Enzymatic Degradation of Lignin by *Trichoderma reesei* extract Using Ethyl Acetate solvent

Mahfuzah Binti Mahmud

F18A0257

A thesis submitted in fulfilment of the requirements for the degree of Bachelor of Sustainable Science (Agrotechnology) With Honours

Faculty of Agro Based Industry

Universiti Malaysia Kelantan

2022

DECLARATION

I hereby declare that the work embodied in here is the result of my own research except for the excerpt as cited in the references.

Mahfuzah

Signature

Student's Name: Mahfuzah Bt Mahmud

Matrix No: F18A0257

Date:

Verified by:

Supervisor Signature

Supervisor's Name:

Stamp:

Date:

ACKNOWLEDGEMENT

All praises to Allah, the most merciful for giving me strength and chances to complete this research for Final Year Project (FYP). I would like to express my gratitude to Universiti Malaysia Kelantan for providing a conducive area, complete materials and apparatus, and helpful laboratory staff. My special appreciation goes to my supervisor, Dr. Mst Laila Naher for her continuous guide, support, advice and give encourage during my lab work. Thank you again for enlightening my thoughts about this research until I have improved my understanding towards the topic.

I am thankful as people surrounds give persistent help and guidance especially to Mr. Basiri Bristone, Ms. Gunawati and Miss Maryam on how to conduct the experiment in the laboratory. I am also grateful for having an understanding FYP mates for always give moral support every time I feel down or having problems due to experiment results.

Aktiviti Lignin daripada *Trichoderma reesei* Ekstrak Menggunakan Pelarut Etil Asetat

ABSTRAK

Biojisim lignoselulosa (LCB) sememangnya merupakan asset boleh diperbaharui yang kebanyakanya terdiri daripada selulosa dan hemiselulosa (polisakarida dan polimer aromatik yang berasal daripada tumbuhan (lignin). Memandangkan biojisim lignoselulosa adalah sisa yang paling banyak daripada pengeluaran pertanian, ia telah diberi perhatian utama sebagai sumber biojisim untuk pengeluaran biobahan api. Menukarkan biojisim lignoselulosa kepada biobahan api untuk bahan api pengangkutan ialah strategi yang berdaya maju untuk meningkatkan keselamatan tenaga sambil mengurangkan kesan terhadap alam sekitar seperti pencemaran udara dan pelepasan gas rumah hijau. Memandangkan permintaan dan penggunaan bahan api meningkat pada setiap tahun, tindakan pantas mesti diambil untuk meningkatkan kecekapan dalam pengeluaran biobahan api daripada sisa lignoselulosa. Selain itu, penciptaan pengeluaran biobahan api generasi kedua daripada sisa lignoselulosa mempunyai banyak faedah dari kedua-dua aspek iaitu alam sekitar dan tenaga. Walau bagaimanapun, perbelanjaan enzim selulase adalah komponen penting kepada kos operasi kilang penapisan bio bahan halus yang menghasilkan biofuel daripada bahan suapan lignoselulosa. Spesies *Trichoderma* dikenali sebagai kulat lignoselulolitik yang mempunyai potensi tinggi dalam merendahkan biojisim tumbuhan melalui aktiviti selulolitik. Spesies kulat ini juga mempunyai keupayaan untuk mengeluarkan pelbagai enzim yang boleh digunakan untuk memecahkan selulosa, kanji dan lipid. *Trichoderma reesei* adalah antara spesies *Trichoderma* yang menjadi pengeluar utama enzim lignoselulolitik dan *T. reesei* mempunyai sistem enzim selulase yang dibangunkan yang berterusan, ekstraselular, dan produktif. *T.reesei* kerana keberkesanannya dalam sintesis enzim pengurai selulosa dan kanji dalam banyak penyelidikan yang telah dijalankan. Eksperimen dalam penyelidikan ini dijalankan untuk melihat aktiviti degradasi lignin oleh ekstrak *Trichoderma reesei* dengan nisbah berbeza menggunakan pelarut etil asetat. Kepekatan *T.reesei* yang digunakan ialah 5 mg, 10 mg, 15 mg dan 20 mg. Degradasi enzimatik lignin dapat dilihat pada kepekatan 10 mg, 15 mg dan 20 mg ekstrak *T.reesei* yang diameter zon halo coklat gelap meningkat apabila kepekatan lebih tinggi.

Kata kunci: Biojisim lignoselulosa, *Trichoderma reesei,* bio bahan api, degradasi lignin, etil asetat.

Enzymatic Degradation of Lignin by *Trichoderma reesei* Extract Using Ethyl Acetate Solvent

ABSTRACT

Lignocellulosic biomass (LCB) is indeed a renewable asset made up mostly of cellulose and hemicelluloses (polysaccharides) and an aromatic polymer derived from plants (lignin). Considering lignocellulosic biomass is the most abundant residual from agricultural production, it has been given major attention as a source of biomass for biofuel production. Converting lignocellulosic biomass to biofuels for transportation fuels is a viable strategy for boosting energy security while reducing the impact on the environment such as air pollutants and emissions of greenhouse gas. Since the demand and consumption of fuel is rising every year, fast action must be taken to increase the efficiency of biofuel production from the lignocellulosic residual. Besides, the creation of second-generation biofuel production from lignocellulosic waste has numerous benefits from both environmental and energy aspects. However, the expense of cellulase enzymes is a significant component to the operating costs of a biorefinery that produces biofuel from lignocellulosic feedstock. *Trichoderma* species is known as lignocellulolytic fungi and has high potential in degrading plant biomass through cellulolytic activity. This fungi species also has the ability to manufacture various enzyme that can be used to break down cellulose, starch and lipid. *Trichoderma reesei* is among *Trichoderma* species that became main producer of lignocellulolytic enzyme and *T. reesei* have a developed cellulase enzyme system that is persistent, extracellular, and productive. *T.reesei* owing to its effectiveness in the synthesis of cellulose- and starch-degrading enzymes in much research that has been conducted. The experiment in this research is conducted to see the degradation activity of lignin by *Trichoderma reesei* extract with different ration using ethyl acetate solvent. The concentration of *T.reesei* used is 5 mg, 10 mg, 15 mg and 20 mg. The enzymatic degradation of lignin can be seen on 10 mg, 15 mg and 20 mg concentration of *T.reesei* extract which the diameter of dark brown halo zone increase while the concentration is higher.

Keywords: Lignocellulosic biomass, *Trichoderma reesei,* biofuel, lignin degradation, ethyl acetate

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

No reference		Page
cm	Centimeter	18
°C	Degree Celsius	23
%	Percent	14
mg	Milligram	30
mm	Millimeter	28
ml	Milliliter	27
<	Less than	32
±	Plus or minus	28
g	Gram	27
$	Dollar	15

LISTS OF ABBREVIATION

No reference		Page
CBP	Consolidated Bioprocessing	19
GH	Glycoside hydrolyse	20
PDA	Potato Dextrose Agar	27
CRD	Complete Randomized Design	31
SPSS	Statistical Package for the Social Science	31
ANOVA	Analysis of Variance	31

LISTS OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Research Background

Lignocellulose is widely available across the world as a renewable organic material, and it is regarded as a valuable chemical compound and act as an important alternative raw material to produce biofuels. The composition of lignocellulose is divided into three major material such as 30% to 50% of cellulose, 25% to 30% of hemicellulose and 15% to 20% of lignin (Gupta et al., 2016; Li, 2014; de Paula et al., 2019). Many industries have used value-added product from the lignocellulose by converting its waste through bioconversion process known as hydrolysis such as fermentation, fiber material and papermaking industry. The process begins with five and six simple carbon sugars that will be produced by enzymatic hydrolysis right after the enzyme access into the lignocellulose residues structure via cellulose then it will be turned into a few products of biotechnological interest. For instance, biofuels, ethanol, methanol, and animal feed are the most common sources produced by this

method. Besides, (Wang et al., 2020) in the past few decades, kinds of chemical, biological and physical pretreatment technologies have been established to maximize the lignocellulose waste's benefit in order to gain high- yield biogas.

Over 14,000 fungi capable of digesting cellulose have been identified by 1976 (Dashtban et al.,2009). Fungi play an important role in the natural decay of lignocellulosic residues by creating a variety of lignocellulolytic enzymes. Fungi have a very effective enzymatic system compared to yeast and bacteria, that allows them to breakdown lignocellulose-containing source materials. The degradation system of fungi is consisting of two part which is intracellular and extracellular. The intracellular part is including the outer cell envelope layer while the extracellular section is having two kinds of enzymes: oxidative, which degrades lignin and hydrolytic, which deteriorates polysaccharides (Andlar et al., 2018). The most effective microorganisms for manufacturing lignocellulolytic enzymes are filamentous fungus (Chen et al., 2020; de Paula et al.., 2019). This filamentous fungus can be found in a variety of places, including the lignocellulose wastes, live plants, and ground. According to (Khelil and Cheba, 2014; Asis et al., 2021), *Trichoderma reesei* and *Trichoderma viride* are able to manufacture hydrolytic enzymes and it has widely applied in the biotechnology industry. *Trichoderma reesei* is a primary workhorse in the manufacture of cellulase, which hydrolyzes lignocellulosic biomass then turn it into bio-based goods. Many researchers have discovered *T.reesei* as an effective cellulase producer for degrading cellulose (Ravikumar et al., 2014 ; Kantharaj et al., 2017). *T.reesei* is perhaps better recognised as a cellulolytic fungus than a lignocellulolytic fungus. Meanwhile, for the good lignin peroxidases manufacturer is among Basidiomycetes fungi to degrading the lignin such

as *Trametes versicolor, Perenniporia medullapanis, Phanerochaete chrysosporium*, and *Pleurotus ostreatus.*

1.2 Problem Statement

The cost for the synthesis process of cellulase enzyme became a main concern as the lignocellulose waste is an important renewable material for biofuel production (Bata et al., 2008; Juneja et al., 2013; Xu and Huang, 2014). According to (Brethauer and Studer, 2015; Kubicek and Kubicek, 2016; Druzhinina and Kubicek, 2017) the step of biomass conversion starts with chipping the plant debris into a smaller size. The biomass is then subjected to a physicochemical preparation or modest chemical to make it more amenable to enzyme breakdown. Lastly, during the enzymatic hydrolysis, cellulose will be converts into D-glucose and balance of present starch will turn into monosaccharides such as L-arabinose, D-xylose and D-glucose. However, when it comes to the enzymatic reaction step, a few enzymes in the cellulase cocktail have insufficient catalytic activity, reducing the quantity of saccharification required (Rabemanolontsoa and Saka, 2016).

Besides, the synthesis and usage of these enzymes is a significant cost component in this process. In addition, the ascomycete *T.reesei* produces nearly all commercially available lignocellulolytic enzymes required for the hydrolysis stage (Bischof et al., 2016). However, in a lignocellulosic biomass-based biorefinery, the cost of commercial enzymes can account

for 20–40% of the total operating expenses as Humbird et al., (2011) has stated in their enzyme generation for ethanol production on-site model.

1.3 Hypothesis

H_0: There is no enzymatic degradation of lignin by *Trichoderma reesei* extract using ethyl acetate solvent.

$H_{1:}$ There is an enzymatic degradation of lignin by *Trichoderma reesei* extract using ethyl acetate solvent.

1.4 Significance of the Study

The result of this study shows that because of the high cost of cellulase preparation and the complex fermentation process, lignocellulosic biomass has developed as an alternate solution to these problems, as well as to lessen the environmental impact of burning rice straw. Other than that, the problem of rising costs of fossil fuels can be tackled by this lignocellulose bioprocessing as an expected solution. For example, based on the collected data, the cellulase market is estimated to be worth $400 million per year in the future as a variety of sectors worldwide start to produce cellulose (Gupta and Verma, 2015; Han et al., 2015).

Besides, it will contribute to the idea of future studies on lignocellulolytic activities of *Trichoderma reesei* which is focusing on the potential of this species to degrade the lignin. Thus far, Nur et al., (2014) state that there is a scarcity of data on the ligninolytic activities of other *Trichoderma* species. Lignin degradation is useful for paper and pulp industry since it lowers the energy usage during the pulping process, removes wood extractives, enhances the paper strength, and reduce the chemical usage.

1.5 Objective

1. To extract the secondary metabolites using ethyl acetate solvent from *Trichoderma reesei.*
2. To observe the enzymatic degradation of lignin activity of *Trichoderma reesei* extract using ethyl acetate solvent.

CHAPTER 2

LITERATURE REVIEW

2.1 Genus Trichoderma spp.

Trichoderma is a filamentous fungus that act as a biocontrol agent as its potential can produce antibiotics and enzyme (Babu et al., 2014; Filizola et al., 2019; Asis et al., 2021), while it is also classified as the most popular culturable fungi in the soil (Shukla and Vankar, 2014). *Trichoderma* are the mycoflora components in soils that can be found at a variety of habitats, including forests, prairie, desert, salt marshes, and agricultural lands (Kamala et al., 2015). According to (Druzhinia et al., 2018; Hewedy, El-Zanaty and Fahmi, 2016) *Trichoderma spp.* has the capacity to create multiple digestive enzymes that break down diverse biopolymers, such as cellulose, starch, lignin, lipids, chitin, and protein, has been used as a technique for degrading lignocellulose biomass, with biofuel being one the most lucrative outcome from the process called hydrolysis.

As mentioned before, *Trichoderma spp.* is an important element in manufacturing biofuel as it also can produce bioethanol through cellulolytic activity which involves process of hydrolyzing cellulosic biomass into the glucose and it will turn into bioethanol after being fermented (Bu et al,2019). Lignocellulolytic fungi from genus *Trichoderma* are good in decomposing plant biomass including highly toxic phenolic compound and it is also act as biological control agent which highly cellulotic activity (Dzuhinina et al., 2018; Nur et al., 2014).

From the research study of comparison between lignocellulolytic activities of *Trichoderma* isolates from Sabah and Antarctic shows that enzymatic degradation of hemicellulose is the highest (7.47 cm) followed by lignin (4.93 cm) and cellulose (2.53 cm) whereas the *Trichoderma* isolate from Sabah always shows higher diameter of halo zone which indicates the lignocellulosic activity (Nur et al., 2014). However, *Trichoderma* species used in this experiment is not stated.

2.1.1 Trichoderma reesei

The lignocellulolytic enzymes producer is mainly conquer by *Trichoderma reesei* (Novy et al., 2019) and the whole set of cellulolytic enzymes is one of the hydrolases generated by *T. reesei*. These enzymes are widely applied in the textile, pulp, paper, food, feed, and biofuel industry as it became an alternative to reduce the enzyme manufacturing

cost (Adav, Chao and Sze, 2012). With unique cellulase profiles and enzyme combinations in the *T.reesei*, it became an excellent host for large-scale heterologous protein synthesis. Pellets, mycelial clumps, and freely disseminated mycelia are morphological characteristics of *T.reesei* that have been linked to enzyme production (Domingues et al., 2000; Vaidyanathan et al., 2003; Adav, Chao and Sze, 2012).

Bioconversion of lignocellulosic wastes is largely begun by microorganisms such as *Trichoderma reesei* and *Aspergillus niger*, according to Hewedy, El – Zanaty, and Fahmi (2016). Besides, ascomycetes (*T.reesei*) generate more cellulolytic enzyme, the growth hormone proteins compare to basidiomycetes (*P. chrysosporium*) on cellulosic and lignocellulosic biomasses (Adav, Chao and Sze, 2012). Researcher has stated that *T. reesei* has been identified as a possible candidate for Consolidated Bioprocessing (CBP), where one microorganism performs the synthesis of lignocellulose into desirable products in a single step (Lynd et al., 2002; Xu, Singh and Himmel, 2009; Zhang and Zhang, 2010).

Furthermore, *T. reesei* may also use all the lignocellulose carbohydrates in the lignocellulose to produce ethanol. However, there is still a lack of industrial cellulosic ethanol manufacturing as the production of cellulose for hydrolysis costs is high. As a result, a new technology of traditional metabolic engineering, post evolutionary engineering and mutagenesis are useful for cellulose production by enhancing the phenotypes of *T.reesei* strains (Xu, Singh and Himmel, 2009). A study conducted by (Druzhinina and Kubicek, 2017), has found that *T.reesei* has a potential in producing a powerful mixture of hydrolysis enzymes for cellulose which became an alternative to cellulose hydrolysis in cost-effective way. *T.reesei* also has been identified as a special fungi for manufacturing cellulase with established technologies and experienced industry for handling it (Bischof et al., 2016).

Enzyme cellulases produced by *T.reesei* are different from other fungi as it have been grouped in six glycoside hydrolase (GH) families such as GH3,GH5,GH6, GH7,GH12, and GH45 (Druzhinina and Kubicek, 2017). Each of these functional GH group have its own task such as catalysis in a processive and rapid mode, contribute to the bulk of hydrolytic turnover, and can further penetrate into the cellulose material.

2.2 Secondary metabolites of *Trichoderma reesei*

Secondary metabolites produced by the genus *Trichoderma* consist of various biological activity (Ghisalberti & Sivasithamparam, 1991; Reino et al., 2008; Vinale et al., 2012). *Trichoderma* metabolites are produced in form of hormones, enzyme, antibiotic compounds, and toxins (Nakkeeran et al., 2021). Besides, *Trichoderma* metabolites act as signal compound also can improve the plant growth (Benítez et al., 2004) by promoting the lateral root growth, producing auxin analogues and enhance the production of plant biomass (Hoyos et al., 2009; Contreras et al., 2009).

2.3 Extraction of secondary metabolites

Secondary metabolites extraction from fungal culture is crucial as during culture fungal spore or mycelia can be affect by abiotic condition in media. Roopashree and Naik (2019) reported that the extraction of metabolites can be based on the partitioning of components between solvent phases and the creation of solid residuals. The solvent volume concentration should be gradient between micelles and eventually zero to achieve equilibrium. The nature and property of the secondary metabolites, mixing ratio, quantity, pH, temperature, selection of solvent and method of solvent preparation affecting the equilibrium state.

2.3.1 Ethyl acetate solvent

Ethyl acetate solvent is formed by acetic acid and ethanol and it act as polar aprotic solvent (PubChem, 2022). Ethyl acetate has lower density than water whereas it features as colourless, transparent liquid with a fruity odour, and the flash point is 24°. It has been used in the extraction process as a choice solvent which good in extracting the polar and non-polar (biphasic actions) and has low toxicity towards test strains. Besides, medium polarity and little toxicity on test strains are two chemical and biological features of ethyl acetate. In the

study of comparison between n-hexane and ethyl acetate for the yield production in the analysis of Sarang Banua ‘s phytochemical shows that ethyl acetate produces highest yield (10.63% w/w) than n-hexane (3.82%) (Simorangkir, Nainggolan and Silaban, 2018).

Ethyl acetate is generally utilised as an extraction solvent for a variety of processes, including decaffeination of coffee and tea; a coatings solvent for paints, lacquers, and varnishes; a carrier solvent for printing inks, adhesives, and nail polish. a pharmaceutical process solvent (Yeşilyurt et al., 2021).

2.4 Tannic Acid Media

Tannic acid is one of the important materials in making Tannic Acid Media and it is widely utilised as a ligninolytic microorganism selection indication (Mukhlis et al., 2013; Asis et al., 2021). Tannic acid is use for primary screening of lignolytic potential of ligninolytic fungi on the plate assay. According to (McSweeney et al., 2001; Silva et al., 2010), tannic acid is a member of the hydrolyzed-tannins group, which has a glucose molecule connected to gallic acid and exhibits antinutritional properties in herbivorous organisms due to its capacity to bind with cellulose, protein, pectin, hemicellulose, and minerals (Silva et al., 2010). Tannic acid also forms complexes with other nitrogen-containing compounds such as nucleic acid, quitin, peptides, and amino acids.

In addition, lignolytic enzymes consist of two grous which is lignin peroxidases (peroxidies) and laccase (polyphenol oxidase) and manganese peroxidase, play an important role in lignin degradation process (Sharma et al, 2017). The ligninolytic fungi will produces polyphenol oxidase enzyme when catechol oxidase and monophenol oxidase catalysed the interaction of poly polyphenol with molecular oxygen and this will result dark brown complex on the media plate. The tannic acid agar plate will be incubated at temperature 25-30°C depending on the fungi used.

2.5 Lignocellulose degradation

Lignocellulose is one of the essential parts to build up the plant cell wall. Plant cell walls are mostly made up of two types of chemicals: lignin and the polysaccharides cellulose, which the main polysaccharides in all lignocellulosic, and the hemicelluloses (Hubbe, 2014). Lignocellulose biomass can be obtained from agriculture byproduct such as sawdust, wood, and sugarcane residues and industrial wates. *Trichoderma* species produce enzymes and use it to bring chemical changes during degrading.

Pretreatment, enzymatic hydrolysis, and fermentation are all steps in the conversion of lignocellulose into biofuels and economically valuable products (Soni, Sharma and Soni,2018). The degradation process occurs in nature with favourable condition to microbial activity otherwise it will interrupt the decomposing process.

2.6 Role of lignocellulose material in industry

Lignocellulosic biomass provides an abundant and relatively affordable raw material for biorefineries (Taylor, 2008; Menon and Rao, 2012; Golecha and Gan, 2016). This lignocellulosic biomass, which includes forest wastes and agriculture leftovers, is often regarded as the world's most abundant natural biopolymer and renewable resource (Singhania et al., 2013). Thus, microbial decomposition of lignocellulosic biomass is a profitable, long-term, and promising method for obtaining important commercial commodities on a massive scale.

In recent times, the need for vehicle fuel has increased as the number of automobiles produced and driven throughout the world has risen. According to reports, 105 billion litres of biofuel were generated in 2010, accounting for around 2.7 percent of all transportation fuels. More than a quarter of the world's transportation fuel needs might be met by biofuels by 2050, according to estimates (Soni, Sharma, and Soni, 2018). Due to the increasing demands and consumption of crude oil that rising the levels of global greenhouse emissions which cause the environment has suffering from climate change (Kantharaj et al., 2017; Stone et al., 2019; Hassan et al., 2019). As global energy challenges worsen and the rising of environmental issues, new bioenergy and other renewable energy sources should be implemented as soon as possible to fulfill the global future energy demands.

Therefore, the exploitation of lignocellulose waste materials must be developed. One of the companies in the Canada named The Logen Corporation lead the bioethanol

manufacture from the lignocellulose biomass and can produce up to 0.52 million gallons of the bioethanol in a year (Dashtban et al., 2009). Although there are other alternatives to create the bioenergy like biodiesel from the vegetable's oils, it will give another impact to world food shortage and food security. Other than that, these limited resources such as restaurant waste oils, leftover frying oils and animal fats used as a raw material for fuel production might be a cost-effective solution, but it may not be enough to supply the growing demand for clean, renewable energy (Abu Yousuf, 2012). For that reason, most of the researcher agree that lignocellulose biomass is regarded to be one of the most promising fuel alternatives (Heaton et al., 2013; Shaheena et al., 2019; Chintagunta et al., 2021), as they are produced by biological photosynthesis from available atmospheric carbon dioxide, sunlight and H_20. Other than that, according to the findings, lignocellulosic ethanol emits 91 percent fewer greenhouse emissions than fossil-based petrol or diesel in transportation applications, compared to only 22 percent for corn-based ethanol (US EPA, 2007).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Raw Materials and Equipment

Raw materials: *Trichoderma reesei* nail, Potato dextrose agar, Potato dextrose broth, Malt extract agar, distilled water

Chemicals: Tannic acid, ethyl acetate, ethanol

Apparatus: Autoclave, Biology Safety Cabinet, Chiller, Incubator, Rotary evaporator machine

3.1.2 Raw Materials Collection

The main substance used is *Trichoderma* nail, malt extract agar, tannic acid, potato dextrose agar and potato dextrose broth. The pure culture of *Trichoderma reesei* and malt extract agar is obtained from Dr Laila's stock for research in Post-graduate laboratory. Meanwhile, other materials and apparatus is collected from the 'Unit Kemudahan Makmal' (UPKeM) in Universiti Malaysia Kelantan.

3.2 Methods

3.2.1 Extraction of secondary metabolites from *T. reesei*

To extract the secondary metabolites from *Trichoderma reesei,* the pure culture of *T.reesei* is grow first on the Potato Dextrose Agar (PDA) medium. Therefore, PDA media is prepared by weighing a 19.5 g of PDA powder than transfer it into 500 ml of distilled water and then autoclave at 121℃ for 15 minutes.

Next, the prepared PDA is transfer into Petri dish to made a media plate and let it cool down before *T.reesei* nail is transfer on the center of the media plate. The culture will be kept in room temperature at 27± ℃ for 6 days. After this, mycelia from culture are

transferred into the media plate and incubated for 10 days in the room temperature. After 10 days, the Potato Dextrose Broth are prepared by mixing 248g of PDB powder with 2000ml of distilled water into the media bottle. Then, pour 400ml of the solution into five 500ml conical flask and autoclave at 121℃ for 15 minutes. Observe each subculture plate and select the plate media that grow and show up the yellowish or greenish color. In aseptic condition, the grown pure culture is transfer into each of 400ml PDB using 5 mm cork borer and the conical flask is cover with aluminum foil and kept at 25± 2°C for 15 days.

After 15 days of fermentation process, the broth is full covered with the fungal mycelia on the top. The size of fungal mycelia grow in the broth is small due to old PDB powder that contain of lower amount of nutrient. So, the mycelia are stirred in broth culture then it is filtered using muslin cloth and filter paper (Whatman filter paper No. 1) for the extraction process. The extraction of secondary metabolites starts with mixing 100ml of ethyl acetate solvents and 500ml of filtered fermentation broth culture into the separation funnel and shake it for 2 minutes. Then, the mixture stand in separation funnel is placed on the stand until two layers of solution were formed which is organic phase and aqueous phase completely separated. The separation funnel's tap is opened to collect the waste (aqueous phase) on the bottom layer and the extract (organic phase) in the upper layer into the 250ml conical flask.

3.2.2 Drying and collecting the secondary metabolites

The excess amount of solvent in the organic phase are evaporated using rotary evaporator machine. The concentrated amount of extract was collected and poured on the petri dish and air drying in the fume hood for 12 days. Meanwhile, the metabolites are observed in each three-day interval to collect the wet metabolites at one place which is at the center of the petri dish in order to make the easier way to harvesting the dry metabolites on day 12. The amount of collected metabolites is weighed as 3g and was kept in the chiller before use it

3.2.3 Preparing a Tannic Acid Media

Three main ingredients were used such as 50g of malt extract, 5g of tannic acid, and 1000ml of distilled to prepare a Tannic acid media (TAM). The ingredients are mix well into the media bottle and sterilize by autoclaving at 115°C for 10 minutes. The disinfected TAM was allowed to cool before being utilised and the balance after used are kept in the chiller.

3.2.4 Isolation of secondary metabolites

The materials used in this method is being seal and sterilize by autoclaving before use it such as distilled water, and glass petri dish. The dried secondary metabolites were weighed 5 mg,10 mg,15 mg and 20 mg in the falcon tube and 1ml of sterile distilled were added. Then, the falcon tubes were mixed using a vortex mixer for about 10 s to allow the metabolites mix in the water. The fluid of the extract was poured in the glass petri dish and were labeled 5 mg, 10 mg, 15 mg and 20 mg each.

3.2.5 Diffusion of filter paper and incubation

The filter paper (Whatman filter paper No. 1) is cut like a disk paper with diameter 18 mm, was placed into beaker and covered with aluminum foil before autoclave. By dipping the filter paper in the metabolite's fluid in each one of the different concentrations by using forceps, it can absorb desirable amount of metabolites and hold it. Then, the filter paper was mounted at the center of a Tannic acid media plate. The media plate was then covered with aluminum foil and was incubated for 5 days at ±30°C in total darkness.

3.2.6 Enzymatic degradation of lignin

Lignin degradation was determined based on the existence of the halo zone on the Tannic acid media. The lignin activity reflects to its concentration whereas the activity is proportional to the concentration applied. The diameter of halo zone is measured on the back side of media plate after 5 days.

3.2.7 Experimental design and statistical analysis

The research experiment was conducted by referring to Complete Randomized Design (CRD) with one control and four treatments with different concentration of specimen and each replicated three times. Analyzation of the result were conducted using statistical software, SPSS version 22 and using Duncan test to compare the mean among the treatments. All collected data were evaluated using one-way ANOVA to see whether there was a significant difference between the means where $p<0.05$. The significance of the difference among treatments is measured based on the 5% of significant level.

CHAPTER 4

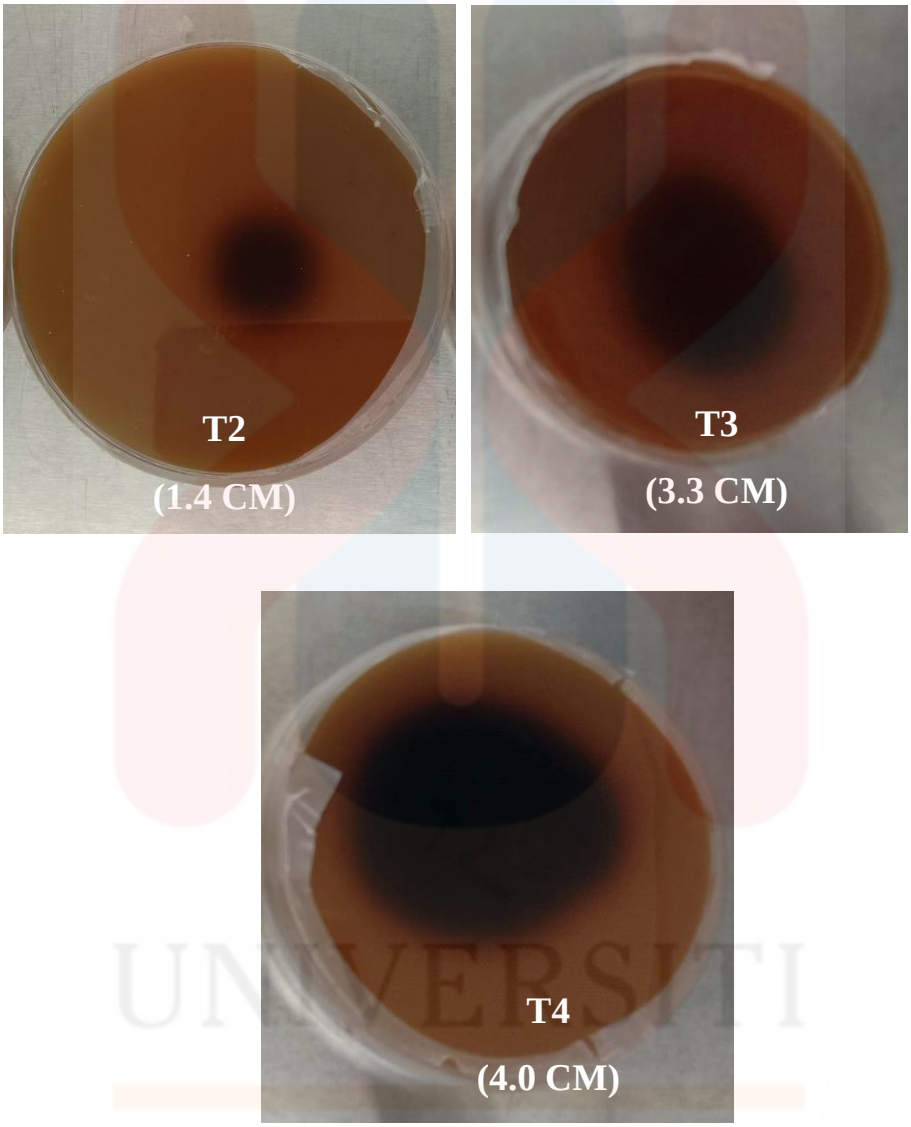
RESULT AND DISCUSSION

4.1 Lignin degradation by *Trichoderma reesei* extract

The result of table 4.1 showed the diameter of dark brown halo zone on each concentration of *T.reesei* extract. The result of the experiment has found that there was significant different between Treatment 1(5 mg), Treatment 2 (10 mg), Treatment 3 (15 mg) and Treatment 4 (20 mg) ($P<0.05$) (Appendix A). Treatment 1 does not show any activities which no dark brown halo zone appears while in Treatment 2, Treatment 3, and Treatment 4 the diameter of dark halo zone is measure 1.333 cm, 2.867 cm, 3.3 cm respectively. However, there is no significant differences between the Treatment 3 whereas the lignin degradation activity is measure 2.867 cm and Treatment 4 is measured 3.3 cm.

A research study from (Asis et al., 2021) show that the *Trichoderma asperellum* degrade lignin more than, *Trichoderma harzianum*.and *Trichoderma reesei* on the tannic acid media. In their research, they put mycelia disk (0.5 cm) of *Trichoderma* strains on each media

while in this study were using filter paper (1.8 cm) dipped into the different concentration of metabolites.



*T2: 10 mg, T3:15 mg, T4: 20mg

Figure 4.1: Enzymatic Degradation of Lignin by *Trichoderma reesei* on each Treatment

Table 4.1: Effect of different concentration of metabolites on the lignin degradation

Treatments	Diameter of halo zone (cm)
T0	0.00 ± 0.00^{a}
T1	0.00 ± 0.00^{a}
T2	1.33 ± 0.503^{b}
T3	2.86 ± 0.40^{c}
T4	3.30 ± 0.75^{c}

Values in the table showed the mean±standard deviation of three replicates. T indicated treatment

*__T0__: Control, __T1__: 5 mg, __T2__: 10 mg, __T3__:15 mg, __T4__: 20mg

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

From this study, it can be concluded that the metabolites of *Trichoderma reesei* show lignin degradation activity on the tannic acid media. The potential of degradation lignocellulosic biomass by the *Trichoderma spp.* can create new renewable resources that fulfill worldwide demands. The cost for producing cellulase enzyme is high due to low productivity of certain microorganism and the quantity of enzyme produced at one time is limited. However, researcher have targeted *Trichoderma reesei* as other alternative in manufacturing cellulase as *T.reesei* produced unique profile of enzyme. Besides, enzyme produced by *T.reesei* have desirable characteristic that able to increase the production yield. With the presence of technology and help from expert, application of *T.reesei* in various bioproduct became easy and accessible for public.

5.2 Recommendation

As for the recommendation, since the potential of *Trichoderma reesei* in degrading lignin material is less studied and lack of information to prove that it can degrade the lignin as efficient as degrading the cellulose, further study need to be carried out the recognition of *Trichoderma* species including *Trichoderma reesei* in enzymatic degradation of lignin. Therefore, according to the study (Wan & Li, 2012; Abdel, Solbiati and Cann, 2013), has stated that white-rot fungi such as *Pleurotus ostreatus*, Phanerochaete *chrysosporium*, *Cyathus stercoreus, Coriolus versicolor,* and Ceriporiopsis *subvermispora* are the most efficient for delignification because they produce ligninolytic extracellular oxidative enzymes.

The use of lignocellulosic wastes has shown to be a significant contender in overcoming insufficient fossil fuel for future, environmental pollution, and shortage of essential bioproduct. So, the exploration, research regarding to lignocellulosic biomass need to be evolved as a means of increasing production and ensuring economic viability.

REFERENCES

Abdel-Hamid, A. M., Solbiati, J. O., & Cann, I. K. (2013). Insights into lignin degradation and its potential industrial applications. Advances in applied microbiology, 82, 1-28.

Adav, S. S., Chao, L. T., & Sze, S. K. (2012). Quantitative secretomic analysis of Trichoderma reesei strains reveals enzymatic composition for lignocellulosic biomass degradation. Molecular & cellular proteomics, 11(7), M111-012419.

Andlar, M., Rezić, T., Marđetko, N., Kracher, D., Ludwig, R., & Šantek, B. (2018). Lignocellulose degradation: an overview of fungi and fungal enzymes involved in lignocellulose degradation. Engineering in Life Sciences, 18(11), 768-778.

Barta, Z., Sassner, P., Zacchi, G., & Reczey, K. (2008). Techno-economic aspects of on-site cellulase production. Hungarian Journal of Industry and Chemistry, 36(1-2).

Babu AG, Shim J, Bang KS, Shea PJ, Oh BT (2014) Trichoderma virens PDR-28: a heavy metal-tolerant and plant growth-promoting fungus for remediation and bioenergy crop production on mine tailing soil. J Environ Manag 132:129–134.

Bischof, R. H., Ramoni, J., & Seiboth, B. (2016). Cellulases and beyond: the first 70 years of the enzyme producer Trichoderma reesei. Microbial cell factories, 15(1), 1-13.

Bredon, M., Dittmer, J., Noël, C., Moumen, B., & Bouchon, D. (2018). Lignocellulose degradation at the holobiont level: teamwork in a keystone soil invertebrate. Microbiome, 6(1), 1-19.

Brethauer, S., & Studer, M. H. (2015). Biochemical conversion processes of lignocellulosic biomass to fuels and chemicals–a review. CHIMIA International Journal for Chemistry, 69(10), 572-581.

Bu, Y., Alkotaini, B., Salunke, B. K., Deshmukh, A. R., Saha, P., & Kim, B. S. (2019). Direct ethanol production from cellulose by consortium of Trichoderma reesei and Candida molischiana. Green Processing and Synthesis, 8(1), 416-420.

Chandra, M. R. G. S., & Madakka, M. (2019). Comparative biochemistry and kinetics of microbial lignocellulolytic enzymes. In Recent Developments in Applied Microbiology and Biochemistry (pp. 147-159). Academic Press.

Chen, Y., Wu, C., Fan, X., Zhao, X., Zhao, X., Shen, T., ... & Wang, W. (2020). Engineering of Trichoderma reesei for enhanced degradation of lignocellulosic biomass by truncation of the cellulase activator ACE3. Biotechnology for biofuels, 13(1), 1-14.

Chernoglazov, V. M., Ermolova, O. V., & Klyosov, A. A. (1988). Adsorption of high-purity endo-1, 4-β-glucanases from Trichoderma reesei on components of lignocellulosic materials: cellulose, lignin, and xylan. Enzyme and Microbial Technology, 10(8), 503-507.

Chintagunta, A. D., Zuccaro, G., Kumar, M., Kumar, S. J., Garlapati, V. K., Postemsky, P. D., ... & Simal-Gandara, J. (2021). Biodiesel Production From Lignocellulosic

Biomass Using Oleaginous Microbes: Prospects for Integrated Biofuel Production. Frontiers in Microbiology, 12.

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Cortés-Penagos, C., & López-Bucio, J. (2009). Trichoderma virens, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. Plant physiology, 149(3), 1579-1592.

Dashtban, M., Schraft, H., & Qin, W. (2009). Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. International journal of biological sciences, 5(6), 578.

Dashtban, M., & Qin, W. (2012). Overexpression of an exotic thermotolerant β-glucosidase in Trichoderma reesei and its significant increase in cellulolytic activity and saccharification of barley straw. Microbial Cell Factories, 11(1), 1-15.

de Paula, R. G., Antoniêto, A. C. C., Ribeiro, L. F. C., Srivastava, N., O'Donovan, A., Mishra, P. K., ... & Silva, R. N. (2019). Engineered microbial host selection for value-added bioproducts from lignocellulose. Biotechnology advances, 37(6), 107347.

de Souza, M. F., da Silva, A. S. A., & Bon, E. P. (2018). A novel Trichoderma harzianum strain from the Amazon Forest with high cellulolytic capacity. Biocatalysis and Agricultural Biotechnology, 14, 183-188.

Domingues, F. C., Queiroz, J. A., Cabral, J. M. S., & Fonseca, L. P. (2000). The influence of culture conditions on mycelial structure and cellulase production by Trichoderma reesei Rut C-30. *Enzyme and Microbial Technology*, *26*(5-6), 394-401.

Druzhinina, I. S., Chenthamara, K., Zhang, J., Atanasova, L., Yang, D., Miao, Y., ... & Kubicek, C. P. (2018). Massive lateral transfer of genes encoding plant cell wall-degrading enzymes to the mycoparasitic fungus Trichoderma from its plant-associated hosts. PLoS genetics, 14(4), e1007322.

Druzhinina, I. S., & Kubicek, C. P. (2017). Genetic engineering of Trichoderma reesei cellulases and their production. Microbial biotechnology, 10(6), 1485-1499.

Fahmi, A. I., Eissa, R. A., El-Halfawi, K. A., Hamza, H. A., & Helwa, M. S. (2016). Identification of Trichoderma spp. by DNA barcode and screening for cellulolytic activity. J Microb Biochem Technol, 8(3), 202-209.

Feng, C., Zeng, G., Huang, D., Hu, S., Zhao, M., Lai, C., ... & Li, N. (2011). Effect of ligninolytic enzymes on lignin degradation and carbon utilization during lignocellulosic waste composting. Process Biochemistry, 46(7), 1515-1520.

Filizola PRB, Luna MAC, de Souza AF, Coelho IL, Laranjeira D, Campos-Takaki GM (2019) Biodiversity and phylogeny of novel Trichoderma isolates from mangrove sediments and potential of biocontrol against Fusarium strains. Microb Cell Fact 18:89.

Golecha, R., & Gan, J. (2016). Cellulosic biorefinery portfolio and diversification: Strategies to mitigate cellulosic biorefinery risks in US Corn Belt. *Energy Strategy Reviews*, *13*, 147-153.

Gusakov, A. V. (2011). Alternatives to Trichoderma reesei in biofuel production. Trends in biotechnology, 29(9), 419-425.

Gupta, A., & Verma, J. P. (2015). Sustainable bio-ethanol production from agro-residues: a review. Renewable and sustainable energy reviews, 41, 550-567.

Han, J., Luterbacher, J. S., Alonso, D. M., Dumesic, J. A., & Maravelias, C. T. (2015). A lignocellulosic ethanol strategy via nonenzymatic sugar production: Process synthesis and analysis. Bioresource technology, 182, 258-266.

Hassan, S. S., Williams, G. A., & Jaiswal, A. K. (2019). Moving towards the second generation of lignocellulosic biorefineries in the EU: Drivers, challenges, and opportunities. *Renewable and Sustainable Energy Reviews*, *101*, 590-599.

Heaton, E. A., Schulte, L. A., Berti, M., Langeveld, H., Zegada-Lizarazu, W., Parrish, D., & Monti, A. (2013). Managing a second-generation crop portfolio through sustainable intensification: Examples from the USA and the EU. *Biofuels, Bioproducts and Biorefining*, *7*(6), 702-714.

Hewedy, O. A., El-Zanaty, A. M., & Fahmi, A. I. (2020). Screening and identification of novel cellulolytic Trichoderma species from Egyptian habitats. BioTechnologia, 101(2), 117-133.

Hoyos-Carvajal, L., Orduz, S., & Bissett, J. (2009). Growth stimulation in bean (Phaseolus vulgaris L.) by Trichoderma. Biological control, 51(3), 409-416.

Huang, J., Chen, D., Wei, Y., Wang, Q., Li, Z., Chen, Y., & Huang, R. (2014). Direct ethanol production from lignocellulosic sugars and sugarcane bagasse by a recombinant Trichoderma reesei strain HJ48. *The Scientific World Journal*, *2014*.

Hubbe, M. A. (2014). Lignocellulose Biodegradation in Composting. Composting for Sustainable Agriculture, 43-66.

Humbird, D., Davis, R., Tao, L., Kinchin, C., Hsu, D., Aden, A., ... & Dudgeon, D. (2011). Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol: dilute-acid pretreatment and enzymatic hydrolysis of corn stover (No. NREL/TP-5100-47764). National Renewable Energy Lab. (NREL), Golden, CO (United States).

Jin, R., Liao, H., Liu, X., Zheng, M., Xiong, X., Liu, X., ... & Zhu, Y. (2012). Identification and characterization of a fungal strain with lignin and cellulose hydrolysis activities. African Journal of Microbiology Research, 6(36), 6545-6550.

Juneja, A., Kumar, D., & Murthy, G. S. (2013). Economic feasibility and environmental life cycle assessment of ethanol production from lignocellulosic feedstock in Pacific Northwest US. Journal of Renewable and Sustainable Energy, 5(2), 023142.

Kantharaj, P., Boobalan, B., Sooriamuthu, S., & Mani, R. (2017). Lignocellulose degrading enzymes from fungi and their industrial applications. *International Journal of Current Research and Review*, *9*(21), 1-13

Keshavarz, B., & Khalesi, M. (2016). Trichoderma reesei, a superior cellulase source for industrial applications. Biofuels, 7(6), 713-721.

Khelil O, Cheba B (2014) Thermophilic cellulolytic microorganism from Western Algerian sources: promising isolates for cellulosic biomass recycling. Procedia Technol 12:519–528.

Khokhar, I., Haider, M. S., Mushtaq, S., & Mukhtar, I. (2012). Isolation and screening of highly cellulolytic filamentous fungi. Journal of applied sciences and environmental management, 16(3).

Kubicek, C. P., & Kubicek, E. M. (2016). Enzymatic deconstruction of plant biomass by fungal enzymes. Current opinion in chemical biology, 35, 51-57.

Kumar, M. R., Kumaran, M. D., Balashanmugam, P., Rebecca, A. I. N., Kumar, D. M., & Kalaichelvan, P. T. (2014). Production of cellulase enzyme by Trichoderma reesei Cefl9 and its application in the production of bio-ethanol. Pak J Biol Sci, 17(5), 735-739.

Liew, K. J., Lim, C. C., Chan, C. S., Wei, K. Y., Salleh, M. M., Sani, R. K., ... & Goh, K. M. (2018). Direct Cellulase Gene Amplification From Hot Spring Using the Guidance of 16S rRNA Amplicon Metagenomics. In Metagenomics (pp. 309-325). Academic Press.

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., & Pretorius, I. S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and molecular biology reviews*, *66*(3), 506-577.

McSweeney, C. S., Palmer, B., McNeill, D. M., & Krause, D. O. (2001). Microbial interactions with tannins: nutritional consequences for ruminants. Animal Feed Science and Technology, 91(1-2), 83-93.

Menon, V., & Rao, M. (2012). Trends in bioconversion of lignocellulose: biofuels, platform chemicals & biorefinery concept. *Progress in energy and combustion science*, *38*(4), 522-550.

Mukhlis SHM, Sariah M, Ismail MR, Habib SH, Kausar H (2013) Potential lignocellulolytic *Trichoderma* for bioconversion of oil palm empty fruit bunches. Aust J Crop Sci 7:425–431

Muratov, G., & Kim, C. (2002). Enzymatic hydrolysis of cotton fibers in supercritical CO 2. Biotechnology and Bioprocess Engineering, 7(2), 85-88.

Nadir, N., Ismail, N. L., & Hussain, A. S. (2019). Fungal pretreatment of lignocellulosic materials. In Biomass for Bioenergy-Recent Trends and Future Challenges. IntechOpen.

Nakkeeran, S., Rajamanickam, S., Karthikeyan, M., Mahendra, K., Renukadevi, P., & Johnson, I. (2021). Antimicrobial secondary metabolites from Trichoderma spp. as next generation fungicides. Biocontrol Agents and Secondary Metabolites, 257-282.

Nakari-Set l , T., & Penttil , M. (1995). Production of Trichoderma reesei cellulases on glucose-containing media. Applied and environmental microbiology, 61(10), 3650-3655.

National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 8857, Ethyl acetate. Retrieved January 22, 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Ethyl-acetate.

Nur, S. S., Shafiquzzaman, S., Ling, W., González, M., & Kumar, S. V. (2014). Lignocellulolytic activities among Trichoderma isolates from Lahad Datu, Sabah and Deception Island, Antarctic. Journal of Microbial and Biochemical Technology, 6(5), 295-302.

Novy, V., Nielsen, F., Seiboth, B., & Nidetzky, B. (2019). The influence of feedstock characteristics on enzyme production in Trichoderma reesei: a review on productivity, gene regulation and secretion profiles. *Biotechnology for biofuels*, *12*(1), 1-16.

Peil, S., Beckers, S. J., Fischer, J., & Wurm, F. R. (2020). Biodegradable, lignin-based encapsulation enables delivery of Trichoderma reesei with programmed enzymatic release against grapevine trunk diseases. Materials Today Bio, 7, 100061.

Rabemanolontsoa, H., & Saka, S. (2016). Various pretreatments of lignocellulosics. Bioresource technology, 199, 83-91.

Roopashree, K. M., & Naik, D. (2019). Advanced method of secondary metabolite extraction and quality analysis. Journal of Pharmacognosy and Phytochemistry, 8(3), 1829-1842.

Shaheena, S., Chintagunta, A. D., Dirisala, V. R., & Kumar, N. S. (2019). Extraction of bioactive compounds from Psidium guajava and their application in dentistry. *AMB express*, *9*(1), 1-9.

Sharma, A., Aggarwal, N. K., & Yadav, A. (2017). Isolation and screening of lignolytic fungi from various ecological niches. Univ. J. Microbiol. Res, 5(2), 25-34.

Silva, I. S., Menezes, C. R. D., Franciscon, E., Santos, E. D. C. D., & Durrant, L. R. (2010). Degradation of lignosulfonic and tannic acids by ligninolytic soil fungi cultivated under icroaerobic conditions. Brazilian Archives of Biology and Technology, 53(3), 693-699.

Silva, J. P., Ticona, A. R., Hamann, P. R., Quirino, B. F., & Noronha, E. F. (2021). Deconstruction of Lignin: From Enzymes to Microorganisms. Molecules, 26(8), 2299.

Simorangkir, M., Nainggolan, B., & Silaban, S. (2018). Secondary Metabolites Phytochemical Analysis of n-Hexane, Ethyl Acetate and Ethanol Extracts of Sarang Banua (Clerodendrum fragrans Vent Willd) Leaves (pp. 18-19). October.

Sood, M., Kapoor, D., Kumar, V., Sheteiwy, M. S., Ramakrishnan, M., Landi, M., ... & Sharma, A. (2020). Trichoderma: the "secrets" of a multitalented biocontrol agent. Plants, 9(6), 762.

Soni, S. K., Sharma, A., & Soni, R. (2018). Cellulases: role in lignocellulosic biomass utilization. Cellulases, 3-23.

Stone, M., Anderson, E., Katahira, R., Reed, M., Muchero, W., Beckham, G. T., & Román-Leshkov, Y. (2019, June). Differences in S/G Ratio in Natural Poplar Variants Do Not Predict Catalytic Depolymerization Monomer Yields: Implications for Lignin Biosynthesis. In *2019 North American Catalysis Society Meeting*. NAM.

Strakowska, J., Błaszczyk, L., & Chełkowski, J. (2014). The significance of cellulolytic enzymes produced by Trichoderma in opportunistic lifestyle of this fungus. Journal of Basic Microbiology, 54(S1), S2-S13.

Taylor, G. (2008). Biofuels and the biorefinery concept. *Energy policy*, *36*(12), 4406-4409.

US EPA. Office of transportation and air quality; May 2007.

Vaidyanathan, S., White, S., Harvey, L. M., & McNeil, B. (2003). Influence of morphology on the near-infrared spectra of mycelial biomass and its implications in bioprocess monitoring. *Biotechnology and bioengineering*, *82*(6), 715-724.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Ruocco, M., Woo, S., & Lorito, M. (2012). Trichoderma secondary metabolites that affect plant metabolism. Natural product communications, 7(11), 1934578X1200701133.

Wang, M., Li, Z., Fang, X., Wang, L., & Qu, Y. (2012). Cellulolytic enzyme production and enzymatic hydrolysis for second-generation bioethanol production. Biotechnology in China III: Biofuels and Bioenergy, 1-24.

Wang, B. T., Hu, S., Yu, X. Y., Jin, L., Zhu, Y. J., & Jin, F. J. (2020). Studies of cellulose and starch utilization and the regulatory mechanisms of related enzymes in Fungi. Polymers, 12(3), 530.

Wan, C., & Li, Y. (2012). Fungal pretreatment of lignocellulosic biomass. Biotechnology advances, 30(6), 1447-1457.

Wu, G., He, R., Jia, W., Chao, Y., & Chen, S. (2011). Strain improvement and process optimization of Trichderma reesei Rut C30 for enhanced cellulase production. Biofuels, 2(5), 545-555.

Xu, Q., Singh, A., & Himmel, M. E. (2009). Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. *Current opinion in biotechnology*, *20*(3), 364-371.

Xu, Z., & Huang, F. (2014). Pretreatment methods for bioethanol production. Applied biochemistry and biotechnology, 174(1), 43-62.

Yeşilyurt, M. K., Erol, D., Yaman, H., & Doğan, B. (2021). Effects of using ethyl acetate as a surprising additive in SI engine pertaining to an environmental perspective. International Journal of Environmental Science and Technology, 1-30.

Zhang, X. Z., & Zhang, Y. H. P. (2010). One-step production of biocommodities from lignocellulosic biomass by recombinant cellulolytic Bacillus subtilis: Opportunities and challenges. *Engineering in Life Sciences*, *10*(5), 398-406.

APPENDICES

APPENDIX A

Table A: Homogeneous subsets for diameter of halo zone (cm) of lignin degradation.

Duncan[a]

Treatment	N	Subset for alpha = 0.05		
		1	2	3
Treatment 0 (Control)	3	.000		
Treatment 1 (5 mg)	3	.000		
Treatment 2 (10 mg)	3		1.333	
Treatment 3 (15 mg)	3			2.867
Treatment (20 mg)	3			3.300
Sig.		1.000	1.000	.260

*__T0__: Control, __T1__: 5 mg, __T2__: 10 mg, __T3__:15 mg, __T4__: 20mg

Means for group in homogeneous subsets are displayed.

Uses Harmonic Mean Sample Size = 3.000

APPENDIX B

Table B: One Way ANOVA for the Diameter of halo zone (cm) for lignin degradation

Treatment		*Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Diameter of halo zone (cm)	Between Groups	28.907	4	7.227	36.622	.000
	Within Groups	1.973	10	.197		
	Total	30.880	14			

APPENDIX C

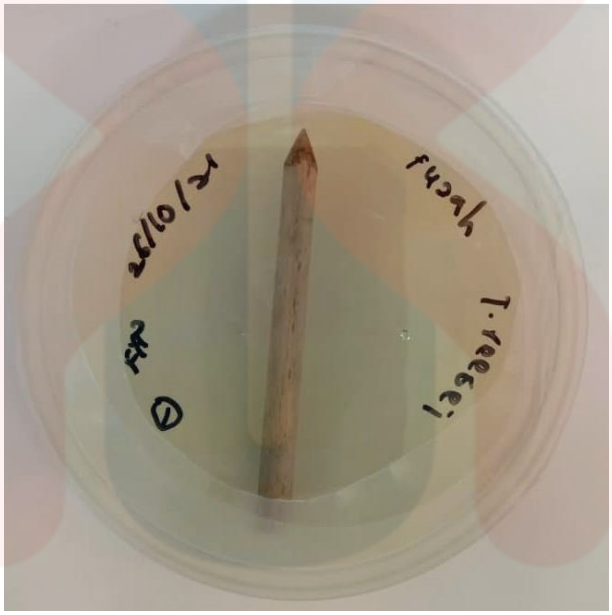
Table C: Raw Data of Lignin Degradation

Diameter of hole zone (cm) / Treatment	Replicate 1	Replicate 2	Replicate 3
T0 (Control)	0	0	0
T1 (5mg)	0	0	0
T2 (10mg)	0.8	1.4	1.8
T3 (15 mg)	2.5	2.8	3.3
T4 (20mg)	2.5	3.4	4.0

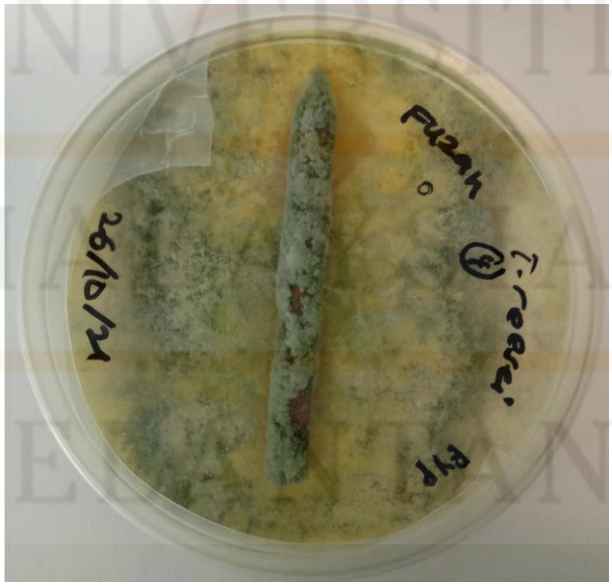
APPENDIX D

Table D: Preparation of Secondary metabolites from *Trichoderma reesei*

Figure D.1: Culturing pure culture of Trichoderma *reesei*

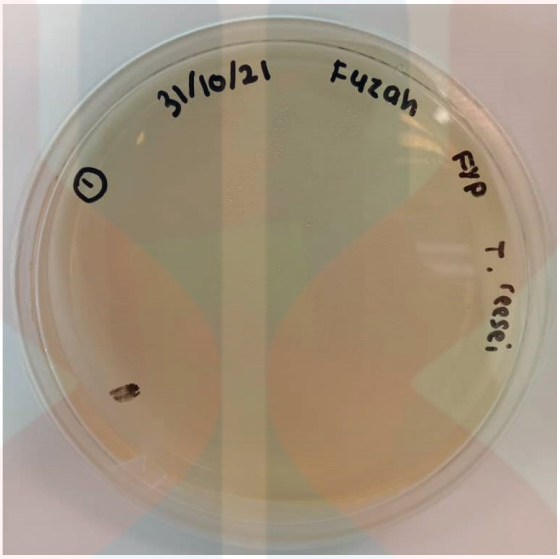


Culture on Day 1

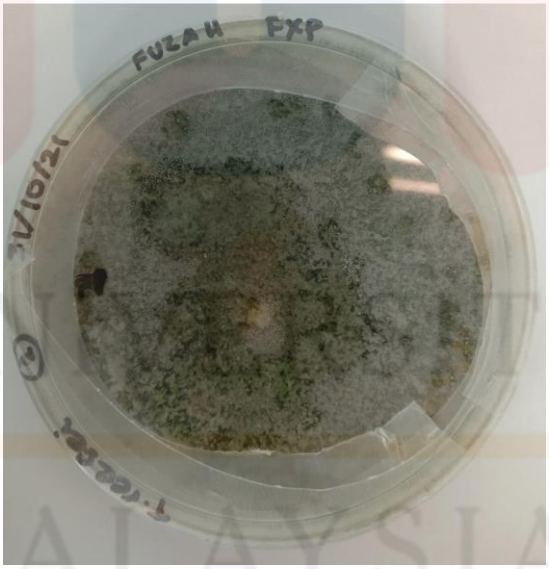


Culture on Day 6

Figure D.2: Subculture of single colony



Subculture on Day 1



Subculture on Day 2

Figure D.3: Growing the T.*reesei* in Potato Dextrose Broth

Figure D.4: Extraction of secondary metabolites using Ethyl acetate

Figure D.5: Evaporation of excess solvent

Figure D.6: Air drying the secondary metabolites in the Fume Hood