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OPTIMISATION OF LIGNIN PEROXIDASE PRODUCTION FROM *PYCNOPORUS* SP.

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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SEPTEMBER 2008

Chairman: **Suraini Abd. Aziz, PHD**

Faculty: **Biotechnology and Biomolecular Sciences**

Ligninase or lignin peroxidase is gaining importance for their biotechnology application due to its integral role in the biodegradation of lignin, lignin related aromatic compounds and the potential use in industrial processes such as biopulping, biobleaching and bioremediation. Lignin peroxidase has been extensively studied and has been reported produced by white rot fungus. In this study, a preliminary study was done to screen for the highest lignin peroxidase producer from five locally isolated white rot fungus using agitated and non-agitated culture condition. The highest lignin peroxidase producer, identified as *Pycnoporus* sp. was selected for the optimisation study. Factorial design approach was chosen to determine the optimum conditions which significantly influence the production of lignin peroxidase by *Pycnoporus* sp. Optimum condition for the highest lignin peroxidase activity of 51.1 U/L was successfully achieved at 24 mM of

nitrogen concentration, agitation speed at 110 rpm, pH 3.5, inoculum concentration of 6 x 10^6 spores/ml and addition of 1 mM inducer (veratryl alcohol) in carbon limited media.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGOPTIMUMAN PENGHASILAN LIGNIN PEROKSIDASE DARIPADA *PYCNOPORUS* SP.

Oleh

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Ligninase atau lignin peroksidase semakin mendapat perhatian di dalam aplikasi bioteknologi di mana ia memainkan peranan penting di dalam bio-penguraian lignin dan juga sebatian aromatik berlignin serta berpotensi di dalam proses-proses industri seperti bio-pulpa, bio-pemutihan dan bioremediasi . Lignin peroksidase, telah dikaji dengan meluas dan dilaporkan dihasilkan oleh kulat pereput putih. Dalam kajian ini, penyaringan awal telah dilakukan untuk memilih kulat pereput putih tempatan yang menghasilkan lignin peroksidase tertinggi melalui kaedah fermentasi kelalang goncang dan statik. Kulat yang menghasilkan aktiviti lignin peroksidase tertinggi dikenalpasti sebagai *Pycnoporus* sp. telah dipilih untuk kajian pengoptimuman. Pendekatan 'Factorial Design' dipilih untuk menentukan keadaan optimum yang mempengaruhi penghasilan lignin peroksidase oleh kulat *Pycnoporus* sp. Keadaan optimum yang menghasilkan aktiviti lignin peroksidase tertinggi 51.1 U/L diperolehi pada kepekatan nitrogen sebanyak 24 mM,

kelajuan goncangan pada 110 psm, pH 3.5, kepekatan inokulum pada 6×10^6 spora/ml dan penambahan penggalak iaitu alkohol veratril di dalam media yang mengandungi sumber karbon yang terhad.

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Thank you.

I certify that an Examination Committee met on [15 September 2008] to conduct the final examination of Zuraidah binti Zanirun on her Master of Science thesis entitled "Optimisation of Lignin peroxidase Production from *Pycnoporus* sp." in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the examination Committee are follows:

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Date: 15 JANUARI 2009

DECLARATION

I declare that the thesis is my original work except for the quotation and citation which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at UPM or other institution.

_____

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Date:

TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DHP	Dehydrogenation polymerizate
LiP	Lignin Peroxidase
%	Percentage
H_20_2	Hydrogen peroxide
MnP	Manganese peroxidases
$CuS0_4$	Copper Sulphate

CHAPTER 1

INTRODUCTIONS

The increasing expansion of agro-industrial activity over the last 40 years has led to the accumulation of a large quantity of lignocellulosic residues all over the world (Villas-Boas *et al*., 2002). Agricultural has played and will continue to play an important economic role in Malaysia. Malaysian agricultural is traditionally based on crop productions, particularly rubber, palm oil, cocoa, pepper, rice and pineapple. By-products derived from the mentioned agricultural sub-sectors have and will continue to provide large quantities of valuable nutrients to sustain livestock, particularly ruminant production in Malaysia. However, they are known mostly containing lignocellulosic materials which is a complex structure of lignin. Removal of lignin is important in order for enzymatic hydrolysis to occur as it acts as a barrier to most of agricultural wastes.

Lignin is a three-dimensional heterogeneous polymer stored in the plant cell wall of all vascular plants. It is the most abundant renewable aromatic biopolymer in the biosphere. Owing to its recalcitrant nature, lignin is remarkably resistant to degradation by most microorganisms, an important factor limiting the rate of degradation of lignocellulosic materials. Filamentous fungi, primarily the white-rot fungi and related basidiomycetous fungi are known to be the most efficient terrestrial micoorganisms capable of catalyzing lignin biodegradation. Studies on lignin biodegradation are important for possible biotechnology application, since lignin polymers are major obstacles to the efficient

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utilization of lignocellulosic materials in a wide range of industrial processes (Eriksson *et al*., 1990).

Although removal of lignin can be done using chemical and physical pre-treatment, it was not a natural process as lignin compound and chemical reagent itself will cause environmental pollution. Therefore, biotechnology methods that are environmental friendly are preferred as a tool in any processes.

Various white-rot fungi isolated have the ability to degrade lignin by producing extracellular oxidative enzymes known as ligninase, a generic name for a group of isoenzymic peroxidases that catalyze the oxidative depolymerization of lignin. Another two extracellular ligninolytic peroxidases (lignin peroxidase, LiP, EC 1.11.1.14 and manganese peroxidase, MnP, EC 1.11.1.13) and one phenoloxidase of laccase type (benzenediol:oxydoreductase, EC 1.10.3.2) also have been intensively studied in various white-rot fungi for lignin biodegradation and dye decolourisation (Maganhotto de Souza Silva *et al*., 2005) . In this study, attempt had been made to focus on lignin peroxidase enzyme production due to it capability to catalyze the oxidation of variety of compounds with high reduction potential (Viral *et al*., 2005). This enzyme was produced by the fungi during secondary metabolism in response to environmental stress and the level of nutrient condition. Thus, several factors were believed can optimize the capability and potential of a white rot fungus producing lignin peroxidase.

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Prior to achieving the objectives listed below, locally isolated fungus was screened to obtain the best lignin peroxidase producer. The potential lignin peroxidase producer was used for further study. Thus, the objectives of this study were:

1) To screen for the best lignin peroxidase producer from locally isolated white rot fungus
2) To optimise the parameters involved in lignin peroxidase production by locally isolated white rot fungus

CHAPTER 2

LITERATURE REVIEW

2.1 Structure and Biosynthesis of Lignin

2.1.1 Structure of Lignin

Lignin is a complex, three-dimensional, phenolic, natural polymer that is responsible for providing structural support to woody plants (Higuchi, 1990; Lewis and Sarkanen, 1998). The most abundant source of carbon is plant biomass, comprised primarily of cellulose, hemicellulose and lignin. Many microorganisms are capable of degrading and utilising cellulose and hemicellulose as carbon and energy sources, however a much smaller group of filamentous fungi has evolved with the ability to breakdown lignin, the most recalcitrant component of plant cell walls. Formed through oxidation and free radical coupling of phenyl alcohol precursors, the insoluble polymer lacks stereoregularity. In constrast to hydrolysable bonds between subunit of other wood polymers (e.g. cellulose and hemicellulose), lignin degradation requires oxidative attack on the carbon-carbon and ether interunit bonds. The lignin polymer encrusts cellulose microfibrils, particularly within the secondary walls. No microbe, including white rot fungi is known to be capable of utilising lignin as a sole carbon or energy sources, and it is believed that lignin depolymerization is necessary to gain access to cellulose and hemicellulose. Extracellular peroxidases and oxidases are thought to play an important role in the initial depolymerisation of lignin and small molecular weight fragments are subsequently

metabolised intracellularly ultimately to water and carbon dioxide (Cullen and Kersten, 2004).

Lignin is biosynthesized by the polymerization of phenylpropanoid precursors initiated by the enzymes peroxidase (Higuchi, 1985) or laccase (Sterjiades *et al*., 1992, 1993). There are three of these precursors, differing in the number of methoxyl groups on the aromatic ring (Figure 2.1). Softwood lignin contains mostly guaiacyl (monomethoxy) units. Hardwood lignin contains roughly equal amounts of guaiacyl and syringyl (dimethoxy) units. Grass lignins contain p-hydroxyphenyl (no methoxyl) units as well as the other two types (Reid, 1995).

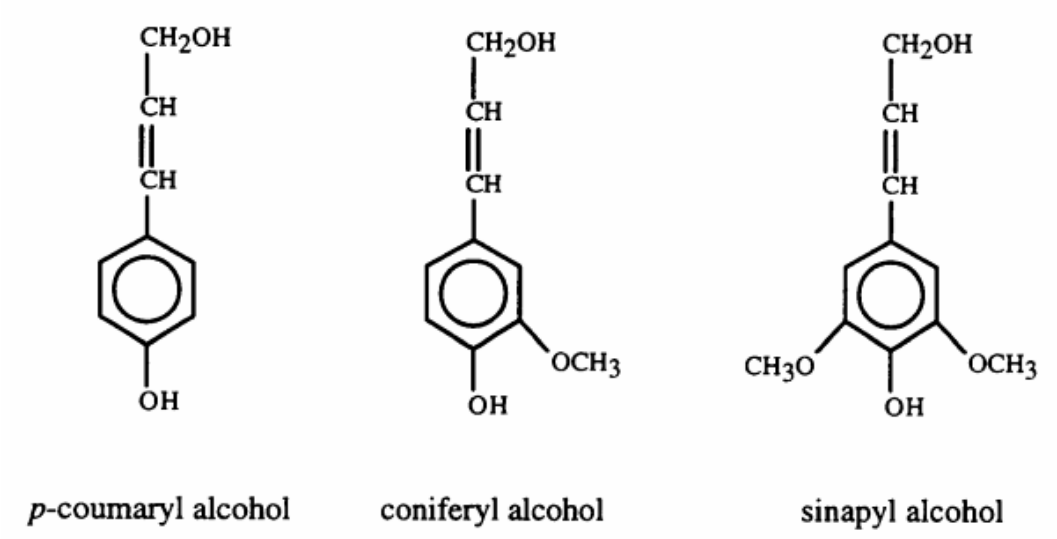

Figure 2.1: Phenylpropanoid precursors of lignin (Grabber, 2005)

Lignin polymerization occurs in the cell walls after the polysaccharides have been deposited and is initiated by a peroxidase- or laccase-mediated one-electron oxidation of the phenylpropanoid precursors to phenoxy radicals. The radicals couple with each other

and with the radicals in the growing lignin polymer in a random fashion to form a complex, cross-linked network (Figure 2.2) of these linkages, six to eight are more important. The single most predominant is the ß-O-4 type between units 1 and 2, 2 and 3, 4 and 5, 6 and 7, 7 and 8, and 13 and 14 and constitutes 40 - 60% of the total linkages in different lignins (Adler, 1977). The other linkages are relatively less numerous and include phenylcoumaran (between units 3 and 4; 6 - 12%), biphenyl (units 5 and 6; 5 - 10%), diarylpropane (units 8 and 9; 5 - 10%), diphenyl ether (units 8 and 10; 4 - 6%), α-aryl ether (units 3 and 13, 15 and 16; 6 - 8%) and pinoresinol (units 10 and 11; < 5%).

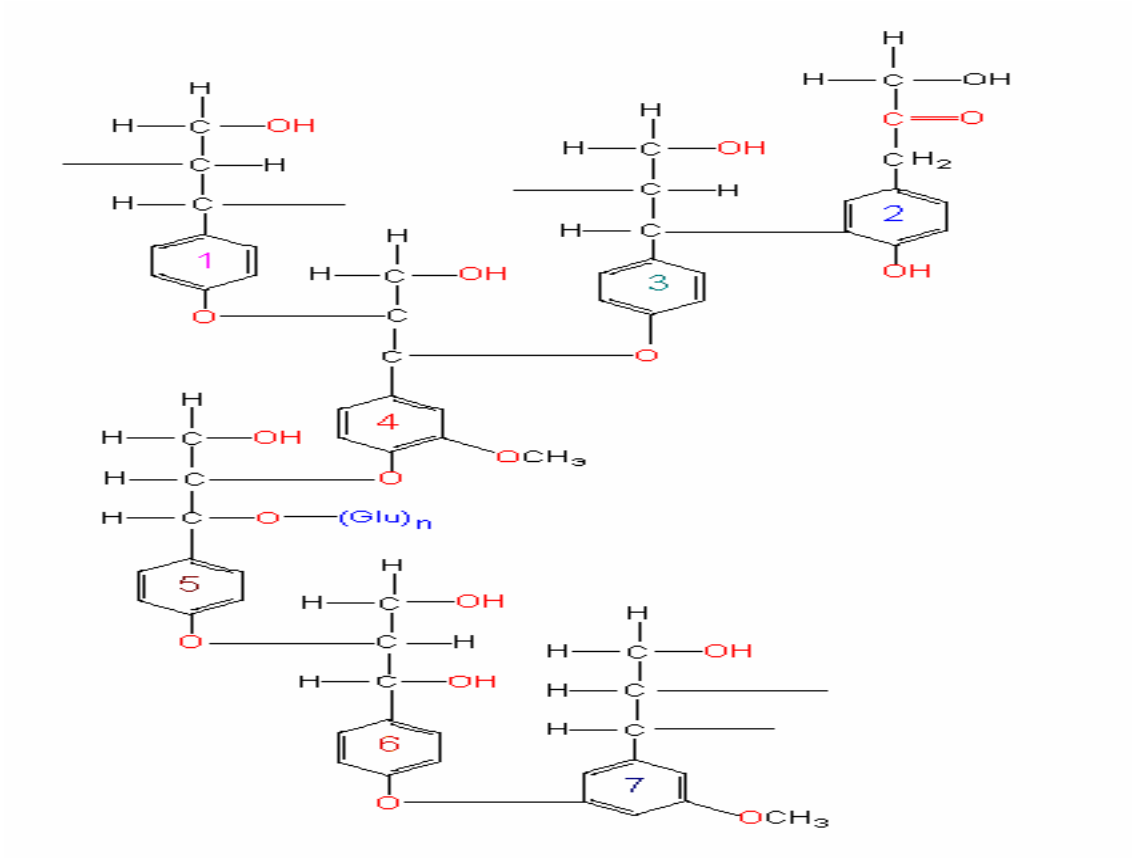

Figure 2.2: Schematic formula for a section of spruce lignin (Adler, 1977)

2.1.2 Biosynthesis of Lignin

Biosynthesis of lignin starts with the fixation of carbon dioxide in plants to form carbon dioxide in plants to form carbohydrates. These are further metabolized via the shikimic acid pathway (Figure 2.3) to tyrosine and phenylalanine (Eriksson *et al*., 1990).

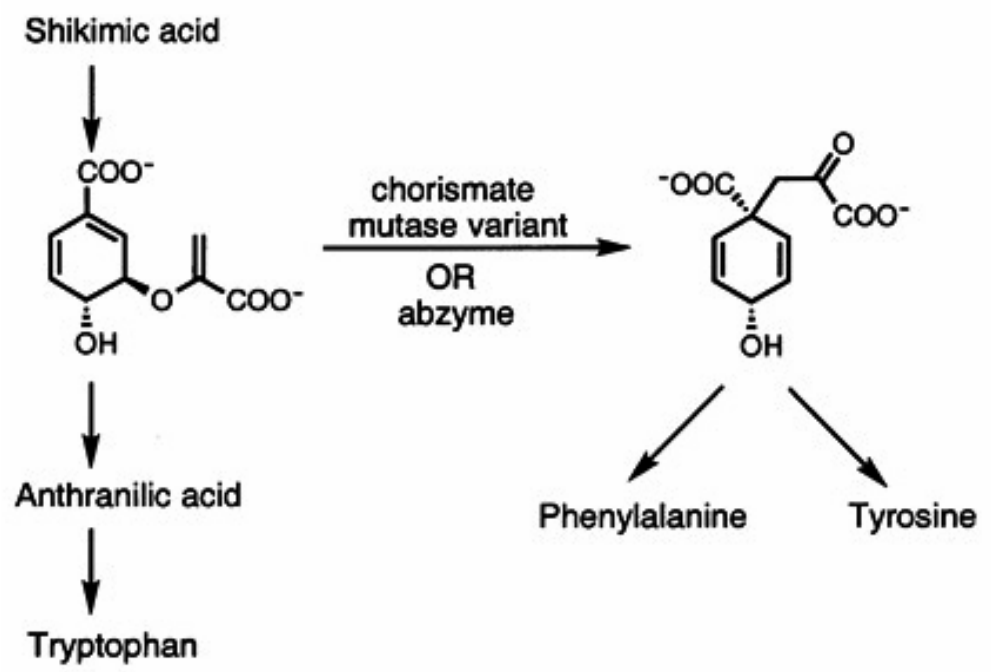

Figure 2.3: Shikimic acid pathway for biosynthesis of lignin

2.2 Lignin biodegradation

Lignin is an insoluble, high molecular weight polymer, so the initial steps in its biodegradation by white rot fungi must be extracellular (Feijoo *et al*., 1995). The final steps in lignin mineralization, ending with the release of CO_2, are likely to occur inside the fungal hyphae. The extracellular reactions must attack lignin in such a way that

fragments were produced and able to diffuse to the hyphae and cross the cell membranes. Previous work in the 1970s unravelled some of the complicated reactions which occur during the extracellular metabolism of the lignin polymer. The extracellular reactions were found to be predominantly oxidative, since the oxygen content of white rotted lignin is generally higher than the control lignin from sound wood (Kirk and Chang, 1975).

There is also a decrease in methoxyl groups and an increase in carbonyl and carboxyl groups. The chemical changes produced by white rot fungi in the lignin polymer include oxidative cleavage of the propanoid side chains between the α- and ß-carbons and between the ß- and γ-carbons, and demethylation and oxidative cleavage of aromatic rings (Chen and Chang, 1985). A progressive depolymerization occurs and releases a wide array of low molecular weight lignin fragments (Chen *et al*., 1982;1983). Lignin biodegradation does not only occur by an orderly removal of the peripheral subunits as single ring compounds; it also involves oxidation of the aromatic rings and side chains in the interior of the polymer, increasing the hydrophilicity and solubility of the polymer core at the same time as fragments of varying sizes are released (Reid, 1995).

2.2.1 Lignin degrading fungi

The only organisms convincingly shown to efficiently degrade lignin are the wood rotting fungi (Eriksson *et al*., 1990; Blanchette, 1991; Daniel, 1994). They can be divided into three groups according to the morphology of wood decay.