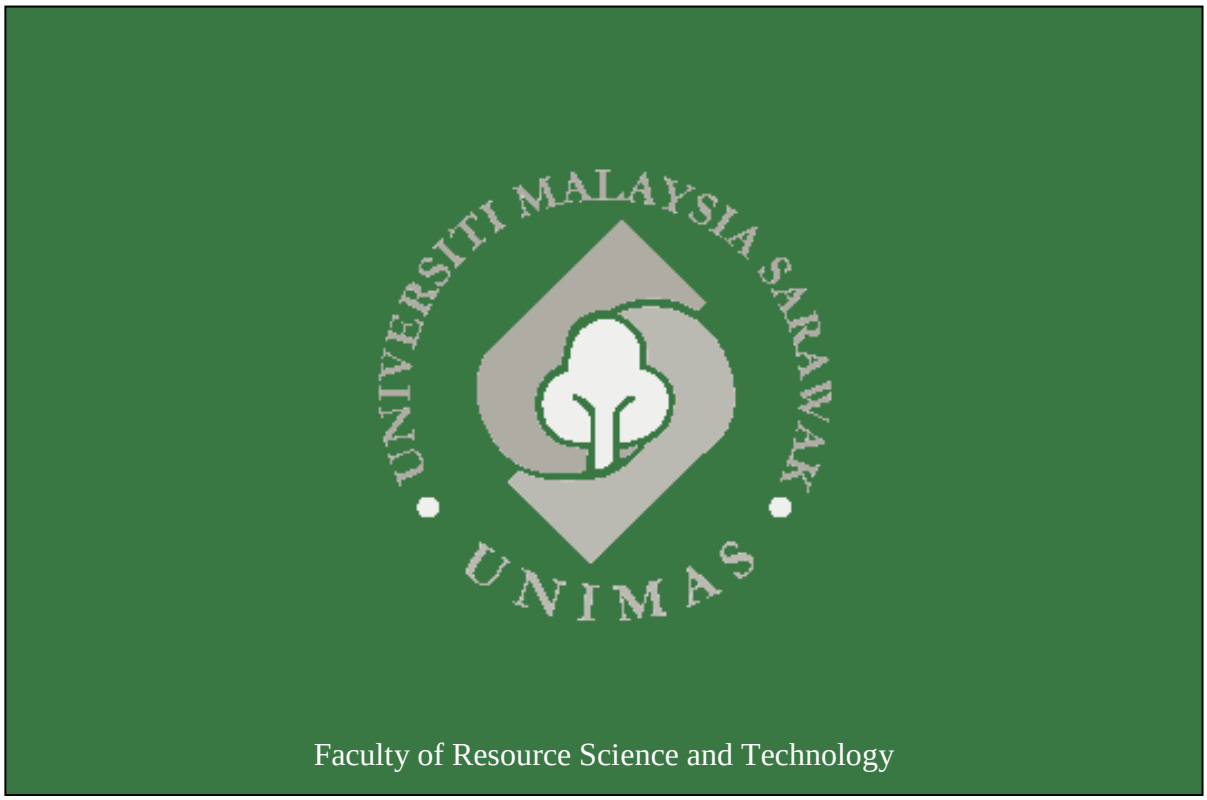

DEVELOPING EFFICIENT BACTERIAL CONSORTIA TO ENHANCE THE BIODEGRADATION OF OIL PALM EMPTY FRUIT BUNCH (EFB) AND SAWDUST LIGNOCELLULOSES WASTE

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Bachelor of Science with Honours

(Resource Biotechnology)

2010

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

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2010

ACKNOWLEDGEMENT

Above all, I am grateful to God for the strength and wisdom He had given to me.

I thank my supervisor, Dr. Awang Ahmad Sallehin, for the opportunity he gave to me. His guidance and encouragement had enabled me to complete this Final Year Project. To the entire unit of Molecular Genetic Lab - Mdm. Sheela Ungau, Farhan, Angel, Hidayah, Lan, Fraser and George, thank you for your support.

To my dearest colleagues – Cass, Jan, Erin, Shalini, Nik, Farith, Chua, Mizi, Praveen, Shahirah, Aalia, Eliane, and also to my lab mates – Q, Jack, Oliver, Ekin, Topek, and Soff, thank you for all the fun we had together. Special thanks to, Aisha, who has taught me much about life for the past 3 years. To Jayne, thank you for being a wonderful friend.

Finally, I dedicate this work to my siblings, Joel, Jody, and Jocy who always have faith in me and to my beloved parents, Joseph Blandoi and Julita Serudin - Both of you are my biggest inspirations. Thank you for the unconditional love.

Joan Alicia Joseph Blandoi

March 2010

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CFU	Colony-Forming Unit
DNS	Dinitrosalicylic acid
EFB	Empty Fruit Bunch
GI	Germination Index
LB	Luria Broth
NA	Nutrient Agar

Developing Efficient Bacterial Consortia to Enhance the Biodegradation of Oil Palm Empty Fruit Bunch (EFB) and Sawdust Lignocelluloses Waste

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ABSTRACT

Empty fruit bunch (EFB) is the lignocellulosic by-product from the oil palm plantation. Without efficient management, EFB could be problematic to the environment. This study aims to develop the microbial consortium for an efficient biodegradation of EFB through windrow composting. Three microbial isolates, *Bacillus licheniformis* P7, *Bacillus amyloliquefaciens* UMAS1002, and *Pseudomonas aeruginosa* IP2 were tested on their ability to degrade EFB based on the parameter time of incubation for 14 days and parameters pH and percentage of inoculums for 10 days. The reducing sugar produced was determined by using Dinitrosalicylic (DNS) method. The best bacterial consortium was inoculated every 10 days of 30-days of EFB composting with uninoculated compost as control. On day 30, the moisture content of inoculated compost is 109.82% with dry mass 0.478 g. The pH is alkaline at 9.68 with bacterial count at 229×10^7 CFU/µl, both lower than control. The reducing sugar produced is 0.477 mg/ml, higher than control and Germination Index (GI) at 1.12 is lower than control. Bacterial consortium AB, consisting of *B.amyloliquefaciens* UMAS1002 and *B.licheniformis* P7 is the best microbial consortium developed for EFB degradation. Inoculation of this consortium into EFB compost has less effect in EFB degradation.

Key words: EFB, bacterial consortium, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, windrow composting

ABSTRAK

Tandan sawit kosong (TSK) merupakan produk sampingan dari kilang kelapa sawit. Tanpa pengurusan yang betul, TSK boleh menyebabkan masalah kepada alam sekitar. Kaedah penghasilan kompos merupakan satu penyelesaian kepada pengurusan TSK. Kajian ini bertujuan untuk mencari konsortium bakteria terbaik untuk diinokulasi ke dalam kompost TSK bagi mempercepatkan proses biodegradasinya. Bacillus licheniformis P7, Bacillus amyloliquefaciens UMAS1002, dan Pseudomonas aeruginosa IP2 diuji mengikut parameter masa inkubasi selama 14 hari serta parameter pH dan peratusan inokulum selama 10 hari. Kaedah asid Dinitrosalisaklik (DNS) digunakan untuk menentukan gula penurun yang dihasilkan. Konsortium bakteria terbaik dipilih untuk diinokulasi ke dalam kompos TSK setiap 10 hari selama 30 hari. Kompos tanpa bakteria dijadikan sebagai kawalan. Pada hari ke-30, kandungan air kompos ialah 109.82% dengan berat kering ialah 0.478 g. pH kompos ialah alkali pada 9.68 dengan bilangan bakterianya ialah 229×10^7 CFU/µl. Kandungan gula penurun ialah 0.477 mg/ml manakala Indeks Percambahan (GI) ialah lebih rendah berbanding kompos kawalan iaitu pada 1.12. Konsortium bakteria AB yang terdiri daripada B.licheniformis P7 dan B.amyloliquefaciens UMAS1002 merupakan konsortium terbaik dan diinokulasi ke dalam kompos TSK. Kesan inokulasi didapati tidak membawa perubahan ketara kepada biodegradasi kompos TSK.

Kata kunci*: TSK, konsortium bakteria, Bacillus licheniformis, Bacillus amyloliquefaciens, kompos*

CHAPTER 1

INTRODUCTION

The Malaysian oil palm industry covers more than 8% of the total land area in Malaysia (Fuad *et al*., 1999). As in 2009, Sarawak has a total oil palm plantation area of 5914.71 km^2 (Chiew, 2009). Together with this development, the industry implements green agriculture such as planting of oil palm trees in terraces, usage of silt pits, and planting of ground legume cover to conserve soil and water to sustain and conserve the environment (Chan, 1999). Despite this, the industry main problem lies in its lignocellulosic by-products which are generated after processing.

In Malaysia, approximately 40 million tonnes of oil palm biomasses such as empty fruit bunch (EFB), trunks, and fronds are produced every year (Kabbashi *et al.*, 2007). Various approaches had been developed to manage these by-products. In the case of EFB, it is being applied as mulch (Alam *et al.*, 2005) and study had proven that carbonizing EFB will produce charcoal (Lim *et al.*, 2004). Through bioconversion, EFB is also suitable for production of fuel ethanol (Lim, 2004).

Although oil palm industry in Malaysia is practising environment-friendly management techniques such as recycling EFB as mulch in plantation area and zero-burning of oil palm residues, some countries do not restrict the burning of these residues (Levine, 1996 (as cited by Howard *et al.*, 2003; Malherbe & Cloete, 2002; Alam *et al.*, 2005). Consequently, this could lead to serious air pollution. Three major factors leading to

the disposal of EFB are identified, namely, due to the complex biochemical structure of the lignocelluloses, the tedious management process of the residues, and less efficient biological techniques compared to chemical digestion technique. The descriptions of each factor are as described below.

The biochemical structure of lignocellulose is very complex. It consists of three components; lignin, cellulose, and hemicelluloses. For degradation to take place, lignin must first be removed for further degradation of cellulose and hemicelluloses. The major setback is due to lignin recalcitrance towards degradation (Hammel, 1997; Howard *et al.*, 2003; Lim, 2004) hence, making large-scale treatment processes time and energy consuming (Malherbe & Cloete, 2002). Despite of its complexity, the lignocellulosic wastes had been studied widely for the past few years because of its importance in the production of various value-added products (Howard *et.al*, 2003).

The management of EFB involving many stages, including storing, transporting, distribution, and treatment, which are very expensive (Schuchardt *et al.*, 2002; Suhaimi & Ong, 2001). This issue also highlighted by Chiew (2009), where the use of EFB as fuel to generate electricity in Malaysia faces difficulties due to inefficient combustion of bulky EFB and transportation problem to the location of power plant.

For the treatment of lignocellulosic wastes in oil palm plantation, it is found out that the chemical method is more efficient than biological techniques (Sunitha & Varghese, 1999). Chemical method is defined as the usage of alkali, acids or salts in the pre-treatment

process of these wastes (Mtui, 2009). Despite being less-selective and toxic, chemical method is preferred due to the limited number of microorganisms capable in complete degradation of the lignocellulosic components, specifically lignin. For biological methods, Phanerochaete *chrysosporium* is the only fungus capable in degrading lignin completely (Crawford, 1981, as cited by Alic & Gold, 1991).

Other microorganisms like actinomycetes are capable in modifying lignin but lack of the capacity to degrade lignocelluloses efficiently (Hammel, 1997). It is also found out that only few filamentous fungi are capable of hydrolyzing cellulose (Niamke & Wang, 2004). In a study by Kaplan & Hartenstein (1980) on synthetic-lignin biodegradation, it is found out that bacteria have limited ability in degrading lignin. Certain microorganisms require other microorganisms to degrade efficiently such as cellulase-producer fungus, *Trichoderma reesei* is incapable of converting cellulose directly into a useful final product individually (Niamke & Wang, 2004).

The management of these biomasses could be problematic if efficient strategy is not implemented. Lignocelluloses biotechnology could play the important roles in management of these biomasses by setting up a low cost, faster method for production of compost and using a safer technique in the treatment process. Optimization stages of composting parameters could also speed up the biodegradation of the lignocellulosic components. According to Mtui (2009), composting is a cheaper method when biological approach is being applied. EFB can be utilized for production of compost.

Therefore, this research aims to apply biological approach in the production of compost by developing the best microbial consortium from three different isolates and to set up an efficient composting technique by shortening the biodegradation process through the inoculation of the best bacterial consortium. Three bacteria were selected for this purpose, namely, *Bacillus amyloliquefaciens* UMAS 1002, *Bacillus licheniformis* P7, and *Pseudomonas aeruginosa* IP2.

These goals are achieved through specific objectives which are to set up the bacterial consortia, to optimize the parameters for composting, to select the best bacterial consortium for lab-scale composting, to perform the compost analyses and to test on the compost maturity.

CHAPTER 2

LITERATURE REVIEW

2.1 Empty fruit bunch

EFB is a by-product of stalks with empty spikelets (Chan, 1999). In the past, EFB was burnt to generate steam at mills and its ash that content is of 30% potassium, can be applied as fertiliser (Ma *et al.*, 1993, as cited by Suhaimi & Ong, 2001). However, when burning method was prohibited to prevent air pollution, EFB is commonly applied as mulch in the oil palm plantation area (Alam *et al.*, 2005; Suhaimi & Ong, 2001). Moreover, incineration destroys any valuable nutrients of the EFB (Singh *et al.*, 1999). In fact, the benefits of applying EFB as mulch had been long known since 1934 (Abdullah *et al.*, 1987, as cited by Chiew & Rahman, 2002).

2.1.1 Composition

Deraman (1993, as cited by Suhaimi & Ong, 2001) stated that EFB is compost of 45 to 55% of cellulose and about 25 to 35% of hemicelluloses and lignin. EFB is also rich in nutrients such as Potassium (K), Nitrogen (N), Magnesium (Mg), and Phosphate (P) (Chiew & Rahman, 2002). These nutrients are recycled back to the soil when applied in oil palm plantation area. According to Singh *et al.* (1990, as cited by Singh *et al.*, 1999), a tonne of EFB is equivalent to 7 kg urea, 2.8 kg rock phosphate, 19.3 kg of muriate of potash, and 4.4 kg of kieserite. The nutrient-rich EFB makes it a suitable organic fertilizer.

2.1.2 EFB utilization

In comparison with the non-mulched planting system, the mulched palms reached maturity earlier 10 months (Chan, 1999). The disadvantages of applying EFB as mulch in the oil palm plantation area include high transportation cost, distribution cost, tedious process of degradation, and its attractiveness for beetles and snakes (Schuchardt *et al.*, 2002). The long process of degradation is due to the lignin content of the EFB. This can be solved by pre-treating EFB to produce compost before applying it to the oil palm plantation area. Example of pre-treatment method is to add Palm Oil Mill Effluent (POME) during composting to speed up the process (Schuchardt *et al.*, 2008). The condition of EFB during mulching can be improved by adding nitrogen and phosphate (Singh *et al.*, 1999). Both composting and mulching techniques could conserve the nutrients of the soil, minimises environmental hazards by replacing chemical fertilizers, and leads for better productivity of oil palm (Chee & Chiu, 1999).

2.2 Lignocellulose components

Lignocellulosic waste is defined as the by-products from the agriculture, forestry, and paper and pulp industry (Lankinen, 2004). Lignocellulose is the composite material formed from the binding of the three types of polymers, found in the cell walls of the vascular tissues of higher land plants (Glazer & Nikaido, 2007). The compositions of these three components in different plants are influenced by genetic and environmental factors (Malherbe & Cloete, 2002). On average, there are 25% of lignin, 45% of cellulose, and 30% of hemicelluloses in trees (Glazer & Nikaido, 2007).

These biomasses which were previously disposed off as wastes are now considered to be valuable sources for production of animal feed, biofuel, compost, soil conditioner,

fertilizer, and to be used in paper and pulp industry (Howard *et al.*, 2003). The lignocelluloses bioconversion process can only be achieved to produce value-added products when the aromatic building blocks and the polysaccharides are removed.

2.2.1 Lignin

Lignin is the most abundant aromatic polymer on earth (Glazer & Nikaido, 2007). Lignin is also ranked the second most abundant renewable biopolymer in nature after cellulose (Lankinen, 2004; Crawford, 1981, as cited by Hammel, 1997). Generally, lignin is consists of the following precursors, namely, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Howard *et al.*, 2003; Lankinen, 2004). Lignin can be further described as softwood lignin, hardwood lignin, or grass lignin (Glazer & Nikaido, 2007).

As lignin is known to be the most recalcitrant part of lignocelluloses, this suits its function to provide rigid structures to plants. For examples, softwoods contain higher lignin compared to hardwoods and allow huge trees with hundred feet tall to remain upright (Glazer & Nikaido, 2007). Other functions of lignin are in the water and nutrient supply and acting as barrier for cellulose and hemicellulose from microbial attack (Hakala, 2007; Hammel, 1997; Paterson, 2008; Crawford & Crawford, 1976). Hammel (1997) also stated that lignin is insoluble in water, pointing out this as a limiting factor that slows down the ligninolysis process. More energy is required to separate lignin from cellulose and hemicellulose. Therefore, the biodegradation of lignin is the rate-limiting step because the process requires high energy (Paterson, 2008). For quantitative lignin degradation studies, radioactive methods are usually used (Li *et al.*, 2009). Generally, this method involves the measurement of ^{14}C-labelled lignins.

2.2.2 Cellulose

Cellulose is the most abundant and renewable organic compound on earth (Glazer & Nikaido, 2007; Bhat & Bhat, 1997). Structurally, it is consists glucose molecules linked together by β-(1,4)-glycosidic bonds which forms the cellobiose as the basic repeating unit (Sanchez, 2009; Glazer & Nikaido, 2007). It is usually in a crystalline form. The non-crystalline form is known as the amorphous regions of cellulose. Three enzymes required to hydrolyse cellulose are endoglucanase, exoglucanase, and *β*-glucosidase that function to restrict monomer between bonds, at the end of the chain, and dimers, respectively (Malherbe & Cloete, 2002).

2.2.3 Hemicellulose

Hemicelluloses are highly branched, non-crystalline heteropolysaccharides consisting of pentoses, hexoses, and uronic acids (Glazer & Nikaido, 2007). The enzymes required for the hydrolysis of hemicelluloses are similar to cellulose. However, more enzymes are required for its hydrolysis since the structure is much more complex compared to cellulose (Malherbe & Cloete, 2002).

2.3 Ligninocellulolytic microorganisms

There are numbers of microorganisms capable in degrading lignocellulosic components. These microorganisms are mainly fungi and bacteria (Howard *et al.*, 2003). Both fungi and bacteria are used as the biological pre-treatment of lignocellulosic wastes (Mtui, 2009).

2.3.1 Bacteria

Bacteria are known to have limited capability in degrading lignin but capable in degrading cellulose and hemicellulose. Bacteria of genera *Alcaligenes*, *Arthrobacter*, *Nocardia*,

Pseudomonas, and *Streptomyces* are able to degrade single ring aromatic substrates (Mahadevan, 1991, as cited by Li *et al.*, 2009). The degradation is achieved by enzyme activity. In a study by Blanchette (1995, as cited by Li *et al.*, 2009), bacteria degrades the cell wall by tunneling, erosion, and cavitation. Tunneling bacteria attack by producing small tunnel to migrate through the cell wall; erosion bacteria attack from the lumen (Holt, 1983, as cited by Li *et al.*, 2009); while cavitation bacteria utilized the products (Singh *et al.*, 1990, as cited by Li *et al.*, 2009).

Bacteria are used as the biological pre-treatment of lignocellulosic biomass. This involves both aerobic and anaerobic systems (Mtui, 2009). Under anaerobic condition, bacteria are incapable to degrade lignin (Glazer & Nikaido, 2007). However, it is found out that bacteria that degrade cell wall by erosion are capable to tolerate near or fully anaerobic conditions (Kim *et al.*, 1996; Bjordal *et al.*, 1999, as cited by Li *et al.*, 2009). These erosion bacteria are typically rod-shaped. It is also suspected that under anaerobic conditions, bacterial consortia had degraded the ^{14}C-labelled lignin (Holt & Jones, 1983, as cited by Li *et al.*, 2009). The process of lignin degradation under anaerobic conditions by bacteria might be slow but it is noted that this process is significant (Li *et al.*, 2009).

2.3.2 Fungi

The degradation of lignocelluloses by fungi is of commercial importance (Malherbe & Cloete, 2002). So far, *Phanerochaete chrysosporium* is the best fungi being studied since it is capable in degrading lignin completely (Crawford, 1981, as cited by Alic & Gold, 1991; Malherbe & Cloete, 2002).

2.4 Composting

Composting is the process or technique involves in the treatment of organic materials that recycles organic matters and nutrients (Rynk & Richard, 2001). The end product of composting is compost, which is described as a nutrient-rich, organic fertilizer and soil conditioner, produced from the biodegradation of lignocelluloses components by microorganisms (Mtui, 2009; Day & Shaw, 2001). The benefits of composting had been known for a long time. Our ancestors had observed that growing crops on a site near rotting of vegetations or manure had resulted in healthy crops compared to other sites (Day & Shaw, 2001). With the current development of green technology, composting is considered important because it is a low-cost technique that could convert lignocellulosic wastes into value-added products.

Composting stimulates environmental awareness worldwide as it can be practised at home or for commercial purposes (Haruta *et al.*, 2005, as cited by Vaz-Moreira *et al.*, 2008). The applications are in the bioconversion process of various agricultural wastes such as sugar wastes (Satisha & Devarajan, 2007), pepper plant waste (Vargas-Garcia *et al.*, 2007), rice straw (Yu *et al.*, 2009), and EFB (Schuchardt *et al.*, 2002).

Composting can be done either using open methods or contained methods (Rynk & Richard, 2001). Windrow and static piles are examples of open methods while horizontal agitated beds and rotating drums are examples of contained methods.

2.4.1 EFB composting

Schuchardt *et al.* (2002) described the rotting process during EFB composting into five steps which are, the chopping of EFB into reduced sizes, the forming of heaps ready for composting, the turning of heaps, the watering of heaps, and the screening of the finished compost. These processes are similar to the method used by Vargas-Garcia *et al.* (2007). It is also reported that EFB can be used as compost within 2 to 12 weeks (Schuchardt *et al.*, 2002).

2.4.2 Composting parameters

The optimization of composting parameters is essential to provide the best condition for production of compost. The parameters are temperature, pH, moisture content, aeration, C:N ratio, and particle size. In traditional composting, these factors were ignored and hence, the final composts were of poor quality (Taiwo & Oso, 2004). Optimum parameters enable the microorganisms to efficiently degrade the composting materials.

2.4.2.1 Temperature

Temperature is an important factor that determines the biological activity of microorganisms (Day & Shaw, 2001). Thermophilic composting is an efficient system because it enables the rapid decomposition of the starting materials and killing any pathogenic microorganisms (Trautmann & Krasny, 1997). Different starting materials results in a different optimum temperature.

2.4.2.2 pH

Composting is relatively insensitive to any pH change (Epstein *et al.*, 1977, as cited by Day & Shaw, 2001). The pH values vary from 5.5 to 8.5 during composting (Trautmann & Krasny, 1997). This is contributed by the microbial activity throughout the course. In the early stage of aerobic composting, the pH usually drops due to the organic acids accumulation (Day & Shaw, 2001) which is the by-products of microorganism digestions (Trautmann & Krasny, 1997). At this stage, the condition is favourable for growth of fungi which are active in lignin and cellulose degradation (Trautmann & Krasny, 1997). The organic acids will be further broken down resulting in the rise of pH (Trautmann & Krasny, 1997).

As the composting process continues, the pH value becomes neutral once these organic acids are converted to methane and CO_2 (Day & Shaw, 2001). A finished compost is in the pH range of 6 to 8 (Trautmann & Krasny, 1997) but usually it is slightly alkaline, which is at pH 7.5 to 8.5 (Day & Shaw, 2001).

In anaerobic composting, the pH tends to be acidic (Trautmann & Krasny, 1997). This is due to the accumulation of organic acids which can limit the microbial activity. It can be prevented by frequent turning to provide aerations.

2.4.2.3 Moisture content

The best moisture content for composting is at 50-60% (Trautmann & Krasny, 1997). Higher moisture content results in nutrients loss in the form of leachate (Day & Shaw, 2001) or causing anaerobic condition in compost due to ineffective diffusion of oxygen (Golueke, 1989; Hamoda *et al*., 1998; McGaughey & Gotass, 1953; Poincelet, 1977 & Wiley, 1957, as cited by Day & Shaw, 2001). In a drier condition, nutrient cannot be solubilised and thus, inhibiting the microbial activity in the compost (Trautmann & Krasny, 1997). According to Sullivan & Miller (2001), when the moisture content of compost increases, the dry mass decreases. High moisture content can be treated by aeration while low moisture content is treated to the addition of water.

2.4.2.4 Aeration

The importance of aeration are to provide oxygen and to remove heat, moisture, CO_2, and other decomposition products which can be generally applied either through passive aeration or forced aeration (Rynk & Richard, 2001). In windrow system, mixing or turning the piles is a way to provide aeration (Krasny & Trautmann, 1997). Turning of piles is an example of passive aeration. In forced aeration, fans and special ducts are required to move air within the composting materials (Rynk & Richard, 2001). Other than balancing the level of oxygen and moisture in the compost, aeration is also required to properly mix the drier and cooler parts to the center of the pile to promote optimal decomposition (Krasny & Trautmann, 1997).

2.4.2.5 C:N ratio

It is important to formulate the starting materials for compost with a suitable C:N ratio. Carbon acts as the energy source and nitrogen is a crucial element in proteins, amino acids,

enzymes, and DNA which is necessary for microbial growth (Trautmann & Krasny, 1997). The suitable carbon-to-nitrogen ratio is at 30:1 and turns 10-15:1 in finished compost (Trautmann & Krasny, 1997).

2.4.3 Maturity and quality

Composts maturity can be indicated through its colour, odour, or through chemical indicators such as C:N ratio (Sullivan & Miller, 2001). Phytotoxicity test is also a method used to indicate the maturity of the compost. This test observed the germination and growth of selected plants. Apart from pH, compost sometimes contains phytotoxic substances such as NH_3, soluble salts, short-chain organic acids (Leege & Thompson, 1997, as cited by Sullivan & Miller, 2001). The presence of these substances could inhibit the growth of plants and therefore, is a suitable method to indicate the maturity of compost. *Lepidium sativum* (Garden cress) is a common species used for this test (Trautmann & Krasny, 1997) but this method had been applied to other species of plants including *Brassica parachinensis* (Chinese cabbage), *Cucumis sativus* (Cucumber), and *Lycopersicon esculentum* (Tomato) (Tiquia *et al*., 1996).

2.4.4 Bulking agent

Bulking agents are needed in the composting of biosolids to promote porosity and good structure (Rynk & Richard, 2001). This is usually applied when the sizes of particles are too small or too compact which prevents effective air circulation in the compost (Trautmann & Krasny, 1997). Examples of bulking agents are wood chips, mixed yard trimmings, sawdust, and finished compost (Naylor, 1996, as cited by Rynk & Richard, 2001).

2.4.5 Reducing sugar

Reducing sugar is one of the recovery products from lignocellulosic biomass (Mtui, 2009). The reducing sugar such as glucose, pentose, and galactose are obtained from the degradation of cellulose in lignocellulosic biomass by cellulases (Mtui, 2009). In a study by Shide *et al*. (2004), wood sawdust was used as a substrate for white rot fungi to produce glucose. The reducing sugar was also being observed in the co-composting of EFB and partially treated POME (Baharuddin *et al*., 2009). This indicates the various range of lignocellulosic biomass can be used as the substrate for reducing sugar production. In both studies, DNS method is being used to analyse the reducing sugar released.

2.4.6 Microbial population

In composting, bacteria are 100 times more widespread than fungi (Poincelet, 1977, as cited by Day & Shaw, 2001). Composting can be achieved by microbial digestion because it supports high population of bacteria (Boulter *et al.*, 2002). Vaz-Moreira *et al*. (2008) had observed various *Bacillus* species in compost such as *Bacillus licheniformis*, *B.subtilis*, *B.bataviensis*.

Various temperature phases also enable different communities of microorganisms to harbour the compost (Trautmann & Krasny, 1997). Thermophilic composting involves three stages in which numbers of bacteria are identified in each stages (Taiwo & Oso, 2004). In latent phase, there will be at least 2000 strains of bacteria and the most noted are such as *Streptococcus* sp., *Vibrio* sp., and *Bacillus* sp. (Burford, 1994, as cited by Day & Shaw, 2001). These mesophilic microorganisms become less competitive once the temperature exceeds 40°C (Trautmann & Krasny, 1997). Corominas *et. al* (1987, as cited by Day & Shaw, 2001) stated that the species from the genera *Bacillus*, *Pseudomonas*,