in different types of fishes (Macian et al., 2004; Sawabe et al., 2004; Thompson et al., 2005). According to Safinaz et al. (2011), Vibrio species affects fishes with Vibriosis, which produces symptoms like “severe congestion at the base of the fins, erosion of the fins, excessive mucous secretion of gills, severe congestion of gills, hemorrhagic ulcerations, linear hemorrhages over different parts of the body and severe congestion or hemorrhagic protrusion of the anal opening”.

4
2.3 Physicochemical Factors

Based on Rheinheimer (1991), the physical and chemical properties of the source have a major influence of the microbial growth and the morphological and physiological of the bacterial population. Moreover, the changes in pH, temperature and salt concentration may also affect the metabolism, and reproduction system of certain microorganism. The aquatic life becomes stressful when one or more of these physicochemical factors have altered. The stressed organisms are more prone to the infection of bacteria, fungus, and other pathogens.

2.3.1 pH

A stable physiological hydrogen ion concentration (pH) is crucial to maintain the aquatic life. pH of most freshwater site apart from estuary that containing fishes ranges from 6 to 9 (Robertson - Bryan, Inc, 2004). The basic process of fishes such as respiration is closely influenced by pH changes. Sub-lethal effect such as declining of the growth rates can occur if the pH is fluctuated. In more serious cases, if the fluctuation of pH were out of the toleration level of the freshwater organism, death would occur. Bacteria can be differentiated into acidophile (pH 1 – 5.9), neutralphile (pH 6 – 9) or alkaliphile (pH 9 – 11) and the adaptation of bacteria vary accordingly to pH (Todar, n. d.). Similarly, changes in pH have a discernible effect on the bacterial enzymes; therefore, exposure to extreme pH escalates the mortality rates of bacteria in pond.
2.3.2 Temperature

Temperature is significant in terms of maintaining the life process of microorganisms. Temperature changes can affect growth rate, nutritional requirement, enzymatic and chemical components of the cell (Rheinheimer, 1991). Basically, microorganisms can be categorized as the psychrophiles (able to grow at 0°C), mesophiles (optimum temperature ~37°C), thermophiles (optimum temperature 45°C to 70°C), and lastly hyperthermophiles (optimum temperature 80°C to 115°C). Increasing in temperature would lead to an increasing of metabolic rate of microorganisms due to the activation of enzymatic reaction. As a result, the Biochemical Oxygen Demand (BOD) level is raised when the temperature is increased to a certain extent. Furthermore, the oxygen solubility drops when the water temperature is raised. The concentration of CO₂ in the water is increased, as the O₂ is decreased, in fact, the aggregation of CO₂ limits the O₂ level in the water. Lastly, the level of toxicity is associated with the changes in temperature. As the temperature increases, more toxic would be emitted from the poisonous substances.

2.3.3 Turbidity

Turbidity is one of the main factors that affect the life of microorganisms. The presence of silt, clay, sextons, organic and inorganic matter, and tiny organisms and plankton have greatly influence the level of turbidity. Sexton is the accumulation of living and dead materials in the water that led to the formation of sediment. Most of the microorganisms treat the sexton as the food source. Therefore, increasing organic suspended materials can lead to accretion of turbidity and finally accompanied by an aggressive growth of the bacterial population (Rheinheimer, 1991). Besides affecting the growth of bacteria, the turbidity causes light to be scattered rather than penetrating directly through the
photosynthetic organisms (APHA, 1992). Thus, the amount of light absorbed by the photosynthetic organisms might be reduced.

2.3.4 Dissolved Oxygen (DO)

Dissolved oxygen (DO) is the most fundamental criterion in water. The carbon dioxide gas from photosynthesis of land and aquatic vegetation is the major source of DO. Aquatic organisms required dissolved oxygen to sustain life. The volume of oxygen needed is varied among species and phase of life. Dissolved oxygen level between 5 to 6 ppm is normally needed for fish’s activities and development. Level 3 ppm and below are insufficient for most aquatic living organisms (LaMotte, n.d.). However, alteration of temperature affects the solubility of oxygen in the water (Canadian Council of Ministers of the Environment, 1999). During summer, with the temperature increases, the DO concentration and solubility on the warm upper water layer decreases. On the other hand, when the temperature drops due to the mixing warm water layer and cold lower water layer, oxygen concentration and level of saturation are increasing. Oxygen lost may be due to several factors, for example, respiration of bacterial, plant and animal, direct chemical oxidation of dissolved organic matter (Canadian Council of Ministers of the Environment, 1999).

2.3.5 Biochemical Oxygen Demand (BOD)

BOD is a measurement of the total oxygen required for the aerobic bacteria when digesting the organic matter. Besides, BOD calculates the chemical oxidation of inorganic materials as well. Temperature, pH, types of microorganisms and kind of organic and inorganic
matter are able to affect the speed of consumption of oxygen in the water (EPA, 2012). Plant detritus, plants and animals carcasses and animal wastes are sources of BOD (EPA, 2012). BOD is mainly to indicate the strength of organic in wastewater and heavily polluted water source, because these types of water are normally containing high amounts of organic matter (National Water Quality and Availability Management, 2003).

2.4 (GTG)$_5$ PCR Fingerprinting

The commonly used method such as the 16S rRNA sequencing, ribotyping, and pulsed-field gel electrophoresis (PFGE) are too arduous to carry out from the classification and identification of Lactic acid bacteria (LAB) (Gevers et al., 2001). Even though randomly amplified polymorphic DNA (RAPD) is the most used PCR-based genomic technique for LAB identification, however, according to Gevers et al. (2001), the downsides of randomly amplified polymorphic DNA (RAPD) are the high discriminatory power and the large applicability within a large group of LAB species that have not been described. Moreover, it has displayed the weak reducibility.

As an alternative to provide a better identification of bacteria species, PCR amplification of repetitive bacterial DNA elements (rep-PCR) is used. It is a straightforward PCR-based technique with several advantages which are high discriminatory power, inexpensive, applicable for huge number of strains and able to identify and type a huge range of bacteria (Gevers et al., 2007; Olive & Bean, 1999; Versalovic et al., 1994). Oligonucleotid primer that designed to be used in the rep-PCR is (GTG)$_5$. By using this technique, the genetic diversity of different isolates can be identified through the DNA fingerprinting. AGE is used to observe the multiple banding patterns after PCR amplification. The banding shows
the profile of various isolates. A dendrogram is plotted to determine the genetic diversity distance between isolates.

2.5 API 20E Kit Test

Rapid and reliable diagnostic tests are required to provide the faultless detection of the bacterial strains in the water source (Coz-Rakovac et al, 2007). This is to effectively control and cure the aquatic animal diseases before a disease outbreak that might be leading to zoonosis. API 20E system’s role is to identify the Enterobacteriaceae and other non-fastidious Gram-negative bacteria. It contains 21 standardized and small-scale biochemical tests and a database. Also, the test kit consists of 21 microtubes comprised of dehydrated substances (bioMérieux, 2002). API 20E ratings are based on three factors. The three factors include “the likelihood of a match between the unknown organism’s profile and computer profile, the relative value between the likelihood of the first and the likelihood of the second choice, and the number of tests against the first choice” (Coz-Rakovac et al, 2007). There are several pathogenic bacterial species, which is in the current identification database of API 20E. The bacteria referred are Acinetobacter spp., Aeromonas hydrophila, A. salmonicida subsp., Salmonicida, Citrobacter freundii, Edwadiella tarda, Escherichia vulneris, Hafnia alvei, Klebsiella pneumonia, Moraxella spp., Pantoea spp., Photobacterium damsel, Plesiomonas shigelloides, Providencia rettgeri, Pseudomonas aeruginosa, P. fluorescens/putida, Salmonella arizonae, Serratia liquefaciens, Serratia plymuthica, Shewanella putrefaciens, Vibrio alginolyticus, V. cholera, V. vulnificus.
3.0 MATERIALS & METHODOLOGY

3.1 Sample Collection and Processing

Sample collection was carried out at Malaysian Mahseer breeding farm near Asajaya, Sarawak on 1st of February 2013. This farm mainly breeds *Tor tambraides* (Kelah/Empurau), *Tor dourenensis* (Semah), and *Barbonymus schwanenfeldii* (Tengadak). Water samples were obtained at three different sites, which are the breeding sites, culture site and sale sites. Overall there were six samples collected where three samples taken from breeding site, one sample from culture site and two samples from the sale site. Water samples were collected by using 50 ml Falcon tube, respectively at three different points; inlet, random spot in the tank, and outlet. Gill and fin swabs were taken from one adult *Tor tambraides* in the breeding tank. The water samples collected from three points were then poured into a new sterile 50 ml Falcon tube and homogenized. All tubes were labeled with the date and source of the samples. All samples were kept at 4°C in an insulated double walled container and were transported to the laboratory for further analysis. The temperature, dissolved oxygen (DO), biochemical oxygen demand (BOD) and pH of samples were also recorded.
3.2 Total Plate Count

1 mL of the homogenized water was pipetted into 9 mL of saline solution to make up the dilution of $10^{-1}$. The process was continued by pipetting 1 mL of solution from dilution $10^{-1}$ into 9 mL of saline solution and labeled as dilution $10^{-2}$. Tail and Fin swabs were mixed with the distilled water and diluted into dilution of $10^{-1}$ and $10^{-2}$. 100 µL from original, dilution $10^{-1}$ and $10^{-2}$ solution were then spread onto non-selective Nutrient agar and MacConkey agar and incubated at $27^\circ$C for 24 hours. Single colonies formed were counted after the overnight incubation.

3.3 Bacterial Isolation

Following the total plate count on MacConkey agar, presumptive Enterobacteroceae appeared as colourless, transparent colonies were isolated and streaked on new nutrient agar and incubated at $27^\circ$C for 24 hours. Double confirmation was done by using NaOH String Test to ensure bacteria colonies isolated are gram-negative. The colonies were emulsified onto a glass slide that contained 10 µL of 0.5M of NaOH. The suspension was mixed and next observed whether there is the formation of a string (Figure 1). The colonies that failed the test were discarded. The positive strands were grown in the nutrient broth at room temperature for 24 hours on the shaker (New Brunswick Scientific Exccela E10 Platform Shaker) at 1200rpm prior DNA extraction.

Figure 1: NaOH String Test
3.4 DNA Extraction

Bacteria were grown overnight in 50 mL nutrient broth at room temperature on the shaker at 1200 rpm. 1.5ml of overnight culture was poured into the 1.5 mL Eppendorf (Eppy) tube. The tube was centrifuged under 10,000 rpm for 5 minutes. After centrifugation, the supernatant was discarded but cell pellet remained. Another 1.5 mL of the overnight culture was poured into the same Eppy tube. The tube was centrifuged under previous conditions. The supernatant was discarded following by addition of 500 µL dH₂O then vortex to mix well. The Eppy tube immersed in boiling water and boiled for 10 minutes. After boiling, the Eppy tube was immediately placed into a box containing ice for 5 minutes. Next, the tube was centrifuged for 10 minutes to suspend the cell debris. The supernatant contained the DNA of the bacteria was transferred to the new Eppy tube for PCR purpose.
3.5 (GTG)$_5$ PCR Fingerprinting

(GTG)$_5$ PCR was conducted according to Gevers et al. (2001). The primer used is (GTG)$_5$ with the sequence of 5’-GTGGTGGTGGTGGTGGT-3’.

The PCR reaction set-up used for this study is shown in Table 1.

<table>
<thead>
<tr>
<th>PCR Compound</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq Polymerase (2U)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>(GTG)$_5$ primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>3 µl</td>
</tr>
<tr>
<td>dNTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>Distilled H$_2$O</td>
<td>9.5 µl</td>
</tr>
<tr>
<td>Buffer (10x)</td>
<td>5 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5 µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25 µl</strong></td>
</tr>
</tbody>
</table>

The amplification of (GTG)$_5$ region was carried out in SenseQuest thermal cycler and the amplification conditions are listed in Table 2.
### Table 2: (GTG)$_3$ PCR amplification conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-denaturation</td>
<td>95°C</td>
<td>7 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>2 minutes</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>36°C</td>
<td>2 minutes</td>
<td>4</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>2 minutes</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>50°C</td>
<td>1 minute</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 minute</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>5 minutes</td>
<td></td>
</tr>
</tbody>
</table>

#### 3.7 Agarose Gel Electrophoresis (AGE)

Five microliter of the amplified PCR product was electrophoresed on 2% agarose gel containing 0.1 µl of 10 mg/mL Ethidium Bromide (EtBr) in 1x Tris-Borate-EDTA (TBE) buffer at 80 V for 1 hour 45 minutes. DNA marker of 1 kb DNA ladder (Promega, USA) was used. After electrophoresed, the gel was visualized using UV transilluminator (Gel Doc). Banding patterns was directly linked to the scoring system under binary data format. Presence of band is scored “1” while absence of band is scored “0”. The software that interprets the data input is RAPDistance Package Version 1.04. By using Dice formulations, the genetic distances between band profiles can be specified (Nei & Li, 1979). After the genetic distances are calculated, the dendrogram of neighbour joining tree (NJTREE) was constructed.
3.8 API 20E Test

The suspicious bacteria strains were isolated and identified with API 20E system (Figure 2). Briefly, bacterial suspension was prepared in saline solution (0.85% NaCl). A colony of pure bacteria was deposited into the saline solution to homogenize the suspension. The bacteria suspension was inoculated into each well with sterile pipette, and incubated at 37°C for 18-24 hours. After incubation, the metabolism caused colour changes that were either spontaneous or by adding the reagents. The results was recorded based on the protocol provided by the manufacturer. The identification and analysis was done by using the Apiweb software (Coz-Rakovac et al, 2007).

![Figure 2: API 20E Identification Kit](image)