ISOLATION AND CHARACTERISATION OF CELLULOLYTIC MICROORGANISMS FOR OIL PALM (*ELAEIS GUINEENSIS*) WASTE MANAGEMENT: EFFECTIVE COMPOSTING OF EMPTY FRUIT BUNCHES FOR SUSTAINABLE CROP CULTIVATION

By

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Abstract

Oil palm, *Elaeis guineensis,* has been increasingly cultivated in the recent decades due to the growing requirement of palm oil, palm kernel oil, biodiesel and other products. Due to the ever increasing demands, wastes produced from the palm oil mill have also been steadily mounted up, becoming an environmental hazard. An estimate of 80 million tonnes of agro-industrial waste is generated by the oil palm industry each year. Moreover, the usage of chemical fertiliser to cultivate these oil palms could be an environmental impact and may pollute the environment. Besides, chemical fertilisers are costly and may consist up to 60% of the cost for the production of fresh fruit bunch in the oil palm plantation. Solutions must be found to overcome the problems and composting has often been proposed. Composting is a biological based process which converts organic waste to organic matter that is known as compost. Composting recycles the waste products produced from the oil palm industry into sustainable fertiliser which can be implemented back into the plantation. However, oil palm empty fruit bunch (OPEFB), one of the major wastes produced, is lignocellulosic thus requires a longer period of time to degrade. Studies had claimed that co-composting OPEFB with cellulolytic microbes that produce cellulase and hemicellulase enzymes degrades the lignocellulose at a faster rate. In this study, (a) soil samples collected from regions in Sarawak were isolated for cellulolytic microorganisms using selective media; (b) the microorganisms isolated were assessed and selected for their cellulose and hemicellulose degrading abilities using 2-cyanoacetamide method; (c) the selected microorganisms were used to form a consortium to be inoculated into composting materials to produce compost, and (d) the compost produced was assessed for its efficiency in enhancing the growth of oil palm seedlings via 6-month pot trial. The indigenous microbial consortium consisting of selected cellulolytic strain *Bacillus* sp. S2*, Bacillus* sp. S8*, Penicillium citrinum, Aspergillus nomius*, and *Penicillium rolfsii* were identified through molecular methods. This indigenous microbial consortium had shown to decompose OPEFB and produce compost as efficient as the commercial microbial agents (containing *Bacillus cereus, Bacillus subtilis,* and *Bacillus thuringiensis*), as the indigenous microbial consortium actually produced compost with higher nutrient contents (35% N, 57% P, and 9% Mg) when compared to the commercial microbial agents. The indigenous microbial consortium had

also completed the composting of OPEFB in 30 days, shorter than a similar study by Baharuddin the composting process of OPEFB with partially treated POME was completed in 60 days. The results from the pot trial also revealed that the compost produced by the indigenous microbial consortium was able to enhance the growth of oil palm seedlings significantly better than no-fertiliser control, and comparable with chemical fertiliser. The compost could also be mixed with half amount of normal chemical fertiliser requirement and able to sustain the growth of seedlings equally well. On the basis of the results obtained in this research, it can be concluded that the indigenous microbial consortium containing the isolated cellulolytic microorganisms isolated from the soil have the potential to decompose OPEFB to produce compost for the use of oil palm cultivation.

Key words: Cellulolytic, empty fruit bunch, palm oil mill effluent, composting, oil palm

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Declaration

I, Mertensia Kho Kia Chen hereby declare that this research study entitled "Isolation and characterisation of cellulolytic microorganisms for oil palm (*Elaeis Guineensis*) waste management: effective composting of empty fruit bunches for sustainable crop cultivation" is original and does not contain any material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome. To the best of my knowledge, this thesis does not contain any material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, I have disclosed relative contributions of the respective workers or authors.

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Mertensia Kho Kia Chen

As the principal coordinating supervisor, I hereby acknowledge and verify that the above-mentioned statements are legitimate to the best of my knowledge.

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Dr Daniel Tan Lee Tung

Conference Presentations

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Table of Contents

List of Abbreviations

°C	Degree Celsius
BLAST	Basic Local Alignment Search Tool
Cm	Centimetre
CMC	Carboxymethylcellulose
DNA	Deoxyribonucleic acid
EFB	Empty fruit bunch
FFB	Fresh fruit bunch
Hr	Hour
NA	Nutrient agar
NB	Nutrient broth
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
POME	Palm oil mill effluent
RBBR	Remazol brilliant blue-R
SBC	Sarawak Biodiversity Centre
UV	Ultraviolet

List of Figures

List of Tables

Chapter

1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Malaysia is currently the second largest palm oil producer in the world. Malaysia produces about 39% of palm oil after Indonesia, who is the world largest palm oil producer, producing approximately 44% of the world's palm oil (Global Palm Oil Conference 2015). In the year 2015, it is recorded that 5.63 million hectares of Malaysian land are covered with oil palms. Sarawak being the largest state in Malaysia, produces 25% of the palm oil industry (Malaysian Palm Oil Board 2015). Sarawak and Sabah are located on the island of Borneo experience humid tropical climate with sunshine throughout the year which makes it an ideal place to plant oil palms.

Despite the great contribution of oil palm has given to the economy in Malaysia, waste produced from the oil palm industry has become a major environmental hazard. According to Hasamudin and Soom (2002), approximately 55 tonnes of dry fibrous biomass matter is produced from each hectare of oil palm plantation. These dry waste matters were usually incinerated by mills to obtain energy (Hasamudin & Soom 2002). However, burning the dry waste contributed greatly to the air quality problem in Malaysia (Mahjoub *et al.* 2013). Hence, sustainable waste management must be sought after to tackle the waste produced from the palm oil industry.

Oil palm empty fruit bunch (OPEFB) represents one of the major dry waste matters produced by the oil palm industry (Abdullah & Sulaiman 2013). OPEFB is a lignocellulosic material as it constitutes three major constituents namely cellulose, hemicellulose and lignin (Lai *et al*. 2015). Cellulose is the most abundant constituent contributing approximately 53% in OPEFB (Baharuddin *et al*. 2009). Cellulose occurs naturally in plants as a polymer which comprises of glucose units joined together by β-1,4-glycosidic bonds. Cellulose chains in linear form are bundled together to form microbrils. The microbils are composed of amorphous and crystalline regions. The crystalline regions in the microbils exhibit strong internal bonding while amorphous regions have a weaker internal bonding (Ramli *et al*. 2015).

Composting of lignocellulosic OPEFB often requires a long period, making it a stumbling block to this otherwise eco-friendly disposal procedure (Esse *et al*. 2001). Thus, solutions in accelerating the composting process must be pursued. For this study, a speedy composting method was developed to recycle the OPEFB waste by converting it into compost. It is one of the most promising ways as it recycles large volume of waste

generated from oil palm mills into compost (Ravikumar *et al*. 2008). According to Fang *et al.* (1999), composting is an acceptable alternative for converting waste into useful eco-friendly fertiliser as it improves soil fertility. Compost contains a full spectrum of essential plant nutrients that can be released slowly over months and years unlike chemical fertilisers (Singh *et al.* 2010). Nutrients in the chemical fertilisers on the other hand, are usually easily washed away by rain falls shortly after being applied to plants causing it to pollute water bodies such as ponds and streams (Sharma & Chetani 2017).

Zeng *et al*. (2009) mention that microbial activity plays an important role in the transformation of organic material into stabilised composting product. Cellulolytic microorganisms that were able to produce cellulases and hemicellulases can be targeted and screened from soil samples using the selective media. Cellulolytic microorganisms which showed proficiencies in producing these enzymes were used to create an indigenous microbial consortium. The indigenous microbial consortium accelerated the composting process of OPEFB in our composting experiment. Based on a previous study, indigenous cellulolytic microorganisms were co-composed with lignocellulosic OPEFB could speed up the composting process to 30 days (Zainudin *et al*. 2013). Without such indigenous cellulolytic microorganisms, the normal composting process may take 60-90 days (Baharuddin *et al*. 2009).

Enzymes produced by microorganism are selected as they have functional significance which suggests much novel application especially for environmentally-friendly industrial purposes (Binod *et al*. 2013). Enzymes that are produced by microorganisms are an essential source of catalysts used in the industry (Ibrahim 2008). Microbial enzymes are favoured more than enzymes derived from plant and animals as they are more stable and have diverse properties (Alves *et al*. 2014). Enzymes have the ability to produce quantitative production and can be typically extracted at a low cost using downstream processes (Pandey *et al*. 2010).

1.2 Literature Review

1.2.1 Malaysia's Oil Palm Industry

Oil palm (*Elaeis guineensis*) is a variant of tropical tree crop which originated from West Africa. It was first introduced to Malaysia as an ornamental plant by the British in early 1870's. It was then commercially planted at the start of the year 1917 at the Tennamaran Estate, Selangor as a means to replace an unsuccessful planting of coffee bushes (WWF 2002). During the 1960's, an agricultural diversification programme was introduced by the government to reduce Malaysia's dependency on rubber and tin which caused a rapid large scale expansion of oil palm cultivation.

Oil palm has since then become the crop of choice for vegetable oil production in the world. For the first 4 years in the market, the young oil palms production is often small and of poor quality, sometimes it may even be not economic to be harvested (Verheye 2010). The production begins to reach a peak from the 6th year onwards until it reaches the age of 20-25 years where it declines in production (Rupani *et al*. 2010). In the industrial plantations, the oil palms will be felled and replanted after 25-30 years. Each fresh fruit bunch (FFB) harvested can weigh up to 50 kg. Verheye (2010) found that the oil is mostly concentrated in the fresh fruit pulp and kernels. The oil content in the fruit pulp is about 50-60% or 20-22% of the bunch weight while the oil content in the fruit kernels is 48-52% or 2-3% of the bunch weight. The freshly harvested FFB must be processed at the oil mill within 48hr after harvesting to avoid reduction of quality of oil produced (Global Palm Oil Conference IUF 2015). A bunch of FFB can have up to 2000 fruits which consist of hard kernel within a shell surrounded by fleshy mesocarp. The oil palm requires a high and year-round rainfall with little or no dry season and stable high temperatures. The vegetative growth of the oil palms is not affected by dry spells or temperatures below 18°C, however, it does reduce the yield obtained from the oil palm (Verheye 2010).

According to Sime Darby Plantation, 62% of Malaysia's land area is covered with one of the world's oldest rainforest (Sime Darby Plantation 2014). As of December 2015, Malaysia's palm oil plantation accounted for a total planted area of 5.64 million hectares. Figure 1.1 shows the anatomy of the area planted with oil palms in the different states in Malaysia. Sabah and Sarawak showed to have the highest oil palm planted area accommodating a percentage of 53% out of the total planted area in Malaysia.

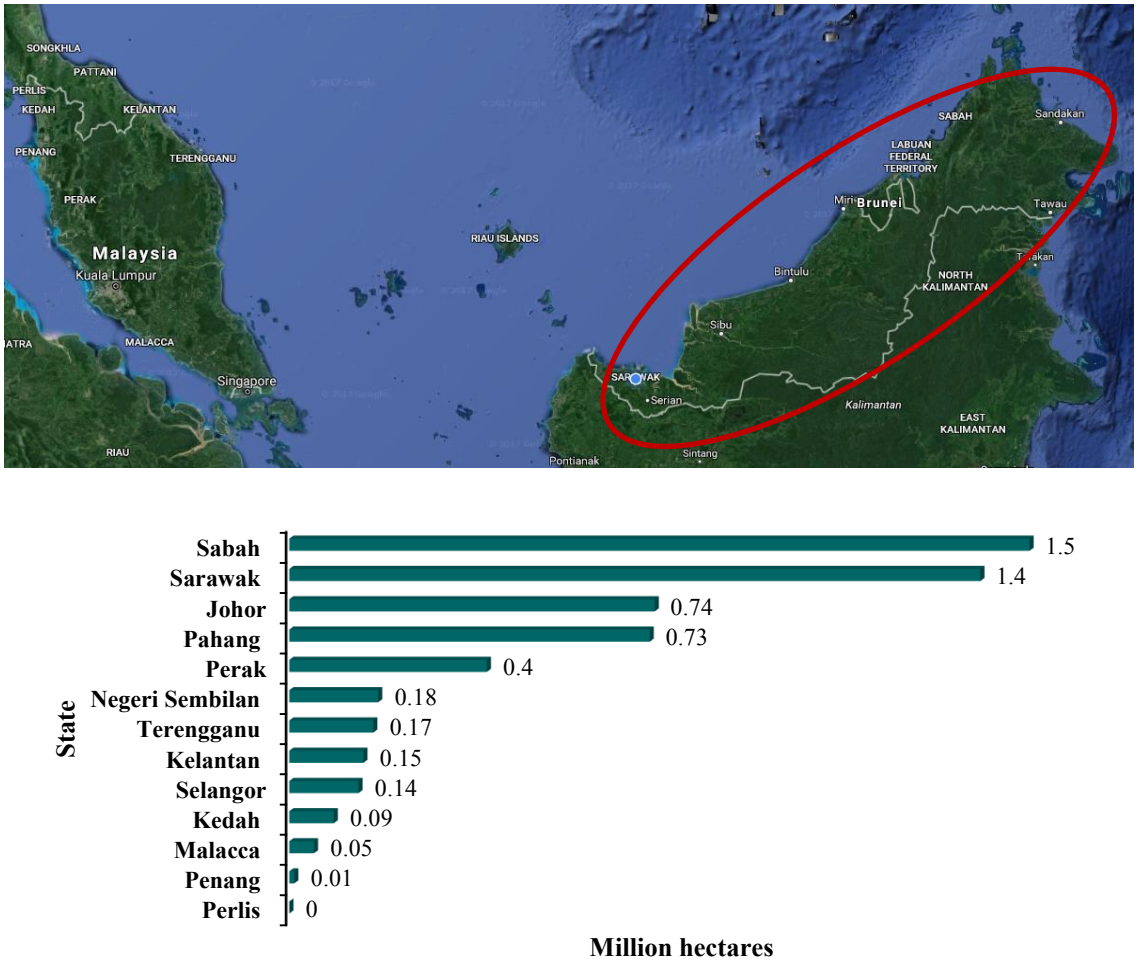


Figure 1.1 Malaysia's oil palm planted area by state at December 2015 (Malaysian Palm Oil Board 2015)

The main producers of palm oil in the world are Malaysia and Indonesia (Lai *et al.* 2015). Indonesia and Malaysia cover about 83% of the total for the palm oil production together in the world as shown in Figure 1.2. The need to expand the production of palm oil was triggered by the high demand for oil palm to use as biodiesel production apart from its traditional use of food preparation. Palm oil is mostly consumed as food items or used to produce soaps, detergents and cosmetics (Lai *et al.* 2015). In recent years, palm oil is also used as feedstock for biodiesel where is it expected to be the leading crop of choice (Global Palm Oil Conference IUF 2015). In the year 2006, Malaysia launched Envodiesel, where 5% of palm oil olein with 95% petrodiesel is mixed together to create a blend. Palm oil can be used as fuel as it is sustainable unlike coal, oil and natural gasses which will run out eventually in term of technical difficulties or high expenses (Halsall 2011). Palm oil produces 1.64 exajoules (1 exa = 10^{18}) of energy when combusted directly

(Halsall 2011). Palm oil was not known to be used as biofuel in the early 1990s. It only began in late 1990 where only 40000 tonnes of palm oil was converted into biofuel. The amount was predicted to increase by 139% by the year 2020 (Lai *et al.* 2015). Therefore, palm oil will be a dominant vegetable oil throughout the world (Sabrina *et al*. 2012).

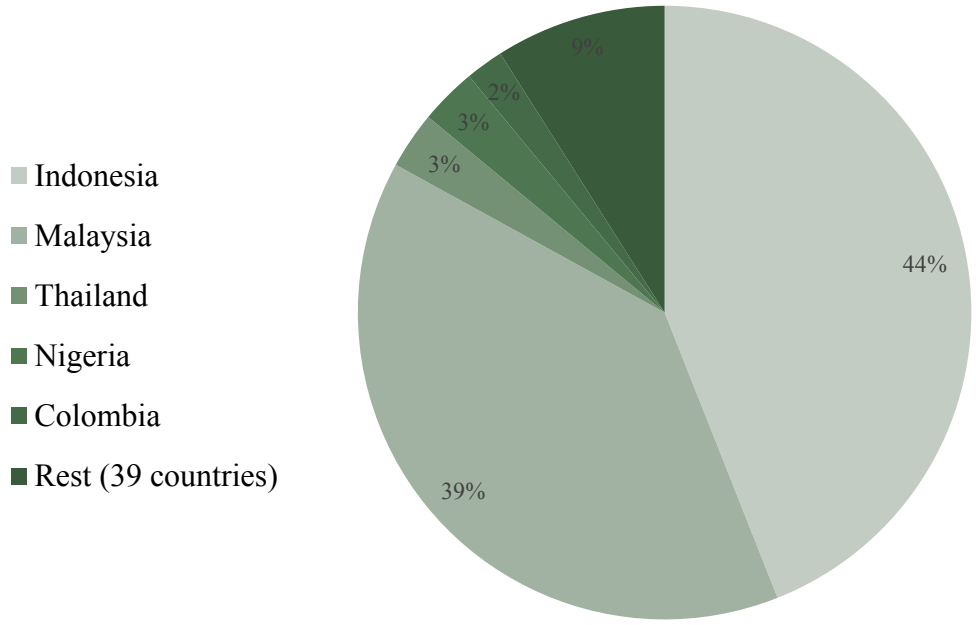


Figure 1.2 Ten largest producers of palm oil in the world for the year 2011 (Global Palm Oil Conference IUF 2015).

About 1 tonne of crude palm oil (CPO) is produced from 5.8 tonnes of FFB (Singh *et al*. 2010). A unique feature of oil palm is where two types of oil can be obtained from it. Palm oil from the flesh of the fruit and also palm kernel oil from the seed or kernel. For every 10 tonnes of palm oil obtained from the process, approximately 1 tonne of palm kernel oil can be obtained too (Malaysian Palm Oil Council n.d.). The two oils have very different composition. Palm oil that is obtained from the mesocarp mainly contains palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (C18:2), and oleic acid (C18:1). Palm oil kernel, on the other hand, contains mainly of lauric acid (C12:0) (Malaysian Palm Oil Board 2011).

The food and agriculture organisations (FAO) define oil crops as those where oil and fats are extracted for producing food products for consumption and industrial purposes (Global Palm Oil Conference IUF 2015). Sime Darby plantations mention that oil palm is the most efficient oil crop in the world (2014). Oil palms bear an average oil yield of

4.08 tonne per hectare per year when compared to other oil-bearing crop as shown in Figure 1.3. Oil palm shows a big advantage as it produces more yield per hectare. Oil palm also uses less land usage when compared to other oil seed crop as shown in Figure 1.3. From the data obtained, palm oil has five more times than efficiency of its next closest competitor: rapeseed and ten times better than soybean. Due to this fact, palm oil has become the most valuable oilseed crop in the world. Not to mention the multiple uses of its oil both in food and other industries has helped boost its value higher.

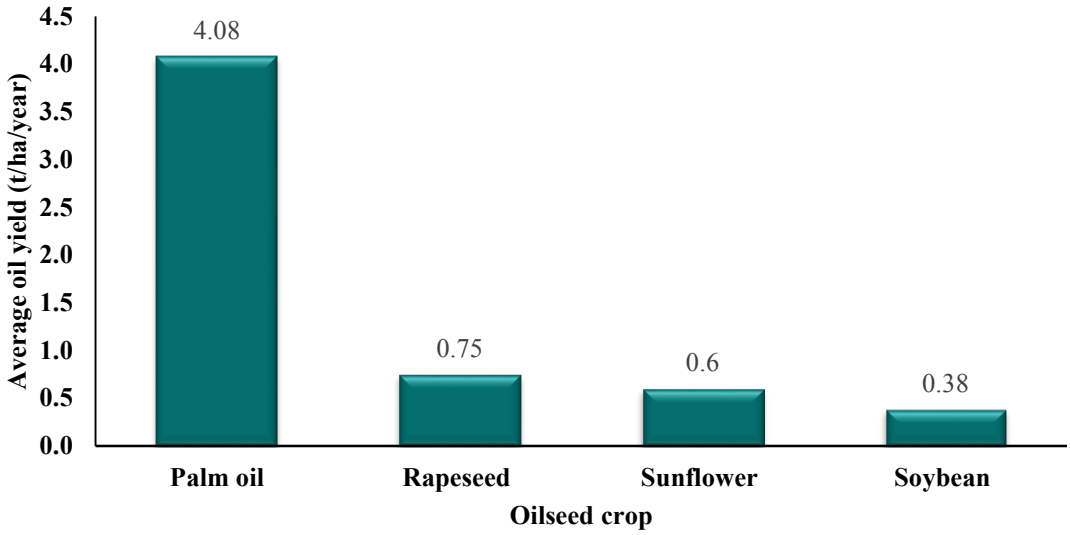


Figure 1.3 Average yield of oil from different oil-bearing crop in the world (Malaysian Palm Oil Board 2011).

1.2.2 Waste Production from the Oil Palm Industry in Malaysia

Oil palm has helped change the scenario of Malaysia in terms of agriculture and economy. Despite the many benefits of oil palms, it has contributes significantly to the environmental degradation from both the input and the output side of its activities (Abdullah & Sulaiman 2013). One of the critical environmental impacts of the input side from the oil palm industry is leaching of chemical fertilisers used into the ground water sources (Rahman *et al*. 2013). On the other hand, the output from the oil palm industry such as the dry biomass and liquid waste has another major environmental impact. The waste produced from processing of palm oil is creating a major environmental danger and entail high disposal cost every year. An oil palm mill can handle an average of 100 metric tonnes of fresh fruit bunches daily generating solid residues and liquid waste (Singh *et al*. 2010). Empty fruit bunches (EFB) and palm oil mill effluents (POME) are

the main waste produced as shown in Figure 1.4. EFB and POME together comprise more than 50% of the waste produced from the processing of oil palm. According to Hassan and Soom (2002), approximately 55 tonnes of dry fibrous biomass matter is produced from each hectare of oil palm plantation. Other than that, an average of 0.9-1.5m^3 POME is generated from each tonne of crude palm oil (CPO) produced (Abdullah & Sulaiman 2013). Sustainable waste management must be sought after to tackle these waste.

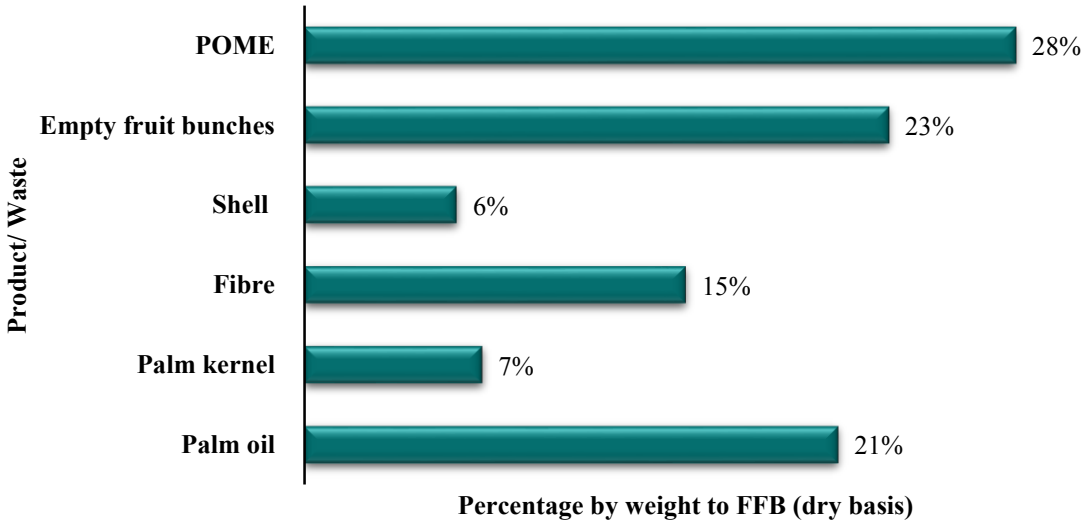


Figure 1.4 Products/wastes from each bunch of fresh fruit bunch (Abdullah & Sulaiman 2013).

Palm oil mill effluents (POME) is the colloidal slurry which consist of water, oil and suspended solid produced during the sterilisation, crude oil clarification and cracked mixture separation processes in the oil palm mill (Yeoh *et al.* 2011; Madaki & Lau 2013). POME contains about 96% water, 0.6% oil, and 4% total solid (Hassan *et al*. 2005). It is considered to be the most expensive and difficult waste to manage as large volumes are generated at a time. POME which has a high content in organic matter is considered non-toxic since no chemicals were added during oil extraction process, however it is identified as an aquatic pollutant when discharged into water bodies (Khalid & Mustafa 1992). In Malaysia, integrating raw POME into anaerobic and aerobic ponds are common methods used in treatment before releasing treated POME into rivers. Unfortunately, the use of anaerobic and aerobic ponds requires vast area. Furthermore, methane which is a greenhouse gas that is 25 times more potent than carbon dioxide that is released during the anaerobic digestion of POME, making it the chief contributor of methane generator to the world global warming (Madaki & Lau 2013).

Oil palm empty fruit bunches (OPEFB) waste product is produced during the milling process when oil seeds are removed from the brunch for oil extraction (Ramli *et al*. 2015). OPEFB are usually either left to rot under oil palm trees to provide organic as mulch or incinerated by mills to obtain energy (Hasamudin & Soom 2002). However, burning the biomass contributed greatly to the air quality problem in Malaysia (Mahjoub *et al.* 2013). The burning of substance releases enormous amounts of carbon dioxide into the atmosphere which is believed to cause greenhouse effect and drastic changes to the environment (Khalil *et al*. 2007). The Malaysian Environmental Air Quality Regulation was introduced in 1978 to overcome the air quality problem caused by incineration of OPEFB. With the new regulation introduced, it prompted mills to find alternative methods of the disposal of the OPEFB waste produced. The environmental awareness also caused an interest among the researchers throughout the world to use the natural fibres to replace the man-made fibres in composite materials (Khalil *et al*. 2007).

1.2.3 Composition of Oil Palm Empty Fruit Bunch

In this study, the focus will be on the oil palm empty fruit bunches (OPEFB) waste generated by the process of manufacturing palm oil as it one of the major wastes produced. OPEFB is a lignocellulosic material which constitutes of three major parts of plants, namely cellulose, hemicellulose and lignin (Sreekala *et al.* 1997; Lai *et al.* 2015). The composition of lignocelluloses depends on the species, the growth condition, the different part of plants and their age. OPEFB of palm oil collectively contains roughly 53% cellulose, 30% of hemicellulose and 17% lignin (Baharuddin *et al*. 2009). Ramli *et al*. (2015) confirmed through testing that OPEFB had similar cellulose, hemicellulose and lignin content across most of its variants. Sreekala *et al.* (1997) investigated the chemical composition of the OPEFB fibre, it was found that the major constituents of the OPEFB were found to be cellulose with the lesser constituent being the lignin. Cellulose content is usually the highest content in all parts of the oil palm tree (Lai *et al.* 2015).

1.2.3.1 Cellulose

Cellulose is the most abundant biological compound in the ecosystem and can be found to be the main component of plants biomass. According to Schmit (2006), the typical fibre wall contains cellulose as its major chemical component. The linear structure of cellulose tends to form intra- or intermolecular hydrogen bonds which form the cellulose microfibrils. These microfibrils promote the aggregation into crystalline. As shown in Figure 1.5, the arrangement of the microfibrils is in an orderly manner. However, there are also regions within these microfibrils which are termed as amorphous due to the less orderly fashion in which they are arranged. When both the amorphous and crystalline cellulose form an arrangement, the resulting formation allows for a stronger and more rigid structure yet still very much flexible. The hydrogen bond also allows for cellulose to be insoluble in most solvents and play a partial role in giving it resistance against microbial degradation (Jørgensen *et al*. 2005). Generally, cellulose degradation is hydrolysed by exoglucanase and endoglucanase. Endoglucanase which is commonly called as cellulase cleaves the glycosidic bonds in the cellulose chain while exoglucanase hydrolyses the cellulose on the free ends of the cellulose chain (Evans *et al*. 2000). Cellulases are conventionally sought from fungi but there is a recent trend in sourcing such enzymes from bacteria which are both good cellulose producers as well as showing rapid growth rate.

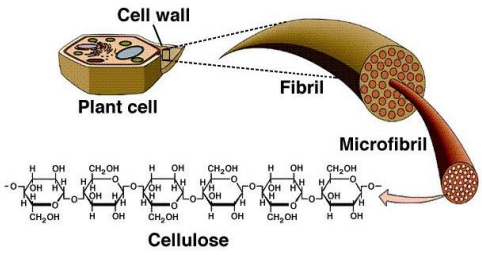


Figure 1.5: Arrangement of fibrils, microfibrils, and cellulose in plant cell walls (Löbmann & Svagan 2017)

1.2.3.2 Hemicellulose

Hemicelluloses is a common name for a combination of different heterogeneous polysaccharides where xylan and glucomannans are the main components (Bastawde 1992). Hemicellulose has a lower degree of polymerization due to having side chains that can be acetylated and are generally amorphous when compared to cellulose (Jeffries 1994). Biologically, hemicellulose plays a vital role in the strengthening of the cell wall by interacting with cellulose as well as lignin in some cases. These structures in

correlation to the widely accepted models of the primary wall have been discussed and due to the nature of hemicellulose being more heterogeneous when compared to cellulose, a much more complex combination of enzymes are needed to degrade it (Schmit 2006). Some examples are endoxylanases, β-xylosidases, endomannanases, β-mannosidases, α-L-arabinofuranosidases and α-galactosidases (Jørgensen *et al*. 2005). The presence of these enzymes has been seen to more common in wood fungi when compared to bacteria (Schmit 2006).

1.2.3.3 Lignin

Known as the third main type of organic polymer which is present in the cell wall, lignin is a made up of dehydrogenative polymerization of three phenyl propane units namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol joined through ether bonds (Jeffries 1994). It functions to provide strength to withstand against compression where the cellulose provides the plant with flexible strength in the cell wall. Lignin also doubles as the bonding component which aids in connecting cells to enable the cell wall of the xylem to harden. This is an important aspect for the xylem to smooth out to transport water from the roots to the leaves. In terms of degradability, lignin is resistant to most microorganisms due to its phenylpropane units within the structure along with the recalcitrant linkages which form between them (Schmit 2006). However, lignin can be effectively broken down through the implementation of white-rot fungi which as a group of filamentous fungi that are capable of producing numerous oxidoreducatases which are enzymes capable of attacking the phenolic structures inside the lignin (Hatakka 2005).

1.2.4 Composting of Oil Palm Empty Fruit Bunch

The boost in palm oil production to the economic growth and rapid development had also contributed to environmental pollution due to the increased waste produced by the milling process (Rupani *et al.* 2010; Singh *et al.* 2010). Ways to treat and dispose of the waste must be sought after to reduce the harmful effect that it has inflicted onto the environment. Researches in Malaysian palm oil board (MPOB) has studied the conversion of oil palm empty fruit bunch (OPEFB) waste into paper-making pulp as OPEFB are categorised as fibrous crops which are lignocellulosic. MPOB has also experimented road making using fibre from OPEFB to produce stone mastic asphalt (SMA) (Hasamudin & Soom 2002). Another way to reduce municipal waste into alternative energy was to convert lignocellulose biomass into ethanol by hydrolysing the biomass into reducing sugar and

fermenting the sugars to ethanol (Sun & Cheng 2002). However, the cost for ethanol production through this method is relatively high.

Composting has often been proposed to reduce agricultural waste products from the oil palm process whereby fertilisers are created from it (Viel *et al.* 1987; Sabrina *et al.* 2012). Composting waste generated from the palm oil mills can be one of the sustainable solutions as it helps recycle the plant nutrients creating a fertiliser (Singh *et al*. 2010). As stated in the Malaysian Biomass Industry Action Plan 2020, the development of bio-fertiliser produced from composting OPEFB would be able to reduce the dependency of chemical fertiliser especially in the palm oil industry (Rahman *et al*. 2013).

Composting involves a biological process where heterogeneous organic wastes are converted into stable and humified organic matter known as compost by the mixed microbial population under controlled environment where optimum conditions such as moisture and aeration are provided (Ahmad *et al*. 2008). The organic matter produced can be stored and applied to the plantations without any environmental effect. Singh (2010) stated compost can be used to stabilise the organic matter for soil amendment and protect the environment from the effect of the oil palm waste products. Additionally, compost has the ability to improve the anion and cation exchange capability, organic matter and microorganism activity (Sharma & Chetani 2017). Composting will recycle the waste products produced from the oil palm industry into sustainable fertiliser which can be implemented back into the plantation. Compost contains a full spectrum of essential plant nutrients. They contain macro and micro nutrients which are released slowly over the months and years unlike synthetic fertilisers (Singh *et al.* 2010).

There are several types of composting methods such as aerated windrow composting, aerated static pile composting, and in- vessel composting. In aerated windrow composting, the composting materials are arranged into rows of long piles called ‘windrow’ and aerated by turning the piles manually or mechanically (Shi *et al*. 1999). This method is suited for composting large volumes of waste. Aerated pile composting can also be used to convert large amounts of waste into compost. Composting materials are mixed together into large piles instead of rows. The pile composting method is aerated by adding layers of bulking agent such as wood chips or newspaper into composting material so that air can have a passage to pass from the bottom to the top of the pile. Another method for pile composting is where the composting materials are placed right on top of piping systems

where air are drawn out from the pipes and delivered directly into the composting materials (Leton & Stentiford 1990). On the other hand, in-vessel composting involves the usage of a drum, silo or similar equipment where composting materials are fed into these vessels. In-vessel composting usually has a mechanism where it turns or agitates the materials placed inside it to provide proper aeration.

In-vessel composting method was chosen to be used to test the indigenous microbial consortium containing selected cellulolytic microorganism in degrading OPEFB during the composting experiment in this thesis study. In-vessel composting method was chosen as it requires less land space than other composting methods. It also enables to provide a controlled environment such as temperature, moisture, and airflow to each composting vessel experimented on during the composting study (Viel *et al*. 1987). In-vessel composting method is self-contained allowing minimal odour and leachate from the composting process.

According to Bernal *et al.* (1998), there are four important stages in composting. The first stage is when the raw materials are freshly laid out and have not gone into the thermophilic process yet. In this initial stage, an explosive growth of the mesophilic microorganisms rapidly uptakes soluble sugars and starches present in the composting material. The active microbial decomposition of the readily available sugar and starch generates heat which then causes the composting process to move into the thermophilic phase. This second stage is usually known as the thermophilic stage, where the thermophilic microorganisms work together to break down the proteins, fats, hemicellulose, and cellulose of the OPEFB rapidly causing the temperature of the compost pile to rise further. The third phase marks the fall in temperature as the bio-oxidative phase ends. In the fourth stage of the composting process, the compost goes through the maturation phase as lignin and other highly resistant compounds are decomposed. It is reported that the composting process must go through the thermophilic phase where the temperature rises above 55°C to sanitise the composting material from pathogens (Gea *et al*. 2005). Microbial activity plays an important role in the transformation of organic material into stabilised and sanitised composting product (Zeng *et al*. 2009). Ekinchi *et al.* (2006) mention that to obtain a successful stabilise and sanitised compost product requires maintaining a suitable environment for the process

which includes the moisture content, oxygen level, C: N ratio, nutrients and temperature given.

Compost protects soil against erosion, enhances soil water retention, reduces soil compaction and acidity, and enhances the soil biological and biochemical activity which establishes an ecological equilibrium in the soil (Sarkar *et al.* 2011). Although compost has its many benefits when applied, large volumes are required to provide sufficient nutrients for plants to grow as compost is low in nutrients. So, compost which is an organic fertiliser should be integrated with chemical fertilisers to give the optimal growth and development when applied to plants. A study by Ahmad *et al.* (2007) showed a higher yield and growth response from wheat and maize when organic and chemical fertiliser were applied together in comparison when the fertiliser was applied individually. The findings from the study implied that a combination of organic and chemical fertiliser could be a more effective and economical way to increase the growth and yield of crop plants than fertilisers alone.

Composting is the most suitable way to manage waste economically and environmentally as it reduces bulk volume of oil palm waste into useful compost which could improve and sustain the soil fertility (Mukhlis *et al*. 2013). For the composting experiment that was carried out in this study, an indigenous microbial consortium of cellulolytic microorganisms was inoculated into the composting material in the beginning of the process. OPEFB was also sprayed with partially treated POME throughout the process. Partially treated POME was added into the compost to maintain the optimum moisture and provide nutrients to the composting material throughout the experiment. A consortium of cellulolytic microorganisms was inoculated into the composting material to accelerate the composting process of oil palm empty fruit bunches (OPEFB). According to a previous study by Zainudin *et al.* (2013), indigenous cellulolytic and hemicellulolytic bacteria from untreated raw POME was co-composed with OPEFB to reduce the composting period. This co-composting experiment was completed in 40 days, shorter in comparison to 60-90 days as reported by Baharuddin *et al.* (2009). Baharrudin *et al.* (2009) composted OPEFB with partially treated POME that did not contain many indigenous microorganisms, thus a longer period could be required to complete the composting process. Apart from the inoculation of lignocellulosic microorganisms, Mukhlis *et al.* (2013) mentioned the importance of selecting effective

lignocellulolytic microorganisms as this could be a crucial step to have a successfully accelerated composting of OPEFB.

1.2.5 Cellulolytic Microorganisms

Cellulolytic bacterium such as Bacillus could be readily isolated from agricultural environments such as soil, compost and animal waste, and often shown to produce both cellulase and xylanase enzymes (Kim *et al*. 2012). Bacillus are gram positive bacteria that are rod-shaped from the member of phylum Firmicutes. *Bacillus* spp. could often be encountered constantly in soil environment, and grown rapidly when cultured in liquid medium, producing spores that are resistant (Shoda 2000). Spores formed by the *Bacillus* spp. can tolerate organic solvents, UV light and high-temperature environment making it able to endure adverse environmental conditions (Arrebola *et al.* 2010).

Bacillus spp. are known to produce various types of enzymes which can be used in various industries. *Bacillus altitudinis* and *Bacillus licheniformis* had been reported to have efficient production of cellulase when incubated in media supplemented with cellulose (Sreeja *et al*. 2013). In a previous study by Lokhande and Pethe (2017), *Bacillus thuringiensis* was tested to have the maximum cellulolytic activity when compared to the other 36 bacterial species isolated in the study. Khianngam *et al.* (2014) isolated 10 cellulase producing microorganisms from oil palm meal which is an oil palm by-product that is rich in fiber and used for the production of the animal feed. Eight out of these 10 isolates belonged to the *Bacillus* spp. while the remaining 2 were from *Paenibacillus* sp. and *Lysinibacillus* sp. respectively. In addition to the ability to produce cellulase, *Bacillus* spp. has also been described to be able to produce hemicellulase apart from cellulase, making them suitable for the composting of oil palm empty fruit bunch (OPEFB) (Kim *et al*. 2012). An article by Archana and Satyanarayana (1997) describe the ability of *Bacillus altitudinis* to produce xylanase enzyme during a soil state fermentation where it was grown on moist solid surface. *Bacillus cereus* have also showed the ability to hydrolyse carbon sources of CMC and xylan when tested on agar plate assays (Sen *et al*. 2012). Aside from cellulase and hemicellulase production, *Bacillus* spp. has a secondary metabolite that enhances the growth of plants and response defence systems in the host plant (Raupach & Kloepper 1998; Arrebola *et al.* 2010).

Fungi are also known to have the ability to degrade lignocellulose as they have efficient enzyme structures which branches onto two enzyme systems: the hydrolytic system, and

the oxidative and ligninolytic system (Sánchez 2009). The enzyme hydrolase produced by certain fungi can degrade polysaccharide, lignin and open phenyl rings. *Phanerochaete chrysosporium* is an example of a known lignocellulosic fungal strain. A study that was carried out by Martinez *et al.* (2004) tested the ability of *P. chrysosporium* to degrade lignocellulose efficiently. From the study, *P. chrysosporium* was able to degrade all major parts of the plant cell. There are only a few known fungi that are able to completely degrade lignin and leave behind crystalline cellulose with a bleaches appearance giving it the term 'white rot'. White rot fungi have the ability to degrade lignin as it involves an extracellular enzyme system which comprises of peroxidase of lignin, laccase and manganese and also hydrogen producing oxidase (Kirk *et al*. 1987). They are commonly found to be an inhabitant of the forest floor among the rotting wood.

Penicillium species is another white rot fungus that has the ability to degrade cellulose, hemicellulose and lignin naturally. Rodriguez *et al.* (1996) tested the ability of the *Penicillium* strains isolated from the forest where all the *Penicillium* strains were showed to be able to degrade cellulose, hemicellulose, and fractions of lignin. In the study of Rodriguez *et al.* (1996), *Penicillium* spp. exhibited to be one of the most powerful lignin degraders among several fungal strains tested. In another study, *Penicillium* was reported to exert high activity of lignocellulose when degrading barley straw (Rosgaard *et al*. 2006). *Penicillium* spp. were also tested for the production of cellulolytic and xylanolytic enzyme where it was compared to *Trichoderma reesei* by Krogh *et al.* (2004) and Penicillium showed to have better higher production activity. Chang *et al.* (2012) reported *Penicillium rolfsii* showed lignocellulolytic ability as it was able to hydrolyse oil palm residue from oil palm trunk to produce bioethanol when compared to other isolated fungus. *Penicillium citrinum* had also shown to produce high amounts of endoglucanases and exoglucanases that are responsible for the composting of cellulose (Suely *et al*. 2014).

Brown rot fungus usually just degrades the cellulose and hemicellulose of cell walls, leaving lignin undigested (Hatakka & Hammel 2010). During the breakdown of cellulose and hemicellulose in moist wood by brown rot fungus, hydrogen peroxide is released which causes the decomposition to increase making the wood appear shrunken with cubicle patterned cracks in a discoloured brown form, hence giving it the name 'brown rot'. *Aspergillus* species are examples of brown rot fungi. *Aspergillus* spp. could produce high level of xylanase which increased the bleachability of eucalyptus pulp in an

environmental process (Khonzue *et al*. 2011). In the previous study, *Aspergillus nomius* exhibited the ability to produce a cellulolytic and xylanolytic enzyme to degrade untreated oil palm trunk (Ang *et al*. 2015). *Aspergillus niger* was used a positive control for the cellulolytic assay during this thesis study as it is a known cellulase and xylanase producer which are usually used as an industrial microbial strain to produce large amounts of these enzymes (Biswas *et al.* 2014). *A. niger* is thermophiles that in temperature range of 35 ± 5°C. *A. niger* showed the ability to produce cellulolytic enzymes in an earlier study as an aid for the fermentation of waste (Santos *et al*. 2011). Thus, *A. niger* was chosen as the positive control for the cellulolytic assay as it is widely used for its cellulolytic enzyme production (Pandey *et al*. 2000).

Soft rot fungus produces cellulase that breaks down cellulose in wood which decolourises and causes crackling pattern to form which has similar appearance to brown rot fungus (Schmit 2006). It is typically found in the Ascomycetes sub-division. Soft rot fungus are less aggressive decomposers when compared to white rot fungus (Vane *et al*. 2005). Soft rot commonly occurs in wood that are exposed to extreme environments (Blanchette *et al*. 2004). Environments that are too wet usually inhibit the growth of brown and white rot fungus but does not limit the growth of soft rot fungus. Soft rot fungus usually favour conditions that are high in moisture and low in lignin content (Levy 1966).

1.2.6 Mechanism of Cellulolytic Microorganisms

Cellulose is usually degraded by cellulolytic enzymes produced from cellulolytic microorganisms. The cellulolytic enzyme consists of three basic type of enzymes which are endoglucanases, exoglucanases and ß-glucosidases (Khianngam *et al*. 2014). Endoglucanase produces oligosaccharides when it catalyses the initial disruption of the internal bonds that are within the cellulose crystalline structure. Endoglucanases are followed by exoglucanases, exoglucanases attack the nonreducing end of oligosaccharide chains that was produced by endoglucanase creating disaccharides. Disaccharides which are the cellobiose fragments of the cellulose is further hydrolysed by ß-glucosidases into glucose, hence completing the hydrolysis of cellulose into glucose (Ang *et al*. 2015).

Hemicellulose in plant cell walls consist of different polysaccharides, xylan represents the major component among the polysaccharides present in hemicellulose (Uffen 1997). According to Li *et al.* (2014), the hemicellulose layer in the oil palm empty fruit bunches (OPEFB) consist mostly of xylan. Hence, cellulolytic microorganisms that produce

xylanases were screened for to degrade the hemicellulose in OPEFB during the composting experiment in this study. Xylanases cleave the 1,4-β-D-xylosidic linkages in xylan randomly, producing xylose, arabinose, galactose and mannose (Collins *et al.* 2005). These sugars produced from the degradation are used by microorganisms for metabolism (Bisaria & Ghose 1981).

1.2.7 Screening for Cellulolytic Microorganisms

Carboxymethylcellulose (CMC) agar and Remazol Brilliant Blue-R (RBBR) xylan agar were chosen to be used for the initial screening of cellulolytic microorganisms from the collected soil samples. For CMC agar, pure isolates were cultured onto CMC agar, stained with 1% Congo red dye and destained with 1M NaCl to detect for visible clear zones. Previous studies have shown that Congo red has a better effectiveness to distinguish cellulase activity on solid medium (Gohel *et al.* 2014). During the staining of the CMC plates, the 1% Congo red dye binds with the polysaccharide which is CMC and forms a visible complex. Cellulases produced by the cellulolytic microbe inoculated into the plates break down the polysaccharide present in the agar medium, replacing the surrounding area of the isolate with smaller monosaccharides and disaccharides (Gupta *et al.* 2012). Therefore, a visible clear zone is formed around the cellulolytic microorganisms as they are unable to bind with the monosaccharides and disaccharides properly, which indicates the presence of cellulolytic bacteria.

Isolates that showed positive (zone-clearing) cellulolytic properties were then cultured on dyed xylan agar. The xylan was dyed with Remazol Brilliant Blue- R (RBB-R) before the screening. Those showed visible clear zones would indicate the presence of xylan degrading microorganisms (Meddeb-Mouelhi *et al.* 2014). Isolates with the ability to produce xylanase were able to degrade the dyed xylan present in the agar medium and form clear zones around the area. The formation of the clear zone on the RBBR-R xylan agar indicated the presence of the cellulolytic microbe that produces xylanase.

Isolated microbial strains which showed positive result in cellulase and xylanase productions were further tested their efficiency by measuring the reducing sugar production. There are two known methods for detecting the reducing sugars in cellulose and hemicellulose activity which are a 2-cyanoacetamide method and the 3,5-dinitrosalicylic acid (DNS) method. DNS is an alkaline solution that reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid that can be read at 510 nm (Breuil & Saddler

1985). For this study, the 2-cyanoacetamide method was chosen to test for the reducing sugar produced by the isolated strains. Jurick *et al.* (2012) tested and found that the 2-cyanoacetamide method is capable of detecting the reducing sugars produced by cellulase as efficient as the DNS assay. Unlike the DNS method, 2-cyanoacetamide method does not require the use of potential carcinogenic and corrosive chemicals; therefore, the assay was not required to be conducted in the fume hood (Jurick *et al.* 2012). The 2-cyanoacetamide method was chosen for its accurate, rapid and non-toxic detection of cellulose and hemicellulose activity. Another advantage of the 2-cyanoacetamide method is that other enzyme activities can also be easily detected by changing the substrate in the growth medium. Carboxymethylcellulose (CMC) was used as a substrate in this study for detecting cellulase activity while xylan was used as a substrate to detect xylanase activity of the isolated microorganisms.

1.2.8 Plant Growth Promoting Microorganisms

Nitrogen, phosphorus, and potassium are the main essential nutrients required by plants to have a normal functioning cell system (Sharma & Chetani 2017). Hence, sufficient amounts of nutrient must be supplemented to plants to ensure optimum growth (Chen 2006). To reduce the adverse effect of chemical fertilisers, new agricultural practices have been developed where agricultural sectors have been creating a mix of chemical fertilisers with compost to help boost the plant growth. Soil samples from the natural environment normally contain plant growth-promoting microorganisms such as nitrogen-fixing microorganisms, phosphate solubilising microorganisms, potassium solubilising microorganisms, and indole acetic acid (IAA) producing microorganisms (López-Mondéjar *et al.* 2016). The presence of these microorganisms is known to aid in enhancing the plant growth when incorporated into the compost to form a bio-fertiliser. For the last couple of decades, plant growth-promoting microorganisms have been used increasingly in several regions of the world as it significantly increases the growth and yield of crops with its inoculation (Chen 2006). Most essential soil nutrients remain in insoluble forms in soils that are unavailable to plants causing nutrient deficiency (Nautiyal 1999). Therefore, plant growth promoting microorganisms play a vital role in converting the nutrients that are in insoluble forms into forms that the plants can absorb. For this study, isolated cellulolytic microorganisms were tested for plant growth promoting ability to enhance the compost produced.

Nitrogen is available molecularly from the atmosphere. However, it cannot be directly assimilated by plants. Nitrogen can only become available for plants through biological nitrogen process that only nitrogen fixing microorganisms can perform (Franche *et al.* 2009). Shortage of nitrogen availability to plants causes the formation of yellow leaves due to the insufficient supply of chlorophyll. The growth of the plants will also be affected as it will appear stunted as nitrogen is and essential nutrients required for cell division and enlargement. *Bacillus* spp., *Rhizobia* spp., F*rankia* spp. and *Penicillium* spp. are some of the examples of nitrogen fixing microorganisms (Moore & Becking 1963; Harper & Lynch 1984). In this study, Jensen's medium was used to screen the isolated cellulolytic microorganisms for the presence of nitrogen-fixing microorganisms. For Jensen's medium, nitrogen-fixing microorganisms grow well on nitrogen-free medium (Ahmad *et al.* 2008). The nitrogen-fixing microorganisms use the nitrogen gas available from the environment to perform cell protein synthesis. The cell protein produced from the synthesis mineralizes into the soil around the plants after the cells have died and therefore, contributes to the nitrogen availability to the plants. Due to the absence of nitrogen source in the agar medium, only nitrogen-fixing microorganisms will grow on the medium. To further confirm the presence of nitrogen-fixing activity, the medium with the nitrogen-fixing microbe could be flooded with bromothymol blue as a colour pH indicator (Gothwal *et al.* 2008). Bromothymol blue have a greenish appearance when it is in its original neutral pH state, yellow in the presence of acid and blue in an alkaline condition. For this study, the change of green to blue indicates the presence of nitrogen-fixing activity by the isolated microorganisms.

Decaying dead plant debris releases phosphate to the soil in organic and inorganic forms that are not readily available for plants to absorb. Organic forms are commonly referred to living substances which contains carbon, proteins, lipids and vitamins while inorganic forms are usually found in non-living substances such as decaying plants which holds minerals (Dibb 2002). Phosphate containing chemical fertilisers that are applied to soil also becomes immobilised and unavailable to plants (Nautiyal 1999). This phenomenon is due to the strong bonds that phosphate has with calcium and magnesium in alkaline condition while having the same strong formation bonds with iron and aluminium in acidic soils (Sridevi *et al.* 2013). Due to the rapid formation of the strong bonds of other elements, plants are not able to absorb the nutrients causing a deficiency in phosphate. Phosphate solubilising plays an important role in maintaining the phosphate balance for

plants. Deficiency in phosphate causes plants to have smaller leaves, weak steam and slower growth development. Some examples of phosphate solubilising strains are *Pseudomonas* spp*., Bacillus* spp*., and Rhizobium* spp. (Peix *et al*. 2001). For this study, phosphate solubilising microorganisms were screened using Pikovskaya's agar media. Calcium phosphate added gave the agar medium a cloudy powdery appearance as it acted as the phosphate for the phosphate solubilising microorganisms to hydrolyse. Dextrose was supplied into the medium for microorganisms to use as an energy source. From the assay, microorganisms that solubilise the phosphate present around its vicinity producing organic acids and showing a halo zone indicated phosphate solubilising activity.

Potassium is one of the three essentials nutrients required by plants. Potassium is available in the soil, however, like phosphate, it is also bound to other minerals making it unavailable for the plant. Potassium solubilising microorganisms can solubilise potassium through their hydroxyl and carboxyl groups that chelate the cations bound to phosphate thus releasing the low molecular weight soluble organic acids that are available for the plant (Chen *et al*. 2006). Potassium is an important nutrient as it helps in the stomatal activity, photosynthesis, protein synthesis and transport of water and nutrient in plants (Zhao *et al.* 2001). Some examples of potassium solubilising microorganisms are *Bacillus* spp. and *Pseudomonas* spp. (Sheng 2005). Aleksandrow agar can be used to screen for potassium solubilising microorganisms in this study. Potassium alumino silicates present in the agar medium acts as the source for potassium salts for the potassium solubilising microorganisms to secrete organic acids. From the test, clear zones that formed around the cellulolytic isolate inoculated into the medium indicate potassium solubilisation activity.

Indole acetic acid (IAA) is an auxin which is a plant hormone that affects the growth of plants. IAA is a product of L-tryptophan that can be produced by plant growth promoting microorganisms (Mohite 2013). IAA induces the growth of longer roots with increased number of hair roots which helps increase the nutrient uptake by plants (Datta & Basu 2000). *Bacillus* spp., *Pseudomonas* spp*., Acetobacter* spp*., Rhizobium* spp., *Aspergillus* spp., and *Penicillium* spp. are some strains that known to produce IAA (Yadav *et al.* 2011; Mohite 2013). During this study, cellulolytic isolates were grown in base medium supplemented with L-tryptophan and tested using Salkowski reagent to determine the concentration of indole-3-acetic acid produced. The IAA production ability by the

cellulolytic microorganisms was judge based on the amount of IAA produced where a higher concentration of IAA gave a more concentrated pink colour.

1.3 Research Aims and Objectives

The aim of this research was to screen and characterise cellulolytic microorganisms isolated from soil samples taken within the region of Sarawak capable of producing cellulase and hemicellulase (xylanase) for use and application as microbial consortium in the composting of oil palm empty fruit bunch (OPEFB) and evaluating the effectiveness of the compost produced. The specific objectives set out to achieve the research aim are:

i. To isolate and characterise cellulolytic microorganisms from soil samples collected in Sarawak;

ii. To evaluate the efficiency of isolated cellulolytic microorganisms in the formulation and development of indigenous cellulolytic microbial consortium; and

iii. To investigate the effectiveness of the indigenous microbial consortium developed in the composting of OPEFB into compost through in-vessel composting method and the compost produced in growth enhancing of oil palm seedlings via pot trial method.

1.4 Thesis Outline

This thesis presented is divided into 5 chapters which are listed as follow:

Chapter 1- Introduction and Literature Review

Chapter 1 provides a brief introduction to the background and a broad review of essential literature related to the study which was reported and described in previous studies by other researchers. The aim and objectives of the research were also conferred in this chapter.

Chapter 2- Screening and Characterisation of Cellulolytic Microorganisms from Sarawak Soil

Chapter 2 describes a detailed screening, isolation and identification of cellulolytic microorganisms which were obtained using selective media. This chapter focuses on the isolation of cellulase and xylanase producing microorganisms from soil samples taken from various location in Sarawak. Isolated cellulolytic microorganisms were also tested for plant growth promoting ability in this chapter.

Chapter 3- Selection of Cellulolytic Microorganisms Based On Enzyme Activity and Optimisation of Selected Microorganisms

Chapter 3 present details on the efficiency of the isolated cellulolytic microorganisms in producing cellulase and xylanase activity using 2-cyanoacetamide method. The purpose of this chapter was to evaluate the effectiveness of the isolated cellulolytic strains as potential candidates to form an indigenous microbial consortium for the composting of OPEFB. The isolates capable of producing comparable cellulolytic activity with positive control strain were selected and used for subsequent experiments. This chapter also focuses on the optimisation of the selected cellulolytic microorganisms to form the microbial consortium.

Chapter 4- Production of Compost Via In-Vessel Composting of Oil Palm Empty Fruit Bunch Using Cellulolytic Microorganisms and Its Enhancement in the Growth of Oil Palm Seedlings

Chapter 4 describes the production of compost by composting of OPEFB using indigenous microbial consortium containing selected cellulolytic microorganisms through in-vessel composting method. During this chapter, the efficiency of the compost in the enhancement of the growth of oil palm seedlings was also described. The knowledge obtained from the study can lead to further investigation along this line of research such as large-scale field application using the indigenous microbial consortium of cellulolytic microorganisms produced in this study.

Chapter 5- General Conclusions, Recommendations, and Future Works

Chapter 5 provides a succinct overview of the findings from the experimental studies presented. Perspective on future research possibilities within this field as well as recommendations is conferred in this chapter.

Chapter

2

SCREENING AND CHARACTERISATION OF CELLULOLYTIC MICROORGANISMS FROM SARAWAK SOIL

2.1 Introduction

The growing oil palm industry in Malaysia has been ever increasing over the last decade due to escalating requirement of palm oil (Rosenani *et al*. 2016). The amount of waste produced by the oil palm industry has been accumulating causing an environmental hazard. Oil palm empty fruit bunch (OPEFB) is one of the major produced by the oil palm industry. OPEFB is a lignocellulose waste produced abundantly in the oil palm industry that consists mainly of cellulose, hemicellulose, and lignin (Mukhlis *et al*. 2013). Ways to tackle the growing waste production is very much needed.

Fungi and bacteria are the main natural agents of cellulose degradation (Jahangeer *et al.* 2005). Cellulolytic microorganisms were targeted in this study due to its ability to degrade cellulose in OPEFB. Microorganisms are favoured for degrading cellulose over the use of chemical treatments due to the harmful effects of the chemical treatments might inflict on the environment (Li *et al.* 2014). Cellulolytic microorganisms that produce cellulase and hemicellulase have exhibited the ability in previous studies to degrade cellulose into simple sugars (Li *et al.* 2014).

In this chapter, soil samples collected from several locations within Sarawak were screened for cellulolytic microorganisms. Soil are chosen as source for obtaining cellulolytic microorganisms as it contains an immense diversity of microorganisms that have yet to be discovered (Torsvik & Øvreås 2002). Cellulolytic microorganisms can be found naturally in the forest soil as they usually consist of the microbial community that degrades most of the organic material that settles on the forest floor (López-Mondéjar *et al.* 2016). Selective media was used in this study to isolate cellulolytic microorganisms from soil samples. Carboxymethylcellulose (CMC) agar was used to detect microorganisms that produced cellulase while dyed xylan agar was used to detect microorganisms which produce xylanase. Cellulolytic microorganisms were selected in this study as a potential candidate to degrade the cellulose and hemicellulose in the OPEFB waste.

The experimental focus of this chapter was to screen, isolate and identify cellulolytic microorganisms from soil samples taken from the forest grounds of Borneo as a potential source to degrade OPEFB waste produced from the oil palm industry.

2.2 Materials and Methods

2.2.1 Sampling Locations and Collections

Forest soil was targeted in this study as cellulolytic microorganisms can be found naturally in the forest ground (López-Mondéjar *et al.* 2016). Upon the authorisation by Sarawak Biodiversity Center, fresh soil samples were collected from 4 targeted locations within the region of Sarawak. A total of 12 soil samples, 3 soil samples from each targeted location were collected in January 2016. Each sample was collected aseptically using sterile tools and placed into its own individual resealable bag which was stored in a polystyrene box containing ice packs at the collection site. The soil samples were then kept in 4°C cold room for not more than 3 days before performing the isolation procedures. The targeted sites are as follow:

(i) Melikin, Serian.

(ii) Semonggok Forest Reserve, Kuching.

(iii) Kubah National Park, Kuching.

(iv) Kampung Panchor Hot Spring, Kuching.

2.2.2 Screening for Potential Microorganisms from Soil Samples

Agar plate assay was used to the initial screening for potential cellulolytic, nitrogen-fixing, phosphate solubilising, potassium solubilising, and indole acetic acid (IAA) producing microorganisms from collected soil samples during this research. Preparation of agar plates for the assays can be found in Appendix Section 1.

2.2.2.1 Serial Dilution

Serial dilution technique was used during the initial screening for cellulolytic microorganisms. A ten-fold serial dilution was carried out for each soil sample using sterilised deionised water.

2.2.2.2 Screening for Potential Cellulolytic Microorganisms Using Selective Media

(I) Screening for Cellulolytic Microorganisms

Carboxymethylcellulose (CMC) agar was used to screen for cellulolytic microorganisms. The dilution series of the diluted soil samples chosen for the initial screening was 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. Dilution 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} was chosen for the initial screening because dilution of 10^{-1} and 10^{-2} gave an overgrowth of

microorganism on medium plates. From the diluted soil samples prepared, 100 µL of diluted soil samples were spread onto the CMC agar evenly. The spread plates were sealed to avoid contamination and incubated under aerobic conditions at 37°C for 3 days. After incubation, the plates were stained with 1% Congo red dye (Fisher Scientific, USA) for around 30 minutes followed by destaining process using 1M sodium chloride (NaCl) for approximately 15 minutes. The plates were then observed for formation of clear zones around the colonies visible on the CMC plates. CMC plates that showed clear zone were kept in 4°C for isolation purposes.

(II) Screening for Xylanase Producing Degrading Microorganisms

RBBR-xylan agar was used to screen for xylanase producing microorganisms. Pure isolated cellulolytic microorganisms that were obtained from the soil samples using CMC agar which showed potential in degrading cellulose were cultured onto the xylan agar. Plates were sealed to avoid contamination and incubated under aerobic conditions at 37°C for 3 days. After 3 days of incubation, the plates were observed for the formation of clear zones around the colonies. Plates that showed clear zone were noted down. The screening analysis for xylanase producing microorganisms were performed in triplicates for each isolate.

(III) Screening for Nitrogen-Fixing Bacteria

Jensen's medium was used to screen for nitrogen fixing bacteria. Pure cellulolytic isolates were inoculated onto fresh Jensen's medium agar and sealed to avoid contamination. The plates were incubated under aerobic conditions at 37°C for 3 days. After 3 days of incubation, the plates were observed for the formation of clear zones around the colonies. Plates that contained cellulolytic microorganisms that showed clear zone were noted down. The screening analyses for nitrogen fixing microorganisms were performed in triplicates for each isolate tested on.

(IV) Screening for Phosphate Solubilising Bacteria

Pikovskaya agar was used to screen for nitrogen fixing bacteria. Pure cellulolytic isolates were inoculated onto fresh Pikovskaya agar plate. The inoculated were sealed to avoid contamination and incubated under aerobic conditions at 37°C for 3 days. After 3 days of incubation, the plates were observed for the formation of clear zones around the colonies. Plates that contained cellulolytic microorganisms that showed

clear zone were noted down. The screening analyses for phosphate solubilising microorganisms were performed in triplicates for each isolate tested on.

(V) Screening for Potassium Solubilising Bacteria

Aleksandrov agar was used to screen for nitrogen fixing bacteria. Pure cellulolytic isolates were inoculated onto fresh Aleksandrov agar and sealed to avoid contamination. The inoculated plates were incubated under aerobic conditions at 37°C for 3 days. After 3 days of incubation, the plates were observed for the formation of clear zones around the colonies. Plates that contained cellulolytic microorganisms that showed clear zone were noted down. The screening analyses for potassium solubilising microorganisms were performed in triplicates for each isolate tested on.

(VI) Screening for Indole Acetic Acid (IAA) Producing Microorganisms

Isolated cellulolytic microorganisms were tested for the ability to produce indole acetic acid (IAA) using the Swalkowski method. Broth cultures of pure cellulolytic microorganisms were inoculated into basic growth medium with an addition of tryptophan (1 g/L). They were incubated at 37°C for 4 days in an incubator shaker (Sartorious Stedim Biotech, Germany). After incubation, the cultures were spun down using a centrifuge machine and 1 mL of the supernatant was transferred aseptically into a universal bottle with proper labelling. In the darkness of the fume hood, 1.5 mL of Swalkoski reagent (2% 0.5 $FeCl_3$ in 35% $HClO_4$ solution) was pipetted into each universal bottle containing the supernatant. The solutions were mixed well and were left to incubate in the dark for 75 minutes before reading it at 525 nm using a spectrophotometer. If the solution turns pink, it indicates production of IAA. However, if the solution turns yellow, it shows that no IAA production occurred. The screening analysis for IAA producing microorganisms were performed in triplicates for each isolate tested on, the results of the study were presented as mean values for the three separate plates.

2.2.3 Isolation of Pure Culture

Each strain that exhibited clear zones surrounding its colony on carboxymethylcellulose (CMC) agar plate was isolated and transferred to its own fresh agar medium. For each bacterial strain, it was picked using sterilised loops, while for fungal strain, isolation was performed using autoclaved 5-mm straw. Subsequent sub-culturing was performed until pure isolates were obtained. The pure isolates on the CMC agar plates were stained and destained to re-confirm the ability of the isolates to degrade CMC. Isolates that exhibited the formation clear zones were kept in 4°C for further analysis. Isolates that did not show clear zones were discarded by decontamination using an autoclave (Hirayama, Japan).

2.2.4 Storage and Preservation

2.2.4.1 Bacterial Culture

Long term storage using glycerol stock was used in this study for maintenance and preservation of the isolated cellulolytic bacteria. For the maintenance of the bacterial culture in glycerol stock, 800 µL of overnight grown cultures in nutrient broth (NB) (Himedia, India) were inoculated into 2.0-mL cryogenic vials (Nest Biotechnology, China) containing sterilised 800 µL of 60% glycerol (HMBG, Germany) to obtain a final glycerol concentration of 30% (v/v). The stocks were mixed carefully by pipetting the mixture using a micropipette (Eppendorf, Germany). The cryogenic vials were labelled, sealed with paraffin film, and placed into a cryo box and kept in a -80°C freezer (Thermo Scientific, USA). Long term storage for each bacterial strain was deposited in 10 replicates. When reviving the stored bacteria, a sterile toothpick or inoculation loop was used to scrape off the solid ice in the cryogenic vials and transfer culture onto fresh nutrient agar (NA) (Himedia, India) which would then be incubated at 37°C.

For short term storage of the bacterial culture, the pure isolates were grown on NA (Himedia, India) plates for 24 hr and stored at 4°C until further requirement for the next experiment. Short term storage for each bacterial strain was also in 10 replicates.

2.2.4.2 Fungal Culture

Long term storage using glycerine agar was used in this study for maintenance and preservation of the isolated cellulolytic fungus. For the maintenance of the fungal culture in glycerine agar, 50 mL of glycerol stock was incorporated into the 1 L of potato dextrose agar (PDA) (Himedia, India) before autoclaving. The autoclaved PDA and glycerol stock solution (HMBG, Hamburg, Germany) was aseptically dispensed into a sterile 90-mm petri dish in a laminar flow cupboard and allowed to cool and harden for 45 minutes before keeping it aside. Pure fungal cultures were cultivated onto their own respective plates for 7 days at 37°C, allowing enough mycelium to develop before storing them. Straws 5 mm in diameter were cut into smaller size slightly shorter than the depth of 2.0 mL cryogenic vial, autoclaved and oven dried. The fungal cultures were stored by plugging the freshly grown cultures into the sterilised straws using forceps until the straw was filled. Once the straw was filled with the mycelium, it was placed into the sterilised 2.0-mL cryogenic vials (Nest Biotechnology, China). The cryogenic vials were labelled and placed into a cryo box where it was kept in the -80°C freezer (Thermo Scientific, USA). Long term storage for each fungal strain was deposited in 10 replicates. When reviving the stored fungus, a sterile toothpick or inoculation loop was used to poke out the fungal cultures out from the straws onto the fresh PDA medium and incubated at 37°C.

Fungal cultures were also preserved using barley grains for storage of 6 months. Clean universal bottles were first filled with 5 g of organic barley and rinsed twice with distilled water. The distilled water was drained and replaced with 1.5 mL of Potato dextrose broth (PDB) (Himedia, India). The universal bottles containing the barley and PDB were autoclaved twice before allowing them to cool prior to inoculation. A straw of 5 mm diameter was used to transfer two 7-day old fungal cultures into the universal bottles containing the sterilised barley and PDB. They were allowed to grow for 2 weeks before placing them in the 4°C fridge. Storage for each fungal strain in barley was performed in triplicates. When reviving the stored fungus, a sterile forceps was used to transfer two grains of barley colonized with the fungal culture onto a sterile PDA medium.

For short term storage of fungal culture, the pure cultures were grown on PDA plates for 3 days and stored at 4°C until required. Short term storage for each fungal strain was in 10 replicates.

2.2.5 Molecular Identification

2.2.5.1 Preparation of Culture Broth for Molecular Identification

(I) Bacterial Culture

Cellulolytic bacterial isolates were inoculated into sterilised nutrient broth (Himedia, India) and shaken overnight at 37°C in an incubator shaker (Sartorious Stedim Biotech, Germany).

(II) Fungal Culture

Cellulolytic fungal isolates were inoculated into sterilised potato dextrose broth (Himedia, India) and shaken overnight at 37°C in an incubator shaker (Sartorious Stedim Biotech, Germany).

2.2.5.2 DNA Extraction

The freeze-and-thaw method (Griffin *et al.* 2002) was used to lyse the isolated microorganisms. From the incubated broth, 1000 µL of the culture broth were transferred into sterilised 1.5-mL microcentrifuge tubes respectively and centrifuged for 10 minutes at 7500 rpm using a microcentrifuge (Eppendorf, Germany). The supernatant was decanted carefully to avoid the disposal of the pellet. When a pellet was observed, it will be re-suspended in 1 mL of sterilised deionised water by mixing using a micropipette. The mixture was then mixed using a vortex (Barnstead Thermolyne, USA) to ensure the pellet was completely re-suspended. The suspension was then subjected to the freeze and thaw cycle of -80°C freezer (Thermo Scientific, USA.) and hot water bath (Memmert, USA). A total of 4 cycles were carried out in a programme illustrated in Table 2.1.

After subjecting the sample to freeze and thaw, it was spun down at 10,000 rpm for 5 minutes using a microcentrifuge (Eppendorf, Germany). The supernatant from the sample was carefully transferred into a fresh sterile microcentrifuge tube, labelled, and stored in the -20°C until further requirement. Only the supernatant was kept because the pellet that contains mostly cellular debris would be decontaminated and discarded.

Table 2.1. DNA extraction - Freeze-and-thaw cycles.

1st cycle:	
8 minutes	Freeze samples at -80°C using the -80°C freezer
3 minutes	Thaw samples at 85°C using a hot water bath
2nd to 4th cycle:	
5 minutes	Freeze samples at -80°C using the -80°C freezer
3 minutes	Thaw samples at 85°C using a hot water bath

2.2.5.3 DNA Amplification

The microbe's DNAs obtained from the extraction process were amplified by using polymerase chain reaction (PCR). MyTaq Red Mix (Bioline, USA) were used along with primers (IDT, USA) to prepare the master mix for PCR amplification. The PCR master mix contained the following: MyTaq Red Mix (25µL), forward primer (1 µL, 20 µM), reverse primer (1 µL, 20 µM), and sterilised MilliQ water (18 µL).

For the bacterial DNA samples, universal primer 8 F and 1541 R was used, while for the fungal DNA samples, universal primer ITS 4 and ITS 5 was chosen. The concentrated primers were mixed with sterile distilled water according to manufacturer's instructions. The primer sequences of the primers used are listed in Table 2.2.

Table 2.2. Primer sequences.

For bacterial DNA samples	
8 F	5'-AGA GTT TGA TCC TGG CTC AG-3'
1541 R	5'-AAG GAG GTG ATC CAG CCG CA-3'76
For fungal DNA samples	
ITS 4	5'-TCC TCC GCT TAT TGA TAT GC-3'
ITS 5	5'-GGA AGT AAA AGT CGT AAC AAG G-3

The prepared master mix was aliquot into labelled sterilised PCR tubes using a micropipette. Each PCR tube contained 45 µL master mix and 5 µL of crude DNA extract was added into its own respective tube. DNA amplification was performed in a PCR machine (Eppendorf, Germany) which was pre-programmed with the following cycling conditions: initial denaturation at 95ºC for 1 minute, followed by 29 cycles of denaturation process at 95ºC for 15 seconds, annealing process at 55ºC for 15 seconds

and extension process at 72ºC for 15 seconds, followed by a final elongation process at 72ºC for 5 minutes. The final PCR products were hold on at 4ºC.

The final PCR product was analysed by gel electrophoresis. Each PCR product was pipetted into wells made from 1% (w/v) agarose gel (Vivantis Technologies, USA) with 3µL of Redsafe (iNtRON Biotechnology, South Korea) for staining of the nucleic acid, allowing the visualization under UV light. The gel electrophoresis was run at 120V for 30 minutes for the bacterial PCR product while 100V for 50 minutes for the fungal PCR product. The voltage and time to run for the bacterial and fungal PCR product were different due to the difference in the base pair, bacteria have about 1500 bp while fungus has 600-700bp. The Bio- Rad Gel Documentation EQ System for DNA/RNA Gel Photos was used to observe and capture the gel image of the PCR product.

2.2.5.4 DNA Analysis

PCR product that showed clear bands in the gel electrophoresis was sent for DNA sequencing. The sequencing of the DNA of the PCR product was performed by First Base Laboratories Sdn Bhd, Selangor, Malaysia. The identification of the microorganisms was then identified by matching the unknown gene sequence obtained to known gene sequences in the GenBank database using Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information (NCBI). Molecular Evolutionary Genetics Analysis Version 7.0 (Mega 7) was used to create a phylogenetic tree by using the maximum likelihood method based on Tamura-Nei model (Tamura & Neil, 1993).

2.3 Results and Discussion

2.3.1 Isolation of Celluloytic Microorganisms

Forest soil was targeted as it contains diverse microorganisms that are responsible for producing enzymes that decompose the cellulose and hemicellulose present in the soil. As reported by López-Mondéjar (2016), approximately 12% of the bacterial colonies isolated from forest soil samples showed to have cellulase producing ability.

The microorganism of interest was distinguished by its ability to form clear zones around the colony that were cultured on the selective media. For the initial screening of cellulolytic microorganisms, diluted soil samples were first cultured onto carboxymethylcellulose (CMC) agar to detect cellulase producing microorganisms. Cultures that exhibited clear zone (Figure 2.1) was isolated and subsequent sub-cultured onto fresh growth medium until pure culture was acquired. These pure isolates were then re-cultured onto CMC agar in triplicates to confirm its cellulase production. Isolates that showed visible clear zones during the CMC plate assay were further cultured onto xylan agar to test if it could degrade the dyed xylan (Figure 2.2), suggesting the presence of xylanase.

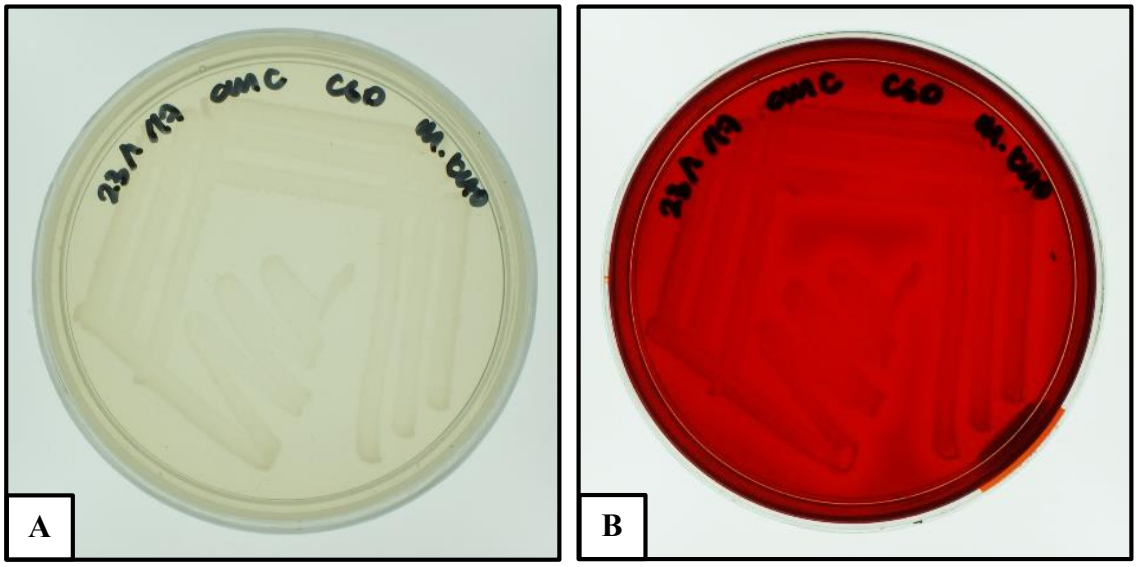


Figure 2.1 Clear zones indicating the presence of cellulase in CMC agar (A: before staining) and (B: after staining).

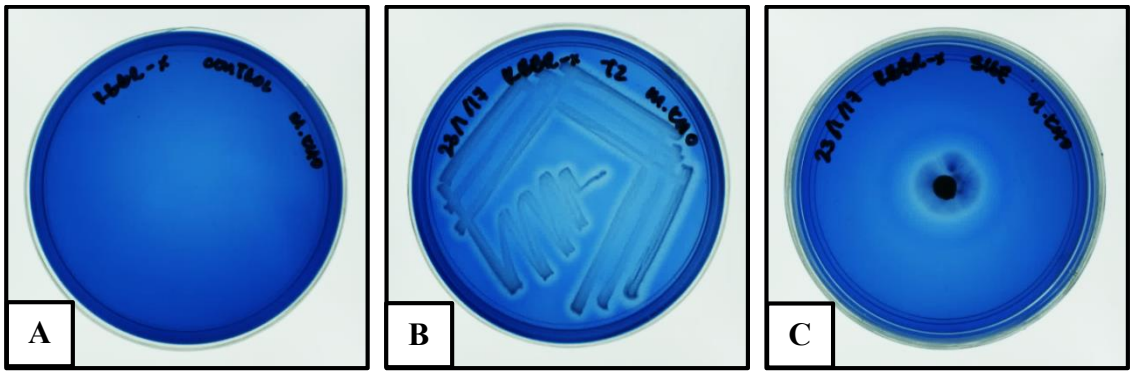


Figure 2.2 Pure isolates showing xylan-degrading activities on dyed xylan agar: (A) control; (B) clear zone formed by bacterial isolates; and (C) fungal isolates when xylanase could be produced.

Isolates which exhibited clear zone in the plate assays expressed a strong evidence of positive results. Isolates which showed clear zone formation on the CMC agar and xylan agar were inoculated onto new CMC plates and kept in glycerol stock which was placed in the -80° freezer for long term storage. A total of 24 cellulolytic isolates were isolated from the soil samples collected from the targeted region which showed positive results in degrading cellulose while 10 out of the 24 isolates demonstrated to degrade xylan. A summary of the ability of the isolates to degrade cellulose and hemicellulose can be seen in Table 2.4.

2.3.2 Screening for Plant Growth-Promoting Microorganisms

N-fixing microorganisms: Jensen's medium was used to screen the isolated cellulolytic microorganisms for the presence of nitrogen-fixing microorganisms. Bromothymol blue in its original pH state turns from green (Figure 2.3A) to blue (Figure 2.3B) when in an alkaline condition which indicates the presence of nitrogen-fixing activity. Bromothymol blue changes from green to yellow when exposed to an acidic environment (Figure 2.3C). The colour change from green to blue indicates the presence of nitrogen-fixing activity by the isolated microorganisms. The results showed that 5 out of 24 isolates showed positive results in performing nitrogen fixing (Table 2.3).

Table 2.3 Summary of isolated cellulolytic microorganisms with cellulolytic degrading properties and growth promoting properties.

	Code	Degradation properties		Growth promoting properties			
		Cellulose	Hemi-cellulose	N-fixing	PO_4^{3-}-solubilizing	K-solubilizing	IAA
Bacterial isolates	S1	+	-	-	-	-	+
	S2	+	+	-	+	-	+
	S3	+	+	-	-	-	+
	S4	+	-	-	-	-	+
	S5	+	-	-	+	-	+
	S6	+	-	+	-	-	+
	S7	+	-	-	-	-	+
	S8	+	+	+	-	-	+
	S9	+	+	-	-	-	+
	S10	+	-	+	+	+	+
	S11	+	-	-	-	-	+
	S12	+	-	-	-	-	+
	S13	+	-	-	-	-	-
	S14	+	+	-	-	-	-
Fungal isolates	F1	+	-		-	-	+
	F2	+	-	+	-	-	+
	F3	+	-	-	-	-	+
	F4	+	+	-	-	-	-
	F5	+	+	-	-	-	-
	F6	+	+	+	-	-	-
	F7	+	-	-	-	-	-
	F8	+	+	-	-	-	+
	F9	+	-	-	-	-	+
	F10	+	+	-	-	-	+

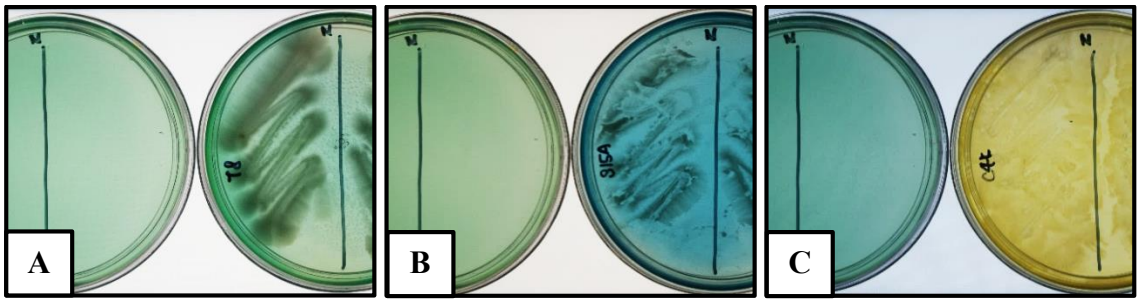


Figure 2.3 Cellulolytic microorganisms grown on Jensen's medium with bromothymol blue dye – (A) neutral state – green; (B) blue – alkaline, and (C) yellow – acidic.

Cellulolytic isolates which exhibited the presence of activity of interest were noted down. From the isolated 24 isolates, five isolates (S6, S8, S10, F2 and F6) showed to have the N-fixing abilities, three isolates (S2, S5 and S10) with phosphate-solubilising property, one isolate (S10) with potassium-solubilising property, and 16 isolates (S1-S12, F1-F3, and F8) with the abilities to produce IAA (Table 2.3).

Phosphate-solubilising microorganisms: Pikovskaya agar was used for the detection of phosphate-solubilising microorganisms. Phosphate is available in both organic and inorganic forms in soil (Gupta *et al*. 2012). However, plants are only able to utilize phosphate that is in the readily available inorganic phosphate form. Therefore, phosphate-solubilising microorganisms play a vital part in converting the phosphate present in the soil into readily available form for the uptake by the plants. As it can be seen from Figure 2.4 (A), phosphate-solubilising activity was detected from the isolated cellulolytic microorganisms when a clear zone was formed around the vicinity of the isolate due to the solubilisation of phosphate. Only 3 out of 14 bacterial isolates showed phosphate-solubilising properties (Table 2.3). None was observed in the fungal isolates.

Potassium-solubilising microorganisms: Aleksandrow agar was chosen as a selective medium to detect potassium-solubilising activity. Potassium-solubilising microorganisms convert insoluble potassium available in the soil into a form that plants can access. As reported by Prajapati and Modi, the use of potassium-solubilising microorganisms were found to resolve potassium from insoluble minerals, therefore, it has been used as an agricultural improvement (2012). From Figure 2.4 (B), clear zones were formed around

isolates that solubilise potassium within its vicinity. From this experiment, only one isolate could solubilise potassium (Table 2.3).

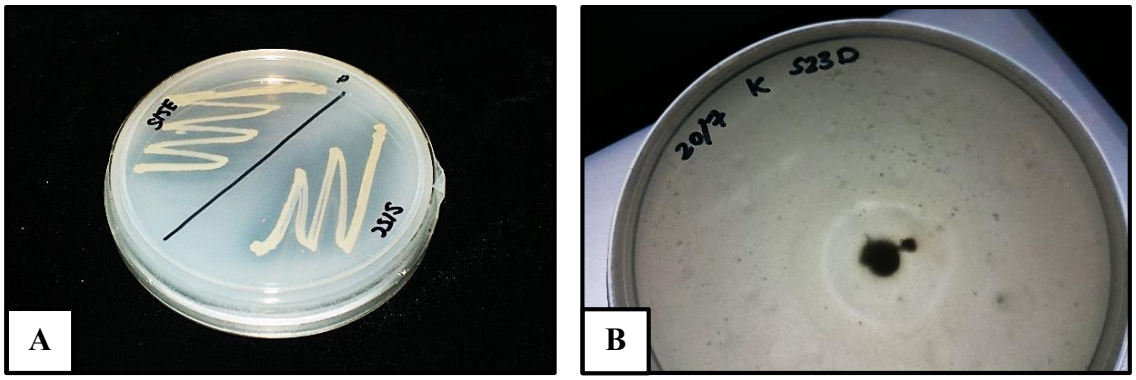


Figure 2.4 Cellulolytic Microorganisms grown on Pikovskaya agar (A) and Aleksandrow agar (B) showed clear zone indicates solubilising property.

IAA-producing microorganisms: According to Mohite (2013), indole acetic acid (IAA) producing microorganisms play an important role in stimulating and facilitating plant growth. Growth promoting microorganisms are able to produce IAA where it is a product of L-tryptophan metabolism. Swalkowski method was used to test the ability of the isolated cellulolytic microorganisms to produce IAA. Such microbe can be detected when the supernatant and reagent turns pink.

From this experiment, 16 isolates out of the 24 isolated cellulolytic microorganisms showed positive results in producing IAA as shown in Table 2.4. Based on the absorbance reading at 525 nm, F8 have the highest production of IAA (Abs = 0.270), followed by S3 (Abs = 0.170) and S2 (Abs = 0.168).

Table 2.4 Indole acetic acid (IAA) produced by cellulolytic microorganisms.

Bacterial isolates			Fungal isolates		
Isolate codes	**Production of IAA**	**Absorbance reading (Abs)**	**Isolate codes**	**Production of IAA**	**Absorbance reading (Abs)**
S1	+	0.141±0.002	F1	+	00.143±0.002
S2	+	0.168±0.001	F2	+	0.052±0.003
S3	+	0.170±0.002	F3	+	0.117±0.003
S4	+	0.156±0.003	F4	-	-
S5	+	0.157±0.002	F5	-	-
S6	+	0.109±0.005	F6	-	-
S7	+	0.110±0.001	F7	-	-
S8	+	0.149±0.001	F8	+	0.270±0.002
S9	+	0.109±0.003	F9	-	
S10	+	0.135±0.009	F10	-	
S11	+	0.059±0.005			
S12	+	0.111±0.002			
S13	-	-			
S14	-	-			

Absorbance value expressed as mean ± standard deviation (n=3).

2.3.3 Molecular Identification of the Isolated Bacteria and Fungus

From the initial screening, 14 bacterial isolates and 10 fungal isolates were identified using molecular identification via DNA sequencing. In the molecular approach, the crude DNA of the 24 isolates was successfully extracted using the freeze and thaw method. Universal primers, forward primer 8 F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 1541 R (5'-AAG GAG GTG ATC CAG CCG CA-3') was chosen for the bacterial isolates identification while forward primer, ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and reverse primer ITS 5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') were used for the molecular identification of the fungal isolates.

Based on the gel electrophoresis results, PCR product size obtained from the bacterial isolates were about 1300-1500 base pairs (Figure 2.5) while the 550-650 base pairs were obtained for the fungal isolates (Figure 2.6).

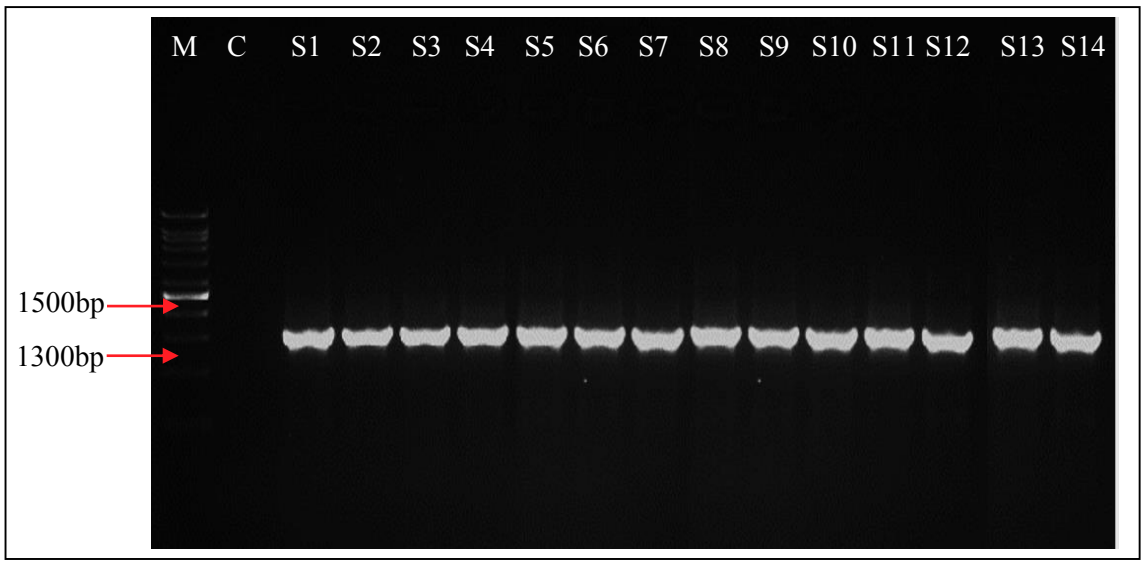


Figure 2.5 DNA bands of the PCR product extracted from the cellulolytic bacterial isolates. Label M represents 1kB DNA Marker; C represents negative control; and S1-S14 represents the DNA bands produced from the bacterial isolates.

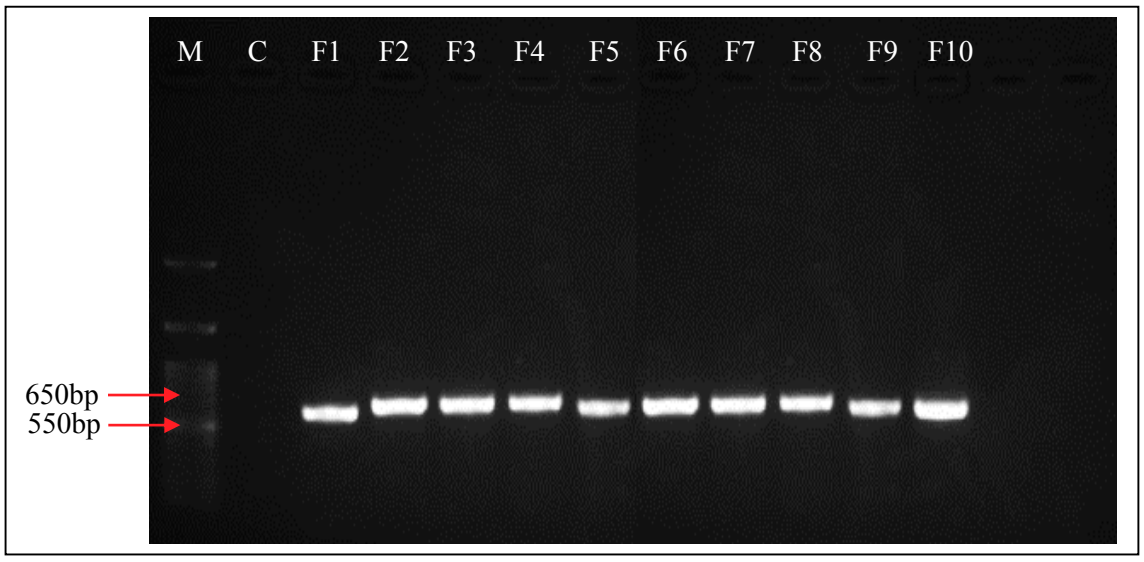


Figure 2.6 DNA bands of the PCR product extracted from the cellulolytic fungal isolates. Label M represents 1kB DNA Marker; C represents negative control; and F1-F10 represents the DNA bands produced from the fungal isolates.

BLAST was used to compare the PCR product sequence obtained in this study to sequences available in the GenBank database, it then calculates the statistical significance of the sequence of the unknown entered to the closest known match sequence (Kent 2002). The sequence matches of the bacterial isolates and the fungal isolates are summarised in Table 2.5 and Table 2.6, respectively.

Table 2.5 Molecular identification of the cellulolytic bacterial isolates.

Code	Strain Name	Closest match	Accession no. of closest match	Base Pair	Query cover (%)	Similarity (%)
S1	*Bacillus* sp. S1	*Bacillus subtilis*	KY285264.1	1455	94	96
S2	*Bacillus* sp. S2	*Bacillus licheniformis*	KX426642.1	1414	90	93
S3	*Bacillus* sp. S3	*Bacillus amyloliquefaciens*	KX379740.1	1481	92	96
S4	*Staphylococcus* S4	*Staphylococcus saprophyticus*	KT441087.1	1488	89	98
S5	*Bacillus* sp. S5	*Bacillus cereus*	KF500919.1	1427	94	96
S6	*Bacillus* sp. S6	*Bacillus toyonensis*	KY750686.1	1387	93	98
S7	*Bacillus* sp. S7	*Bacillus subtilis*	KY123860.1	1496	90	98
S8	*Bacillus* sp. S8	*Bacillus altitudinis*	KC172054.1	1500	88	97
S9	*Bacillus* sp. S9	*Bacillus aerius*	JX981918.1	1329	97	97
S10	*Pseudomonas aeruginosa*	*Pseudomonas Aeruginosa*	KC790300.1	1381	98	99
S11	*Bacillus* sp. S11	*Bacillus licheniformis*	FJ493045.1	1345	99	95
S12	*Bacillus methylotrophicus*	*Bacillus methylotrophicus*	KU146560.1	1431	83	99
S13	*Bacillus* sp. S13	*Bacillus atrophaeus*	KY287785.1	1459	87	96
S14	*Bacillus* sp. S14	*Bacillus subtilis*	KX426659.1	1357	95	95

Identification was based on sequencing data obtained using NCBI nucleotide BLAST database.

The closest match of the sequences was based on the query cover percentage (%) in base pairs and identity percentage (%) which were no less than 80% for both criteria. The accession number, base pair, query cover and, similarity of the closest match species to the sequences were recorded in Table 2.5 and Table 2.6.

Table 2.6 Molecular identification of the cellulolytic fungal isolates.

Code	Closest match	Accession no. of closest match	Base Pair	Query cover (%)	Similarity (%)
F1	*Fusarium fujikuroi*	KT192406.1	557	97	99
F2	*Fusarium proliferatum*	GU066655.1	555	98	99
F3	*Fusarium oxysporum*	KF751873.1	556	97	99
F4	*Penicillium coprophilum*	AF033469.1	579	97	99
F5	*Trichoderma harzianum*	KY750325.1	618	98	99
F6	*Penicillium citrinum*	KM979730.1	548	99	99
F7	*Penicillium oxalicum*	GU078430.1	587	96	99
F8	*Aspergillus nomius*	DQ467991.1	593	98	99
F9	*Endomelanconiopsis endophytica*	GQ469968.1	579	96	99
F10	*Penicillium rolfsii*	KM246748.1	581	98	99

Identification was based on sequencing data obtained using NCBI nucleotide BLAST database.

Only isolates which showed sequences that had a similarity of ≥99% were named using the closest match species name identified using BLAST. In the past, the sequenced isolates which showed 97.0% to 99.5% of similarity when BLAST was named with the closest match (Stackebrandt & Goebel 1994). However in recent years, more papers have recommended and supported the idea where a similarity of ≥99% was required to name the strain to the closest match species name (Keswani & Whitman 2001; Clarridge & Alerts 2004). Thus, strain name for each isolate which had a similarity of <99% were named after the genus followed by its code name for convenience and were referred as such throughout the thesis. Out of the 14 bacterial isolates that were searched against the GenBank database, 12 isolates were found to be from the *Bacillus* species. The identified strains were listed in Table 2.7. The isolates identified consist of the following genus: *Bacillus, Staphylococcus, Pseudomonas, Fusarium, Endomelanconiopsis, Penicillium, Aspergillus, and Trichoderma*.

Table 2.7 The closest match of strains identity obtained for the isolated isolates after comparison with GenBank database.

No	Label	Domain	Strains identity obtained	Number of isolates	Strain species
1	S1/S7/ S14	Bacteria	*Bacillus subtilis*	3	*Bacillus* spp.
2	S2/S11	Bacteria	*Bacillus licheniformis*	2	*Bacillus* spp.
3	S3	Bacteria	*Bacillus amyloliquefaciens*	1	*Bacillus* spp.
4	S4	Bacteria	*Staphylococcus saprophyticus*	1	*Staphylococcus* spp.
5	S5	Bacteria	*Bacillus cereus*	1	*Bacillus* spp.
6	S6	Bacteria	*Bacillus toyonensis*	1	*Bacillus* spp.
7	S8	Bacteria	*Bacillus altitudinis*	1	*Bacillus* spp.
8	S9	Bacteria	*Bacillus aerius*	1	*Bacillus* spp.
9	S10	Bacteria	*Pseudomonas aeruginosa*	1	*Pseudomonas* spp.
10	S12	Bacteria	*Bacillus methylotrophicus*	1	*Bacillus* spp.
11	S13	Bacteria	*Bacillus atrophaeus*	1	*Bacillus* spp.
12	F1	Fungus	*Fusarium fujikuroi*	1	*Fusarium* spp.
13	F2	Fungus	*Fusarium proliferatum*	1	*Fusarium* spp.
14	F3	Fungus	*Fusarium oxysporum*	1	*Fusarium* spp.
15	F4	Fungus	*Penicillium coprophilum*	1	*Penicillium* spp.
16	F5	Fungus	*Trichoderma harzianum*	1	*Trichoderma* spp.
17	F6	Fungus	*Penicillium citrinum*	1	*Penicillium* spp.
18	F7	Fungus	*Penicillium oxalicum*	1	*Penicillium* spp.
19	F8	Fungus	*Aspergillus nomius*	1	*Aspergillus* spp.
20	F9	Fungus	*Endomelanconiopsis endophytica*	1	*Endomelanconiopsis* spp.
21	F10	Fungus	*Penicillium rolfsii*	1	*Penicillium* spp.

The molecular phylogenetic trees were constructed based on the sequences obtained from cellulolytic and hemicellulolytic bacterial and fungal isolates of the current study and known closely matched species' sequences available in the GenBank. The phylogenetic tree for cellulolytic and hemicellulolytic bacteria and fungi in Figure 2.7 and Figure 2.8 shows the position of the isolates. The phylogenetic trees were generated using Molecular Evolutionary Genetic Analysis version 7.0 (MEGA7) software with the adaption of the maximum likelihood method based on Tamura-Nei model (Tamura & Neil 1993). A phylogenetic tree was constructed to organize the biological data obtained from the isolates to structure classifications and to have an insight of the evolution of the isolates have gone through (Kumar *et al.* 2016). A phylogenetic tree is a diagram that paints the lines of the evolutionary origin of the different species, genes or organisms which derive from a common ancestor (Baum 2008).

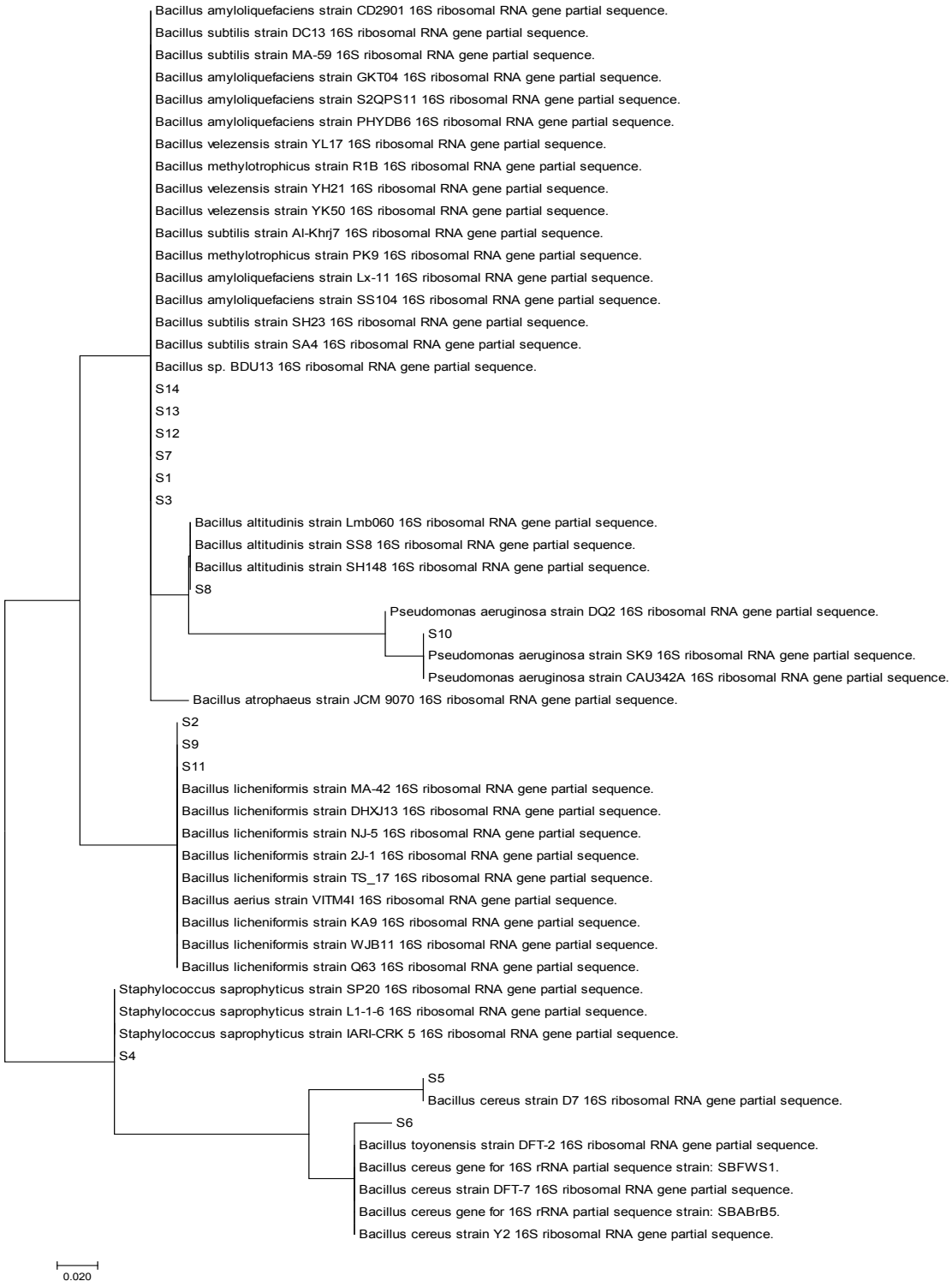


Figure 2.7 The molecular phylogenetic tree showing the position of the cellulolytic bacterial isolates.

*Data based on their partial sequence which was constructed using the Molecular Evolutionary Genetic Analysis version 7.0 (MEGA7) software with the adaption of the maximum likelihood method based on Tamura-Nei model.

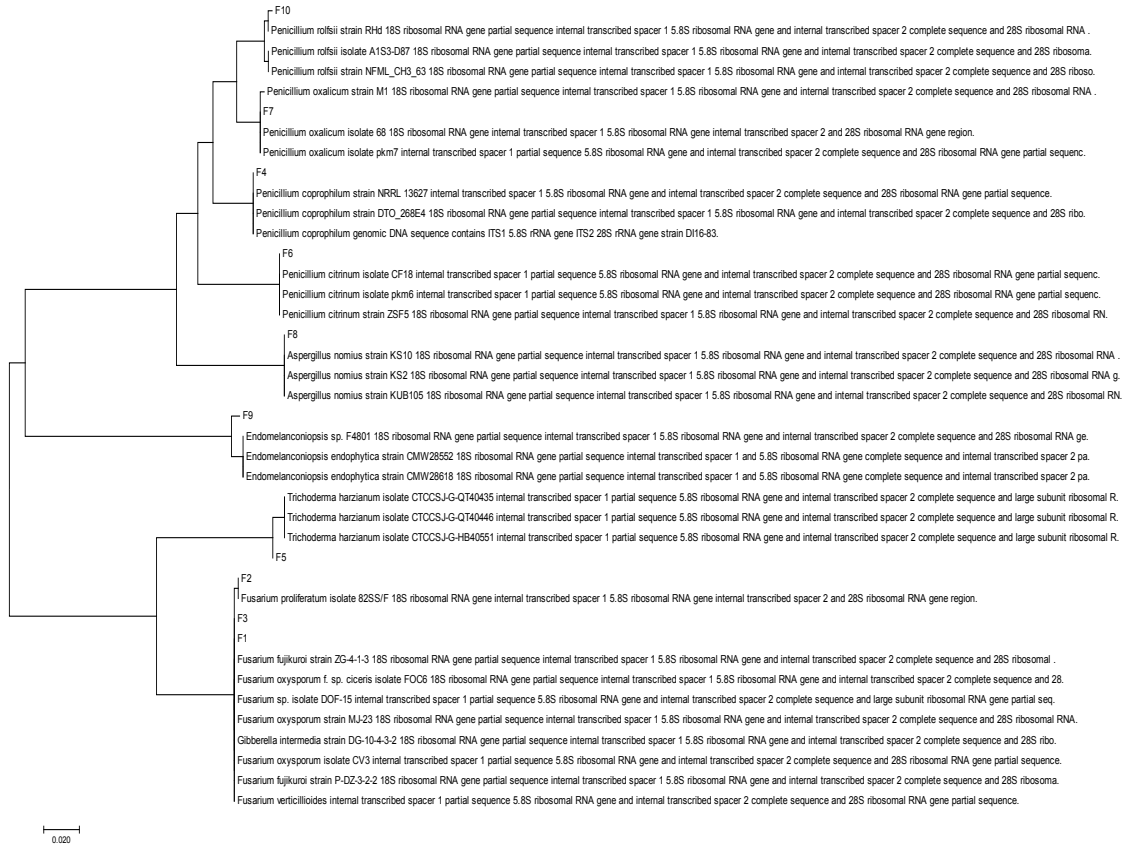


Figure 2.8 The molecular phylogenetic tree showing the position of the cellulolytic fungal isolates.

*Data based on their partial sequence which was constructed using the Molecular Evolutionary Genetic Analysis version 7.0 (MEGA7) software with the adaption of the maximum likelihood method based on Tamura-Nei model.

2.4 Summary

This chapter focused on the screening and characterization of cellulolytic microorganisms. The cellulolytic microorganisms were successfully isolated from the soil samples collected from the local targeted area using selective media such as Carboxymethylcellulose (CMC) agar and Remazol Brilliant Blue-R (RBBR) xylan agar. The desired microorganisms were distinguished by its ability to exhibit visible clear zones around the isolates that were cultured on the selective media. Out of the 24 isolates studied, all 24 isolate was able to produce cellulose, while only 10 isolates were produced xylanase.

The cellulolytic microorganisms isolated out from the soil samples were further analysed for their ability to be plant growth-promoting microbes – based on their abilities to make available three of the plant macronutrients namely the nitrogen (N), phosphate (P) and potassium (K), as well as their abilities to produce indole acetic acid (IAA), a plant growth enhancing hormone. Jensen's medium was used to detect microorganisms that were able to perform nitrogen fixation. Pikovskaya agar was chosen for the detection of phosphate-solubilising activity by the cellulolytic microorganisms while Aleksandrow agar was used as a detector for potassium-solubilising microorganisms. The isolated microorganisms were also tested for IAA production using the Salkowski method. From the 24 isolates, five isolates showed to have the abilities to perform nitrogen fixing, three isolates were able to solubilise phosphate, one isolates exhibited the ability to solubilise potassium, and 16 isolates produced IAA.

The molecular identification of the 24 isolates was identified by using the freeze and thaw method for the DNA extraction and polymerase chain reaction (PCR) for the amplification of the DNA. Based on the comparison with the GenBank database, the identified isolates were classified to be members of the genus of *Bacillus, Staphylococcus, Pseudomonas, Fusarium, Endomelanconiopsis, Penicillium, Aspergillus and, Trichoderma.* Further evaluations for the 24 different strains were carried out and the results would be described in the following chapters. The efficiency of the 24 different strains as potential cellulolytic and hemicellulolytic microbes to degrade oil palm empty fruit bunches (EFB) was evaluated in Chapter 3.

Chapter

3

SELECTION OF CELLULOLYTIC MICROORGANISMS BASED ON ENZYME ACTIVITY AND OPTIMISATION OF SELECTED MICROORGANISMS

3.1 Introduction

Environmentally friendly ways to overcome the increasing oil palm empty fruit bunch (OPEFB) waste produced by the growing oil palm industry in Malaysia must be seek. Composting of OPEFB is recommended to manage wastes that are large in volume (Zainudin *et al.* 2013). Composting reduces the bulk volume of OPEFB waste into useful compost which is economically and environmentally friendly (Mukhlis *et al.* 2013). Nevertheless, as OPEFB is lignocellulosic material, a longer time would be required to degrade completely (Baharuddin *et al.* 2009). OPEFB contains roughly 53% cellulose, 30% of hemicellulose and 17% lignin (Baharuddin *et al*. 2009). Hence, the addition of cellulolytic microorganisms would help to accelerate the composting of OPEFB (Zainudin *et al.* 2013).

Microbes that are cellulolytic have the potential to degrade OPEFB as they produce certain enzymes such as cellulase and hemicellulase. The cellulase and hemicellulase produced by theses microbes have the ability to help in the hydrolysing process of the cellulose and hemicellulose in OPEFB into simple sugars (Ang *et al*. 2015). In this chapter, isolated cellulolytic microorganisms that were obtained from collected soil samples that were discussed in Chapter 2 were tested for its ability degrade cellulose and hemicellulose using the 2-cyanoacetamide method. The 2-cyanoacetamide method can be used to detect glucose level produced by the cellulolytic microorganisms when it degrades cellulose and hemicellulose into simpler sugars (Jurick *et al.* 2012). Carboxymethylcellulose (CMC) was used as a substrate in this study for detecting cellulase activity while xylan was used as a substrate to detect hemicellulase activity in the cellulolytic isolates. Isolates which showed the highest cellulase and hemicellulase activities were chosen for further testing as a potential candidate to form an indigenous microbial consortium of degrading microorganisms to degrade OPEFB waste for further experiments.

Antagonism among the selected cellulolytic strains that were chosen based on their degrading abilities was evaluated and discussed in this chapter. The selected strains were grown together to detect any mutual inhibitions in a test for potential antagonism. This test was conducted to prevent any growth inhibition that to be inflicted among the selected cellulolytic microorganisms when inoculated together as a consortium (Ghai *et al.* 2007).

A study by Hamzah *et al.* (2012) indicated that physical parameters such as initial medium pH and incubation temperature would play important roles to promote microbial biomass production. Each of the selected cellulolytic strains were optimised for its ideal growth condition in different parameters. The selected strains were placed into differently tuned growth mediums and temperature environments to find its ideal growth condition based on its growth rate which would be the number of cells that were produced per volume of growth medium.

The aim of this chapter was to analyse the efficiency of the selected microorganisms for its cellulase and hemicellulase activities. Strains which showed to have the highest activity were further tested for antagonism activity and optimised to find its ideal incubation condition. Final selected strains were used to form an indigenous microbial consortium for further investigations.

3.2 Materials and Methods

3.2.1 Measurement of Enzyme Activity of Isolated Cellulolytic Microorganisms

3.2.1.1 Detection of Glucose Level Produced by Cellulolytic Microorganisms

The cellulase and hemicellulase activities of the isolated cellulolytic microorganism were detected using 2-cyanoacetamide method adapted from Jurick *et al.* (2012). This method is based on measurement of glucose level liberated from the Carboxymethylcellulose (CMC) and xylan substrates by cellulases and hemicellulases respectively.

(I) Preparation of Standard

Glucose standards were first prepared by dissolving glucose (Sigma-Aldrich, USA) into 50 mL sodium acetate (Sigma-Aldrich, USA) buffer pH5.0 with the concentration of 0, 5, 25, 50, 100, 200, 400, and 800 mg/mL^{-1}. Each concentration was measured in triplicates into clean sterile universal bottles with a corresponding blank. The glucose standard with 0 mg/mL^{-1} concentration was used as a blank for the assay. The blank value for the standard was subtracted from the readings to adjust the background. The water bath was first set to 80°C. From the prepared glucose solution, 200 µL of it was added to 400 µL of 1% 2-cyanoacetamide (Acros Organics, USA) solution and 2mL of 100mM borate (Sigma-Aldrich, USA)

with pH9.0 in sterile universal bottles. The solution was boiled for 10 minutes in the water bath (Memmert, USA) after being mixed thoroughly using a vortex (Barnstead Thermolyne, USA). The boiled solution was allowed to cool to room temperature and read at 276 nm using a multiplate reader (BioTek Synergy HT, USA) to determine its absorbance.

(II) Analysis On the Glucose Level Produced by Cellulolytic Microorganisms

For the determination of sugars produced by the microorganisms in the different substrate, the selected microorganisms were first cultured into carboxymethylcellulose (CMC)/xylan broth media. The basic broth medium used can be referred to Table 2.1 where 8 g/L of CMC/xylan substrate was measured depending on the requirement. Overnight bacterial broth culture were inoculated into the CMC/xylan broth and incubated for 3 days before performing the cellulase/hemicellulase bioassay. As for fungus, 5 days old fungal broth culture was inoculated into the CMC/xylan broth and incubated for 5 days before performing the cellulase/hemicellulase bioassay. The cultures were incubated in the condition of 37°C with shaking at 120 rpm in an incubator shaker (Sartorious Stedim Biotech, Germany). After incubation, 200 µL of the culture was added to 400µL of 1% 2-cyanoacetamide solution and 2 mL of 100 mM borate with pH9.0 in sterile universal bottles. The solution was boiled for 10 minutes in the water bath after being mixed thoroughly using a vortex. The boiled solution was allowed to cool to room temperature and read at 276 nm using a multiplate reader (Sartorious Stedim Biotech, Germany) to determine its absorbance. CMC broth was used as a blank for cellulase bioassay while xylan broth was used for the hemicellulase bioassay. The absorbance readings obtained from the cellulase/hemicellulase bioassay were used to determine the glucose produced by the cellulolytic microorganisms. The absorbance readings from the bioassay and the standard curve of glucose were used to calculate the glucose produced by the isolated cellulolytic microorganisms. The cellulase/hemicellulase analyses were performed in triplicates for each sample.

3.2.2 Screening for Antagonistic Activity of Selected Strains

Antagonistic activity of the selected based on the bioassay for cellulase and hemicellulase activity were tested for antagonism against each other. Antagonistic activity was experimented based on the protocol explained by Ghai *et al.* (2007). The selected strains used for further investigation are as follow:

(i) *Bacillus* sp. S2 (Bacterium)
(ii) *Bacillus* sp. S8 (Bacterium)
(iii) *Penicillium citrinum* F6 (Fungus)
(iv) *Penicillium rolfsii* F10 (Fungus)
(v) *Penicillium coprophilum* F4 (Fungus)

Antagonism properties of selected strains were tested by using the dual culture technique. Fresh nutrient agar (NA) plates were chosen to test for the antagonism activity. The NA plates were divided into two sections where each selected strain was grown with each other. Selected bacterial strains were streak onto one side of the agar plates using a sterile loop while 5 mm diameter of 5 days old mycelia was placed on the other side of the agar plate. The plates were incubated at 37°C for 3 days and observed for antagonistic activity by the selected strain on each other. The antagonistic activity analysis was performed in triplicates for each plate.

3.2.3 Optimisation of Bacterial and Fungal Strains

Selected cellulolytic strains were cultured in different conditions to test for its optimal growth environment. Fresh broth cultures for the selected cellulolytic strains were prepared before carrying out the experiment. Bacterial strains were cultured by inoculating them into individual 10 mL nutrient broth media while fungal strains were inoculated into individual 10 mL potato dextrose broth media. The cultured nutrient broth was incubated overnight while the fungal strains were grown for 5 days in an incubator shaker with shaking at 120 rpm with the temperature set at 37°C ± 1°C.

3.2.3.1 pH

The influences of different pH ranging from pH5.0 to pH8.0 with an interval of 0.5 were tested on the selected cellulolytic strains. The initial pH of the growth medium used was attuned with the use of 1 N NaOH and 1 N HCl. The bacterial strains were grown overnight in tuned nutrient broth incubated at 37°C ± 1°C with an agitation of 120 rpm while the fungal strains were grown for 5 days in tuned potato dextrose broth incubated

at the same temperature and agitation as the bacterial strain. The optical density of the bacterial cultures was read at 600 nm in triplicates using spectrophotometer (Thermo Scientific, USA). Sterilised clean nutrient broth was used as a blank for the setting of the spectrophotometer. For the fungal cultures, the experimental broth medium was dried in the oven to obtain its mycelial dry weight g/L. The technique for obtaining the mycelial dry weight for detecting the growth of fungal culture was modified from the method mentioned by Zhu *et al.* (2008). Fresh PDB growth medium was used as a reference blank for the fungal cultures.

3.2.3.2 Temperature

The optimal temperature of the selected cellulolytic strains was tested by incubating the selected cellulolytic strains in different temperature ranging from 35 to 45°C ±2 with an interval of 5°C and detecting the influence of it. The bacterial strains were grown overnight in nutrient broth incubated at 37°C ± 1°C with an agitation of 120 rpm while the fungal strains were grown for 5 days in potato dextrose broth incubated at the same temperature and agitation as the bacterial strain. The optical density of the bacterial cultures was read at 600 nm in triplicates using spectrophotometer (Thermo Scientific, USA). Sterilised clean nutrient broth was used as a blank for the setting of the spectrophotometer. For the fungal cultures, the experimental broth medium was dried in the oven to obtain its mycelial dry weight g/L. The technique for obtaining the mycelial dry weight for detecting the growth of fungal culture was modified from the method mentioned by Zhu *et al.* (2008). Clean PDB growth medium was used as a reference blank for the fungal cultures.

3.2.4 Microbial Growth Rate of Selected Microorganisms

3.2.4.1 Bacterial Strain

Fresh broth cultures for the selected bacterial cellulolytic strains were prepared before carrying out the experiment. Bacterial strains were cultured by inoculating them into individual 10mL nutrient broth media. The cultured nutrient broth was incubated overnight in an incubator shaker and shaken at 120 rpm with the temperature set at 37°C ± 1°C. The optical density of the cultures for this experiment was read at 600 nm using spectrophotometer (Thermo Scientific, USA). Sterilised clean nutrient broth was used as a blank for the setting of the spectrophotometer.

(I) Indirect Growth Measurements

To begin the indirect growth measurement of the selected bacterial strains, 10 mL of overnight culture prepared was poured into its own individual shake flask containing 90 mL of sterilised nutrient broth. It was mixed well and its initial optical density, time zero, was taken immediately and noted down. The optical density reading of each bacterial strain was taken every 1 hr interval in triplicates for the first 8 hr right after the inoculation. The final optical density reading was taken at time 24 hr. For each reading, 1000 µL of the culture broth was pipette into its own cuvette and discarded immediately after taking its reading. For each time point, the optical density reading of the cultures was taken in triplicates.

(II) Direct Growth Measurements

The colony forming unit of the selected bacterial strains was obtained through the direct growth measurement method and total plate count. The direct growth measurement was carried out along with the indirect growth measurements. From time zero to time 8 hr, 1 mL of culture broth from each bacterial strain cultured in shake flask was taken every 1hr interval and a ten-fold dilution was carried out using sterilised deionised water. To perform the ten-fold dilution, 9 mL of sterilised deionised water was allocated into seven, 15-mL centrifuge tube for each sample taken from the shake flask culture, and labelled 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} respectively. The 1 mL culture broth taken from the shake flask culture was pipetted into tube 10^{-1} and mixed well by pipetting up and down with a minimum of 5 times. From tube 10^{-1}, 1 mL of the mixture was transferred to the tube labelled 10^{-2}, and mixed well with the same method as mentioned before. These steps were repeated until tube 10^{-7}. For the dilution 10^{-5}, 10^{-6}, and 10^{-7}, 100 µL was transferred respectively onto each nutrient agar plate in triplicates, and spread evenly using a sterile cotton swab. The plates were incubated at 35°C for one day in the incubation room. In the second day, the distinct colonies on each plate were counted. The colony forming unit per millilitre (CFU/mL) was calculated according to the equation 3.1 below:

Equation 3.1 CFU/mL = a number of colonies per mL plated/ Dilution.

3.2.4.2 Fungal Strain

(I) Indirect Growth Measurements

For the indirect growth measurement of the selected fungal strains, the dry weight of the mycelium produced from the incubating the fungal strains in broth was observed. The indirect growth measurement of the fungal strains was modified from the protocol explained by Zhu *et al.* (2008). Potato dextrose broth (PDB) was first prepared by sterilizing 100 mL of in 250 mL baffled flasks. The sterilised PDB were inoculated with the selected 5-day old fungal agar plate culture. For the transfer of fungal cultures into PDB, the straw technique was used to transfer one 5-mm diameter fungal culture into the broth. The inoculated baffled flask was incubated at 35°C and shaken at 120 rpm for 5 days. After 5 days of incubation, the mycelial biomass from the broth culture was harvested by vacuum filtration with pre-fried and weighed Whatman No.1 filter paper. The mycelium on the filter paper was dried at 80°C for 24 hr and weight using a weighing balance. The growth of each fungal strain was estimated by the dry weight of the mycelium. Indirect measurement of the selected fungal strains was carried out in triplicates.

(II) Direct Growth Measurements

Direct growth measurement of each selected fungal cellulolytic strain was carried out by measuring its growth diameter on potato dextrose agar over the span of 7 days. Fresh fungal culture plates were first prepared by inoculating pure cultures onto individual potato dextrose agar plates using the sterile straw technique and grown for 5 days at 35°C in the incubator. For the direct growth measurement of fungal strains, the sterile straw technique was adapted to transfer 5mm diameter of fungus from the fresh culture prepared onto sterile potato dextrose agar plates and incubated at 35°C. The growth diameter of the fungus during incubation day 5 was observed. Direct measurement of the selected fungal cellulolytic strains was carried out in triplicates.

3.2.5 Data Analysis

The bioassay for detecting the glucose level produced by cellulolytic microbes, optimisation and microbial growth rate of selected microbial strains were performed in triplicates. The results of these studies were presented as mean values for the triplicates.

3.3 Results and Discussion

3.3.1 Measurement of Enzyme Activity by Selected Cellulolytic Microorganisms

The strains isolated from soil samples that were identified were tested for its cellulose and hemicellulose activity using the 2-cyanoacetamide method to detect the glucose level produced. Carboxymethylcellulose (CMC) was used as a substrate in this study for detecting cellulose activity while xylan was used as a substrate to detect hemicellulase activity in the isolated microorganisms. A standard curve was first created using glucose with acetate buffer of pH5.0 to make a concentration of 5, 25, 50, 100, 200, 400, 800 and 1000 mg mL^{-1}. Acetate buffer used for the construction of the standard curve as it is a commonly used buffer for enzyme assay including cellulase (Jurick *et al.* 2012). Construction of the standard curve yielded a R^2 value of 0.9985 and a linear equation of y = 0.0015x + 0.0083. Standard curve of glucose constructed using the 2-cyanoacetamide method can be referred to in Appendix Section 2.

The 2-cyanoacetamide bioassay was used to detect the reducing sugar produced by cellulase and hemicellulase producing microorganisms. The glucose value produced by the isolated microbial strains was calculated based on the equation y = 0.0015x + 0.0083 and absorbance reading obtained from the bioassay. *Aspergillus niger* (NBRC 33023) was used as positive control for this study due to it is commonly used to produce cellulase and hemicellulose commercially (Ang *et al*. 2015). Figure 3.1 summarises the reducing sugar produced by each isolated strain from cellulose and hemicellulose respectively.

Cellulose is the most abundant carbohydrate found in nature and is a major structural material in plant cell walls (Chen 2014). About 45-50% of the plant cell wall of the oil palm empty fruit bunch (OPEFB) consists of cellulose (Baharuddin *et al*. 2009). Therefore, the CMC which derived from cellulose was used as a substrate to detect cellulase activity of the isolated microbial strains to seek out which strains has the best degrading capability to degrade cellulose. As illustrated in Figure 3.2, isolated strains that produced the most amount of glucose from degrading CMC was *Bacillus* sp. S2 (185.13 mg mL^{-1}) followed by *Penicillium citrinum* F6 (162.24 mg mL^{-1}), *Penicillium rolfsii* F10 (160.47 mg mL^{-1}), *Bacillus* sp. S8 (140.47 mg mL^{-1}), and *Aspergillus nomius* F8 (129.80 mg mL^{-1}). *Bacillus* sp. S7 and *Fusarium oxysporum* F3 showed to have the least glucose production which was 3.13 mg mL^{-1} during the cellulase bioassay. For the cellulase activity, *Bacillus* sp. S2 and *Bacillus* sp. S8, *P. citrinum* F6, and *P. rolfsii* F10

showed the ability to produce higher amount of glucose than the positive control, *A. niger* which had the glucose value of 134.02 mg mL^{-1}.

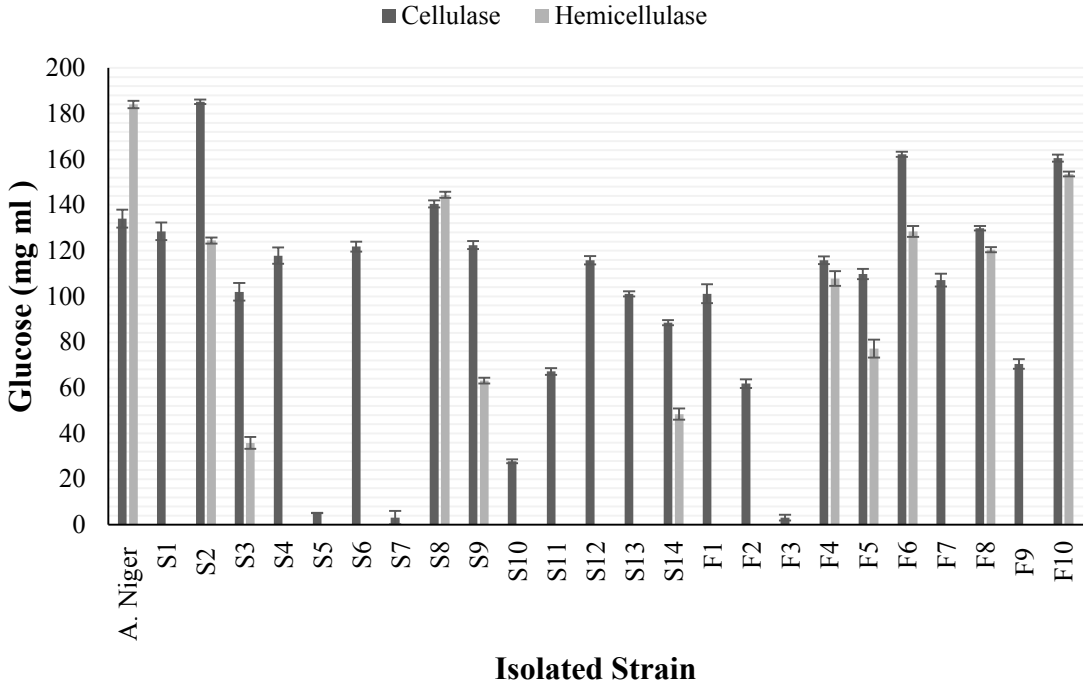


Figure 3.1 Glucose produced by cellulase and hemicellulase producing microorganisms as detected using 2-cyanoacetamide method.

The hemicellulose of empty fruit bunches in oil bunch consists mainly xylan (Li, Ng & Wu 2014). Therefore, the bioassay for testing hemicellulase activity of the isolated strains was carried by replacing the substrate to xylan. The hemicellulase bioassay showed only 10 out of the 24 different strains having the ability to degrade both CMC and xylan which was shown in Figure 3.1. Among the 10 strains tested for its hemicellulase activity, *Penicillium rolfsii* F10 exhibited the highest level of glucose at 153.58 mg mL^{-1}, followed by *Bacillus* sp. S8 (144.47 mg mL^{-1}), *Penicillium citrinum* F6 (128.46 mg mL^{-1}), *Bacillus* sp. S2 (124.45 mg mL^{-1}), *Aspergillus nomius* F8 (120.47 mg mL^{-1}), *Penicillium coprophilum* F4 (107.80 mg mL^{-1}), *Trichoderma harzianum* F5 (77.13 mg mL^{-1}), *Bacillus* sp. S9 (63.13 mg mL^{-1}), *Bacillus* sp. S14 (48.47 mg mL^{-1}), and lastly, *Bacillus* sp. S3 (35.80 mg mL^{-1}). *A. niger* which was used as the positive control for the hemicellulase activity obtained a glucose value of 184.02 mg mL^{-1} which was higher than all the strains tested.

From the bioassay for testing the cellulase and hemicellulase activity of the isolated microorganisms, the 14 cellulolytic isolates which produced the lowest glucose level from degrading CMC was excluded from further analysis. Cellulose composes the first layer of the oil palm fibre waste, therefore, the microorganisms with the highest cellulose degrading ability were prioritised. After confirming 10 cellulolytic microorganisms with the highest cellulose degrading abilities, the hemicellulase productions of these isolates were observed. From the observation, the top five cellulolytic strains which produced among the highest cellulase activities were also found to exhibit the high hemicellulase productions. Therefore, the top five isolated strains namely *Bacillus* sp. S2 and *Bacillus* sp. S8, *Penicillium citrinum* F6, *Aspergillus nomius* F8, and *Penicillium rolfsii* F10 showed the most prominent results in producing the highest cellulase and hemicellulase activities during the experiment were chosen as to form an indigenous microbial consortium for the purpose of composting of oil palm empty fruit bunches. Indigenous consortium of a mixed bacterial and fungal strains was often chosen as it seemed more effective in composting waste such as reducing the time required for such process (Mukhlis *et al*. 2013).

The chosen strains consisted of a mix population of bacterium and fungus from the genus *Bacillus, Penicillium* and *Aspergillus* which have been discussed in Chapter 2 for their genetic identification. Similar species had also been previously studied by other researchers on cellulolytic activities. *Bacillus* spp. isolated from agriculture environments had been reported to exhibit the ability to convert cellulose and hemicellulose into soluble sugars (Kim *et al*. 2012). *Penicillium* spp. had also been studied more recently as it has been described to be a promising strain in the bioethanol industry for its capability in producing cellulases (Schneider *et al.* 2016; Amore *et al.* 2013). *Aspergillus* spp. are known cellulolytic microorganisms as they have been industrially used to produce large amounts of cellulolytic enzymes such cellulases and hemicellulases (Vries *et al*. 2001). The select five cellulolytic strains were therefore in line with the previous findings, and further test were therefore conducted before using them as inoculum to accelerate the composting process.

3.3.2 Screening for Antagonism Activity Among Selected Strains

Dual culture plate assay was adapted to be used for this study to test for the presence of antagonism among the selected cellulolytic strains. Dual culture plate assay is an established technique used to discover antagonism among strains. The selected strains, *Bacillus* sp. S2 and *Bacillus* sp. S8, *Penicillium citrinum* F6, Aspergillus *nomius* F8, and *Penicillium rolfsii* F10 were tested for the antagonism effect on each other. The selected strains were cultured among each other on fresh nutrient agar (NA) and incubated for 3 days at 37°C. The growth of the selected strains when grown with each other was visually observed from the dual plate assay. The dual plate assays were observed for the presence of growth inhibition. As shown in Figure 3.2, no antagonistic activities were observed on each selected strain when grown together. The selected cellulolytic strains *Bacillus* sp. S2 and *Bacillus* sp. S8, *P. citrinum* F6, *A. nomius* F8, and *P. rolfsii* F10 showed no antagonistic activity against each other. The results obtained served as an indication that the selected isolated strains had no antagonism properties. Therefore, the selected strains were of potential to be used as the cellulolytic degrading microorganism consortia in the following in-vessel composting study discussed in Chapter 4 as it will not inflict any growth inhibition when formed as the microbial consortium.

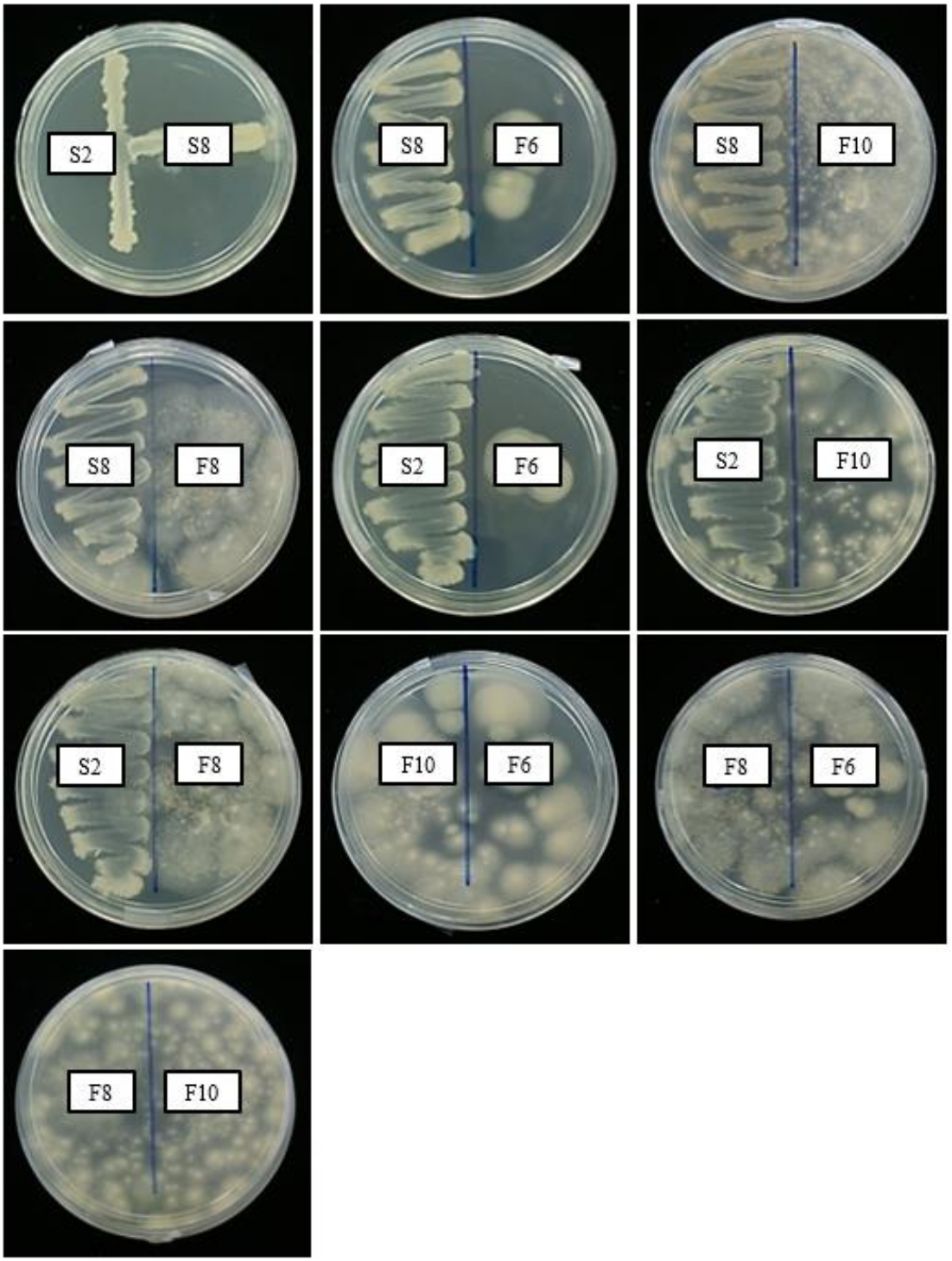


Figure 3.2 Antagonistic activity evaluations were performed for the 5 selected cellulolytic strains: *Bacillus* sp. S2, and *Bacillus* sp. S8, *P. citrinum* F6, A. *nomius* F8, and *P. rolfsii* F10.

3.3.3 Optimisation of Bacterial and Fungal Strains

A study by Hamzah *et al.* (2013) indicated that physical parameters such as initial medium pH and incubation temperature play an important role to promote microbial biomass production. For this study, the selected degrading microorganisms *Bacillus* sp. S2 and *Bacillus* sp. S8, *Penicillium citrinum* F6, *Aspergillus nomius* F8, and *Penicillium rolfsii* F10 were tested for their optimum pH and incubation temperature by incubating the selected microorganisms in growth mediums tuned to different pH and different temperature conditions.

3.3.3.1 pH

(I) Bacterial Strain

The optimisation of pH for the selected bacterial strain *Bacillus* sp. S2 and *Bacillus* sp. S8 was carried out. The selected strains were tested for the optimum growth rate when incubated overnight in nutrient broths with pH ranging from pH5.0 to pH8.0 with an interval of 0.5 as illustrated in Figure 3.3. The growth medium was attuned with the use of 1 N NaOH and 1 N HCl. The absorbance of overnight culture that was incubated in different pH nutrient broth mediums was read using a spectrophotometer at a wavelength of 600 nm. Fresh nutrient was used as a blank for adjusting the background. The optimal incubation pH of growth medium for the selected microorganisms is shown in Figure 3.4. The maximum absorbance reading that could be observed from the assay was produced by *Bacillus* sp. S8 with the absorbance value of 1.519 when incubated at pH6.0. However, *Bacillus* sp. S8 also exhibited the lowest absorbance value of 0.577 when grown at pH8.0. It can be said that *Bacillus* sp. S8 grows the best when grown in the growth medium of pH6.0 when incubated in different pH condition. From the pH optimisation of *Bacillus* sp. S2, the strain also exhibited to prefer the growth condition of pH 6.0 giving an absorbance reading of 1.188. From the graph bar of *Bacillus* sp. S2, *Bacillus* sp. S2 showed to favour the growth conditions that have a pH value of lower than pH6.0 as it grows better in acidic conditions compared to the alkaline condition. From the pH optimisation of the selected bacterial strains, it can be concluded that both bacterial strains *Bacillus* sp. S2 and *Bacillus* sp. S8 favoured the growth medium condition of pH6.0±0.5.

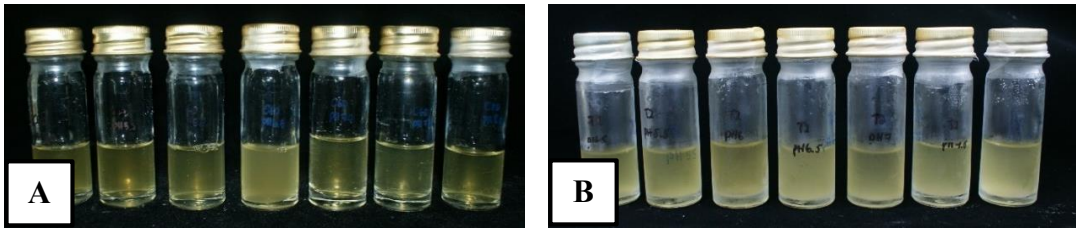
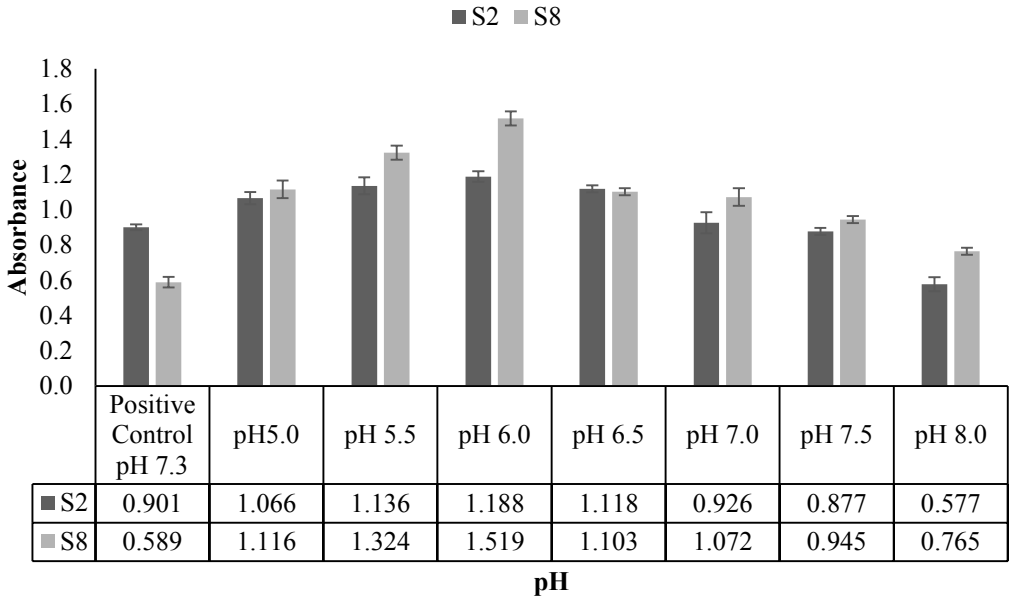


Figure 3.3 The selected bacterial strain (A) *Bacillus* sp. S2 and (B) *Bacillus* sp. S8 exhibited higher growth rates when placed in growth condition of pH6.0.



	Positive Control pH 7.3	pH5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
S2	0.901	1.066	1.136	1.188	1.118	0.926	0.877	0.577
S8	0.589	1.116	1.324	1.519	1.103	1.072	0.945	0.765

Figure 3.4 Effect of different pH on the growths for selected bacterial strain *Bacillus* sp. S2 and *Bacillus* sp. S8.

(II) Fungal Strain

The pH optimisation for the three selected fungal strain *P. citrinum* F6, *A. nomius* F8, and *P. rolfsii* F10 was carried out. The selected strains were tested for the optimum growth rate when incubated for 5 days in potato dextrose broth (PDB) ranging from pH5.0 to pH8.0 with an interval of 0.5 as shown in Figure 3.5. From visual observation, it can be seen that more mycelium is produced when the fungal strains were incubated in their optimum pH condition. The PDB growth medium for the fungal strains was attuned with the use of 1 N NaOH and 1 N HCl. The mycelial dry weight obtained from the assay was tabulated in Figure 3.6. The dry weight of fungal grown in PDB obtained from the analysis was used to adjust the background weight.

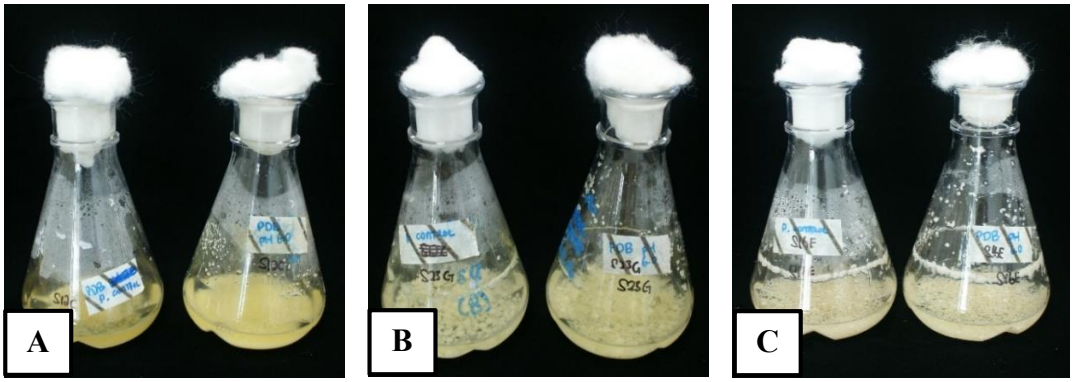
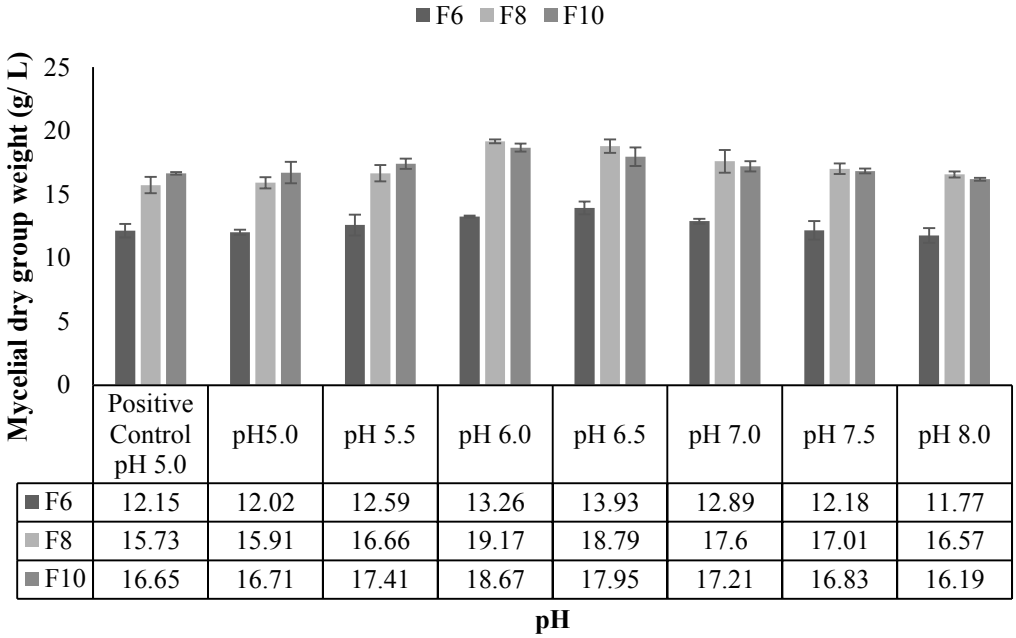


Figure 3.5 Selected fungal strain (A) *P. citrinum* F6, (B) *A. nomius* F8, and (C) *P. rolfsii* F10 showed to favour the growth condition of pH6.5 where more mycelia were produced.



	Positive Control pH 5.0	pH5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
F6	12.15	12.02	12.59	13.26	13.93	12.89	12.18	11.77
F8	15.73	15.91	16.66	19.17	18.79	17.6	17.01	16.57
F10	16.65	16.71	17.41	18.67	17.95	17.21	16.83	16.19

Figure 3.6 Effect of different pH on the growths of selected fungal strain *P. citrinum* F6, *A. nomius* F8, and *P. rolfsii* F10.

From the pH optimisation of the fungal strains, *A. nomius* F8 produced the heaviest mycelial dry weight of 19.17g after incubation at pH6.0 for 5 days in the incubator shaker followed by *P. rolfsii* F10 which exhibited a mycelial dry weight of 18.67g when grown in pH6.0. From this information, it shows *A. nomius* F8 and *P. rolfsii* F10 favours the pH growth condition of pH6.0. From the graph bar of *A. nomius* F8, *A. nomius* F8 grows better in conditions above pH6.0 than when placed in growth conditions below pH6.0. This displays *A. nomius* F8 prefers to grow in more alkaline

conditions compared to the acidic condition it was placed in. When the pH optimisation was tested on *P. citrinum* F6, the fungal strain exhibited a preference of growth condition with pH6.5 producing a mycelial dry weight of 13.93 g/L. However, it showed the lowest mycelial dry weight of 11.77 g/L when grown in pH8.0. From the optimisation of the fungal strain, it can be concluded that the selected fungal strains preferred to be grown in growth condition with pH6.0±0.5.

3.3.3.2 Temperature

(I) Bacterial Strain

The optimisation for the selected bacterial strain *Bacillus* sp. S2 and *Bacillus* sp. S8 was carried out. For the initial stage of optimisation of the selected bacterial strains, the bacteria were allowed to grow for overnight in pH6.0 nutrient broth media at temperatures ranging from 35°C to 45°C with an interval of 5°C as it can be seen in Figure 3.7. The optimal incubation temperature obtained from the assay for the selected bacterial strains was shown in Figure 3.8.

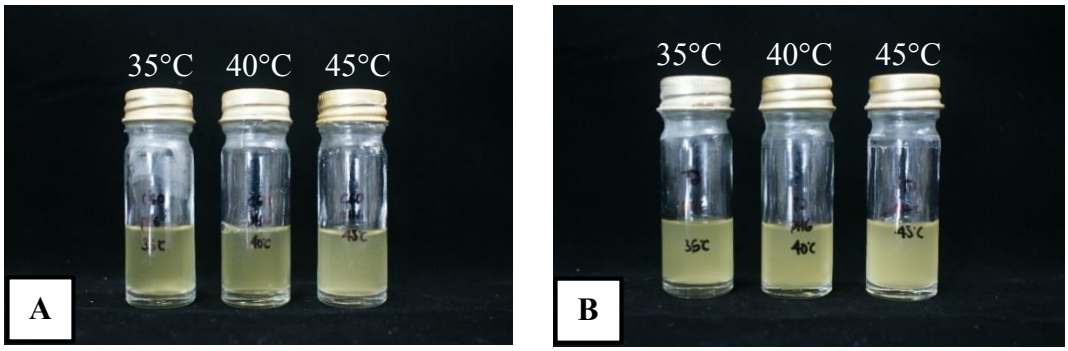
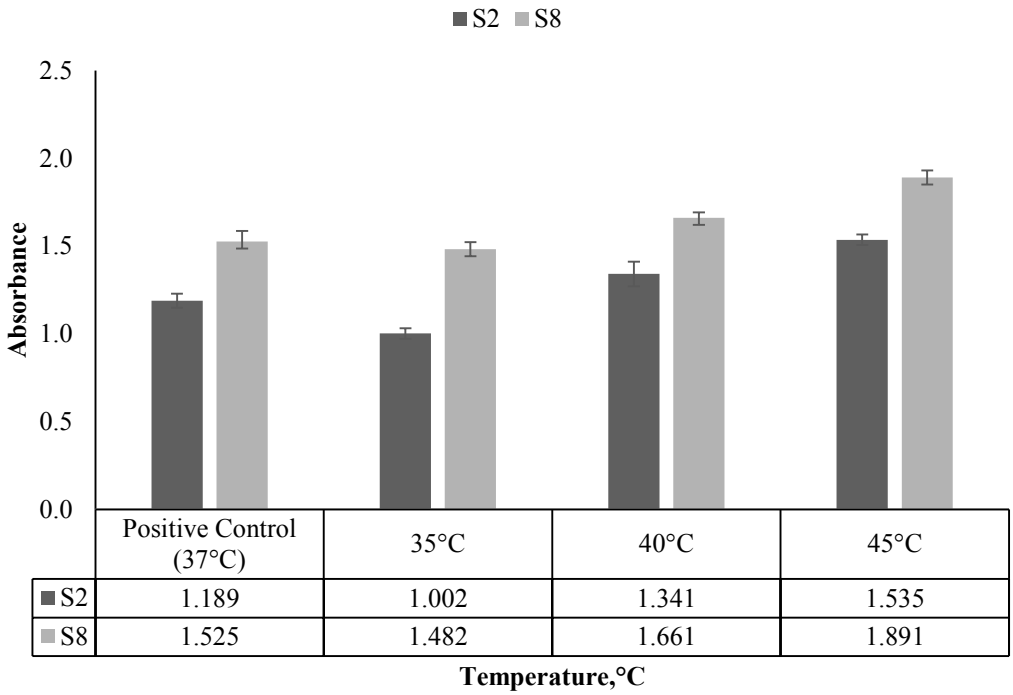


Figure 3.7 The selected bacterial strain (A) *Bacillus* sp. S2 and (B) *Bacillus* sp. S8 exhibited higher growth rates when placed in a 45°C condition based on higher absorbance readings.



	Positive Control (37°C)	35°C	40°C	45°C
S2	1.189	1.002	1.341	1.535
S8	1.525	1.482	1.661	1.891

Figure 3.8 Effect of different temperature on the growth for selected bacterial strain *Bacillus* sp. S2 and *Bacillus* sp. S8.

From the observation of the temperature optimisation for the selected bacteria strains, it can be observed that the bacterial strain grew better when placed in thermophilic condition. Both selected bacterial strains exhibited a higher absorbance reading when incubated at the growth condition of 45°C where *Bacillus* sp. S2 gave an absorbance reading of 1.535 while *Bacillus* sp. S8 had an absorbance reading of 1.891. *Bacillus* sp. S2 showed the lowest absorbance reading of 1.189 when grown at the temperature of 35°C. From the temperature optimisation assay for the selected microorganisms, it can be concluded that *Bacillus* sp. S2 and *Bacillus* sp. S8 favours the growth condition of 45°C as it shows a higher growth rate when incubated in that temperature condition.

(II) Fungal Strain

The optimisation of temperature for the selected fungal strains *P. citrinum* F6, *A. nomius* F8, and *P. rolfsii* F10 was carried out. The selected fungal strains were incubated in pH6.0 potato dextrose broth (PDB) medium placed at different temperatures ranging from 35°C to 45°C with an interval of 5°C for 5 days in an incubator shaker. As can be seen from Figure 3.9, it can be visually observed the

mycelium produced from the fungal strains when placed in different temperatures differ in size and volume. *P. citrinum* F6 produced fine mycelium when grown in 35°C while *A. nomius* F8 and *P. rolfsii* F10 exhibited larger sized mycelium. Mycelium was present in both *A. nomius* F8 and *P. rolfsii* F10 5-day old cultured baffled flask when grown in temperature of 40°C. This indicates the capability of *A. nomius* F8 and P. *rolfsii* F10 to grow in higher temperature growth condition. The dry weight of the mycelium produced by the fungal strains from 5 days of incubation in different temperatures was illustrated in Figure 3.10.

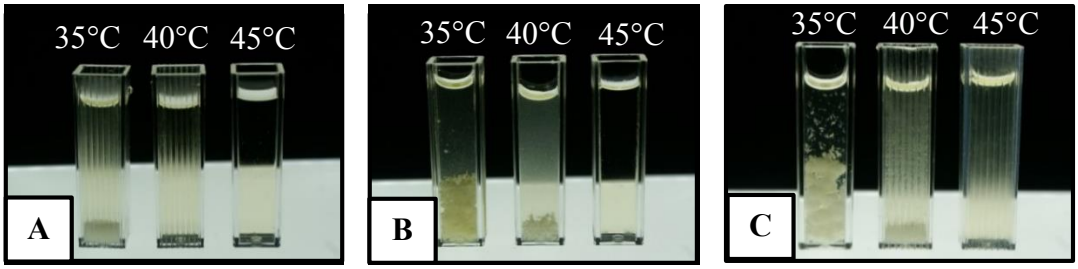


Figure 3.9 Selected fungal strain (A) *P. citrinum* F6, (B) *A. nomius* F8, and (C) *P. rolfsii* F10 showed to favour an incubation temperature of 35°C where more mycelia were produced.

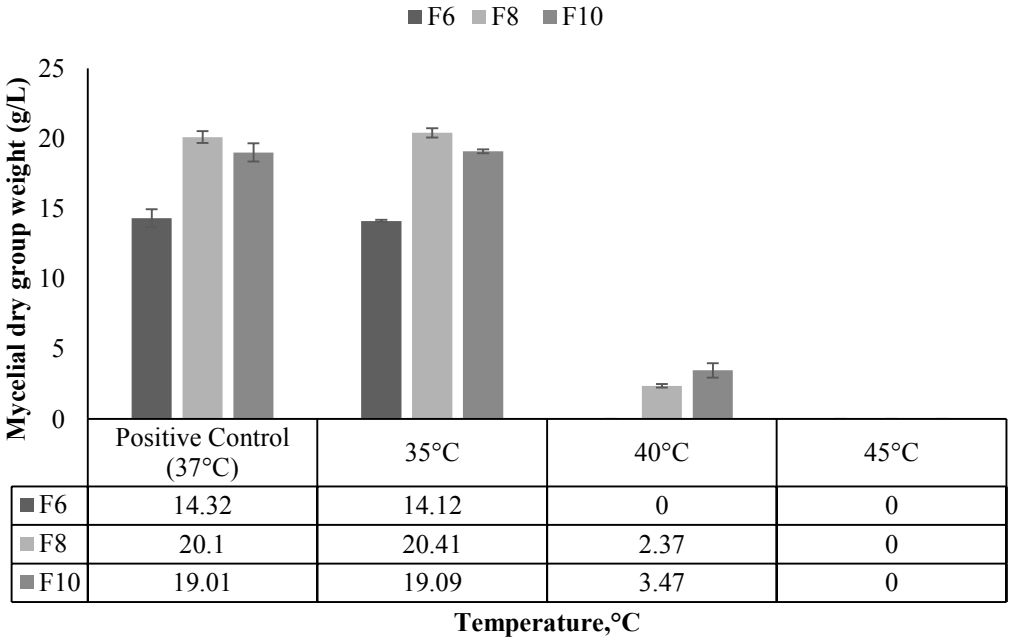


Figure 3.10 Effect of different temperature on the growth of selected fungal strains *P. citrinum* F6, *A. nomius* F8, and *P. rolfsii* F10.

From the temperature optimisation of the selected fungal strains, *A. nomius* F8 exhibited to have the heaviest mycelial dry weight of 20.41 g per 100 mL when incubated in the growth condition of 35°C followed by *P. rolfsii* F10 with mycelial dry weight of 19.09 g per 100 mL. *A. nomius* F8, and P. rolfsii F10 produced mycelium when grown at the temperature of 40°C indicating the ability of the two out of the three selected fungal strains to grow at a higher temperature. However, *P. citrinum* F6 was not able to grow when it was placed in the growth condition of 40°C and 45°C. *P. citrinum* F6 produced a mycelial dry weight of 14.12 g when incubated at 35°C. From the optimisation of the selected fungal strain, *P. citrinum* F6, Aspergillus *nomius* F8, and *P. rolfsii* F10, it can be concluded that the three selected strains favoured the growth conditions of 35°C.

3.3.3.3 Optimal Growth Conditions of Selected Microorganisms

The optimal growth condition of the 5 selected microorganisms was tested based on the condition of pH and temperature. From the pH optimisation assay of the selected strains, the data obtained from it exhibited that both selected bacterial and fungal strains favoured the growth conditions of pH6.0±0.5 when incubated in tuned basic broth mediums that consist of different pH conditions ranging from pH5.0 to pH8.0. During the optimisation of temperature for the selected strains, the two selected bacterial strains showed to favour the growth of condition of thermophilic temperature of 45°C while 2 out of the 3 selected fungal strains were able to grow in the temperature condition of 40°C. From the analysis, the selected fungal strains preferred to be incubated in the temperature condition of 35°C. After observing data from all optimisation, the growth rate of the microorganisms can be seen to slowly increase after each element was adjusted to the optimum condition. The summary of the increasing growth rate of the selected microorganisms can be seen from Table 3.1 for the selected bacterial strain and Table 3.2 for the selected fungal strain.

Table 3.1 Summary of increasing growth rate of selected bacterial strains after optimisation.

	***Bacillus* sp. S2 (Bacteria), Absorbance reading (Abs.)**	***Bacillus* sp. S8 (Bacteria), Absorbance reading (Abs.)**
NB pH7.3, 37°C	0.901 ± 0.006	0.589 ± 0.011
NB pH6.0, 37°C	1.188 ± 0.010	1.118 ± 0.002
NB pH6.0,45°C	1.535 ± 0.004	1.891 ± 0.005

Table 3.2 Summary of increasing growth rate of selected fungal strains after optimisation.

	***P. citrinum* F6, Mycelial Dry Weight (g)**	***A. nomius* F8, Mycelial Dry Weight (g)**	***P. rolfsii* F10, Mycelial Dry Weight (g)**
PDB pH5.0, 37°C	12.15 ± 0.01	15.73 ± 0.05	15.65 ± 0.04
PDB pH6.0, 37°C	13.01 ± 0.09	19.17 ± 0.01	18.67 ± 0.05
PDB pH6.0, 35°C	14.12 ± 0.04	20.41 ± 0.01	19.09 ± 0.01

3.3.4 Microbial Growth Rate of Selected Microorganisms

The growth of the selected cellulolytic bacterial and fungal strain was analysed. A different method of the test was used due to the growth difference of bacteria and fungus. The aim of this experiment was to observe the growth rate of the selected strain and also the amount of cell that will be inoculated into the in-vessel composting that will be discussed in Chapter 4.

3.3.4.1 Bacterial Strain

Evaluations of the growth kinetics of the selected bacterial strains *Bacillus* sp. S2 and *Bacillus* sp. S8 were conducted based on indirect growth measurement technique and the direct growth measurement technique. For the indirect growth measurement technique, the bacterial strains were grown in their own individual flask containing 90 mL of nutrient broth and 10 mL overnight broth culture. The flask was incubated at 35°C in the incubator shaker and observed. A spectrophotometer was used to measure the turbidity of the culture at 600 nm wavelength. During the direct growth measurement technique, the viable count or plate count of the bacterial strains was carried out as can be seen in Figure 3.11. The growth kinetics characteristics of the selected bacterial strains were recorded in Table 3.3.

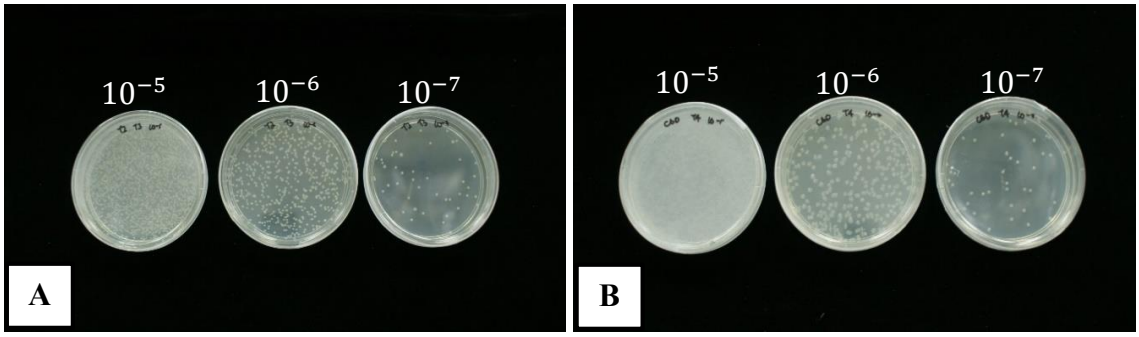


Figure 3.11 Viable count or plate count was used for direct growth measurement technique of the selected bacterial strains (A) *Bacillus* sp. S2 and (B) *Bacillus* sp. S8.

Table 3.3 Growth kinetics characteristics of the selected bacterial strains *Bacillus* sp. S2 and *Bacillus* sp. S8 grown in NB at 35 °C, 120 rpm.

Strain	Specific growth rate, k [hr-1]	Doubling time, Dt [hr]	Absorbance value of bacteria at 24 hr [OD_{600}]	Colony forming units at 24 hr [CFU/mL]
***Bacillus* sp. S2**	0.2102	3.30	1.279	$4.04x10^7$
***Bacillus* sp. S8**	0.3311	2.10	1.580	$4.52x10^7$

The growth kinetic characteristics for the selected bacterial strain *Bacillus* sp. S2 and *Bacillus* sp. S8 was evaluated based on spectrophotometer assay and total plate count techniques. The formula shown in Equation 3.2 was used to calculate the specific growth rate of the bacterial strains based on the absorbance reading obtained from the spectrophotometer assay.

$$\textbf{Equation 3.2} \quad \mu = \frac{ln\frac{OD_{600_{t2}}}{OD_{600_{t1}}}}{t_2 - t_1}$$

The specific growth rate is a quantitative measurement used to measure the increase of cell mass over a specific time. *Bacillus* sp. S8 showed to have a higher a specific growth rate of 0.1209 hr^{-1} than *Bacillus* sp. S2. The doubling time for each bacterial strain was also studied by calculating the doubling time based on the value obtained from the specific growth rate. The doubling time is the period of time required for the bacterial strain to double its quantity. The doubling time of the bacterial strain is proportional to the specific growth rate. In other words, the higher the specific growth rate, the shorter

the doubling time is required by the bacterial strain to double its quantity size. From Table 3.3, *Bacillus* sp. S8 showed to have a shorter doubling time of 1.20 hr than *Bacillus* sp. S2. This shows that *Bacillus* sp. S8 takes 2.10 hr to double its population while *Bacillus* sp. S2 requires 3.30 hr to double up its quantity, which is 1.20 hr slower than *Bacillus* sp. S2. The absorbance reading of the two selected bacterial strains along with the colony forming unit after 24 hr of incubation were recorded in Table 3.3. *Bacillus* S8 showed to have a higher absorbance and colony forming unit after 24 hr of incubation than *Bacillus* sp. S2. From this study, it can be concluded that *Bacillus* sp. S8 has a higher growth rate than *Bacillus* S2.

3.3.4.2 Fungal Strain

Evaluations of the growth kinetics of the three selected fungal strains *Penicillium citrinum* F6, *Aspergillus nomius* F8, and *Penicillium rolfsii* F10 were conducted based on indirect growth measurement technique and the direct growth measurement technique. For the indirect growth measurement technique, the fungal strains were grown in their own individual flask containing 90 mL of potato dextrose broth and 10 mL 5-day old broth culture. The flask was incubated at 35°C in the incubated in an incubator shaker for 5 days as can be seen in Figure 3.12 and the mycelium biomass was obtained by filtering it using filter paper. The dry weight of the mycelium was obtained after 24 hr of oven dry. During the direct growth measurement technique, a sterile 5mm straw was used to transfer 5 days old agar plate culture onto fresh potato dextrose agar (PDA). The inoculated PDA plates were incubated at 35°C for 5 days. On the 5^{th} day of incubation, the diameter of the fungal culture was measured and recorded. The growth kinetics characteristics of the 3 selected fungal strains were recorded in Table 3.4.

The growth kinetics characteristics for the selected fungal strains *P. citrinum* F6, A. *nomius* F8, and *P. rolfsii* F10 were evaluated based on the dry weight of the mycelium obtained from 5 days old culture and the growth diameter of the 5-day old fungal culture on PDA. From Table 3.4, *A. nomius* F8 showed to have the highest value of mycelial dry weight with the value of 20.58 g from the assay while *P. citrinum* F6 has the lowest mycelial dry weight value of 14.79 g. This shows that *A. nomius* F8 grows faster than the other selected fungal strains, therefore, producing more mycelium in a shorter period of time. *A. nomius* F8 also exhibited higher growth rate on PDA plate, having a diameter of 8.4cm which is higher than *P. citrinum* F6 and *P. rolfsii* F10 by 2.6 cm and 1.2 cm

respectively. From this study, it can be concluded that *A. nommius* F8 has the highest growth rate followed by *P. rolfsii* F10 and *P. citrinum* F6.

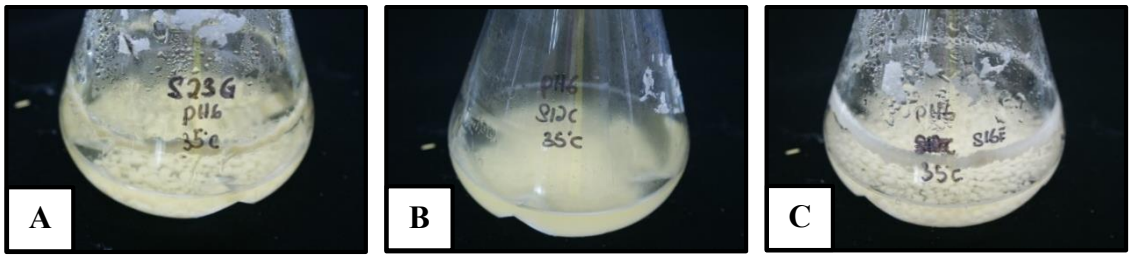


Figure 3.12 The dry weight of the mycelium obtained from the 5-day old culture broth of (A) *A. nomius*, (B) *P. citrinum*, and (C) *P. rolfsii* was used for the indirect growth measurement technique to measure the fungal growth.

Table 3.4 Growth kinetics characteristics of the selected fungal strains, *P. citrinum* F6, A. *nomius* F8, and *P. rolfsii* F10 grown in PDB at 35 °C, 120 rpm.

Strain	Mycelial Dry Weight after 5 days (g)	Growth Diameter at Day 5 (cm)
***Penicillium citrinum* F6**	14.79 ± 0.63	5.8 ± 0.12
***Aspergillus nomius* F8**	20.58 ± 0.66	8.4 ± 0.24
***Penicillium rolfsii* F10**	19.37 ± 0.42	7.2 ± 0.19

3.3.5 Selected Final Strains for Development of Indigenous Microbial Consortium

The selected cellulolytic strains intended for the formulation of indigenous microbial consortium for in-vessel composting were pre-tested for their abilities to secrete cellulases and hemicellulases based on the glucose level detected from the degradation of carboxymethylcellulose (CMC) and xylan respectively. The selected strains were also tested for antagonism effect among each other to prevent the possibility of mutual growth inhibition during in-vessel composting. These selected strains were chosen based on their abilities to degrade CMC and xylan as discussed earlier (Figure 3.13). The selected strains had exhibited high glucose level among the 24 cellulolytic strains isolated from soil, and had a comparable result to the positive control *Aspergillus niger* (NBRC 33023). The selected cellulolytic strains also showed no antagonistic activity among each other. Hence, these five strains were confirmed to be used as indigenous microbial consortium for the composting of oil palm empty fruit bunch (OPEFB).

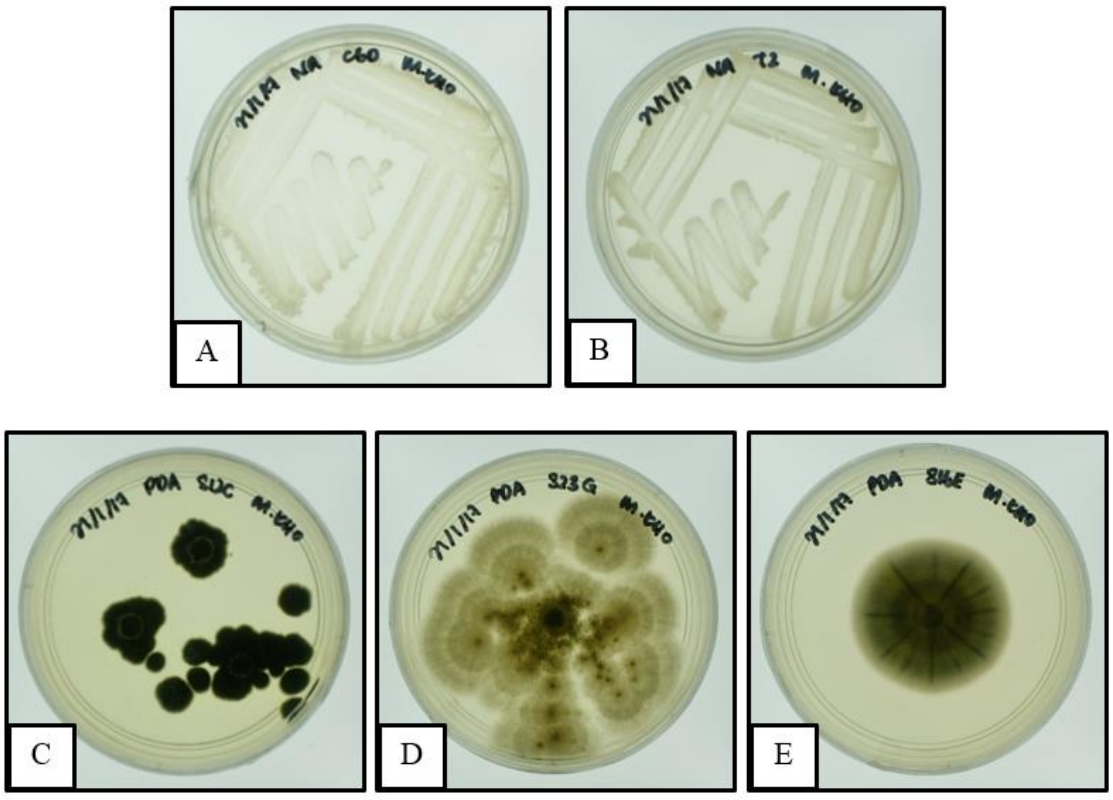


Figure 3.13 The selected 5 strains (A) *Bacillus* sp. S2, (B) *Bacillus* sp. S8, (C) *P. citrinum* F6, (D) *A. nomius* F8, and (E) *P. rolfsii* F10 were chosen for the formulation of the degrading microorganisms consortia for the in-vessel composting discussed in Chapter 4.

3.4 Summary

The study of Chapter 3 was focused on the cellulase and hemicellulase enzyme productions by the selected cellulolytic microorganisms, mutual antagonism within these selected microorganisms, and also the growth rate of the respective microorganisms. The cellulase and hemicellulase activities of the isolated microorganisms were successfully confirmed using the 2-cyanoacetamide method. After performing the cellulase and hemicellulase assays, *Bacillus* sp. S2, *Bacillus* sp. S8, *Penicillium citrinum* F6, *Aspergillus nomius* F8, and *Penicillium rolfsii* F10 were chosen as they exhibited among the highest glucose levels. These selected cellulolytic microorganisms were used to form an indigenous microbial consortium for the composting of oil palm empty fruit bunch(OPEFB) during an in-vessel composting in Chapter 4. Antagonism evaluations were performed among these strains where all the respective strains were paired up and cultured onto the same nutrient agar plate and observed for any mutual growth inhibition. From the evaluation, no antagonistic activities were present in any of the selected strains when paired and grown together. Investigations of the optimum growth condition for the selected microorganisms were also performed. The selected microorganisms were grown in different pH ranging from pH5.0 to pH8.0 with an interval of 0.5. They were also grown in a different temperature of 35°C, 40°C, and 45°C. From the analysis, both selected bacterial and fungal strains showed preference to be grown in a pH of pH6.0. The bacterial strains showed to favour growth in thermophilic temperature of 45°C while the fungal strains preferred to grow in 35°C. However, two out of three fungal strains, namely *A. nomius* F8 and *P. rolfsii* F10, were able to grow in 40°C. Upon further investigation of the bacterial strains, *Bacillus* sp. S8 showed to have the higher growth rate when compared to *Bacillus* sp. S2. For the fungal strains, *A. nomius* F8 showed to have a higher growth rate, followed by *P. rolfsii* F10, and *P. citrinum* F6. The efficiency of the five selected strains was tested in Chapter 4 in the in-vessel composting experiment.

Chapter

4

PRODUCTION OF COMPOST VIA IN-VESSEL COMPOSTING OF OIL PALM EMPTY FRUIT BUNCH USING CELLULOLYTIC MICROORGANISMS AND ITS ENHANCEMENT IN THE GROWTH OF OIL PALM SEEDLINGS

4.1 Introduction

The oil palm industry has become one of the most important economic contributors in Malaysia (Abidemi *et al.* 2006). As a result, the oil palm industry produces a large amount of solid waste which consists of lignocellulosic materials such as fronds, trunks, and empty fruit bunches (Harun *et al.* 2013). Oil palm empty fruit bunches (OPEFB) is one of the major dry biomass wastes produced by the oil palm industry (Abdullah & Sulaiman 2013). OPEFB are usually burned to generate power for mills, however, this act has caused environmental pollution. Therefore, sustainable waste management for the reduction of this environmental hazardous waste must be explored.

Composting of agroindustrial wastes such as OPEFB had been suggested to be the most practical method for managing the disposal large volume of these wastes (Zainudin *et al.* 2013). Composting is biodegradation of organic waste turns into stable and humified organic material by a mixed microbial population that occurs under controlled conditions distinguishes itself from natural rotting (Sarkar *et al.* 2011).

Composting is the most suitable way to manage waste economically and environmentally as it reduces bulk volume of oil palm waste into useful compost which improves and sustain soil fertility (Mukhlis *et al.* 2013). According to Baharuddin, *et al.*, OPEFB is a lignocellulosic material that usually contains 53% cellulose, 30% hemicellulose and 17% lignin which makes it require a longer composting period (2009). Cellulolytic microorganisms are the key to depolymerise the lignocellulosic material in OPEFB at a faster rate (Mukhlis *et al.* 2013). Thus, the addition of effective cellulolytic microorganisms to the compost would accelerate the composting process. In this composting experiment, the indigenous microbial consortium containing a variety of cellulolytic microorganisms as discussed in Chapter 3 were inoculated into the OPEFB composting material for in-vessel composting. The objective of the experiment was to test the efficiency of the indigenous microbial consortium in the composting of OPEFB. The compost produced from the experiment was further analysed for its effectiveness in the enhancement of the growth of oil palm seedlings via small scale pot trial.

Oil palm seedling has been cultivated using topsoil as a growing medium during the nursery stage. However, using top soil as a growth medium for long term is not practical as growing oil palms would require more nutrients as they grow (Rosenani *et al.* 2016). For the optimum production yields from crops, sufficient and balanced nutrients must be

available for the plants' absorption. Therefore, fertilisers are required to supplement the nutrients already present in soil (Chen 2006). Chemical fertilisers are usually used as an essential nutrition for the crops to get a higher yield. However, in recent years, the yields have become almost static where no crop yields have increased even with the application of the optimum level of chemical fertilisers (Ahmad *et al.* 2007). Furthermore, high energy cost inputs are required for the production of chemical fertiliser. From this scenario, it is highly likely that the use of compost accompanied with chemical fertiliser could be an effective alternative approach to improving the crop yields. According to Ahmad *et al.* (2008), fortification of compost with chemical fertiliser enhances the effectiveness of both compost and chemical fertiliser as it reduced the amount of fertiliser required and also improves the quality of the compost. Apart from that, compost can also help to improve the organic matter status and physiochemical properties of the soil when applied together with chemical fertiliser which gives an addition of nutrients to the plants (Ahmad *et al.* 2007).

Several papers had reported the application of compost to crop productions that exhibited beneficial effects (Gil *et al.* 2008). Compost had been established to have potential ability to enhance water retention, reduce soil compaction and acidity, and also enhance the biological activities in the soil (Sarkar *et al.* 2011). However, compost is comparatively low in nutrient content, so larger volumes are required to provide sufficient nutrients to the growth of plants (Chen 2006). Traditionally, farmers would have to add several tonnes per hectare of compost to their plants due to the poor nutrient of compost (Ahmad *et al.* 2008). In recent practices, it is rather common to integrate compost with chemical fertiliser. An evaluation of soil quality by Dutta *et al.* (2003) showed to a positive effect on the soil health and hence plant health when organic fertiliser and chemical fertiliser were integrated together.

For this chapter in the research study, the indigenous microbial consortium created that contained the selected cellulolytic microorganisms isolated from the collected soil samples were tested for its efficiency in degrading OPEFB during the in-vessel composting study. This experiment was carried out to successfully create an indigenous microbial consortium of degrading microorganisms that can help degrade OPEFB waste in a shorter period of time to produce compost. The compost was further tested for its effectiveness in the enhancement of the growth of oil palm seedlings via small scale pot

trial. The aim was to develop an effective soil supplement by combining compost and chemical fertiliser to increase the growth of oil palms seedlings.

4.2 Materials and Methods

4.2.1 In-Vessel Composting

The composting experiment was conducted in a small-scale in-vessel system placed on black plastic covering in an empty land located in Kuching, Sarawak. A total of nine 200-L vessels were used for the composting of the oil palm empty fruit bunch (OPEFB) and partially treated palm oil mill effluent (POME). The composting vessels were bought and fabricated by a local company named Lien Ann Engineering Sdn Bhd, Kuching, Sarawak. Holes were drilled at the sides of the composting vessels holes to ensure proper aeration and doors latch was made to the vessels to obtain samples and data throughout the composting study. Stands with wheels were also custom made by Lien Ann Engineering Sdn Bhd, composting vessels were placed on top of the roller stand to allow proper rotation of the vessels.

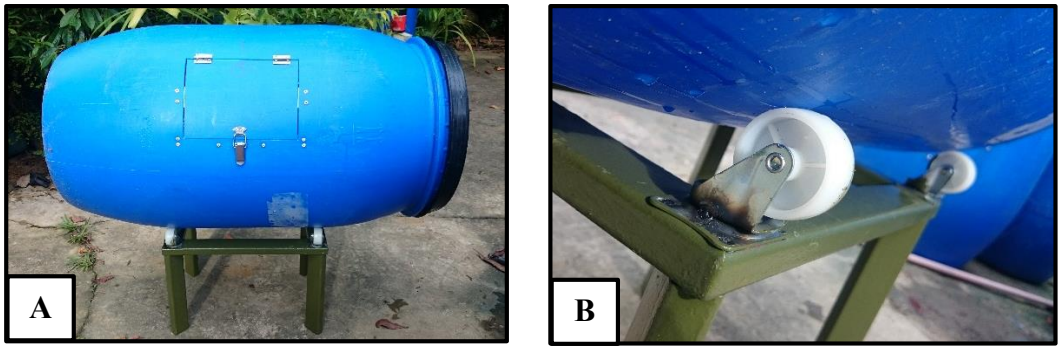


Figure 4.1 Composting vessels were fabricated with door latches (A) and placed on stand stands with rollers (B) to allow easy rotation.

4.2.1.1 Preparation of The Commercial Microbial Agents

The positive control used in this study was a commercially available composting indigenous microbial consortium constituted of *Bacillus* spp. which were *B. cereus, B. subtilis,* and *B. thuringiensis.* The degrading microbe's consortium was obtained in a concentrated dry powder form that was stored at room temperature. For the revival of the

commercial microbial agents, 1.5 g of the concentrated powder was inoculated into 6 replicates of 1500 mL sterilised nutrient broth (Himedia, India) in baffled shake flask (Pyrex, USA), separately. After incubating the indigenous microbial consortium for overnight in the incubator shaker (Sartorious Stedim Biotech, Germany) with a condition of 37°C with shaking at shaken at 180 rpm, they were allocated into three 3-L beakers, each beaker (Duran, UK) contained 500 mL of degrading microbe from each baffled shake flask. They were mixed thoroughly to produce 3 sets of 3-L positive control degrading microbe's consortia for each positive control composting vessel in the composting study.

4.2.1.2 Preparation of Indigenous Microbial Consortium

The following five cellulolytic microorganisms were selected to be used as the indigenous microbial consortium for the composting study:

(i) *Bacillus* sp. S2 (Bacterium)
(ii) *Bacillus* sp. S8 (Bacterium)
(iii) *Penicillium citrinum* F6 (Fungus)
(iv) *Aspergillus nomius* F8 (Fungus)
(v) *Penicillium rolfsii* F10 (Fungus)

The five selected cellulolytic microorganisms were first grown individually in 180 mL fresh basic broth. The cellulolytic bacterial strains were grown in nutrient broth (Himedia, India) while fungal strains were incubated in potato dextrose broth (Himedia, India) with pH6.0 in 250 mL baffled shake flask (Pyrex, USA) incubated at 35°C shaken with agitation of 120 rpm. The bacterial cellulolytic microorganisms were grown overnight while the fungal cellulolytic microorganisms were grown for 5 days before inoculating them into 1620 mL sterilized broth tuned to pH6.0 in baffled shake flask (Pyrex, USA), separately. The freshly grown cellulolytic microorganisms in 180 mL broth medium were poured directly into 1620 mL individual growth medium to ensure a standardized amount of inoculum is inoculated into the broth to ensure consistency of inoculation. The bacterial cellulolytic microorganisms were grown overnight while the fungal cellulolytic microorganisms were grown for 5 days at 35°C with shaking at 180 rpm in the incubator shaker (Sartorious Stedim Biotech, Germany). After incubating the cellulolytic microorganisms, 600mL of culture broth from each cellulolytic microorganism was measured and poured into three 3-L beakers (Duran, UK) to create a consortium of

cellulolytic microorganisms. They were mixed thoroughly to produce 3 sets of 3-L manipulated variable cellulolytic indigenous microbial consortium for each manipulated composting vessel in the composting study.

4.2.1.3 Transportation of Inoculum to In-Vessel Composting Site

The inoculum for the in-vessel composting was packed into labelled plastic bottles with the caps screwed tightly and sealed with paraffin film. The bottles were wipe down with 70% ethanol and placed into a Styrofoam box with sponges surrounding the bottles to prevent the bottles with the inoculums from toppling over and leaking. The Styrofoam box was taped with a hazard warning sign accompanied with the material transfer form and biosafety level of containment. The name and telephone number of the person responsible for handling the inoculums were also written onto the material transfer form.

4.2.1.4 Raw Materials

The composting material, oil palm empty fruit bunch (OPEFB) and partially treated palm oil mill effluent (POME) were obtained from the sponsoring company, Daitoku Sdn Bhd. The OPEFB were obtained after oil palm fruits were removed from the fresh fruit bunch (FFB) during the milling process (threshing). After the threshing process, the OPEFB were shredded into approximately 5 cm length loose fibrous material by using a shredder machine. The palm oil mill effluent used in this study was collected from an anaerobic pond which was used to moisten the OPEFB.

The raw OPEFB and partially treated POME were analysed for its physio-chemical properties before the experiment. Freshly shredded EFB was sent to i-Testchem Laboratory Services, Kota Samarahan, Kuching to analysed for moisture, pH, organic carbon, nitrogen, phosphate, potassium, magnesium and its C: N ratio while freshly collected partially treated POME was analysed for its pH, nitrogen, chemical oxygen demand (COD), biological oxygen demand (BOD), oil and grease, total solids, phosphate, and potassium.

4.2.1.5 Preparation of The Composting Vessels and Experimental Design

For the composting raw materials, 30 kilograms of oil palm empty fruit bunch (OPEFB) was weighed and placed into each individual composting vessel. Approximately 10 L of partially treated palm oil mill effluent (POME) was sprayed evenly onto the EFB into each composting vessel. The substrate ratio of OPEFB to partially treated POME was made to 3:1 as the method was adapted from a related study by Mohammad *et al*. (2014).

After ensuring that the OPEFB was evenly moistened by the partially treated POME, 3 L of inoculum was inoculated into each vessel. Three test groups were carried for the composting study as shown in Table 4.1, each experimental group was carried out in triplicates where the composting vessels were arranged in a completely randomised design.

Table 4.1 Experimental groups for the in-vessel composting study.

Experimental group	Description
Control	OPEFB, partially treated POME, sterilised broth.
Commercial microbial agents	OPEFB, partially treated POME, commercial microbial strains.
Indigenous microbial consortium	OPEFB, partially treated POME, cellulolytic indigenous microbial consortium that was discussed in Chapter 3.

4.2.1.6 Composting Analysis Throughout the Study

A moisture level of 65-75% was maintained throughout the composting process by spraying partially treated palm oil mill effluent (POME) on the oil palm empty fruit bunch (OPEFB) when necessary. To ensure proper aeration was provided, the compost vessels were turned over every 2 days to allow air to enter the compost and give proper mixing. The C: N ratio and temperature of compost were monitored throughout the 60-day composting process. A hygrometer was used to check the temperature and humidity of the surrounding area.

4.2.1.7 Sampling

A 500g compost sample was collected at different locations in the composting vessel: bottom, core, and surface. The samples collected in triplicates were air dried and kept in sterile clear polyethene resealable bag at 4°C.

4.2.1.8 Sample Analysis

Compost samples were sent to i-Testchem Laboratory Services, Kota Samarahan, Kuching to analysed for the C: N ratio every 15 days throughout the 60 days in-vessel composting study. Final compost sample collected at the end of the composting study was analysed for moisture, pH, organic carbon, nitrogen, phosphate, potassium, and magnesium.

4.2.2 Pot Trial

4.2.2.1 Preparation of Additive

Chemical fertiliser - Chemical fertiliser chosen for this pot trial study was a standard palm oil fertiliser formula used in Malaysia with the nutrients ratio of 14:7:9 (Rosenani *et al*. 2016).

Compost - Compost was obtained from the compost produced during the in-vessel composting study discussed in Chapter 4.

4.2.2.2 Preparation of Oil Palms and Experimental Design

Oil palm seedlings that were 5-month old of age were selected for the pot trial study. The 5-month old oil palm seedlings were purchased from Sarjoh Plantation Nursery Sdn. Bhd, Kuching, Sarawak and transplanted into a soil mixture that consists of top soil and sand with the ratio of 3:1 (Tchapda & Oscar 2016). The soil used to plant the oil palms were bought from Pelangi Landscaping and analysed for its physio-chemical properties. The oil palms were watered with approximately 1.5 L of tap water for each plant daily for irrigation and monitored for a month before beginning the treatment.

After 30 days, 40 oil palms with the best condition were selected and treated with different treatments. The selected oil palms were uprooted and replanted into new bigger polybags. Pot trial experiment was conducted in a black netting roofing shed to assess the effect of compost and chemical fertiliser on the growth of 6 months old oil palms. The selected oil palms were divided into four different treatment groups with 10 oil palms seedlings per treatment group for the pot trial study as shown in Table 4.2.

The oil palms with different treatments were arranged in the net house in a completely randomised design with 10 replicates for each treatment with ambient light and temperature provided. For each pot, approximately 1.5 L of tap water was watered to each pot daily in the morning for irrigation.

Table 4.2 Treatment groups for pot trial study.

Experimental Group	Description	Amount of additive
Control	The oil palms were planted without the presence of any added nutrients.	No additives were added.
Chemical fertiliser	The oil palms were fertilised with chemical fertiliser.	20 g of NPK 20-10-10 chemical fertiliser.
Compost	The oil palms were fertilised with compost only.	100 g of enriched compost.
Compost-chemical mixed fertiliser	The oil palms were fertilised with a mix of compost and chemical fertiliser.	10 g of enriched compost and 5 g of NPK 14:7:9.

4.2.2.3 Application of Additive

Chemical fertiliser group - The chemical fertiliser was measured out beforehand and poured onto the top layer of the soil after transplanting.

Compost group- The compost was incorporated in the soil for each pot by mixing it with the top 10 cm soil at the time of transplanting.

Compost-chemical mixed fertiliser group- The compost was supplemented with chemical fertiliser by integrating the measured compost and chemical fertiliser together. It was then incorporated into the soil by mixing it with the top 10 cm soil at the time of transplanting.

4.2.2.4 Oil Palm Data

(I) Initial Physical Examination

The oil palms initial physical examination was carried out and recorded before the start of the pot trial. The measurements and observations were:

(i) The number of opened leaves was counted.

(ii) The height of the trees where it was measured by beginning from the base of the stem which was right above the soil surface to the first leaf internode that was encountered along the stem of the oil palm (Figure 4.2)

(iii) The measurement of the stem girth was performed by using a cotton string to measure the girth diameter of the oil palm bulb (Figure 4.2).

4.2.2.5 Observations of Oil Palms Seedlings at End of Pot Trial Experiment

Oil palm plants were measured for its physical properties (number of leaves, height and girth measurement of oil palm) and harvested at the 6th month of experiment for analysis. Freshly harvested oil palm leafs were sent to Sarawak Plantation Services Sdn. Bhd. in Kuching, Sarawak to performed foliar analysis in accordance to MS 677: Parts III, IV, V, VI, VII: 1980 to analysed for the percentage of nitrogen, phosphate, potassium, magnesium, calcium, and boron based on the dry leaf matter (*Malaysian Standard* nd.).

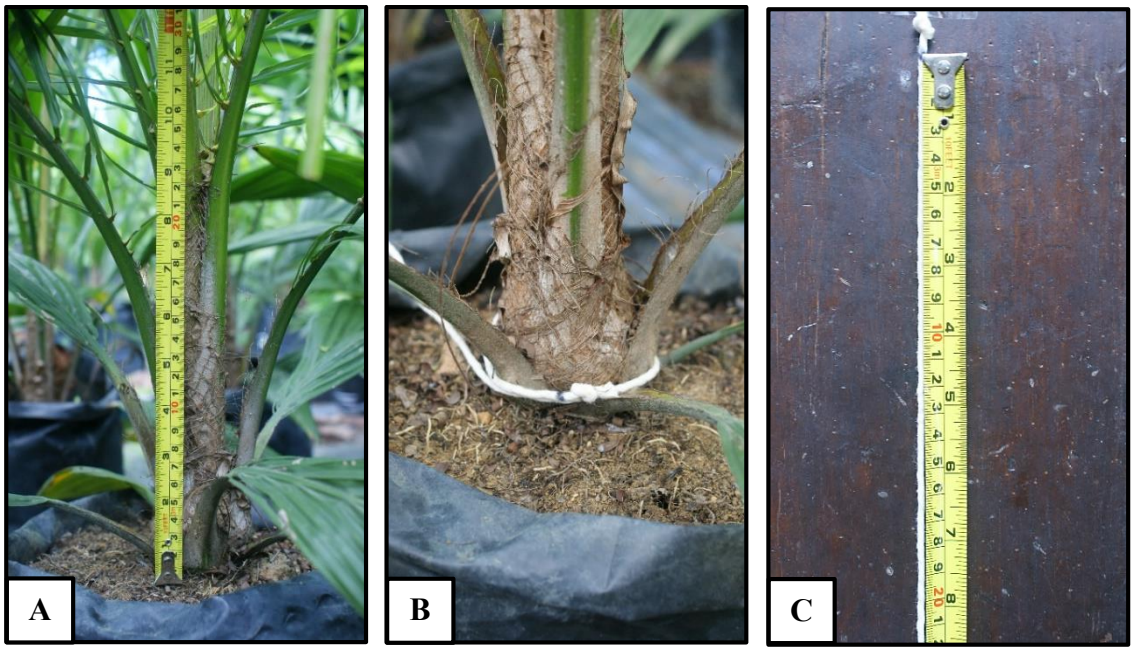


Figure 4.2 Technique used to measure the height (A) and stem girth (B&C) of the oil palm.

4.3 Results and Discussion

4.3.1 In-Vessel Composting

4.3.1.1 Characteristics of Raw Materials and Final Compost

Freshly shredded oil palm empty fruit bunch (OPEFB) contains a relatively large amount of cellulose and lignin. Therefore, requiring a longer period of time to decompose compared to other composting material (Baharuddin *et al*. 2009). Throughout the composting process, different colour and texture of the OPEFB inoculated with commercial microbial agents and indigenous microbial consortium was observed as it slowly degraded (Figure 4.3). The OPEFB was light brown and in the form of long rough fibrous material at the beginning of the experiment. The OPEFB slowly turned darker in colour and shorter in length as it degraded throughout the experiment. Matured compost at the end of the experiment exhibited a blackish appearance accompanied with a texture similar to soil.

Figure 4.3 Physical changes of oil palm empty fruit bunch at Day 0(A), Day 20(B), and Day 60(C).

Oil palm empty fruit bunches (OPEFB) has a low moisture content but high carbon to nitrogen ratio (C: N ratio) while partially treated palm oil mill effluent (POME) has the opposite characteristic as can be observed from Table 4.3. Thus, by mixing these two materials together, it can provide adequate nutrients and moisture content for microorganisms to grow and degrade the composting material. According to Baharuddin *et al.,* OPEFB and partially treated POME complement each other during the co-composting as the high carbon and low nitrogen content available in the OPEFB balances the partially treated POME which has low carbon but high nitrogen content (2009). The value of the parameters recorded in Table 4.3 showed some variations in the chemical

oxygen demand (COD), biological oxygen demand (BOD), oil and grease and total solids. The variations could be due to the unpredictable weather condition and inconsistent discharge from mill operations (Yacob *et al*. 2005).

Throughout the study, partially treated POME was added to the composting material (OPEFB) to maintain a moisture level of 65-75%. According to Liang and Das (2003), the ideal range for moisture content in a compost is 60-75% as it provides maximum microbial activities. Therefore, partially treated POME was added to each OPEFB in the composting vessels every subsequent 3 days to replenish the water loss due to the evaporation to the environment to maintain the microbial activities (Baharuddin *et al*. 2010). Partially treated POME was added to composting vessel until day 50 to avoid the final compost to be too wet. The final compost sample collected on day 60 from all the experimental groups had a moisture content of 60-65% (Table 4.3).

Each composting vessel was turned over every two days to maintain proper distribution of the moisture and build-up of heat during the composting period. Turning of the composting vessel was also carried out to provide a proper supply of aeration for the composting materials. The composting materials in the vessel were made sure to have proper aeration to prevent the slowdown of decomposition due to low oxygen level. Low oxygen level increases the opportunity of the composting material to absorb ammonia which will lead to immobilisation (Baharuddin *et al*. 2010).

Table 4.3 Properties of shredded OPEFB, partially treated POME and final compost for the three experimental test groups at Day 60.

Parameters	Raw materials		Final Compost		
	Shredded OPEFB	Partially treated POME	Control	Commercial microbial agents	Indigenous Microbial consortium
Moisture (%)	18.97 ± 1.33	95.30 ± 2.10	60.1 ± 11.40	62.43 ± 5.29	64.67 ± 1.29
pH	5.57 ± 0.31	7.70 ± 0.40	7.47 ± 0.29	8.77 ± 0.47	8.50 ± 0.10
Carbon (%)	39.87 ± 0.21	19.10 ± 1.90	32.93 ± 3.05	30.27 ± 2.99	30.70 ± 1.87
Nitrogen (%)	1.33 ± 0.12	2.80 ± 0.60	2.01 ± 0.25	2.25 ± 0.28	3.04 ± 0.37
C:N ratio	30.05 ± 2.58	7.90 ± 3.60	17.00 ± 1.15	13.33 ± 1.53	10.00 ± 1.73
COD ($mg\ L^{-1}$)	-	1338.00 ± 226.64	-	-	-
BOD ($mg\ L^{-1}$)	-	263.00 ± 15.92	-	-	-
Oil and grease ($mg\ L^{-1}$)	-	18.00 ± 2.20	-	-	-
Total solid ($mg\ L^{-1}$)	-	2744.00 ± 159.63	-	-	-
Composition of nutrients and metal elements (%)					
Phosphate	0.15 ± 0.00	4.23 ± 0.60	0.25 ± 0.04	0.28 ± 0.06	0.46 ± 0.07
Potassium	0.64 ± 0.01	1.80 ± 0.30	1.17 ± 0.25	1.31± 0.06	1.14 ± 0.13
Magnesium	0.20 ± 0.01	0.90 ± 0.10	0.40 ± 0.05	0.55 ± 0.05	0.60 ± 0.05

4.3.1.2 Physiochemical and Biochemical Changes in Composting

(I) Temperature Variation

The most important indicator that can be used to check the efficiency of the composting process is temperature (Li *et al.* 2008). The ambient temperature around the composting site was measured to be around 24-30°C throughout the study. Figure 4.4 shows the temperature profile of the compost vessels thought the 60-day composting study. The average temperature of the oil palm empty fruit bunch (OPEFB) and partially treated palm oil mill effluent (POME) in the composting vessel at the start of the compost study after inoculation was approximately 21°C. On

the second day of fermentation, the temperature of the composting vessels that contained the commercial microbial agents and indigenous microbial consortium increased sharply to about 65°C. The temperature of the compost spiked up due to the explosive growth of the mixed population and active microbial decomposition of the raw material generating heat. During this phase, the dominant microorganisms attack the soluble, readily biodegradable compounds which are relatively small size with high content of available nutrients (Fourti *et al.* 2008). The composting vessel that was inoculated with the commercial microbial agents and indigenous microbial consortium maintained in a thermophilic phase from day 3 to day 28 with a temperature around 40-63°C. According to Wong *et al.* (2001), most studies on composting had reported that the optimum temperature range for effective decomposition of raw material was 50-70°C where 60°C was regarded as the most satisfactory level.

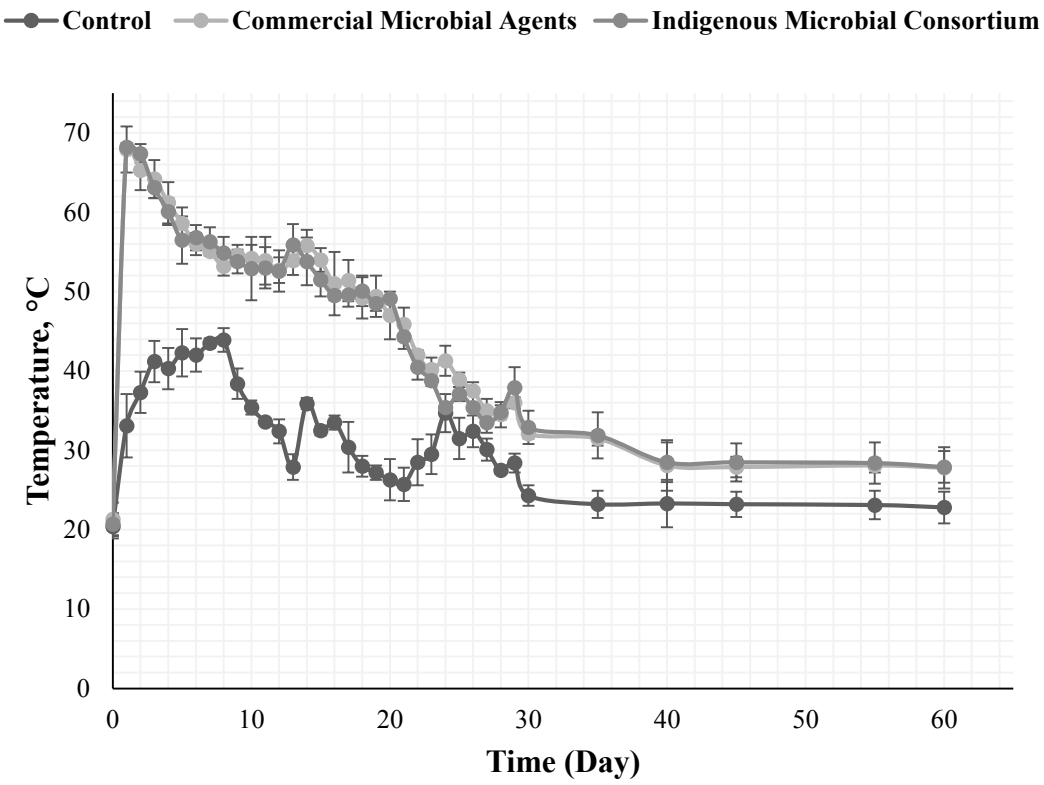


Figure 4.4 Compost temperature profile for 60 days of in-vessel composting.

Several reports have claimed the temperature of the composting process must reach a temperature of 55°C and above to kill pathogens and sanitize the compost (Gea *et al*. 2005). According to Hoitink and Fahy (1986), diseases caused by pathogens can

be controlled through composting as pathogens in the waste would be killed. The composting process of the composting vessel inoculated with commercial microbial agents and indigenous microbial consortium met the sanitary requirement without requiring extra heat application as it remained in the thermophilic phase for approximately 25 days of the experiment. Baharuddin *et al.* (2010) stated that a temperature of 45-55°C must be maintained in a composting process to ensure maximum biodegradation of the raw materials. During the thermophilic stage, cellulose and hemicellulose of the OPEFB would be broken down by the thermophilic bacteria and heat-tolerant fungi. The temperature of the composting vessel inoculated with commercial microbial agents and indigenous microbial consortium decrease gradually after thermophilic phase and entered a curing phase. During the curing phase, lignin would be slowly degraded by the mesophilic microorganisms in the compost. Throughout the composting study, the composting vessel that was used as the control entered the lower range of thermophilic phase from day 3 to day 9 with the highest temperature of 44°C recorded during day 9 before falling back into the mesophilic phase where a temperature around 22-38°C was maintained until the end of the experiment. This was mainly because no microorganisms were inoculated into it. Therefore, not much microbial activity was observed in the control vessel. The temperature of all the experimental group remained the same after day 40 despite turning of the vessel for another 20 days.

(II) Carbon to Nitrogen Ratio Observation

The nitrogen content of the entire experimental group increased gradually throughout the composting process while the carbon content of the compost decreased throughout the treatment. The nitrogen content of the indigenous microbial consortium group rose the highest among the entire experimental group where it was originally 1.0% and increased to 3.0%. It was followed by the commercial microbial agents experimental group where the nitrogen content increased from 1.0% to 2.3%. The control group had the lowest increase in nitrogen content which was 1.0% to 2.0%. The carbon content of the composting material of the composting material was decreased from 40.0% to 33.0% (control), 30.0% (commercial microbial agents), and 31.0% (indigenous microbial consortium) whereby indigenous microbial consortium had the most decrease. The phenomenon of the increase of nitrogen

content and a decrease of carbon could be attributed from the microbial activity on the cellulose substrate and nitrogen, which increased the microbial protein and humic substance (Thambirajah *et al.* 1995). A lower carbon content that is accompanied with a high nitrogen value gives a low C: N ratio for the compost.

Wong *et al.* (2001) mentioned that the carbon to nitrogen ratio (C: N ratio) can be used as one of the indicators to determine the maturation of the compost. C: N ratio of a compost can also be used in determining the stability apart from the maturity of the compost (Baharuddin *et al*. 2010). The C: N ratio was calculated from the carbon and nitrogen content of the composting material from the composting study was used to measure the rate of decomposition of the raw materials. The C: N ratio analysed from the samples collected from the compost vessels on specific time point was illustrated in Figure 4.5. The experimental group for commercial microbial agents and indigenous microbial consortium showed to decreased in C: N ratio and reach a stable condition of 13:1 and 12:1, respectively. An article by Mohammad *et al.* (2014) mentions that compost with a C: N ratio of not higher than 20 could be considered to have reached a satisfactory maturation level. However, Jiménez and García (1992) reported that compost that exhibited a stable ratio of 15:1 and below is often preferable.

The duration of composting was focused on as it is a critical criterion in order to make the composting method fast and feasible for the management of oil palm waste. The C: N ratio profile of the composting experiment using indigenous microbial consortium exhibited to satisfactory results in which better maturity level in a shorter period of time were achieved, which was in line with to other related studies. Studies by other researchers reported by Zainudin *et al.* (2013) to obtain a C: N ratio of 12.4 after 40 days of composting OPEFB with cellulolytic and hemicellulolytic rich untreated rich POME while Baharuddin *et al.* (2009) managed to get a C: N ratio of 12.7:1 after 60-day of co-composting OPEFB with partially treated POME. The indigenous microbial consortium in this experiment exhibited to have a conspicuous result as it exhibited a C: N ratio of 12:1 after 30-day composting when compared to the other researchers study showing its ability to degrade OPEFB effectively.

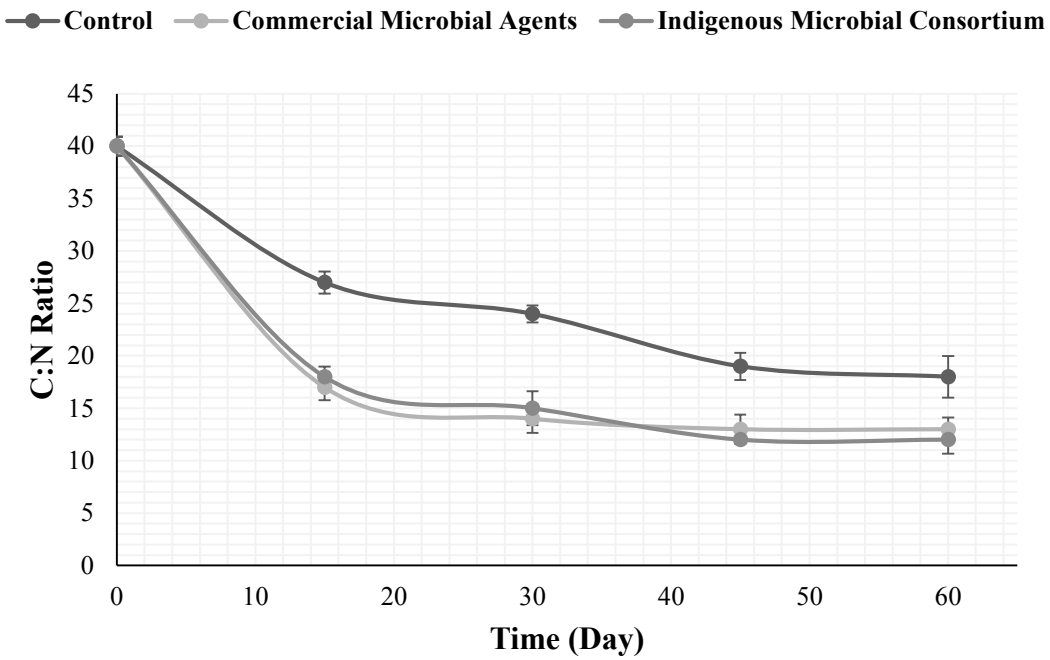


Figure 4.5 Compost C: N ratio profile throughout 60 days of in-vessel composting.

(III) Effect of pH Throughout the Composting Study

Figure 4.6 shows the pH profile of three experimental groups throughout the 60- day in-vessel composting study. The pH of the composting material for the commercial microbial agents and indigenous microbial consortium gradually increase and remained in the pH range of 8.8 to 9.8 from day 3 to day 24. The pH of the commercial microbial agents and indigenous microbial consortium experimental groups increased in the initial stage of the composting as the temperature rises. The increment in pH might due to the increase in ammonia level produced by the biochemical reactions of composting materials that contained nitrogen (Baharuddin *et al*. 2010). Additionally, the partially treated palm oil mill effluent (POME) supplemented to the oil palm empty fruit bunch (OPEFB) could have also contributed to the slightly alkaline condition. POME has a higher pH than OPEFB (Table 4.2), therefore the pH of the composting material increased slightly over time and until reaching a stable pH. Furthermore, bacteria that often dominates during the thermophilic phase in the composting process are not acid tolerant (Baharuddin *et al*. 2010). For this study, the pH for the

commercial microbial agents and indigenous microbial consortium finally decrease to a stable pH of 8.8 and 8.5, respectively in the compost. Fully developed compost have been reported to have a pH value of 8-9 (Sundberg *et al.* 2004). Therefore, the compost produced by commercial microbial agents and indigenous microbial consortium would deem to have reached a matured stage.

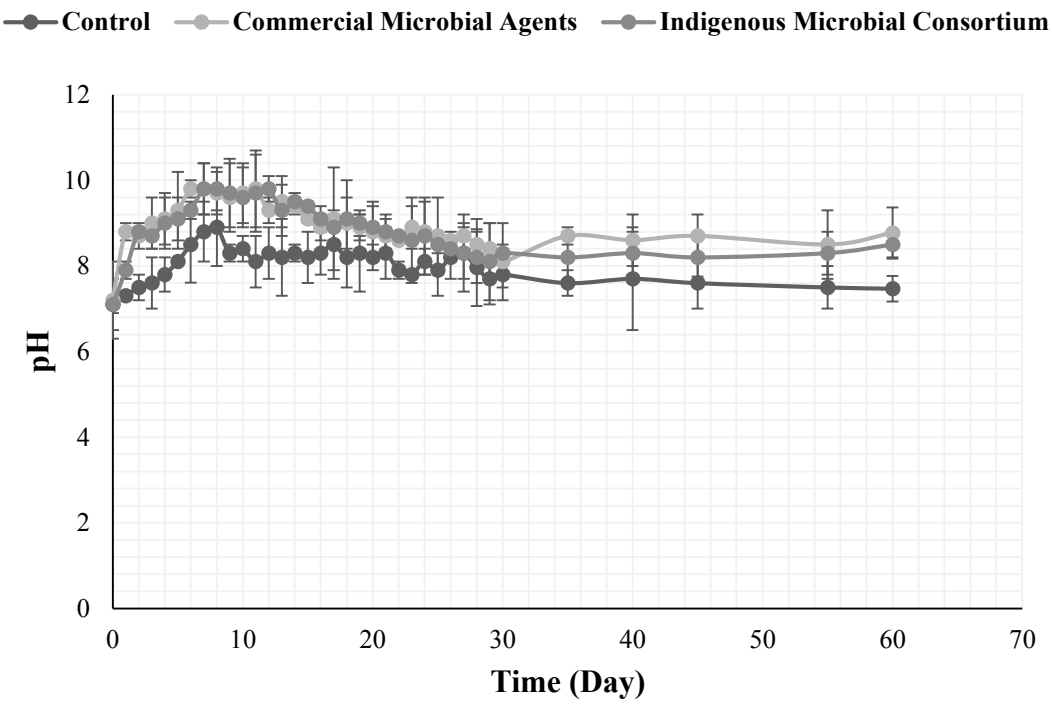


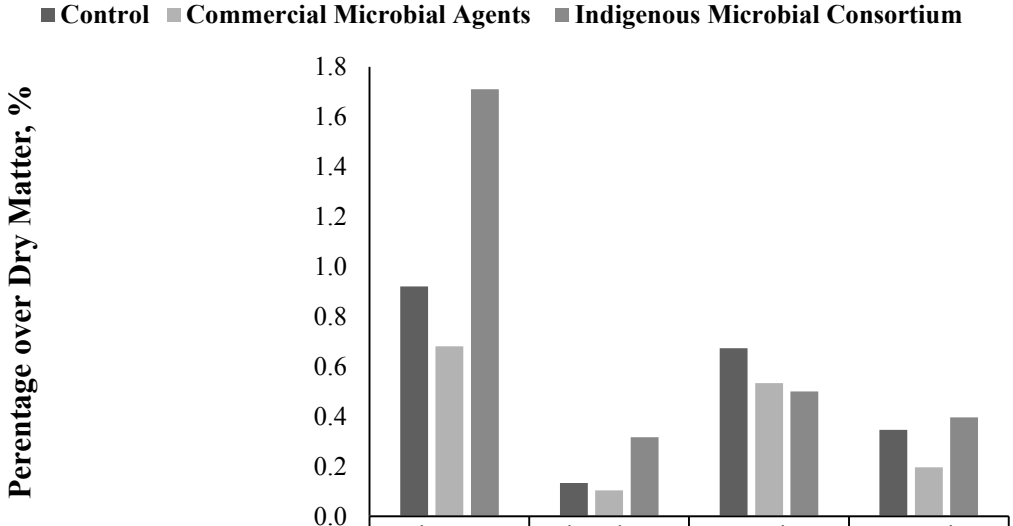
Figure 4.6 Compost pH profile for 60-day of in-vessel composting.

(IV) Nutrient Changes of the Compost Produced

For this study, the percentage of nitrogen, phosphate, potassium, and magnesium present in the compost at the initial and final stage were analysed. The increment of the nutrients in the compost for all experimental group during the composting study can be seen from Figure 4.7.

All four nutrients tested from the collected compost samples showed an increase in nutrients percentage in for each experimental group. All experimental groups exhibited an increase in nutrient content as these could had been contributed by the nutrients available in the partially treated POME that was sprayed onto the

composting material throughout the composting study. Partially treated POME contains appreciable amounts of nitrogen, phosphate, potassium, and magnesium which are vital nutrient elements for the growth of plants (Bala *et al.* 2014). Partially treated POME used in the experiment was tested to have 2.80% of nitrogen, 4.23% of phosphate, 1.80% of potassium and 0.90% of magnesium. Nonetheless, part of the nutrients from partially treated POME could also be used for the growth of microorganisms during the composting process (Baharuddin *et al*. 2010).



	Nitrogen	Phosphate	Potassium	Magnesium
Control	0.920	0.134	0.673	0.347
Commercial Microbial Agents	0.680	0.104	0.533	0.197
Indigenous Microbial Consortium	1.710	0.317	0.500	0.397

Figure 4.7 Nutrient increments of the composts after 60 days of in-vessel composting.

The compost vessels inoculated with the indigenous microbial consortium exhibited to have the highest increase in nitrogen (1.71%), phosphate (0.32%), potassium (0.50%), and magnesium (0.40%) contents among the different experimental groups. The selected cellulolytic microorganisms which consisted of *Bacillus, Penicillium* and *Aspergillus* species were chosen to form the indigenous consortium showed to have nitrogen-fixing and phosphate solubilising as studied and discussed in Chapter 2. These indigenous microbes could have contributed in the increased of nutrient level in the compost.

The nutrients available in the compost that was inoculated with the commercial microbial agents showed lowest nutrient increments of nitrogen (0.68%), phosphate (0.10%), potassium (0.53%), and magnesium (0.20%) among the three experimental groups. The commercial microbial agents used constituted of Bacillus species microbes. Bacillus spp. are found to be one of the most dominant group that contributes highly to the breakdown of cellulose in oil palm empty fruit bunch (OPEFB) as it takes up the nutrient existing in the composting material rapidly (Krishnan *et al.* 2016). Due to the quick uptake of nutrients, the commercial microbial agents could have contributed to the lower nutrient percentage.

The control of the composting study had an increase of nutrients by 0.92% in the nitrogen, 0.13% in phosphate, 0.67% potassium, and 0.35% in magnesium. The control of the experimental group had a higher nutrient content than the commercial microbial agents group in the experiment as no microbial inoculant was inoculated into the control group. Thus, nutrients were only used slightly since no extra microbes was inoculated into it resulting in a higher nutrient increment than the commercial microbial agents group.

The nutrient analysis results of the indigenous microbial consortium experimental groups obtained from the composting study showed a more prominent nutrient increment compared to the other experimental group, hence making it a potential indigenous microbial consortium for the composting of OPEFB in large scale.

4.3.2 Pot Trial

For the end this study, the aim was to develop an effective soil supplement by combining compost and chemical fertiliser to increase the growth of oil palms seedlings. This was to reduce the dependency on chemical fertiliser. 6-month old oil palms were treated with 4 different treatments as shown in Table 4.2. No further nutrients were added to the oil palm seedlings after the commencement of the experiment. The oil palms were watered daily to overcome the water loss due to evaporation (Baharuddin *et al*. 2009). Sufficient water was provided to the oil palm seedlings as water deficiency may cause growth rate to decrease and also an uneven distribution of nutrients in different parts of plants (Cheng *et al.* 2011). Furthermore, fertilisation is most effective when the oil palms are not water-stressed (Sands & Mulligan 1990). Throughout this experiment, the increases in height, diameter of stem, and number of leaves of the oil palm seedlings were observed (Figure 4.1). The results obtained from the study will be presented and discussed in the following sections.

4.3.2.1 Physical Observations After 6-Month of Pot Trial Experiment

(I) Leaf

The number of fully opened leaves of the oil palms was counted as it was useful as one of the indicators to observe the improvement in the growth of treated oil palms. The number of leaves on the oil palms was counted in 3rd and 6th month. Only leaves that have fully opened were counted while the leaves that have not opened were left out of the calculation. The average number of leaves that were counted on the oil palms was tabulated in Table 4.4.

P value of each treatment group was calculated using one-way ANOVA that is available IBM SPSS Statistics 23. From the observation of Table 4.4, only oil palms that were treated with compost group and compost-chemical mixed fertiliser group showed to have significant differences to p-value ($p \leq 0.05$) at the 3rd month of treatment. The oil palms treated with chemical fertiliser did not show a significant difference to the control group ($p > 0.05$) during this period. The oil palms treated with chemical fertiliser and the control group produced 3 new leaves each during the 3rd month. As for 6th month, the treatment groups and the new leaf count (in parenthesis) were: chemical fertiliser (5 leaves), compost group (8 leaves) and

compost-chemical mixed fertiliser group (8 leaves). These showed that oil palms treated with compost produced more new leaves than the untreated oil palm (control).

Table 4.4 Numbers of new fully opened leaves of oil palms over a 6-month period.

	Average Numbers of New Fully Opened Leaves of Oil Palm Seedlings (cm)			
	Control	**Chemical fertiliser**	**Compost**	**Compost-chemical mixed fertiliser**
3rd Month	3 ± 0.00**b**	3 ± 0.00**b**	5 ± 0.32**a**	5 ± 0.19**a**
6th Month	4 ± 0.42**b**	5 ± 0.48**a**	8 ± 0.00**a**	8 ± 0.00**a**

** Note:

Each value is the mean ± standard deviation of the 10 oil palms from each experimental group.

Each value that is significantly different from p value where $p \leq 0.05$ is labelled with the letter 'a' while the label letter 'b' indicates a value that does not have a significant difference to the p-value.

This study showed that that compost may enable more new leaves to be produced. Compost, either used *per se* or in combination with chemical fertiliser, would both enhance newer leaf growth as early as 3rd month, while the chemical fertiliser may only enhance such growth in the 6th months.

(II) Height

During the pot trial study, the height of the oil palm was used as one of the indicators to detect the growth of the trees. The height of the treated oil palm was observed and measured using a normal tape measure shown in Figure 4.1. The height obtained from the observation was recorded in unit cm. The height of oil palms was recorded every three months throughout the pot trial study. The data obtained from the experiment was tabulated in Table 4.5. P value of each treatment group was calculated using one-way ANOVA that is available IBM SPSS Statistics 23.

In the 3rd month of treatment, chemical fertiliser, compost and compost-fertiliser groups exhibited significantly differences with the p-value where $p \leq 0.05$ were noted

when compared to the control group. Control group did not show a significant difference to the p-value in the 3rd month of treatment. The control group took a longer period of time to exhibit a significant difference to the p-value only at the 6th month.

Table 4.5 Height of oil palms over a 6-month period.

	Height of Oil Palm Seedlings (cm)			
	Control	**Chemical fertiliser**	**Compost**	**Compost-chemical mixed fertiliser**
3rd Month	2.98 ± 0.42**b**	5.27 ± 0.63**a**	3.92 ± 0.48**a**	4.52 ± 0.26**a**
6th Month	3.96 ± 0.47**a**	10.35 ± 0.42**a**	8.72 ± 1.33**a**	9.67 ± 0.32**a**

** Note:

Each value is the mean ± standard deviation of the 10 oil palms from each experimental group.

Each value that is significantly different from p value where $p \leq 0.05$ is labelled with the letter 'a' while the label letter 'b' indicates a value that does not have a significant difference to the p-value.

In the 3rd month, chemical fertiliser group showed average height of 5.27 ± 0.63 cm. It was followed by oil palms treated with compost-chemical mixed fertiliser (4.52 ± 0.26 cm), compost (3.92 ± 0.48 cm) and lastly, control (2.98 ± 0.42 cm). All treatment groups, be it compost, chemical fertiliser and their combination, show to improve the height at 3rd month. During the 6th month of pot trial, except for the control group, all groups showed an increase of approximately 5 cm from the 3rd month. Although the chemical fertiliser group showed the higher increase, it is not significantly different from the compost and compost-chemical mixed fertiliser group. In this experiment, the growths of plant height were equally good in all treatment groups. If data were to be obtained beyond 6th month, a clear effect of compost on height could be concluded.

(III) Girth

Stem girth is an important aspect of oil palms as it is during assessment as it shows the potential number of fruit bunches an oil palm can carry at one time (Abidemi, Akinrinde & Obiqbesan 2006). Hence, the changes in the stem girth were measured and used as one of the growth indicator for the oil palms during this pot trial study. A cotton string was used to obtain the stem girth of the oil palm. The cotton string length that went around the stem base was measured with a measuring tape. The same tape measure was used for the measurement of height and stem girth. The data obtained from the study was tabulated in Figure 4.6. P value of each treatment group was calculated using one-way ANOVA that is available IBM SPSS Statistics 23.

Table 4.6 Stem girths of oil palms over a 6-month period.

	Stem Girths of Oil Palm Seedlings (cm)			
	Control	**Chemical fertiliser**	**Compost**	**Compost-chemical mixed fertiliser**
3rd Month	5.37 ± 0.37**b**	5.54 ± 0.48**b**	7.47 ± 0.55**a**	8.11 ± 0.14**a**
6th Month	8.46 ± 0.28**a**	13.72 ± 0.68**a**	15.62 ± 0.69**a**	16.82 ± 0.28**a**

** Note:

Each value is the mean ± standard deviation of the 10 oil palms from each experimental group.

Each value that is significantly different from p value where $p \leq 0.05$ is labelled with the letter 'a' while the label letter 'b' indicates a value that does not have a significant difference to the p-value.

Both the compost group and compost-chemical mixed fertiliser group showed fast effects in stem girth growth as significant stem growths were noted in 3rd month of pot trial with significant p-values ($p \leq 0.01$) in comparison to control group. The control and chemical fertiliser group however only showed significant differences ($p \leq 0.01$) later at 6th month towards the end of the pot trial.

Oil palms treated with compost produced in this study thus showed to enhance the growth of stem girth better than the other experimental groups. Oil palms treated with compost-chemical mixed fertiliser exhibited a good growth of stem girth of 16.82 ± 0.28 cm at the 6th month which were almost doubled the sizes of the stem girths of the control group. The control group that had no fertiliser showed the smallest stem girths when compared to the other experimental groups throughout the study. From the study, the compost group exhibited to a better girth growth when compared to the chemical fertiliser group. The mix of compost and chemical fertiliser exhibited conspicuous results compared to the other treatment groups as it could have provided a better source of nutrients to the oil palm seedling for stem girth growth.

(IV) Nutrient in Oil Palm Leaf

The measurement of nutrient levels in oil palm leaf or foliar analysis was used as an indicator to test the efficiency of the treatment on the growth of the treated oil palms. Foliar analysis was used to determine whether the fertilisers applied were utilised by the oil palms (Pushparajah 1979). Freshly harvested leaves were sent to Sarawak Plantation Services Sdn. Bhd. in Kuching, Sarawak to performed foliar analysis in accordance to MS 677: Parts III, IV, V, VI, VII: 1980 to analysed for the percentage of nitrogen, phosphate, potassium, and magnesium based on the dry leaf matter. From the sampling of the oil palms leaves, the foliar analysis obtained were tabulated in Table 4.7.

Table 4.7 Nutrient levels in oil palm leaves over a 6-month period.

	Nutrient in Oil Palm Leaf (%)			
	Control	**Chemical fertiliser**	**Compost**	**Compost-chemical mixed fertiliser**
Nitrogen	1.506 ± 0.03**b**	1.834 ± 0.01**a**	1.927 ± 0.05**a**	2.011 ± 0.01**a**
Phosphate	0.121 ± 0.03**b**	0.142 ± 0.03**a**	0.154 ± 0.02**a**	0.166 ± 0.01**a**
Potassium	1.285 ± 0.03**b**	1.786 ± 0.02**a**	1.469 ± 0.05**a**	1.606 ± 0.02**a**
Magnesium	0.160 ± 0.04**b**	0.179 ± 0.03**a**	0.305 ± 0.05**a**	0.236 ± 0.01**a**

** Note:

Each value is the mean ± standard deviation of the 10 oil palms from each experimental group.

Each value that is significantly different from p value where $p \leq 0.05$ is labelled with the letter 'a' while the label letter 'b' indicates a value that does not have a significant difference to the p-value.

P value of each treatment group was calculated using one-way ANOVA that is available IBM SPSS Statistics 23. Treated oil palms (chemical fertiliser, compost, and compost-chemical mixed fertiliser) exhibited to have a significant ($p \leq 0.01$) difference from the control group in all the nutrients tested from the foliar analysis.

Leaves from oil palms treated with chemical, compost, and compost-chemical mixed fertiliser were 1.83%, 1.93% and 2.01% respectively, all significantly higher in nitrogen percentage than control which was 1.51%. There was no significant difference among each of the three treatment groups, albeit the compost seemed to provide higher nitrogen nutrient than chemical fertiliser and could have help to contribute to the highest nitrogen level recorded in the compost-chemical mixed fertiliser group. Thus, compost could probably comparable to chemical fertiliser in supplementing nitrogen to oil palm. Nitrogen is one of the major nutrients required by oil palms which known to be responsible for increasing the growth and fruiting. Furthermore, nitrogen also known to promote the growth of leaf and number of bunches with a heavier weight.

In the foliar analysis for phosphate content, the findings were similar to nitrogen content that all treatments groups were significantly different from the control, albeit there was no significant difference among the treatment groups. The phosphate contents for chemical, compost and chemical-compost mixed fertiliser groups were 0.14%, 0.15% and 0.17% respectively, while the control had 0.12% phosphate content as it was not supplemented with any additional nutrients. Although couldn't be proven significant, the phosphate content was higher for the compost group if compared to chemical fertiliser group, and this could have contributed to the highest phosphate level in the compost-chemical mixed fertiliser group. This observation is similar to nitrogen content. This result thus indicating that compost may substitute part of the phosphate requirement by oil palm which is conventionally being provided by chemical fertiliser. Phosphate is one the nutrients required by oil palms for

improvement in the bunch production rate and weight, thus, increasing the yield that could obtain from the oil palm fruit bunch.

The potassium contents in the leaves for the treatment groups were significantly different to control, albeit there was no significant difference noted among the treatment groups. The potassium contents for chemical, compost and chemical-compost mixed fertiliser groups were 1.79%, 1.47% and 1.61% respectively, while the control had 1.29% potassium content. Unlike nitrogen and phosphate, the potassium content was actually lower for compost group if compared to chemical fertiliser group, although the results could not be proven significant. This lower potassium could have pulled down the potassium level in compost-chemical mixed fertiliser group. Thus, compost produced in this study may only be able to provide potassium at a lower level to the oil palm. Potassium is an essential nutrient to oil palm as it helps the oil palm in a similar way with phosphate as it also increases the yield of the oil palm.

As for magnesium, the results were similar to nitrogen and phosphate where compost was shown to provide high level of such nutrient, although the results could not be proven significant. The magnesium contents for chemical, compost and chemical-compost mixed fertiliser were 0.18%, 0.31% and 0.24% respectively, all significantly more than the 0.16% recorded for the control.

It is interesting to note that potassium may play a role in the increment of height in oil palm seedlings in this study. Chemical fertiliser, which showed the largest height increment among the different treatments (Table 4.5), also exhibited to have the highest potassium percentage from the foliar analysis (Table 4.7).

Nonetheless, from the foliar analysis, even though chemical fertiliser showed to have contributed to the highest percentage of potassium, oil palms treated with compost gave exceptional results in high levels of nitrogen, phosphate, and magnesium when compared to chemical fertiliser group and control. The compost seemed to be an equally good source of nutrients to be supplemented to the oil palm seedlings in comparison to chemical fertiliser. From this study, it can also be seen that compost-chemical mixed fertiliser exhibited an exceptional result as it may have combined the good properties of both chemical fertiliser and compost.

(V) Physical Observation of Oil Palms After 6-Month of Experiment

The pot trial experiment was carried out in an open environment at a vacant field. The condition of the surrounding environment was similar to a plantation estate. Unpredictable weather was inflicted onto the oil palms during the trial. Thus, the results obtained from the experiment might be influenced by such factors. The observation of the physical appearance of the oil palm was recorded at the end of the 6-month timeframe of the pot trial. The physical appearance of the treated oil palms (chemical fertiliser, compost, and compost-chemical mixed fertiliser) were placed next to the untreated oil palm (control) for comparison purposes as illustrated in Figure 4.8.

Figure 4.8 Physical appearances of oil palms treated with different treatments which were Chemical fertiliser (A), compost (B), and compost-chemical mixed fertiliser (C) after 6 months of the experiment.

Oil palms treated with the control group were not treated with any additional nutrient. As can be seen in Figure 4.8, oil palms which did not receive any additional nutrient were much smaller in size. It also can be visually seen to be smaller when placed next to treated oil palms. This experiment supported the notion that sufficient and balanced nutrient must be supplemented for the optimum growth of oil palm as suggested by Chen (2006). Chemical fertiliser showed to have the highest height increment when shown in Table 4.5. The physical appearance of oil palms treated with chemical fertiliser appeared to be taller in height when compared to oil palms treated with compost. Oil palms treated with compost however appeared to be physically larger due to larger stem girth and leafier. Oil palms treated with compost mixed with chemical fertiliser were actually slightly larger than both the compost group and chemical fertiliser group. The oil palms treated with compost had leafier and larger stem than the chemical fertiliser group. Hence, from the pot trial study, it

can be said that the compost produced in this study showed to have a better enhancement on the overall growth of oil palms in stem girth and leaves. Conversely, the integration of chemical fertiliser with compost had a better influence on the height of oil palms.

A clearer effect of the compost could be concluded if data were to be obtained beyond 6th months. The compost enriched with chemical fertiliser would continuously show a better influence on the growth of oil palms. Nutrients in chemical fertiliser are often washed away by rain falls shortly after being applied (Sharma & Chetani 2017). Unlike chemical fertilisers, compost conditions the soil to enable the release nutrients to plants slowly over the time span of months and years (Singh *et al.* 2010).

4.3.2.2 Treatment Which Showed Potential as A Soil Supplement

Oil palms that were 6 months of age were treated with four different treatments for a 6-month period. The four groups were the control group (no fertiliser), chemical fertiliser group, compost group, and compost-chemical mixed fertiliser group. From the pot trial experiment, oil palms treated with the compost produced in this study gave an exceptional result when compared to the positive control (chemical fertiliser) and negative control (no fertiliser). The compost-chemical mixed fertiliser group, followed by compost group, showed to have the best influence on increasing the growth of the oil palm seedlings. This development could be due to the growth enhancement abilities of the cellulolytic microorganisms that were inoculated into the compost group during the composting experiment. These cellulolytic microorganisms which showed various growth enhancement abilities were discussed in Chapter 2. The mix population of bacteria and fungi from the genus *Bacillus, Penicillium* and *Aspergillus* were used in the indigenous microbial consortium for the composting of oil palm empty fruit bunch (OPEFB). *Bacillus* spp. were known to have growth enhancement abilities (Arrebola *et al.* 2010; Raupach & Kloepper 1998). The *Bacillus* sp. S2 and S8 isolated in this study exhibited nitrogen fixing and phosphate solubilising ability respectively. Both these *Bacillus* spp. and fungal isolate *Aspergillus* sp. F8, and *Penicillium* sp. F10 had also shown to produce indole acetic acid (IAA) when tested using the Swalkowski method. Lastly, *Penicillium* sp. F8 used in this study had shown nitrogen fixing ability.

Even though compost group and compost-chemical mixed fertiliser group showed a lower height increment than the chemical fertiliser group of 1.63 cm and 0.68 cm based on the

mean height respectively, both compost group and compost-chemical mixed fertiliser group exhibited to have the better influence on stem girth, leaf production, as well as demonstrating high level of essential nutrient contents in the foliar analysis. Thus, the compost produced by this indigenous microbial consortium could also be an effective soil supplement. By supplementing chemical fertiliser with compost, the oil palm seedlings had exhibited slightly better results than the compost when applied *per se*.

In this pot trial study, the integration of compost and chemical fertiliser was shown to improve the growth of oil palm seedling in different aspects. Related studies had also reported that mixing chemical fertiliser with compost would give positive effect to the soil and the plants (Dutta *et al.* 2003; Ahmad *et al.* 2008). The oil palms treated with compost-chemical mixed fertiliser group would be leafier and having thicker stem girth. Hence, the oil palms treated with compost-chemical mixed fertiliser group could be more stable and able to sustain heavier fruit bunches (Abidemi *et al.* 2006). The integration of compost and chemical fertiliser could also help reduce the dependency of chemical fertilisers by 50% for the cultivation of oil palms.

4.4 Summary

The research study of Chapter 4 was focused on the testing the efficiency of the indigenous microbial consortium that was composed of the selected cellulolytic microorganisms isolated from the collected soil samples in degrading oil palm empty fruit bunches (OPEFB) into compost. OPEFB and the indigenous microbial consortium was composed along with partially treated palm oil mill effluent (POME) that helped moisten the compost and supply nutrients for the degrading microorganisms to thrive. The composting study was carried with three different experimental groups which were the control group, commercial microbial agents group, and indigenous microbial consortium group. The composting study was carried out using an in-vessel composting method that studied for 60 days. The composting vessel was turned and sprayed with partially treated POME three times a week to ensure sufficient aeration and moisture level was provided for the growth activities of the degrading microorganisms. Different parameters were analysed throughout the compost study such as moisture, pH, temperature, C: N ratio, and nutrient changes. The pH, temperature, and C: N ratio were used to measure the composting rate of the raw materials. The indigenous microbial consortium group showed to have a comparable result to the commercial microbial agents group. The indigenous microbial consortium group entered the thermophilic phase on the second day along with the commercial microbial agents group and successfully obtained a C: N ratio of 12:1 and 13:1 respectively on day 30 which indicated the compost have reached a matured level. The indigenous microbial consortium group also exhibited higher nutrient increments of 35% N, 57% P, and 9% Mg when compared to the commercial microbial agents group. From the results obtained from the composting experiment, it can be said that an effective indigenous microbial consortium of degrading microorganisms was successfully created in this study as it degrades OPEFB waste into compost group with the help of their enzymes in a shorter period of time of 30days.

In the second half of this chapter, the efficiency of the compost group produced during the composting experiment waste tested for its enrichment potency in the growth of oil palm seedlings. Oil palms seedling that was 6-month of age were obtained and treated with four different treatments namely control, chemical fertiliser, compost, and compost-chemical mixed fertiliser. For compost-chemical mixed fertiliser, only half of the amount

of the chemical fertiliser and compost group used in chemical fertiliser and compost group were used to integrate it together. The pot trial was carried out within a 6-month timeframe. The different parameter of the oil palms seedlings was observed throughout the study. The number of fully opened leaves, stem girth, and height of the oil palms were measured at 3rd and 6th month. At the end of the 6-month pot trial, the leaves were harvested for foliar analysis to obtain its nitrogen, phosphate, potassium, and magnesium contents. From the study, the control group, which has no added nutrient applied to it, exhibited the lowest growth rate based on its growth parameters in comparison with treatment groups. Chemical fertiliser groups showed highest in plant height when compared to the other treatments, followed by compost-chemical mixed fertiliser. However, compost-chemical mixed fertiliser group showed the highest leaf production and stem girth, both significant at $p \leq 0.05$. Compost-chemical mixed fertiliser group also exhibited significant highest nutrient content based on foliar analysis ($p \leq 0.01$). Thus, compost-chemical mixed fertiliser group showed to have the ideal overall good influence on the growth of the oil palm seedlings in this study.

Chapter

5

GENERAL CONCLUSIONS, RECOMMENDATIONS, AND FUTURE WORKS

5.1 General Conclusion

5.1.1 Aims of Thesis

The main aim of this thesis was to isolate cellulolytic microorganisms which produce cellulases and xylanases from Sarawak soil for the composting of oil palm empty fruit bunch (OPEFB) waste produced by the oil palm industry. The rapid development of oil palm industry during recent decades has contributed to a significant amount of wastes that would eventually become a major environmental hazard. Ways to treat and dispose of these wastes must be sought after to reduce the harmful effect that it may inflict to the environment. Therefore, this thesis proposed the use of cellulolytic microorganisms to convert OPEFB waste into compost group through composting.

This thesis reports on the approach to isolate and identify the cellulolytic microorganisms obtained from soil samples. The cellulolytic microorganisms were evaluated for its cellulase and xylanase activities through several bioassays. From the results obtained from the bioassays, five cellulolytic strains which showed high cellulolytic activity were selected to create the indigenous microbial consortium for in-vessel composting of OPEFB. The compost produced was evaluated for its effectiveness in growth enhancement of oil palm seedling via a pot trial. This chapter summarises the results obtained during this research study that has been described in details in chapter 2, 3, and 4. Future works and recommendation of this study were also discussed in this chapter.

5.1.2 Screening and Isolation of Cellulolytic Microorganism

Selective media were used to target microorganisms capable of degrading cellulose and xylan. Carboxymethylcellulose (CMC) agar was chosen as the primary screening and isolation for cellulase producing microorganisms. Microorganisms which showed the ability to degrade cellulose were further tested for its ability to degrade xylan using dyed xylan agar. These strains which exhibited cellulolytic activity on both CMC and xylan were further tested for growth promoting abilities such as nitrogen-fixing, phosphate solubilising, potassium solubilising and indole acetic acid production. Molecular identification of these cellulolytic strains were members of the genus of *Bacillus, Staphylococcus, Pseudomonas, Fusarium, Endomelanconiopsis, Penicillium, Aspergillus* and, *Trichoderma*. The identified cellulolytic strains were further tested through several assays.

Cellulase and xylanase activity of these 24 strains were tested using 2-cyanoacetamide method. *Aspergillus niger* (NBRC 33023) was used as a control strain for the assay. From the isolated strains, five strains showed to have produce high level of cellulases and xylanases in comparison to *A. niger* (NBRC 33023). These five strains were paired and tested respectively for mutual inhibition activity where no antagonism was shown. These five selected cellulolytic strains that showed high cellulase and xylanase productions and no mutual antagonism were selected to form an indigenous microbial consortium for the in-vessel composting study. These five strains which formed the indigenous microbial consortium are *Bacillus* sp. S2 (bacterium), *Bacillus* sp. S8 (bacterium), *P. citrinum* (fungus), *A. nomius* (fungus), and *P. rolfsii* (fungus).

5.1.3 Evaluating Efficiency of Cellulolytic Microorganisms as an Indigenous Microbial Consortium in Degrading OPEFB Via In-Vessel Composting Method to Produce Compost

The efficiency of the indigenous microbial consortium comprised of cellulolytic strains was evaluated for composting of oil palm empty fruit bunch (OPEFB) waste. Three different experimental groups were carried out in triplicates via a 60-day in-vessel composting study: control, commercial microbial agents, and indigenous microbial consortium. The composting vessel was turned and sprayed with partially treated POME 3 times a week to ensure sufficient aeration and moisture level was provided for the growth of the microorganisms. Parameters analysed throughout the compost study were moisture level, pH, temperature, C: N ratio, and nutrient changes.

The indigenous microbial consortium showed comparable efficiency to the commercial microbial agents in composting of OPEFB, as both experimental groups progressed rapidly into the thermophilic phase on the second day. These indicated that that the composting was in good progress. Both groups also reached the mature level on day 30 with a C: N ratio of 13:1 and 12:1 respectively. According to Jimennez and Perez (1992), compost which exhibited a stable ratio of 15:1 and below could be considered as a satisfactory maturation level of compost. The indigenous microbial consortium produced the highest nutrient increment during composting when compared to the other experimental groups. The composting experiment showed that indigenous microbial consortium had successfully degraded OPEFB in a shorter period of time of 30 days when compared to the conventional composting processes that normally require 60-90 days.

5.1.4 Small Scale Pot Trial to Evaluate the Growth Enhancement Potency of Compost Group in Oil Palm Seedlings

A pot trial was conducted to evaluate the efficiency of the compost group on the enhancement of the growth of oil palm seedlings. 6-month oil palm (*Elaeis guineensis*) seedlings purchased from Sarjoh plantation, Sarawak. Four different treatment groups were tested namely control, chemical fertiliser, compost, and compost-chemical mixed fertiliser. Compost-chemical mixed fertiliser group consisted of half the amount of the chemical fertiliser used in chemical fertiliser group, and full amount of compost at the same level that would be used in the compost group. The pot trial employed a completely randomised design with 10 replicates for each treatment. This 6-month trial was aimed to observe the number of fully opened leaves, stem girth, and plant height of oil palm seedlings, as these parameters were often used as growth indicators for oil palms. At the end of the pot trial, the leaves of the oil palm seedlings were also sent to analyse the nitrogen, phosphate, potassium, and magnesium contents.

From the pot trial study, control group without any fertiliser exhibited the lowest growth parameters were compared to the other treatment groups. Chemical fertiliser showed to have the highest in plant height, which was then followed by compost-chemical mixed fertiliser. Nonetheless, chemical fertiliser seemed to be inferior in comparison to compost for leaf production, stem girth growth and leaf nutrient level. Oil palm seedling from the compost group showed to have a better growth of leaves and stem girth, and higher leaf nutrient when compared to the chemical fertiliser group. Interestingly, when compost mixed with chemical fertiliser in the compost-chemical mixed fertiliser group, the best growth of leaves and stem girth were recorded. Compost-chemical mixed fertiliser group also exhibited the highest nutrient content of nitrogen, phosphate, potassium, and magnesium in the foliar analysis. Hence, compost-chemical mixed fertiliser group exhibited to have the best overall influence on the growth enhancement of oil palm seedlings when compared to the other treatments in this pot trial.

5.2 Future Works

Through the findings from this study, composting using indigenous microorganisms shows to be a good and sustainable way to recycle oil palm industry waste into useful compost fertiliser. A mixture of compost with chemical fertiliser had been proven to improve the growth of oil palm seedlings in a pot trial. The selected cellulolytic strains of *Bacillus* sp. S2*, Bacillus* sp. S8*, Penicillium citrinum, Aspergillus nomius, and Penicillium rolfsii* had formed an efficient indigenous microbial consortium that could degrade oil palm empty fruit bunch (OPEFB) in an in-vessel composting study in 30 kg scale. Further investigation of the effectiveness of indigenous microbial consortium in degradation of OPEFB into compost group in larger scale should be studied such as the large scale windrow composting plant. The compost produced from the composting process should be further analysed for its potential enhancement in the growth of oil palms in field trials in plantations for a longer period of time.

Additionally, further works on the improvement of the composting process such as incorporation of fly ash into the composting material could be carried out. Fly ash is a waste material produced from coal power due to the production of energy plants through the use of coal, thus often considered an environmentally harmful substance. However, Mupambwa *et al*. (2015) had claimed that fly ash contains a high amount of essential plant nutrients such as phosphate, but the limited bioavailability of the nutrients itself has prevented the direct implementation in agriculture. If the harmful elements in the fly ash can be minimised while maintaining the phosphate content, it can be used in the agriculture. Fly ash contains very high salinity which in turns is affiliated with high pH. It also incurs boron toxicity which limits microbial activity within the soil when it is assimilated directly to the soil. Although high in phosphate content, fly cash still is lacking of many important plant nutrients and unsuitable in pH. However, a research done by Ravikumar *et al*. (2008) suggests that it may be possible to incorporate compost along with fly ash with the aid of earthworms as a bio-remediation method to convert the fly ash into beneficial nutrients which include nitrogen and phosphate, it also might help improve the pH balance. Incorporating fly ash into the composting process would be a good way to help reduce more types of waste products while increasing the total phosphate concentration in the compost.

Furthermore, the enhancement of the cellulolytic enzyme ability of the selected cellulolytic microorganisms from the soil samples to form the indigenous microbial consortium during this thesis study could be studied. A paper by Adsul *et al*. (2007) investigated on the improvement of *Penicillium* spp. to stimulate higher cellulase production. *Penicillium* spp. showed an increased production of cellulose enzymes through repeated process of mutating the strain through EMS followed by UV-irradiation. This idea can be adapted to increase the cellulase production of the cellulolytic microorganisms analysed in this thesis study. Efficient and improved cellulolytic strains that can produce potent cellulose enzymes would help to break down the OPEFB waste at a faster rate. This will also address the lengthy time needed for composting as it is one of the major limitations of composting process.

5.3 Concluding Statements

This thesis aimed towards isolating indigenous cellulolytic microorganisms that have the abilities to produce cellulases and hemicellulases for the composting of oil palm empty fruit bunch (OPEFB) waste. Selective media were used to screen and isolate a total of 24 cellulolytic microorganisms from soil samples taken from various locations within Sarawak. Several bioassays were also performed to eventually select five effective cellulolytic strains to create an indigenous microbial consortium to degrade OPEFB via in-vessel composting within 30 days. The compost produced was shown to be able to improve oil palm growth based on significant improvement in leaf growth, stem girth and nutrient level, either applied *per se*, or in combination with chemical fertiliser. The aims and objectives of this study have been successfully achieved. The findings obtained from this study suggested that the isolated strains were potential OPEFB degraders to produce compost for the growth enhancement of oil palm seedlings.

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Appendix

Section 1 Media Preparation

Carboxymethylcellulose (CMC) Medium Preparation

CMC medium was prepared using the modified recipe as shown in Table 1. The addition of agar-agar powder was added to the medium only when a solid growth medium was required.

Table 1: Ingredients required for the preparation of CMC agar.

Ingredients	Amount per litre
Magnesium Sulfate Heptahydrate, $\boldsymbol{MgSO_4.7H_2O}$	0.5 g
Potassium chloride, *KCl*	1.0 g
Ammonium dihydrogen phosphate, $\boldsymbol{NH_4H_2PO_4}$	1.0 g
Yeast powder	1.0 g
Glucose	1.0 g
Carboxymethylcellulose powder	8.0 g
Agar-agar powder	17.0 g
Deionised water	1000 mL

* Chemicals used in the media were from Sigma-Aldrich, USA.

Xylan Agar Preparation

(i) Preparation of Dyed Xylan

Xylan for the xylan agar was first prepared by dying the xylan from beechwood powder with Remazol Brilliant Blue-R (RBBR) dye. The ingredients used in preparing the dyed xylan are as shown in Table 2.

Table 2: Ingredients required for preparing dyed xylan.

Ingredients	Amount per preparation
Xylan, from beechwood	2.5 g
Remazol Brilliant Blue –R	2.5 g
Deionised water, $\boldsymbol{dH_2O}$	60 mL
Sodium hydroxide solution, *NaOH* (1.5g in 20mL sterile $\boldsymbol{dH_2O}$)	20 mL
Sodium acetate solution, $\boldsymbol{C_2H_3NaO_2}$ (0.675g in sterile $\boldsymbol{dH_2O}$)	20 mL
96% ethanol	200 mL
Wash solution (660mL ethanol, 330 mL $\boldsymbol{dH_2O}$, 1.35g sodium acetate)	1 L
Acetone	50 mL

* Chemicals used in the media were from Sigma-Aldrich, USA except for xylan from beechwood which was produced by Himedia, India.

Xylan and Remazol Brilliant Blue-R (RBBR) dye were first mixed together in a 500 mL beaker containing 60 mL of deionised water. The RBBR-xylan solution was stirred using a magnetic stirrer that was placed on a hot plate (Favorit, Malaysia) with its heating plate off. At room temperature (25°C), 20 mL of sodium acetate solution was added drop wise every 5-minute interval while leaving the mixed solution to stir continuously. After mixing in the sodium acetate solution, 20 mL of sodium hydroxide solution was poured into it and left to stir for another 90 minutes at room temperature (25°C). To precipitate the RBBR-xylan, 200 mL of 96% ethanol was added to the mixture. The precipitate of the RBBR-xylan was obtained by using a vacuum flask and Whatman filter paper No. 1. The precipitate was washed sequentially with 1 L of wash solution until a colourless filtrate was present. The final precipitate was washed with 100 mL 75% ethanol followed by 50 mL of acetone. The precipitate was left to dry in a fume hood (Lab Craft Company, USA) overnight then kept in a sterile container until required for analysis.

(ii) Preparation of Xylan Agar

Xylan agar was prepared using the modified recipe from CMC agar as shown in Table 1. The CMC powder was replaced with 2 g/L of dyed xylan. The addition of agar-agar powder was added to the medium only when a solid growth medium was required.

Preparation of Nutrient Agar (NA)

NA (Himedia, India) was prepared by suspending 28.0 g of the NA powder in 1L of distilled.

Preparation of Potato Dextrose Agar (PDA)

PDA (Himedia, India) was prepared by suspending 39.0 g of the PDA powder in 1 L of distilled water.

Preparation of Jensen's Agar Medium

Jensen's medium (Himedia, India) was prepared by suspending 39.1 g of the Jensen's medium powder in 1 L of distilled water.

Preparation of Pikovskaya Agar

Pikovskaya agar (Himedia, India) was prepared by suspending 31.3 g of the Pikovskaya mediums powder in 1 L of distilled water.

Preparation of Aleksandrov Agar

Aleksandrov agar (Himedia, India) was prepared by suspending 29.60 g of Aleksandrov medium powder in 1 L of distilled water.

Preparation of Nutrient Broth (NA)

The NB (Himedia, India) was prepared by suspending 13.0 g of the NB powder in 1 L of distilled water.

Preparation of Potato Dextrose Broth (PDB)

PDB (Himedia, India) was prepared by suspending 24.0 g of the PDB powder in 1 L of distilled water.

Sterilization and Storage of Growth Mediums

All growth mediums, chemicals, and glassware used in this study were sterilised by autoclaving at 121°C, 103.42 kPa for 20 min using an autoclave machine (Hirayama-HVE-50, Japan). Growth mediums containing agar that has been sterilised were placed into 60°C oven until required while growth mediums in broth were kept tightly capped on the clean bench working area.

Dispensation of Growth Mediums

Sterilised mediums that contained agar were aseptically dispensed into a sterile 90mm petri dish in a laminar flow cupboard (ESCO, Singapore) to avoid contamination. The sterilised medium was mixed well by inverting the Duran Schott bottle before dispensing its content into the petri dishes. The petri dishes containing the freshly poured agar was left open to dry for 45 minutes in the laminar flow cupboard before being closed and kept aside until required.

Section 2 Standard curve of glucose constructed using the 2-cyanoacetamide method.