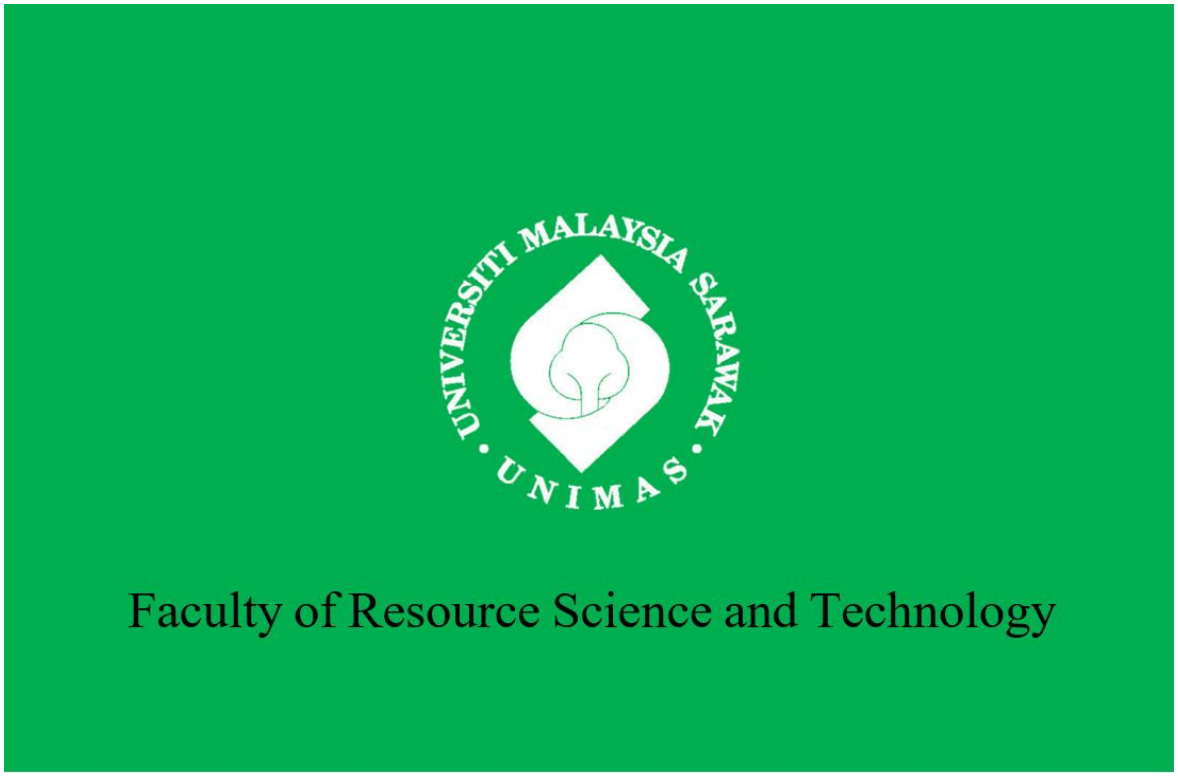

Microbial Profiling of Dropsy Diseased Empurau (*Tor tambroides*)

Fong Jia Khi (69745)

Bachelor of Science with Honours
(Resource Biotechnology)
2022

Microbial Profiling of Dropsy Diseased Empurau (*Tor tambroides*)

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A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours
(Resource Biotechnology)

SUPERVISOR: DR CHUNG HUNG HUI

Programme of Resource Biotechnology
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UNIVERSITI MALAYSIA SARAWAK
2022

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Acknowledgment

The author would like to express the thanks to all individual and parties that have been involved helping in completing this Final Year Project (FYP) directly and indirectly. Special thanks are dedicated to supervisor, Dr Chung Hung Hui for his guidance, encouragement and moral support. He gives advice and invaluable comments which help a lot in completing this thesis. In addition, high appreciation to Faculty of Resource Science and Technology for providing the author this opportunity to embark on this project. Furthermore, the author like to extend her sincere thanks to master candidate, Cindy Kho Jia Yung, for her precious assistance, advice, and concerns throughout the whole duration in completing my thesis. Last but not least, the author would like to express her sincere thanks to all her coursemates and family who have patiently extended all sort of help for accomplishing this report.

Microbial Profiling of Dropsy Diseased Empurau (*Tor tambroides*)

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ABSTRACT

The intentions of this study were to identify and compare the microbial community between skin samples of dropsy diseased and health Empurau to reveal the potential pathogen of dropsy disease which reduce yield by increasing the mortality of Empurau. Skin samples of healthy and dropsy-diseased fish was harvested and subjected to serial dilution before they were plate on different agar (Aeromonas agar, nutrient agar, EMB agar, MacConkey agar). The colonies formed on the plates were picked as much as possible based on their morphological difference and inoculated onto the patch plate for single colony isolation. A total of 10 isolates from healthy fish skin samples (Aeromonas agar: 4; nutrient agar: 2; EMB agar: 3; MacConkey agar: 3) and 12 isolates from diseased fish skin sample (Aeromonas agar: 3; nutrient agar: 2; EMB agar: 2; MacConkey agar:3) were then identified by morphological characteristics, Gram staining and string test. Among these 22 isolates, only one was Gram positive coccus and the 21 isolates were all Gram negative rod-shaped bacterial. The isolates also subjected to PCR and agarose gel electrophoresis before sending them for molecular 16s rDNA sequencing which targeting the V3-V4 region with 450 bp to 550 bp. Overall, there were 10 types of different bacteria species had been identified. They were from genus of pseudomonas (59.0%), Citrobacter (32.0%) and Staphylococcus (4.5%) with one uncultured bacterium (4.5%) which might be resulted from mixed cultured of colony. Further research on the above-mentioned potential pathogen for dropsy disease should be conducted to maintain the high yields of empurau to maximise profit.

Key words: Empurau (*Tor tambroides*), dropsy disease, microbial profiling

ABSTRAK

Tujuan kajian ini adalah untuk mengenal pasti dan membandingkan komuniti mikrob antara sampel kulit yang berpenyakit dropsy dan sihat untuk mendedahkan pathogen berpotensi untuk menyebabkan penyakit dropsy yang mengurangkan hasil ikan dengan meningkatkan kematian Empurau. Sampel kulit ikan yang sihat dan berpenyakit dropsy diambil dan tertakluk kepada pencairan bersiri sebelum diletakkan di atas agar-agar yang berbeza (agar Aeromonas, agar nutrien, agar EMB, agar MacConkey). Koloni yang terbentuk pada plat telah dipetik sebanyak mungkin berdasarkan perbezaan morfologinya dan diinokulasi pada plat tampalan untuk pengasingan koloni tunggal. Sebanyak 12 koloni daripada sampel kulit ikan yang sihat (Aeromonas agar: 4; nutrient agar: 2; EMB agar: 3; MacConkey agar: 3) dan 10 koloni daripada sampel kulit ikan yang berpenyakit (Aeromonas agar: 3; nutrient agar: 2; EMB agar: 2; MacConkey agar:3) kemudiannya dikenal pasti melalui ciri morfologi, pewarnaan Gram dan ujian rentetan. Di antara 22 koloni ini, hanya satu merupakan kokus Gram positif dan 21 koloni yang lain semuanya bakteria berbentuk batang yang Gram negatif. Pengasingan juga tertakluk kepada PCR dan elektroforesis gel agarose sebelum menghantarnya untuk penjujukan molekul 16s rDNA yang menyasarkan rantau V3-V4 dengan 450 bp hingga 550 bp. Secara keseluruhannya, terdapat 10 jenis spesies bakteria yang berbeza telah dikenalpasti. Mereka adalah daripada genus pseudomonas (59.0%), citrobacter (32.0%) dan staphylococcus (4.5%) dengan satu bakteria yang tidak dibiakkan (4.5%) yang mungkin terhasil daripada kultur campuran koloni. Kajian lanjut mengenai potensi patogen yang dinyatakan di atas untuk penyakit dropsy perlu dijalankan untuk mengekalkan hasil empurau yang tinggi untuk memaksimumkan keuntungan.

***Kata kunci**: Empurau (Tor tambroides), penyakit dropsy, profil mikrob*

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List of Abbreviations

DNA	Deoxyribonucleic acid
EMB	Eosin methylene blue
HEPA	High efficiency particulate air
LB	Luria Broth
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
TBE	Tris/Borate/EDTA
UV	Ultraviolet radiation

CHAPTER 1:

INTRODUCTION

Humans rely heavily on fisheries production, which includes both capture and aquaculture. According to Oliva-Teles (2021), fish consumption has increased significantly over the last few decades, reaching an average of roughly 20 kg per capita. In Malaysia, freshwater fisheries and fish culture are a rapidly growing sector. The aquaculture sector in the country produces 391,000 t of farmed organisms per year and is estimated generated around USD 700 million in economic value in 2019 (Azra et al., 2021).

The species stated in the title, *Tor tambroides* is also known in Sarawak as "Empurau" and in Peninsular Malaysia as "kelah merah" (Ingram et al., 2007). This species is a critically endangered and highly sought-after game, food, and ornamental fish. Due to the species' declining population, it has become the most valuable freshwater fish species in Malaysia, with market prices fetch up to RM 750 per kg in the local market and between RM 800 and RM 850 per kg in the export market (Lee et al., 2014). Empurau are now classified as a threatened species in the wild as a result of environmental deterioration, and considerable interest has been raised in empurau breeding for conservation and aquaculture purposes.

Ingram et al. (2007) investigated the ecological and economic impacts, as well as the feasibility of *Tor tambroides* for pond culture. They also induced artificial spooning and reared broodstock in pond farms. Along with pond technology, the dietary protein and fat requirements for feeding have been extensively researched (Ng et al. 2008). However, there is a lack of analyses to filling knowledge gaps regarding diseases in this species. Similar to other animals, fish can also suffer from various type of disease. For aquaculturists, fish disease is one of the most significant sources of monetary loss. Fish disease outbreaks raise production

costs because of the investment lost in dead fish, the expense of treatment, and the slowed growth that occurs while the fish is recovering from the sickness.

According to Assefa and Abunna (2018), diseases account for over half of all production losses in aquaculture, with disease severity being higher in developing nations. The annual revenue loss caused by disease can amount to as much as $6 billion dollars (Assefa & Abunna, 2018). Dropsy disease is the common infection that frequently affected the freshwater fishes. This form of infection in fish is caused by a combination of factors, including the environment, the host, and unique factors for each pathogen, such as its virulence (Roberts et al., 2009). The most significant symptom of the disease is the presence of coelomic distension in varied degrees of severity in fish that have been clinically affected (Densmore, 2019). Densmore (2019) also stated that scaled fish that have been severely afflicted by the disease may have protrusion of the scales, which causes them to stand erect from the body surface ("pinecone" appearance), resulting in lepidorthosis. A wide variety of cultured fishes are affected by the disease, which results in mortality and morbidity that bring a huge lose in aquaculture sector.

Empurau in freshwater fisheries are a cost and time consume sector in Malaysian economy and yet desire high yields to maximise profit. Unfortunately, dropsy disease reduce yield by increasing the mortality of empurau fish. To avoid this, it is necessary to take action based on scientifically derived microbial profiling of the dropsy diseased empurau. Utilization of different microbial profiling methods should be able to reveal the microbial communities that are likely to be involved in the dropsy disease. The ability to identification of existence of a disease-causing organism in fish quickly will have significant economic benefits to our country Malaysia.

The main objectives of this study are to:

1. To identify the diverse collection of microorganisms in the skin sample of dropsy diseased Empurau.
2. To compare the microbial community between skin samples of dropsy diseased and health Empurau.

CHAPTER 2:

LITERATURE REVIEW

2.1 *Tor tambroides*

T. tambroides, *T. duoronensis*, and *T. tambra* are the three *Tor* species that have been identified in Malaysia, according to published reports (Chang, 2000; Ng, 2004; Eddy, 1997; Mohsin & Ambak, 1983). The species examined under the study, *Tor tambroides*, which also known as Empurau fish in the local language for its ability to produce "unforgettable flavour" in Chinese characters. The species is a member of the Cyprinidae family. Figure 2.1 showed the taxonomic hierarchy of *Tor tambroides*.

Taxonomic Hierarchy

Kingdom	Animalia – Animal, animaux, animals
Subkingdom	Bilateria
Infrakingdom	Deuterostomia
Phylum	Chordata – cordés, cordado, chordates
Subphylum	Vertebrata – vertebrado, vertébrés, vertebrates
Infraphylum	Gnathostomata
Superclass	Actinopterygii – ray-finned fishes, spiny rayed fishes, poisson épineux, poissons à nageoires rayonnées
Class	Teleostei
Superorder	Ostariophysi
Order	Cypriniformes – cyprins, meuniers, minnows, suckers
Superfamily	Cyprinoidea
Family	Cyprinidae – carps, minnows, carpas y carpitas, carpes et ménés, carps and minnows, carpes, ménés, carpitas, carpas
Genus	Tor Gray, 1834
Species	Tor tambroides (Bleeker, 1854)

Figure 2.1: Taxonomic hierarchy of *Tor tambroides* (Adapted from Integrated Taxonomic Information System, n.d.)

Tor tambroides is an omnivorous eater that feeds on molluscs, plants, tiny fish, and insects in addition to other foods (Ingram et al., 2007). It is noteworthy that it has a distinct feeding preference for fruits with a high protein-to-energy level, such as the illipe, which also known as the engkabang fruit (*Shorea macrophylla*), that fulfil the favour of *T. tambroides* (Abdul Rahman & Basri, 2013; Kamarudin et al., 2014). It is widely believed that the intake of illipe fruit contributes to the distinct flavour of Malaysian empurau fish, which is a delicacy (Frost & Sullivan 2015).

2.1.1 Morphology of *T. tambroides*

It was Bleeker (1854) who made the first morphological description of *T. tambroides*, which characterized it as having four dorsal spines, eight soft rays on the dorsal side, three anal spines, five soft rays on the anal side, and vertebrae on the back. According to Siraj et al. (2006), the most distinguishing characteristics of *T. tambroides* in the field are the presence of a fleshy and thicker lip, median lobe when observed from the dorsal side of the fish, a pointed snout when observed from the dorsal side of the fish, and black coloration at the edge of scales of this species.

The presence of the short median lobe which is not extending to a line connecting inner corner of mouth is the key differentiating characteristic of this species of fish (Mohsin & Ambak, 1983). Besides, Sim et al. (2007) in their project found that the species also can be differentiated from the other Tor species according to its scale characteristic of larger size and reddish colour. In 2018, Zulfami et al. (cited in Lau et al., 2021) described the characteristic of the *T. tambroides* from the perspective of axial skeleton. Zulfami et al. (2018, cited in Lau et al., 2021) stated that within the weberian apparatus, this fish possessed one os urostyles vertebrae, eighteen pairs of costales vertebrae, nineteen abdominal vertebrae vertebrae, sixteen caudal vertebrae vertebrae, four axial vertebrae bones, and one os urostyles vertebrae bone. Some other characteristics of *T. tambroides* that are shared by other Tor species include a sub-terminal mouth location, a pointed rostrum hood, and a caudal fin lobe that is equal in length (Jaafar et al., 2021).

Figure 2.2: The image of *T. tambroides.* (Adapted from Dawum, 2019.)

2.1.2 Habitat and Distribution of *T. tambroides*

The natural environment where *T. tambroides* thrive should consists of clear, swift waterways with rocky bottoms (Shreshtha 1997). The wild *T. tambroides* prefers to live in the upper reaches of rivers that are swift-flowing, whose waters are highly oxygenated, transparent, clean, and whose bottoms are stony, pebbly, or rocky in nature (Sungan et al., 2006). The larger fish may be found in the deeper pools, while the smaller fish can be found in the shallower sections and minor tributaries of the river. *T. tambroides* is restricted to a small number of river systems in Sarawak, including the Limbang, Baram, Rejang, and Batang Ai rivers (Sungan et al., 2006).

The species are distributed throughout southeast Asia from Indonesia to southern China and sharing a comparable biogeographical distribution with *T. douronensis* and *T. tambra* across freshwaters of Malaysia (Pinder et al. 2019). Based on Figure 3, Perak River, Pahang River and Sarawak River has recorded the existence of *T. tambroides*.

Figure 2.3: The geographic distribution of Tor species in the Southeast Asian region (Adapted from Jaafar et al., 2021).

In the unfortunate case of the natural habitat, many of the higher reaches or watershed areas have been adversely damaged, particularly as a result of the large-scale land-based developmental projects. Because of the environmental problems such as river pollution, deforestation, and the construction of logging roads in rural areas, as well as upper stream development, the natural habitats of Empurau have been gradually degraded. As a result, the fish has become the preferred species for fish farmers to raise in order to prevent extinction. The degradation of *T. tambroides'* natural habitat, along with an increase in public demand for this fish's flesh, has resulted in the fast rise of empurau farms in Malaysia. As for Malaysia, there are several fish farm have developed to culture and grow the species, for example, A Farm Agrotech at Negeri Sembilan, Borneo Empurau Farm & Resort Sdn Bhd, LTT Aquaculture Sdn Bhd and Puri Johan Aquaculture Sdn Bhd at Sarawak.

2.1.3 Economic Value of *T. tambroides*

Tor tambroides sell for up to RM 750 per kg locally and between RM 800 and RM 850 per kg internationally (Lee et al., 2014). At this relatively high price, Empurau is readily converted into a Malaysian export trade from the fishing industry, with a promising market segment both locally and internationally. For example, Borneo Empurau Farm & Resort Sdn Bhd had open market to exports the live empurau fish to Singapore, China, Hong Kong, and Taiwan (Lam, 2021).

The importance of *T. tambroides* is demonstrated by its distinct flavour and creamy texture, which is said to be related to the engkabang fruit that the fish eat. An engkabang tree, on the other hand, bears fruit every four to five years (Banji, 2013). Even though the price of engkabang fruit is only RM0.80 per kilogramme (Banji, 2013), the farm owner will normally buy a large quantity and store it to ensure that the fruit is available for fish feeding during the time that the engkabang tree takes to develop fruit. Another reason the build-up the high price of *T. tambroides* is that it required a harvest time of three years for the fish to reach a harvesting

weight of 2kg or more (Lam, 2021). This takes a lot more time and money than the tilapia which was discovered by Borrego-Kim et al. (2020), and its optimal harvest time is six to seven months or 181 to 196 days.

2.2 Dropsy Disease

Dropsy is defined as an accumulation of fluid within a fish's body cavity or internal organs by Sharma et al. (2012). The accumulation of fluid resulting in swollen abdomen that might be confused with the normal appearance of gonadal development particularly prior to and during the spawning season (Hoole et al., 2008). It is possible to look through the fish's scales and readily identify dropsy disease depends on its distinct physical traits. Coelomic distension is present in varied degrees of severity in fish that have been clinically affected. As a result of the coelomic distension, scaled fish that have been severely afflicted by the disease may have protrusion of the scales, which causes the scales to stand erect from the body surface, resulting in lepidorthosis, known as a "pinecone" appearance (Densmore, 2019).

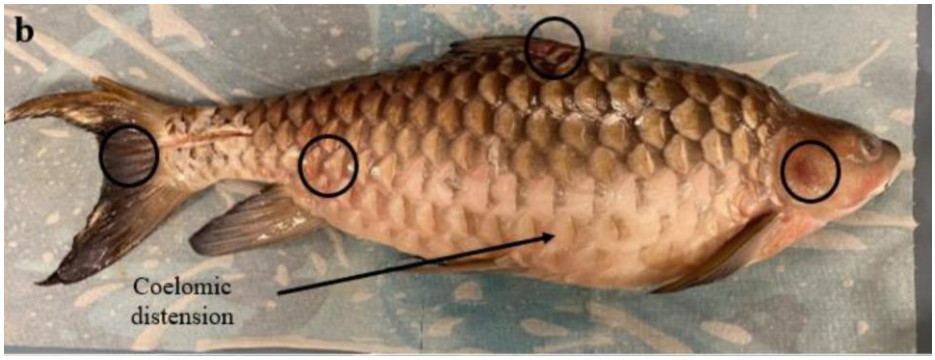

Figure 2.4: Diseased empurau fish with coelomic distension at the arrow pointed part and petechial haemorrhage circled in image. (Adapted from Lau et al., 2021)

Fish suffering from dropsy diseases will find that it is extremely difficult to swim due to the enlarged state of their bodies, making it tough for them to move around and breathe properly as normal (Haryo & Nurhidayati, 2020). Furthermore, Sharma et al. (2012) summarised that exophthalmic condition, inflammation of the gut, and haemorrhagic ulcer

develop on the skin and fins of fish are the symptom that presence on fish that are suffering from dropsy disease. Dropsy is not peculiar to any one taxonomic group of fishes, but it is particularly well-known among pond farmed and hobbyist cyprinids, where it is very common in every species of fish.

2.2.1 Causes of Dropsy Disease

Dropsy disease can be caused by a range of different agents, both infectious and non-infectious. As described by Densmore (2019), dropsy is connected with infectious disease processes that are caused by viral, mycotic, or bacterial infection. Free serous fluid accumulation in the coelomic cavity of many fish species has been linked to the presence of pathogenic aeromonad, pseudomonad, and vibrio bacteria. Densmore (2019) also stated that *Rhabdovirus carpio*, the causative agent of spring viremia of carp (SVC), is another of the more recognised causative agents associated with dropsy in some cyprinids and other fishes, and it is one of the more recognised causative agents associated with dropsy in some cyprinids and other fishes.

Dropsy linked with SVC has also been referred to as infectious abdominal dropsy (IAD), which is a more specific term (Densmore, 2019). Additionally, parasitism of the coelomic cavity or the coelomic organs, as well as infections of mixed etiologies, have been related with dropsy also. A variety of etiological agents for infectious dropsy have been identified and are summarised in Table 2.1.

The major cause of dropsy disease is due to a variety of microorganism as stated above, but sometime kidney infection may also contribute to the disease as kidney significance in osmoregulation (Haryo & Nurhidayati, 2020). An infection blocks the tube draining the kidney, causing the liquid wastes from the fish to back up and enlarge the kidney like a grape (Dropsy, n.d.). Furthermore, stressors such as sudden changes in water temperature may lead fish to

bacterial disorders such as dropsy, where the change may be favourable to the bacteria growth. Water conditions that are too turbid might potentially cause dropsy symptoms in fish. Inadequate nutrition is yet another source of stress. In most cases, a single or short-term exposure to stress will not impair the fish's capacity to fight infection and will not harm them (Dropsy, n.d.). However, in the case of stress exposure has occurred over an extended period of time, or a number of stress factors have arisen simultaneously aside with the decline of fish immunity will cause the dropsy disease.

Table 2.1: Potential etiological agents for infectious dropsy.

Pathogen	Type of pathogen	Resources
Aeromonas hydrophila	Gram negative bacteria	(Shome et al. 1996)
A. salmonicida	Gram negative bacteria	(Bootsma et al. 1977; Kumar et al. 1986)
Rhabdovirus carpio	Virus	(Fijan et al. 1971; Fijan 1972)
Saprolegnia sp.	Water moulds/Fungus	(Havelka 1974)
Myxosporidian sp.	Protozoa	(Kumar et al. 1986)
Aeromonas veronii biovar sobria	Gram negative bacteria	(Sreedharan et al. 2011)
Pseudomonas fluorescens	Gram negative bacteria	(Swain et al., 2008)

2.3 Microbial Profiling

Studying the ecology, distribution, morphology, mechanism, and relationships of microbial communities can be accomplished through the use of microbial profiling (Janvier et al., 2007). Approaches for studying microbial communities are varies. The dramatic decrease in the cost and increase in throughput of DNA sequencing over the past as made it possible for the majority of research groups to map the microbial community composition in fields of interest (Janvier et al., 2007).

2.3.1 Physiological Profiling

Physiological profiling is one of the culture-dependent approach of microbial profiling. It cultivates the microorganism from pathological material on medium, followed by identification using morphology of the microorganism (Austin, 2019). Nutrient Agar medium is commonly used as general-purpose medium for the cultivation of broad range of bacteria. Besides on NA medium, MacConkey agar (MAC) also frequently used as a selective and differentiating agar for growing gram-negative bacterial species. There is also Aeromonas agar which is a selective medium used for the detection and enumeration of Aeromonas spp.. Eosin methylene blue agar (EMB) is a selective and differential medium that usually used to isolate faecal coliforms.

Microorganisms are very small organisms, and thus profiling through physical appearance of microorganism can only be done by observing it under microscope. Colonial morphology such as colour, shape, size, margin and elevation can place an organism in a single family, genus, or even species level (Bullock & Aslanzadeh, 2013). Margin of microbial when looking on its colony can be divided into entire, undulate, filamentous, curled and lobate while the elevation of bacteria included raised, flat, convex, crateriform and umbonate.

For the cell shape of bacteria, it comes in four basic shapes which are spherical or cocci, rod-shaped or bacilli, arc-shaped or vibrio, and spiral or spirochete (Zhou & Li, 2015). However, bacteria sometime show cellular arrangement or grouping. According to Zhou and Li (2015), cocci can be grouped in pairs to form diplococci, whereas streptococci is the name of cocci that are arranged in chains form. The cocci can be also presence in a groups of four (tetrads) or eight (sarcina), or grape-like clusters, depending on the plane of cellular division (staphylococci).

2.3.2 Biochemical Profiling

In biochemical profiling, the microbial identity is tested by biochemical test and then doing the comparison to known diagnostic schemes. Biochemical characterisation is accomplished through culture, which yields vast quantities of cells from a clonal population and enables for the performance of any number of functional tests (Austin, 2019). The biochemical test generally uses a single-enzyme rapid test, which is a set of tests that detects the presence or absence of a single enzyme or a biochemical reaction within a few seconds to a few minutes (Bullock & Aslanzadeh, 2013). These tests are relatively affordable, simple to administer, and can provide valuable preliminary data that is utilised to identify the next steps in the microbial identification process. Rapid enzyme tests are an important part of both conventional as well as commercial microbial identification systems. Furthermore, these tests can be used to presumptively identify organisms down to the genus or even species level (Bullock & Aslanzadeh, 2013).

Besides, the antibiotics sensitivity profiles or Kirby-Bauer disc diffusion antibiotic sensitivity test is used to detect whether an antibiotic is effective to treat a given bacteria. If the antibiotic is said to be effective against the bacteria at a particular concentration, the bacteria will not grow when the concentration of the agar at that point is more than the effective concentration (Bhargav et al., 2016). This region of no bacterial growth is called as the Zone of Inhibition. There is a marked difference and easily visible to the naked eye. The measurement of the diameter of this Zone of Inhibition will conform if the antibiotic is effective in treating the bacteria.