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INVESTIGATION OF THE PRODUCTION AND CHARACTERIZATION OF LIGNOCELLULOSE-DEGRADING ENZYMES FROM VIETNAMESE FUNGI

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INTRODUCTION

Plant biomass, the largest renewable source of carbohydrates on Earth, is photosynthesized at a rate of 200 billion tons per year, of which 60% is lignocellulose. Vietnam possesses abundant plant resources, generating a large quantity of lignocellulose-rich agricultural and forestry by-products (such as wood, straw, and bagasse). However, most of this biomass is either burned or discarded, causing environmental pollution. Lignocellulose, with its robust structure composed of cellulose (35–50%), hemicellulose (15–30%), and lignin (10–25%), can be utilized in the bio-based and circular economy, for example, in the production of biofuels. Conventional physico-chemical methods for lignocellulose treatment often consume large amounts of energy and produce hazardous waste. To overcome these limitations, biological conversion using microorganisms, particularly fungi, has emerged as a promising approach due to its environmental friendliness and high efficiency. Fungi from the Basidiomycota and Ascomycota phyla have the ability to synthesize hydrolytic enzymes (e.g., acetyl esterase) and oxidative enzymes (e.g., lignin peroxidase, laccase, and cellobiose dehydrogenase). These enzymes break down lignin, enhancing the accessibility of cellulose and releasing structural units such as monosaccharides and organic compounds, which serve as feedstocks for sustainable production. In this study, these enzymes are referred to as the fungal biomass pretreatment enzyme group.

The doctoral dissertation entitled *"**Investigation of the production and characterization of lignocellulose-degrading enzymes from Vietnamese fungi**"* focuses on fungal strains isolated from natural forests in Vietnam (Muong Phang – Dien Bien, Cuc Phuong – Ninh Binh). The study involves the isolation, purification, and characterization of enzymes, as well as the

investigation of their potential applications in the bioconversion of lignocellulose-rich biomass.

❖ **Research objectives:**

- To isolate and select fungal strains capable of producing lignocellulose-degrading enzymes (including lignin peroxidase, laccase, acetyl esterase, cellobiose dehydrogenase, and unspecific peroxygenase);

- To purify and characterize the physicochemical properties, substrate specificity, and catalytic efficiency of the enzymes obtained in lignocellulose bioconversion.

❖ **Research scope and content:**

1. Isolation and selection of fungal strains with high activity in synthesizing biomass pretreatment enzymes.
2. Purification and characterization of fungal biomass pretreatment enzymes.
3. Investigation of the lignocellulose bioconversion potential using individual enzymes and synergistic "*enzyme cocktails*".

❖ **Novel contributions of the dissertation:**

- Successfully isolated and established a collection of 56 fungal strains belonging to 19 families within the phyla Basidiomycota and Ascomycota. Notably, a new fungal species, *Candolleomyces eurysporus* (family Psathyrellaceae, order Agaricales), was identified;

- Purified five enzymes, including: Acetyl esterase (*Ls*AE) and lignin peroxidase (*Ls*LiP) from *Lentinus squarrosulus* MPN12; Cellobiose dehydrogenase (*Cau*CDH) from *Coprinellus aureogranulatus* MPG14; Laccase (*Pleu*Lac) from *Pleurotus pulmonarius* MPN18; Unspecific peroxygenase (*Ceu*UPO) from *Candolleomyces eurysporus* CP22. Comprehensive physicochemical characterization, substrate specificity profiling, and catalytic kinetics were conducted for each enzyme;

- Optimized the enzyme cocktail composition for the biocatalytic conversion of lignocellulose (specifically rice straw) into *C5* and *C6* monosaccharides and gluconic acid, demonstrating potential for biotechnological applications.

❖ **Scientific and practical significance:**

This dissertation successfully isolated fungal strains with high production capacity of biomass pretreatment enzymes. These enzymes exhibit strong potential for practical application in the conversion of lignocellulosic biomass into monosaccharides and structural building blocks, contributing to the development of a sustainable bioeconomy in the future.

CHAPTER I. LITERATURE REVIEW

1.1. Overview of Lignocellulose and its role in the development of the bioeconomy

1.1.1. Structure of Lignocellulose

Lignocellulose is a sustainable and complex polymer found in the plant cell wall, composed of three main components: cellulose (40–50%), hemicellulose (20–40%), and lignin (20–35%). The proportions of these components vary depending on the source; for example, hardwood contains approximately 45–47% cellulose, whereas wheat straw contains only around 30%. Hemicellulose accounts for 35–50% in grasses but decreases to about 25% in rice straw. Lignin content ranges from 30–60% in softwoods, yet is reduced to only 6–10% in pulp. These compositional differences influence the physical and chemical properties of lignocellulose, as well as its processing pathways, making it suitable for applications in biofuels, composite materials, and biochemicals.

1.1.2. Lignocellulosic biomass resources

Lignocellulosic biomass plays a crucial role in the bioeconomy, serving as a raw material for biotechnological processes and industrial applications.

Based on its origin and composition, lignocellulose can be categorized into hardwood, softwood, agricultural crops (such as cereals and grasses), and herbaceous plants. Vietnam, with its well-developed agricultural and forestry sectors, generates hundreds of millions of tons of lignocellulosic biomass annually from agroforestry production.

1.1.3. The Role of Lignocellulose in the development of the bioeconomy

Lignocellulose conversion plays a vital role in the development of the bioeconomy due to its ability to utilize renewable biological resources as substitutes for fossil fuels, while enabling the production of value-added products.

1.2. Lignocellulose-converting enzymes and microbial sources of enzyme biosynthesis

The complex structure of lignocellulose, characterized by the tight association between cellulose, hemicellulose, and lignin, poses significant challenges to its conversion. Lignocellulose-converting enzymes, including oxidative enzymes (e.g., laccase, lignin peroxidase, unspecific peroxygenase) and hydrolytic enzymes (e.g., cellulases, hemicellulases), play a crucial role in this process. Oxidative enzymes break down the recalcitrant lignin network, facilitating the access of hydrolytic enzymes to depolymerize cellulose and hemicellulose, thereby releasing monosaccharides and oligosaccharides. The synergistic action between these enzyme classes is essential in determining the overall efficiency of lignocellulose degradation.

1.2.1. Oxidative enzyme group

Laccase (EC 1.10.3.2) is a polyphenol oxidase that contains multiple copper atoms in its catalytic center and is commonly referred to as a multi-copper polyphenol oxidase.

Lignin peroxidase (LiP, EC 1.11.1.14) is a monomeric hemoprotein that plays a key role in the oxidation of lignin compounds. LiP isozymes are capable of directly oxidizing a wide range of organic substances.

Unspecific peroxygenase (EC 1.11.2.1), commonly referred to as UPO, is an oxidative enzyme belonging to the heme-thiolate peroxidase family, known for its ability to catalyze peroxygenation reactions. UPO is capable of directly oxidizing lignin in lignocellulosic biomass by utilizing hydrogen peroxide as the oxidant, without the need for mediators, unlike many other oxidative enzymes.

Cellobiose dehydrogenase (CDH, EC 1.1.99.18) is an extracellular enzyme synthesized by various wood-decaying fungi. This enzyme efficiently oxidizes soluble cellodextrins, mannodextrins, and lactose into their corresponding lactones via a unique mechanism that utilizes a broad range of electron acceptors, including quinones, phenoxyl radicals, Fe^{3+}, Cu^{2+}, and triiodide ions. CDH exhibits strong and specific adsorption to cellulose, and contains two distinct functional domains: one harboring flavin adenine dinucleotide (FAD) and the other heme, which can be separated through limited proteolysis.

1.2.2. Hydrolytic enzyme group

1.2.2.1. Glycoside hydrolase families

Glycoside hydrolase (also known as glycosidases or glycosyl hydrolases; EC 3.2.1.-) represent a large and widespread group of enzymes that catalyze the hydrolysis of glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Among these, one of the most important enzyme systems is cellulase, a complex that includes endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and cellobiase (EC 3.2.1.21).

1.2.2.2. Carbohydrate esterase group

Acetyl (xylan) esterase: The efficient conversion of lignocellulose requires a cocktail of hydrolytic enzymes—including cellulases, xylanases, and carbohydrate esterases—that work synergistically to break down the complex polymer structure. Among the carbohydrate esterases involved in lignocellulose deconstruction, acetyl esterase plays an essential role by removing acetyl groups from hemicellulose, enhancing the accessibility of other enzymes.

1.3. Characteristics of fungal Lignocellulose-degrading enzymes

Fungal lignocellulose-degrading enzymes possess significant potential due to their superior ability to break down lignin, cellulose, and hemicellulose more efficiently than enzymes derived from bacteria or plants. Fungi are capable of producing a diverse array of powerful lignocellulolytic enzymes, particularly those involved in lignin degradation, which is crucial for converting lignocellulosic biomass into value-added products. While bacteria generally grow faster and may produce enzymes more rapidly, they often lack the enzymatic diversity and efficiency required for comprehensive lignocellulose breakdown. In contrast, fungi-especially species within the phyla Basidiomycota and Ascomycota-are uniquely equipped with both oxidative and hydrolytic enzymes, enabling them to play a key role in biomass conversion and sustainable biotechnological application.

CHAPTER II. MATERIALS AND METHODS

2.1. Materials

Fungal samples were collected during the rainy season (October) from Cúc Phương National Park (Ninh Bình) and Mường Phăng (Điện Biên), and preserved at 4ºC in the Laboratory of Experimental Biology, Institute of Natural Products Chemistry.

Rice straw samples were collected from Củ Chi, Ho Chi Minh City. After harvest, the straw was washed with clean water, sun-dried until the

moisture content was ≤15%, then cut into short pieces (0.5–2 mm) and stored at room temperature (25–30ºC).

Lignin was isolated from the above-mentioned dried rice straw following the method described by Đỗ Hữu Nghị et al.

Commercial enzymes, including cellulase and glucuronoxylanase (Cell/Xyl), derived from *Trichoderma reesei*, were provided by AB Enzyme (Darmstadt, Germany). These enzymes exhibit optimal activity at pH 5.0 and a temperature of 40ºC.

2.2. Methods

2.2.1. Isolation of fungi
2.2.2. Identification of fungal strains
2.2.3. Investigation of fungal enzyme biosynthesis
2.2.4. Effects of various factors on the growth and enzyme production of selected fungal strains
2.2.5. Enzyme activity assay methods
2.2.6. Purification of natural enzymes
2.2.7. Characterization of purified enzymes
2.2.8. Biocatalytic conversion of lignin-based materials
2.2.9. Biocatalytic conversion of rice straw using an enzyme cocktail
2.2.10. High-Performance Liquid Chromatography (HPLC)
2.2.11. Optimization of enzyme cocktail composition for rice straw bioconversion using response surface methodology (RSM).

CHAPTER III: RESULTS

3.1. Isolation and screening of fungal strains capable of producing highly active Lignocellulose pretreatment enzymes

3.1.1. Fungal isolation

A total of 56 fungal strains belonging to the phyla Basidiomycota and Ascomycota were isolated from fruiting body samples collected in ecological regions of Cuc Phuong National Park (Ninh Binh) and Muong

Phang (Dien Bien). The fungal isolates were purified and identified based on morphological characteristics and molecular biological methods.

3.1.2. Screening of enzyme-producing fungal strains

The biosynthetic capacity of 56 selected fungal strains to produce lignin peroxidase (LiP), cellobiose dehydrogenase (CDH), laccase (Lac), acetyl esterase (AE), and unspecific peroxygenase (UPO) was evaluated based on their ability to transform specific substrates corresponding to each enzyme. Culture supernatants were clarified by removing biomass and impurities before enzyme activity was determined. Comparative analysis of enzymatic activity revealed significant differences in enzyme production among the isolated fungal strains. Notably, strain MPN12 demonstrated the highest biosynthetic capacity for both AE and LiP enzymes. Additionally, strain MPG14 exhibited superior production of CDH, while strain MPN18 was the most efficient producer of Lac. UPO activity was found to be highest in strain CP22. Based on these findings, strains MPN12, MPG14, MPN18, and CP22 were selected for further studies on culture conditions for enzyme production, purification, biochemical characterization, and application in lignocellulose biomass conversion.

3.1.3. Identification of high enzyme-producing fungal isolates

3.1.3.1. Nucleotide sequence analysis of strain MPN12

The nucleotide sequence obtained from the ITS region of strain MPN12 was compared against sequences in the GenBank database using the BLAST tool. The analysis revealed a high sequence identity of 99.76% with *Lentinus squarrosulus* (accession number: GU001951). To further confirm the phylogenetic placement, a maximum likelihood (ML) phylogenetic tree was constructed using representative species from the genus *Lentinus*. The resulting tree showed that strain MPN12 clustered tightly with Lentinus squarrosulus, forming a distinct clade with strong bootstrap support (>98%),

indicating a close evolutionary relationship. These results support the identification of strain MPN12 as closely related to *Lentinus squarrosulus*, sharing a common origin based on high genetic similarity.

3.1.3.2. Identification of strain MPN18 based on its sequence analysis

The nucleotide sequence obtained from strain MPN18 was compared against the GenBank database using the BLAST tool. The results revealed that the ITS sequence of MPN18 shared 100% identity with *Pleurotus pulmonarius* strain MH395979. A phylogenetic tree was constructed using the Maximum Likelihood (ML) method to further examine the genetic relationship among selected *Pleurotus* species. Strain MPN18 clustered in the same clade as Pleurotus pulmonarius, forming a distinct group with 100% sequence similarity and a strong bootstrap support value of 99%. These findings confirm that strain MPN18 is genetically identical to *Pleurotus pulmonarius* and likely shares a common evolutionary origin with this species.

3.1.3.3. Identification of strain MPG14 based on ITS sequence analysis

The ITS sequence obtained from strain MPG14 was analyzed for sequence similarity using the BLAST tool against the GenBank database. The results showed that strain MPG14 exhibited 100% sequence identity with *Coprinellus aureogranulatus* (accession number GQ249274). This high degree of similarity suggests that MPG14 shares a common taxonomic origin with *Coprinellus aureogranulatus*.

3.1.3.4. Identification of strain CP22 based on ITS sequence and phylogenetic analysis

The ITS sequence of strain CP22 showed 100% identity with *Candolleomyces euryspous* (accession number NR172427) in the GenBank database. A phylogenetic tree based on ITS sequences of CP22 and related *Coprinellus* species was constructed using the Maximum Likelihood (ML)

method. The results indicated that CP22 and *C. euryspous* formed a distinct clade with a bootstrap value of 100%, indicating a strong genetic relationship. Therefore, based on molecular identification, CP22 is classified as *Candolleomyces euryspous*, a white-rot fungus belonging to the family Psathyrellaceae.

3.1.4. The study of cultivation conditions for enzyme production from fungi.

3.1.4.1. Effect of temperature and pH

Temperature and pH are two critical factors affecting fungal growth and development. Within the temperature range of 23–37°C, all fungal strains were able to grow, with the optimal temperature for MPN12, MPN18, and MPG14 being 27°C, and for CP22 being 29°C. Regarding pH, the strains grew within the range of 4.0–8.0, with the optimal pH for MPN18, MPG14, and CP22 being 6.0, and for MPN12 being 5.5. Outside these optimal temperature or pH ranges, the growth rate decreased significantly.

3.1.4.2. Effect of nitrogen sources on fungal growth and enzyme production

Experiments to determine suitable nitrogen sources for biomass development and enzyme production in fungal strains showed that organic nitrogen sources (peptone and yeast extract) promoted better growth compared to inorganic nitrogen sources (KNO_3, $(NH_4)_2SO_4$, $NaNO_3$). Strain MPN12 grew best in yeast extract, but exhibited the highest AE enzyme activity in peptone medium. Strain MPN18 achieved both the highest biomass and laccase activity in yeast extract. Strain MPG14 showed the best growth in yeast extract, while CDH enzyme activity peaked in peptone. Strain CP22 grew best in peptone, but UPO activity was highest in yeast extract. In summary, yeast extract and peptone are suitable nitrogen sources

for these fungal strains, depending on the specific enzyme targeted for production.

3.2. Study on the purification and characterization of lignin peroxidase, laccase, acetyl esterase, cellobiose dehydrogenase, and unspecific peroxygenase enzymes from fungi

3.2.1. Investigation of enzyme purification

3.2.1.1. Purification of acetyl esterase from L. squarrosulus MPN 12 (LsAE)

After the purification process, a total enzyme protein yield of 17.0 mg was obtained, corresponding to 391 U, with a specific activity of 23 U/mg and a recovery yield of 7.5%. The purification fold reached 12.1 times compared to the initial crude extract (Table 3.1). The purified enzyme fraction was stored at -20ºC and used for subsequent studies on enzyme protein characterization and in vitro transformation of lignocellulose-rich materials. SDS-PAGE analysis of the fraction with the highest AE activity after purification showed a distinct protein band corresponding to *Ls*AE with a molecular weight (M_W) of 41.4 kDa.

Table 3.1. Purification of *Ls*AE from *L. squarrosulus* MPN12.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2709	5214	1,9	100	1,0
DEAE Cellulose	210	2540	12,1	48,9	6,4
Sephadex G-100	57	789	13,8	15,2	7,3
HiTrap™ Q XL	17	391	23	7,5	12,1

3.2.1.2. Purification of cellobiose dehydrogenase from C. aureogranulatus MPG 14 (CauCDH)

The fungal strain under study synthesized a significant amount of *Cau*CDH during solid-state fermentation with straw, yielding a crude extract with a total *Cau*CDH activity of 1817.1 U (measured using DCIP as the electron acceptor) (Table 3.2). Throughout the purification process, enzyme activity at each step was evaluated, and specific activity as well as purification fold were calculated as shown in the table.

Table 3.2. Purification of *Cau*CDH from *C. aureogranulatus* MPG14.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	1817,1	2635,6	0,69	100	1,0
Siêu lọc 10 kDa	1687,1	2239,3	0,75	92,8	1,1
DEAE Sepharose	889,2	231,5	3,84	48,9	5,6
Superdex G100	549,2	25,7	21,33	30,2	30,9
HiTrap™ Q FF	411,8	14,3	28,86	22,7	41,9

After a three-step purification process (including ion-exchange chromatography and size-exclusion chromatography), the remaining amount of *Cau*CDH represented only 22.7% of the original activity, but the enzyme was purified 41.9-fold from the crude culture filtrate, reaching a specific activity of 28.86 U/mg (total 411.8 U). The purity of *Cau*CDH after the final step on the HiTrap Q FF column was assessed by SDS-PAGE analysis, which revealed a single prominent band with a molecular weight of 109 kDa.

3.2.1.3. Purification of laccase from Pleurotus pulmonarius MPN 18 (PleuLac)

After the purification process, a total of 19 mg of purified enzyme protein was obtained, corresponding to 532.0 U, with a yield of 8.3% and a purification fold of 9.6. This purified enzyme fraction was subsequently used for further studies on the biochemical properties of the enzyme protein (Table 3.3). SDS-PAGE analysis of the purified protein revealed a single protein band corresponding to *Pleu*Lac with a molecular weight (M_W) of approximately 35 kDa.

Table 3.3. Purification of *Pleu*Lac from *P. pulmonarius* MPN18.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2243	6432	2,9	100	1.0
DEAE Cellulose	205	3011	14,7	46,8	5,1
Sephadex G-100	54	889	16,5	13,8	5,7
HiTrap™ Q XL	19	532	28	8,3	9,6

3.2.1.4. Purification of lignin peroxidase from L. squarrosulus MPN 12 (LsLiP)

The crude extract containing *Ls*LiP enzyme was purified through three consecutive chromatography steps: ion-exchange chromatography using a DEAE Cellulose column, gel filtration chromatography on a Sephadex G-100 column, and finally, ion-exchange chromatography on a HiTrap™ Q XL column. Upon completion of the purification process, 20 mg of enzyme protein was obtained, with a total activity of 566 U, a specific activity of 28.3 U/mg, a recovery yield of 15.7%, and a purification fold of 47.1 compared to the initial crude extract (Table 3.4). The purified protein was stored at −20ºC for subsequent studies. The purification results after two

anion-exchange chromatography steps and one gel filtration step showed a protein band with an approximate molecular weight of 53 kDa.

Table 3.4. Purification of *Ls*LiP from *L. squarrosulus* MPN12.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	5400	3600	0.6	100	1.0
DEAE Cellulose	350	1250	4	35	6.7
Sephadex G-100	80	831	10.4	23	17.3
HiTrap™ Q XL	20	566	28.3	15.7	47.1

3.2.1.5. Purification of unspecific peroxygenase from Candolleomyces euryspous CP22 (CeuUPO)

At the end of the purification process, a total of 19 mg of enzyme protein was obtained, corresponding to a total activity of 534 U. The specific activity of the enzyme increased to 28.1 U/mg, with a purification yield of 23.2% and a purification fold of 45.3 compared to the crude extract. The purity of the protein, as assessed by SDS-PAGE, showed a distinct single band at approximately 40 kDa.

Table 3.5. Purification of *Ceu*UPO from *C. euryspous* CP22.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	3700	2300	0.62	100	1.0
DEAE Cellulose	280	1110	3.9	42.8	6.3
Sephadex G-75	83	787	9.5	34.2	15.3
Mono Q™	19	534	28.1	23.2	45.3

3.2.2. Enzyme Characterization

3.2.2.1. Characterization of acetyl esterase (LsAE) from Lentinus squarrosulus MPN12

The acetyl esterase *Ls*AE exhibited optimal activity at pH 5.5 and 35ºC. The thermal stability of the enzyme was assessed at pH 5.5 and 35ºC by incubating the enzyme for varying durations (from 0 to 120 minutes) at different temperatures (4, 25, 40, and 60ºC). The enzyme retained relatively high stability at 4ºC and 25ºC after 1 hour of incubation; however, a gradual decrease in activity was observed in the following hour. The purified enzyme also showed good stability under acidic conditions (pH 5.0), maintaining its activity after 6 hours of incubation with no significant loss compared to the initial activity. In contrast, under neutral conditions (pH 7.0), a steady decline in enzymatic activity was observed over the 1 to 6-hour incubation period.

3.2.2.2. Characterization of cellobiose dehydrogenase (CauCDH) from Coprinellus aureogranulatus MPG14

The CDH enzyme (*Cau*CDH) exhibited maximum activity at 50ºC and pH 5.5 compared to other tested temperatures. Thermal stability of CDH was evaluated at pH 5.5 and 50ºC by incubating the enzyme for various durations (from 0 to 120 minutes) at different temperatures (25, 40, and 70ºC). The enzyme remained relatively stable at 25ºC after 12 hours of incubation; however, its activity progressively declined at 70ºC. The purified enzyme also demonstrated good stability under acidic conditions (pH 4.0), with no significant loss in activity observed after 12 hours of incubation. In contrast, at neutral pH (pH 8.0), enzyme activity markedly decreased after 2 hours of incubation, and was almost completely lost after 8 hours.

3.2.2.3. Characterization of laccase (PleuLac) from Pleurotus pulmonarius MPN18

The laccase enzyme PleuLac exhibited the highest activity at 40ºC and pH 5.5 compared to neighboring temperature conditions. The thermal stability of the enzyme was evaluated at pH 5.5 and 40ºC by incubating the enzyme for various durations (from 0 to 120 minutes) at different temperatures (25, 40, and 70ºC). The enzyme remained relatively stable at 25ºC after 120 minutes of incubation. However, its activity gradually declined at 40ºC and significantly decreased at 70ºC, retaining only approximately 20% of its initial activity after 120 minutes of incubation. In addition, *Pleu*Lac demonstrated good stability under acidic conditions (pH 5.0), with no noticeable loss in enzymatic activity after 6 hours of incubation compared to the initial value.

3.2.2.4. Characterization of lignin peroxidase (LsLiP) from Lentinus squarrosulus MPN12

The highest activity of LiP was observed at pH 5.0 and 35ºC when compared to adjacent temperature conditions. The thermal stability of LiP was evaluated at pH 5.0 and 35ºC by incubating the enzyme for different durations (0-120 minutes) at various temperatures (4, 25, 40, and 60ºC). The enzyme retained relatively high stability at 4ºC and 25ºC after 1 hour of incubation; however, its activity gradually decreased during the second hour. After 2 hours, the residual activity dropped to approximately 60% of the initial value.The purified enzyme also exhibited good stability under acidic conditions (pH 4.0), showing no apparent loss in activity after 1 hour of incubation, with only a slight decrease observed after 3 hours. In contrast, at neutral pH (pH 6.0), LiP activity declined steadily over the 1 to 6-hour incubation period.

3.2.2.5. Characterization of unspecific peroxygenase (CeuUPO) from Candolleomyces euryspous CP22

To determine the optimal pH of *Ceu*UPO, enzyme activity was assessed across a pH range of 2.0 to 10.0 using three different substrates: ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate)], veratryl alcohol (VA), and 2,6-dimethoxyphenol (DMP). The findings indicate that the optimal pH for *Ceu*UPO activity varies depending on the substrate. Specifically, *Ceu*UPO exhibited the highest catalytic activity toward ABTS under acidic conditions (pH 5.0), whereas optimal activity toward VA and DMP was observed around neutral pH (pH 7.0). These results suggest that *Ceu*UPO possesses good adaptability to a wide pH range, and that its catalytic efficiency is substrate-dependent.

3.2.3. Catalytic kinetics of the enzyme

3.2.3.1. Catalytic kinetics of LsAE

The kinetic parameters of the purified *Ls*AE enzyme from *Lentinus squarrosulus* MPN12 were determined using *p*-nitrophenyl acetate as the substrate, based on the Lineweaver–Burk plot. Substrate concentrations ranging from 0.3 mM to 1.5 mM were used to determine the maximum reaction velocity (V_{max}). The results showed a Michaelis constant K_m of 4,03 µM and a V_{max} of 312 µM/min. The catalytic turnover number (k_{cat}) was calculated to be 10 s^{-1}, resulting in a catalytic efficiency (k_{cat}/K_m) of 2,48 $\mu M^{-1} s^{-1}$.

3.2.3.2. Catalytic kinetics of CauCDH

Michaelis–Menten constants (K_m, ranging from 117,1 to 1.354.000 µM) and catalytic efficiencies (k_{cat}/K_m, ranging from 25,8 x 10^4 to 21,7 $M^{-1} s^{-1}$ for cellobiose and maltose, respectively) were determined for all tested substrates. The highest substrate affinity (K_m = 117,1 µM) and catalytic

efficiency (k_{cat}/K_m = 25,8 x 10^4 M^{-1} s^{-1}) were observed for cellobiose, identifying it as the most favorable substrate for *Cau*CDH.

3.2.3.3. Catalytic kinetics of PleuLac

The kinetic parameters of the purified *Pleu*Lac enzyme were determined using ABTS as the primary substrate. Substrate concentrations ranging from 0.1 mM to 1.0 mM were used to establish the maximum reaction velocity (V_{max}). The resulting Michaelis constant K_m was found to be 1,35 µM, which is notably lower than the K_m values reported for laccases from other fungal strains.

3.2.3.4. Catalytic kinetics of LsLiP

The *Ls*LiP enzyme demonstrated effective oxidative catalytic activity toward various substrates, including DCP, guaiacol, ferulic acid, veratryl alcohol, vanillic acid, and dimethoxyphenol. Among the phenolic compounds tested, *Ls*LiP exhibited the highest specificity toward DCP, with a relative activity reaching up to 240%, followed by veratryl alcohol at 180%. In contrast, the enzyme showed significantly lower activity toward other aromatic compounds such as guaiacol, ferulic acid, and vanillic acid. The kinetic parameters of purified *Ls*LiP were determined using DCP as the primary substrate. Substrate concentrations ranging from 0.3 mM to 1.5 mM were applied to establish the maximum reaction rate (V_{max}). The results revealed a Michaelis constant K_m of 2,45 µM and a V_{max} of 285 µM/min. The catalytic turnover number (k_{cat}) for the peroxidase reaction was calculated to be 12 s^{-1}, yielding a catalytic efficiency (k_{cat}/K_m) of 4,85 μM^{-1} s^{-1}.

3.2.3.5. Catalytic kinetics of CeuUPO

The catalytic kinetic parameters of the purified *Ceu*UPO enzyme were determined for three different substrates: ABTS, veratryl alcohol (VA), and 2,6-dimethoxyphenol (DMP). The results indicated that *Ceu*UPO exhibited the highest substrate affinity toward ABTS, as evidenced by the

lowest Michaelis constant K_m of 39 μM, followed by DMP (K_m = 117 μM) and VA (K_m = 693 μM). These findings suggest that *Ceu*UPO catalyzes reactions with ABTS most efficiently, demonstrating both high affinity and favorable substrate–enzyme interaction compared to the other tested substrates.

3.3. Investigation of enzyme-based biocatalysis in lignocellulosic biomass transformation

3.3.1. Investigation of lignin oxidation catalyzed by the purified LsLiP enzyme from L. squarrosulus

Lignin derived from rice straw, exhibiting a dark brown color, was used as a substrate for biocatalytic transformation by the *Ls*LiP enzyme from *Lentinus squarrosulus* MPN12. FTIR spectral analysis was performed to evaluate the structural changes in lignin before and after enzymatic treatment with *Ls*LiP. Notably, significant alterations were observed in the fingerprint region (1600–1000 cm^{-1}) of the lignin spectrum following treatment, indicating modifications in the stretching vibrations associated with various functional groups within the lignin structure. A marked decrease in the intensity of the peak at 1115 cm^{-1}-characteristic of planar deformation of syringyl units-suggests degradation of the lignin macromolecule. Additionally, the emergence of new peaks at 1080 and 1039 cm^{-1} in the treated sample indicates the formation of C–O bonds, typically associated with primary alcohols. These FTIR findings confirm the pivotal role of *Ls*LiP in catalyzing the transformation of lignin from plant biomass, highlighting its potential in lignocellulosic bioconversion processes.

3.3.2. Rice straw biomass valorization using a synergistic enzyme Cocktail

3.3.2.1. Investigation of individual factors affecting conversion efficiency

The objective of this experimental evaluation was to determine the optimal values of enzyme-to-substrate activity ratios (U g^{-1}) for individual

enzymes within the "*enzyme cocktail*" used in the bioconversion of lignocellulose into monosaccharides.

Experimental results identified suitable operating ranges and the experimental center points for subsequent optimization modeling. Specifically, the optimal activity range and center value for the cellulase/xylanase (Cell/Xyl) component were found to be 12–24 U g^{-1} and 18 U g^{-1}, respectively. For acetyl esterase (AE), the optimal range was 25–45 U g^{-1} with a center value of 35 U g^{-1}. For the cellobiose dehydrogenase (CDH) enzyme, the appropriate activity range was 40–60 U g^{-1}, with an optimal center value of 50 U g^{-1}.

3.3.2.2. Experimental design-based optimization of enzyme dosages in Lignocellulosic bioconversion

The basic levels and ranges of the reaction parameters were established based on preliminary studies involving single-factor experiments, in which the activity-to-substrate ratios (U g^{-1}) of the individual enzymes-Cell/Xyl, AE, and CDH-were selected as independent variables. In general, the most significant increase in bioconversion products (i.e., monosaccharides and carboxylic acids) was observed at enzyme-to-substrate ratios of 18 U g^{-1} for Cell/Xyl, 35 U g^{-1} for AE, and 50 U g^{-1} for CDH. Higher enzyme dosages resulted in only marginal improvements in product release, as previously described. Accordingly, three activity levels for each enzyme were selected as input factors for the experimental design matrix. These levels were defined as follows: 12, 18, 24 U g^{-1} for Cell/Xyl, 25, 35, 45 U g^{-1} for AE and 40, 50, 60 for CDH, representing the low (−1), center (0), and high (+1) levels, respectively.

The dependent variables, i.e., the yields (Y) of glucose (mg g^{-1}), xylose (mg g^{-1}), and gluconic acid (mg g^{-1}), were experimentally determined and are presented in the experimental design matrix. The regression models

for these response variables were evaluated through analysis of variance (ANOVA) using F-values, p-values, and coefficients of determination (R^2). Second-order polynomial equations were established for each response (Y_1, Y_2, and Y_3) in relation to the independent variables. The ANOVA results demonstrated that all three models corresponding to the target responses are statistically significant and well-fitted. The F-values for Y_1, Y_2, and Y_3 were 84.44, 110.35, and 38.60, respectively, with all p-values < 0.05, indicating high statistical significance. Moreover, the R^2 values of 0.9835, 0.9850, and 0.9858 for Y_1, Y_2, and Y_3, respectively, indicate that the models explain the majority of the variability in the experimental data. These results confirm the predictive accuracy and reliability of the developed regression models, demonstrating a high degree of agreement between observed and predicted values.

Table 3.6. Second-order polynomial equation models for glucose (Y_1), xylose (Y_2), and gluconic acid (Y_3) concentrations.

Response	Model equation	R^2 value	p value
Y_1	$Y_1 = 159{,}54+5{,}56A+16{,}48B+6{,}29C-7{,}13A^2-15{,}84B^2-6{,}52C^2$	0,9835	< 0,0001
Y_2	$Y_2 = 63{,}23+2{,}95A+6{,}07B+2{,}57C-2{,}46A^2-7{,}08B^2-1{,}89C^2$	0,9850	< 0,0001
Y_3	$Y_3 = 5{,}01+0{,}71A+0{,}48B+0{,}17C-0{,}21AB-0{,}64A^2-0{,}33B^2-0{,}36C^2$	0,9858	0,0004

3.3.2.3. Response surface analysis and model validation

The second-order polynomial model was analyzed and visualized using Design Expert 7.0.0 software. The X and Y axes of the 3D response surface plots represent the interactions between process parameters, including the interaction between Cell/Xyl and AE (CDH at 50 U g^{-1}); Cell/Xyl and CDH (AE at 35 U g^{-1}); and AE and CDH (Cell/Xyl at 18 U g^{-1}). It can be observed that the two-factor interactions influenced all

objective functions in the descending order of AB > BC > AC. This trend is consistent with the descending influence of the individual parameters in the order of B > A > C (corresponding to the coded variables AE, Cell/Xyl, and CDH, respectively), as shown in the second-order equations in Table 3.7.

Desirability function-based optimization and model validation were employed to optimize the objective functions following the enzymatic catalytic process, with the expectation that Y_1, Y_2, and Y_3 could reach their maximum values. As a result, ten experimental options were identified, among which the best scenario for maximizing the objective functions was predicted. These experimental values were compared with the predicted values to validate the model. The differences between the theoretical and experimental objective function values were negligible, further confirming that the constructed model was consistent with the experimental conditions. Upon optimization of the three dependent variables, the optimal levels of the factors were determined to be 19.2 U g^{-1} for Cell/Xyl, 38.8 U g^{-1} for AE, and 52.8 U g^{-1} for CDH after 48 hours of substrate incubation at 45°C and pH 5.0. Accordingly, the objective functions were determined to be 165.18 ± 3.19, 64.21 ± 1.22, and 5.17 ± 0.13 mg g^{-1} (product/substrate, w/w), corresponding to the *C-6* (glucose), *C-5* (xylose), and gluconic acid, respectively.

Table 3.7. Coded and actual values of the independent variables.

Coded variable			**Actual variable**		
A	**B**	**C**	**Cell/xyl**	**AE**	**CDH** (U g^{-1})
0,2	0,38	0,28	19,2	38,8	52,8

Table 3.8. Response values and experimental results obtained under optimal conditions.

Dependent variables	**Optimal value**

	Experiments	**Theoretical prediction**
Y_1 – Glucose (mg g^{-1})	165,18 ± 3,19	165,69
Y_2 – Xylose (mg g^{-1})	64,21 ± 1,22	65,57
Y_3 – Gluconic acid (mg g^{-1})	5,17 ± 0,13	5,26

In this study, the application of an experimentally optimized model successfully identified the optimal conditions for the enzyme cocktail (CDH, AE, and Cell/Xyl) in the bioconversion of rice straw substrate, aiming to enhance the efficiency of *C-5* and *C-6* monosaccharide (glucose and xylose) production. This finding further confirms that the enzyme cocktail facilitates the hydrolysis of polysaccharides into mono- and dimers more effectively. The presence of the auxiliary enzymes (CDH and AE) enables the main enzyme complex (Cell/Xyl) to more efficiently cleave polymer chains into lower-degree oligosaccharides, thereby allowing Cell/Xyl to complete the conversion process more readily.

CONCLUSION

From the results of this study, the following conclusions are proposed:

1. A total of 56 fungal strains belonging to the phyla Basidiomycota and Ascomycota were isolated and screened. Among them, 38 strains exhibited acetyl esterase (AE) activity, 25 strains showed lignin peroxidase (LiP) activity, 35 strains demonstrated cellobiose dehydrogenase (CDH) activity, 31 strains exhibited laccase (Lac) activity, and 24 strains showed unspecific peroxygenase (UPO) activity. Strains with high enzyme activities were identified to the species level for further investigation, including: MPN12 (AE and LiP activities of 3,650.8 U/L and 39.3 U/mL, respectively), MPG14

(CDH activity of 77.4 U/L), MPN18 (Lac activity of 7,659 U/L), and CP22 (UPO activity of 41.2 U/mL).

2. Five enzymes were purified and their physicochemical properties (molecular weight, specific activity, stability, optimal catalytic conditions) and substrate specificity were determined, including: AE and LiP from *Lentinus squarrosulus* MPN12 (designated LsAE, 41.4 kDa; and LsLiP, 53 kDa); CDH from *Coprinellus aureogranulatus* MPG14 (CauCDH, 109 kDa); Lac from *Pleurotus pulmonarius* MPN18 (PleuLac, 35 kDa); and UPO from *Candolleomyces euryspous* CP22 (CeuUPO, 40 kDa).

3. The application of these enzymes in lignocellulose conversion from rice straw was investigated. *Ls*LiP from *Lentinus squarrosulus* MPN12 effectively degraded lignin, resulting in significant structural changes in the 1600–1000 cm^{-1} region (FTIR spectrum). Additionally, the process of biomass hydrolysis was optimized using an enzyme cocktail consisting of CDH, AE, and Cell/Xyl, which efficiently converted rice straw into *C-5* and *C-6* monosaccharides (glucose and xylose). The optimized model demonstrated strong predictive agreement, with the proposed optimal enzyme loading ratios being 19,2:38,8:52,8 (U/g) for Cell/Xyl, AE, and CDH, respectively.

RECOMMENDATIONS

The research results demonstrate the significant potential of fungal biodiversity in Vietnam for the biosynthesis of lignocellulose-degrading enzymes. Therefore, it is recommended that future research should not only focus on the discovery of novel enzymes but also on the optimization of enzyme cocktails to further improve the efficiency of lignocellulosic biomass conversion. These studies should be extended to pilot-scale and larger-scale applications, in order to evaluate the feasibility and scalability of applying this enzymatic technology in industrial production systems.

LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

1. Do Huu Nghi, Harald Kellner, Enrico Büttner, Le Mai Huong, Le Xuan Duy, Vu Dinh Giap, **Dang Thu Quynh**, Tran Thi Nhu Hang, An Verberckmoes, Ludo Diels, Christiane Liers and Martin Hofrichter. Cellobiose dehydrogenase from the agaricomycete *Coprinellus aureogranulatus* and its application for the synergistic conversion of rice straw. Appl Biol Chem, 2021, 64-66. Doi: https://doi.org/10.1186/s13765-021-00637-y.
2. Dinh Giap Vu, Huu Nghi Do, Huu Cuong Le, **Thu Quynh Dang**. Lignin peroxidase from the white-rot fungus *Lentinus squarrosulus* MPN12 and its application in the biodegradation of synthetic dyes and lignin. BioResources, 2022, 17.3. Doi: 10.15376/biores.17.3.4480-4498.
3. Vu Dinh Giap, **Dang Thu Quynh**, and Do Huu Nghi. Investigation of lignin peroxidase (LiP) production from fungi grown on liquid culture medium. Vietnam Journal of Biotechnology 19(4):771-778. Doi: 10.15625/1811-4989/15738.
4. **Dang Thu Quynh**, Nguyen Huy Hoang, Nguyen Ngoc Lan, Le Viet Hoang, Do Huu Nghi. Cloning, Experession, and Characterization of a Laccase from the White Rot Fungi *Pleurotus pulmonarius* MPN18. VNU Journal of Science: Natural Sciences and Technology, 2023. Doi: https://doi.org/10.25073/2588-1140/vnunst.5312.
5. Vu Dinh Giap, **Dang Thu Quynh**, and Do Huu Nghi. Screening carbohydrate esterase and oxidase enzyme from fungi isolated in Cuc Phuong (Ninh Binh) and Muong Phang (Dien Bien). Proceedings of the 3rd national scientic conference of Vietnam Museum system, 2021.
6. Do Huu Nghi, **Dang Thu Quynh**, Enrico Büttner, Christiane Liers, Harald Kellner, Martin Hofrichter. Investigation of the lignocellulose-degrading enzyme system from the fungus *Candolleomyces eurysporus* and purification, characterization of selected properties of a putative proxygenase. National Conference on Biotechnology Science and Technology, 2023.