Utilisation of Leaf Litter from *Cinnamomum iners* (Wild Cinnamon) for Laccase Production by *Marasmius clodophyllus* UMAS MS8

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ABSTRACT

Laccase is still not widely applied in industry because of the limitation in its production. Production of laccase is very expensive by using chemical media. As a consequence of that, researchers opt for alternative ways to produce laccase affordably by using agricultural waste such as wheat bran, oil cakes and fruit peels which are easy to find and affordable. In this research, *Marasmius cladophyllus* UMAS MS8 was examined for its ability to produce laccase by using *Cinnamomum iners* as a substrate by using two fermentation methods, which were submerged fermentation (SmF) and solid-state fermentation (SSF). The fungi were maintained on 2% (w/v) malt extract broth solution at 4 ℃. Fermentation was carried out with 10 g of *C. iners* and glucose minimal media as the liquid medium in two fermentation methods. In SSF , glucose minimal (GM) media was used to moisten the *C. iners* to 70% (v/w). However, in SmF, 100 mL of the media was added to the *C. iners.* The fermentation process was carried out for 12 days with sampling at three-day interval. Laccase activity was determined based on 1 mmol of 2,2- azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) assay. The result obtained showed that *M. cladophyllus* could not produce laccase in both SSF and SmF. However, if to compare between these two methods, SSF was more favourable condition for the *M. cladophyllus* to grow since the laccase reading of SSF (0.0285 U/g) was higher than SmF (0.00574 U/g) method after 12 days of fermentation. Bradford assay was obtained to confirm that there were protein produced by the *M. cladophyllus* but not laccase.

Keyword: *Marasmius cladophyllus* UMAS MS8, solid-state fermentation, submerged fermentation,laccase

ABSTRAK

Lakase masih tidak digunakan secara meluas dalam industri kerana terhad dalam penghasilannya. Penghasilan lakase sangat mahal dengan penggunaan media kimia. Oleh hal yang demikian, penyelidik memilih alternatif lain untuk menghasilkan lakase dengan harga yang berpatutan dengan menggunakan sisa pertanian seperti dedak gandum, kek minyak dan kulit buah yang mudah didapati dan berpatutan. Dalam penyelidikan ini, Marasmius cladophyllus UMAS MS8 *telah dikaji kebolehannya menghasilkan lakase dengan menggunakan* Cinnamomum iners *sebagai substrat dengan menggunakan dua kaedah penapaian, iaitu penapaian terendam (SmF) dan penapaian keadaan pepejal (SSF). Kulat dikekalkan pada larutan kaldu ekstrak malt 2% (b/v) pada 4 ℃. Penapaian telah dijalankan dengan menggunakan 10 g* C. iners *dan media glukosa minimum sebagai medium cecair untuk dua kaedah penapaian. Dalam SSF, media glukosa minimum (GM) digunakan untuk melembapkan* C. iners *kepada 70% (v/b). Manakala, dalam SmF, 100 mL media telah ditambah ke dalam* C. iners*. Proses penapaian dijalankan selama 15 hari dengan persampelan pada selang tiga hari. Aktiviti lakase ditentukan berdasarkan 1 mmol 2,2- azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) assay. Setelah menyelesaikan proses penapaian,* M. cladophyllus *tidak dapat menghasilkan lakase sama ada untuk penapaian terendam mahupun penapaian keadaan pepejal. Walau bagaimanapun, penapaian keadaan pepejal lebih sesuai untuk pertumbuhan* M. cladophyllus *kerana bacaan lakase (0.00285 U/g) lebih tinggi berbanding dengan penapaian terendam (0.00574 U/g) selepas fermentasi selama 12 hari. Ujian Bradford telah diperoleh untuk memastikan terdapat protein yang dihasilkan oleh* M. cladophyllus *namun bukan lakase.*

Kata kunci: Marasmius cladophyllus UMAS MS8*, penapaian keadaan pepejal, penapaian terendam, lakase*

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LIST OF ABBREVIATIONS

ABTS	(2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt)
BSA	Bovine Serum Albumin
DMSO	Dimethyl sulfoxide
ED_{50}	Median Effective Dose
GM	Glucose minimal media
kDa	Kilodaltons
KH_2PO_4	Potassium dihydrogen phosphate
MIC	Minimum Inhibitory Concentration
MEB	Malt extract broth
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
$Na_2HP0_4.2H_2O$	Disodium hydrogen phosphate dihydrate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaNO_3$	Sodim nitrate
$(NH_4)_2SO_4$	Ammonium sulfate
nm	Nanometer
RBBR	Remazol Brilliant Blue R
SmF	Submerged fermentation
SSF	Solid-State fermentation
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background

Marasmius cladophyllus is a white-weathered Basidiomycota fungus. *M. cladophyllus* may degrade lignin in lignocellulose by producing lignolytic enzymes which include laccase (Risdianto,H, 2008).*M. cladophyllus* can acquire cellulose and hemicellulose after decomposing the lignin component, which is then converted into glucose molecules that are easily digested by microorganisms and used as a growing medium for their development.

Laccases copper-containing enzymes are produced by various plants, bacteria, fungi, and insects. Fruiting body formation, sporulation and dye production are all enhanced by fungal laccases. While oxidising phenolic and nonphenolic substrates, laccase can also deplete molecular oxygen to water (Hoegger et al., 2006; Góralczyk-Bińkowska, et al., 2019) due to the presence of copper in laccase.

Laccase production is constrained by an inadequacy of a productive expression structure and escalating production costs. Laccase production has its limitation of production due to the expensive chemical media used. Additionally, the operational costs are very costly to meet the standard of increasing industrial demand. Therefore, alternative sources and techniques of laccases are necessary for producing the enzyme quickly and affordably. Enzyme production can be increased by modifying the culture medium composition, transforming the process conditions, or supplementing with chemical inducers, like as metal ions and aromatic compounds. However, studies from Birhanli & Yeşilada (2017) and Zhu et al., (2016) state that metal ions and aromatic compounds can synergistically stimulate laccase production with the use of bio-waste, the production costs are reduced whilst product

concentrations increase. Laccase has been successfully produced from a variety of agricultural by-products, including fruit peels, husks, bagasse, and oil cakes (Panda et al., 2016).

Cinnamomum iners leaves litter are one of the plantation wastes that can be used as a substrate for laccase production.There is no report yet on the use of leaf litter as the subsrate for laccase production. According to Humphreys & Chapple (2002), *C. iners* leaves contain cinnamic acid, generated from phenylalanine, serves as a necessary component for lignin molecules. As a result of lignin breakdown and allelopathy, fungus would be exposed to cinnamic acid. Hence, in this research,the lignin induced *M. cladophyllus* to produce laccase.

There are two fermentation methods that were tested out in this project using *C. iners* as fermentation substrate for laccase production namely submerged fermentation method and solid-state fermentation method. Submerged fermentation method is in which microorganisms grow in a liquid broth medium while solid-state fermentation is conducted on a solid substrate with a low moisture source material.

Hence, it is hypothesised that *C. iners* can be used as a substrate for the production of laccase by *M. cladophyllus* UMAS MS8. Solid-state fermentation is also hypothesised to have a higher laccase production by the fungus as compared to submerged fermentation.

1.2 Objectives

a) To determine whether *M. cladophyllus* is capable of using *C. iners* as substrate for the production of laccase.

b) To compare the perfomance of submerged fermentation and solid-state fermentation in the production of laccase using *C. iners.*

CHAPTER 2

LITERATURE REVIEW

2.1. *Marasmius cladopyllus* UMAS MS8

Marasmius cladophyllus is a fungus species pertaining to the Marasmiaceae family. It can be usually found in the South and Central of America. A distinguishing feature of the mushroom is the gills distinctive ventricose appearance.

Marasmius cladophyllus UMAS MS8 was initially isolated as an endophytic fungus from *Melastoma malabathricum* (Ngieng et al., 2013). Endophytic fungi live in plant tissues without infecting or killing the host cell. Due to their symbiosis, endophytes synthesize nearly same chemicals to those produced by the host plant (Woods, et al., 2017). The uses of continuous transfer of the fungal isolate which referred as isolate UMAS MS8, on malt extract agar (MEA) can help to sustain it.

M. cladophyllus UMAS MS8 can decolorize a variety of textile dyes, including RBBR, in both liquid and solid media (Ngieng et al., 2013). Extensive studies on the dye decolorisation ability of *Marasmius* sp., such as isolate UMAS MS8, have been reported infrequently. Potential causes include the poor production of lignin-modifying enzymes and the inability of certain *Marasmius* sp. to decolorize dyes initially discovered for various *Marasmius* sp. (Leung & Pointing, 2002; Lucas et al., 2008).

Nevertheless, *Marasmius* sp., more specifically *Marasmius quercophilus*, has been discovered to generate laccase, and endeavours have been undertaken to purify the enzyme for use in the breakdown of xenobiotic aromatic compounds (Dedeyan et al., 2000, Farnet et al.,2002, Farnet et al., 2004).

Additionally, it has been shown that *Marasmius scorodonius* produces a new peroxidase capable of decomposing beta-carotene (Scheibner et al., 2008). *Marasmius* sp. has provided a report of a rare single copper laccase capable of decolorizing numerous dyes, including RBBR (Schückel et al., 2011). Hence, in this study, *M. cladophyllus* was used to produce laccase without the presence of decolourized dye and feeds the *M. cladophyllus* by using the leaves litter of *C. iners.*

2.2. *Cinnamomum iners*

Cinnamomum iners is very similar to *Cinnamomum paraneuron* and *Cinnamomum intidum*. *C. iners* is also commonly called as wild cinnamon, clove cinnamon, *kayu manis*, *medang teja* and *teja lawang*. It is belonged to Lauraceae family. *C. iners* is a tree that can reach a height of 18m and has a thick, bushy canopy. It flowers periodically, and the tree is flushed with cream-colored inflorescences that have a terrible sticky odour which then make hoverflies, beetles, and other small insects to be attracted to this plant (Jake,2018). Birds, bats, and squirrels eat the berries and disseminate the seeds. Additionally, *C. iners* is the host plant to butterflies such as Common Mime and Common Bluebottlem (Jake, 2018).

The leaves are tri-veined and alternately oriented. The immature leaves are a shade of reddish pink. According to Corner (1997), it may be distinguished from other *Cinnamomum* species by its glabrous, slightly cinnamon-scented, oblong leaves.

The leaves of the *C. iners* contained cinnamaldehyde and eugenol that have an antioxidant activity. Research from Lee et al. (2002) states that cinnamaldehyde's inhibitory effects on nitric oxide synthesis have proved that cinnamaldehyde can inhibit both nitric oxide production and the expression of inducible nitric oxide synthase. On top of that, arabinoxylan also a chemical compound found in *C. iners* which creates excessively viscous

aqueous solutions and have a large water capacity that can be useful in fermentation process (Foschia et al., 2013).

C. iners has been useful for both as a natural herb and as a landscape plant. The leaves are frequently used in traditional medication to treat fever, gastrointestinal disturbances, and as a diaphoretic (Pengelly, 2021). It is believed to be essential in curing rheumatism by adding boiled leaves in bath water on a daily routine. Headache and fever can be relieved by drinking leaf juice. According to Chooi (2004), the crushed leaves can be applied on the damaged area of cuts and bruises. Apart from that, because of the fragrant scent and substantial mucilage level, the leaves have also been widely applied as joss sticks and insect repellents (Jantan et al., 1992). The leaves are beneficial to sweeten drinking water in rural regions. The leaves are also valuable as a raw material in production and industrial such as plastic, gum, paper, tyres, and glass.

2.3 Laccase

Laccases are the enzyme systems that are the most ancient and well-studied (Williamson,1994). Laccases were initially discovered upon the Japanese lacquer tree *Rhus vernicifera* in 1883 (Yoshida,1883). These enzymes are composed of 15–30% carbohydrate and have a molecular mass of 60–90 kDa. These are 1,4-benzenediol: oxygen oxidoreductases (EC 1.10.3.2) containing copper that are produced in vascular plants and microbes. Laccase also a glycosylated polyphenol oxidases with four copper ions per molecule which oxidise phenolic and related compounds by one electron and reduce oxygen to water (Nunes & Kunamneni,2018). When a laccase oxidises a substrate, it lost one electron and commonly creates a free radical that can be either oxidised or undergo nonenzymatic processes such as hydration, disproportionation, and polymerization (Shraddha et al., 2011).

2.3.1 Sources of Laccase

Laccases are more abundant in fungus compared in higher plants. Laccase is produced by basidiomycetes such as *Phanerochaete chrysosporium, Theiophora terrestris,* and *Lenzites betulina* (Bello et al., 2021), as well as white-rot fungi including *Phlebia radiate*, *Pleurotus ostreatus*, and *Trametes versicolor*. *Trichoderma* species, including *T. atroviride, T. harzianum*, and *T. longibrachiatum* are laccase producers. Laccase from *Monocillium indicum* was the initial laccase from Ascomycetes that has been identified with peroxidase activity (Thakker et al., 1992). However, there are also some species of fungi that does not produce laccase for instance, Zygomycetes and Chytridiomycetes (Morozova et al., 2007).

2.3.2 The Effect of Carbon and Nitrogen Sources to Laccase

Carbon sources such as maltose, glucose, fructose, and lactose are frequently utilised for laccase production. Excessive glucose and sucrose inhibit the beginning of laccase synthesis. This issue of enzyme synthesis has been addressed by using polymeric substrates such as cellulose (Lee et al., 2004). Nitrogen sources such as yeast extract, peptone, urea, $(NH_4)_2SO_4$, and $NaNO_3$ are frequently utilized. Although nitrogen deficiency induces laccase formation (Keyser et al., 2021), some nitrogen sources have no effect on the enzyme's efficiency (Leatham & Kent Kirk,1983). Based on Buswell et al. (1995) studies, it has been reported that the increment of laccase activity can be achieved by employing a high carbon-to-nitrogen ratio whilst Monteiro & De Carvalho (1998) research, the increment of laccase activity can be accomplished by using a low carbon-to-nitrogen ratio.

2.3.3 Usage of Laccase

Food and beverage production companies, laccase is being applied in the clarification of beer, wine, and fruit juices to eliminate undesirable phenolic compounds that causes browning or darkening, haze production, and turbidity formation (Bilal et al., 2019). Due to the low thermostability and acidic working pH characteristics, laccase have been widely used in pulp and paper industry for the ecologically sustainable manufacturing of high grade bleached cellulosic pulps without the utilization of toxic substances (Singh et al., 2015). In pharmaceutial industry, laccases have shown substantial potential in the production of anticancer and antibiotic medications, as well as in the formation of melanin, prostaglandins and allergenicity control (Chaurasia et al., 2016). Recent study by Coelho et al. (2019) have created a laccase biosensor for the detection of dopamine in synthetic biological specimens, demonstrating good selectivity even in the presence of uric acid, ascorbic acid, and other phenolic compounds.

2.4 Enzyme Assay

Enzyme assays have a function to detect a specific enzyme, to demonstrate its presence or absence in a specific sample, such as an organism or a tissue, and to evaluate the amount of the enzyme present in the sample. The activity of enzymes is highly dependent on specific variables such as temperature, pH, nature, glucose concentration, and ion strength (Bisswanger,2014). While enzymes perform optimally in their optimal conditions, deviations from the optimal result in a decrease in activity, depending on the degree of the aberration.

2.4.1 Continuous Assay

One type of enzyme assay includes continuous assay. The term "continuous assay" refers to an assay that provides a consistent measurement of procedure. Continuous assays

are the most efficient because they provide the reaction rate with no workload. Continuous assays enable online monitoring of the enzymatic reaction and offer the advantage of allowing the reaction's progress curves to be tracked immediately (Geueke & Kohler,2010).

2.4.2 Spectrophotometry Assay

Spectrophotometric assay is the example of the continuous assay that will be used in this research. The method of this assay is to determine the absorption of light from the assay solution. When the light is in the observable area, the colour of the assay changes, which is known as a colorimetric assay. ultraviolet light is commonly utilized in spectrophotometry assays because NADH and NADPH absorb UV light only in reduced forms instead of oxidised forms. As a result, an oxidoreductase using NADH as a substrate can be tested by measuring the decrement in UV absorbance at 340 nm as it uses up the coenzyme.

2.4.3 ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt) Assay

According to Góralczyk-Bińkowska et al. (2020), the laccase activity can be determined by using ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) as substrate and determining the absorbance difference due to the action of enzyme on the substrate using a Specord 200 spectrophotometer in one minute. Sondhi et al. (2014) states that at 420 nm of the molar absorption coefficient (ε) for ABTS is $36{,}000\text{M}^{-1}\ \text{cm}^{-1}$. Figure 2.4.3 shows the result of colour changes to green at the end of the product when using ABTS substrate.

Figure 2.4.3 A green end-product of ABTS substrate (Guan et al., 2015)

ABTS is a water-soluble HRP substrate that, when used in conjunction with peroxidase, produces a green end-product. The green product exhibits two distinct absorbance peaks at 410 and 650 nm (Hosseini,2018). Despite its relatively larger redox potential and intensify the oxidation of aromatic compounds (Farnet et al.,2004). Bourbonnais et al. (1998) and Eichlerova et al. (2012) found that ABTS demonstrates 40 times greater sensitivity to laccase activity than the 3,4-DihydroxyL-phenylalanine L-DOPA assay.

2.4.4 Bradford Coomassie Blue G-250 Assay

The Coomassie Blue G-250 is a reddish brown dye with a maximum absorbance of 465 nm that shifts to 595 nm when attached to protein (Becker et al., 1996 ; Goldring, 2019). The dye reacts with proteins in the Bradford assay, resulting in a colour transition. The dye reacts with arginine and, to a lower proportion, lysine, histidine, tyrosine, tryptophan, and phenylalanine residues in proteins to generate the blue colour in acidic conditions (Goldring, 2019). Over a 10-fold concentration level, the extinction coefficient of a dye-albumin complex solution is constant (Becker et al., 1996).

Bradford assay is a much simpler technique as compared to Lowry assay. It takes only one reagent and five minutes to conduct an assay as opposed to three reagents and 30-

40 minutes for Lowry assay (Becker et al., 1996). Besides, the dye-protein complex absorbance of Bradford assay is very persistent and does not demand the critical time required as compared to the Lowry assay. Bradford assay also does not influenced by many compounds that limiting the Lowry assay application.

2.5 Submerged Fermentation

Submerged fermentation is a process where microorganisms grow in a liquid broth medium that has been modified with the necessary nutrients for optimal microbe development. This is attained by nurturing the specified microorganisms thoroughly in a locked reactor that consists of the fermentation medium and a high level of oxygen (Doriya et al., 2016).

SmF is a faster process than solid-state fermentation (SSF) to rise the laccase production yield (Songulashvili et al., 2007). Physiological control of laccase synthesis by SmF is substantially simpler than control via SSF (Elisashvil et al., 2008). The other advantages of SmF includes a good heat and mass transfer, better diffusion of microorganisms and comercially available in large scale (Doriya, 2016). The addition of various agriculture lignocellulosic wastes to a chemically synthesized carbon source in SmF contributed to the laccase successful synthesis by white-rot fungus (Stajić, et al., 2006). According to Songulashvili et al. (2012) previous research, it was demonstrated that *C. unicolor* C-139 growing on wheat bran in SmF has produced laccase at an extraordinarily high level of 416.4 UmL^{-1}. This is the earliest study that examines SmF laccase production on using wheat bran, which is an affordable medium.

Submerged fermentation process has been used for citric acid fermentation by using batch fermentation method (Moore et al., 2020). In 2020, United States of America has the highest citric acid production compared to other country which are China, Africa, Asia and

Europe (*IHS Markit's Chemical Economics Handbook* – Citric acid, 2020). In addition, antibiotics are the most common bioactive chemicals synthesized from microorganisms through fermentation. The use of submerged fermentation was widely spread in the early stages of production. Penicillin from *Penicillium notatum* was the first antibiotic to be economically generated by fermentation (Ravichandran & Vimala, 2012).

2.6 Solid-State Fermentation

Solid-state fermentation has commonly used technology for the synthesis of microbial metabolites (Tsuchiya et al., 1994). SSF is conducted on a solid substrate with a low moisture content, which leading to an increase of product concentration with a comparatively low energy requirement (Maruyama et al., 2000). The substrate absorbs the vital water content in SSF in a solid substrate, which supplies supplemental benefits for microbe production and transfer of oxygen (Robinson et al., 2001).

Industrially, metabolites are primarily produced via SmF because to the simplified down streaming process associated with SmF than SSF (Sadhukhan et al., 1999). Although SmF is a simpler process to operate than SSF, the microbial mycelial structure is well adapted for the generation of microbial secondary metabolites in SSF (Suryanarayan,2003).

Thus, SSF has various benefits, including ease of gaseous transmission, reduced water usage, the utilisation of cellulosic waste, pH control, and the usage of smaller fermenters, which requires lesser work for downstream processing (Robinson et al., 2001).

Solid pollutants that contribute to pollution and are a rich source of cellulose and protein may be used as substrates for SSF. Particularly in Japan, SSF is commonly used in the manufacturing of a variety of products, including amylase, protease, and soy sauce. Additionally, a large amount of SSF-related equipment has been successfully constructed and modified in Japan for the manufacture of a variety of industrially significant microbial

metabolites (Cooper et al., 1981). Meanwhile, Biocon Company in India has begun using the SSF technique for enzyme manufacturing over two decades ago (Suryanarayan, 2003). Figure 2.6 shows an overview of the submerged fermentation and solid-state fermentation.

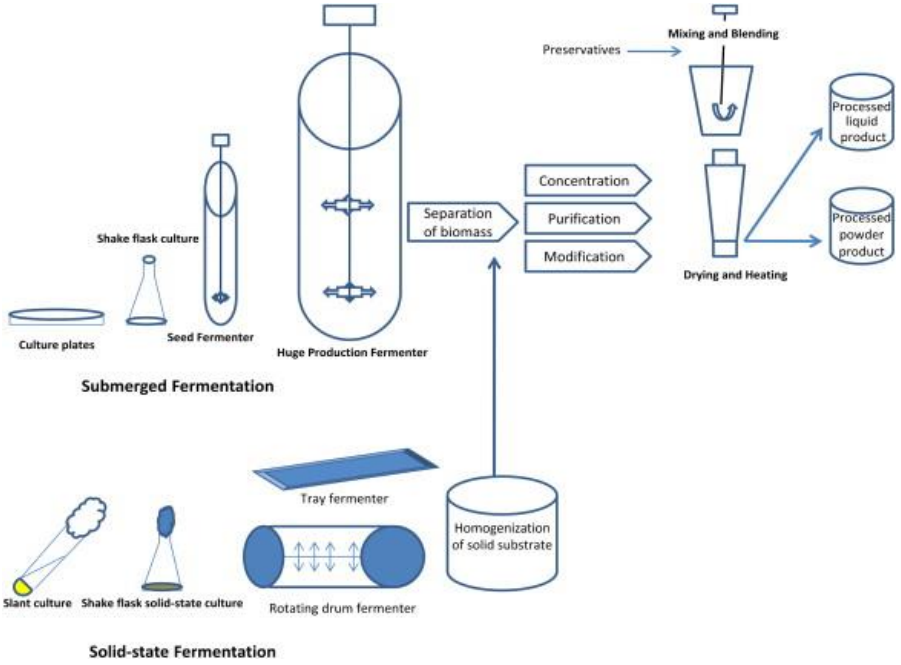

Figure 2.6. An overview of the submerged fermentation process and solid state fermentation process (Singhania et al., 2015)

CHAPTER 3

MATERIALS AND METHODS

3.1 Methods

3.1.1 Leaf Litter Preparation

C. iners leaves were collected at the UNIMAS campus area, Kota Samarahan, Sarawak. The leaves litter *of C. iners* were sorted out from their stems. Then, the leaves litter were washed and rinsed. After rinsing the leaves litter, they was dried in the oven overnight at 70℃. Then, they were crushed and grinded by using blender to blend them into powdered form. No water was poured into the blender to maintain the dryness of the leaves. This powdered leaves sample were transferred into airtight glass for the experiment and was used as the substrate for laccase production.

3.1.2 Inoculum Preparation of the Media

M. cladophyllus was obtained from Molecular Genetic Laboratory, Faculty of Science Resource and Technology,University of Malaysia Sarawak. Inoculum preparation of *M. cladophyllus* were obtained by growing the UMAS MS8 in the Malt Extract Agar (MEA) plate for 7 days. After 7 days of subculture, 3 plugs of mycellium were added into 400 mL of 2% (w/v) Malt Extract Broth (MEB) and were incubated for 7-10 days at the rotary shaker (Songulashvili et al., 2015).

After 7-10 days of cultivation and more fungal biomass were produced, the contents in the flask were filtered to separate the broth from fungal biomass. Then, the fungal biomass was weighed out at 6.46 g- 8g (0.138g -0.168 g of fungal dry weight) which were used as inoculum to start the production fermenter (Songulashvili et al., 2015).