Faculty of Resource Science and Technology

Utilization of Leaf Litter from *Filicium decipiens* (Japanese Fern Tree) for Laccase Enzyme Production by *Marasmius cladophyllus* UMAS MS8

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Utilization of Leaf Litter from *Filicium decipiens* (Japanese Fern Tree) for Laccase Enzyme Production by *Marasmius cladophyllus* UMAS MS8

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Programme of Resource Biotechnology
Faculty of Resource Science and Technology
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ABSTRACT

Marasmius cladophyllus has the ability to produce laccase for the degradation of lignin. Lignocellulosic residues used as substrate are important lignin sources and become the potential inducer for laccase production. To date, limited study on the ability of leaf litter from *F. decipiens* to be used as substrate for fermentative enzyme production have prompted the investigation on the ability of *M. cladophyllus* to produce laccase using *F. decipiens* as a substrate through both solid-substrate fermentation (SSF) and submerged fermentation (SmF). Submerged fermentation resulted in the highest laccase activity throughout the 12-days fermentation period, with maximum activity attained at 0.508 U/g on the twelfth day of fermentation. However, for solid-substrate fermentation, the highest average reading was 0.086 U/g but there was no laccase activity present as no colour change in the assay was observed. The findings obtained in this study confirmed the potential approach using cheap lignocellulosic waste, which is leaf litter of *F. decipiens* for laccase production by *M. cladophyllus* through submerged fermentation.

Key words: *Marasmius cladophyllus,* lignin, laccase, solid-substrate fermentation, submerged fermentation

ABSTRAK

Marasmius cladophyllus *mempunyai kebolehan untuk menghasilkan enzim lakase melalui proses penguraian lignin. Lebihan lignosellulosa yang digunakan sebagai substrat merupakan sumber lignin yang penting dan berpotensi menjadi penggalak untuk penghasilan lakase. Kekurangan kajian terhadap kebolehan daun gugur dari* Filicium decipiens (F. decipiens) *menarik minat banyak penyelidik untuk mengkaji kebolehan* M. cladophyllus *dalam penghasilan lakase melalui penggunaan* F. decipiens *sebagai substrat melalui proses penapaian, iaitu penapaian substrat pepejal dan penapaian tenggelam. Penapaian terendam menghasilkan aktiviti lakase yang tertinggi sepanjang dua belas hari penapaian, mencapai aktiviti maksimum 0.508 U/g pada hari ke-dua belas penapaian. Untuk penapaian substrat pepejal, purata bacaan tertinggi adalah 0.086 U/g tetapi tiada aktiviti lakase dikesan kerana tiada perubahan warna yang berlaku. Penemuan yang dicapai di dalam kajian ini mengesahkan pendekatan menggunakan lebihan lignosellulosa yang murah iaitu daun-daun gugur berpotensi untuk menghasilkan lakase daripada* M. cladophyllus *melalui penapaian tenggelam.*

***Kata kunci**:* Marasmius cladophyllus, *lignin, lakase, penapaian substrat pepejal, penapaian terendam*

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LIST OF ABBREVIATIONS

ABTS	2,2-azino-bis-[3-ethylthiazoline-6-sulfonate]
CH_3COONa	Sodium acetate
$CuSO_4.5H_2O$	Copper (II) sulfate pentahydrate
F. decipiens	*Filicium decipiens*
FRST	Faculty of Resource Science and Technology
G. lucidum	*Ganoderma lucidum*
KH_2PO_4	Potassium dihydrogen phosphate
Lac	Laccase
LiP	Lignin peroxidase
M. cladophyllus	*Marasmius cladophyllus*
MCOs	Multicopper oxidases
MEA	Malt extract agar
MEB	Malt extract broth
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
MnP	Manganese peroxidase
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic dehydrate
NaOH	Sodium hydroxide
PDA	Potato dextrose agar
P. martensii	*Penicillium martensii*
P. pinodella	*Peyronellaea pinodella*
P. taeda	*Pinus taeda*
SD	Standard deviation
SMF	Submerged fermentation

SSF	Solid-substrate fermentation
UNIMAS	Universiti Malaysia Sarawak

CHAPTER 1

INTRODUCTION

1.1 Study Background

M. cladophyllus is the endophytic fungi that belongs to the family Tricholontataceae and is a member of ligninolytic fungi. This white-rot fungi are capable of producing extracellular oxidative enzymes namely manganese peroxidase (MnP), laccase (Lac), and lignin peroxidase (LiP) (Hasnul *et al.,* 2015). *M. cladophyllus* UMAS MS8 is the fungus that has been isolated successfully from *Melastoma malabathricum* (Ngieng *et al.,* 2013). Laccase enzymes which in particular belong to the superfamily of multicopper oxidases (MCOs) are able to catalyze the oxidation of several key compounds including phenolics, and non-phenolic substances (Vantamuri & Kaliwal, 2016). In biogeochemical cycles, fungal communities and the activity of its ligninolytic enzyme breaks down the plant cell wall polymers, as well as the lignin (Rosales-Castillo *et al.,* 2017). All MCOs reduce oxygen to water without the production of harmful by products. The ability of laccase to oxidize a wide range of phenolic and non-phenolic compounds causes this enzyme to be used in numerous amount of biotechnological processes such as bioremediation, nanobiotechnology, biosensor, paper and pulp industry, and also biodegradation (Yang *et al.,* 2017). In textile industries, the water release is polluted with dyes and it is detected to be one of the top ten contaminating sources of water bodies (Kishor *et al.,* 2021). With the use of laccases, the enzyme provides an efficient alternative in the decolourization and detoxification of dyes before discharging the water back into the environment (Góralczyk-Bińkowska *et al.,* 2020).

For harvesting enzymes, usually a fermentation method will be used. Fermentation is one of the methods that can be used in generating enzymes for industrial purposes.

Production of enzymes to meet increasing industrial demand however is very costly as expensive growth media are needed. Therefore, in this study, cheap substrate which is leaf litter will be tested for laccase enzyme production via two fermentation methods, which are solid-substrate fermentation (SSF) and submerged fermentation (SmF). Specifically, both SSF and SmF will be used to identify the possibility of *M. cladophyllus* to produce laccase enzymes using leaf litter from *Filicium decipiens (F. decipiens)* or commonly known as Japanese Fern Tree as the growth substrate. SSF involves the use of solid medium to harvest enzymes on the surface of the substrate in the absence or presence of a small amount of liquid (Banat *et al.,* 2021). As for SmF, it is the fermentation of microorganisms as a suspension in liquid medium on which the nutrients are dissolved as particulate solids (Martáu *et al.,* 2021). Fermentation method is commonly conducted by using malt extract agar (MEA) and potato dextrose agar (PDA) (Goud *et al.,* 2020). However, the limitation of those commercial media for fermentations are their high operating cost, which causes researchers to opt for other alternatives in producing laccases using leaf litter. Since *F. decipiens* is one of the most popular plant species that is planted around Universiti Malaysia Sarawak (UNIMAS), the leaf litter will be collected and will be used as substrate for laccase biosynthesis. By using both fermentation methods, which are SSF and SmF, it is hypothesized that the Japanese fern tree can be used in the production of laccase from *M. cladophyllus.* However, the fermentation method that is expected to be the most suitable for the production of laccase enzymes from *F. decipiens* is the SSF.

1.2 Objectives

The goals of this study are:

a) To determine whether *M. cladophyllus* can utilize the leaf litter as the substrate for the production of laccase enzymes.

b) To compare between solid-substrate fermentation and submerged fermentation in the production of laccase enzyme by *M. cladophyllus.*

CHAPTER 2

LITERATURE REVIEW

2.1 *Filicium decipiens* (Japanese Fern Tree)

Figure 2.1 shows *Filicium decipiens,* or Japanese fern tree that belongs to the Phylum Tracheophyta and Family Sapindaceae. This dioecious tree has thick and round canopies, and also large and fern-like leaves (Sharmila *et al.,* 2017). *F. decipiens* is native to humid forests of African (Ethiopia, Kenya, Malawi, Mozambique, Tanzania, and Zimbabwe) and Asian (India and Sri Lanka) countries and is considered as ornamental trees because it is cultivable in the tropical and humid subtropical climate regions (Sharmila *et al.,* 2017). Aside from the fern-like leaves, the resinous leaves of *F. decipiens* also have large and pinnate compound leaves. According to the study conducted by Zainudin *et al.* in 2012, *F. decipiens* was identified as one of the five most popular species that dominates the area in UNIMAS. The leaves of this plant have a smooth surface, shiny at the bottom of the leaves and also occupy a wavy edge (Monichan, 2021). Leaves of *F. decipiens* will be collected and will be used as substrate for microbial enzyme production, which is the laccase biosynthesis (Góralczyk-Bińkowska *et al*., 2020).

Figure 2.1: *Filicium decipiens* with resinous fern-like leaves which occupy six to eight pairs of leaflets. (Retrieved from Akila & Priya, 2019).

2.2 Solid-substrate fermentation (SSF)

Several industries such as pharmaceuticals, food industry, and textile industry uses SSF as their fermentation methods in producing the metabolites of the microorganisms, by using solid support instead of liquid medium (Kumar *et al.,* 2021). SSF involves the growth of organisms on solid substrates with the absence of water. The solid support, or the substrate commonly used in SSF needs to have the property to absorb or contain water, and contain low moisture levels (Magro *et al.,* 2019). The usual substrates used in SSF includes wheat bran, sugar cane bagasse, and teas waste are as illustrated in **Figure 2.2.**

Figure 2.2: Substrates commonly used in solid-substrate fermentation that contain low moisture levels. (Retrieved from Magro et al., 2019).

The advantages in using SSF is that this method does not cause damage to the environment because of the minimum amount of waste and liquid effluent produced (Gonzalez, 2012). Other than that, simple and natural solids can be used as the substrate such as rice husk, sago hampas, saw dust, and soy hull (Wang *et al.,* 2019). In this experiment, the substrate of choice is leaf litter from *F. decipiens* (**Figure 2.2.1**). When using SSF, the production costs is low despite the high product yield. Based on a study conducted by Salomão *et al.* in 2019, the enzyme activity of cellulase was produced by different fungi, namely *Trichoderma koningii, Penicillium* sp., and *Rhizomucor* sp., using sugarcane bagasse as the substrate. This study proves that the utilization of sugarcane bagasse is feasible and offers a low-cost opportunity. Since leaf litter from *F. decipiens* and the wheat bran is the natural solids, the cost when conducting laccase production from leaf litter substrate can also be minimized.

However, SSF also have its downside, where only microorganisms that can tolerate low moisture content can be used. The growth of the organisms occurs slowly, hence the product formation is also limited (Leite *et al.,* 2021). In this study, the fungal isolate will be used for SSF and tested for the production of laccase enzyme from leaf litter substrate since fungi are optimally active in very low water activity (Valle *et al.,* 2022).

Figure 2.2.1: Erlenmeyer flask that has been inoculated with isolate UMAS MS8 using leaf litter as the substrate in SSF for the optimization of laccase production.

Other than enzyme production, SSF are also used in other industries, such as industrial chemicals, pharmaceuticals, and also used in the production of microbial products such as fuel, feed, and foods (Ślo *et al.,* 2021). In bioprocessing, the application of SSF covers bioleaching, bioremediation, bio-pulping, and bio-beneficiation (Manan & Webb, 2017).

2.3 Submerged fermentation (SmF)

SmF is the fermentation methods that manufacture the biomolecules where enzymes and other reactive compounds are being submerged in liquids as illustrated in **Figure 2.2.** The figure shows the *Saccharomyces cerevisae* that exists as suspension and submerged completely in liquid broth. The growth of the microorganism in the liquid medium exists as suspension, in which the nutrients is dissolved in commercial media (Fatemeh *et al.,* 2019). The development of the microorganisms occurs in liquid broth, where in this study, the liquid broth of choice is the liquid minimal media consisting of several nutrients. Generally, the nutrients of the liquid broth result in the production of the industrial enzymes and other products (Das & Kumar, 2018). A microorganism will be placed in the closed flask used in conducting SmF, supplied with high volume of oxygen, and then the bioactive compounds

will be secreted into the broth (Namnuch *et al.,* 2020). In this study, the liquid minimal media will be poured into the Erlenmeyer flask and isolate UMAS MS8 will be placed inside the flask. The enzyme production will occur when the nutrients in the broth interact with the fungal isolate. As a result, metabolites such as laccase enzyme will be secreted into the fermentation broth.

Figure 2.3: Submerged fermentation showing that the yeast exists as a suspension in nutrient broth. (Retrieved from Sharma et al., 2018).

There are two most common methods of SmF, which are fed-batch fermentation and continuous fermentation. For fed-batch fermentation, the growth nutrients will be added into the culture while the continuous fermentation is when the sterilized liquid nutrients are continuously added into the bioreactor, at the same rate (Campos, 2020). The application of SmF can be seen in extracting the secondary metabolites that is needed to be used in liquid form (Zhang *et al.,* 2019).

The advantages in using SmF in the study are high yield production, low cost, and also easy purification of products (Liu *et al.,* 2020). When conducting the study, the aim is to produce high amount of laccase enzymes by submerged cultures while decreasing the

production costs. The disadvantages of SmF on the other hand are lower concentration of products and more complex fermentation equipments (Sharma *et al.,* 2017).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials needed for this study includes: 2% (w/v) malt extract broth (MEB), glucose minimal media containing (per liter) 2 g peptone, 2 g yeast extract, 5.5 g glucose·$1H_2O$, 0.8 g of potassium dihydrogen phosphate (KH_2PO_4), 0.25 g of sodium phosphate dibasic dehydrate ($Na_2HPO_4 \cdot 2H_2O$), 0.45 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), and 0.25 g of copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), sodium hydroxide (NaOH), acetic acid (CH_3COOH), 1 mmol of 2,2-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS), and 20 mM of sodium acetate (CH_3COONa) buffer.

3.2 Methods

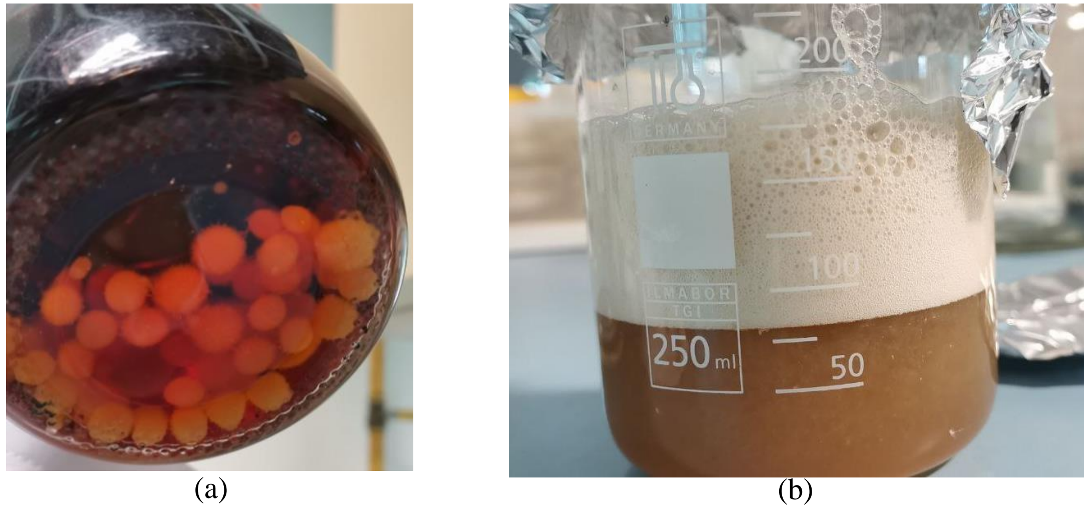
3.2.1 Preparation of leaf litter substrate

Japanese fern trees were identified and the fresh leaves were collected based on the leaf morphology at UNIMAS FRST as illustrated in **Figure 4.1.** Upon collection, the leaves were washed thoroughly with water and were left overnight in the oven at temperature 80ºC. Subsequent to the washing and drying process, the grinding process was conducted until the leaves turned into a fine powder (**Figure 3.2.1**). The fine powder of the leaves was used as the substrates for laccase enzyme production. (Ngieng *et al.,* 2013).

Figure 3.2.1: Leaves of *F. decipiens* that were ground until it turns into fine powder which were used as a substrate for the fermentation processes.

3.2.2 Fungus and inoculum preparation using *M. cladophyllus* UMAS MS8

M. cladophyllus UMAS MS8 was obtained from the Molecular Genetic Laboratory of the Faculty of Resource Science and Technology (FRST) UNIMAS. The isolate UMAS MS8 (or fungal isolate) was cultivated on malt extract agar (MEA) for 7 days before inoculating 2 malt-agar plugs in a stock bottle containing 400 mL of 2% (w/v) malt extract broth (MEB) on a shaker operating at 140 rpm and room temperature. The incubation of isolate UMAS MS8 were done for 7 days until more fungal biomass was obtained (**Figure 3.2.2 a**). After 7 days of incubation, the contents inside the stock bottle were filtered to separate the broth from the fungal biomass. The fungal biomass was blended in glucose minimal media containing (per liter): 5.5 g of glucose·$1H_2O$, 0.8 g of KH_2PO_4, 0.25 g of $Na_2HPO_4 \cdot 2H_2O$, 0.45 g of $MgSO_4 \cdot 7H_2O$, 0.25 g of $CuSO_4 \cdot 5H_2O$, 2.0 of yeast extract, and 2.0 g of peptone. As shown in **Figure 3.1.2 b,** the blended isolate UMAS MS8 with glucose minimal media (referred to as homogenized UMAS MS8) were then used as inoculum to start the production fermenter (Songulashvili *et al.,* 2015).



(a) (b)

Figure 3.2.2: The preparation of isolate UMAS MS8 using fungal biomass that were grown in MEB for 7 days on incubator shaker at room temperature (a) and homogenized isolate UMAS MS8 with glucose minimal media used as the inoculum in fermentation processes (b).

3.2.3 Fermentation methods

The fermentation media that were used in SSF and SmF were glucose minimal media containing the defined medium as mentioned in **Section 3.2.2** with the adjusted pH of 5.5 by adding 5M of NaOH before autoclaving at 121℃ for 15 minutes. However, different volumes of minimal media were used in both SSF and SmF (100 mL for SmF and 70% (v/w) for SSF). All of the fermentations were carried out in 250 mL Erlenmeyer flask. Additional attempt was made by replacing the inoculum with 8 agar-plugs (5 mm in diameter). Biotic and abiotic controls were also prepared by inoculating isolate UMAS MS8 agar plugs with glucose minimal media in biotic flask while flask with leaf litter substrate and glucose minimal media only was treated as abiotic control. (Nur Adila Muradi *et al.,* 2017).

3.2.3.1 Solid-substrate fermentation (SSF) of isolate UMAS MS8 on leaf litter substrate in 250 mL Erlenmeyer flask.

In 250 mL Erlenmeyer flasks that were prepared in triplicate, 10 g of leaf litter substrate was added in each flask before inoculating 6.14 mL – 8 mL (0.126 g – 0.168 g of fungal dry weight) of the blended fungus depending on the moisture content of the substrate in all flasks. The flasks were covered with aluminium foils and were left on static for 12 days