UNIVERSITI
MALAYSIA
KELANTAN

CELLULOSE ENZYME ACTIVITY FROM *TRICHODERMA REESEI* EXTRACT USING HEXANE SOLVENT

NUR FATHIHAH BINTI SUHAIMI KAMAL

F18B0309

A thesis submitted in fulfillment of the requirement for the degree of Bachelor of Applied Science (Agrotechnology) with Honors

UNIVERSITY MALAYSIA KELANTAN

Faculty Of Agro-Based Industry University Malaysia Kelantan

2022

Cellulose Enzyme Activity From *Trichoderma Reesei* Extract Using Hexane Solvent

Nur Fathihah Binti Suhaimi Kamal
F18B0309

A thesis submitted in fulfillment of the requirement for the degree of Bachelor of Applied Science (Agrotechnology) with Honors

Faculty Of Agro-Based Industry
University Malaysia Kelantan

2022

DECLARATION

As a result, I declare that the work embodied in this report is the result of the original research and has been submitted for a higher degree to any university or institution.

_____

Name: Nur Fathihah Binti Suhaimi Kamal

Matric No: F18B0309

Date: 27 February 2022

_____

Supervisor's Name: Dr. Mst Laila Naher

Stamp:

Date:

ACKNOWLEDGMENT

All praise to Allah SWT for this valuable opportunity and excellent health that were necessary to complete this final year project. My deepest condolence I gave to my supervisor, Dr. Mst. Laila Naher for her expertise sharing, guidance, and supervision extended to me throughout this period.

I take this opportunity to express my gratitude towards all lab assistants and staff of the Faculty of Agro-based Industry, Ph.D. student Mr. Bristone and Maryam, for their help and supervision during lab work and machinery handling. Besides, they are permitted to use the lab space and facilities fully.

I am genuinely grateful for the tremendous moral support and attention are given by my family when I was at my lowest point and felt discouraged during this journey. Thank you to all of my friends for truthfully lending their hands whenever I need their bits of help and advice. My sense of gratitude also goes to all parties and individuals who directly or indirectly lend their assistance to ease my venture. I will never make it to the end without any bits of help and attention given by lecturers, staff, families, and friends.

Aktiviti Enzim Selulosa Daripada Ekstrak *Trichoderma reesei* Menggunakan Pelarut Heksana

ABSTRAK

Trichoderma ialah genus kulat bawaan tanah yang mempunyai sejarah panjang yang penting dari segi ekonomi dalam pertanian. Spesies genus berfilamen ini digunakan terutamanya sebagai racun kulat bio dan pengubah tumbesaran tumbuhan. Oleh itu, kajian ini dijalankan untuk melihat aktiviti enzimatik selulosa daripada spesies *Trichoderma reesei*. Spesies genus berfilamen ini digunakan terutamanya sebagai racun kulat bio dan pengubah tumbesaran tumbuhan. Oleh yang demikian, kajian ini dilakukan untuk melihat aktiviti enzimatik selulosa daripada species *Trichoderma reesei*. Tertumpu kepada aktiviti enzimatik selulosa kerana masalah industri enzim selulosa seperti kos penyediaan enzim sintetik. Kajian ini dimulakan dengan pengumpulan sampel diikuti dengan penyediaan Potato Dextrose Agar (PDA) dan Potato Dextrose Broth (PDB), pengasingan *T.reesei*, kultur sebenar dan pemerhatian morfologi *T. reesei*. Kemudian, pengekstrakan metabolit sekunder daripada *T. Reesei*. Proses pengasingan dilakukan dengan menggunakan corong pemisah. Seterusnya, mesin penyejat putar digunakan untuk mengekstrak larutan heksana dengan takat didih 69ºC sehingga ia kering. Selepas itu, ekstrak disimpan di bawah serombong wasap untuk pengeringan udara selama lima hari. Untuk menentukan aktiviti selulolitik Jensen Media (JM) digunakan. Cakera kertas penapis steril (15.7 mm) direndam dalam larutan kawalan pada kepekatan berbeza 5 mg, 10 mg, 15 mg, dan 20 mg larutan pengekstrakan. Didapati terdapat perbezaan yang ketara dalam diameter zon halo setiap rawatan. *T.reesei* menghasilkan diameter kecil zon halo ialah Rawatan 2 (10 mg/5ml) ialah 20 mm. Untuk Rawatan 3 (15 mg/5ml) dan Rawatan 4 (20 mg/5 ml) didapati membentuk zon halo pembersihan yang lebih besar daripada 50 mm hingga 60 mm selepas ditapis dengan merah Congo. Spesies Trichoderma mesti dikenal pasti dengan tepat kerana strain ini berpotensi untuk digunakan sebagai agen biokawalan untuk mencegah penyakit dan meningkatkan hasil dalam tanaman pertanian.

Kata kunci: *Trichoderma reesei*, aktiviti enzimatik selulosa, metabolit sekunder, Jensen Media (JM), diameter zon halo.

Cellulose Enzyme Activity From *Trichoderma Reesei* Extract Using Hexane Solvent

ABSTRACT

Trichoderma is a genus of a soil-borne fungus with a long history of being economically important in agriculture. This filamentous genus species are mainly used as bio fungicides and plant growth modifiers. Therefore, this study was conducted to look at the enzymatic activity of cellulose from *T.reesei* species. The focuses on enzymatic activity of cellulose because of the problem for cellulose enzyme industry such as synthetic enzyme preparation cost. The investigation started with sample collection, followed by Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) preparation, *T.reesei* isolation, actual culture, and *T.reesei* morphological observation. Then, the extraction of secondary metabolites from *Trichoderma Reesei*. The isolation process is performed using a separator funnel. Next, a rotary evaporator machine was used to extract the solution with a boiling point of 69ºC until it was dry. After that, the extract was stored under a fume hood for air drying for five days. To determine the cellulolytic activity Jensen Media (JM) was used. Sterile filter paper discs (15.7 mm) were immersed in a control solution at different concentrations of 5 mg, 10 mg, 15 mg, and 20 mg of extractor solution. It was found that there was a significant difference in the diameter of the halo zone of each treatment. *T.reesei* produced the minor diameter of halo zone is Treatment 2 (10 mg/5ml) is 20 mm. For the Treatment 3 (15 mg/5ml) and Treatment 4 (20 mg/5 ml) were found forming larger clearing halo zones from 50 mm to 60 mm after straining with Congo red. *Trichoderma* species must be accurately determined because these strains can be employed as a biocontrol agent to prevent disease and boost yield in agriculture crops.

Keywords: *Trichoderma reesei,* enzymatic activity of cellulose, secondary metabolites, Jensen Media (JM), diameter of halo zone.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS/SYMBOLS/NOMINATIONS AND TERMS

		Pages
T.reesei	*Trichoderma reesei*	1
g	Gram	2
C_6H_{14}	Hexane	7
T. viride	*Trichoderma viride*	8
mg/mL	Milligram per Milliliter	
mL	Milliliter	8
CBH	Cellobiohydrolase	7
EG	Endo-1,4-glucanase	7
XYN	Xylanase	7
°C	Degree Celsius	8
pH	Potential of Hydrogen	9
PDB	Potato Dextrose Broth	19
PDA	Potato Dextrose Agar	19
%	Percentage	20
BCAs	Biocontrol Agent	11
IPM	Integreted Pest and Disease	12
JM	Jensen Media	21
K_2HPO_4	Dipotassium Hydrogen Phosphate	21
CMC	Carboxymethyl Cellulose	21
$CaCO_3$	Calcium Carbonate	21

$Fe_2(SO_4)_3$	Iron (III) Sulfate	21
NaCl	Sodium Chloride	21
$MgSO_4$	Magnesium Sulfate	21
Na_2MoO_4	Sodium Molybdate	21
mm	Milimetre	21
CRD	Complete Randomized Design	22
ANOVA	Analysis of Variance	22
SPSS	Statistical Package for the Social Science	22

CHAPTER 1

INTRODUCTION

1.1 Research Background

Trichoderma spp is a filamentous fungus that is found all over the environment, especially in soil, plant matter, decayed plants, and wood. *Trichoderma reesei* is the species from Hypocreales class in, Ascomycota Phyllum which is a highly productive industrial cell factory fungus to synthesize cellulolytic enzymes for biofuel and other purposes. This filamentous genus species are mainly used as bio fungicides and plant growth modifiers. *Trichoderma* is a fungus that has evolved to fit into various ecological niches. Due to its extraordinarily diversified metabolism, which allows it to catabolize an extensive range of substrates while also creating a diverse range of secondary metabolites (Vicente et al., 2022).

Trichoderma reesei secretes a complete mixture of cellulolytic enzymes to degrade crystalline to glucose. *T.reesei* is an asexual clonal line produced from a tropical saprophytic Ascomycete *Hypocrea jecorina* population (Kuhls et., 1996). Because of the massive amount of available knowledge, multiple genomic data, and gene manipulation approaches, *T. reesei* research is relatively advanced compared to other *Trichoderma spp*. Cellulose is the most prevalent polysaccharide since it is the major component of plant

biomass. In plants, it forms the complex and stiff structure of the plant cell walls with hemicelluloses, lignin, and different extractives (protein, lipids, waxes, terpenes, phenols, alcohols, and alkanes). 7000–15,000 glucose units are joined together by b-1,4-glycosidic linkages in cellulose, a linear polymer (Fengel., et al 1989).

Research has been greatly aided in recent years by sequencing the genomes of three strains that represent the most appropriate uses of this species. According to Martinez et al., (2008), despite its relevance in industrial cellulose production, the genome of *T. reesei*, the industrial workhorse, found that it had the fewest genes encoding cellulolytic and hemicellulolytic enzymes. The species of *T. reesei* plays the most significant role in industrial cellulase production, with the ability to create up to 20 g L1 cellulases (Peterson et al., 2012). The enzymes deficient in *T. reesei* may thus be advantageous to plant pathogens and mycoparasites in their natural habitat. Still, it will not stimulate cellulose and hemicellulose hydrolysis if the core set of enzymes is produced in the necessary quantities (Kubicek, 2013). Furthermore, several *Trichoderma* species have been shown to have positive effects on plants at low concentrations, promoting plant growth and development and activating defense responses to abiotic conditions and pathogens (Vicente et al., 2022).

1.2 Problem Statement

Trichoderma species, a hyphomycetes genus, are found all over the environment, but mainly in soils. *Trichoderma* is a genus of filamentous ascomycetes commonly used in industrial applications due to their capacity to produce enormous quantities of extracellular lignocellulose-degrading hydrolases (Mach et al., 2003). This fungus species is utilized or encountered in various human activities, including commercial enzyme production and natural plant disease treatment. The conversion of native cellulose to glucose monomers is a complicated process requiring the synergistic action of cellulolytic bacteria' extracellular enzymes. For species belonging to the *Trichoderma* genus, the enzyme systems that can break down native cellulose have been intensively explored. *Trichoderma reesei*, which is highly specialized in the efficient degradation of plant cell wall cellulose, has produced most of the cellulolytic enzymes described thus far (Strakowska et al., 2014).

Consequently, the cost of enzyme synthesis remains a significant bottleneck because sure of the generated enzymes have poor catalytic activity under industrial circumstances. The rate of hydrolysis of particular enzymes in the released enzyme mixture is restricted. The ascomycete *Trichoderma reesei* produces nearly all of the lignocellulolytic enzyme combinations required for the hydrolysis stage (Druzhinina et al., 2017). The findings show additional players in cellulose degradation, including non-catalytic proteins secreted from various carbon sources, transporters, transcription factors, carbohydrate-active enzyme (CAZymes), and *T. reesei's* regulatory network in reaction

to cellulose and sophorose (dos Santos Castro et al., 2014). As a result, *T. reesei* has undergone extensive research into the structure and mechanism of the enzymes involved in regulating their expression and the pathways of their production and secretion. Hence, lignocellulolytic activities were performed to identify the celluloses activity of *Trichoderma reesei.*

1.3 Hypothesis

$H_{a:}$ There is no enzymatic activity of cellulose that will be extracted from *Trichoderma reesei*.

$H_{b:}$ There is an enzymatic activity of cellulose that will be extracted from the *Trichoderma reesei* strain.

1.4 Significance of The Study

This study's findings will redound to look for an alternative source of new and efficient crop yield by extracting the mycotoxin from fungus by using hexane solvents from the fungal strain *T. reesei*. In addition, investigating and identifying *T. reesei* is needed as these strains can be potentially used as a biocontrol agent to prevent disease and are helpful for the crop industry. Among the many species utilized as biocontrol agents, including fungi and bacteria, the fungal genus *Trichoderma* spp generates a variety of enzymes that are important for biocontrol activities, such as cell wall breakdown, tolerance to biotic or

abiotic stimuli, and hyphal development (Waghunde et al., 2016). By modifying *T. reesei's* metabolism to create large amounts of celluloses for plant cell-wall disintegration, the data will aid in the development of industrial *T. reesei* strains. Since the fungal strains under investigation have strong antimicrobial properties and promote growth, this study has significant consequences for identifying *T. reesei* genus morphological characteristics by conducting the lignocellulolytic activities.

1.5 Scope of Study

The scope of this study is to identify and determine the enzymatic activity of cellulose based on the presence of colony appearance and the diameter of the halo zone surrounding the inoculated area. There are four treatments with different concentrations of mycotoxin that dilute with 5 ml of distilled water.

1.6 Research Objectives

The objective of this study are:

1) To extract the secondary metabolites from *Trichoderma reesei* using Hexane solvent.
2) To identify the cellulolytic enzyme activity from the secondary metabolites of *Trichoderma reesei* hexane extract.

The concentration of extraction has been designed as follow:

T1: 100% water (Used as the control in this study)

T2: 5 mg of extract and 5 ml distilled water

T3: 10 mg of extract and 5 ml distilled water

T4: 15 mg of extract and 5 ml distilled water

T5: 20 mg of extract and 5 ml distilled water

Notes: T- treatment

CHAPTER 2

LITERATURE REVIEW

2.1 General Characteristics of *Trichoderma reesei*

Colonies of *Trichoderma* spp. in culture were characterized to be floccose, tufted green, developing rapidly, and sporulating better under incandescent light than otherwise, often releasing spores in bands (Domsch et al. 1980). According to Gams and Bissett (1998), *Trichoderma* spp. is known for its branched conidiophores that bear bright green conidia.

Furthermore, *Trichoderma reesei* breaks down natural cellulose using a series of cellulolytic enzymes such as Cellobiohydrolase (CBH) and endo-1,4-glucanase (EG), as well as hemicellulases like xylanase (XYN), are enzymes that work together to complete biomass hydrolysis (Singhania et al., 2013). These enzymes exhibit a typical bidomain structure of fungal cellulases, both acting primarily as exoglucanases, releasing cellobiose from the ends of high molecular weight cellulose chains (Teeri et al., 1995). *T.reesei* produces two main enzymes: high-titer cellulases and chitinase, and its synergistic action allow lignocellulosic biomass to be saccharified into soluble, fermentable sugars (Greene et al., 2015). According to Druzhinina et al. (2011); Kubicek

et al. (2011), *Trichoderma reesei* is likely to descend from a mycoparasitic fungus specializing in lignocellulose breakdown.

2.1.1 Favorable Condition for *Trichoderma Reesei*

Trichoderma harzianum thrives at temperatures ranging from 30^0C to 37^0C, while *Trichoderma koningii* thrives at 32^0C to 35^0C (Danielson and Davey). *Trichoderma spp* should be injected into the soil whenever soil temperatures reach 15^0C. The life cycle of Trichoderma is around 28 days. It is self-replicating, but like with any lifecycle, it eventually becomes debilitated. *Trichoderma* spp actively colonizes the root zone, making it harder for pathogens to compete for space and nutrients. *Trichoderma* spp parasitizes pathogenic fungus by coiling around their hyphae and then producing enzymes that disintegrate the pathogen's cell walls. Zehra et al., (2017) proposed that the *Trichoderma* genus strains studied could grow in a pH range of 2.0 to 6.0, with an optimal pH of 4.0. The presence of heavy metals affects the mycelial development of *Trichoderma* strains.

2.2 Cultivation of Filamentous Fungi

The filamentous fungus produces a wide range of proteases of biotechnological uses and various substrate specificities. A proteolytic study of industrial enzyme producers like *Trichoderma reesei* (Daranagama et al., 2019). Cultivating other microorganisms and filamentous fungi typically begins with inoculating conidia into a growth medium and is accomplished in shake flasks, top bench fermenters, or on agar plates. In addition, fungi, as a heterotrophic organism, organic matter is necessary for growth and as a source of energy. Examples of growth media include potato – dextrose, malt extract, and soybean flour. Temperature, humidity, pH, and shaking speed influence the filamentous fungi's metabolism and development (Svahn, S. 2015).

2.3 Morphological characterization of *Trichoderma Reesei*

Trichoderma spp. are excellent colonizers of their environments, as evidenced by their effective use of the substrate available as well as their ability to secrete antimicrobial metabolites and enzymes. They can work in various conditions, including the diverse and lush habitat of a tropical rain forest and the dark and sterile environment of a biotechnological fermentor or shake flask. Under all of these circumstances, they respond to their surroundings by regulating their growth, conidiation, and enzyme production. They, therefore, adapt their lifestyle to the current circumstances, which can be used for

the advantage of humankind (Schuster, & Schmoll 2010). The genus produces a wide range of pigments, ranging from bright greenish-yellow to reddish. However, some are colorless. Similarly, conidial pigmentation varies from colorless to varied green tints, with grey or brown thrown in for good measure (Gams and Bissett 1998). Trichoderma spp. is characterized by rapid development, usually brilliant green conidia, and a conidiophore structure that is repetitively branched (Gams and Bissett 1998).

2.4 Uses of *Trichoderma Reesei* in agricultural

Trichoderma spp. has a wide range of properties that could be useful in agriculture, including alleviating abiotic stresses, improving physiological response to pressures, reducing nutrient uptake in plants, increasing nitrogen-use efficiency in various crops, and improving photosynthetic efficiency. This genus has spread worldwide as general plant protectants and growth boosters and in several industrial activities (Waghunde et al., 2016). *Trichoderma reesei* micromorphology was recently researched using a confocal laser scanning microscope to see the fungus's branching pattern that produces cellulolytic enzymes (Novy et al., 2016). Hence, *Trichoderma* spp. may utilize a broad range of carbon and nitrogen sources and make several enzymes to break down complex plant polymers into simple sugars for energy and growth.

2.5 Uses of *Trichoderma Reesei* as a biopesticide

Bio-fungicide consists of beneficial fungi and bacteria that colonize and attack pathogens from plants and prevent the diseases they cause. Using microbial antagonists, biological control of infections or crop pests has been an environmentally friendly alternative to chemical pesticides. Biological control is experiencing a resurgence of interest and research in microbiological balance to control soil-borne plant diseases and develop a more efficient farming system. *Trichoderma* genus is one of the greatest bioagents for biological control, as it is effective against a broad spectrum of soil and foliar diseases. According to Woo et al. (2016), more than 50 different *Trichoderma*-based agricultural products are created in various nations and offered to farmers to improve crop yields. *Trichoderma* spp. based biocontrol agents as a relatively new type of biocontrol agent (BCAs). Nearly 20 *Trichoderma* species operate as bioagents against various soil-borne and foliar plant diseases. The most important species that act as potential antagonists include *Trichoderma hamatum, Trichoderma longibrachiatum, Trochoderma harzianum, Trichoderma pseudokoningii, Trichoderma viride, Trichoderma atroviride, Trichoderma koningii, Trichoderma polysporum,* and *Trichoderma reesei* (Monaco et al. 1991). *Trichoderma spp*. has been widely studied as a potential biocontrol agent for controlling several plant pathogens among different antagonistic fungi used for plant disease management (Agrios, 2005; Mukherjee et al., 2012). Parasitism, competition, and antibiosis are several mechanisms of *Trichoderma*

spp. to inhibit the growth and proliferation of hazardous diseases. *Trichoderma* spp. has long been used as a biocontrol agent in Integrated Pest Management (IPM) to control pests and diseases in an environmentally friendly manner (Monte, 2001).

2.6 Lignocellulolytic activities

Lignocellulosic materials such as cellulose, hemicellulose, and lignin are composed of various cellulases and are widely used to bioprocess different plant materials. These materials are commonly used in the bioprocessing of various plant materials such as bio-fuel, feed additives, and chemical feedstocks (Behrendt et al., 2000). Lignocellulosic is the most abundant renewable organic carbon resource on Earth, storing around half of the energy produced by plants during photosynthesis. Lignocellulose comprises three densely interconnected polymers: cellulose, hemicellulose, and lignin, which make up about 98 percent of the dry weight of lignocellulose (Mosier N et al., 2005). *Trichoderma reesei* and its various mutant strains produce a lot of cellulases and are commonly used as a source of carbohydrate-active enzymes. Several studies have shown that *T. reesei* co-cultures have higher enzyme activities than monocultures, particularly when it comes to β -glucosidase activity (Sperandio et al., 2021).

2.6.1 Enzymatic activity

The goal of measuring enzyme activity is to determine the amount of enzyme present under specific conditions to compare activity between samples and research labs (Scopes, 2001). It's crucial to figure out what role extracellular enzymes produced by *Trichoderma* species play. Extracellular hydrolytic enzymes were significant in mycoparasitism and antibiosis, both essential aspects of pathogen biocontrol (Bhale, 2012). Many extracellular enzymes are produced quite efficiently by *Trichoderma* species. *Trichoderma* spp is utilized to make cellulase and other enzymes that break down complex polysaccharides in commercial production (Bhale, 2012). It is commonly used for these purposes in the food and textile sectors. For example, cellulose from these fungi is employed in the "stoning" of denim fabrics to create stone-washed denim, a soft, whitened fabric. The enzymes are also utilized in chicken feed to make barley and other crops more digestible hemicelluloses (Harman, 2006). Because of their high secretary capacity and inducible promoting features, *Trichoderma* species are widely exploited in industrial applications. (Mach et al., 2003).

2.6.2 Cellulolytic activity

Cellulose is the most overall biomass on Earth, and it provides energy to numerous microbes. The natural breakdown of the crystalline portion is greatly dependent on the

degree of processivity of the degrading enzymes (i.e., the extent of continuous hydrolysis without detachment from the substrate cellulose). As a result, developing procedures for the effective treatment and exploitation of cellulosic waste as an inexpensive carbon source has been of substantial commercial significance. Cellulases are an essential tool for obtaining the many benefits of biomass usage (Lynd et al., 2002). According to Klinke et al. (2004), lignocelluloses are commonly processed with alkalis or acids to release cellulose during the cellulose processing process. Over the last two decades, the ability of enzymes to remain active in the presence of organic solvents has gotten a lot of attention. There are several advantages to utilizing enzymes in organic solvents or aqueous solutions incorporating organic solvents rather than water alone, including higher nonpolar substrate solubility and the exclusion of microbial contamination in the reaction mixture (Ogino & Ishikawa, 2001)

2.7 Solvent uses for extraction

The distribution of a solute between two immiscible liquid phases in contact with each other is the term for solvent or liquid to liquid extraction. Solvent extraction techniques are used in various fields, including inorganic and organic chemistry, large-scale industrial separations, analytical chemistry, pharmaceutical and biochemical industries, and waste management. Solvent extraction is also a valuable tool for

investigating the fundamentals of equilibrium and kinetics in complicated formation processes. In separation technologies, extraction processes have become a standard procedure (El-Nadi, Yasser. 2017).

CHAPTER 3

METHODOLOGY

3.1 Materials

The chemicals and reagents/media used in the laboratory are Hexane solvent, Jensen Media (JM) [10g Dipotassium Hydrogen Phosphate, 2.0g Calcium Carbonate, 2.0g Carboxymethyl Cellulose, 0.1g Iron (III) Sulfate, 0.5 Sodium Chloride, 0.5 Magnesium Sulfate and 0.005 Sodium Molybdate], Congo red aqueous solution, Potato dextrose agar (PDA), Potato dextrose broth (PDB).

3.1.1 Apparatus

The apparatus used for this activity includes a petri dish, weighing machine, Bunsen burner, gloves, parafilm, media bottle, cork borer, 50 mL beaker, 50 mL volumetric, conical flask, measuring cylinder, stainless steel spatula, aluminum foil, and separator funnel and clamp and rotary evaporator machine.

3.2 Methods

3.2.1 Potato Dextrose Agar (PDA) Preparation

Potato Dextrose Agar (PDA) is used to cultivate *Trichoderma reesei* strain. Potato starch, potato infusion, and dextrose are general purposes mediums supplemented with acid or antibiotics to inhibit fungus growth. Potato infusion provides a nutrient base for most fungi's luxuriant growth, and dextrose serves as a growth stimulant.

39.0 grams of potato dextrose agar powder HIMEDIA brand was weighed, suspended in 1 liter of purified water, and mixed thoroughly in a media bottle. It needs to shake well to dissolve the powder completely. Next, the media bottle autoclave at 121^0C for 15 minutes. Before dividing the medium, it needs to cool the base to 45 until 50ºC and then aseptically add on the appropriate amount of potato dextrose agar medium into a sterile petri dish. All plates were sealed with parafilm, labeled, and put in a chiller to allow the media to solidify.

3.2.2 Pure culture of *Trichoderma reesei*

Trichoderma reesei is culture from *T.reesei* nail and were obtained from postgraduates lab and then cultured aseptically on a fresh potato dextrose agar petri dish to grow the pure culture and incubated at room temperature (25ºc) for 6 days. The subculture has been done on PDA media to obtain the pure colonies of *T. reesei*. After six days, mycelium samples were taken from the colonies using the wire loop and streaked onto PDA culture. The pure culture process had been done under aseptic conditions and left at room temperature for five days.

3.2.3 Potato Dextrose Broth (PDB) Preparation

For PDB (Potato Dextrose Broth) preparation, 24.0 grams of PDB powder from the HIMEDIA brand were weighed and mixed in 1000 ml distilled water. It needs to shake well until dissolved and autoclaved for 15 minutes at 121^{0}c. The pure culture developed in a liquid culture of 400 mL potato dextrose broth in a 500 ml conical flask and incubated at 25°C in a temperature room. Next, the conical flask need to cover with aluminum foil.

Figure 3.1: Preparation of Potato Dextrose Broth

3.2.4 Broth Culture of *Trichoderma reesei*

With a conical flask containing PDB, the broth culture process was done. Inside laminar airflow, using a 7mm cork borer, the part from the old culture of *T. reesei is cut* and put onto the sterile Potato Dextrose Broth solution. The mycelia were inoculated into 500 ml of the conical flask containing 400 ml of sterile PDB. The conical flask was covered back by using aluminum foil. After 15 days of fermentation, the broth contains the fungal mycelia.

Figure 3.2: Potato Dextrose Broth contains mycelia

3.2.5 Extraction of secondary metabolites from *Trichoderma Reesei*

The 15 days potato dextrose broth culture of *T. reesei* was used for the extraction method. The separation process was done by using a separation funnel. The separation funnel is used to separate (partition) the mixture's components into two immiscible solvent phases of differing densities using liquid-liquid extractions. The 100 ml of hexane (C_6H_{14}**)** was added until the solution reached 200 ml. The stopcock was adjusted horizontally, and the mixture was poured into a separatory funnel. The extraction process was repeated three times. All apparatus is meticulously planned, operated, cleaned, and maintained to avoid contamination by unwanted microbes. Next, a rotary evaporator machine was used to extract the solution with a boiling point of 69^0c until it was dried. After that, the extract was kept under the fume hood for air drying for five days.

Figure 3.3: Separation funnel process

Figure 3.4: The extraction process

Figure 3.5: Drying the extraction in the flame hood

3.2.6 Lignocellulolytic activities

3.2.6.1 Enzymatic activity of cellulose

To determine the cellulolytic activity Jensen Media (JM) was used. The ingredient of Jensen media is 20g agar, 20g sucrose, 1.0g dipotassium hydrogen phosphate, 2.0g calcium carbonate, 2.0 CMC, 0.1g Iron (III) Sulfate, 0.5g sodium chloride, 0.5g magnesium sulfate, 0.005g sodium molybdate and 1000 mL distilled water. It needs to shake well until dissolved and autoclaved for 15 minutes at 121ºc and poured into Petri dish. The experiment was performed in triplicates. A sterile filter paper disk (15.7 mm) are soaked in a control solution at different concentration of 5 mg, 10 mg, 15 mg, and 20 mg mycotoxin solution. Use the forceps and then press them against the side of the plate to drain any excess fluid. Then, the filter paper disk was placed at the center of the Petri dish of Jensen media, seized using a parafilm, and incubated at 28^0C for seven days.

Table 3.1: The concentration of mycotoxin each treatment

TREATMENT	CONCENTRATION OF EXTRACTION
T0 (CONTROL)	Water
T1	5 mg
T2	10 mg
T3	15 mg
T4	20 mg

Notes: T: Treatment, mg: Milligram

3.2.6.2 Color changing

After incubation for seven days, under sterile conditions, the plates were flooded with Congo red solution (dissolve 0.5 g of Congo red dye in 50 ml of distilled water and add 50 ml of ethanol) for 15 minutes and then effused. Next, 1 M Sodium chloride (NaCl) solution was overflowing on the plate for 15 minutes and effused. The inoculated area appeared yellowish, evidenced by the diameter of a clear zone showing cellulase activity around the filter paper disk was recorded.

3.3 Experimental design and statistical analysis

The experiment was designed based on Complete Randomized Design (CRD) with control and four different treatments. Each treatment had three replicates. All the data were analyzed using one-way ANOVA to establish the significant difference between the means where $p<0.05$. The calculations were done using statistical software, SPSS statistics version 26.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of Pure Culture of *Trichoderma reesei*

Trichoderma reesei culture was used for this study. Many mycelial fungi are known to have a high potential for cellulase production. *T.reesei* rapidly grow mycelia on PDA plate in seven days in temperature room. The morphological from Figure 4.1, shows the color of the spore started from whitish to yellowish and then changed to the dense dark yellow-greenish colored colony. The colony textures were compact, and hyphae were very thick.

Figure 4.1: Isolation of Pure Culture of *Trichoderma reesei*

4.2 Screening of Lignocellulolytic activities of *Trichoderma reesei*

Enzymatic degradation of cellulose was screened using Jensen media (JM). The lignocellulolytic activities of the isolates were measured based on the diameters of the halo zones produced when degrading cellulose and lignin, respectively. This diagnostic assay can identify *Trichoderma* as it has polyphenol oxidase when endoglucanases when Jensen media is used for cellulose. It was found that there was a significant difference in the diameter of the halo zone of each treatment. Figure 4.2 shows the degrading cellulose activities producing clear halo zone of *T.reesei*. *Trichoderma reesei* produced the minor diameter of halo zone is Treatment 2 (10 mg/5ml) is 20 mm. For the Treatment 3 (15 mg/5ml) and Treatment 4 (20 mg/5 ml) were found forming larger clearing halo zones from 50 mm to 60 mm after straining with Congo red. Optimization of the time course is of prime importance for cellulases biosynthesis by fungi (Kuhad, et al., 1997).

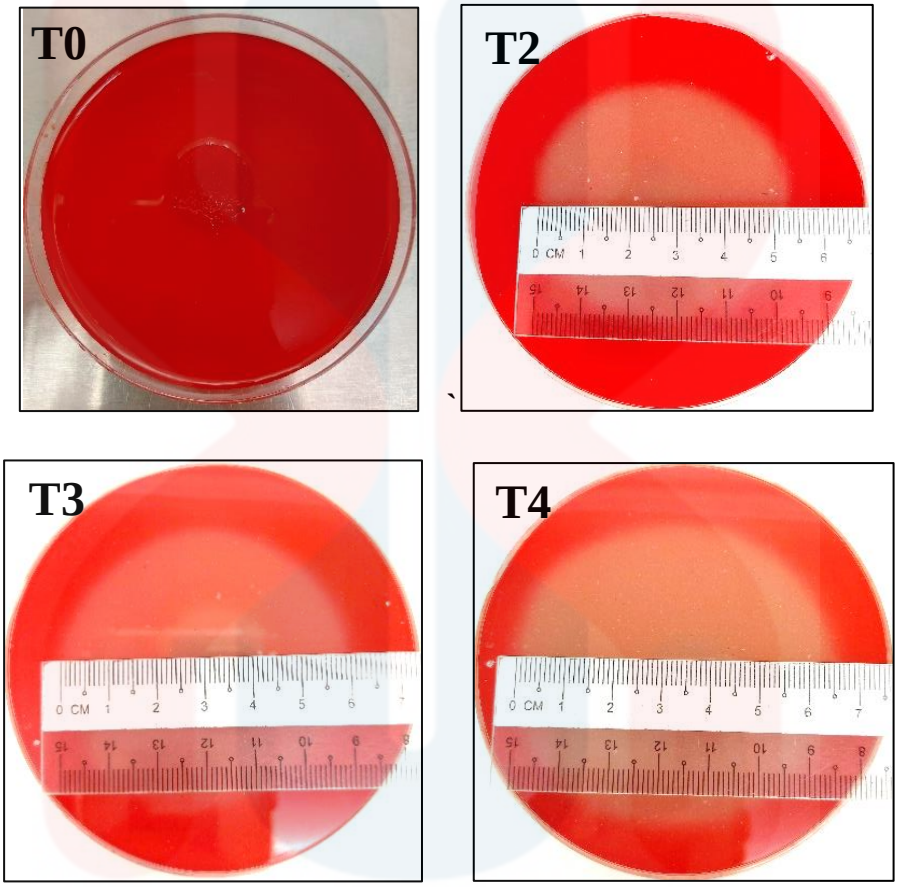
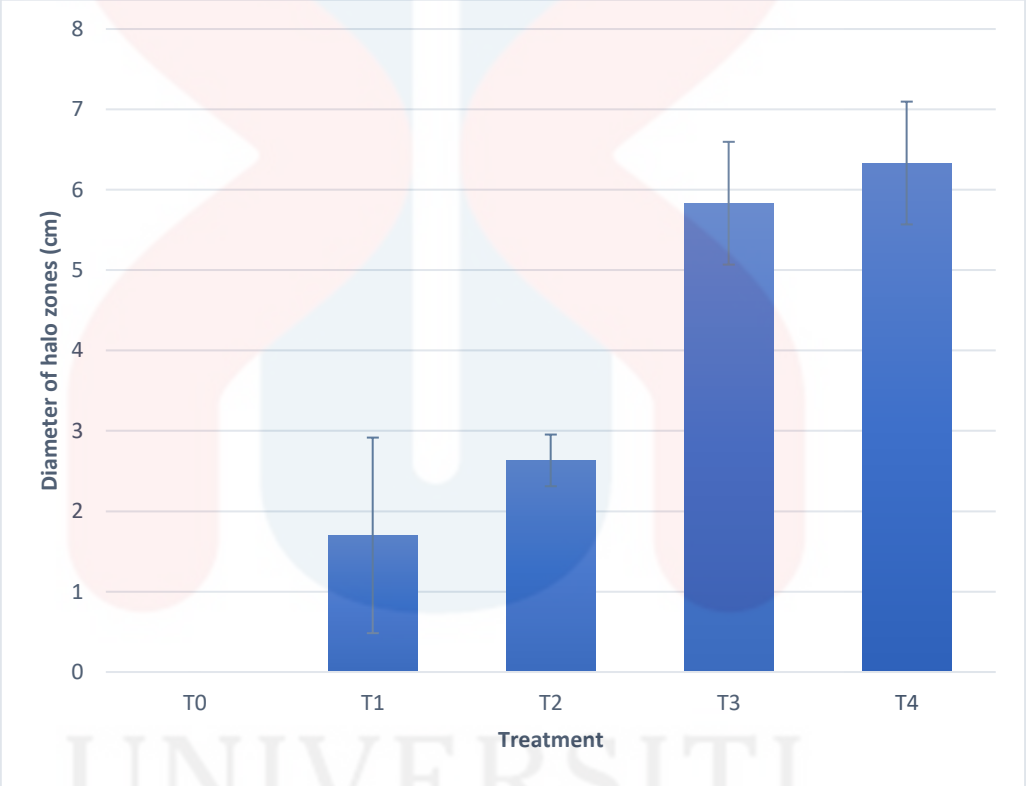


Figure 4.2: Degrading cellulose activities producing clear halo zone of *T.reesei*.

4.3 Growth performances of cellulolytic activity of *Trichoderma reesei*

Figure 4.3 shows the result of stipe length halo zones with a diameter for each treatment. The shortest stipe length showed in Treatment 1 (5 mg mycotoxin concentration) had a mean of 1.7 cm, followed by Treatment 2 (10 mg mycotoxin concentration) with a standard of 2.63 cm. Treatment 3 (15 mg mycotoxin concentration)

and Treatment 4 (20 mg mycotoxin concentration) had average stipe length with a mean of 5.83 cm and 6.33 cm, respectively.



* **T0:** Control (Water), **T1:** 5mg concentration, **T2:** 10mg concentration, **T3:** 15mg concentration, **T4:** 20mg concentration

Figure 4.3: Growth performances of the diameter of halo zones of enzymatic activity of cellulose and vertical bars represent standard deviation (SD) of the mean

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Trichoderma spp. has a wide range of properties useful in agriculture, such as reducing abiotic stresses, improving physiological stress responses, reducing nutrient uptake in plants, increasing nitrogen-use efficiency in various crops, and assisting in agriculture improving photosynthetic efficiency. Fungal cellulase is one of the hot enzymes, owing to its high demand in the biodiesel industry. *Trichoderma* spp. genomes have been thoroughly studied and found to have numerous essential genes and produce a wide range of expression patterns, allowing these fungi to adapt to a wide range of conditions like soil, water, dead tissues, etc. Also, lignocellulose is a mixture of cellulose, hemicellulose, lignin, pectin, and other minor compounds. It is the most abundant renewable biomass, created by plants directly from CO2 through photosynthesis, and amounts to around 200 billion metric tonnes worldwide (Seiboth, B et al., 2011).

Cultivation of extraction from *Trichoderma reesei* on Jensen Media (JM) shows an active activity of cellulose. The lignocellulolytic activity shows that the fungal adaptation mechanism can enhance performance. The results reported in this work indicate that *T.reesei* fungus can successfully be cultivated under submerged fermentation

to produce lignocellulolytic the significance of this new strain. The *Trichoderma* genus has evolved broad metabolic pathways that lead to numerous enzymes and secondary metabolites, allowing it to thrive in various environments. According to Harman et al., (2004), commercially relevant enzymes including amylases, cellulases, 1-3 beta glucanases, and chitinases have been intensively investigated, and the technology is constantly being upgraded.

5.2 Recommendation

The mycelial extract of *Trichoderma reesei* is beneficial in further research and can be commercialized. These plant cell wall degrading enzymes are now used for the saccharification of cellulosic plant biomass to simple sugars for biofuel production, in addition to their well-established applications in the pulp, paper, food, feed, and textile processing industries (Bouws et al., 2008). It is strongly advised that such modified strains be evaluated on suitable lignocellulose substrates. Also included should be an in vitro saccharification experiment using the enzymes produced in these cultivations. Further research is needed to look into the experimental benefit of the *Trichoderma reesei* strain.

REFERENCES

Asis, Azriah & Shahriar, Saleh & Naher, Laila & Saallah, Suryani & Fatihah, Hasan & Kumar, Vijay & Siddiquee, Shafiquzzaman. (2021). Identification patterns of Trichoderma strains using morphological characteristics, phylogenetic analyses and lignocellulolytic activities. Molecular Biology Reports. 48. 10.1007/s11033-021-06321-0.

Buchert, Ranua, M. Siika-aho, M., Pere, Viikari, (1994). *Trichoderma reesei* cellulases in bleaching of kraft pulp. Applied Microbiology and Biotechnology 40. 941-945

Behrendt, C.J., Blanchette, R.A., Akhtar, M., Enebak, S., Iverson, S. and Williams, D. (2000) Biomechanical pulping with Phlebiopsis gigantea reduced energy consumption and increased paper strength. Tappi Journal, 83, 65.

Bhale, Udhav & Rajkonda, J. (2012). Enzymatic activity of *Trichoderma* species. J. Novus Natural Science Research.. 1. 1-8.

Bouws, H.; Wattenberg, A. & Zorn, H. (2008). Fungal secretomes--nature's toolbox for white biotechnology. Applied Microbiology and Biotechnology, Vol.80, No. 3, pp. 381-388, ISSN 1432-0614

Bourgaud, F., Gravot, A., Milesi, S., & Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant science*, *161*(5), 839-851.

Calvo, A. M., Wilson, R. A., Bok, J. W., & Keller, N. P. (2002). Relationship between secondary metabolism and fungal development. *Microbiology and molecular biology reviews*, *66*(3), 447-459.

dos Santos Castro, L., Pedersoli, W. R., Antoniêto, A. C. C., Steindorff, A. S., Silva-Rocha, R., Martinez-Rossi, N. M., Faça, V. M. (2014). Comparative metabolism of cellulose, sophorose and glucose in Trichoderma reesei using high-throughput genomic and proteomic analyses. *Biotechnology for biofuels*, *7*(1), 1-18.

Domsch KH, Gams W, Anderson TH (1980) *Trichoderma* pero. Ex Fries, 1821. In compendium of soil fungi, Vol 1 pp: 368-377, 794-809, Academic Press, London, New York

Daranagama, N.D., Shioya, K., Yuki, M. et al. Proteolytic analysis of *Trichoderma reesei* in celluase-inducing condition reveals a role for *Trichoderma* pepsin (TrAsP) in cellulase production. J Ind Microbiol Biotechnol **46,** 831–842 (2019). https://doi.org/10.1007/s10295-019-02155-9

Derntl, C., Mach-Aigner, A. R., & Mach, R. L. (2014). Chapter 21 - *Trichoderma* Proteins with Disruption Activity on Cellulosic Substrates. In V. K. Gupta, M. Schmoll, A. Herrera-Estrella, R. S. Upadhyay, I. Druzhinina, & M. G. Tuohy (Eds.), Biotechnology and Biology of Trichoderma (pp. 309-317). Amsterdam: Elsevier.

Druzhinina, Irina & Kubicek, Christian. (2017). Genetic engineering of *Trichoderma reesei* cellulases and their production. Microbial biotechnology. 10. 10.1111/1751-7915.12726.

El-Nadi, Yasser. (2017). Solvent Extraction and Its Applications on Ore Processing and Recovery of Metals: Classical Approach. Separation & Purification Reviews. 46. 10.1080/15422119.2016.1240085.

Fengel, D., Wegener, G., (1989). Wood Chemistry, Ultra-structure, Reactions, Walter de Gruyter, Berlin.

Greene, E. R., Himmel, M. E., Beckham, G. T., & Tan, Z. (2015). Chapter Three - Glycosylation of Cellulases: Engineering Better Enzymes for Biofuels. In D. C. Baker & D. Horton (Eds.), Advances in Carbohydrate Chemistry and Biochemistry (Vol. 72, pp. 63-112): Academic Press.

Gams, W., & Bissett, J. (1998). Trichoderma and Gliocladium. Basic biology, taxonomy, and genetics (Vol. 1, pp. 1-34). Bristol, PA: Taylor & Francis.

Gams W, Bissett J (1998) Morphology and identification of *Trichoderma*. In: Harmann GE, Kubicek CP (eds) Trichoderma and Gliocladium. Taylor and Francis, London, pp 3–34

Horgan, K. A., & Murphy, R. A. (2011). Pharmaceutical and chemical commodities from fungi. Biology and Applications, 147.

Harman, G. E. Overview of mechanisms and uses of *Trichoderma spp*. Phytopathology, (2006); 96(2): 190-194.

Harman GE, Howell CR, Viterbo A (2004). *Trichoderma* species- opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. 2:43-56.

Jacqueline A. T, Bianca V. R., Bruna de A. M, Christiano P. G & Marília A. F. (2020). use of the versatility of fungal metabolism to meet modern demands for healthy aging, functional foods, and sustainability. *Journal of Fungi*, 6 (223), 1-27.

Kubicek, C. P. (2013). Systems biological approaches towards understanding cellulase production by *Trichoderma reesei*. Journal of Biotechnology, *163*(2), 133-142. doi:https://doi.org/10.1016/j.jbiotec.2012.05.020

Klinke, H. B., Thomsen, A. B., & Ahring, B. K. (2004). Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Applied microbiology and biotechnology, 66(1), 10-26.

Kanthal, L., Dey, A., Satyavathi, K., & Bhojaraju, P. (2014). GC-MS analysis of bio-active compounds in methanolic extract of *Lactuca runcinata* DC. *Pharmacognosy Research*, *6*(1), 58. https://doi.org/10.4103/0974-8490.122919

Kubicek, C.P., et al. (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life Style of *Trichoderma*. *Genome Biology*, 12,R40. https://doi.org/10.1186/gb-2011-12-4-r40

Kim, H. K. and Verpoorte, R. (2010). Sample preparation for plant metabolomics. Phytochemical Analysis 21(1): 4-13.

Kuhad, R.C., Singh, A. and Eriksson, K.E.L. (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. *Advances in Biochemical Engi-neering/Biotechnology*, **57**, 45-125. doi:10.1007/BFb0102072

Kuhls, K., Lieckfeld, E., Samuels, G., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Borner, T. and Kubicek, C. (1996). Molecular evidence that the asexual industrial fungus *Trichcoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proc. Natl. Acad. Sci. 93:7755-7760.

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.

Mach, R. L. and Zeilinger, S.. Regulation of gene expression in industrial fungi: *Trichoderma*. Appl. Microbial. *Biotechnol*., (2003);60: 515-522

Mach, Robert & Zeilinger, Susanne. (2003). Regulation of gene expression in industrial fungi: *Trichoderma. Applied microbiology and biotechnology*. 60. 515-22. 10.1007/s00253-002-1162-x.

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M: Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour Technol* 2005, 96:673–686

Monaco C, Perello A, Alippi HE, Pasquare AO (1991) Trichoderma spp.: biocontrol agent of *Fusarium spp*. and *Sclerotium rolfsii* by seed treatment. Adv Hort Sci 5(3):92–95

Mukherjee, D., Lahiri, S., Mukherjee, D. and Billing, T.K. (2012), "Leading virtual teams: how do social, cognitive, and behavioral capabilities matter?",Management Decision, Vol. 50 No. 2,pp. 273-290.

Martinez, D., Berka, R. M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S. E., Brettin, T. S. (2008). Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. Hypocrea jecorina). *Nat Biotechnol*, *26*(5), 553-560. doi:10.1038/nbt1403

Monte E (2001) Understanding Trichoderma: between biotechnology and microbial ecology. Int Microbiol 4:1–4

Marvin C. McMaster (2008). GC/MS A Practical User's Guide Second Edition, Wile-Interscience.

Mukhopadhyay, R., & Kumar, D. (2020). Trichoderma: a beneficial antifungal agent and insights into its mechanism of biocontrol potential. Egyptian Journal of Biological Pest Control, 30(1), 1-8.

Nigam, P. (1994). Process selection for protein-enrichment: fermentation of the sugar industry byproducts molasses and sugar beet pulp. *Process Biochemistry* 29, 337-342

Novy V, Schimd M, Eibinger M, Petrasek Z, Nidetzky B (2016) The micromorphology of *Trichoderma reesei* analyzed in cultivations on lactose and solid lignocellulosic substrate, and its relationship with cellulase production. *Biotechnol for Biofuels* 9. https://doi.org/10.1186/s13068-016- 0584-0

Ogino, H., & Ishikawa, H. (2001). Enzymes which are stable in the presence of organic solvents. *Journal of bioscience and bioengineering*, 91(2), 109-116.

Potato Dextrose Agar (PDA) for fungal cultures. (2021). Hardydiagnostics.com. https://catalog.hardydiagnostics.com/cp_prod/Content/hugo/PotatoDextroseAgar.htm

Peterson R, Nevalainen H. *Trichoderma reesei* RUT-C30 - thirty years of strain improvement. Microbiology. (2012);158:58–68.

Piepenbring, M., Hofmann, T.A., Miranda, E., Caceres, O. and Unterseher, M. (2015). 'Leaf shedding and weather in tropical dry seasonal forest shape the phenology of fungi - Lessons from two years of monthly surveys in southwestern Panama. *Fungal Ecology, 18*, 83-92.

Preechasuth, K., Anderson, J., Peck, S., Brown, A. and Gow, N. (2015). Cell Wall protection by the Candida albicans class I chitin synthases. Fungal Genetics and Biology, 82, 264-276.

Qian, Y., Zhong, L., Sun, Y., Sun, N., Zhang, L., Liu, W.,Zhong, Y. (2019). Enhancement of Cellulase Production in *Trichoderma reesei* via Disruption of Multiple Protease Genes Identified by Comparative Secretomics. *Frontiers in Microbiology*, 10. doi:10.3389/fmicb.2019.02784

Rank,C., Larsen, T.O.andFrisvad, J.C.Functional Systems Biologyof Aspergillus inMachida,M.,and Gomi, K. (Eds.). *Aspergillus*: Molecular Biology and Genomics, (2010), Caister Academic Press, Hethersett,NR,UK.ISBN:9781904455530.

Strobel, G. A., and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiol.* Mol. Biol. Rev. 67, 491–502. doi:10.1128/MMBR.67.4.491-502.200

Scopes, R. K. (2001). Enzyme activity and assays. *e LS*.

Schuster, A., & Schmoll, M. (2010). Biology and biotechnology of *Trichoderma*. *Applied microbiology and biotechnology*, 87(3), 787-799. doi:10.1007/s00253-010-2632-1

Sperandio, G.B., Filho, E.X.F. An overview of *Trichoderma reesei* co-cultures for the production of lignocellulolytic enzymes. *Appl Microbiol Biotechnol* **105,** 3019–3025 (2021). https://doi.org/10.1007/s00253-021-11261-7

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., & Pandey, A. (2013). Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresource technology, *127*, 500-507.

Sushanto Gouda, Gitishree Das, Sandeep Kumar Sen, & Jayanta Kumar Patra. (2016, September 29). Endophytes: A Treasure House of Bioactive Compounds of Medicinal Importance. ResearchGate; Frontiers. https://www.researchgate.net/publication/308756060_Endophytes_A_Treasure_House_of_Bioactive_Compounds_of_Medicinal_Importance

Strakowska, Judyta & Błaszczyk, Lidia & Chełkowski, Jerzy. (2014). The significance of cellulolytic enzymes produced by *Trichoderma* in opportunistic lifestyle of this fungus. Journal of basic microbiology. 54. 10.1002/jobm.201300821.

Seiboth, B., Ivanova, C., & Seidl-Seiboth, V. (2011). Trichoderma reesei: a fungal enzyme producer for cellulosic biofuels. *Biofuel production-recent developments and prospects*, 309-340.

Teeri, T. T., Koivula, A., Under, M., Reinikainen, T., Ruohonen, L., Srisodsuk, M., Alwyn Jones, T. (1995). Modes of action of two *Trichoderma reesei* cellobiohydrolases. In S. B. Petersen, B. Svensson, & S. Pedersen (Eds.), *Progress in Biotechnology* (Vol. 10, pp. 211-224): Elsevier.

Vicente, I., Baroncelli, R., Hermosa, R., Monte, E., Vannacci, G., & Sarrocco, S. (2022). Role and genetic basis of specialised secondary metabolites in Trichoderma ecophysiology. *Fungal Biology Reviews, 39*, 83-99. doi:https://doi.org/10.1016/j.fbr.2021.12.004

Waghunde, Rajesh & Shelake, Rahul & Sabalpara, Ambalal. (2016). *Trichoderma*: A significant fungus for agriculture and environment. African journal of agricultural research. 11. 1952-196. 10.5897/AJAR2015.10584.

Zehra A, Dubey MK, Meena M, Upadhyay RS (2017) Effect of different environmental conditions on growth and sporulation of some *Trichoderma species*. J of Environ Biol 38:197–203

APPENDIX A

Table A: Raw data of diameter of halo zone of *T.reesei* cellulose producing

TREATMENT/REPLICATE	CONCENTRATION (mg)	DIAMETER OF HALO ZONE (cm)
T0R1	Control	0
T0R2	Control	0
T0R3	Control	0
T1R1	5 mg	1.5 cm
T1R2	5 mg	1.5 cm
T1R3	5 mg	1 cm
T2R1	10 mg	2.5 cm
T2R2	10 mg	3.0 cm
T2R3	10 mg	2.4 cm
T3R1	15 mg	6.5 cm
T3R2	15 mg	5.0 cm
T3R3	15 mg	6.0 cm
T4R1	20 mg	6.5 cm
T4R2	20 mg	5.5 cm
T4R3	20 mg	7.0 cm

APPENDIX B

Table B: Diameter of the halo zone

		Subset for alpha = 0.05		
TREATMENT	**N**	**1**	**2**	**3**
T0	3	.000		
T1	3		1.700	
T2	3		2.633	
T3	3			5.833
T4	3			6.333
SIG		1.000	154	428

APPENDIX C

Table C: ANOVA

Diameter of the halo zone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	88.540	4	22.135	40.245	.000
Within Groups	5.500	10	.550		
Total	94.040	14			

APPENDIX D

Table D: Means Plot