Characterization of Cellulolytic Fungi in Peat Soil for Potential Application and Biodegradation of Rice Husk

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ABSTRACT

Cellulases are a group of hydrolytic enzymes that capable of degrading cellulose to the smaller glucose units. Nine isolates were isolated from samples taken from peat soil by growing on Potato Dextrose Agar (PDA). Cellulolytic activities were measured qualitatively and quantitatively by using 0.1% Congo Red and 3,5-dinitrosalicylic acid method, respectively. All isolates were morphologically characterized and the highest cellulase-producing fungus, F9 was selected for species identification by molecular technique. F9 has been identified as *Penicillium citrinum* was used to proceed the solid state fermentation. The highest cellulolytic activities were shown on day five with; FPase, 1.474 IU/ml; CMCase, 2.663 IU/ml; and Avicelase, 0.449 IU/ml.

Key words: Cellulolytic fungi, cellulose degradation, solid state fermentation.

ABSTRAK

Selulase adalah satu kumpulan enzim hidrolitik yang boleh menguraikan sellulos kepada unit-unit gula yang kecil. Sembilan dapatan telah didapati daripada sampel yang telah diambil daripada tanah gambut dan ditumbuhkan di atas medium agar kentang (PDA) dan dipilih di atas medium agar carboxymethylcellulose (CMC) bersama garam mineral. Kualiti dan kuantiti aktiviti selulolitik diukur dengan masing-masing menggunakan kaedah Congo Red 0.1% dan 3,5-dinitrosalicylic acid. Kesemua dapatan telah dikaji cirri-ciri morfologinya dan fungus yang menghasilkan paling banyak selulase telah dipilih untuk mengetahui nama spesisnya. F9 diketahui sebagai Penicillium citrinum telah digunakan di dalam penapaian pepejal. Aktiviti selulase yang paling tinggi ditunjukkan pada hari kelima dengan aktiviti; FPA, 1.474 IU/ml; CMC, 2.663 IU/ml; dan Avicel, 0.449 IU/ml.

Kata kunci: *Fungi selulolitik, penguraian selulos, penapaian pepejal.*

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CI	Chloroform/Isoamyl alcohol
CMC	Carboxymethylcellulose
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
EDTA	Ethylenediaminetetraacetic acid
FPA	Filter Paper activity hydrolase
HCl	Hydrochloric acid
ITS	Internal Transcribed Spacer
$MgCl_2$	Magnesium chloride
Na_2CO_3	Sodium Carbonate
PCI	Phenol/Chloroform/Isoamyl alcohol
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
SDS	Sodium Dodecyl Sulphate Polyacrylamide gel
SSF	Solid State Fermentation
SmF	Submerged Fermentation

CHAPTER 1

INTRODUCTION

Malaysia is well known for its agricultural sector and one of world paddy producers (Van, 2004). Malaysia has more than 0.5 million ha of rice fields in which 0.2 million ha are double-cropped (Rahman, 2003). Meaning that, the remains are single-cropped. Hence, the annual yields are still low. Majority of farmers who work in rice field have low income since they only earn their income from the production of rice. Nowadays, the government is looking into other alternatives to develop other beneficial efforts by utilizing the products and by products from rice and the same time, the alternatives can help to increase farmers' incomes.

It is not surprising that since the rice sector in Malaysia is growing, we should not forget that rice wastes also would be increased as byproducts. The production of rice subsequently produce wastes including rice husk, rice mill, rice effluent and many more. Therefore, the agricultural wastes should be managed properly.

An effort that has been studied and done where rice husk is treated with concentrated chemical to give other valuable products (Rahman, 1992). Instead of using chemical treatment, biological treatment must be carried out as this treatment is much friendly to the environments. The recycling organic wastes by burning will dispose a global warming by emitting greenhouse gases. There is a source stated that biomass burning of agricultural wastes is one of the sources of the emission of methane (CH_4) (Basri *et al.,* 2006). Methane is

one of the greenhouse gases after CO_2 and N_2O. These consequences should be overcome in order to reduce the pollution of water, soil and air.

The Third National Agricultural Policy (1998-2010) focuses on agricultural programmes which aim at high productivity while ensuring conservation and utilization of natural resources. In this study, it was purposely worked with cellulolytic fungi with a reason to support the sustainable agriculture in Malaysia by utilizing the waste products especially produced from rice field that is rice husk for an example represented for other agricultural wastes.

Rice husk is primarily structured by cellulose that can be degraded by certain hydrolytic enzymes called cellulases. Cellulases are classified as hydrolytic enzymes which are capable to degrade cellulose to the smaller glucose unit (Pothiraj, 2006). Cellulases can be naturally produced by some microorganisms especially fungi where predominantly colonized at cellulosic area especially in soil. Soil is the place where many cellulosic materials are accumulated due to the dead plants and animals as well.

Cellulolytic fungi which can be obtained from peat soil will be identified and further look into their potential application and biodegradation of cellulosic materials such as rice husk. In addition, there was a study had been done that rice husk can be used as substrates for cellulases activity (Milala *et al.*, 2005). Degradation of cellulosic materials such as rice husk by cellulolytic fungi is one of the efforts that called as biological treatment which involved environmentally friendly process. The cellulose degradation which produced reducing sugars, glucose as reducing sugar can be furthered study to downstream process such as bioethanol

production. Bioethanol production is a fermentation process that utilized microorganisms such as cellulolytic fungi to convert glucose to ethanol. Moreover, the utilization of glucose by the process of fermentation is one of the most popular technologies exploited by industrial sector.

The objectives of this study are to isolate cellulolytic fungi from peat soil. Their morphological characterization would be studied. The highest cellulases-producing fungi would be identified by molecular technique for species identification and would be used to proceed the cellulose degradation under the solid state fermentation.

CHAPTER 2

LITERATURE REVIEW

2.1 Cellulolytic fungi

Cellulose along with hemicellulose comprises the major part of all growing plants which are difficult to be digested (Smith *et al.,* 1983). Cellulose is a linear polymer of anhydroglucose which is linked by β-1,4-glucoside bonds (Xianzhen Li, 1997). Therefore, many enzymes involve in cellulose degradation. Cellulases are a group of hydrolytic enzymes that capable of degrading cellulose to smaller sugar components like glucose (Onsori *et al*., 2004). Reviewed studies had proved that cellulases can be classified into three main types: those are endoglucanase, exoglucanase and β-glucosidase. These three major enzymes are required in the degradation or depolymerization of cellulose where exoglucanases release cellobiose units from crystalline cellulose, endoglucanases degrade regions of amorphous cellulose and β-glucosidases degrade short oligosaccharides such as cellobiose to glucose (Wood & Ingram, 1992). Table 1 showed the three major enzymes in the cellulose degradation with enzyme number, trivial names, substrate and product as well (Jeffries, 1987).

Table 1: Enzymes of the cellulose complex.

Systematic name	**Enzyme no.**	**Trivial names**	**Substrate and product**
1,4-β-D-glucan Cellobiohydrolase	(EC 3.2.1.91)	exoglucanase, cellobiohydrolase	crystalline cellulose, cellobiose
Endo-1,4-β-D-glucan 4-glucanohydrolase	(EC 3.2.1.4)	endoglucanase, β-glucanase	amorphous cellulose, celloologosaccharides
β-D-glucoside glucohydrolase	(EC 3.2.1.21)	Cellobiase, β-glucosidase	cellobiose, triose glucose

These enzymes also can be secreted by several types of fungi and bacteria (Onsori *et al.*, 2004). However, fungi more successful in degrading of cellulosic materials since they can either grow on the surface or penetrate into the cellulosic materials by using hyphae (Boer *et al.*, 2004). They are called as saprotrophic fungi since they are able to decompose organic matters such as celluloses. Saprotrophic fungi decompose cellulosic materials by secreting those cellulases from the formation of the hyphae (Smulski, 1996).

Early years, studies on cellulolytic fungi were more concentrated on cellulolytic species identifications. There are many cellulolytic fungi had been identified through several years ago. From year to year, new cellulolytic fungi have been recognized. Mostly, reviewed studies of cellulolytic fungi come from the genus of *Aspergillus sp.*, *Trichoderma sp.*, *Penicillium sp.* (Mahmood *et al.,* 2006). However, there are other species which have been classified as cellulolytic fungi had been found such as *Sporotrichum pulverulentum, Mycothecium verrucaria* and many more. As technology goes up, the characteristics of cellulolytic fungi itself being carried out and further recently, many researchers come out with

studying on the potential of cellulolytic fungi to overproduce the three important enzymes as widespread issue (Kaur, 2007).

Natural ecological niche of celulolytic fungi is soil where they can survive and grow on organic wastes (Latge, 1999). Therefore, cellulolytic fungi can easily obtained form soils (Kader *et al.*, 1999) especially soil under crop plantation due to the accumulation of cellulosic materials wasted from plants. Peat soil is an example that always being selected as the place to isolate cellulolytic fungi especially under crop plantation of oil palm (Prasertsan *et al.*, 1992), agave (Punnapayak *et al.*, 1999), sago (Pothiraj *et al.,* 2006) plantation. Cellulolytic fungi can also be obtained from soil in the forest sites (Kader *et al.*, 1999).

2.2 Isolation and screening of cellulolytic fungi

The isolation of cellulolytic technique developed Dr. Elwin Reese and Dr. Mary Mandels from 1955 until 1980 (Jeffries, 1996) is always used by researchers, nowadays. Cellulolytic fungi can be cultured, enriched and maintained on Potato Dextrose Agar (PDA) as the culture media with optimum temperature of incubation ranged from 28^{o}C until 30^{o}C. Other microorganisms can also be enriched on the PDA plates. However, cellulolytic fungi can be selected by growing them with the addition of trace metal stock. Cellulolytic fungi must be pure-cultured before growing on selective media. Selective media for cellulolytic fungi is carboxymethylcellulose (CMC) agar media. CMC is needed as carbon source. There was a modification of the isolation technique where treatment with antibiotic can be added to allow only fungi to be grown. Antibiotics such as ampicillin, penicillin, streptomycin and carbenicillin will be added on agar media before it solidified to kill other microorganisms especially bacteria.

In order to ensure the cellulolytic fungi have been isolated and able to produce cellulases, screening method has been developed by Teather *et al.* (1981) and still being used in recent studies. They introduced the screening method by using Congo red. The CMC agar media that have grown with fungi as pure culture will be flooded with an aqueous solution of Congo red. Congo red acts as an indicator for β-D-glucan degradation in the agar medium. After several minutes, clear-zone of hydrolysis on the agar medium can be seen indicates that β-D-glucan (cellulose) degradation has occurred. The more the diameter of clear-zone indicates the more cellulose has been degraded.

2.3 Internal Transcribed Spacer (ITS) primers

In the chromosomal site which known as nucleolar organizing region, there is a major part known as major rDNA transcript. Ribosomal DNA or rDNA can be found as parts of repeat units that are arranged in tandem arrays. Each repeat unit consists of transcribed region and non-transcribed spacer region (Sharma *et al.*, 2007). The transcribed region is highly transcribed and easily amplified by using universal PCR primers designed from highly conserved regions bordering the internal transcribed spacer (ITS) and its small size (600-700bp) enable easy amplification of ITS region due to the high copy number. By using primers ITS1 and ITS4, the internal transcribed spacer that bordered 5.8S ribosomal DNA can be highly amplified. 5.8S ribosomal DNA is the region which is useful region of the fungal genome for determining species-specific (Lennon *et al.*, 1994).

2.4 Cellulase enzymatic assays

Enzymatic assay that will be carried out in this study is cellulolytic assays in which to determine cellulose activities of cellulolytic fungi. Cellulases can be classified into three types: exoglucanase; endoglucanase and; cellobiase.

The determination of the overall cellulase activities can be determined by filter paper assay (Jie Liu *et al.*, 1996). Mandel also had described the determination of overall cellulase activity by filter paper assay. Filter paper assay is always used by researchers to determine the overall cellulase activities.

Each of enzymes can be determined by using different analysis:

a. Carboxymethylcellulase (CMCase) for endoglucanase,

   Endoglucananase activity can be analyzed by Carboxymethylcellulase (CMCase). Diluted enzyme samples will be added before incubating for 15 minutes at 50^{o}C. The reaction will be terminated by adding 3ml of DNS reagent, and the amount of reducing sugars released will be determined as described below in DNS method. The absorbance should be measured by the spectrophotometer at 540nm (Vicini *et al.*, 2003).

b. Avicel for exoglucanase,

Exoglucanase activity can be determined by Avicellase by incubating diluted enzyme with the avicel soluiton for 2 hours at 50^{o}C. The reaction will be terminated by placing by heating in boiling water bath for 10 minutes. After centrifugation, the reducing sugars present in the supernatant will be determined by the DNS method. The absorbance should be measured by the spectrophotometer at 540nm (Vicini *et al*., 2003).

c. ρ-nitrophenyl-β-D-glucoside for β-glucosidase,

β-glucosidase can be determined by mixing and incubating the diluted enzyme with ρ-nitrophenyl-β-D-glucoside solution for 15 minutes at 37^{o}C. Na_2CO_3 is added to the mixture in order to stop the reaction. The absorbance should be measured by the spectrophotometer at 420nm (Berghem & Pettersson *et al*., 1974).

2.5 DNS method

In order to measure enzymatic activities, measurement on their product such as reducing sugar by a method known as Dinitrosalicyclic acid (DNS) method can be used. Reducing sugar assays such as the Neslon-Somogyi method or the Dinitrosalicylic acid (DNS) method are used to assay for the product sugars. Reactions are carried out by mixing and incubating a dilution of the enzyme preparation with a known amount of substrate at a buffered pH and set temperature. DNS method has been available since 1955. However, it is still useful for the quantitative determination of reducing sugar. According to Frost (2004) who studied on the determination of glucose concentration, his biochemistry students had carried two difference ways on glucose determination. Those are nonenzymatic assay and enzymatic assay. The nonenzymatic assay indicates the presence of all reducing sugar while the enzymatic assay is specific for D-glucose. However, both the reducing sugar and the specific glucose will be measured by using spectrophotometer. A standard calibration curve (absorbance versus reducing sugar or glucose concentration) was generated for both assays to determine reducing sugar and glucose concentration. DNS method is said to be a nonenzymatic assay where only indicates the presence of reducing sugar instead of specific glucose.

The standard calibration gradient for glucose analysis is used in calculating the glucose concentration:

$$\text{Glucose concentration (g/L)} = \frac{\text{Optical density (OD) X df}}{\text{m}}$$

m = the standard calibration gradient for glucose analysis.

df = dilution factor for enzyme.

Optical density (OD) is the reading obtained from the spectrophotometer. Df indicates as dilution factor where the dilution factors are different for each different solid state fermentation time.

2.6 Mechanism of cellulose degradation

The mechanism of cellulose degradation is a sequential multienzymes system made up the three enzymes (Arumugam, 2005). In the first step of cellulose degradation described by John (1983), endoglucanase act randomly over the exposed surfaces of crystalline cellulose to form open chain termini (Arumugam, 2005). The exposed non-reducing termini are then hydrolyzed by exoglucanase and produce cellobiose. Endoglucanase and exoglucanase act by breaking the glucosidic linkage (Wang, 2007). Cellobiose formed is a dimer with a β-1,4 bond as opposed to maltose, a complement with an α-1,4 bond (Wang, 2007). Subsequently, the cellubiose will be cleaved by β-glucosidase to form glucose (Arumugam, 2005) by breaking the β-1,4 glucosidic bond (Wang, 2007). Eventhough the mechanism described is in sequential, these three enzymes act in a synergistic manner (Onsori *et al.*, 2004). The convertion of cellulose to glucose can be summarized in Figure 1.

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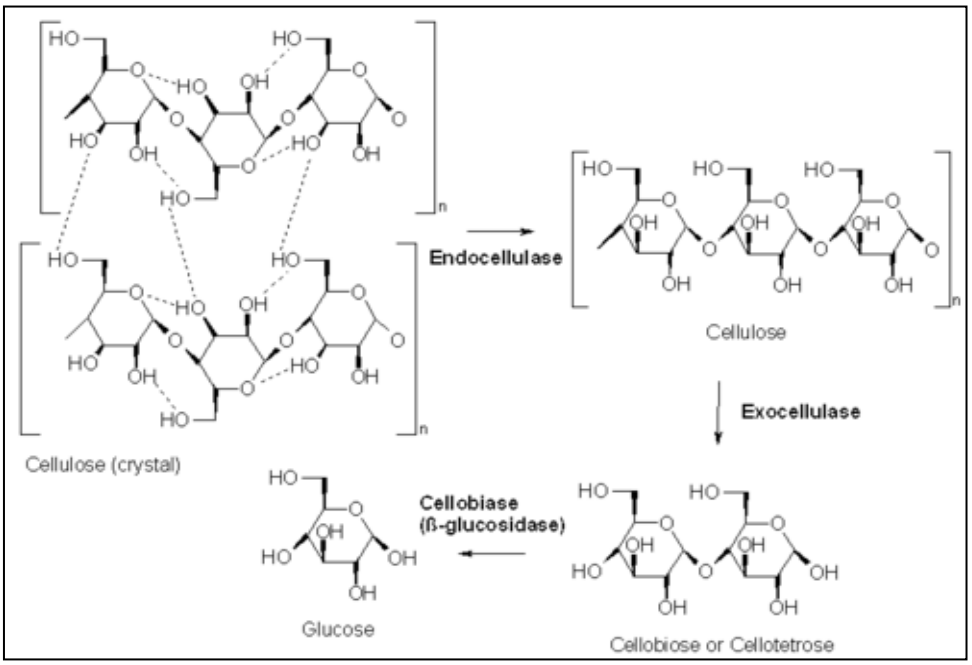

Figure 1: Mechanism of cellulose degradation.

2.7 Importances of cellullolytic fungi

Since the production of native cellulolytic fungi is low especially in *Trichoderma reesei*, there was a study carried by Oinonen (2002) where the production of endoglucanase was enhanced by using the technique of DNA recombination where endoglucanse gene obtained from *T. reesei* was inserted into *E. coli* as the host cell and further increasing the copy number of the endoglucanase gene. The overproduction of endoglucananse by the endoglucanase-transformant strain increased fourfold above that of the host strain. This overproduction of endoglucanase is important in textile industry for biostoning and also for finishing of cellulosic fibers. In their study, they tested on denim fabric. Biostoning of fabric means the use of cellulases in place of, or in addition to, the use of pumice stones for the treatment of denim fabric to impart a stonewashed effect Endoglucanase showed the most effective of the main cellulases to remove colour from denim fabric and the high endoglucanase activity proved to improve the stonewashing effect.

One of the main carbohydrates components of cell wall are cellulose. Enzyme such as cellulose (endoglucanase, exoglucanase and β-glucosidase) must exist in order to breakdown cellulose. It is well known that we are surrounded by a lot of organic materials and it can be imagined if it did not rot, the earth would be covered with masses of dead degradation (Highley, 2007). Cellulolytic fungi are also widely used in pulp and paper industry (Arumugam, 2005) and food industry such as Indonesian ‘tempeh’, Japanase ‘koji’ and French ‘blue cheese’ (Raimbault, 1998).

2.8 Downstream processes

The production of glucose can be applied in many industrial processes since enzyme is very important and has great economic values. Since there were many studies had reported that there are a lot of fungi are able to produce cellulases, many efforts have been done due the abundant waste of renewable cellulosic materials on the earth. One of the most popular of downstream process towards cellulose degradation is bioethanol production.

Basically, the process of fermentation is used where reducing sugar or glucose is converted to ethanol. In addition, there is a special process known as solid state fermentation where the crude fermented products such as agricultural wastes may be directly used as the source of the production of cellulases (Pandey *et al.,* 1998). For an instance, Xiros *et al.* (2005) have studied on the direct bioconversion of brewer's spent grain to ethanol by using *Fusarium oxysporum. F. oxysporum* have been reported to attain the ability of fermenting cellulose from the brewer's spent grain directly to ethanol. The fermentation of cellulose is done in bioreactor n order to produce mass production of ethanol. Usually, ethanol production is be measured by High Performance Liquid Chromatography (HPLC).