PRODUCTION OF CELLULASE BY *TRICHODERMA* SP. PRO-A1 AND *BACILLUS CEREUS* B1 USING PALM KERNEL CAKE (PKC) IN SOLID-STATE FERMENTATION

TENGKU NORSALWANI BINTI TUAN LAH

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by

TENGKU NORSALWANI BINTI TUAN LAH

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Tengku Norsalwani Tuan Lah

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LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ANOVA	Analysis of Variance
B. cereus	*Bacillus cereus*
CMCellulose	Carboxymethylcellulose
DDGS	Distillers dry grain with solubles
DNS	Dinitrosalicylic acid
FPase	Filter paper cellulase
ISO	International Organization for Standardization
LSD	Least significant difference
NA	Nutrient agar
NDF	Neutral detergent fiber
MSW	Municipal solid waste
OPEFB	Oil palm empty fruit bunches
PDA	Potato dextrose agar
PKC	Palm kernel cake
SEM	Scanning electron microscope
sp.	Species
SSF	Solid state fermentation
T.reesei	*Trichoderma reesei*
w	Weight

LIST OF SYMBOLS

FPU	Filter paper unit
N	Normality
NaOH	Sodium hydroxide
Min	Minute
psi	Pounds per square inch
rpm	Revolution per minute
%	Percentage

LIST OF PUBLICATIONS

T.L. Tengku Norsalwani and N.A. Nik Norulaini (2012). Utilization of lignocellulosic wastes as a carbon source for the production of bacterial cellulases under solid state fermentation. *International Journal of Environmental Science and Development*, 3(2), 136-140.

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Nik Norulaini N. Ab. R., Moftah, B.N.M., Lah, T.N.T,Nawi, A.H.,Kadir, M.O.A. and Aliyu-Paiko, M. (2012). Maximizing glucose production from palm kernel cake (PKC) from which residual oil was removed supercritically via Solid State Fermentation (SSF) method using Trichoderma Reesi isolate Pro-A1. The Internet Journal of Microbiology. 10(1), DOI: 10.5580/2a8

Tengku Norsalwani Tuan Lah, Nik Norulaini Nik Ab. Rahman, Nurul Jannah Hasnan, Moftah Massaud Ben Name, Hideyuki Nagaoh and Mohd Omar Ab.Kadir (2012). Cellulase activity in solid state fermentation of palm kernel cake with *Trichoderma* sp. *Malaysian Journal of Microbiology*, 8(4), 235-241.

Tengku Norsalwani Tuan Lah and Nik Norulaini Nik Ab. Rahman (2011). Utilization of palm kernel cake (PKC) and vegetable waste for the cellulase production by *Trichoderma* sp. on optimized parameters. School of Distance Education Postgraduate Colloquium, 19-20 November 2011.

Tengku Norsalwani Tuan Lah and Nik Norulaini Nik Ab. Rahman (2010). Measurement of the cellulase activity with the application of *Trichoderma* sp. on the palm kernel cake under solid state fermentation. School of Distance Education Postgraduate Colloquium, 20-21 November 2010.

PENGHASILAN ENZIM SELULASE OLEH *TRICHODERMA* SP. PRO-A1 DAN *BACILLUS CEREUS* B1 MENGGUNAKAN HAMPAS ISIRUNG KELAPA SAWIT (PKC) DI DALAM FERMENTASI KEADAAN PEPEJAL

ABSTRAK

Hampas isirung kelapa sawit, bahan buangan daripada pemprosesan minyak kelapa sawit adalah sumber serat yang berpotensi untuk diet manusia. Walau bagaimanapun, bahan-bahan selulosa tidak dapat dicerna sepenuhnya dan tidak sesuai untuk kegunaan manusia. Pemecahan polisakarida bukan kanji menjadikan ia lebih sesuai untuk dijadikan aditif makanan. Penyelidikan ini bertujuan menyiasat penggunaan kulat *Trichoderma* sp. Pro-A1 dan bacteria *Bacillus cereus* B1 dalam proses degredasi bahan berselulosa dalam hampas isirung kelapa sawit oleh selulase yang wujud dalam organisma tersebut. Aktiviti selulase ditentukan melalui kaedah fermentasi keadaan pepejal substrat oleh *Trichoderma* sp. Pro-A1 dan *B. cereus* B1. Jenis dan komposisi inokulum yang digunakan merangkumi *Trichoderma* sp. Pro-A1 mycelium pepejal, konidia berkepekatan berbeza daripada kultur pepejal dan turasan kultur cecair, juga berkepekatan berbeza. Bacteria *B.cereus* B1, melibatkan kaedah pengkulturan yang berbeza iaitu secara pertumbuhan pertama dan pertumbuhan kedua (diuji dengan potensi sel yang berbeza). Selain itu, kesan saiz zarah substrat keatas aktiviti enzim melibatkan saiz < 250 mm, ≥ 250 mm hingga < 500 mm dan ≥ 500 mm hingga < 1 mm turut dikaji. Aktiviti enzim selulase maksimum yang diperoleh adalah sebanyak 5.95 FPU g^{-1} substrat untuk hampas isirung kelapa sawit dan 10.77 FPU g^{-1} substrat untuk hampas isirung kelapa sawit nyah-minyak menggunakan $2x10^8$ spora/ml inokula *Trichoderma* sp. Pro-A1 daripada turasan

kultur cecair. Aktiviti maksimum sebanyak 6.22 FPU g^{-1} substrat telah diperolehi dengan menggunakan $1x10^6$sel/ml *B. cereus* B1 kultur kedua pada hampas isirung kelapa sawit nyah-minyak dan 2.15 FPU g^{-1} substrat pada hampas isirung kelapa sawit. Aktiviti selulase yang tinggi dipaparkan oleh inokulum tunggal *Trichoderma* sp. Pro-A1 berbanding inokulum tunggal *B. cereus* B1 atau inokulum campuran *B. cereus* B1 dan *Trichoderma* sp. Pro-A1. Saiz substrat yang paling sesuai digunakan untuk penghasilan enzim selulase adalah 500 μm untuk hampas isirung kelapa sawit. Perbezaan ketara pada aktiviti selulase mikrob dapat dilihat pada setiap jenis inokulum, kepekatan inokulum dan jenis substrat dan saiz zarah yang diuji. Ini menunjukkan bahawa komponen selulosa yang terdapat didalam PKC boleh ditukarkan kepada gula ringkas oleh inokulum individu *Trichoderma* sp. Pro-A1 dan *Bacillus cereus* B1 dan sekaligus menjadikannya sesuai untuk digunakan sebagai bahan makanan dan senang dihadam oleh haiwan ternakan.

PRODUCTION OF CELLULASE BY *TRICHODERMA* SP. PRO-A1 AND *BACILLUS CEREUS* B1 USING PALM KERNEL CAKE (PKC) IN SOLID-STATE FERMENTATION

ABSTRACT

Palm kernel cakes, waste from oil palm processing are potential fiber source for human diets. However, the cellulosic materials are indigestible and unsuitable for human consumption. Breaking the non-starch polysaccharides can make it more amenable as food additives. This research aims to investigate the use of fungus Trichoderma sp. Pro-A1 and bacteria *Bacillus cereus* B1 in breaking down the cellulosic materials in defatted and undefatted palm kernel cakes from the action of the cellulase inherent in these organisms using solid state fermentation (SSF). The SSF substrates were inoculated with three types of inocula of fungus Trichoderma sp. Pro-A1 which are mycelia biomass, conidia suspensions of varying concentrations (from solid culture) and fungal filtrate. B. cereus B1 inocula are cells from first seeding culture and cells from second seeding culture. Substrate particle size effect on cellulase activity was examined with sizes < 250 mm, ≥ 250 to <500 mm and ≥ 500 mm to < 1 mm. Maximum cellulase activity obtained by Trichoderma sp. Pro-A1 was about 5.95 FPU g^{-1} substrate for raw PKC and 10.77 FPU g^{-1} substrate for defatted PKC inoculated with 2 x 10^8spores/ml crude fungal filtrate. Cellulase activity was highest using 1 x 10^6 cells/ml of B. cereus B1 second seeding with 6.22 FPU g^{-1} substrate on defatted PKC and 2.15 FPU g^{-1} substrate on raw PKC. Higher cellulase activity achieved from sole inoculation with Trichoderma sp. Pro-A1 compared to either B. cereus B1 or combined. PKC particle size of 500 μm was best for the production of cellulase. The results have thus clearly distinguished the

different levels of cellulase activities by each microbe based on inocula types, concentration of the inocula, and substrate type and particle size. The cellulose components of the PKC can be better broken down by sole inoculation with Trichoderma sp. Pro-A1 and Bacillus cereus B1 to make the PKC more amenable as feed ingredients which can easily be consumed by livestock.

CHAPTER 1

INTRODUCTION

1.1 General

The bioconversion of solid organic substrates or wastes into useful products is not a new concept. Many scientific research and developments have been carried out with the intention to maximize the utilization of organic solid substrate for the production of food, feed, fuels, fertilizers, medical products and so on (Iluyemi, 2003).

Lignocellulosic wastes are abundant in nature and valuable as an alternative energy sources. The compositions of the biomass wastes differ. Typically, it comprises of cellulose (35-50% w/w), hemicelluloses (20-35% w/w), lignin (10-25% w/w) (Saha, 2003; Nigam *et al*., 2009). Lignocelluloses from agricultural crop wastes has been utilized and converted into useful products such as corn husk, sugarcane bagasse, rice straw, palm kernel cake (PKC), rice husk, rubber wood dusts and many other wastes materials (Pang *et al*., 2006a).

PKC is an excess residue generated from the palm kernel oil extraction process that is available in large quantities. PKC is considered a cheap organic raw material in Malaysia as well as other countries where oil palm is one of their main economic contributors. At present, Malaysia is one of the biggest producers of palm oil with the cultivated area estimated at about 5 million hectares and the crude palm oil production recorded as 18.9 million tonnes in 2011 (MPOB, 2012b, 2012c). The

production of PKC has been increasing concomitantly every year. This is indicated by the difference in production between the year of 2010 and 2011 which is closed to 0.15 million tonnes (MPOB, 2012a). The PKC therefore symbolizes an underutilized palm oil industry wastes in Malaysia.

PKC is made up mainly of lignocellulosic materials predominantly cellulose that can be hydrolyzed to glucose. Over the past decades, enzyme-based technologies have stimulated the interest in inducing in situ production of enzymes rather than inoculating with commercially available enzymes to keep cost of productions down. There has been inadequate reference on the hydrolyzing of agro wastes using the enzymes produced in situ through solid state fermentation (SSF) according to Hong *et al*. (2011). Malaysia invested a great deal of money yearly on a vast variety of enzymes meant for local industries usage as well as for research purposes. If Malaysia is to expand the enzyme production industries, the used of SSF would be the preferred method of enzyme productions since it is cheap and economical (Pandey, 1992).

Solid-state fermentation (SSF) is defined as the fermentation involving solids in absence (or near absence) of free water, provided the substrate must possess enough moisture for growth and metabolism of micro-organisms (Pandey, 2003). Intensive action of cellulolytic enzymes in the degradation of lignocellulosic materials to monomeric sugars is essential because sugars can be used as the raw material for various biotechnological production processes (Juhasz *et al*., 2005).

Enzymes, most often reported metabolites produced via solid state fermentation (SSF) include cellulases, xylanases, lipases, β-Glucosidases, mannanases, phytases, proteases, lignin-degrading enzymes and pectinases. Apart from enzymes production, agro industrial wastes also have been used as substrates in SSF for the other secondary metabolites. The abundant agro industrial wastes mainly from oil palm industry will give a huge benefit for countries like Malaysia (Lee *et al.,* 2011).

Cellulase is an enzyme that can be used to hydrolyze cellulose to form glucose and other commodity chemicals. It can be divided into three types which are endoglucanase (endo-1, 4-β-D-glucanase, EG, EC 3.2.1.4), cellobiohydrolase (exo-1, 4-β-D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1,4-β-D-glucosidase, BG, EC 3.2.1.21) (Hong *et al*., 2001; Li *et al*., 2006). Cellulolytic enzyme systems can be generated by various microorganisms namely aerobic and anaerobic bacteria (Anderson *et al*., 2012), white rot and soft rot fungi (Schmidt and Czeschlik, 2006) and anaerobic fungi (Bravo-Martins *et al*., 2009). The soft rot fungus *Trichoderma* sp. especially *Trichoderma reesei* (Kim and Hong, 2001; Uzbas *et al*., 2012) is the most generally studied microorganism in the cellulolytic enzymes systems. In these cases, a lot of studies have been performed involving enzymes with cellulolytic activity as a promising means of obtaining energy, chemicals, and single-cell protein from the cellulose, an abundant renewable resource. Therefore, the cellulase system of the fungus *Trichoderma* sp. has been comprehensively studied (Mach and Zeilinger, 2003; Saloheimo and Pakula 2012).

Fungal cellulases are produced in huge quantity, which consists of all the multi-enzyme system components with different specificities and mode of action such as endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidase, acting in synergism for complete hydrolysis of cellulose (Knowles *et al*., 1987; Wood and Garcia- Campayo, 1990; Ogawa *et al*., 1991; Bhat and Bhat, 1997; Lusta *et al*., 1999; Busiswa, 2007).

Several bacterial genera have also been discovered to secrete cellulolytic enzymes, even though not all of these are able to hydrolyze crystalline forms of cellulose. Among the bacteria, *Bacillus* species produce a variety of extracelcellular polysaccharide hydrolysing enzymes (Priest, 1977; Bhat and Bhat, 1997). A few researchers have found *Bacillus* strains capable of hydrolyzing carboxymethyl cellulose (CMC), but there are no well-documented reports of *Bacillus* species being able to degrade the more crystalline cellulosic substrates. The *Bacillus* species studied include strains of *B. amyoliquefaciens*, *B. licheniformis*, *B. sp. AC-1*, *B. halodurans C-125*, *B. circulans*, *B. pumilus strain*, *B. sphaericus JS1 and B. Subtilis ZJF-1A5* (Ariffin *et al*., 2006). The bacterial specific cellulolytic activity is found to be dependent on the source of occurrence (Sexana *et al*., 1993).

1.2 Problem statement

Cellulases have drawn interests from scientific communities due to its multiple applications in various fields. Those includes starch processing, animal food production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry, and textile industry (Adsul *et al*., 2007; Kaur *et al*., 2007). Steps must be taken to reduce the cost such as using

microorganisms that produced cellulolytic enzymes in cellulose breakdown to glucose. Hence, it is important to search for microorganisms that can produce an enormous amount of cellulase (Kotchoni and Shonukan, 2002).

Enzymes from filamentous fungi used to recover cellulose from lignocellulosic biomass are an example of green method for glucose production. Cellulolytic enzymes from a filamentous fungus, *Trichoderma sp*. have been rigorously studied due to its ability to produce a great amount of enzymes needed for complete hydrolysis of crystalline cellulose (Kubicek, 1992; Teeri *et al*., 1992).

Malaysia produces a large quantity of agricultural wastes such as palm kernel cake, sugar cane bagasse, rice husk, rubber wood dusts and many other wastes materials with the volume of approximately 5 million tonnes annually and has been predicted to double by the year 2010 (Pang *et al.*, 2006a). Usage of cheap and easily available substrates such as palm kernel cake and *Trichoderma* sp. is a means of obtaining cellulase at a reduced cost as has been found in many studies which have indicated that the use of carbon source in cultivation will cut the cost massively (Beg *et al*., 2000; Senthilkumar *et al*., 2005). The lignocellulosic biomass is an excellent carbon source for microbial enzyme production which covers the areas of industrial, environmental and food biotechnology.

A large amount of available PKC should be efficiently manipulated for domestic use as the main energy and protein sources for ruminant and non-ruminant feedings. The usage of fungi in the breakdown of PKC was proven to enhance the nutritional value of PKC. Ramin *et al*. (2010) found that there was an increase in

crude protein concentration and decrease in neutral detergent fiber (NDF) and acid detergent fiber (ADF) on PKC after 10 days solid of state fermentations with respective fungi. Solid state fermentation and enzyme treatments with microbes such as fungus and bacteria are potentially beneficial in breaking down the cellulose chain in PKC to make it more digestible as dietary ingredient for ruminant (Ramin *et al*., 2008; 2010). Solid state fermentation is a cultivation of microorganism in a wet solid substrate without the presence of free water. It provide suitable growth environment for microbes as its condition is similar to the natural habitat. Furthermore, by using the solid state fermentation (SSF) in cellulase production will lower the cost due to its lower operating cost (Xia and Cen, 1999). SSF has been proven to be successful in producing cellulase by using various agricultural substrates by-products and microbial cultures.

1.3 Objectives

1. To determine the cellulase activity in raw palm kernel cake (PKC) and defatted palm kernel cake (dPKC) as substrates under solid states fermentation (SSF) inoculated with *Trichoderma* sp. Pro-A1 and *Bacillus cereus* B1
2. To elucidate the effect of inocula types and concentration and substrate particle size on cellulase activity.
3. To determine the differences in cellulase activity by *Trichoderma* sp. Pro-A1 and *Bacillus cereus* B1 mono-culture and co-cultures on substrates.

CHAPTER 2

LITERATURE REVIEW

2.1 Lignocellulosic wastes

Lignocellulose is a major structural constituent of woody plants and non-woody plants such as grass. It is a renewable resource and organic in nature. Lignocellulose consists of hemicellulose, lignin and cellulose. Industries such as pulp and paper, timber and agriculture have generated huge lignocellulosic waste (Pothiraj *et al*., 2006). This is detrimental to the environment if not properly managed.

Unfortunately, a large amount of the lignocellulosic waste are regularly disposed of by burning, which can be considered as a worldwide phenomenon (Fauziah *et al*., 2010). On the other hand, it has far-reaching and wide applications in various industries such as those in food, animal feed, chemicals, brewery and wine, fuel, textile and laundry, pulp and paper and agriculture.

The lignocellulosic wastes are a major component of the agriculture waste and deforestration by-products which are the cheapest source of cellulose (Chahal, 1985). Agriculture related industries generate abundant residual plant biomass. This waste can likely be converted into the production of numerous value added products including biofuels, chemicals, and cheap energy sources for fermentation, improved animal feeds and human nutrients (Mohd *et al.*, 2003).

In Malaysia, one of the lignocellulose sources is palm oil trees which have lifespan of about 25 to 30 years. Re-planting of the trees had started since 1985 and had produced wastes such as empty fruit bunches, decantered sludge, palm kernel cake and so on, in large quantities. Based on projected 2005 production, around 30 million metric tonnes of oil equivalent of non-palm oil dry biomass matter will be theoretically generated from Malaysian palm oil plantations (Fauziah *et al*., 2010). Various research have been made in the related fields such as the production of panel materials, paper and pulp (Wan and Law, 2011) palm kernel meal for the livestock feed (Atil, 2009) and energy and biofuels (Sylvester and Elijah, 2012).

2.1.1 Palm kernel cakes

The size of palm fruits is approximately the size of a small plum and appear in a large bunches weighing 10-20 kg (Plate 2.1). A bunch may contain up to 2000 individual fruits with an individual weight of 3-30 g and 2-5 cm in size (Corley and Tinker, 2003; Vaughan *et al.,* 2009)

Plate 2.1 Oil palm fruits

Each fruit comprised of a hard kernel (seed) inside a shell (endocarp) surrounded by a fleshy mesocarp. An oil extraction process must consist of both the pulp which is known as palm oil-edible oil and the kernel which is called palm kernel. The extractable palm oil from the mesocarp comprises of almost 20% (w/w) of the fruits total weight and another 5% (w/w) from the palm kernel oil (from the nut) (Henderson and Osborne, 2000; Sumathi *et al.*, 2008).

Palm kernels are by-products from oil palm mills. The kernels comprise about 45-48% (by weight) of the palm fruits *Elaeis guineensis*. Based on the wet basis, each kernel contains about 47-50% by weight of oil which their properties and characteristics quite different from palm oil but similar to coconut oil (Thin and Pek, 1985). Palm kernel cakes (PKC) are produced as a byproduct after the palm kernels have been crushed to extract palm kernel oil. The PKC still contains about 5-10% (w/w) residual oil after the extraction. Most of the PKCs are being exported to be processed as animal feed. It is found to be unsuitable for human consumption because of the residual oil.

Plate 2.2 Oil palm fruits cross section

(Available at http://www.etawau.com/OilPalm/Elaeis_guineens.htm)

At present, Indonesia is the largest supplier of palm oil, followed by Malaysia with both account for 85% of the total palm oil production (Johan, 2012; Bernama, 2012). Recently, plant oil has enjoyed popularity in demand. As such, to cope with the increasing demand, the production of palm oil has doubled up. Indonesia has seen a tremendous increase in production between the years 2000-2005 (Basiron, 2007) and this trend is expecting to increase in the coming years. With the increase in palm oil production also comes an increase in PKC production.

Currently, PKC is only used as animal feed. This is due to its lack of nutrient, moderate protein content and poor amino acid. It is best described as a medium quality feed due to its deficiency in lysine, methionine and tryptophan (O'Mara *et al.,* 1999; Ramachandran *et al*., 2007). Fast deterioration, increase in production has all contributed to the large amounts of PKC being discarded. This phenomenon has led to the environmental problems in a country such as Indonesia (Sundu *et al.,* 2006). For ethanol production, the use of PKC (apart from renewing energy) will likely to increase the nutritional value. This is mainly because a by-product after undergoing fermentation will cause higher protein content because of the removal of complex polysaccharides and enrichments with yeast cell protein. Moreover, excess PKC could be more effectively utilized and analysis has shown that more than 60% (w/w) of PKC is cell wall component, consisting of 58% (w/w) of mannan, 12% (w/w) of cellulose and 4% (w/w) of xylan (Jaafar and Jarvis, 1992).

Solid state fermentation of PKC with enzyme extracts has been able to break down the complex carbohydrate fractions in PKC. Research conducted by Lawal *et al*., (2010) involving 7 days solid state fermentation on PKC with multi-enzyme

complexed from fungi had shown a reduction in cellulose and hemicelluloses content in PKC. They also reported an increased in level of soluble sugars as well as crude protein, phosphorus and energy in PKC after undergoing solid state fermentation with fungi. Digestibility of nutrients including feed conversion and weight gain were significantly improved in birds that received diets based on SSF PKC with fungi (Lawal *et al*., 2010).

PKC has been used as a substrate in the production of many microbial enzymes such as cellulase (Pang *et al*., 2006b; Lee *et al*., 2011), β-glucosidase (Abdeshahian *et al*., 2010), alpha amylase, metalloprotease, tannase and mannanase (Ramachandran *et al.,* 2004; Sabu *et al*., 2005; Sumantha *et al*., 2005). PKC is a derivative product consists of 12% to 18% (w/w) crude fiber and 15%- 18% (w/w) crude protein (Awaludin, 2001). Besides, it also contains 12% (w/w) cellulose (Dusterhoft *et al*., 1992). This PKC composition is suitable to support good microbial growth and enzyme production. The use of PKC as substrates for enzyme productions at the same time can enhance the nutritional value of the PKC and its suitability to be used as feed ingredients. PKC have recently generated much interest in its potential use in fish diets due to its availability and low production costs. In recent years, the cost of imported feed ingredients continued to rise due to increased global demand. The rising cost of imported ingredients such as fish meal and wheat flour had significantly affected the local fish farmers. Many of local aquaculture enterprises are suffering losses. Those who are mainly affected are the one who breed lower-value fish species such as catfish, tilapia and carps. At present, there is a great interest within the animal feed industry to reduce costs by using locally available feed ingredients like palm kernel cake (Imandi *et al*., 2010).

2.1.1 (a) Oil extraction from palm kernel cake (PKC) using soxhlet

The amount of residual oil in PKC depends on the efficiency of oil extraction from the kernels. Removal of oil by solvent extraction is more efficient if compare to mechanical extraction with PKC undergo solvent extraction contains oil ranging from 2-4% (w/w) while latter consists of 6-8% (w/w) of oil content (Alimon and Wan, 2012). A raw palm kernel contains 45% of oil content. After being screw-pressed, there is still remaining of about 8% of oil in what is dubbed as palm kernel cake. It is not suitable for human consumption. However, it can be removed through supercritical fluid extraction or sohxlet to concurrently produce a by-product. This by-product, also known as palm kernel meal (PKM) has fiber content with no invasive oil or has been converted to oil-less residual.

Defatted palm kernel cake (palm kernel cake with residual oil removed) is a useful source of protein and energy which contains (% w/w): dry matter (90); crude protein (16.1); ether extract (0.8); crude fiber (15.2); Ash (4); N-free extract (63); calcium (0.29); phosphorous (0.71) and metabolized energy—N7/ kg (6.2) (Sabu *et al*., 2005).

In Malaysia, productions of PKC are mostly from the expeller extraction. Solvent extraction also has been used to obtain palm kernel oil especially in large plants and is considered expensive thus; the screw press extraction (expeller) becomes the major attraction for most mills. In direct screw pressing, kernels must undergo several processes before extracting oil. The process includes the seed preparation steps of size reduction, flaking and steam conditioning prior to

mechanical extraction. The mechanical wear and tear, maintenance expenses and electricity are the things that were taken into account. For the solvent extraction, production costs rely on solvent loss and energy used in solvent recovery and pelletizing. However, oil recovery is more complete than in screw pressing (Thin and Pek, 1985).

Defatted palm kernel cake has a longer storage time than palm kernel cake. Rich in sources of protein and energy both can be used as livestock feed especially in Europe. Table 2.1 shows that palm kernel cake contains low moisture content. Having an average amount of protein and high fiber content will reduce the nutrient solubility (Chong *et al*., 1998). Generally, PKC is made up of 27% (w/w) of hemicellulose such as polysaccharide mannan. It is made up of mannose units with some galactose and 78% (w/w) non-polysaccharides (Dusterhoft *et al*., 1992). The scanning electron micrograph of a palm kernel cake demonstrates a hive like structure (see Plate 2.2 below).

Palm kernel cake has an average level of crude protein and fibre but packed with energy. The percentage of extract that is non-nitrogenous is slightly more than 50% (v/v), 31% (w/v) acid detergent fiber and 72% (w/w) neutral detergent fiber. The cell wall (testa) comprises of (w/w): 56% mannose, 12% glucose, 4% xylose and 14% galactose.

Table 2.1 Nutrient content of palm kernel cake (dry weight)

Nutrient	Mean
Dry matter, %	93.76
Crude protein, %	16.58
Crude fiber, %	14.61
Ether extract, %	8.03
Energy, kcal/kg	4688
Ash, %	4.91
Calcium, %	0.41
Phosphorus total, %	0.77
Neutral detergent fiber, %	70.07
Acid detergent fiber, %	43.08
Hemicelullose, %	26.98
Free nitrogen extract, %	49.32
Metabolizable energy, MJ/kg	7.54

Source: Chong *et al*., 1998.

Defatted palm kernel cake is:

i. High in metabolizable energy and crude fiber that are good sources of energy.

ii. Aflotoxin free, hence safe for consumption, no toxic chemicals, heavy metals, pesticides and dioxins

iii. High dry matter in PKC encourages intake while discourages growth of microbes and moulds

iv. Easy availability – Oil palm industry in Malaysia produces voluminous amount of PKC

v. Cost is relatively affordable

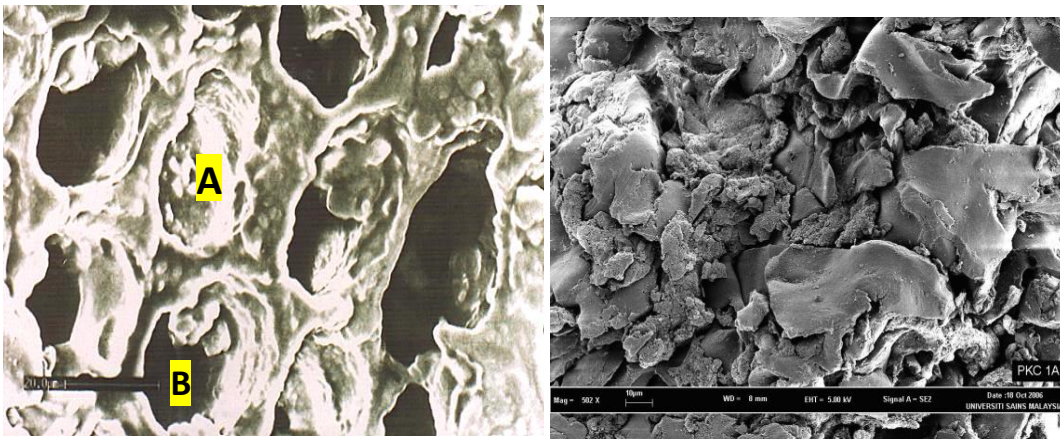

Plate 2.3 Palm kernel cake before complete removal of residual oil A) Oil residue within the cell of palm kernel cake B) Empty cavities of palm kernel cake where the oil has been removed

The presence of residual oil in the cell structures (A) and empty cavities (B) revealing where oil has been removed was shown on Plate 2.3.

2.2 Cellulose

Cellulose is a linear homopolymer made up of β-(1-4)-linked glucose residues and uridine diphosphoglucose, ((UDP)-glucose) molecules and also performs as a substrate for cellulose biosynthesis. One glucose residue, known as an anhydroglucose is a monomer of cellulose. The dimer, two glucose residues β-(1-4)-linked, called cellobiose is the repetitive structural units of the cellulose chain (Plate 2.3).

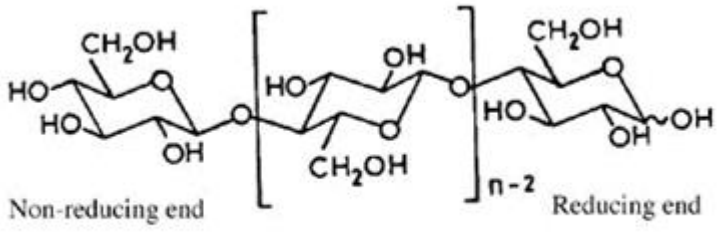

Figure 2.1 Cellulose structure

Fiber source: http://www.afma.org/fiber.html (Accessed on 10 February 2012)

The degree of polymerization is based on the number of monomers which create each cellulose chain (Brown *et al*., 1996; Delmer, 1999). Two different ending groups can be found in each cellulose chain edge (Figure 2.1). A non-reducing group is present at the end of the chains, where a closed ring structure is found. Meanwhile, a reducing group with both an aliphatic structure and a carbonyl group is found at the other end of the chains. The cellulose chain is thus a polarized molecule. The β-(1-4) linkage between glucose residues is different with the-(1-4) linkage as it occurs in starch, provided cellulose with unique structural features.

Cellulose is a water-insoluble polymer which consists of a rigid linear structure. Controlled cellulose biosynthesis allows the arrangement of extensive linear chains which can be aligned side-by-side, forming fibers that have a great mechanical strength. As a result, tension resistance of cellulose is similar to that of steel (Eckardt, 2003). Cellulose occurs in nature as the main structural component that build up the cell wall and responsible for many of its typical behaviour.

Cellulose consists of insoluble, linear chains of β-(1→4)-linked glucose units with an average degree of polymerization of about 10 000 units but it can also be as low as 15 units (Eveleigh, 1987). It consists of highly crystalline regions and amorphous (non-crystalline) regions. This in turn forms a high tensile structure which is generally resistant to enzymatic hydrolysis particularly the crystalline regions (Walker and Wilson, 1991).

2.3 Cellulolytic enzyme production

The main barrier for the biological pre-treatment process of the raw materials in industries is due to the high cost of commercial cellulase enzyme being used at the enzymatic hydrolysis stage (Fang *et al*., 2009). It was suggested that the production of the enzyme to be carried out in-situ and used directly for the saccharification process. Commercial cellulase productions usually utilize high performance or genetically modified fungi such as *Aspergillus* sp., *Trichoderma* sp. and *Acremonium* sp. etc. High activity cellulase production depends on the fungi, substrate, substrate morphology and inducer.

There are lots of potentials to be explored in cellulases and hemicellulases. Industries such as chemicals, fuels, animal feed, textile, brewery and wine, pulp and paper, textile and laundry and agriculture will be the main beneficiaries with the application of these two enzymes. Apart from numerous applications, these two enzymes also have biotechnological potential (Bhat, 2000; Sun and Cheng, 2002; Wong and Saddler, 1992a, b; Beauchemin *et al*., 2001, 2003). Cellulases, hemicellulases and pectinases have wrapped up 20% of market trading of industrial

enzymes which is trading at approximately USD 1 billion and even more amazingly, it is projected to increase in the range of 1.7 to 2.0 billion dollars by the year 2005 (Bhat, 2000; Ashish and Deepak, 2005).

The use of carbon sources such as Avicel, cotton, Solca Floc or commercial crude cellulose pulp would be too costly to produce cellulolytic enzymes (Doppelbauer *et al.*, 1987; Moosavi-Nasab and Majdi-Nasab, 2007). Other investigators have recommended an alternative for enzyme production which is the use of municipal solid waste (MSW) or a fraction of this. This would actually be one of the cheapest carbon sources imaginable. However, to the best of our knowledge, no reports exist in the literature that MSW has been successfully employed for cellulase production.

The advantage of cellulolytic enzymes has been observed in an enzymatic hydrolysis and cellulose waste fermentation to convert cellulose to glucose, ethanol, fructose and other chemical component (Emert and Katzen, 1980, Alexander *et al*., 2009). Enzymatic hydrolysis had been used worldwide in industries compared to acid hydrolysis due to the medium operational conditions, high yields, pure yields and cost-savings (Borriss, 1987). Acids can breakdown the long chain cellulose to release the sugars through hydrolysis reaction. However, due to their high specificity, cellulase can achieve higher yield of glucose from cellulose (Wyman, 2004). A portion of pretreated biomass can be used to feed a fungus or other organism that produces cellulase that can then be added to pretreated solids to release glucose from cellulose (Wyman, 2008).

Enzymes, a specific biocatalysts is preferred over acid or alkaline since it can operate under milder conditions. Besides, it is environmental-friendly which is not the case with acid and alkaline. The technologies are there for the bioconversion of lignocelluloses to ethanol and other chemical product. However, some tweaking must be done to the production side ensure that it can be priced competitively lower than other source. We can start by minimizing feedstock cost by focusing on agricultural residues and waste materials. Next, we can look at pretreatment to improve bioconversion, its capital cost as well as production of enzymes for depolymerization of complex raw material. These processes are expensive since it entails three steps which are process design, system optimization and model development. Processing normally involves the use of biocatalysts, whole microorganisms or their enzymes or enzymes from other organisms. It will be able to synthesize or bioconvert raw materials into new products. Apart from that it also has the ability to recover/ purify such bio-products and subsequent downstream modifications (Howard *et al*., 2003).

Cellulolytic enzymes had been produced by many types of microorganisms such as fungi, actinomycetes, microbacteria and bacteria (Goksoyr and Eriksen, 1980, Lee *et al*., 2002). Fungus, in general is 50000 times more hydrolytic if compared with an active cellulolytic bacteria (Saddler *et al*., 1987)

2.3.1 Cellulase

Cellulases are responsible for the hydrolysis of cellulose. It is a complex mixture of enzyme proteins with different specificities to hydrolyse glycosidic bonds. Cellulases have three major enzyme activity classes (Goyal *et al*., 1991; Rabinovich

et al., 2002a, b). They are endoglucanases or endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Endoglucanases, often called carboxymethylcellulose (CM)-cellulases, are planned to initiate random attack. These attacks were meant for multiple internal sites in the amorphous regions of the cellulose fibre opening up sites. This will lead to subsequent attack by the cellobiohydrolases (Wood, 1991). One of the major components of fungal cellulase system is cellobiohydrolase or sometimes referred to as exoglucanase. It accounts for 40-70% of the total cellulase proteins and it can hydrolyse highly crystalline cellulase (Esterbauer *et al.,* 1991).

Dimers and monomers at the end of the glucose chain, β-glucosidase hydrolyse glucose dimers and in some cases cello-oligosaccharides are normally broken down by cellobiohydrolases to glucose. Generally, endoglucanases works vis-à-vis cellobiohydrolases but the mechanism remains unclear (Rabinovich *et al.,* 2002b). Microorganisms have multiple distinct variants of endoglucanases and exoglucanases (Beldman *et al*., 1987; Shen *et al*., 1995). Most cellulases are multi- domain proteins similar to that of hemicellulases.

Cellulase and β-glucosidase are active enzymes involved in the depolymerization of cellulose in co-operation with the other enzymes. Cellulase enzymes helped in digestion of food, green tea extraction, modification of food tissue, improved food quality and extraction of various products from plants (Toyama, 1969). Cellulolytic enzymes also assist in the enzymatic hydrolysis and fermentations of cellulosic waste to glucose, ethanol, fructose and other chemicals (Emert and Katzen, 1980; Alexander *et al*., 2009).

Enzymatic hydrolysis has been preferred in the industry rather than acid hydrolysis because it has so many advantages such as low operational costs, high and purified yield and cost-saving (Borriss, 1987; Enari, 1983; Teeri, 1997). However, enzymatic hydrolysis involved the high usage of cellulase enzyme and expensive purified enzymes. According to Teeri (1997), most of the cellulolytic enzymes applications nowadays imply the applications of raw non-specific enzymes rather than purified enzymes. The large amount of the enzyme involved in various applications is due to the low specific activity of cellulase which influences the enzyme cost.

Three kinds of enzymes involve in the digestion of cellulose, i.e.: (1) endo-β-D-1,4-glucanase which randomly hydrolyzes cellulose into cellodextrin, cellobiose, and glucose; (2) exo-β-D-1,4-glucanase which removes cellobiose from the non-reducing end of cellulose chain; and (3) β-D-1,4-glucosidase which breakdowns cellobiose into two glucose molecules. Beside the action in the different areas of the cellulose structure, the activity of the enzymes to digest cellulose is much influenced by the structure of cellulose that contains crystalline and amorphous areas (Purwadaria, 1995).

2.4 Fungus *Trichoderma* sp.

Trichoderma sp. has been known for a long time since 1865 (Bisby, 1939). Its taxonomy and species identification were unclear until 1969 (Rifai, 1969). The species concepts and biodiversity in *Trichoderma* sp. fungi have been extensively reviewed by Druzhinina and Kubicek (2005). The authors have stated that

Trichoderma sp. fungi are difficult to distinct morphologically. However, the phylogenetic classification has rapidly reached 100 (Druzhinina *et al*., 2006) and it is expected to increase consistently. Fungi, which can develop a great amount of cellulase and xylanase, appear to be the most effective taxonomic group that are accountable for the breakdown of lignocellulosics (Kvesitadze *et al*., 1999). Varieties of cellulolytic fungi inhabit different types of agricultural wastes and are responsible in the degradation of natural crystalline cellulose into simple sugar (Domsch and Gans, 1972; Dashtban *et al*., 2009). Their ability to degrade the crystalline cellulosic materials depends on the presence of complete cellulase activity in adequate quantity (Immanuel *et al*., 2006). The complete cellulase can be produced by those strains that have the ability to degrade cellulose due to the synergism in endoglucanase, exoglucanase and β-glucosidase activities. Under suitable conditions, fungus grows in a woody sample through hyphal proliferation.

The ability of fungus to degrade is mainly dependent on the hyphal capability to penetrate cell walls involving enzymes that can degrade cellulose, lignin and other complex contents. Enzymes will be absorbed through fungi cell wall to substrate and it will transform the existing polysaccharides and lignin. Based on types of degradations, fungi that can degrade cellulose can be divided into three classes which are brown, white rot and soft rot. Brown rot will degrade woody polysaccharides with a little modification to lignin and will cause wood to become brownish and decayed. White rot fungi will degrade lignin woody polysaccharides to become whitish and soft after the swelling. Soft rot has a capability to degrade polysaccharides and lignin which will reduce the wood strength (Fengel and Wegener, 1984; Hatakka and Hammel, 2010). Soft rot usually attack a moist wood or

tree. Decaying process will start from the outer layer (surface) and enter the inner part of the tree once the outer layer had been destroyed.

Lignin degradation requires oxidative attack on the carbon-carbon bonds and ether inters unit bonds. Degradation of lignin will enable access to the carbohydrate polymers of plant cell walls for use as carbon and energy sources. Lignolytic enzymes such as manganese peroxidase, laccase and cellobiose dehydrogenase are some examples of microbial enzymes that are involved in lignin degradation. Previous research showed that white-rot fungus *Phanerochaete chrysosporium* is able to degrade lignin. The enzymes from white rot fungi that catalyze the initial depolymerization of lignin are extracellular and unusually nonspecific (Brambl and Marzluf, 2004).

Mostly, research involved the production of cellulolytic enzymes has been done using *Trichoderma reesei* and its mutant (Montenecourt and Eveleigh, 1979; Reesei and Mandels, 1984; Kubicek, 1992, Peterson and Nevalainen, 2012; Liu *et al.*, 2013) due to its ability of producing large amount of cellulose. The *Trichoderma* species have been used regularly in the enzymatic measurement because of the ability in producing the complete cellulase systems which consists of all components involved in hydrolysis of crystalline cellulose and produce high cellulose protein.

2.4.1 Cellulase production by *Trichoderma* sp.

In recent years, one of the most important biotechnological applications is the conversion of agricultural wastes and all lignocellulosics into products of commercial interest such as ethanol, glucose and single cell products (Ojumu *et al*., 2003). The key element in bioconversion process of lignocellulosics to these useful products is the hydrolytic enzymes, mainly cellulases (Ojumu *et al.,* 2003; Fan *et al.,* 1980; Immanuel *et al*., 2006). The mechanism of saccharification of crystalline cellulose at the macromolecular level by fungal cellulase is given in Figure 2.2