MICROBES IDENTIFICATION FROM PALM OIL WASTE FOR NATURAL DECOMPOSER

BY

LIM ZHENG XIN

A project submitted to the Department of Chemical Science

Faculty of Science

Universiti Tunku Abdul Rahman

In partial fulfilment of the requirements for the degree of

Bachelor of Science (Hons) Chemistry

May 2018

ABSTRACT

MICROBES IDENTIFICATION FROM PALM OIL WASTE FOR NATURAL DECOMPOSER

Lim Zheng Xin

Oil Palm empty fruit brunch (OPEFB) sludge is produced from palm oil milling process which has not been completely used in palm oil industry. OPEFB sludge is a palm oil waste that can cause environmental pollutions when there is an inappropriate disposal to the surrounding. The concept of locally isolated microorganisms can be implied to biodegradation of EFB sludge is economically practicable and environmentally friendly. From this research, there were two microbes being isolated locally from EFB sludge. The isolated microbes were characterized by colonial morphology, microscopic morphology and some biochemical tests to identify the type of microbes. Two isolates which labelled as OM1 and WM2 were chosen for further characterization. OM1 was gram negative bacteria while WM2 was gram positive yeast. Both isolates gave same positive biochemical tests of sucrose, glucose, fructose, mannitol and xylose fermentation test and Voges-Proskauer test. Oppositely, both isolates gave similar negative biochemical test of lactose fermentation test, citrate test and indole test. Isolate WM2 was chosen for 16S rDNA gene sequencing to species level with nucleotide-Blast program. Molecular Evolutionary Genetics Analysis (MEGA) 6 software was

used for phylogenetic analysis for the construction of phylogenetic tree. The isolate WM2 showed 100% identical to *Pichia occidentalis* strain. In future, *Pichia occidentalis* can be utilized to decompose furfural and hydroxymethylfurfural (HMF) in lignocellulose hydrolysate oil palm empty fruit bunch sludge. Thus, the biological detoxification of furfural and HMF can be successfully convert to less harmful composites.

ABSTRAK

IDENTIFIKASI MIKROBES DARI MINYAK KELAPA SAWIT UNTUK DECOMPOSER NATURAL

Lim Zheng Xin

Enapcemar buah kelapa sawit kosong (OPEFB) dihasilkan daripada proses pengilangan minyak kelapa sawit yang belum sepenuhnya digunakan dalam industri minyak sawit. Enapcemar OPEFB adalah sisa minyak kelapa sawit yang boleh menyebabkan pencemaran alam sekitar apabila terdapat pelupusan yang tidak sesuai untuk sekelilingnya. Konsep mikroorganisma yang terpencil secara tempatan boleh disyorkan untuk biodegradasi enapcemar EFB secara ekonomi praktikal dan mesra alam. Dalam kajian ini, terdapat dua mikrob yang diasingkan secara tempatan dari enapcemar EFB. Mikrob terpencar dicirikan oleh morfologi kolonial, morfologi mikroskopik dan beberapa ujian biokimia untuk mengenal pasti jenis mikrob. Dua isolat yang dilabelkan sebagai OM1 dan WM2 telah dipilih untuk pencirian lanjut. OM1 adalah bakteria gram negatif manakala WM2 adalah ragi gram positif. Kedua-dua isolat memberikan ujian biokimia positif yang sama terhadap ujian fermentasi sukrosa, glukosa, fruktosa, manitol dan xilosa dan ujian Voges-Proskauer. Sebaliknya, kedua-dua isolat mempunyai ujian biokimia negatif yang sama terhadap ujian fermentasi laktosa, ujian sitrat dan ujian indole. Isolat WM2 telah dipilih untuk pengenalan gen 16S rDNA sehingga tahap spesies dengan

program nukleotida-Blast. Perisian Analisis Genetik Evolusi Molekular (MEGA) 6 digunakan untuk analisis filogenetik untuk pembinaan pokok phylogenetic. Isolat WM2 menunjukkan 100% identiti dengan strain *Pichia occidentalis*. Pada masa depan, *Pichia occidentalis* boleh digunakan untuk mengurai furfural dan hydroxymethylfurfural (HMF) dalam lignocellulose hydrolyzate enapcemar buah kelapa sawit kosong. Oleh itu, detoksifikasi biologi furfural dan HMF berjaya diubahkan menjadi komposit yang kurang berbahaya.

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere gratitude to my final year project supervisor, Dr. Sim Yoke Leng and co-supervisor, Dr. Kuan Chee Hao for her/his patience, enthusiasm, motivation, immense knowledge and most importantly her/his unceasing support for my final year project. Her/His guidance assisted me a lot in completing the research and thesis.

Apart from that, I would like to address my appreciation to my friends, lab mates and lab officers for the thought-provoking discussion and assists me for completion of research and thesis. Indeed, they have provided a friendly and cooperative work environment as well as useful feedback and insightful comments on my work. Moreover, I would like to thank my family members for their unconditional support, both financially and emotionally throughout my degree.

Last but not least, my deepest appreciation was addressed to Universiti Tunku Abdul Rahman (UTAR) for providing me a comfortable environment and sufficient advanced facilities to carry out my final year project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

(LIM ZHENG XIN)

APPROVAL SHEET

This project report entitled **"MICROBES IDENTIFICATION FROM PALM OIL WASTE FOR NATURAL DECOMPOSER"** was prepared by **LIM ZHENG XIN** and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Chemistry at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Dr. Sim Yoke Leng) Date: ……

Supervisor

Department of Chemical Science

Faculty of Science

Universiti Tunku Abdul Rahman

_____

(Dr. Kuan Chee Hao) Date: ……

Co-Supervisor

Department of Agricultural and Food Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **LIM ZHENG XIN** (ID No: **15ADB02674**) has completed this final year project entitled **"MICROBES IDENTIFICATION FROM PALM OIL WASTE FOR NATURAL DECOMPOSER"** under the supervision of **Dr. SIM YOKE LENG (Supervisor)** from the Department of Chemical Science, Faculty of Science and **Dr. KUAN CHEE HAO (Co-Supervisor)** from the Department of Agricultural and Food Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

_____

(LIM ZHENG XIN)

TABLE OF CONTENTS

CHAPTER 3

MATERIALS AND METHODS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBRREVIATIONS

EFB	Empty fruit bunch
POME	Palm oil mill effluent
PKS	Palm kernel shell
OPEFB	Oil palm empty fruit bunch
SPO	Sludge palm oil
FFA	Free fatty acids
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
16S rDNA	16 subunit ribosomal ribose nucleic acid
PCR	Polymerase chain reaction
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
ITS	Internal transcribed spacer
MEGA	Molecular Evolutionary Genetics Analysis
MSA	Mannitol salt agar
DMAB	p-dimethylaminobenzaldehyde
LB	Luria Broth
PDA	Potato dextrose agar
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine
MR-VP	Methyl red-Voges Proskauer
KOH	Potassium Hydroxide

TME	True metabolisable energy
H_2O_2	Hydrogen peroxide
HMF	Hydroxymethylfurfural
rpm	Revolutions per minute
w/v	Weight/volume
sp.	Species
mL	Millilitre
µL	Microlitre
V	Volts

CHAPTER 1

INTRODUCTION

1.1 Oil palm

1.1.1 Palm oil industry

Nowadays, palm oil industry acts as an important role as a main support to the economy of a country, especially in many developing countries. Many sectors such as environment, economic and social are gaining benefits from the production of palm oil as the palm oil industry progresses (Zabid and Abidin, 2015). Oil palm tree (Elaeis guineensis) which is originally from West Africa initially grows in the wild and later was developed into an agricultural crop. Oil palm is considered as a famous crop that grown in tropical countries as it produces the greatest oil crop yield in the world (Murphy, 2014).

Oil palm can be found in almost 43 countries in tropical regions of Southeast Asia, Africa and South America although it was originated in Africa. Among palm oil production countries, two major producers of palm oil in the world are Indonesia and Malaysia which contributed 21.1 and 17.6 million tonnes respectively. However, Malaysia still embraces the title as the world's largest crude palm oil

exporter (Mahat, 2012). Currently, Malaysia constitutes to 39 % of palm oil production and 44% of exports in the world (Shevade and Loboda, 2019).

1.1.2 Palm oil production

Fresh fruit bunches (EFB) is harvested from the oil palm trees. Firstly, crude palm oil is extracted from the fruit during the processing in the palm oil mill. For the second stage of processing, the crude palm oil undergoes further refined to get a wide range of palm products of specified quality. Solid (stearin) and liquid (olein) fractions of various melting characteristics of palm oil can be obtained by fractionated, using simple crystallization and separation processes (Basiron, 2007). Two different type of oils that being produced from oil palm fruit are palm oil and palm kernel oil. Palm oil is produced from the mesocarp while palm kernel oil is produced from the seed or kernel. About 1 tonne of palm kernel oil is obtained for every 10 tonnes of palm oil (Abdullah and Sulaiman, 2013). Palm oil can be used as a source of affordable edible and non-edible oils, bio-composites, nutritious and other pharmacological products. Palm oil products that considered edible occupy 80% while non-edible use for palm oil product only 20% which is used in chemical manufacturing. Currently, palm oil constitutes for about 5% of the production of bio-diesel in the world (Otieno, et al., 2016).

1.1.3 Oil palm biomass waste

Biomass can be defined as the organic matters or compounds which can be processed from crops, forestry or aquatic life. Sewage and municipal solid waste are also considered as biomass. According to Sanagi (2018), palm oil plantations produced an estimated of 85.5% agricultural wastes in Malaysia. The oil palm biomass waste contains of mainly empty fruit bunches (EFB), shell and kernels, fronds, leaves and trunks. From oil palm industry, the solid biomass wastes can be generated in two ways. Firstly, the solid waste can be produced from oil palm plantations which generated in the form of harvested trunks and pruned fronds. Secondly, EFB, mesocarp fiber and PKS are the solid waste that can be produced from the palm oil extraction mills (Awalludin, et al., 2015).

Palm oil mill effluent will be generated also during the production of palm oil which contributed about 65.35 million tons per year (Oseghale, Mohamed and Chikere, 2017). The high demand of palm oil production in Malaysia causes the palm oil waste difficult to treat and hard to manage in short time. Consequently, environment problem will be occurred when there is inappropriate treatment of palm oil waste due to significantly amount of palm oil waste (Khatiwada, Palmén and Silveira, 2018).

1.1.4 Oil palm empty fruit bunch (OPEFB)

Oil palm empty fruit bunch is a secondary product of the palm oil milling process, left over after the fruits from the fresh fruit bunches have been removed for oil extraction. EFB is a product with fibrous texture and is in wet condition when it is in a raw state. After the processing of EFB, it usually contains 30 to 35 % lignocellulose, 1 to 3 % residue oil, and roughly 60 % of moisture. Similar to other lignocellulosic biomass, EFB consists of 44.2 % of cellulose, 33.5 % hemicellulose and 20.4 % lignin in nature (Jinn, et al., 2015). It can be said that an approximately of 230 kg of EFBs would be generated during the processing of every 1 tonne of oil palm fresh fruit bunch. Oil palm empty fruit bunch is yet to be fully utilized economically, handling in the palm oil mill also consumes unproductive cost and energy. EFB suitable for bioconversion into value added product as it can be obtained in huge amount, easily available and rich in lignocellulose (Shahriarinour, et al., 2011).

1.1.5 Oil palm empty fruit bunch (OPEFB) sludge

Oil palm empty fruit bunch sludge is a by-product that generated from sludge settling after the extraction of palm oil during the crude palm oil milling process. The sludge consists of 1 to 1.2 % of residual oil which unable to be extracted out from the sludge by mechanical mean. Therefore, it will discharge into the effluent treatment ponds (NORHAYATI, 2013). Sludge palm oil (SPO) is the recovered oil

from sludge pit of palm oil milling process. It has the characteristic of dark brown in colour, bad odour and maintain solid state at 25°C. There are two major constituents in SPO which are free fatty acid (FFA) and neutral oil. PSO can act as the substrate for microbes that present in the natural environment. The sludge is a low quality oil which unable to use directly as food source due to high free fatty acids contents which ranging from 40 to 80% (by weight) (Wafti, Harrison, and Choo, 2012).

From the EFB sludge, it consists of 10 % of crude protein, 10 % of crude fat, 12 % of crude fiber, 54 % of soluble carbohydrate and the remaining is water. An estimation of 6.8 million tons of palm oil sludge discharged each year (Shacklady, 1983). As Sinurat (2003) stated, it shows that sludge can be used in the feed of cattle, pigs and poultry. However, limitation of sludge in feed due to high fiber contents and low nutrient digestibility. By undergo fermentation process, the fiber content of sludge can be reduced with the increasing in protein, amino acids and True metabolisable energy (TME).

1.2 Microorganisms

Microorganisms or microbes which commonly recognized as microscopic organisms, can be existed all around the earth. There are different types of organisms such as bacteria, fungi, yeast, viruses, algae, archaea and protozoa. Most and the microorganisms have no harmful threat to humans, plants and animals

where they are widely used in our daily life including helping in decomposition, decaying process and digestion of food However, a small number of microorganisms will cause negatively influence in our lives, result in infection, bad smell and product damaged (Batt, 2016). Microorganisms can be isolated from any environment conditions due to they have ability of strong adaption to environmental changes and deterioration (Khan, 2014). Microorganisms play an important role in the biogeochemical cycling of numerous elements, and can used in various environmental and industrial applications. They can be used in degradation and removing of contaminants from water, soil and air which convert low grade resources and waste streams into value added products (Kaksonen, 2018).

1.2.1 Importance of microorganisms

Previous researched shows there are microorganisms that can be isolated and used in palm oil waste treatment. Palm oil waste are degraded in a slow process when in natural conditions. Treatment of palm oil waste must be focused on to prevent any impact to our ecosystem. Hence, microorganisms are a need to use in order to speed up the rate of recovery of the polluted environment. Therefore, microbes isolated and identification from palm oil waste such as EFB sludge was done in this research. Hence, EFB sludge can be converted into value added product before proceed to the next steps (Gandahi and Hanafi, 2014).

1.3 Problem Statement

Based on Abdullah and Sulaiman (2013) studies, large amount of EFB was disposed by combustion which caused air pollution. During the digestion of POME, odor released into surrounding air, hence, the air quality in the surrounding area become worst. From Metosen (2009) reviews, he stated that as the EFB sludge produced significantly from empty fruit brunch, the disposal of EFB sludge on land can cause soil contamination and accumulation of heavy metal in crops. Due to high content of nutrients and organic matter in EFB sludge, soil contamination occurred when disposal of sludge in agriculture. During raining, the toxic organic matter flow from the soil into lake water which causes water pollution, and as a result affect aquatic life. Hence, microbial practice is an alternative way to minimize EFB sludge waste that produced from palm oil milling process.

1.4 Objectives of study

The purpose of this research was to identify the potential microbes that can be found in EFB sludge. The objective of this research was:

i. To isolate potential microbes from EFB sludge
ii. To perform colonial morphology, microscopic morphology and biochemical tests on isolated microbes to characterize them easily
iii. To identify isolated microbes to species level via 16S rDNA sequencing

CHAPTER 2

LITERATURE REVIEW

2.1 Waste production in Malaysia

Waste can be defined as unusable, undesirable or castoff materials which itself does not have any value and are thrown away. In Malaysia, the amount of wastes is continuously increasing due to industrial development, population growth and urbanization (Zaved, et al., 2008). Wastes can separate into 3 major categories which are solids, liquids and gases. However, the most challenging waste are liquid and solid wastes. Solid and liquid waste can produce from industrial, mining, household, agricultural and plantation. There are several liquid wastes such as wastewater, oils or grease (FOG), used oil, and harmful household liquids while solid waste such as food waste, plastic, garbage and sludge from waste water treatment plant.

2.1.1 Food waste

Food waste is an organic waste which can be generated from numerous sources such as food processing industry, domestic and kitchens and restaurants. Based on the statistics from Solid Waste Corporation of Malaysia (SWCorp), it showed that generation of food waste in Malaysia reached 15,000 tonnes daily in 2015. There

are negatively impact on the accessibility of food to others if the amount of food waste is not in control. Famine and malnutrition will occur among the people when over one third of worldwide produced food is wasted.

2.1.2 Agricultural waste

Agriculture can be defined as the manufacture of food stuff and corresponding stocks through farming. Agricultural waste was generated from a variety of agricultural processes. Composition of agricultural waste will base on the organization and variety of agricultural activities. They can be in the form of liquids, slurries, or solids. The waste includes animal dungs, crop waste and fertilizer that run- off from fields. Crop residues are produced in significant amount and it is less likely to be used as a source of renewable biomass in agriculture (Nagendran, 2011). Production of agriculture is growing which give rise to an increase amounts of livestock waste, crop deposits from agricultural and agro-industrial by-products. It is predicted that approximately 998 million tonnes of agricultural waste is generated annually (Obi, Ugwuishiwu and Nwakaire, 2016).

2.1.3 Palm oil waste

From oil palm plantation, the oil that extracted contributes only 10% of the total biomass generated while the remaining is considered as wastes. About 50 to 70 tonnes of biomass residues are generated from each hectare of oil palm plantation.

The wastes that produced contains empty fruit bunch (EFB), mesocarp fruit fiber (MF), palm oil mill effluent (POME) and etc. EFB can be obtained from the residual of fruit bunch after the fruit are removed from fresh fruit bunch. Palm oil mill effluent (POME) can be obtained during oil mining and cleaning processes in the mill. Environmental problems will be occurred with undesirable managing of raw effluent into river streams. 2.5 tonnes of POME is produced with the extraction of every one tonne of crude palm oil in milling process (Singh, et al., 2010).

2.2 Earlier study of food waste treatment using microorganism

Microorganisms can use up and decrease the quantities of food wastes due to food wastes contain many organic compounds. According to An, Park and Oh (2018), food waste can be treated by using different kind of microorganisms including aerobic and anaerobic fermentation. Bacteria is the most common microorganisms that can be found in food wastes and it can be used for food waste treatment. *Pseudomonas spp.*, *Xanthomonas spp.*, *Bacillus spp.*, and *Stearothermophilus spp.* which known as mesophilic and thermophilic bacteria are often found in food wastes. It is stated that thermophilic bacteria show good growth and undergo food wastes decomposition effectively as the temperature increased to 50°C during fermentation process. Moreover, thermophilic bacteria can produce hydrogen from food waste decomposition. In addition, thermophilic *Bacillus subtilis* that isolated from food waste exhibit strong proteolytic and cellulolytic activities which have a stronger ability to degrade food waste.

2.3 Earlier study of agricultural waste treatment using microorganism

Composting can define as a main process of stabilizing agricultural wastes during the break down of decomposable components by microorganisms. Based on Zhang, et al. (2014), they stated that white-rot fungi have ability to degrade lignocellulose in agricultural waste into water, carbon dioxide and other low molecular mass compounds. White-rot fungi, *Phanerochaete chrysosporium* was able to decompose lignin and a wide-ranging variation of aromatic compounds as it was known as the most active ligninolytic organisms. This species also give rise to several effects on the microbial enzyme activities, removal of organic contaminants and reduction of toxic heavy metals during composting of agricultural waste. Therefore, *Phanerochaete chrysosporium* can give rise to lignocellulose biodegradability and the value of the compost products can be improved as well.

2.4 Earlier study of empty fruit bunch treatment by microorganism

The main enzymes that used in degradation of lignin were lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). From researched, white rot fungi (WRF) including *Trametesversicolor*, *Trametes hirsuta*, *P. ostreatus* and *P. radiata* were described as lignin degrading microorganisms which able to produce all the enzymes. Nevertheless, fungi that contain ligninolytic enzymes had low constancy on high working temperature, high pH condition and high substrate conditions. This is due to the delignification process required a higher temperature above 70 ℃.

Moreover, *Paenibacillus sp*., *Bacillus sp*., and *Streptomyces sp*. were recognized as the bacterial enzymes with ability to degrade lignin. The three species have the ability to produce the major enzyme. Furthermore, *Ureibacillus terrunus*, *Nocardiopsis sp*., *S. violaceorubidus* and *Streptomyces sp.* which known as thermophilic bacteria were isolated from EFB (Lai, et al., 2017).

2.5 Earlier study of palm oil mill effluent treatment by microorganism

According to Bala, et al. (2018), microorganisms that can be discovered in POME has the ability to utilize carbon source exist in the POME. Since palm oil mill effluent consists of oily characteristic, it was a suitable environment for lipolytic microorganisms to grow in it. These microorganisms utilized the oil as its carbon source. *Micrococcus luteus*, *Stenotrophomonas maltophilia*, *Bacillus cereus* and *Bacillus subtilis* were isolated from palm oil mill effluent. They have the capability to degrade lipid (oil) as carbon source with the enzyme lipase. These species are known as oil degrading microorganisms as they able to hydrolyze lipid content. There microbes are able to break down oil in POME and decompose organic matters into water and carbon dioxide.

2.6 Cellulose

2.6.1 Structure and properties

Cellulose can be defined as the most abundant biopolymers on earth which is the major structural component of the cell wall of plant biomass (Rudnik, 2012). Cellulose consists of 44.44% carbon, 6.17% hydrogen, and 49.39% oxygen. Cellulose is a linear organic polysaccharide which involve the combination of many glucose monosaccharides with the chemical formula $(C_6H_{10}O_5)n$ link together through β(1→4)-glycosidic bonds. Cellulose has the characteristic of odorless, tasteless, low solubility in water and organic solvents, chiral and biodegradable (Dahman, 2017). Since cellulose is a polysaccharide polymer, its backbone consists a large amount of hydroxyl groups. With the presence of these hydroxyl groups, cellulose can form a compact crystalline structure with well-ordered hydrogen bonding network. Moreover, some of the cellulose chains have disorderly rearrangement which cause amorphous region of cellulose (Sun, et al., 2016). The cellulose structure is shown in Figure 2.1.

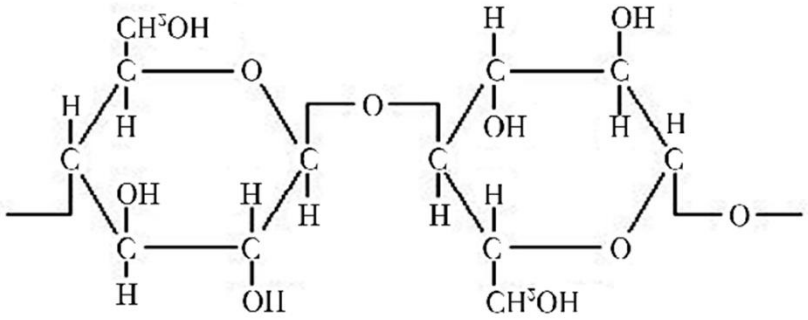


Figure 2.1: Structure of cellulose

2.6.2 Cellulase

Cellulase is an enzyme produced by the fungi or bacteria and which will undergo cellulolysis. This process is known as hydrolysis of cellulose. The major cellulase enzyme are endocellulase, exocellulase and beta-glucosidase. Cellulose molecule can break down into monosaccharides such as beta-glucose or shorter polysaccharides by enzyme cellulase (Garg, et al., 2016). Cellulases enzymes have the ability to hydrolyze β -1,4 linkages in cellulose chains. Due to the strongly binding of cellulose molecules between each other's, breakdown of other polysaccharides such as starch is relatively easier compare to cellulolysis. Currently, the usage of cellulases in huge variation of manufacturing purposes such as fabric industry, pulp and paper industry, nutrition industry and etc. are increasing in the world (Zhang and Zhang, 2013).

2.6.3 Cellulase producing microorganisms

Cellulose producing microorganisms can be differentiate into aerobic, anaerobic, mesophilic or thermophilic. The major source of enzyme cellulase is filamentous fungi but it is rarely to be used due to high production costs. Compare with fungi, bacteria have the potential to be used in the production of cellulase as it has a high growth rate and short generation time (Khatiwada, et al., 2016). Based on Hii, Yeap, and Mashitah (2012), small amount of microorganism can produce cell-free enzymes that have ability to degrade crystalline cellulose. *Aspergillus* and

Trichoderma species are the fungal that originally produced commercial cellulases. For bacteria, *Bacillus sp.* were found unable to hydrolyze crystalline cellulose completely while *Clstridium* species capable in degrading highly crystalline cellulose.

2.6.4 Earlier study of cellulase producing microorganisms in palm oil industry

According to Krishnan, et al. (2017), microbes that isolated from co-composting of EFB and POME is *Bacillus subtilis* which is a potent thermophilic cellulolytic bacterium. The optimum temperature and pH of cellulase enzyme activity of *Bacillus subtilis* was at 50 °C and pH 7.2. These conditions are ideal for the breakdown of cellulose, which contributes 45.8% of EFB-POME compost. Based on Fadzilah and Mashitah (2010), it stated that Palm Oil Mill effluent can act as a potential substrate for the cellulase production. The isolates that found in POME is the soft rot fungus *Trichoderma*, particularly *T. reesei*. *Trichoderma* is recognized as a fungal that give high activities of overall cellulases, which small quantities of β-glucosidase will be produced, subsequent in the accumulation of cellobiose. It can act as a strong inhibitor in the cellobiohydrolase activity.

2.7 Lignin

Lignin is a three-dimensional compound, phenolic, natural polymer that have the ability to provide structural support to woody plants. It can be considered as the richest polymer synthesized by plants and have significantly amount of renewable aromatic resource on earth. Moreover, its function by increasing rigidity to the plant cell wall and defends plants from pathogenic microorganisms (Siong, 2009). Lignin is the major structural constituent of lignocellulosic biomass. As a biopolymer, it is considered as unusual due to its different in elements and absence of a well-defined primary structure. It has the characteristic of insoluble in water and rich in aromatic subunits (Lu, 2017).

2.8 Hemicellulose

Hemicellulose is defined as a group of heterogeneous polysaccharides with equatorial configuration of β- (1→4)-linked backbones which are formed through biosynthetic pathway different from cellulose. Hemicellulose have the same function as cellulose which act as supporting material in the cell wall. It consists of multiple sugar units which is not similar as starch and cellulose that contain one glucose units. The monomer units in hemicellulose are xylose, mannose, galactose, rhamnose, and arabinose units. In addition, hemicellulose can undergo chemical or enzymatic processes to hydrolyzed itself into sugar constituents (Ji, et al., 2011).

2.9 Isolation technique on microbes

2.9.1 Streak plate method

The streak-plate technique is a simple mechanical isolation which used to separate pure cultures of bacteria, or colonies, from varied populations. This technique often used to isolate discrete colonies to decrease the number of organisms in the inoculums. This method is also known as a technique of dilution which a small amount of culture can be spread to the agar surface. By using this technique, several types of colony are spread onto the agar medium surface, continue transferring the colony from an old, partially contaminated medium to a fresh new medium. Lastly, pure single colony will end up in the agar plate. Two major streak patterns which are three sector "T streak " and four quadrant streaks are the most often used (Sanders, 2012). Figure 2.2 shows the three sector "T streak " and four quadrant streak methods.

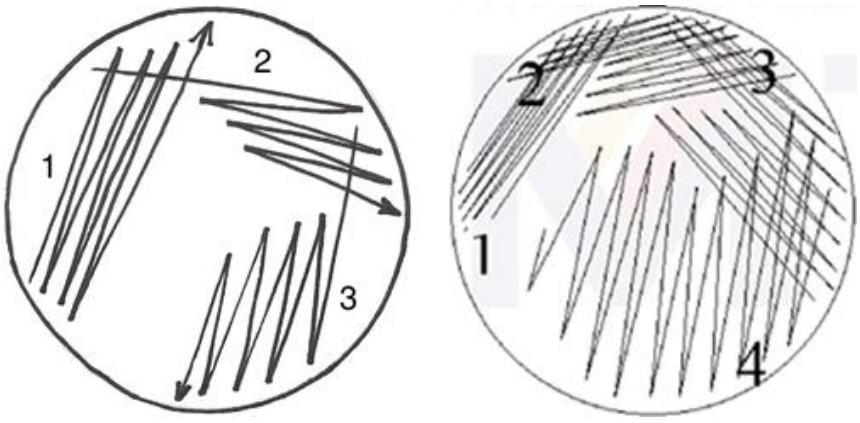


Figure 2.2: Three sector "T streak " (left) and four quadrant streak (right)

2.10 Characterization of isolates

Microbial life is astonishingly varied and microorganisms can be found anywhere in the planet. Some of the microorganisms can survive in extreme temperature and environment (Donlan, 2002). Different type of microbes has different kind of patterns and characteristics. Pure colony can be characterized by observing the colony and microscopic morphology. Normally, gram staining is used to determine the isolate whether it is gram positive or gram negative and followed by observing under power microscope (Beveridge, 2001). The colony morphology of the isolates can be determined by their colour, texture, surface appearance and sizes of the colony. Biochemical tests are then used to evaluate the metabolic properties of colony. Lastly, the pure colony is done with DNA extraction and PCR amplification to obtain 16S rDNA gene sequencing with the construction of phylogenetic tree (Garibyan and Avashia, 2013).

2.10.1 Colonial morphological characterization of isolates

Colonial morphological characterization can be done to observe the features of a pure colony of growing on the surface of agar medium. The colonial morphological characterization of each isolates was examined by pigmentation, shape, surface appearance, opacity, elevation, texture, margin and emulsifiability Every microbe has different pigmentation that can differentiate themselves from others. The shape of isolates can be classified into circular, irregular, filamentous and rhizoid.

Regarding on the surface appearance, it includes shiny with smooth or rough and dull with smooth or rough surface. Moreover, isolates can have opacity of transparent, opaque and translucent and texture of dry, mucoid, moist and etc. In addition, the elevation of isolates includes raised, convex, flat, umbonate and crateriform while the margin includes entire, undulate, filiform, curled and lobate (Abiola and Oyetayo, 2016).

2.10.2 Gram staining reaction

Gram staining is a technique that used to differentiate Gram-positive and Gram-negative bacteria with the usage of different staining method which was staining with a crystal violet–iodine complex and a safranin counterstain. Gram positive and negative microorganism is determined by the structure of the organism's cell wall. Bacteria that can resist decolorization with the retain of primary stain (crystal violet) is determined as gram positive. Oppositely, bacteria that unable to resist decolorization and retain of counterstain (safranin) is determined as gram negative. Complex of crystal violet iodine (CV-I) will form. Acetone is used as a decolorizer which decolorize the crystal violet stain on the isolate. Gram negative organism have thin peptidoglycan layer which cause the CV-I complex being washed away by decolorizer so it will stain the pink or red colour of safranin. Oppositely, gram positive organism has thick peptidoglycan layer which trapped CV-I complex inside without being washed away by decolorizer so it will stain the primary purple stain of crystal violet (Thairu, Nasir and Usman, 2014).

2.11 Molecular characterization of isolates

Molecular identification techniques are used to perform the different classification and characterization of a pure colony. Extraction of the genomic DNA, polymerase chain reaction (PCR), DNA sequencing and phylogenetic analysis are the commonly used method for the molecular characterization of isolate.

2.11.1 DNA extraction

DNA isolation is a method that use for DNA purification from an isolate by using either physical or chemical methods. DNA extraction methods include three types which consists of extraction of organic, Chelex extraction and solid phase removal. These methods can yield isolated DNA constantly, however quality and the quantity of DNA yielded are different from each of them. Pure DNA sample have the ratio of absorbance at 260 nm and absorbance at 280 nm (A260/A280) is 1.8. Contamination of sample by protein or organic solvent will be occurred when the ratio is less than 1.8 (Tan and Yiap, 2009).

2.11.2 Polymerase chain reaction (PCR)

PCR is a common amplification method that used to create a plenty source of a specific segment of DNA from only a small quantity of initial material. Polymerase chain reaction enable the generation of duplicates DNA sequence and 16S

ribosomal DNA (rDNA)- can used for the identification of microbes. The sequenced that already analyzed can be compared with the other identified sequence in the database by using Basic Local Alignment Search Tool (Lorenz, 2012). Agarose gel electrophoresis can be used for the separation of size for PCR products to confirm PCR successfully generated the anticipated DNA target region. DNA ladder is a molecular weight marker that consists of known size DNA fragments which travel on the gel along the PCR products. It is used for the comparison of the size(s) of PCR products (Lee, et al., 2012).

2.11.3 Phylogenetic analysis

Phylogenetic analysis can be refer as phylogenetic tree or evolutionary tree which the construction of branching diagram display the evolutionary relationships between numerous biological species according to their phylogeny. It also can be constructed by referring to the similarities and differences in their physical or genetic features (Ziemert and Jensen, 2012). There are 2 types of tools to construct phylogenetic trees, such as iTOL and TaxonTree. iTOL a is tools that create the images of trees, with features such as tree annotation and the demonstration of horizontal gene transfers. But basic zooming and pruning restrict its interaction options. Oppositely, TaxonTree is also a tool for visualization of tree with a higher degree of interaction, but large trees are hard to visualize correctly due to the linear node-link tree layout (Santamaría and Therón, 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1 Research Framework

There were seven phases to be conducted in this research: sample collection, centrifugation, microbes isolation, colonial morphological studies of isolates, staining reaction of microbes, biochemical characterization and identification of microbes and identification of microbes via 16S rDNA sequencing. Aerobic condition was maintained to ensure successful growth of microbes.

3.1.1 Materials

In this research, the raw materials which was Oil Palm Empty Fruit Bunch Pressed Liquor (OPEFBPL) was collected from United Oil Palm Industries Sdn. Bhd in Nibong Tebal, Pulau Pinang, Malaysia. The chemicals used for glycerol preparation were LB broth and glycerol solution. The chemicals used for gram staining were methanol, crystal violet, sodium bicarbonate buffer, gram iodine, acetone, safranin dye and oil. The chemicals used for catalase and oxidase test were 3 % hydrogen peroxide and 1% oxidase reagent. The chemicals used for carbohydrate fermentation test were starch, sucrose, xylose, galactose, fructose, lactose, glucose, mannitol, carbohydrate, phenol red, peptone and sodium chloride.

The chemicals used for urease test were Christensen's urea agar and Stuart's urea both. The chemicals used for citrate test was Simmons Citrate Agar while for MRVP test was MR-VP broth. The chemicals used for indole test were peptone, sodium chloride and tryptophan while for mannitol salt agar plate test was Mannitol Salt Agar.

3.1.2 Apparatus

The apparatus used in this research included inoculating loop, conical flask, culture tube, petri dish, microcentrifuge tube, pipette tips, pipette tip box, Bunsen burner, igniter and microscope slide.

3.1.3 Equipment and instruments

In this research, the equipment and instruments used were Centrifuge Machine, (Brand: Eppendorf, Model: 5430), Incubator Oven and Biosafety Cabinet (Brand: Telstar).

3.2 Sample collection

The OPEFB pressed liquor samples were collected from United Oil Palm Industries Sdn. Bhd in Nibong Tebal, Pulau Pinang, Malaysia. The samples were mixed homogenously and transferred into a sterile plastic container. The plastic container

was tightly screwed and transported to the laboratory facilities in Universiti Tunku Abdul Rahman located in Kampar.

3.3 Centrifugation

Approximately 40 mL of OPEFB pressed liquor was poured into a centrifuge tube. The centrifuge tube was placed in the centrifuge machine (Eppendorf, Model 5430). The speed of machine was set to 7830 rpm for 10 minutes. After centrifugation, three layers were formed: oil layer, aqueous layer and EFB sludge. The EFB sludge was separated and stored at -80 ℃ for isolation of microbes.

3.4 Isolation of microbes

3.4.1 Inoculum preparation

The OPEFB sludge obtained from centrifugation was directly streaked on the surface of agar. Inoculated plates were incubated at two different temperatures of 25 ℃ and 32 ℃ for 7 days (Davis, Joseph and Janssen, 2005). The growth of microbes was observed daily. Different morphological structure of yeast and bacterial colonies was picked and sub-cultured on a fresh PDA to obtain a pure culture. Sub-cultured of colony was repeated until same morphological of same colonies present in the plate. Pure colony was picked and dipped into a centrifuge tube containing LB broth using a sterile inoculation loop. The tube was gently

swirled and covered loosely with a sterile cap. The microbes were then incubated in a shaking incubator with 250 rpm at 35 ℃ for 24 hours.

3.4.2 Microbes glycerol stock preparation

500 µL of the overnight culture was added to 500 µL of 50% sterile glycerol in a 2 mL labelled sterile screw cap microcentrifuge tube. The cultures were gently mixed and stored in temperature of -80 ℃. To recover the microbes from glycerol stock, sterile inoculation loop was used to scrape some of the frozen glycerol stock off of the top. The frozen glycerol stock was not encouraged to unthaw as subsequent freeze and thaw cycles will reduce the shelf life of the microbes cultures in the glycerol stock (Heller and Spence, 2019).

3.5 Characterization of isolates

3.5.1 Colonial morphological and cultural characterization of the isolates

There was different type of microbes that isolated but each of them will give different-looking colonies. Hence, the colonial morphological characterization of each microbes was studied and observed to identify the microbes species. The colonial morphological characterization of each isolates was examined by pigmentation, shape, surface appearance, opacity, elevation, texture, margin and emulsifiability (Bai, et al., 2010).

3.5.2 Gram staining

The purpose of gram staining was to differentiate the bacteria either in gram negative or gram positive (O'Toole, 2016). Firstly, a loopful of pure colony from agar culture plate was transferred to the microscopic slide using sterile inoculation loop. A droplet of tap water was drop on the colony. The colony was smeared to emulsify with tap water and allowed it to air dry. Methanol was used to rinse though the slide in order to fix the smear. Methanol fixation is used rather than heat fixation to preserve the morphology of host cells. Crystal violet was added to the fixed culture. Sodium bicarbonate buffer was added to prevent the formation of crystal violet crystals on the smear. The slide was rinsed with tap water for 30s and allowed to air dry. Next, the addition of the gram iodine solution to the smear was allowed to set for 1 min before rinsing with tap water. Acetone was used to decolorize the smear until the dark purple colour was faded to colourless then only rinsed under tap water to stop the decolourization process. Lastly, the red dye safranin was added to the smear and allowed to set for 30s before rinsing under tap water. The slide was allowed to air dry before observed under power microscope. The slide was examined under power microscope with objective power of 4x, 10x, 40x and 100x (Oil immersion) magnification (Sandle, 2004).

3.6 Biochemical characterization of isolates

Biochemical tests were conducted on each of the isolates to determine their species based on the differences in biochemical activities (Holding and Collee, 1971). There were 8 types of biochemical tests being performed such as catalase test, oxidase test, carbohydrate fermentation test, urease test, citrate test, methyl red test, Voges-Proskauer test, indole test and mannitol test.

3.6.1 Catalase test

This test is conducted to determine the existence of catalase enzyme in an isolate., A small amount of a well-isolated 18 to 24 hours fresh colony was picked and placed onto the microscope slide by using a sterile inoculating loop. 3 % hydrogen peroxide was dropped on the isolates and the formation of bubbles were observed. Immediate effervescence showed positive result of catalase test while if there was no bubbles formation indicate catalase negative (Reiner, 2010).

3.6.2 Oxidase test

The presence of enzyme cytochrome c oxidase can be detected from this test. A small amount of well-isolated 18 to 24 hours fresh colony was picked by using wooden stick and rubbed on a piece of filter paper. A few drops of 1 % of oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) was added to the smear.

The colour of isolate was observed. Development of purple colour of isolate within 10 s indicated positive oxidase result while no colour change of isolate after 10s indicated negative oxidase result (Tarrand and Gröschel, 1982).

3.6.3 Carbohydrate fermentation test

This test was to examine the capability of isolate in fermentation of specific carbohydrate. 8 carbohydrates such as starch, sucrose, xylose, galactose, fructose, lactose, glucose and mannitol were used to conduct fermentation test. Carbohydrate was excluded in all the phenol red carbohydrate broth ingredients and the ingredients were mixed in 800 mL of distilled and deionized water for the preparation of base broth. 4.5 mL of base broth was added to the culture tube and autoclaved at 121 ℃ for 15 minutes. Let the broth cooled in 50 ℃ water bath before the addition of carbohydrate. 10 g of carbohydrate was dissolved in 200 mL of distilled water to prepare carbohydrate solution. The carbohydrate solution was then filtered through bacteria-retaining membrane for sterilization. 0.5 mL of filtrate was added to each of the culture tubes and mixed well. The final pH of the medium was in the range of 7.2 to 7.6 and light red in colour (Schreckenberger and Blazevic, 1976).

By using inoculation loop, a loopful of isolate was picked from the agar medium into each of the culture tubes. The content in the tube was gently swirled to ensure well mixing. The control culture and inoculated cultures were incubated at 35 ℃

for 18 to 24 hours. In this test, the pH indicator used was phenol red. Positive fermentation test was indicated with the yellow colour solution in culture tubes which means there were sufficient acid being produced during fermentation occurred. Oppositely, red or pink colour solution shown negative fermentation test (Reiner, 2012).

3.6.4 Urease test

The purpose of this test was to identify the microbes which able to hydrolyze urea with the production of ammonia and carbon dioxide in the presence of enzyme urease. Christensen's urea agar and Stuart's urea broth were used in this test.

For Christensen's urea agar, 5 mL of the medium was added to each of the culture tubes. The tubes were allowed to slant during cooling until the solidification of agar occurred. The prepared media had long slant but short butt and was yellow-orange colour. Heavy inoculum isolates from 18 to 24 hours was used to streak the entire slant surface. The tubes were then incubated in 35 ℃ incubator with loosened caps. The colour changes of slant were observed for 18 to 24 hours. Pink colour of the slant indicated the production of urease (Qadri, et al., 1984).

For Stuart's urea broth, each of the culture tubes was poured with 5 mL of the broth. The prepared media was yellow-orange colour. Heavy inoculum isolates from 18 to 24 hours was used to inoculate the broth. The content in the tubes was gently

swirled to ensure well mixing. The tubes were then incubated in 35 ℃ incubator with loosened caps. The colour changes of broth were observed for 18 to 24 hours. Pink colour of the broth indicated the production of urease (Roberts, et al., 1978).

3.6.5 Citrate test

The aim of this test was to determine the isolate which have the capability to ferment sodium citrate as its energy and the main source of carbon with the citrate permease enzyme. 5 mL of the sodium citrate agar medium was added to each of the culture tubes. The tubes were allowed to slant during cooling until the solidification of agar occurred. The prepared media had long slant but short butt and was in deep forest green colour. Heavy inoculum isolates from 18 to 24 hours was used to streak the entire slant surface. The tubes were then incubated in 35 ℃ incubator with loosened caps. The colour changes of slant were observed for 18 to 48 hours. Some of the microbes need up to 7 days of the incubation. Prussian blue colour of the slant indicated citrate positive while no changes in colour indicated citrate negative test (Aditi, Rahman and Hossain, 2017).

3.6.6 Methyl Red and Voges-Proskauer (MR-VP) test

MR-VP broth was obtained as directly from manufacture. A lightly inoculum of pure colony was picked from the agar medium and suspended into the 5 mL MR-VP broth in culture tube. The control and test cultures were incubated in 35 ℃ incubator with loosened caps for 48 hours.

For methyl red test, new sterile culture tube was added with 2.5 mL of the broth with 48 hours incubation. A few drops of methyl red solution were added to the broth. The test cultures were compared with the control culture. The observation was being recorded. A positive MR test is indicated by the formation of red colour broth due to the production of high acid. Oppositely, the yellow colour broth remained unchanged indicated negative MR test due to the production of low acid.

For Voges-Proskauer test, the remaining 2.5 mL of the broth after 48 hours incubation was transferred to a new sterile culture tube. 0.6 mL of Baritt's reagent A (5 % w/v α-napthol in absolute ethanol) and 0.2 mL of Baritt's reagent B (40 % w/v KOH in deionized water) were added to the culture broth. The tube was gentle swirled for 30 s to 1 minute. The tube was allowed to stand for at least 30 minutes. The test cultures were compared with the control culture and the observation was being recorded. Positive result of VP test was indicated by the changing of yellow colour broth into copper red colour while no changing in yellow colour of the broth indicated negative result of VP test (Abiola and Oyetayo, 2016).

3.6.7 Indole test

The purpose of this test is to identify the capability of an organism which can degrade the amino acid tryptophan by enzyme tryptophanase with the production of compound indole. Tryptone broth was autoclaved at 121 ℃ for 15 minutes. By using inoculation loop, a loopful of pure colony was picked and suspended into cooled broth in culture tubes. The control and test cultures were incubated in 35 ℃ incubator with loosened caps for 48 hours. A few drops of Kovac's reagent was added to the tubes. The test cultures were compared with the control culture and the observation was being recorded. Development of red colour ring on top of the medium layer indicated indole positive while the development of cloudy layer in the top layer indicated indole negative (Vashist, Sharma and Gupta, 2013).

3.6.8 Mannitol Salt Agar plate test

Mannitol salt agar (MSA) is both a selective and differential medium used in determining the ability of isolate to ferment the sugar mannitol. A well-isolated pure colony was picked form the agar medium by using sterile inoculation loop and streaked on the mannitol salt agar surface. The plate was incubated in 25 ℃ incubator for 24 to 48 hours. Any changes of colour in plate was observed (Shields, and Tsang, 2006)

3.7 DNA Extraction

The centrifugation machine was set with the speed of 12,000 rpm, duration of 10mins and temperature of 4°C. Centrifugation was done on the overnight culture to pellet the cells. Then, the supernatant was castoff and treatment were done on the pellet according to gram negative or positive bacteria. TaKaRaMiniBEST Bacterial Genomic DNA extraction kit was used for DNA extraction for the culture by following the instructions of the manufacturer (Abdelhai, Hassanin and Sun, 2016).

3.8 Polymerase chain reaction (PCR) amplification

The section of ITS1-5.8S-ITS2 was amplified by using primer sets of forward primer ITS1 (5’ – TCC GTA GGT GAA CCT GCG G – 3’) and reverse primer ITS4 (5’ – TCC TCC GCT TAT TGA TAT GC – 3’) (White, et al., 1990). 20 mL reaction volume consists of 0.2 mM of each primer, 0.2 mM of deoxynucleotides, 1.5 mM of $MgCl_2$, 1X PCR buffer, and 1 unit of DyNAzymeTM EXT DNA polymerase (Finnzymes, Finland) together with 5 mL extracted DNA of isolate WM2. The parameter of PCR involved of an initial denaturation at 95uC for 5 min, and continued for 30 cycles of denaturation at 94uC for 30 seconds. The final extension at 72uC for 10 min was done after annealing at 58uC for 30 seconds, followed by the extension at 72uC for 1 min. 1% (w/v) agarose gel was used to electrophoresed the PCR product at 120 V for 30 min. After that, purification was

done and Sanger sequencing (Apical Scientific Sdn Bhd, Malaysia) was used to determine DNA nucleotide sequence. FinchTV was used to inspect and manually trimmed the base and quality of sequenced ITS. Lastly, MEGA6 was used to aligned the trimmed sequences in order to obtain the clear sequence with complete ITS1-5.8S-ITS2 region (Yew, et al., 2014).

3.9 DNA sequencing and data mining

The nucleotides sequences of WM2 were discovered by using Nucleotide-BlAST program which is a Basic Local Alignment Search Tool which can be found from the website at the National Center for Biotechnology (NCBI). It was a tool that used to compare the information in primary biological sequence such as amino acid sequences of proteins or the sequences of DNA and RNA nucleotides. The blast results with the highest five hits were collected to prevent any untrue information that cause by the errors recorded in the database of NCBI GenBank. ITS sequences of all species collected were then randomly mined from the NCBI GenBank for their complete ITS sequences with at least two sequences verified for each species, except when there was only one record in the database (Yew, et al., 2014.).

3.10 Phylogenetic analysis

All of the ITS sequences of the related species were recovered from the database of NCBI and the strains that do not belong to *Pichia occidentalis*, were undergo

phylogenetic analysis. All data mined ITS sequences of several sequence arrangement was produced with the usage MUSCLE that incorporated with MEGA6 software with default settings. Next, MEGA6 was used to performed the maximum parsimony phylogenetic analysis of strain (Jill and Langdale, 2006). Lastly, construction of phylogram of combined gene analysis with the sequenced ITS gene region was done.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Microbes isolation from EFB sludge

All of the inoculated plates were incubated and the growth of each isolates were observed from 3 to 7 days of incubation. There were 6 pure microbes being isolated from EFB sludge. 2 fungus, 2 mold, 1 bacterium and 1 yeast were isolated. Figure 4.1 shows the appearance of the pure isolates of bacterium and yeast labelled as OM1 and WM2. Figure 4.2 shows the appearance of pure isolates of mold labelled as BM1 and GM2. Figure 4.3 shows the appearance of pure isolates of fungus labelled as WF1 and YF2. The growth of all isolates at 25 ℃ and 32 ℃ were observed and recorded in Table 4.1.

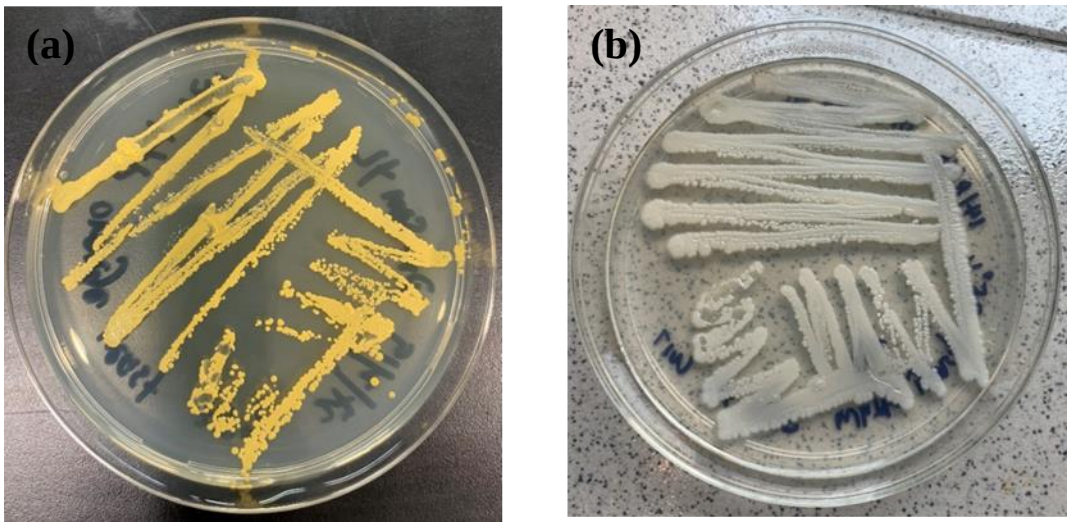


Figure 4.1: The appearance of the (a) bacterium OM1 and (b) yeast WM2

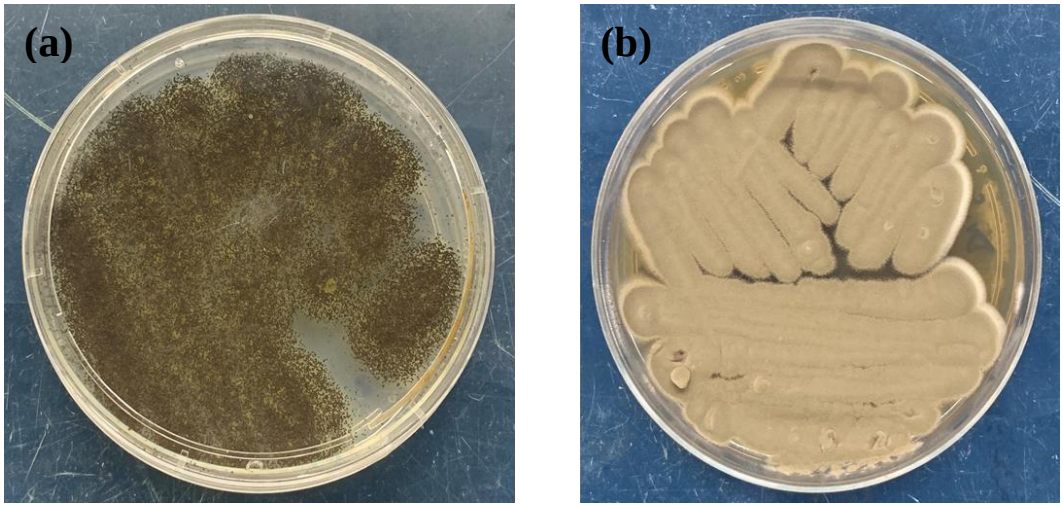


Figure 4.2: The appearance of the mold. (a) BM1 and (b) GM2

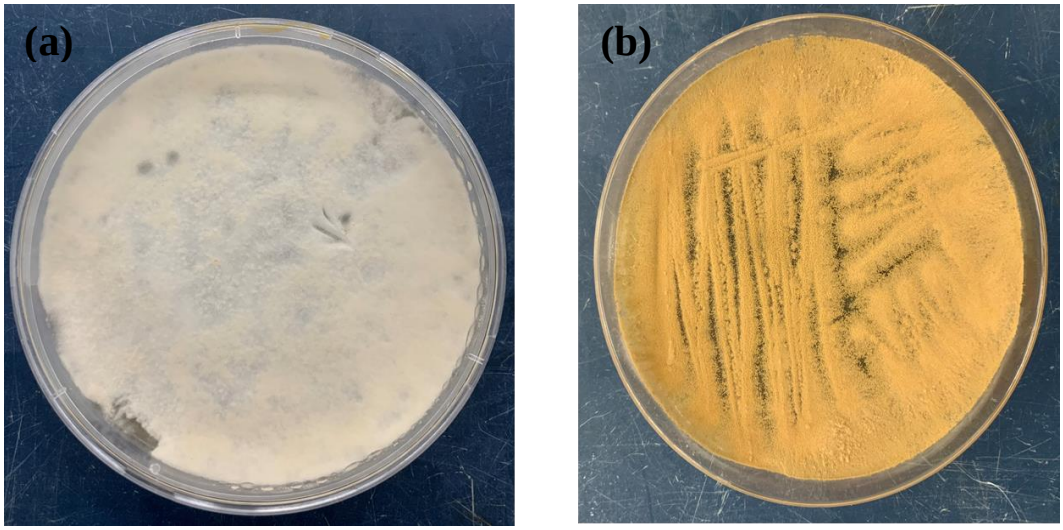


Figure 4.3: The appearance of the fungus. (a) WF1 and (b) YF2

Table 4.1: The growth of microbes at different temperature

Isolates	**Growth Temperature**	
	25 ℃	32 ℃
OM1	+	+
WM2	+	+
BM1	+	+
GM2	+	+
WF1	+	+
YF2	+	+

‘+’indicated positive growth while ‘-’ indicated negative growth

4.2 Colonial morphological characterization of microbes

Colonial morphological characterization of each isolates can be examined by pigmentation, shape, surface appearance, opacity, elevation, texture and margin. All isolates were able to grow on the PDA agar within incubation period of 3 to 7 days and incubation temperature of 25 ℃ and 32 ℃. The pigmentation of each isolates included orange, white, black, green and yellow colour. Isolates OM1 was circular in shape with convex elevation while isolate WM2 was irregular in shape with umbonate elevation. Isolate BM1 and isolate GM2 were in threadlike and filamentous shape with umbonate elevation. Moreover, both isolate WF1 and YF2 was filamentous in shape with flat elevation. Regarding on the opacity, all of the isolates were opaque. For the surface appearance of isolates, it can be divided into

2 groups which were shiny with smooth surface and dull with smooth surface. Both isolate OM1 and WM2 had mucoid texture but difference in their margin. Isolate BM1 had cottony texture with entire margin while isolate GM2 had velvety texture with filiform margin. In addition, isolate WF1 has cottony texture with entire margin while isolate YF2 has powdery texture with entire margin. Among all, bacterium and yeast which designed as OM1 and WM2 were chosen for further characterization in this research. Table 4.2 shows the colony morphological characterization of each isolates.

Table 4.2: Colony morphological characterization of each isolates

Isolates	**Colony characterization**						
	Pigmentation	Shape	Surface Appearance	Opacity	Elevation	Texture	Margin
OM1	Orange	Circular	Shiny with smooth surface	Opaque	Convex	Mucoid	Entire
WM2	White	Irregular	Dull with smooth surface	Opaque	Umbonate	Mucoid	Lobate
BM1	Black	Threadlike	Dull with smooth surface	Opaque	Umbonate	Cottony	Entire
GM2	Green	Filamentous	Dull with smooth surface	Opaque	Umbonate	Velvety	Filiform
WF1	White	Filamentous	Shiny with smooth surface	Opaque	Flat	Cottony	Entire
YF2	Yellow	Filamentous	Shiny with smooth surface	Opaque	Flat	Powdery	Entire

From the Table 4.2, OM1 have the colonial characterization with orange pigmentation, surface appearance of shiny and smooth surface, opaque, convex elevation, mucoid texture with smooth and entire margin. Based on the reviews from Landell, et al. (2009), they stated that *Cryptococcus bromeliarum sp.* was an orange colony with mucoid texture and entire margin which matched the characteristics of isolate OM1. However, isolate OM1 cannot identified to be *Cryptococcus bromeliarum sp.* due to the lacking of other features such as surface appearance, elevation, opacity and shape. Based on Wang and Bai, (2004) studies, they described that *Sporobolomyces jilingensis sp.* produced colony of orange pigmentation with smooth surface appearance. Moreover, the colony can grow in temperature within 17 ℃ to 33 ℃ and had characteristic of mucoid texture and entire margin. Hence, the characteristic of *Sporobolomyces jilingensis sp.* described matched with the colony morphology characteristics of isolate OM1. According to another literature review by Hizbullahi, et al. (2018), they reported that *Salinococcus roseus* has morphological characteristics of circular in shape, convex elevation, smooth surface, mucoid texture and occurred in orange pigmentation. The characteristic of *Salinococcus roseus* also matched with the colony morphology characteristics of isolate OM1. Therefore, isolate OM1 might be preliminary characterized as *Sporobolomyces jilingensis sp.* and *Salinococcus roseus* based on the similar characteristic described.

From the observation, isolate WM2 have the colonial characterization with white pigmentation, surface appearance of dull and smooth surface, opaque, umbonate elevation, mucoid texture with lobate margin. From Franco, et al. (2012), they stated that *Issatchenkia occidentalis* (synonym = *Pichia occidentalis*) is a white colony with colony morphology characteristic of irregular in shape, opaque, an umbonate elevation and lobate margin. Hence, the characteristic of *Pichia occidentalis* described matched with the colony morphology characteristics of isolate WM2. Besides, based on Lu, Guo and Liu (2018) reviews, they described *Bacillus subtilis* produced colony of white pigmentation with dull and smooth surface. It had colony morphology characteristic of irregular shape and mucoid texture. Therefore, isolate WM2 might be *Pichia occidentalis* or *Bacillus subtilis* due to the similar characteristic described.

4.3 Gram reaction and cell morphological characterization of isolates

The gram staining reaction and cell morphological characterization of isolates were summarized in Table 4.3. Isolate OM1 was gram negative and had long single rod cell shape while isolate WM2 was gram positive and had oval shaped cell. Figure 4.4 shows gram staining reaction and shape of cell of both isolates under light microscope with 100x magnification. From the microscopic observation, isolate WM2 had a larger size of cell compared to isolate OM1. Hence, isolate WM2 was suspected as yeast due to larger cell size while isolate OM1 was suspected as bacteria due to smaller cell size.

Table 4.3: Cell morphological characterization of isolates

Isolates	**Cell morphological characterization**	
	Shape of cell	Gram reaction
OM1	Long single rod	-
WM2	Oval-shaped	+

‘+’ indicated positive reaction while ‘-’ indicated negative reaction

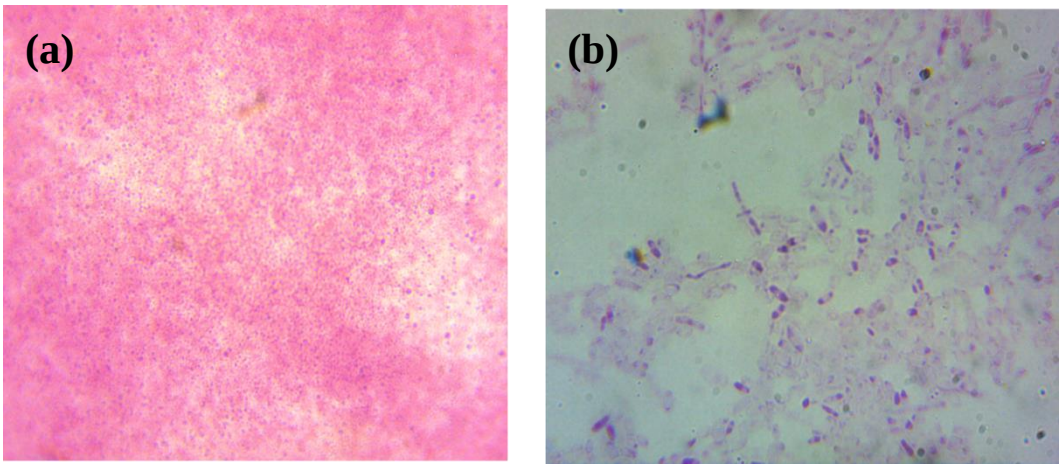


Figure 4.4: Gram staining reaction and shape of cell isolates. (a) OM1 (b) WM2

There were a range of gram-negative bacteria with rod-shaped or bacillus such as *Escherichia coli, Salinococcus roseus, Paracoccus rnarcusii* and *Pseudomonas brassicacearum* species (Huang, et al., 2008). *Salinococcus roseus* was decribed as rod-shaped bacterium with the orange colonies. The colonies had morphological characteristics of circular in shape, convex elevation, smooth surface, mucoid texture (Hizbullahi, et al., 2018). Moreover, *Paracoccus rnarcusii* formed cocci to short rods but had morphological characteristics of smooth surface with flat elevation which was an orange colony (Harker, Hirschberg and Oren, 1998). In addition, *Pseudomonas brassicacearum* was described as an orange colony with single and rod-shaped cells (Ivanova, et al., 2009). Hence, isolate OM1 can be suspected as *Salinococcus roseus*, *Paracoccus rnarcusii* and *Pseudomonas brassicacearum.* In the other hand, *Pichia*, *Cryptococcus* and *Saccharomyces* were gram positive yeast like cells that had characteristic of spherical or oval shaped (Okungbowa, Dede and Isikhuemhen, 2009). *Cryptococcus* was described as spherical or thick-walled yeast-like cell and it was white in colour. For *Pichia* species, it described as a white colony, having dull and smooth surface with oval shaped cell (Franco, et al., 2012). From the microscopic characteristics described above, isolate WM2 was suspected as *Pichia* species or *cryptococcus* species. *Saccharomyces* was not identified as isolate WM2 was due to its cream colour not similar as the white colour of isolate WM2.

4.4 Biochemical characterization of isolates

There were several biochemical tests that conducted to identify and characterize the isolates such as catalase test, oxidase test, carbohydrate fermentation test, urease test, citrate test, methyl red test, Voges-Proskauer test, indole test and mannitol salt agar test.

4.4.1 Catalase test

The catalase test is the breakdown of hydrogen peroxide to produce oxygen and water. Microbes that have ability to produce enzyme catalase can undergo cellular detoxification. The reaction can be observed by the vigorously bubbling after the addition of hydrogen peroxide to the isolates indicates the presence of catalase enzyme (Hansen and Stewart, 1978), as shown in Equation (4.1):

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2 \qquad (4.1)$$

From the observation, isolate OM1 had the formation of bubbles while no formation of bubbles for isolate OM2 after the addition of hydrogen peroxide. Isolate OM1 showed a positive catalase test while isolate WM2 showed negative catalase test. Positive catalase test indicated presence of catalase enzyme in the isolate which break down hydrogen peroxide into oxygen and water. Figure 4.5 shows the observation of both isolates before and after the addition of H_2O_2 for catalase test.

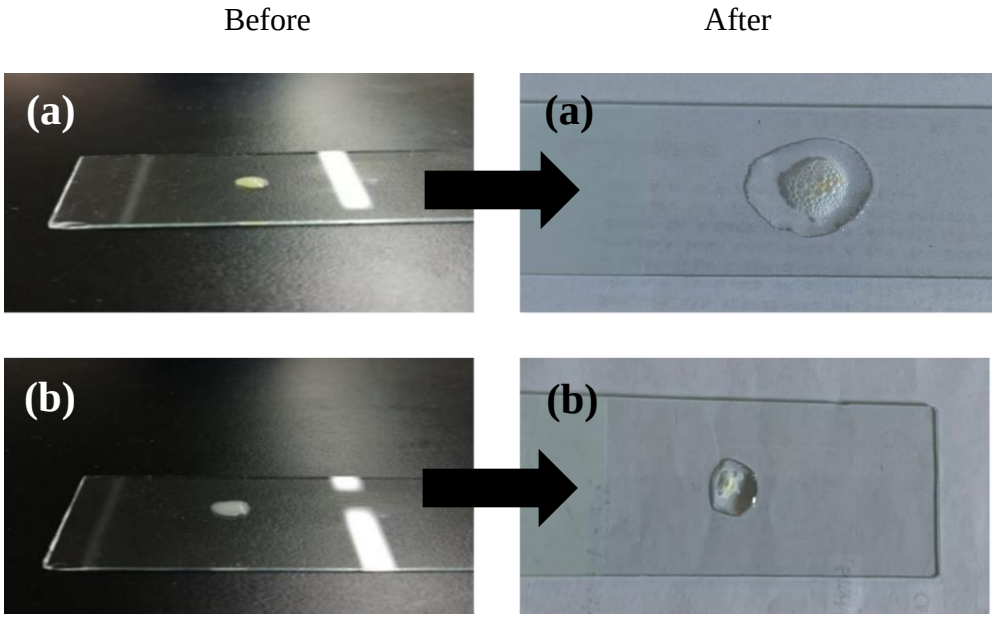


Figure 4.5: Addition of H_2O_2 for catalase test (a) OM1 (b)WM2

4.4.2 Oxidase test

Organisms that perform respiration will contain the cytochrome C oxidase enzymes in their electron transport chain. This enzyme functions by transferring electrons to oxygen, which can reduce oxygen into water or hydrogen peroxide. N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) was a reagent which known as an artificial electron acceptor in oxidase test. Cytochrome C oxidase have the ability to oxidize the reagent which change the colour from colourless to purple compound which known as indophenols blue (Yadav, et al., 2013). Hence, the positive result is the colourless reagent change to purple colour while negative result is indicated with no change in the colour of reagent.

From the observation, both isolates OM1 and WM2 showed negative result as the colour of isolate remain unchanged. Negative result indicated there was absent of cytochrome C oxide, which was an enzyme of bacterial electron transport chain. Hence, both isolates cannot oxidize the oxidase reagent (tetramethyl-p-phenylenediamine) to indophenols which was purple in colour. Figure 4.6 shows the observation of both isolates for oxidase test.

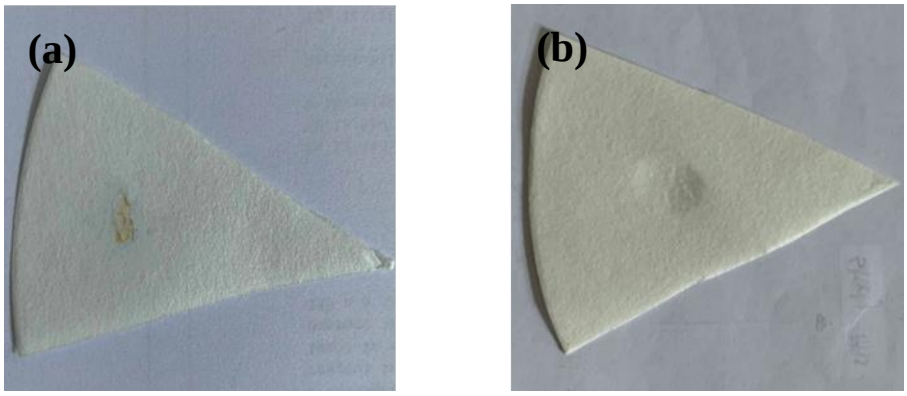
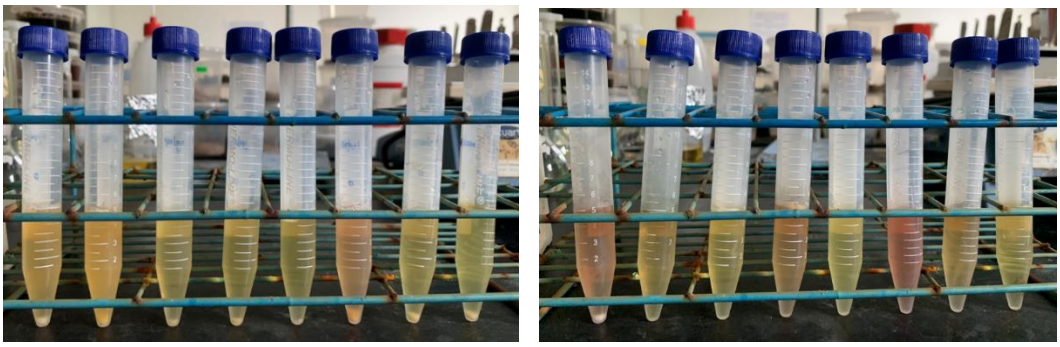


Figure 4.6: Oxidase test for (a) OM1 and (b) WM2

4.4.3 Carbohydrate fermentation test

This test was to examine the capability of isolate in fermentation of specific carbohydrate. In this test, phenol red acts as a pH-sensitive indicator to identify whether there is acid production during fermentation process. The positive result is the changing of colour to yellow when there is production of acid that lower the pH. Oppositely, microbes that only utilize the peptone will produce alkaline by-products (Rutter, 1970).

From the observation, isolate OM1 showed strong fermentation with xylose, galactose, fructose, glucose and mannitol followed by weak fermentation with starch and sucrose and no fermentation with lactose. For isolate WM2, it showed strong fermentation with xylose, fructose and mannitol followed by weak fermentation with sucrose and glucose and no fermentation with starch, galactose and lactose. During fermentation process, acid will be produced which caused the change of orange-yellow medium into yellow colour indicated positive result. Negative result was indicated by red colour medium. Figure 4.7 shows the observation of carbohydrate fermentation test for both isolates after 72 hours incubation period. Table 4.4 shows the result of carbohydrate fermentation test for both isolates with the incubation period of 72 hours.



(a) **(b)**

Figure 4.7: Carbohydrate fermentation tests of (a) OM1 and (b) WM2 with 72 hours incubation. The medium was arranged from left to right (starch, sucrose, xylose, galactose, fructose, lactose, glucose and mannitol).

Table 4.4: Result of carbohydrate fermentation test for isolate OM1 and WM2 with 72 hours incubation

Types of carbohydrate	**Isolates**	
	OM1	WM2
Starch	+	-
Sucrose	+	+
Xylose	++	++
Galactose	++	-
Fructose	++	++
Lactose	-	-
Glucose	++	+
Mannitol	++	++

‘++’ indicated strong fermentation; ‘+’ indicated weak fermentation and ‘-’ indicated no fermentation

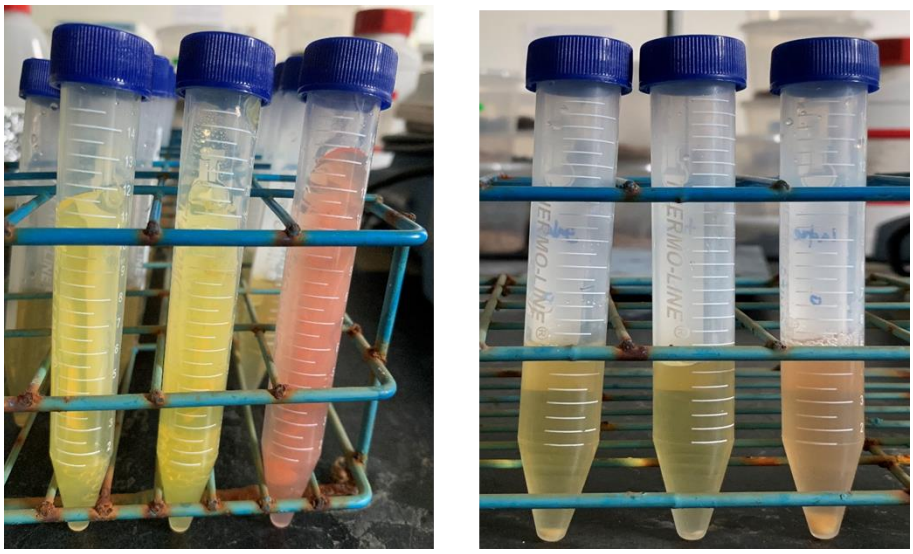
4.4.4 Urease test

The purpose of this test was to identify the microbes which able to hydrolyze urea with the production of ammonia and carbon dioxide in the presence of enzyme urease. Urease test can be performed by two method which were Chistensen's Urea Agar test and Struart's Urea Broth test. The medium of urease test consists of 2% urea and a pH indicator which is phenol red. The production of ammonia will cause the medium to become alkaline, result in a rise of pH. Hence, there will be a changing in yellow colour to pink colour in the medium. Stuart's urea broth that used in this test is considerably high in buffer content which need significantly amount of ammonia to increase the pH above 8.0 and there will be a colour change occurred. Moreover, Christensen's urea agar is a medium with low buffer content which contains peptones and glucose. Urease activity can be observed by using this medium as it provides the growth of many enterobacteria (Brink, 2010). The equation is shown in equation (4.2):

$$(NH_2)_2CO + H_2O \rightarrow CO_2 + 2\ NH_3 \qquad (4.2)$$

From the observation, isolate WM2 showed positive urease result for both test while isolate OM1 showed negative result for both of the test. Positive urease test was indicated by the color changing of medium from yellow to pink while colour remained for medium indicated negative urease test. Hence, urease enzyme was

present in isolate WM2 which can hydrolyze urea into ammonia and carbon dioxide. Figure 4.8 shows the observation of both isolates for urease test.



(a) **(b)**

Figure 4.8: Urease test for both isolates. (a) Chistensen's Urea Agar and (b) Struart's Urea Broth. The medium was arranged from left to right (control, isolate OM1 and isolate WM2)

4.4.5 Citrate test

The aim of this test was to determine the isolate which have the capability to ferment sodium citrate as its energy and the main source of carbon with the citrate permease enzyme. Citrate is known as the only carbon source which the oxaloacetate and acetate can be obtained through the cleavage of citrate by citrate-metabolizing organisms in Simmons citrate agar. Firstly, the conversion of

oxaloacetate to pyruvate and CO_2 by oxaloacetate dehydrogenase is occurred. Next, sodium carbonate which is an alkaline product being produced with the conversion of carbon dioxide. This give rise to an alkaline pH shift and the positive result is observed with the pH indicator which is bromothymol blue change its originally deep forest green colour (neutral) to Prussian blue (alkaline) (Van Hofwegen, Hovde, and Minnich, 2016).

From the observation, both isolate OM1 and WM2 showed negative citrate test as no changing of deep forest green medium into Prussian blue. Hence, both isolates cannot utilize sodium citrate in the medium as its only carbon source. Figure 4.9 shows the observation of both isolates for citrate test.

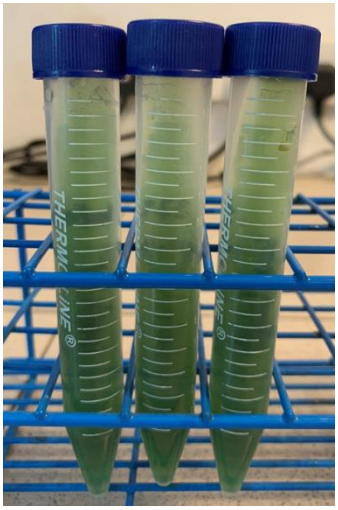


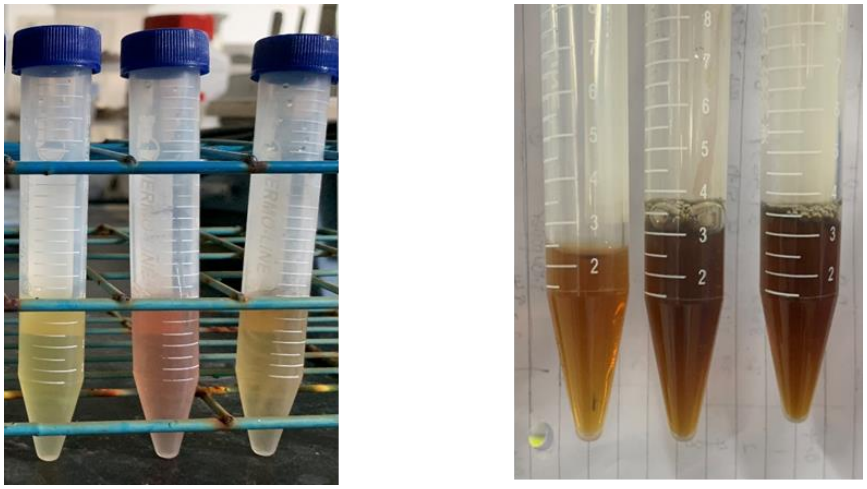
Figure 4.9: Citrate test on different isolates. The medium was arranged from left to right (control, isolate OM1 and isolate WM2)

4.4.6 Methyl red and Voges-Proskauer test

Both of the tests originally were performed to differentiate among members in family Enterobacteriaceae, but other groups of bacteria including Actinobacteria can also be characterized. For the MR test, it is used to examine the isolate which have ability to generate acid end products from glucose fermentation. Positive MR test is obtained when the culture medium shows a red colouration due to the production of high acid, resulting in lowering of pH to 4.4. Oppositely, the yellow colouration in culture medium indicate MR negative due to less acid production. For Voges-Proskauer test, acetyl methyl carbinol is produced from butanediol pathway during the fermentation of sugar. During the catalyzation reaction by α-naphthol with the presence of KOH, diacetyl was obtained from the oxidation of acetoin. Guanidine group that linked with peptone molecules were interacted with diacetyl in the medium. Hence, a copper-red coloured product was formed which indicated positive result of VP test (Aditi, Rahman and Hossain, 2017).

For methyl red test, isolate OM1 showed positive MR result with the indication of red colouration while isolate WM2 showed negative MR result with the indication of yellow colouration. Hence, OM1 have the ability to produce acid during fermentation of glucose. For Voges-Proskauer test, both isolates showed positive result. Positive result of VP test was the changing of yellowish-brown medium to copper red colour while no changing colour in medium indicated negative VP test.

Hence, both isolates had the ability to digest glucose to acetyl methyl carbinol. Figure 4.10 shows the observation of MR-VP test on both isolates.



(a) **(b)**

Figure 4.10: MR-VP test on both isolates. (a) MR test (b) VP test. The medium was arranged from left to right (control, isolate OM1 and isolate WM2)

4.4.7 Indole test

The purpose of this test is to identify the capability of an organism which can degrade the amino acid tryptophan with the production of compound indole. With the presence of tryptophanase enzyme, amino acid tryptophan can perform deamination and hydrolysis process. A by-product from metabolism of tryptophan is produced indicate the detection of indole compound by reacting indole and p-dimethylaminobenzaldehyde (DMAB) in acidic conditions which a red dye rosindole will form. Development of red colour ring on top of the medium layer indicates positive indole test after the addition of Kovac's reagent. Oppositely,

indole negative result is observed with the yellow or slightly cloudy in the medium layer (Li, et al., 2016).

From the observation, both isolates OM1 and WM2 showed indole negative result with the development of yellowish or cloudy ring in the top layer of medium. Hence, enzyme tryptophanase was absent in both isolates which they cannot split amino acid tryptophan into compound indole. Figure 4.11 shows the observation of both isolates for indole test.

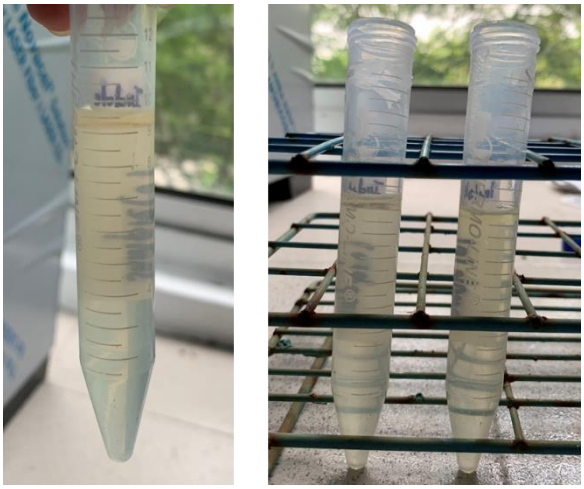


Figure 4.11: Indole test on both isolates. The medium was arranged from left to right (control, isolate OM1 and isolate WM2)

4.4.8 Mannitol salt agar plate test

Mannitol salt agar (MSA) is both a selective and disparity medium used in the isolation of staphylococci. The medium consists of 7.5% sodium chloride and the bacteria that able to withstand high salt concentrations can grow in MSA. Sodium

chloride acts as an essential electrolyte supplier for transport and osmotic balance. MSA can also be used to determine the ability of isolate to ferment the sugar mannitol in the medium. Phenol red indicator in the medium is used to detect whether there is fermentation process occur on mannitol. The positive result is indicated when there is a formation of yellow area surrounding the isolated colony. This is due to the acid production which causes the pH of the medium drop when fermentation of mannitol occurred. Oppositely, an isolate that cannot ferment mannitol but can withstands the high salt concentration, the surrounding of isolate will remain red due to the breakdown of peptone. This result in negative MSA test (Aryal, 2016).

From the observation, OM1 showed negative test on mannitol salt agar plate test with the indication yellow colouration surround the isolate while WM2 showed positive result with the indication red colouration surround the isolate. Positive result showed the ability of isolate to ferment mannitol in mannitol salt agar plate test. Figure 4.12 shows the observation of both isolates for mannitol salt agar plate test.

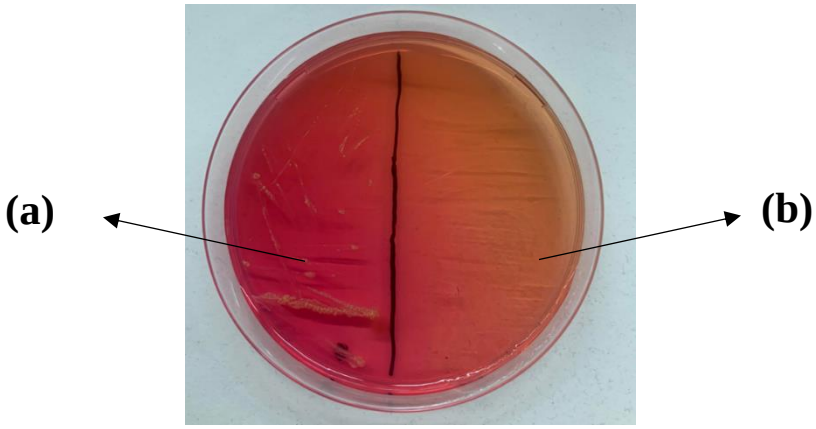


Figure 4.12: Mannitol salt agar plate test on both isolates. (a) OM1 (b) WM2

4.5 Identification of isolates via 16S rDNA gene sequencing

Isolate WM2 was selected for the identification of genotypic. Isolate WM2 was subjected to 16S rDNA gene sequencing to identify its species.

4.5.1 PCR amplification and DNA sequencing for isolate WM2

Preliminary characterization of isolate WM2 was confirmed by using PCR amplification of ITS gene region. Primer sets of forward primer ITS1 (5' – TCC GTA GGT GAA CCT GCG G – 3') and reverse primer ITS4 (5' – TCC TCC GCT TAT TGA TAT GC – 3') were used in PCR amplification of ITS gene region for isolate WM2. The partial PCR sequencing of 16S rDNA gene sequencing of isolate WM2 for ITS1 and ITS4 were show in Figure 4.13 and Figure 4.14. Nucleotide-BlAST program which is a Basic Local Alignment Search Tool from the website at the National Center for Biotechnology (NCBI) was used to search against the ITS nucleotide sequences of isolate deposited in NCBI-nucleotide database. The blast results with the highest five hits were collected from the to prevent any untrue information that cause by the errors which recorded in the database of NCBI GenBank. Both ITS1 and ITS4 sequence showed 100% identical to *Pichia occidentalis* stain FIR004. Figure 4.15 shows the colour key for alignment scores of matching sequences for both ITS1 and ITS4. Figure 4.16 shows the distribution of the top 100 Blast Hits on 100 subject sequences for ITS1 and Figure 4.17 shows the distribution of the top 100 Blast Hits on 100 subject sequences for ITS4. Figure

4.18 shows the alignment statistic for matching partial sequence of ITS1 with partial sequence of *Pichia occidentalis* small subunit ribosomal RNA gene. Figure 4.19 shows the alignment statistic for matching partial sequence of ITS4 with partial sequence of *Pichia occidentalis* small subunit ribosomal RNA gene.

GNNGCNNNNN	NNATACTCTT	CCACACGTGC	GTGAGCGCAA
GCAAAACACG	AAAAAACTGT	AGTACGAGAG	TCAAAACAAA
CCAAAAAACA	AAACTTTCAA	CAACGGATCT	CTTGGTTCTC
GCATCGATGA	AGAGCGCAGC	GAAATGCGAT	ACCTAGTGTG
AATTGCAGCC	ATCGTGAATC	ATCGAGTTCT	TGAACGCACA
TTGCGCCCTC	TGGTATTCCG	GAGGGCATGC	CTGTTTGAGC
GTCGTTTCCT	TCTTGCTTGC	GAGCAGAAAT	GGGGGGGCCC
TGGCATTGGG	GCCGCTCTGA	AAAGAAACGT	TGCGGGCGAA
GCGAACTATG	AGTAGGACGC	TTGGCCGCCG	AACTTAATAC
ATAAGCTCGA	CCTCAAATCA	GGTAGGAATA	CCCGCTGAAC
TTAAGCATAT	CAATAAGGCG	GAGGAAA	

Figure 4.13: Partial PCR sequencing of 16S rDNA gene sequencing of isolate WM2 for ITS1

NNNNNNNNNN	NNNCNACNGA	TTTGAGGTCG	AGCTTATGTA
TTAAGTTCGG	CGGCCAAGCG	TCCTACTCAT	AGTTCGCTTC
GCCCGCAACG	TTTCTTTTCA	GAGCGGCCCC	AATGCCAGGG
CCCCCCCATT	TCTGCTCGCA	AGCAAGAAGG	AAACGACGCT
CAAACAGGCA	TGCCCTCCGG	AATACCAGAG	GGCGCAATGT
GCGTTCAAGA	ACTCGATGAT	TCACGATGGC	TGCAATTCAC
ACTAGGTATC	GCATTTCGCT	GCGCTCTTCA	TCGATGCGAG
AACCAAGAGA	TCCGTTGTTG	AAAGTTTTGT	TTTTTGGTTT
GTTTTGACTC	TCGTACTACA	GTTTTTTCGT	GTTTTGCTTG
CGCTCACGCA	CGTGTGGAAG	ATGTTAAATC	ACAGTAATGA
TCCTTCCGCA	GGTCACCCTA	CGGAAGAGGG	ATCATAACT

Figure 4.14: Partial PCR sequencing of 16S rDNA gene sequencing of isolate WM2 for ITS4

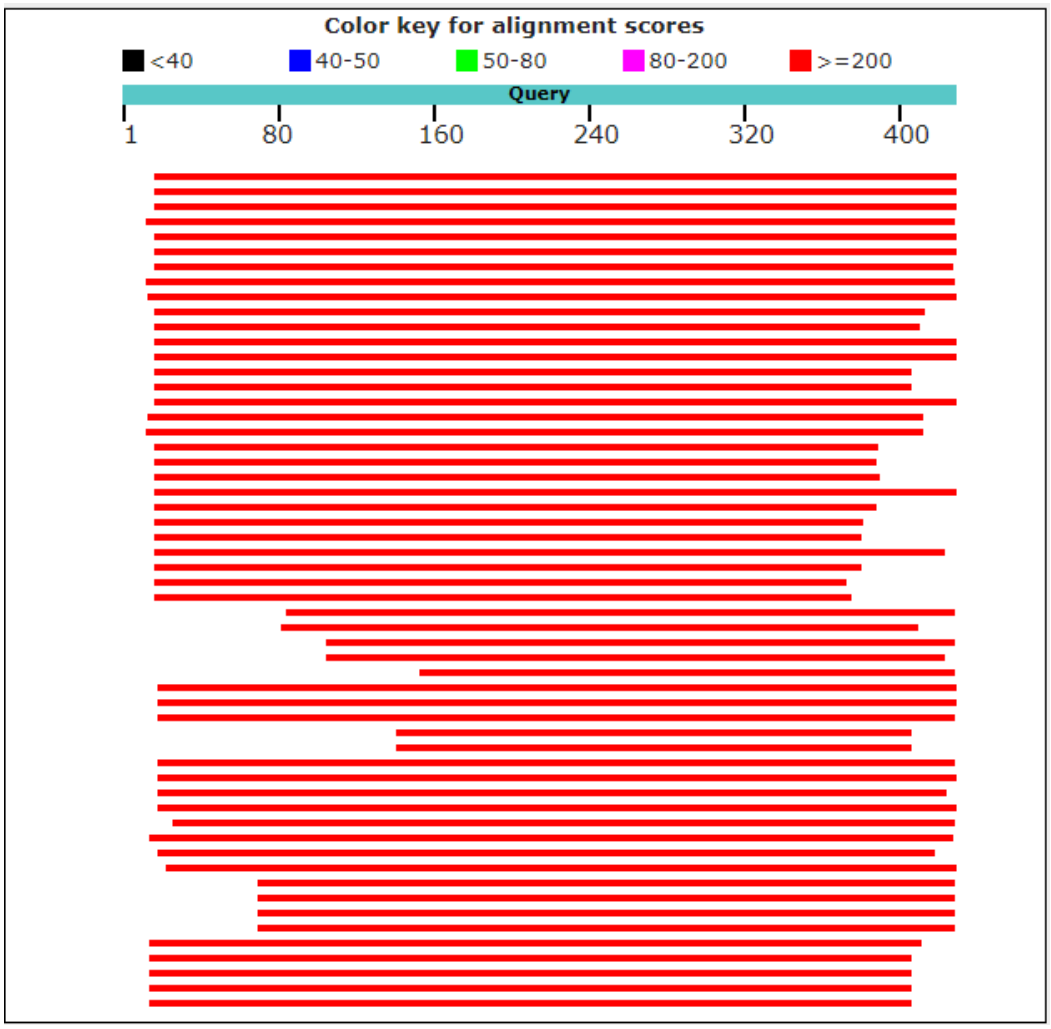


Figure 4.15: The colour key for alignment scores of matching sequences for both ITS1 and ITS4

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☐	Pichia occidentalis isolate KOP7 18S ribosomal RNA gene, partial sequence; internal trans	752	752	96%	0.0	99.76%	KY849376.1
☐	Pichia occidentalis strain YPD D3 internal transcribed spacer 1, partial sequence; 5.8S ribo	752	752	96%	0.0	99.76%	KY816890.1
☐	Pichia occidentalis strain PMM08-2452L isolate ISHAM-ITS_ID MITS1079 internal transcri	752	752	96%	0.0	99.76%	KP132530.1
☐	Saccharomycetes sp. LY5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal	752	752	96%	0.0	99.52%	KJ535099.1
☐	Pichia cecembensis isolate M20 18S ribosomal RNA gene, partial sequence; internal trans	752	752	96%	0.0	99.76%	EU315768.1
☐	Saccharomycete sp. SCH-47 isolate B2 18S ribosomal RNA gene, partial sequence; intern	752	752	96%	0.0	99.76%	EU315761.1
☐	Uncultured saccharomycete clone 2 internal transcribed spacer 1, partial sequence; 5.8S r	749	749	95%	0.0	99.76%	EF087980.1
☐	Pichia occidentalis isolate LBI-65 internal transcribed spacer 1, partial sequence; 5.8S ribo	747	747	96%	0.0	99.28%	MH879824.1
☐	Pichia cecembensis strain DBMY404 18S ribosomal RNA gene, partial sequence; internal	743	743	96%	0.0	99.04%	KJ706621.1
☐	Issatchenkia occidentalis isolate SB517 internal transcribed spacer 1, partial sequence; 5.8	730	730	92%	0.0	100.00%	DQ872864.1
☐	Pichia occidentalis isolate SSFG-1 10-3II2 internal transcribed spacer 1, partial sequence;	725	725	91%	0.0	100.00%	MK123421.1
☐	Pichia cecembensis strain DBMY245 internal transcribed spacer 1, partial sequence; 5.8S	721	721	96%	0.0	98.30%	KJ706462.1
☐	Pichia cecembensis strain DBMY722 18S ribosomal RNA gene, partial sequence; internal	719	719	96%	0.0	98.30%	KJ706939.1

Figure 4.16: The distribution of the top 100 Blast Hits on 100 subject sequences for ITS1

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☐	Pichia occidentalis strain F028 18S ribosomal RNA gene, partial sequence; internal transcr	739	739	92%	0.0	99.51%	KY580388.1
☐	Uncultured saccharomycete clone 2 internal transcribed spacer 1, partial sequence; 5.8S r	739	739	92%	0.0	99.51%	EF087980.1
☐	Candida inconspicua isolate H137 18S ribosomal RNA gene, partial sequence; internal tra	737	737	92%	0.0	99.51%	KU238836.1
☐	Saccharomycete sp. SCH-47 isolate B2 18S ribosomal RNA gene, partial sequence; intern	737	737	92%	0.0	99.26%	EU315761.1
☐	Candida inconspicua strain L11-1 18S ribosomal RNA gene, partial sequence; internal tran	736	736	92%	0.0	99.26%	DQ681370.2
☐	Candida inconspicua strain DBN38 18S ribosomal RNA gene, partial sequence; internal tra	734	734	91%	0.0	99.50%	KT207004.1
☐	Candida inconspicua internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA	734	734	91%	0.0	99.50%	GU237054.1
☐	Issatchenkia occidentalis 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rI	734	734	92%	0.0	99.26%	FM199961.1
☐	Pichia occidentalis strain 39 internal transcribed spacer 1, partial sequence; 5.8S ribosoma	730	794	95%	0.0	97.87%	GQ254805.1
☐	Pichia cecembensis isolate M20 18S ribosomal RNA gene, partial sequence; internal trans	721	721	92%	0.0	98.53%	EU315768.1
☐	Pichia occidentalis isolate KOP7 18S ribosomal RNA gene, partial sequence; internal trans	715	715	88%	0.0	100.00%	KY849376.1
☐	Candida inconspicua isolate LY6 internal transcribed spacer 1, partial sequence; 5.8S ribos	715	715	88%	0.0	100.00%	KJ535100.1
☐	Pichia cecembensis strain DBMY404 18S ribosomal RNA gene, partial sequence; internal	710	710	92%	0.0	98.04%	KJ706621.1
☐	Pichia occidentalis isolate ZJB-09162 18S ribosomal RNA gene, partial sequence; internal	708	708	92%	0.0	98.04%	JN872840.1

Figure 4.17: The distribution of the top 100 Blast Hits on 100 subject sequences for ITS4

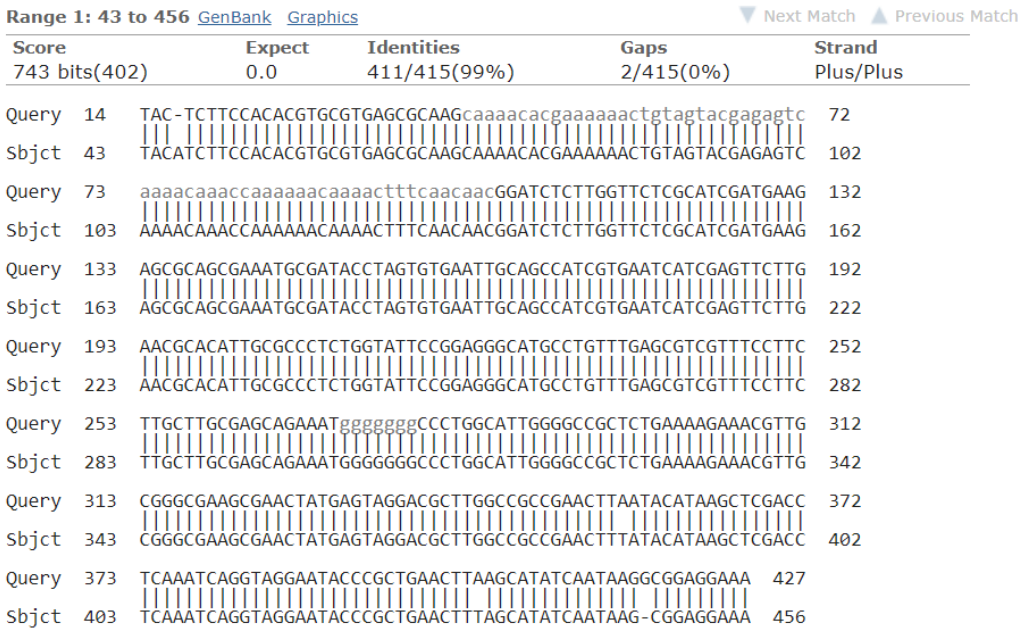


Figure 4.18: The alignment statistic for matching partial sequence of ITS1 with partial sequence of *Pichia occidentalis* small subunit ribosomal RNA gene

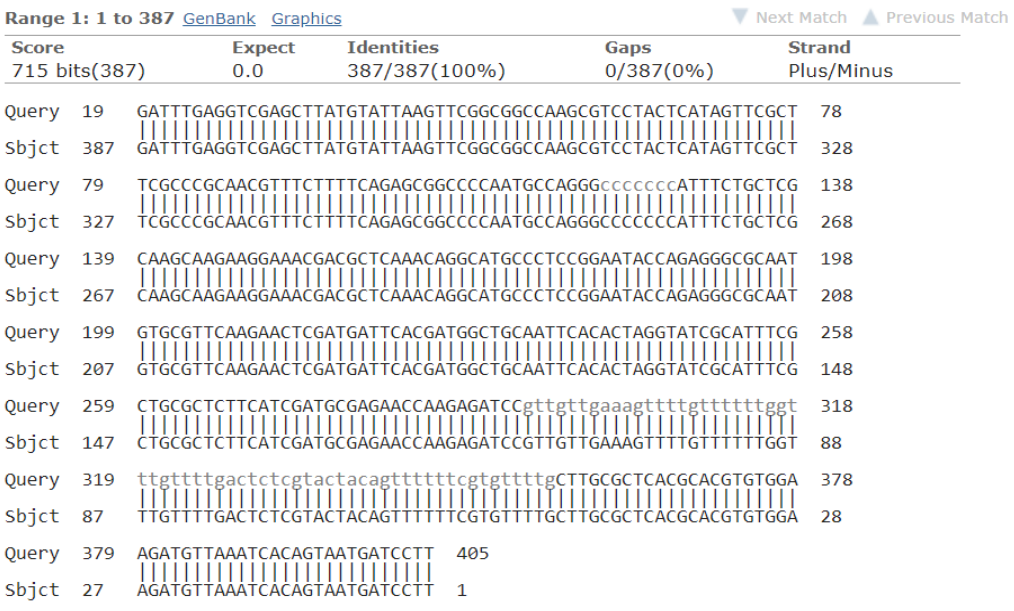


Figure 4.19: The alignment statistic for matching partial sequence of ITS4 with partial sequence of *Pichia occidentalis* small subunit ribosomal RNA gene

4.5.2 Phylogenetic analysis of sequence

Phylogenetic analysis is to study the relationship among isolated microbes. It is used for determining the relatives of the isolated microbes, identify the functionality of an isolated microbes and trace the origin of an isolated microbes. The branching pattern in a phylogenetic tree imitates how species or other groups grew from a sequence of mutual ancestors. From the phylogenetic tree, it showed that isolate WM2 was tightly clustered together with *Pichia occidentalis.* Figure 4.20 shows the phylogenetic tree and the relationship of isolate WM2 with 10 reference strains.

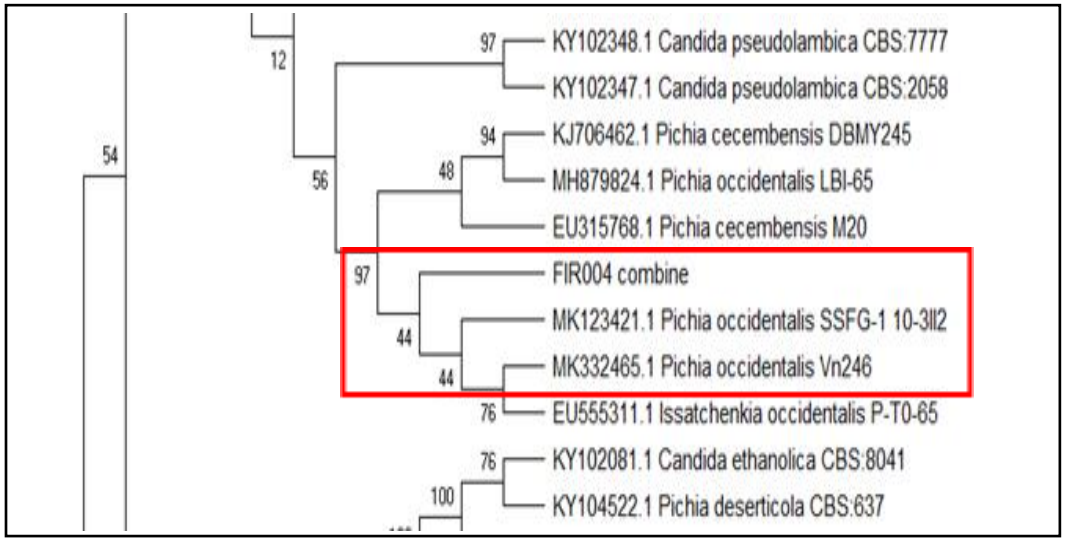


Figure 4.20: Phylogenetic relationship of isolate WM2 with 10 reference strains

4.6 Discussion on isolate WM2

Isolate WM2 was able to grow in incubation temperature of 25 ℃ and 32 ℃. It was white in colour and had characteristic of irregular shape, dull and smooth surface, opaque, umbonate elevation, mucoid texture with lobate margin. It was gram positive with oval shaped cell which was larger in size when observed under light microscope. For biochemical tests, isolate WM2 showed positive tests with urease test, Voges-Proskauer test, mannitol salt agar plate test and carbohydrate fermentation test of sucrose, glucose, fructose, mannitol and xylose. Oppositely, it showed negative biochemical tests with catalase test, oxidase test, citrate test, methyl red test, indole test and other carbohydrate fermentation test of starch, galactose and lactose. From the characterization of isolate WM2, *Pichia* species was the most prevalent microbes determined as the colonial morphological characterization, microscopic morphology and biochemical testing result were similar. Hence, isolate WM2 was subjected to 16S rDNA gene sequencing to identify its species. From the result obtained, isolate WM2 showed 100% identical to *Pichia occidentalis* strain.

Pichia species is described as a genus of yeasts from the *Saccharomycetaceae* family with sphere-shaped, oval, or oblong acuminate cells. The anamorphs of the species of *Pichia* are various *Candida* species. The active species were *Pichia anomala*, *Pichia guilliermondii*, *Pichia norvegensis*, and *Pichia ohmeri* (Doctor Fungus, 2018). In palm oil industry, *Pichia occidentalis* acts as a biocatalyst, which

undergoes the transesterification reaction was implemented for biodiesel production from palm oil waste (Zahan and Kano, 2018). The microbial species found in EFB sludge, *Pichia occidentalis*, has the ability to degrade hydrocarbon in the EFB. By degrading carbon sources from palm oil waste, it can reduce environmental pollution (Soleimaninanadegani, and Manshad, 2014). Moreover, *Pichia occidentalis* was used to produce yeast biomass from palm oil waste. EFB sludge contains polysaccharides which are sugar that can undergo fermentation process by using *Pichia occidentalis* (Izah, 2017). Furthermore, furfural and HMF in lignocellulose hydrolysate oil palm empty fruit bunch sludge can be easily decomposed with the usage of *Pichia occidentalis*. Hence, the biological detoxification of furfural and HMF can be successfully convert to less harmful composites (Zhang, et al., 2013).

CHAPTER 5

CONCLUSION

The objectives of this research were reached, which were:

a) 2 potential microbes which labelled as isolate OM1 and isolate WM2 being isolated from EFB sludge
b) The characterization of both isolates was determined by colonial morphology, microscopic morphology and biochemical testing
c) Identification of isolate WM2 to species level via 16S rDNA gene sequencing was done and showed 100% identical to *Pichia occidentalis* stain.

From the biochemical tests conducted, isolate OM1 is good as a fermenter and can have ability to break down hydrogen peroxide reagent while isolate WM2 is also good as fermenter and have ability to hydrolyze urea-containing waste. Table 4.5 shows the summary of biochemical tests on both isolates.

This research involves the preliminary characterization of isolates to check the species of microbes and its function in EFB sludge. Only one isolate which was isolate WM2 was chosen to identify via 16S rDNA gene sequencing to species level. From the information in phylogenetic tree, isolate WM2 was showed to be clustered with *Pichia occidentalis* strain.

Table 4.5: Summary of biochemical tests on both isolates

Biochemical tests		**Isolates**	
		OM1	WM2
Catalase test		+	-
Oxidase test		-	-
Carbohydrate fermentation test	(a) Starch	+	-
	(b) Sucrose	+	+
	(c) Xylose	+	+
	(d) Galactose	+	-
	(e) Fructose	+	+
	(f) Lactose	-	-
	(g) Glucose	+	+
	(h) Mannitol	+	+
Urease test		-	+
Citrate test		-	-
Methyl red test		+	-
Voges-Proskauer test		+	+
Indole test		-	-
Mannitol salt agar plate test		-	+

‘+’ indicated positive result while ‘-’ indicated negative result.

The future study of this research is the usage of other potential microbes that isolated from the EFB sludge and undergo identification up to species level for characterization. The isolates can be further done to investigate the growth, biological detoxification of hemicellulose hydrolysates and have the potential to produce cellulase or lipase as biodegrading agent. Moreover, the isolation and finding of more microbes can help to reduce to cost of waste management.

REFERENCES

Abdelhai, M.H., Hassanin, H.A. and Sun, X., 2016. Comparative study of rapid DNA extraction methods of pathogenic bacteria. *Am. J. Biosci. Bioeng.*, 4, pp.1-8.

Abdullah, N. and Sulaiman, F., 2013. The oil palm wastes in Malaysia. *Biomass Now-Sustainable Growth and Use*. IntechOpen.

Abiola, C. and Oyetayo, V.O., 2016. Isolation and biochemical characterization of microorganisms associated with the fermentation of Kersting's groundnut (Macrotyloma geocarpum). *Research Journal of Microbiology*, 11, pp.47-55.

Aditi, F.Y., Rahman, S.S. and Hossain, M.M., 2017. A study on the microbiological status of mineral drinking water. *The open microbiology journal*, 11, pp.31.

An, B., Park, M.K. and Oh, J.H., 2018. Food waste treatment using Bacillus species isolated from food wastes and production of air-dried Bacillus cell starters. *Environmental Engineering Research*, 23(3), pp.258-264.

Aryal, S., 2016. Mannitol Salt Agar for the isolation of Staphylococcus aureus. [online]. Available at: http://www.microbiologyinfo.com/mannitol-salt-agar-for-theisolation-of-staphylococcus-aureus/ [Accessed 1 April 2019].

Awalludin, M.F., Sulaiman, O., Hashim, R. and Nadhari, W.N.A.W., 2015. An overview of the oil palm industry in Malaysia and its waste utilization through thermochemical conversion, specifically via liquefaction. *Renewable and Sustainable Energy Reviews*, 50, pp.1469-1484.

Bai, N., Bae, E., Bhunia, A.K., Robinson, J.P. and Hirleman, E.D., 2010. Morphology Characterization of Bacterial Colonies for Predicting Forward Scattering Patterns. *In Optical Sensors*. Optical Society of America.

Bala, J.D., Lalung, J., Al-Gheethi, A.A.S., Hossain, K. and Ismail, N., 2018. Microbiota of Palm Oil Mill Wastewater in Malaysia. *Tropical life sciences research*, 29(2), pp.131.

Baron, S., 1996. *Classification--Medical Microbiology*. University of Texas Medical Branch at Galveston.

Basiron, Y., 2007. Palm oil production through sustainable plantations. *European Journal of Lipid Science and Technology*, 109(4), pp.289-295.

Batt, C. A., 2016. Microorganisms. *Reference Module in Food Science*.

Beveridge, T.J., 2001. Use of the Gram stain in microbiology. *Biotechnic & Histochemistry*, 76(3), pp.111-118.

Brink, B., 2010. Urease Test Protocol. Laboratory Ptotocols. Washington, DC: American Society of Microbiology. Available at: http://www.asmscience.org/docserver/fulltext/education/ptotocol/protocol.3223.pdf?expires=1554204446&id=id&accname=guest&checksum=0D0889B53D5D2A5A2F431D3DFDB98399 [Accessed 2 April 2019].

Dahman, Y., 2017. *Nanotechnology and Functional Materials for Engineers*. Elsevier.

Davis, K.E., Joseph, S.J. and Janssen, P.H., 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. *Appl. Environ. Microbiol*., 71(2), pp.826-834.

Doctor Fungus, 2018. *Pichia* species. [online]. Available at: https://drfungus.org/knowledge-base/pichia-species/ [Accessed 1 April 2019].

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. *Emerging infectious diseases*, 8(9), pp.881.

Fadzilah, K. and Mashitah, M.D., 2010. Cellulases production in palm oil mill effluent: effect of aeration and agitation. *Journal of Applied Sciences*(Faisalabad), 10(24), pp.3307-3312.

Franco, W., Pérez-Díaz, I.M., Johanningsmeier, S.D. and McFeeters, R.F., 2012. Characteristics of spoilage-associated secondary cucumber fermentation. *Applied Environment Microbiol*., 78(4), pp.1273-1284.

Gandahi, A.W. and Hanafi, M.M., 2014. Bio-composting oil palm waste for improvement of soil fertility. *In Composting for Sustainable Agriculture*, pp. 209-243.

Garg, R., Srivastava, R., Brahma, V., Verma, L., Karthikeyan, S. and Sahni, G., 2016. Biochemical and structural characterization of a novel halotolerant cellulase from soil metagenome. *Scientific reports*, 6, pp.39634.

Garibyan, L. and Avashia, N., 2013. Research techniques made simple: polymerase chain reaction (PCR). *The Journal of investigative dermatology*, 133(3), pp.e6.

Hansen, S.L. and Stewart, B.J., 1978. Slide catalase: a reliable test for differentiation and presumptive identification of certain clinically significant anaerobes. *American journal of clinical pathology*, 69(1), pp.36-40.

Harker, M., Hirschberg, J. and Oren, A., 1998. Paracoccus marcusii sp. nov., an orange gram-negative coccus. *International Journal of Systematic and Evolutionary Microbiology*, 48(2), pp.543-548.

Heller, A.A. and Spence, D.M., 2019. A rapid method for post-antibiotic bacterial susceptibility testing. *PloS one*, 14(1), p.e0210534.

Hii, K.L., Yeap, S.P. and Mashitah, M.D., 2012. Cellulase production from palm oil mill effluent in Malaysia: Economical and technical perspectives. *Engineering in Life Sciences*, 12(1), pp.7-28.

Hizbullahi, M.U., Farouq, A.A., Baki, A.S., Abdulkadir, N. and Mustapha, G., 2018. Production and characterization of orange pigment produced by Halophilic bacterium Salinococcus roseus isolated from Abattoir soil. *Journal of Microbiology & Experimentation*, 6(6), pp. 238–243.

Holding, A.J. and Collee, J.G., 1971. Chapter I Routine biochemical tests. *In Methods in microbiology*, 6, pp. 1-32.

Huang, K.C., Mukhopadhyay, R., Wen, B., Gitai, Z. and Wingreen, N.S., 2008. Cell shape and cell-wall organization in Gram-negative bacteria. *Proceedings of the National Academy of Sciences*, 105(49), pp.19282-19287.

Ivanova, E.P., Christen, R., Bizet, C., Clermont, D., Motreff, L., Bouchier, C., Zhukova, N.V., Crawford, R.J. and Kiprianova, E.A., 2009. Pseudomonas brassicacearum subsp. neoaurantiaca subsp. nov., orange-pigmented bacteria isolated from soil and the rhizosphere of agricultural plants. *International journal of systematic and evolutionary microbiology*, 59(10), pp.2476-2481.

Izah, S.C., 2017. Potentials of Yeast Biomass Production from Food Processing Wastes Effluents. *EC Nutrition*, 8(3), pp.72-74.

Ji, X.J., Huang, H., Nie, Z.K., Qu, L., Xu, Q. and Tsao, G.T., 2011. Fuels and chemicals from hemicellulose sugars. *In Biotechnology in China III: biofuels and bioenergy*, pp. 199-224.

Jill, H.C. and Langdale, J.A., 2006. A step by step guide to phylogeny reconstruction. *The Plant Journal*, 45(4), pp.561-572.

Jinn, C.M., Ling, C.K., Wen, C.E., Tahir, P.M., Hua, L.S., Chen, L.W., Chuah, L. and Maminski, M., 2015. Empty fruit bunches in the race for energy, biochemical, and material industry. *In Agricultural Biomass Based Potential Materials*, pp. 375-389.

Kaksonen, A., 2018. Editorial for Special Issue "Microorganisms for Environmental and Industrial Applications". *Microorganisms*, 6(3), 62.

Khan, M.A., 2014. Microbiological solution to environmental problems-A Review on bioremediation. *International Journal of Pure Applied. BioScience*, 2(6), pp.295-303.

Khatiwada, D., Palmén, C. and Silveira, S., 2018. Evaluating the palm oil demand in Indonesia: Production trends, yields, and emerging issues. *Biofuels*, pp.1-13.

Khatiwada, P., Ahmed, J., Sohag, M.H., Islam, K. and Azad, A.K., 2016. Isolation, screening and characterization of cellulase producing bacterial isolates from municipal solid wastes and rice straw wastes. *Journal of Bioprocessing & Biotechniques*, 6, pp.280-285.

Krishnan, Y., Bong, C.P.C., Azman, N.F., Zakaria, Z., Abdullah, N., Ho, C.S., Lee, C.T., Hansen, S.B. and Hara, H., 2017. Co-composting of palm empty fruit bunch and palm oil mill effluent: microbial diversity and potential mitigation of greenhouse gas emission. *Journal of Cleaner Production*, 146, pp.94-100.

Lai, C.M.T., Chua, H.B., Danquah, M.K. and Saptoro, A., 2017, June. Isolation of thermophilic lignin degrading bacteria from oil-palm empty fruit bunch (EFB) compost. In IOP conference series: materials science and engineering (Vol. 206, No. 1, p. 012016). IOP Publishing.

Landell, M.F., Inacio, J., Fonseca, A., Vainstein, M.H. and Valente, P., 2009. Cryptococcus bromeliarum sp. nov., an orange-coloured basidiomycetous yeast isolated from bromeliads in Brazil. *International journal of systematic and evolutionary microbiology*, 59(4), pp.910-913.

Lee, P.Y., Costumbrado, J., Hsu, C.Y. and Kim, Y.H., 2012. Agarose gel electrophoresis for the separation of DNA fragments. *Journal of visualized experiments: JoVE*, (62).

Li, Q., Chen, X., Jiang, Y. and Jiang, C., 2016. Cultural, physiological, and biochemical identification of actinobacteria. *In Actinobacteria-Basics and Biotechnological Applications*. IntechOpen.

Lorenz, T.C., 2012. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *Journal of visualized experiments: JoVE*, (63).

Lu, Y., Lu, Y.C., Hu, H.Q., Xie, F.J., Wei, X.Y. and Fan, X., 2017. Structural Characterization of Lignin and Its Degradation Products with Spectroscopic Methods. *Journal of Spectroscopy*, 2017.

Lu, Z., Guo, W. and Liu, C., 2018. Isolation, identification, and characterization of novel Bacillus subtilis. *Journal of Veterinary Medical Science*, pp.16-0572.

Mahat, S.B.A., 2012. The palm oil industry from the perspective of sustainable development: A case study of Malaysian palm oil industry. *Graduate School of Asia Pacific Studies, Ritsumeikan Asia Pacific University of Japan.*

Muhamad, H., Ai, T.Y., Khairuddin, N.S.K., Amiruddin, M.D. and May, C.Y., 2014. Life cycle assessment for the production of oil palm seeds. *Tropical life sciences research*, 25(2), p.41.

Murphy, D.J., 2014. The future of oil palm as a major global crop: opportunities and challenges. *J Oil Palm Res*, 26(1), pp.1-24.

Nagendran, R., 2011. Agricultural waste and pollution. In Waste (pp. 341-355). Academic Press.

Norhayati, S., 2013. Optimization of enzymatic sludge palm oil recovery from palm oil mill effluent using response surface. *Journal of Oil Palm Research*, 25(3), pp.348-356.

Obi, F.O., Ugwuishiwu, B.O. and Nwakaire, J.N., 2016. Agricultural waste concept, generation, utilization and management. *Nigerian Journal of Technology*, 35(4), pp.957-964.

O'Toole, G.A., 2016. Classic spotlight: how the Gram stain works. *Journal of Bacteriology*, 198(3128), pp.00726-16.

Okungbowa, F.I., Dede, A.P.O. and Isikhuemhen, O.S., 2009. Cell morphology variations and budding patterns in Candida isolates. *Advances in Natural and Applied Sciences*, 3(2), pp.192-196.

Oseghale, S.D., Mohamed, A.F. and Chikere, A.O., 2017. Status Evaluation of Palm Oil Waste Management Sustainability in Malaysia. *OIDA International Journal of Sustainable Development*, 10(12), pp.41-48.

Otieno, N.E., Dai, X., De Barba, D., Bahman, A., Smedbol, E., Rajeb, M. and Jaton, L., 2016. Palm oil production in malaysia: An analytical systems model for

balancing economic prosperity, forest conservation and social welfare. *Agricultural Sciences,* 7(02), p.55.

Qadri, S.M., Zubairi, S.A.B.I.H.A., Hawley, H.P. and Ramirez, E.G., 1984. Simple spot test for rapid detection of urease activity. *Journal of clinical microbiology*, 20(6), pp.1198-1199.

Reiner, K., 2010. Catalase test protocol. Washington, DC: American Society of Microbiology. Available at: http://www.asmscience.org/docserver/fulltext/education/protocol/protocol.3226.pdf?expires=1557382243&id=id&accname=guest&checksum=78FCA05BA22AD10087D555787B86E6BC [Accessed 1 April 2019].

Reiner, K., 2012. Carbohydrate Fermentation Protocol. Washington, DC: American Society of Microbiology. Available at: http://www.asmscience.org/docserver/fulltext/education/protocol/protocol.3779.pdf?expires=1554406867&id=id&accname=guest&checksum=8FCCEB60B84AF820DE62744B39E11AD4 [Assessed 1 April 2019].

Roberts, G.D., Horstmeier, C.D., Land, G.A. and Foxworth, J.H., 1978. Rapid urea broth test for yeasts. *Journal of clinical microbiology*, 7(6), pp.584-588.

Rudnik, E., 2012. 10 Compostable Polymer Materials: Definitions, Structures, and Methods of Preparation. *Handbook of Biopolymers and Biodegradable Plastics: Properties, Processing and Applications*, p.189.

Rutter, J.M., 1970. A study of the carbohydrate fermentation reactions of Clostridium oedematiens (Cl. novyi). *Journal of medical microbiology*, 3(2), pp.283-289.

Sanagi, M.M., 2018. Recent advances in the preparation of oil palm waste-based adsorbents for removal of environment pollutants- a review. *Malaysian Journal of Analytical Sciences*, 22(2), pp.175-184.

Sanders, E.R., 2012. Aseptic laboratory techniques: plating methods. *Journal of visualized experiments: Journal of Visualized Experiments*, (63), p.e3064

Sandle, T., 2004. Gram's Stain: History and Explanation of the Fundamental Technique of Determinative Bacteriology. *IST Science and Technology Journal*, (54), pp3-4

Santamaría, R. and Therón, R., 2009. Treevolution: visual analysis of phylogenetic trees. *Bioinformatics*, 25(15), pp.1970-1971.

Schreckenberger, P.C. and Blazevic, D.J., 1976. Rapid fermentation testing of anaerobic bacteria. *Journal of clinical microbiology*, 3(3), pp.313-317.

Shacklady, C.A. ed., 1983. The Use of Organic Residues in Rural Communities: Proceedings of the Workshop on Organic Residues in Rural Communities Held in Denpasar, Bali, Indonesia, 11-12 December 1979, Under the Auspices of the Indonesian Government Institute of Sciences (LIPI), the Government of the Netherlands, and the United Nations University (Vol. 7). United Nations University Press.

Shahriarinour, M., Ramanan, R.N., Wahab, M.N.B.A., Mohamad, R.B., Mustafa, S. and Ariff, A.B., 2011. Improved cellulase production by Aspergillus terreus using oil palm empty fruit bunch fibre as substrate in a stirred tank bioreactor through optimization of the fermentation conditions. *BioResources,* 6(3), pp.2663-2675.

Shevade, V.S. and Loboda, T.V., 2019. Oil palm plantations in Peninsular Malaysia: Determinants and constraints on expansion. *PloS one,* 14(2), p.e0210628.

Shields, P. and Tsang, A.Y., 2006. Mannitol salt agar plates protocols. Washington, DC: American Society of Microbiology. Available at: http://www.asmscience.org/docserver/fulltext/education/protocol/protocol.3034.pdf?expires=1554407236&id=id&accname=guest&checksum=76960809725C5E440EECA2FDF6BC2474 [Accessed 1 April 2019].

Singh, R.P., Ibrahim, M.H., Esa, N. and Iliyana, M.S., 2010. Composting of waste from palm oil mill: a sustainable waste management practice. Reviews in Environmental Science and Bio/Technology, 9(4), pp.331-344.

Sinurat, A.P., 2003. Utilization of palm oil sludge for poultry feed. WARTAZOA. *Indonesian Bulletin of Animal and Veterinary Sciences*, 13(2), pp.39-47.

Siong, T.K., 2009. Biodegradationof Lignin in Oil Palm Empty Fruit Bunch (EFB) Using Igninolytic Fungi (Doctoral dissertation, Universiti Malaysia Sarawak).

Soleimaninanadegani, M. and Manshad, S., 2014. Enhancement of biodegradation of palm oil mill effluents by local isolated microorganisms. *International scholarly research notices*.

Sun, S., Sun, S., Cao, X. and Sun, R., 2016. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. *Bioresource technology*, 199, pp.49-58.

Tan, S.C. and Yiap, B.C., 2009. DNA, RNA, and protein extraction: the past and the present. *BioMed Research International*.

Thairu, Y., Nasir, I.A. and Usman, Y., 2014. Laboratory perspective of gram staining and its significance in investigations of infectious diseases. *Sub-Saharan African Journal of Medicin*e, 1(4), p.168.

Van Hofwegen, D.J., Hovde, C.J. and Minnich, S.A., 2016. Rapid evolution of citrate utilization by Escherichia coli by direct selection requires citT and dctA. *Journal of bacteriology*, 198(7), pp.1022-1034.

Vashist, H., Sharma, D. and Gupta, A., 2013. A review on commonly used biochemical test for bacteria. *Innovare Journal of Life Sciences*, 1(1), pp.1-7.

Wang, Q.M. and Bai, F.Y., 2004. Four new yeast species of the genus Sporobolomyces from plant leaves. *FEMS yeast research*, 4(6), pp.579-586.

White, T.J., Bruns, T., Lee, S.J.W.T. and Taylor, J.L., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, 18(1), pp.315-322.

Yadav, S., Garg, K., Prakash, A. and Rai, A., 2013. Detection and Biochemical Characterization of Microorganisms in Milk and Cocoa powder samples by FTIR and subsequent production of Bacteriocin from Lactobacillus. *Int. J. Drug Dev. & Res*, 5(1), pp.310-320.

Yew, S.M., Chan, C.L., Lee, K.W., Na, S.L., Tan, R., Hoh, C.C., Yee, W.Y., Ngeow, Y.F. and Ng, K.P., 2014. A five-year survey of dematiaceous fungi in a tropical hospital reveals potential opportunistic species. *PloS one*, 9(8), p.e104352.

Zabid, M.F.M. and Abidin, N.Z., 2015, December. Palm oil industry: A review of the literature on the modelling approaches and potential solution. *In AIP Conference Proceedings*, 1691(1), p. 030004.

Zahan, K. and Kano, M., 2018. Biodiesel production from palm oil, its by-products, and mill effluent: A review. *Energies*, 11(8), p.2132.

Zaved, H.K., Rahman, M.M., Rahman, M.M., Rahman, A., Arafat, S.M.Y. and Rahman, M.S., 2008. Isolation and characterization of effective bacteria for solid waste degradation for organic manure. CURRENT APPLIED SCIENCE AND TECHNOLOGY, 8(2), pp.44-55.

Zhang, D., Ong, Y.L., Li, Z. and Wu, J.C., 2013. Biological detoxification of furfural and 5-hydroxyl methyl furfural in hydrolysate of oil palm empty fruit bunch by Enterobacter sp. FDS8. *Biochemical engineering journal*, 72, pp.77-82.

Zhang, J., Zeng, G., Chen, Y., Liang, J., Zhang, C., Huang, B., Sun, W., Chen, M., Yu, M., Huang, H. and Zhu, Y., 2014. Phanerochaetechrysosporium inoculation shapes the indigenous fungal communities during agricultural waste composting. Biodegradation, 25(5), pp.669-680.

Zhang, X.Z. and Zhang, Y.H.P., 2013. Cellulases: characteristics, sources, production, and applications. *Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers*, pp.131-146.

Ziemert, N. and Jensen, P.R., 2012. Phylogenetic approaches to natural product structure prediction. *In Methods in enzymology*, 517, pp. 161-182.