SCREENING AND ISOLATION OF LIGNOCELLOLYTIC FUNGI FROM MANGROVE SOILS

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Bachelor of Science with Honours (Resource Biotechnology) 2013
Screening and Isolation of Lignocellulolytic Fungi from Mangrove Soils

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In fulfilment of the requirements for the degree of Bachelor of Science with Honour

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Declaration

I hereby declare that this thesis is based on my original work except for quotation and citation, which has been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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<tr>
<td>cm</td>
<td>Centimere</td>
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<tr>
<td>Lac</td>
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<td>LiP</td>
<td>Lignin Peroxidase</td>
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<td>ME</td>
<td>Malt Extract</td>
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<td>ml</td>
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<td>Mn(II)</td>
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<td>Mn(III)</td>
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<td>MnP</td>
<td>Manganese Peroxidase</td>
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<td>nm</td>
<td>Nanometre</td>
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<td>PDA</td>
<td>Potato Dextrose Agar</td>
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<td>µl</td>
<td>Microlitre</td>
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<td>%</td>
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<td>(v/w)</td>
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<td>(w/w)</td>
<td>Weight per weight</td>
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<td>°C</td>
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SCREENING AND ISOLATION OF LIGNOCELLULOLYTIC FUNGI FROM MANGROVE SOILS

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ABSTRACT

Lignocellulolytic fungi play an important role in bioremediation of many environmentally persistent pollutants such as aromatic xenobiotics, heterocyclic aromatic hydrocarbons, chlorinated aromatic compounds and various dyes which are used in textile industries and pulping industries. Lignin is a compound which is held together by strong chemical bonding. This made it difficult to be degraded. However, studies had found that, lignocellulolytic fungi can solve these environmental problems by secreting their non-specific lignocellulolytic extracellular enzymes. In this research, a lignocellulolytic fungus SHY 43 was isolated from mangrove soils and was identified as *Penicillium* sp. It showed 92.6% of Remazol Brilliant Blue R dye decolourisation which indicates the presence of ligninolytic activities. It also performed well in cellulase screening test whereby it had the highest total endoglucanase activity on day 8 which was 3.074 U/mL. It was then used in the solid state fermentation (SSF) using oil palm empty fruit bunch (OPEFB) as the substrate. There were four enzymes analysed during the enzyme assays, which were laccase, manganese peroxidase, lignin peroxidase and cellulase. Among these four enzymes, only cellulase activity was detected whereas the other three ligninolytic enzymes were deactivated. Total endoglucanase activity peaked at day 9 with a value of 0.067 U/mL.

Keywords: Lignocellulolytic fungi, mangrove soils, *Penicillium* sp., oil palm empty fruit bunch (OPEFB), enzyme assays

ABSTRAK

Kulai lignosehidolis is memainkan peranan penting dalam bioremediasi alam sekitar yang mengandungi pencemaran yang berasal dari xenobiotik aromatik, hidrokarbon aromatik heterosistik, sebati aromatik berbilin dan pelbagai pewarna yang digunakan dalam industri tekstil dan industri pulp. Lignin merupakan komponen yang mempunyai ikatan kimia yang kuat. Ini menyebabkan sulit untuk diterakam. Walaupun begitu, kulai lignosehidolis mampu menangani masalah ini melalui penghancuran enzim luar sel yang dihasilkan. Dalam kajian ini, kulai lignosehidolis SHY 43 yang di osingkan dari tanah bumi pelabuhan diberi spesies *Penicillium*. Penyairwarman sebanyak 92.6% Remazol Brilliant Blue R oleh kulai menunjukkan kehadiran aktiviti enzim lignosehidolis. Semasa penerikan, kulai juga menunjukkan aktiviti yang tinggi dalam aktiviti endoglucanase pada hari ke-8 iaitu 3.074 U/mL. Lignin kemudian di gunakan di dalam fermentasi secara pelepah menggunakan tannin kosong kelapa sawit (EFB) sebagai substrat. Terdapat empat enzim yang di analisis iaitu: laccase, manganese peroxidase, lignin peroxidase dan cellulase. Di antara empat enzim ini, hanya aktiviti endoglucanase memberikan reaksi positif dan diketahui sebagai enzim lignosehidolis yang lain memberikan keputusan negatif. Jumlah aktiviti endoglucanase adalah paling tinggi direkomendasi pada hari ke-9 dengan nilai 0.067 U/mL.

Kata kunci: Kulai lignosehidolis, tanah paya bakau, *Penicillium* sp., tannin kosong kelapa sawit, ujian enzim
1.0 Introduction

Lignin is the second most abundant organic polymer which is renewable that found on earth. It is usually found in all vascular plants’ cells. Lignin is very important in giving the shape and reinforces the cell wall which prevents the cell wall of the plants from collapsing (McCray, 1991). In addition, degradation of lignin is rather hard because it is very resistant due to its structure. It is held together with strong chemical bonds and a lot of internal hydrogen bonds (McCray, 1991). The structure of lignin is a common phenylpropane structure (a benzene ring with a 3 carbon tail) (McCray, 1991).

Environmental pollutants are cleaned up by bioremediation using fungi as one of the option (Husaini et al., 2008). Biodegradation of lignin by white rot fungi involves the degradation of aromatic xenobiotics, heterocyclic aromatic hydrocarbons, synthetic high polymers, chlorinated aromatic compounds and various dyes which are environmentally persistent pollutants (Ohkuma et al., 2001). Other than that, when papers are produced from wood, the final product that contain lignin is undesirable due to its hydrophobicity and colour which made the lignin as a waste product during pulping process (Javor et al., 2000). Thus, degradation of lignin is required to reduce the accumulation and pollution by lignin. White rot fungi is selected to degrade lignin because it produced extracellular enzymes, namely lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) which involved in the depolymerisation of lignin (Ohkuma et al., 2001). Besides that, white rot fungi have a high degradability to lignin as it has strong oxidative activity and their ligninolytic enzymes are low in substrate specificity (Ohkuma et al., 2001).
The objectives of this research are to screen, isolate and characterize the best lignocellulolytic fungi from mangrove soils. In addition, further studies on the lignocellulolytic enzyme biodegradation activities was performed by employing the selected lignocellulolytic fungi as inoculums in the solid state fermentation (SSF) of oil palm empty fruit bunch (OPEFB).
2.0 Literature Review

2.1 Mangrove

Mangroves are mainly found in tropics and subtropics tidal areas where it is a saline coastal sediment habitat. Mangrove forest locates in a distinctive and extreme environment which is in the boundary between land and sea (Zakaria et al., 2010). The environmental condition of mangrove is unique and extreme due to the high levels of salinity, high temperature, muddy anaerobic condition and extreme tides (Zakaria et al., 2010). The studies of mangrove soils involve few important physical and chemical properties, which are pH (H⁺ concentration), salinity, particle size and Eh (Redox potential) (Kathiresan, 2007). Majority of the mangrove soils have a pH value between pH 6 to pH 7. However, there are also some mangrove soils that have a pH as low as 5 (Kathiresan, 2007). Mangrove is one of the most valuable habitats which provide economic, ecological, scientific and cultural resources (Khan et al., 2009).

Mangrove forest has a diverse ecology where it is habitats for various animals, including mammals, snakes, migratory birds, crabs, tunicates and other benthic marine invertebrates (Ellison, 2008). All organisms that found in mangrove areas can withstand a wide range of salinity, thus they are being called as euryhaline-able animals (Maikut, 2004). Mangrove can shield the inner zone from severe damage of winds and waves because it is a naturally flexible plant (Maikut, 2004). Thus, it saves the inner zone from tsunami and hurricanes. Besides that, mangrove trees gather sediments in shallow water areas to support its root structure. This help the soil built up along tropical coast lines which can withstand storms (Maikut, 2004).
Majority of the mangrove soils has loose sediments and they are either muddy or sandy (Zakaria et al., 2010). Besides that, mangrove roots, trunks and branches are submerged in mangrove soils which attract large groups of fungi and bacteria (Zakaria et al., 2010). The principal degrader of plant debris is the fungal community when compared with other microorganisms that were found in mangrove environment. It takes part in the early phases of decomposition (Zakaria et al., 2010).

2.2 Lignocellulolytic fungi

Lignocellulolytic enzyme catalyses the breakdown of lignin and cellulose components. Lignocellulolytic fungi can be divided into 3 major groups, which are white rot, brown rot and soft rot (Mtui, 2012). The extracellular enzymes system which are non-specific and non-stereoselective enable lignocellulolytic fungi to degrade lignin (Husaini et al., 2010). White rot fungus part in the degradation of lignin and cellulose. Naturally, it is the most abundant wood degraders (Hammel, 1997). Usually it caused the rotted wood to feel moist, soft, spongy or stringy (Mtui, 2012). The rotted wood will appear in white or yellowish colour (Mtui, 2012). White rot fungi is the most efficient lignocellulolytic fungi compare with others (Husaini et al., 2010). Brown rot degrades cellulose and hemicellulose (carbohydrates) in wood and left the wood lignin brownish in colour. However, the degradation by brown rot occurs only in the areas which are directly adjacent to their growth (Mtui, 2012). According to Mtui (2012), soft rot grow much slower than white and brown rot. The decay of the wood of living tree that is caused by soft rot does not cause serious structural damage as what white and brown rot did.
Among these three groups of lignocellulolytic fungi, white rot fungi are the most common and the major degrader of lignin components in wood. The species of Basidiomycetes are the most abundant wood decomposer that can be found in all forest type (Groposo & Leita, 2005), especially in hardwood forest, tropical forest and temperate coniferous forests. As compared to other bacteria and fungi, white rot fungi are able to degrade lignin completely to produce carbon dioxide and water (Husaini et al., 2010). Besides that, the only organism that can degrade phenylpropanoid and lignin is the basidiomycetous white rot fungi (Sundaramoorthy et al., 2005). Due to this reason, white rot fungi are found to be a potential asset for biopulping process. This is because during the making of papers from wood, the final product that contain lignin is undesirable due to its hydrophobicity and colour which make the lignin as a waste product during pulping process (Javor et al., 2000). Thus, degradation of lignin is required to reduce the accumulation and pollution of lignin.

Lignocellulolytic fungi plays important roles in the bioremediation of polluted soils, detoxification of textile waste, waste water treatment, pulping, de-inking, decolourisation and degradation of recalcitrant, highly toxic phenolic compounds and various dyes which are environmentally persistent pollutants (Ohkuma et al., 2001). Besides that, the enzymes produced by white rot fungi are able to breakdown agricultural chemicals which are xenobiotic compounds, such as Dieldrin, Simazine, DDT, Parathion and Trifuralin (Mtu, 2012).

Lignocellulolytic fungi secretes few enzymes which play an important roles in degrading lignin and cellulose. These enzymes are lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (Lac) and cellulose.
2.3 Lignin Degrading Enzymes

Lignin degrading enzymes is also called lignolytic enzymes. Based on the study of Mtui, 2012, white rot fungi is found to be the most common and major degrader of lignin if compared with brown and soft rot. White rot fungi complete its degradation process by secreting three main enzymes, namely lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac).

LiP and MnP are classified as peroxidase enzymes while Lac is an oxidase enzyme (Mtui, 2012). Peroxidases use hydrogen peroxide \((\text{H}_2\text{O}_2)\) as its co-substrate. It catalyses the oxidation process resulted in the formation of free radicals (e.g. phenoxy and aryl cation radicals), reactive cations \((\text{Mn}^{3+})\) and anions \((\text{OCl}^-)\). LiP is the first discovered as the ligninolytic enzyme (Tien & Kirk, 1983; Hammel, 1997). Hydrogen peroxide \((\text{H}_2\text{O}_2)\) oxidises LiP producing a two-electron deficient intermediate. This intermediate then performed two one-electron oxidations of the donor substrate to return to its resting state (Hammel, 1997). The devastation of lignin and humic substances, the oxidation of toxic compounds and nonspecific defence are involved in these oxidation processes by peroxidases (Mtui, 2012). The oxidase enzyme on the other hand are involved in the conversion of organic compounds and materials oxidatively. Laccase is one of the oxidase enzyme that is produced by various fungi where its active sites contain copper (Mtui, 2012).

Laccase is under the category of oxidase enzymes. According to Hammel, 1997; Mtui 2012, it belongs to blue copper oxidases and polyphenol oxidases (para-benzenediol:dioxygen oxidoreductase). The active site of laccase contained 4 copper ion (Mtui, 2012). Besides that, Mtui 2012 states that while the abstracted electrons is
transferred to dioxyglu, laccase will catalyse the oxidation of one-electron of numerous substrates preferably of phenolic and aromatic amines (Mtui, 2012).

2.4 Cellulase

Cellulase is the enzyme that hydrolyses cellulose. It is classified as hydrolases which means the enzyme responsible in catalyse hydrolysis on the chemical bonds of a compound. According to Mtui 2012, there are various hydrolase that responsible in cellulolytic reactions, such as endoglucanases, endo-1,4-β-glucanase, carboxymethyl cellulase (CMCs), exoglucanases, exocellulases, endo-1,4-beta-D-glucanase, β-1,4-glucanase, β-1,4-endoglucan hydrolase, cellobiohydrolases (CBH), celludextrin, β-glucosidases and other groups. Hemicellulose is a polymer of pentose sugar and is under the group of cellulose. It can be hydrolase by hemicellulases (xylanases and β-D-xylosidases) (Mtui, 2012).

Based on the studies of Ravindran et al., 2010; Mtui, 2012, there are 3 types of enzymes that is categorised under cellulases, namely β-endoglucanase, β-exoglucanase and β-glucosidase. These enzymes from wide range of pH (pH 4 to 12) of Chaetomium sp. (a type of marine fungi) shows substantial enzymes activities and heat stabilities when its substrates are agricultural and industrial wastes and in a submerged liquid and solid culture conditions (Ravindran et al., 2010; Mtui, 2012).

There are extensive studies carried out on the production of fungal cellulases from plant biomass feed (Mtui, 2012). According to Ng et al., 2010 and; Mtui, 2012, N-terminal sequencing is used to determine the major enzymes involved. It has been
proofed and demonstrated that *D. cadaria* is a promising fungus in the production of biofuels from the biodegradation of lignocellulosic materials.

However, there are some limiting factors which affect the commercial enzyme production. The high price of refined substrate is one of the most reasons. The refined substrate such as cellulose and hemicellulose can be directly used as food or feed (Mtui, 2012). There are some inexpensive alternative carbon and nitrogen sources that used to reduce the cost of production of hydrolases (Mtui, 2012). The examples of the alternatives are wood residues (sawdust and paper mill junks), grasses, waste papers, residue from agriculture (stalks, straw and nutshells), domestic waste (lignocellulosic litter and sewage) and residues from food industry (Mtui, 2009; Mtui, 2012).
3.0 Materials and Methods

3.1 Soil sampling from mangrove

The mangrove soil was collected near the roots of mangrove tree and near pneumatophores where studies believed these areas trap the most decaying lignocellulosic substrates. The soil was collected and placed into sterile plastic bags. Soil sample A and B were collected near the root of mangrove tree while soil sample C and D were collected near pneumatophores. The collected soils were brought back to laboratory and stored at 4 °C refrigerator and processed the next day.

3.2 Serial dilution

Serial dilution was done. First of all, 1 g of the soil sample was transferred into 10 mL sterile distilled water in a bottle, to give a dilution of 1:10. Then, 1 mL of the mixture was transferred to fresh bottle 2 which contained 9 mL of sterile distilled water, which gave a dilution of 1:100. The suspension was mixed well so that the soils were spread evenly in the solution. The above steps were repeated to get a dilution of 1:1,000.

The isolation of fungus was performed using pour plate method. Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) used to grow the fungi were adjusted to pH 8.0, salinity 0.2% and supplemented with 0.05% chloramphenicol. Each sample was done in duplicates using this method and incubated at 28 °C. Fungal isolates were distinguished by its colony morphology and were subcultured periodically for further analysis.

3.3 Screening of lignocellulolytic fungi
Remazol Brilliant Blue R (RBBR) decolourisation test was used to screen for lignolytic enzymes production from the fungi while Congo red staining of carboxymethylcellulose (CMC) agar was used to test for cellulase production. These screening tests were carried out in both solid (qualitative) and liquid (quantitative) state.

3.3.1 Qualitative test: RBBR decolourisation test on solid state medium

This method was done according to Chroma et al. (2002) with some modification. First of all, the fungi culture (around 1 g of wet weight) were grown on glucose minimal medium which containing RBBR (Vyas & Molitoris, 1995; Kacerorá et al. 1998). Glucose minimal (GM) media was prepared in 1 litre which contained the following: 1 g K$_2$HPO$_4$, 10 mg ZnSO$_4$. 7H$_2$O, 5 mg CuSO$_4$. 5H$_2$O, 0.5 mg MgSO$_4$. 7H$_2$O, 10 mg FeSO$_4$. 7H$_2$O, 0.5 g KCl, 10 g glucose which served as carbon source, 3 g NaNO$_3$ which served as sole source of nitrogen and 20 g of agar. RBBR was added into agar from stock solution to final concentration of 200 mg L$^{-1}$. The pH of the agar was adjusted to pH 5.5. After that, 5 mm agar plug from 5 days old fungal culture was inoculated into the GM agars and kept in dark at room temperature. Then, the dye decolourisation activity of each tested strain was evaluated by measuring the diameter of clearing zone around the fungus culture.
3.3.2 Quantitative analysis: RBBR decolourisation test in liquid state medium

A total of 15 positive decolourising fungal strains that shown in the solid agar medium were selected to proceed with quantitative liquid state decolourisation experiments. Decolourisation test using liquid medium was carried out by using GMM media but excluding agar. First of all, 20 mL of GMM was added into a 100 mL Erlenmeyer flask. Then, RBBR from the stock solution was added into GMM to give a final concentration of 200 mgL$^{-1}$. In the Erlenmeyer flask, two pieces of 5 mm agar plugs from a 5 days old fungal culture was inoculated into the GMM media supplemented with RBBR dye. This flask was prepared in duplicates and incubated in dark at room temperature. The incubation periods of the flasks were 20 days with 4 days intervals of sampling. During each time of the sampling, the whole culture will be harvested and centrifuged at 6,000 rpm for 10 minutes. This process is to separate fungal mycelium from culture medium. The supernatant was taken to measure the decolourisation rate using spectrophotometer. The decolourisation of the dye was measured by monitoring the absorbance of the dye in the culture medium at its maximum absorption wavelength. The maximum absorption wavelength of RBBR is 595 nm. The percentage of decolourisation was determined by using the formula below (Gomaa & Mornaz, 2011):

$$\text{Percentage of decolourisation (\%) = } \frac{A(\text{initial}) - A(\text{final})}{A(\text{initial})} \times 100\%$$

- $A_{\text{initial}}$ is the absorbance of the maximum absorbance wavelength of sample flasks on Day 0.
- $A_{\text{final}}$ is the absorbance of the maximum absorbance wavelength of sample flasks on Day 20.
3.3.3 Qualitative analysis: Congo red staining on carboxymethylcellulose (CMC) agar

First of all, minimal salt medium with 0.2% yeast extract, 0.1% KH₂PO₄, 0.1% MgSO₄, 0.5% CMC and 0.5% agar was prepared and autoclaved. Then, the medium were transferred into Petri dishes. The CMC agar was inoculated with test fungus. After inoculation, the Petri dishes were incubated in darkness at 25°C. After 2 days, staining process was carried out to stain the agar plate. First of all, 1% w/v aqueous Congo red was flooded on the plate and then left for 15 minutes. Then, the stain was poured off and the agar surface was washed with distilled water. After that, 1 M NaCl was flooded on the agar plate for 15 minutes to de-stain. The NaCl was discarded and the agar plate was observed. CMC degradation around the colonies appeared as yellowish-opaque area while the undegraded CMC remains red colour. The diameter of the clearing zone was measured and recorded for all the test fungal strains.

3.3.4 Quantitative test: Endoglucanase assay - Carboxymethylcellulose (CMC) method

This test was used to test the ability of degradation of cellulose according to Pointing (1999). First of all, production of cellulase was carried out by using minimal salt medium (0.2% Yeast extract, 0.1% KH₂PO₄, MgSO₄ and 0.5% CMC) was prepared and 40 mL of the aliquots was transferred to glass culture bottles and autoclaved. Then, 4 fungus strains that showed positive results in both RBBR and Congo red staining test were used to inoculate the bottles which contained the liquid medium respectively and an un-inoculated bottle was used as controls. The bottle caps were loosely fit so that there was sufficient gaseous exchange. The bottles were incubated in dark at room temperature and examined every 2 days interval for an incubation period of 10 days.
Every 2 days, the enzyme produce was harvested by centrifuge at 15,000 g for 20 minutes. The supernatant obtained was the crude enzyme preparation which was used in Dinitrosalicylic acid (DNS) test.

DNS assay was used to determine the total cellulolytic activity of enzymatic extract. First of all, the reaction mixture was prepared by adding 0.5 mL of the enzyme extract with 0.5 mL of 0.1 M sodium acetate pH 5.0 which supplemented with 1% carboxymethylcellulose. The suspension was incubated for 1 hour at 37 °C. The blank was boiled enzyme. The reactions were stopped by the adding of 1.0 mL of DNS colour reagent and incubate for 10 minutes in boiling water. Then, it was cool to room temperature. After that, 1 mL of Rochelle salt was added to stabilize the developed colour. The suspensions were measured by using spectrophotometer at the absorbency of 540 nm. The cellulase or CMC activity is defined as activity of 1 mM glucose produced per minute under optimum assay conditions.
3.4 Identification and characterisation

3.4.1 Microscopic identification

The morphology of the selected isolated fungi was view under microscope. The selected isolated fungus was based on the criteria where the best strain that have the highest decolourisation rate in RBBR. These selected fungi were viewed under microscope to observe the structure of the fungal mycelium and types of fruiting body produced.

Microscopic slides were prepared by using Lactophenol blue staining. The procedures were according to standard method from Shimeld and Rodgers (1999).

First of all, there were few important steps that need to be done before transferring the fungal culture onto the slides. An inoculating needle which is usually a thin needle or wire at the end of along a pencil-like handle was dipped in alcohol and flamed with gas flame until it glows bright red. Then, the needle was cooled for about 15 seconds. This cooling procedure was important because transferring cultures using a red hot needle will kill the fungus.

Then, the needle was entered an opened-wide enough Petri dish which contains the fungus culture. A small portion of the colony margin was cut out with the heat-sterilized needle. After that, the square of colony margin was transfered to the sterile slide. Then, the slide was stained using a drop of Lactophenol blue and covered with cover slip. Finally, the slide was placed under the microscope and observed. The