การทำให้บริสุทธิ์ และลักษณะสมบัติของแลคเคสจากราไวท์รอต WR77 และการประยุกต์ในการฟอกสีของสีย้อมสังเคราะห์

นางสาวปาจรียา ส่งเสริม

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PURIFICATION AND CHARACTERIZATION OF LACCASE FROM WHITE-ROT FUNGUS WR77 AND ITS APPLICATION IN DECOLORIZATION OF SYNTHETIC DYES

Miss Pajareeya Songserm

A Thesis Submitted in Partial Fulfillment of the Requirements
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Department of Microbiology
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ปาจรียา ส่งเสริม: การทำให้บริสุทธิ์และลักษณะสมบัติของแลกเคสจากราไวท์รอต WR77 และการประยุกต์ในการฟอกสีของสีย้อมสังเคราะห์ (PURIFICATION AND CHARACTERIZATION OF LACCASE FROM WHITE-ROT FUNGUS WR77 AND ITS APPLICATION IN DECOLORIZATION OF SYNTHETIC DYES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.ประกิตติ์ สิน สีหนนทน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร. อภิชาติ กาญจนทัต, 65 หน้า.

เป็นที่ทราบกันดีว่าแลกเคสที่สร้างโดยราไวท์รอตมีความสามารถในการย่อยสลายสีย้อมสังเคราะห์ได้ ดังนั้นในงานวิจัยนี้ได้ทำการคัดกรองราไวท์รอตที่มีกิจกรรมของแลกเคสสูงและมีสมบัติใหม่ๆ เพื่อนำไปประยุกต์ใช้ในการฟอกสีของสีย้อมสังเคราะห์ให้มีประสิทธิภาพมากยิ่งขึ้น ได้ทำการคัดเลือกราไวท์รอตที่สร้างแลกเคสโดยเลี้ยงบนอาหารแข็งที่ผสม 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) พบว่าราไวท์รอตสายพันธุ์ WR77 สามารถผลิตแลกเคสได้ดีที่สุด โดยทดสอบผลหาแหล่งคาร์บอนและไนโตรเจนของการผลิตแลคเคสโดยราไวท์รอตสายพันธุ์ดังกล่าว พบว่าเมื่อเลี้ยง WR77 ในอาหารเหลวที่ผสมด้วย 1% แกลบ และผสมกับ 0.5 กรัม/ลิตร ไดแอมโมเนียมทาเตรต และ 0.01 กรัม/ลิตร เปปโตน เป็นแหล่งคาร์บอนและไนโตรเจนจะมีค่ากิจกรรมของแลกเคสสูงสุด เมื่อทำแลกเคสให้บริสุทธิ์โดยใช้เทคนิคการตกตะกอนโปรตีนด้วยเกลือแอมโทเนียมซัลเฟต, โครมาโทรกราฟีแบบแลกเปลี่ยนประจุ และเจลฟิวเตรชันโครมาโทกราฟี ตามลำดับ แลกเคสบริสุทธิ์จะมีค่ากิจกรรมของเอนไซม์ 617.12 ยูนิต/มิลลิกรัมของโปรตีน มีน้ำหนักโมเลกุลประมาณ 75.2 กิโลดาลตัน แลกเคสที่บริสุทธิ์ของ WR77 มีความเสถียรในช่วงค่าความเป็นกรด-ด่างตั้งแต่ 4.0 ถึง 11.0 และที่อุณหภูมิ 40 องศาเซลเซียส ค่า Km และ Vmax เป็น 447.93 มิลลิโมลลาร์ และ 104.17 ไมโครโมล/นาที/มิลลิกรัมของโปรตีน ตามลำดับ เมื่อใช้ ABTS เป็นสับสเตรท โลหะไอออน 5.0 มิลลิโมลลาร์ Hg^{2+} และ 1.0 มิลลิโมลลาร์ Fe^{2+} ยับยั้งการทำงานของเอนไซม์ในขณะที่ 10 มิลลิโมลลาร์ Cu^{2+} กระตุ้นการทำงานของแลกเคสได้ 9.7% WR77 สามารถฟอกสีย้อมสังเคราะห์ Ambifix Blue H3R ได้ 98% ภายใน 8 วัน, Ambifix Yellow H3R ได้ 24% ภายใน10 วัน และ Ambifix Red HE3B ได้ 50% ภายใน 18 วัน และพบว่าแลกเคส (5 ยูนิต/มิลลิลิตร) สามารถฟอกสีย้อม Ambifix Blue H3R ได้ 65% ภายใน15 นาที และ Malachite Green ได้ 80% ภายใน 24 ชั่วโมง

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต.....

สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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PAJAREEYA SONGSERM: PURIFICATION AND CHARACTERIZATION OF LACCASE FROM WHITE-ROT FUNGUS WR77 AND ITS APPLICATION IN DECOLORIZATION OF SYNTHETIC DYES. ADVISOR: ASSOC. PROF. PRAKITSIN SIHANONTH, Ph.D., CO-ADVISOR: APHICHART KARNCHANATAT, Ph.D., 65 pp.

It is well known that laccase produced by white rot fungi were able to decolorize synthetic dyes. Therefore, in this research, white rot fungi were screened for high laccase activity with new characteristic. Selective white rot fungi produced high laccase activity by growing on medium supplemented with 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS). White rot fungus strain WR77 showed highest laccase activity among other isolates. WR77 was tested for the effect of carbon source and nitrogen source on highest laccase production. One percent (w/v) rice chaff, 0.5 g/L di-ammonium tartrate and 0.01 g/L peptone were supplemented in the medium as carbon and nitrogen sources showed highest laccase activity. Purification WR77 laccase by ammonium sulphate precipitation, Q-sapharose anion-exchange chromatography, and Sephadex G-75 gel filtration chromatography showed enzyme activity as 617.12 U/mg protein. The enzyme has molecular mass of approximately 75.2 kDa as determined by SDS-PAGE. Purified WR77 laccase remained active at pH range 4.0 to 11.0 at 40°C for up to 120 min of incubation time. The Km value of enzyme for ABTS as substrate was 447.93 mM and its corresponding Vmax value of 104.17 μmol/min/mg protein. 5.0 mM Hg^{2+} and 1.0 mM Fe^{2+} inhibited WR77 laccase activity whereas 10 mM Cu^{2+} ions stimulated WR77 laccase activity by 9.7%. WR77 could decolorized 98% Ambifix Blue H3R was in 8 days, 24% Ambifix Yellow H3R in 10 days and 50% Ambifix Red HE3B in 18 days. Crude laccase with concentration 5 U/ml could also decolorize 65% Ambifix Blue H3R within 15min and 80% Malachite Green within 24hr.

Department : Microbiology Student's Signature .....
Field of Study : Industrial Microbiology Advisor's Signature .....
Academic Year : 2010 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

%	percentage
°C	degree celsius
µg	microgram
µl	microlitre
A	Absorbance
BLAST	Basic local alignment search tool
BSA	bovine serum albumin
cm	centimeter
Da	Dalton
EDTA	Ethylenediamine tetraacetic acid
ESI/MS/MS	Electrospray ionisation/Mass spectrometry/Mass spectrometry;
g	gram
hr	hour
kDa	kilodaton
l	litre
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MW	molecular weight
N	normal
nm	nanometer
NaCl	Sodium chloride
PAGE	polyacrylamide gel electrophoresis
PDA	Potato dextrose agar
rpm	revolution per minute
SDS	sodium dodecyl sulfate
TEMED	*N,N,N',N'*-tetramethyl ethylenediamine

TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
U	Unit activity
V	Volt
V/V	volume by volume
W/V	weight by volume

CHAPTER I

INTRODUCTION

Lignin was a complex oxyphenyl propanoid polymer, found in all vascular plants including herbaceous species, which provides rigidity, support, and protection to the plants (Dean and Eriksson, 1992). Lignin polymer comprises of a variety of monomers connected by various C–C and C–O–C nonhydrolyzable bonds with irregular arrangement of successive monomeric and intermonomeric bonds (Alder, 1977). Therefore, it is not susceptible to hydrolytic attack. However, it is degraded by various microorganisms, particularly, white-rot fungi. This degradability of white rot fungi is due to the strong oxidative activity of their ligninolytic enzymes consist of the three kinds of extracellular phenoloxidases, namely, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) (Hatakka, 1994; Arora and Sharma, 2010). Moreover, the enzymes have low substrate specificity allow them to degrade either lignin than a wide range of pollutants, such as chlorinated aromatic compounds, heterocyclic aromatic hydrocarbons, synthetic high polymers and various dyes (Barr and Aust, 1994)

Dyes are extensively used in several industries, including textile, paper, printing, leather-dyeing, cosmetic, pharmaceutical and food industries (Erkurt *et al*., 2007). It was estimated that 10-15% of the dyes were released into the environment during manufacturing and usage (Vaidya and Datye, 1982). These dyes were not only toxic to human animal or some aquatic life but also inhibit sunlight penetration and reduce photosynthetic action within ecosystems (Aksu, 2005). Moreover, dyes usually had a synthetic origin and complex aromatic molecular structures that make them difficult to biodegrade (Forgacs *et al*., 2004). Therefore, the ways to remove these dyes have been extensively studied. Decolorisation of these dyes by physical or chemical methods, including adsorption, coagulation/flocculation, ion exchange, oxidation and electrochemical methods had economic and methodological disadvantages (Erkurt *et al*., 2007). Alternatively, dye decolorization using microbial enzymes had received great attention in recent years due to its efficient application (Abadulla *et al*., 2000 and Couto *et al*., 2005). Fungus had proved to be a suitable

organism for the treatment of textile effluent and dye removal. The fungal myceлıa have an additive advantage over single cell organisms by solubilising the insoluble substrates by producing extracellular enzymes (Kaushik and Malik, 2009).

White rot fungi had been demonstrated for decolorization of synthetic dyes mediated by their lignolytic enzymes such as lignin peroxidase, manganese peroxidase and laccase. Generally, the white rot fungi contain either of the above or all the three types of above lignolytic enzymes. Most of the white rot fungal strains produced laccase as the main enzyme during dye decolorization process (Murugesan *et al*., 2007). Laccases (EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase) were multi-copper oxidases. They catalyse the oxidation of a variety of phenolic and organic substrates coupled with the reduction of molecular oxygen to water (Thurston, 1994).

To date, numerous fungal strains had been tested for decolorization and mineralization of various dyes (Forgacs *et al*., 2004). Unfortunately, the majority of dyes are chemically stable and resistant to microbiological attack. Therefore, expanding the spectrum of microorganisms with laccase activities and isolating novel laccase with different physicochemical and catalytic properties for the applications of laccase in decolorization is need. In this study, we selected the white rot fungi strain which had high laccase activity. Then we purified and characterized the enzyme. In addition, the selected strain and their laccase was evaluated for its decolorization capability against synthetic dyes.

CHAPTER ll

LITERATURE REVIEWS

2.1 Dye history

People had made use of dye since prehistoric times, for example in decorating their bodies, in colouring the furs and skins that they wore and in the painting which adorned their cave dwellings. Of course, in those days the colours that were used were derived from natural resources. The dyes used to colour clothing were commonly extracted ether from vegetable sources, including plants, trees, roots, seeds, nuts, fruit skins, berries and lichens, or from animal sources such as crushed insects and mollusks, such as ochre and haematite, which were dug from the earth, ground to a fine powder and mixed into a crude binder (Christie, 2001).

In 1856 William Henry Perkin, in the attempt to synthesize quinine, discovered and patented a substance with excellent dyeing properties that later would come to be known as Aniline Purple, but which was later to become known as Mauveine (Welham, 2000). During the several years following the discovery of Mauveine, research activity in dye chemistry intensified, especially in Britain, Germay, and France. For the most part, chemists concentrated on aniline as the starting material, adopting a largely empirical approach to its conversion into coloured compounds, and this resulted in the discovery of several other synthetic textile dyes, such as Magenta, introduced in 1859 and Bismarck Brown, introduced in 1861. Towards the end of the 19th century, ten thousand new synthetic dyes had been developed and manufactured (Christie, 2001).

In the latter part of the 20th century, new types of dyes and pigments for the traditional applications of textiles, leather, plastics, paints and printing inks continued to be developed and introduced commercially but at a declining rate. At the same time, during this period, research effort in organic colour chemistry developed in new directions, sustained by the opportunities presented by the emergence of a range of novel applications demanding new types of colorants. These colorants have commonly been termed ‘functional dyes’ because the applications require the dyes to

perform certain functions beyond simply providing colour. The applications c functional dyes include some of the more recently developed reprographic technique, such as electrophotography and ink-jet printing, a wide range of electronic applications including optical data storage, liquid crystal displays, lasers and solar energy conversion, and a range of medical uses (Christie, 2001).

2.2 Dye classification

Colorants may be classified usefully in two separate ways, either according to the method of application or their chemical structure. Most of the commercial dyes are classified in terms of color, structure or method of application in the Colour Index (C.I.), a publication produced by the Society of Dyes and Colourists, Bradford, England. Each dye is assigned to a C.I. generic name determined by its application and color. The 15 Color Index different application classes are listed in Table 2.1

Table 2.1 Color index application classes

Application Class	Characteristics
Acid dyes	Highly water-soluble due to the presence of sulphonic acid groups. From ionic interactions between the protonated functionalities of the fibers ($-NH_3^+$) and the negative charge of the dyes. Also Van-der-Waals, dipolar and hydrogen bonds are formed. The most common structures are azo, anthraquinone and triarylmethane.
Reactive dyes	From covalent bonds with –OH, -NH or –SH group in cotton, wool, silk and nylon. The problem of colored effluents associated to the use of these dyes is due to the hydrolysis of the reactive groups that occurs during the dyeing process. The most common structures are azo, metal complex azo, anthraquinone and phthalocyanine.

Table 2.1 (continued) Color index application classes

Application Class	Characteristics
Direct dyes	Their flat shape and length enables them to bind along-side cellulose fibers and maximize the Van-der-Waals, dipole and hydrogen bonds. Only 30% of the 1600 structures are still in production due to their lack of fastness during washing. The most common structures are almost always sulphonated azo dyes.
Basic dyes	Basic dyes work very well on acrylics due to the strong ionic interaction between dye function groups such as $–NR_3^+$ or $=NR_2^+$ and the negative charges in the copolymer. The most common structures are azo, diarylmethane, triarylmethane and anthraquinone.
Mordant dyes	Mordant are usually metal salts such as sodium or potassium dichromate. They act as "fixing agent" to improve the color fastness. They are used with wool, leather, silk and modified cellulose fibers. The most common structures are azo, oxazine or triarylmethane.
Disperse dyes	Non-ionic structure, with polar functionality like $–NO_2$ and –CN that improve water solubility, Van-der-Waals forces, dipole forces and the color. They are usually used with polyester. The most common structures are azo, nitro, anthraquinones or metal complex azo.
Pigment dyes	These insoluble, non-ionic compounds or salts, representing 25% of all commercial dye names, retain their crystalline or particulate structure throughout their application. The most common structures are azo or metal complex phthalocyanines.

Table 2.1 (continued) Color index application classes

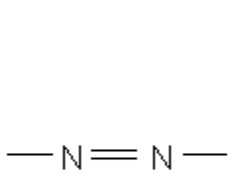
Application Class	Characteristics
Vat dyes	Vat dyes are insoluble in water, but may become solubilized by alkali reduction (sodium dithionite in the presence of sodium hydroxide. The produced *leuco* form is absorbed by the cellulose (Van-der-Waals forces) and can be oxidized back, usually with hydrogen peroxide, to its insoluble form. The most common structures are anthraquinones or indigoids.
Ingrain dyes	The term ingrain is applicable to all dyes formed in *situ*, in or on the substrate by the development, or coupling, of one or more intermediate compounds and a diazotized aromatic amine. In the color Index the sub-section designated ingrain is limited to tetra-azaporphin derivatives or precursors.
Sulphur dyes	Sulphur dyes are complex polymeric aromatics with heterocyclic S-containing rings representing about 15% of the global dye production. Dyeing with sulphur dyes (mainly on cellulose fibers) involves reduction and oxidation processes, comparable to vat dyeing.
Solvent dyes	Non-ionic dyes that are used for dyeing substrates in which they can dissolve as plastics, varnish, ink and waxes. They are not often used for textile processing. The most common structures are diazo compounds that undergo some molecular rearrangement, triarylmethane, anthraquinone and phthalocyanine.

Table 2.1 (continued) Color index application classes

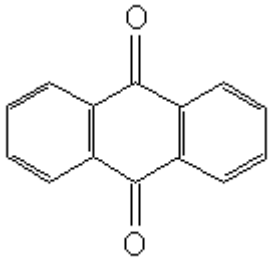
Application Class	Characteristics
Other dye classes	Food dyes are not used as textile dyes. Natural dyes use in textile—processing operations is very limited. Fluorescent brighteners mask the yellowish tint of natural fibers by absorbing ultraviolet light and weakly emitting blue light. Not listed in a separate class in the Color Index, many metal complex dyes can be found (generally chromium, copper, cobalt or nickel). The metal complex dyes are generally azo compounds.

Reference: Christie (2001)

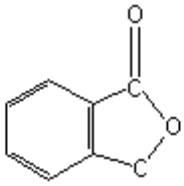
In the chemical classification method, Based on the chemical structure or chromophore, 20-30 different dye groups can be identified. Azo (monoazo, diazo, triazo, polyazo), anthraquinone, phthalocyanine and triarylmethane dyes are quantitatively the most important chromophores (fig. 2.1).



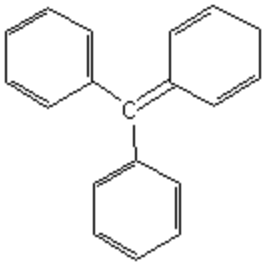
Azo



Anthraquinone



Phthalocyanine



Triarylmethane

Fig. 2.1 The most important chromophores

2.3 Dye removal techniques

Dyes are extensively used in several industries, including textile, paper, printing, leather-dyeing, cosmetic, pharmaceutical and food industries (Erkurt *et al.*, 2007). It is estimated that 10-15% of the dyes were released into the environment during manufacturing and usage (Vaidya and Datye, 1982). Most of them are toxic, mutagenic and carcinogenic. Moreover, they are unusually resistant to degradation.

A wide range of methods has been developed for the removal of synthetic dyes from waters and wastewaters to decrease their impact on the environment. The major methods for the removal of synthetic dyes involve physical and/or chemical processes, such as adsorption on inorganic or organic matrices, decolorization by photocatalysis, and/or by oxidation processes. Moreover, biological processes,

microbiological or enzymatic decomposition, have received great attention in recent years due to its efficient application microbiological or enzymatic decomposition (Hao et al., 2000). In the following chapters an overview of the most important techniques is presented.

2.3.1 Adsorption

The use of any adsorbent, whether ion-exchanger, activated carbon or high-surface-area inorganic material, for removing species from a liquid stream depends on the equilibrium between the adsorebed and the free species. Dye effluents are multicomponent mixtures with different absorption degrees and concentrations. In same cases weaker bounds are formed with the adsorbent and some material can be released back into the stream (Southern, 1995).

The range of adsorbents described in the literature for this application covers the range of activated carbons, high-surface-area inorganic materials, synthetic ion-exchange resins and cellulose-based adsorbents such as chitin (poly-N-acetylglucosamine), synthetic cellulose and other fiber-based bioadsorbents. Standard ion exchange systems have not been widely used for treatment of dye effluents due to the high cost of organic solvents to regenerate the ion-exchanger, and due to the extremely large inorganic load of the effluent (Southern, 1995 and Robinson *et al*, 2001a). Activated carbon is reasonably effective at removing many different dyes from aqueous streams (Robinson *et al*, 2001a).

However, the effective cost of the high-temperature regeneration process, including the replacement cost and the waste sludge yield, makes their regeneration unattractive to the small companies (Faria *et al*. 2004). Also, Forgacs *et al.* (2004) discussed that the adsorption processes are generally not selective, the other components of the wastewater can also be adsorbed by the support and the competition among the adsorbates can influence the dye binding capacity of supports in an unpredictable manner. Moreover, an adsorption process removes the synthetic dyes from wastewater by concentrating them on the surface retaining their structure practically unchanged. When the support is to be regenerated, the fate of the resulting concentrated solution of dyes presents a problem that is not satisfactorily solved. Even the mineralization of dyes on the surface of support cannot be achieved. Large-scale applications based on the adsorption process have to take into consideration the problems discussed above.

2.3.2 Photocatalytic

The photocatalytic or photochemical degradation processes are gaining importance in the area of wastewater treatment, since these processes result in complete mineralization with operation at mild conditions of temperature and pressure. The photo-activated chemical reactions are characterized by a free radical mechanism initiated by the interaction of photons of a proper energy level with the molecules of chemical species present in the solution, with or without the presence of the catalyst (Gogate and Pandit, 2004). The radicals can be easily produced using UV radiation. UV light has been tested in combination with H_2O_2, TiO_2, Ferton reagent, O_3 and other solid catalysts such as for the decolorization of dye solutions (Hao *et al.*, 2000). While the UV/ H_2O_2 process appeared too slow, costly and little effective for potential full-scale application, the combination UV/TiO_2 seems more promising. With UV//TiO_2 treatment, a also highly mineralized (Forgacs *et al.*, 2004). Because UV penetration in dye solution is limited due to the highly colored nature of the effluents, the best use of UV technology is a post-treatment after ozonation (Vanderviere *et al*, 1998).

2.3.3 Ozonation

Ozone is a very powerful and rapid oxidizing agent that can react with most species containing mulitiple bonds (such as C=C, C=N, N=N, etc.) and with simple oxidizable ions such as S^{2-}, to form oxyanions such as SO_3^{2-} and SO_4^{2-} (Gogate and Pandit, 2004). Ozone rapidly decolorizes water-soluble dyes but with non-soluble dyes (Vat dyes and disperse dyes) react much slower. Furthermore, textile-processing wastewater usually contains other refractory constituents that will react with ozone, thereby increasing its demand (Muthukumar *et al*. 2005). After ozone treatment seems logical the use of biological methods for reaching a complete mineralization (Krull *et al*., 1998). A major limitation of the ozonation process is the relatively high cost of ozone generation process coupled with its very short half-life (Gogate and Pandit, 2004).

2.3.4 Microbiological decomposition

Microbial decolourization being cost-effective is receiving much attention for remove of synthetic dyes from industrial effluents (Stolz, 2001). The process is relatively inexpensive, the running costs are low and the end products of complete mineralization are not toxic. Large number of species has been tested for decoloration and mineralization of various dyes (Forgacs, 2004). In particular fungus has proved to be a suitable organism for the treatment of textile effluent and dye removal. The fungalmycelia have an additive advantage over single cell organisms by solubilising the insoluble substrates by producing extracellular enzymes. Due to an increased cell-to-surface ratio, fungi have a greater physical and enzymatic contact with the environment. The extra-cellular nature of the fungal enzymes is also advantageous in tolerating high concentrations of the toxicants. Unfortunately, the majority of these compounds are chemically stable and resistant to microbiological attack. The isolation of new strains or the adaptation of existing ones to the decomposition of dyes will probably increase the efficacy of bioremediation of dyes in the near future (Kaushik and Malik, 2009).

2.3.5 Enzymatic decomposition

The character of enzymes and enzyme systems in microorganisms that are suitable for the decomposition of dyes has been extensively investigated. Effort has been devoted to the separation, isolation and testing of these enzymes. Exact knowledge of the enzymatic processes governing the decomposition of dyes is important in the environmental protection both from theoretical and practical points of view. Also, the employment of enzyme preparations shows considerable benefits over the direct use of microorganisms. Commercial enzyme preparations can be easily standardized, facilitating accurate dosage. The application is simple and can be rapidly modified according to the character of the dye or dyes to be removed (Kaushik and Malik, 2009).

Ligninolytic enzymes from white rot fungi, including lignin peroxidase, manganese peroxidase and laccase have been demonstrated for decolorization of synthetic dyes. In particular laccase from *Pleurotus ostreatus* seemed to increase up to 25% the degree of decolorization of individual commercial triarylmethane, anthraquinonic, and indigoid textile dyes using enzyme preparation (Abadulla *et al*., 2000). On the contrary, manganese peroxidase was reported as the enzyme involved

in dye decolorization by *Phanerochaete chrysosporium* (Chagas and Durrant, 2001) and lignin peroxidase for *Bjerkandra adusta* (Robinson *et al*., 2001b)

2.4 White rot fungi

White rot fungi are basidiomycetes, which cause white rot decay, are able to degrade lignin in wood. Lignin forms a matrix surrounding the cellulose in woody cell walls, which protects the hemicellulose and cellulose, together this is called holocellulose, from microbial depolymerization. White-rot fungi are the only known organisms that can completely break down the lignin to carbon dioxide and water. However, lignin cannot be degraded as a sole source of carbon and energy (Leatham, 1986). Degradation of lignin by white rot fungi enables them to gain access to the holocellulose, which is their actual carbon and energy source. Presumably, this is the real purpose for lignin degradation. This degradability of white rot fungi is due to the strong oxidative activity of their ligninolytic enzymes consist of the three kinds of extracellular phenoloxidases, namely, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). The ligninolytic system of white-rot fungi is not homogenous. Different white-rot fungi have been shown to possess one or more enzymes (Arora and Sharma, 2010). The same unique non-specific mechanisms allow them are directly involved not only in the degradation of lignin in their natural lignocellulosic substrates but also in the degradation of various xenobiotic compounds (Field *et al*., 1993; Pointing, 2001) including dyes (Robinson, *et al*., 2001a). Therefore, white rot fungi and their enzymes are thought to be useful not only in some industrial processes like biopulping and biobreaching but also in bioremediation.

Marco-Urrea et al. (2009) studied the potential of four white rot fungi (*Trametes versicolor*, *Ganoderma lucidum*, *Irpex lacteus* and *Phanerochaete chrysosporium*) to degrade selected pharmaceuticals (10 mg L^{-1}) including ibuprofen (IBU), clofibric acid (CLOFI) and carbamazepine (CARBA). Whereas IBU was extensively degraded by all the fungi tested, *T. versicolor* was the only strain able to degrade either CLOFI (~91%) and CARBA (~58%), although the latter was also degraded by *G. lucidum* (~47%). Thus, the results show that white rot fungi, and more especially T. versicolor, are suitable candidates to apply to the clean-up of waters contaminated with CLOFI, IBU and CARBA.

Revankar and Lele (2007) studied the decolorization of recalcitrant dyes by *Ganoderma* sp. WR-1 which was isolated from bark of dead tree. They found that

maximum decolorization (96% of 100 ppm amaranth) was achieved in 8 h with optimized medium containing 2% starch and 0.125% yeast extract.

Eichlerova *et al*. (2007) found that *B. adusta* strain CCBAS 232 was able to decolorize a number of chemically different synthetic dyes (Orange G, Amaranth, Remazol Brilliant Blue R, Cu-phthalocyanine and Poly R-478) at relatively high concentrations of 2-4 gL^{-1} both on solid and in liquid medium.

2.5 Laccases

Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) are a large group of multicopper oxidases. An enzyme of this group was first discovered by Yoshida in the exudates of the Japanese lacquer tree *Rhus verniczfera*. (Yoshida, 1883). They also occured in fungi (Mayer and Staples 2002), bacteria (Givaudan *et al*., 1993; Claus, 2003) and insects (Kramer, 2001). They catalyse oxidation of a surprisingly wide variety of organic and inorganic compounds, including diphenols, polyphenols, substituted phenols, diamines and aromatic amines, with the concurrent reduction of one molecule of oxygen to two water (Thurston, 1994). Because of their oxidative capabilities, these enzymes played an important role in many industrial and environment fields such as textile dye bleaching, pulp bleaching, effluent detoxification, synthesis of polymers, biosensors, and bioremediation (Mayer and Staples, 2002)

2.5.1 Laccase structure and mechanism of action

Laccases is a monomeric, dimeric or tetrameric glycoprotein. The molecular weight ranging from 60–80 kDa, with usually comprise 520–550 amino acids (Thurston, 1994).. They contain 4 atoms of copper which are distributed into three sites (T1, T2, T3; Fig. 2). The 4 Cu atoms differ from each other in their characteristic electronic paramagnetic resonance (EPR). The T1 site contains the type 1 blue copper, which coordinates a cysteine, and is responsible for the blue color of the enzyme. Due to its high redox potential, type 1 copper is the site where substrate oxidation takes place. The T2 site contains a type 2 copper, which shows no absorption in the visible spectrum and reveals paramagnetic properties in EPR studies. In the T3 site, a binuclear center spectroscopically characterized by an electron adsorption at 330 nm (oxidized form) and by the absence of an EPR signal as the result of the anti-ferromagnetic coupling of the copper pair. (Claus, 2004). The reactions catalysed by

laccases proceed by the T1 copper is reduced by the substrate being oxidized and the extracted electrons are transferred to the T2/T3 site, where molecular oxygen is reduced to water (Mougin *et al*., 2003) (Fig. 3)

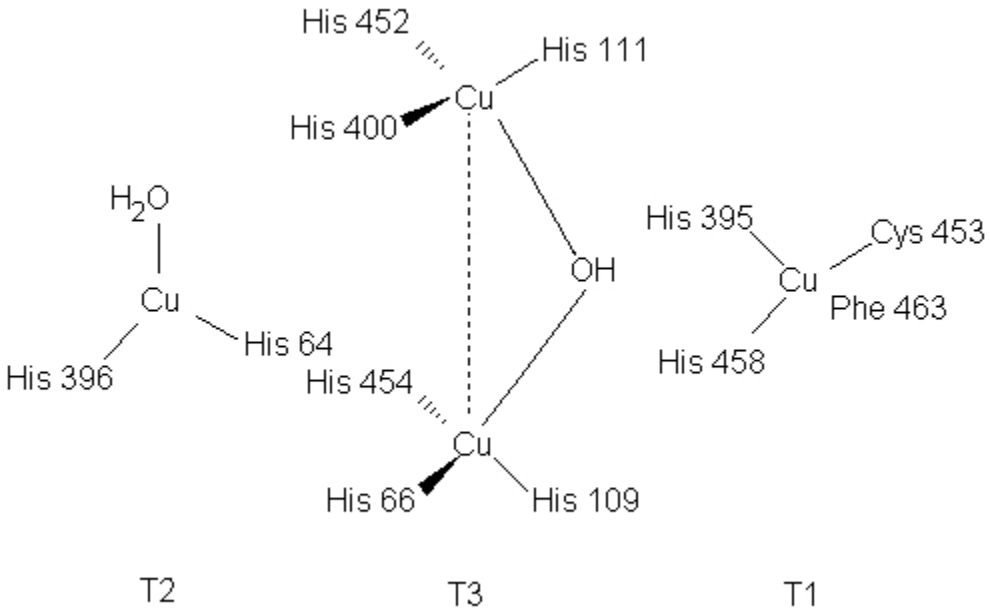


Fig. 2.2 Model of the catalytic cluster of the laccase from *Trametes versicolor* consist with four copper atoms. (Riva, 2006)

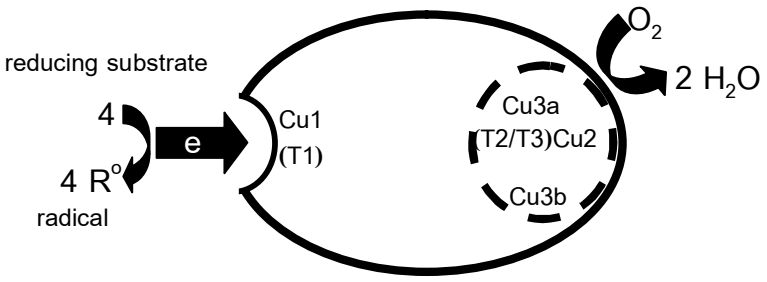


Fig. 2.3 Schematic mechanism of action of fungal laccases (Mougin *et al*., 2003)

2.5.2 Laccase properties

Current knowledge about the structure and physico-chemical properties of fungal laccase proteins is based on the study of purified proteins. Table 2 shows the examples of fungal laccases that have been purified and been more or less characterized.

Table 2.2 Some characteristics of purified fungal laccases

Fungal	MW (kDa)	pI	Temp. (^{o}C)	pH	Km (mM)	Reference
Agaricus blazei	66	4.0		2.0	63	Ullrich *et al. (*2005)
Cantharellus cibarius			50	4.0		Ng and Wang (2004)
Chalara paradoxa	67			4.5	770	Robles *et al.* (2002)
Coprinus cinereus	58	4.0	60–70	4.0	26	Schneider *et al*. (1999)
Coprinus friesii	60	3.5		5.0	41	Heinzkill *et al.* (1998)
Coriolus hirsutus	78	4.2	45		8	Lee and Shin (1999)
Cyathus stercoreus	70	3.5		4.8		Sethuraman *et al.* (1999)
Daedalea quercina	69	3.0	70, 55	2.0	38	Baldrian (2004)
Hericium echinaceum	63		50	5.0		Wang and Ng (2004c)
Lentinula edodes Lcc1	72	3.0	40	4.0	108	Nagai *et al.* (2002)
Phellinus ribis	152			5.0	207	Min *et al*. (2001)
Polyporus pinsitus	66			3.0	22	Heinzkill *et al.* (1998)
Volvariella volvacea	58	3.7	45	3.0	30	Chen *et al.* (2004)

Baldrian (2006) concluded about properties of fungal laccases which have been studied. The summarize showed that typical fungal laccase is a protein of approximately 60–70 kDa with acidic isoelectric point around pH 4.0 (Table 2.3).

Table 2.3 Properties of fungal laccases

Properties	n	Median	Q25	Q75	Min	Max
MW (Da)	103	66000	61000	71000	43000	383000
pI	67	3.9	3.5	4.2	2.6	6.9
Temperature optimum (^{o}C)	39	55	50	70	25	80
pH optimum						
ABTS	49	3.0	2.5	4.0	2.0	5.0
2,6-Dimethoxyphenol	36	4.0	3.0	5.5	3.0	8.0
Guaiacol	24	4.5	4.0	6.0	3.0	7.0
Syringaldazine	31	6.0	4.7	6.0	3.5	7.0
KM (mM)						
ABTS	36	39	18	100	4	770
2,6-Dimethoxyphenol	30	405	100	880	26	14720
Guaiacol	23	420	121	1600	4	30000
Syringaldazine	21	36	11	131	3	4307
kcat (s^{-1})						
ABTS	12	24050	5220	41460	198	350000
2,6-Dimethoxyphenol	12	3680	815	6000	100	360000
Guaiacol	10	295	115	3960	90	10800
Syringaldazine	4	21500	18400	25500	16800	28000

n, number of observations; Q25, lower quartile; Q75, upper quartile.

Reference: Baldrian (2006)

2.5.3 Laccase in dye decolorization

It has been known for two decades that wild laccases catalyze the direct oxidation of phenols and amines such as chlorophenols and dyes to produce oligomers (Mougin *et al*, 2003). The capability of laccases to act on chromophore compounds such as azo, tiarylmethane, anthraquinonic and indigoid dyes leads to the suggestion that they can be applied in industrial decolorization processes (Kirby *et al*, 2000). Recently, a number of studies have focused on the dye degradation capacities of fungal laccases and shown that these laccases are indeed major dye-decolorising enzymes in many white rot fungi (Svobodova, 2008).

Baldrian (2004) reported that purified laccase from *Daedalea quercina* was able to decolorize the synthetic dyes Chicago sky blue, poly B-411, remazol brilliant blue R, trypan blue and reactive blue 2. While only 9% decolorization of reactive black 5 was detected after a 24-h incubation, the enzyme decolorized 74% of remazol brilliant blue R, 65% of Chicago sky blue 6B, 59% of reactive blue 2, 59% of poly B-411 and 42% of trypan blue at the same time point. Chicago sky blue 6B and reactive blue 2 were degraded more than 50% within 6 h.

Erkurt *et al.* (2007) studied the decolorization of Remazol Brillant Blue Royal (RBBR) and Drimaren Blue CL-BR (DB) by three white rot fungi named as *Pleurotus ostreatus*, *Coriolus versicolor* and *Funalia trogii*. The result showed that *Funalia trogii* was found to be the most effective fungus in decolorization of the tested dyestuffs, resulting in almost complete color removal at the end of 48 h of incubation period. Fungus from which the highest laccase activity was obtained was also found to decolorize the dyes most efficiently. In addition to this, compared with control groups, laccase activity was increased by dye addition. Therefore, it is concluded that laccase exists in decolorization mechanism. Results of activity staining showed that laccase is the only enzyme that is responsible for decolorization of DB and RBBR. Furthermore, it was observed that it is not required to use hydrogen peroxide or mediator for decolorization process of RBBR and DB by *Pleurotus ostreatus*, *Coriolus versicolor* and *Funalia trogii*.

Li *et al*. (2009) found that the crude culture from *R. lignosus* W1 had the ability to decolorise both the anthraquinone dye RBBR and the triphenylmethane dye MG, with 39mg L^{-1} MG being removed within 2 h and 160 mg L^{-1} RBBR within 1 h

at 60 ◦C and pH 4.5. Thus, this laccase would seem to be a good candidate for application in dye decolorization and textile effluent biotreatment.

Murugesan *et al.* (2007) studied the lignolytic enzyme production from *Ganoderma lucidum* by solid state fermentation (SSF) of wheat bran (WB), a natural lignocellulosic substrate. They found that *Ganoderma lucidum* produced laccase as the dominant lignolytic enzyme. Also, Crude enzyme shows excellent decolorization activity to anthraquinone dye Remazol Brilliant Blue R (RBBR) without redox mediator whereas diazo dye Remazol Black-5 (RB-5) requires a redox mediator. Polyacrylamide gel electrophoresis (PAGE) of crude enzyme confirms that the laccase enzyme was the major enzyme involved in decolorization of either dyes. In the presence of 1mM *N*-Hydroxybenzotriazole (HBT) maximum decolorization of RB-5 (50 mg l^{-1}) was achieved within 1 and 2 h by 62.0 and 77.4%, respectively by the crude laccase (25Uml^{-1}). In the case of RBBR, the crude enzyme (25Uml^{-1}) showed 40% decolorization during 2 h incubation without HBT. However, the addition of 1m MHBT enhanced the decolorization nearly to 2.3-fold and the maximum decolorization of 92.4% were observed within 2 h.

CHAPTER III

EXPERIMENTAL

3.1 Materials and Chemicals

2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, ABTS (Sigma, Canada)

Acetic acid (Merck Ag Darmstadt, Germany)

Acrylamind (Plusone Pharmacia Biotech, Sweden)

Bis-acrylamide (Promega, USA)

Bovine serum albumin (Sigma, USA)

Bromophenol Blue (USB, USA)

Coomassie Brilliant Blue G-250 (USB, USA)

Di- Potassiumhydrogen phosphate (Merck Ag Darmstadt, Germany)

Ethylenediaminetetraacetic acid, EDTA (Sigma, USA)

Ethanol (Merck Ag Darmstadt, Germany)

Ethyl acetate (Ajax Finechem, New Zealand)

Hydrochloric acid (J.T. Baker, USA)

Potassium dihydrogen phosphate (Merck Ag Darmstadt, Germany)

Sodium azide (Merck Ag Darmstadt, Germany)

Sodium chloride (Merck Ag Darmstadt, Germany)

Sodium hydroxide (Merck Ag Darmstadt, Germany)

Standard Molecular Weight Marker (Sigma, U.S.A)

Tetramethylethylenediamine, TEMED (Plusone Pharmacia Biotech, Sweden)

Tris (USB, U.S.A)

3.2 Equipment

Autopipette (Pipetman, Gilson, France)

Dialysis bag (Snake Skin Dialysis Tubing, Pierce, U.S.A)

Electrophoresis unit (Hoefer mini VE, Amersham Pharmacia Biotech, Sweden)

Freeze dryer (Labconco, U.S.A)

High Speed Refrigerated Centrifuge (Kubota 6500, Japan)formance Liquid

High Performance Liquid Chromatography (Spectra system/spectra series, Fortune Scientific)
Hot plate stirrer (HL instrument, Thailand)
Laminar Flow (Safety Lab, Asian Chemical and Engineering Co., Ltd., Thailand)
LC/MS/MS mass spectrometry
Microcentrifuge (Tomy MTX-150)
Orbital Shaker (OS-10 Biosan, Latvia)
pH meter (Mettler Toledo, U.S.A)
Pipette tips (Bioline, U.S.A)
Spectrophotometer (Synergy HT Biotek, USA)
Speed vacuum centrifuge (Heto-Holten, Denmark)
Vortex mixer (Vortex-Genie2, Scientific Industries, U.S.A)
Water Bath (NTT-1200 Tokyo kikakikai, Japan)
384-well microtter plate (greiner, USA)

3.3 Organism

The 30 strains of white rot fungi obtained from the culture collection of the Microbiology Department, Faculty of Science, Chulalongkorn University, Thailand. The fungus was grown on 2% (w/v) malt extract agar at 25oC for 7 days and maintained at 4^{o}C until used. The fungus was subcultured every 2 or 3 months.

3.4 Selection of laccase-producing fungal stain

All fungal strains were activated on 2% (w/v) malt extract agar for one week. The ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt)-agar medium (Pointing, 1999) were used for selection of laccase-producing stains. Each plate was inoculated with one agar plug (8 mm diameter) obtained from the edge of actively growing mycelium of each test fungus and incubated for 7 days at room temperature. A positive reaction for laccase producing strains was indicated by the green zone around the colony. The width of green zone was considered to be directly related to the amount of extracellular laccase produced. The strain having the highest ratio of green zone diameter (cm) to colony diameter (cm) was selected.

3.5 Effect of various carbon and nitrogen sources on the laccase production

3.5.1 Effect of various carbon sources on the laccase production

The selected fungal strain was precultured on 2% (w/v) malt extract agar for one week. Laccase production was performed in 250 ml flasks containing 100 ml of LME basal medium (LBM) that was added 1% of one of the investigated carbon sources (rice chaff, baggasse, rice bran, rice straw and sawdust), incubated at 25^{o}C, for 20 days, on a rotary shaker at speed 120 rpm. The initial pH of the medium was adjusted to 5.5. Every 2 days of cultivation, the mycelium was removed from liquid culture by filtration by using a whatman no.1 and filtrate were used to estimate laccase activity. The best carbon sources for production of laccase was selected. Also, the effect of carbon source concentration was estimated using concentrations of 0.5, 1.0 and 2.0% in the same condition that described above. All experiments were done in triplicate.

3.5.2 Effect of various nitrogen sources on the laccase production

For the study of the effect of the inorganic nitrogen sources, di-amonium tatrate ($C_4H_{12}N_2O_6$) in the LBM with the best carbon source for Laccase production was replaced by one of the inorganic nitrogen sources (ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), ammonium hydrogen phosphate ($(NH_4)_2PO_4$), ammonium persulfate $(NH_4)_2S_2O_8$) , in a nitrogen concentration of 0.5 gL^{-1}). While, the study of the effect of the organic nitrogen sources, without yeast extract in the LBM with the best carbon source for laccase production was replaced by one of the organic nitrogen sources (peptone, urea, soy bean and corn steep), in a nitrogen concentration of 0.01 gL^{-1}). The effect of different concentrations of the best nitrogen sources on the laccase production was studied in a medium with 0.25, 0.5 and 1.0 gL^{-1} of inorganic nitrogen, and 0.005, 0.01, and 0.02 gL^{-1} of organic nitrogen sources. All experiments were done in triplicate.

3.6 Enzyme assays

Laccase (EC 1.10.3.2) activity was measured at 30 ^{o}C using 1mM ABTS as the substrate. The assay mixture (1 ml) contained 880 ml of 100 mM sodium acetate buffer (pH 5.0), 100ml of ABTS stock (final concentration 1 mM), and 20ml of appropriately diluted crude enzyme. The absorbance increase of assay mixture was monitored at 420 nm ($E420$ = 36.0mM−1 cm−1) in a spectrophotometer (Murugesan et. al., 2007). One unit was defined as the amount of enzyme that oxidized 1 mmol of ABTS per minute.

3.7 Protein amount determination

Protein contents were determined by Bradford's procedure as described by Bollag (1996). Bovine serum albumin (BSA) was used as standard, and a standard curve derived from the average of three determinations of a 5-30 μg/ml BSA concentration series was created for every determination. The working procedure began with 50 μl of samples which were two-fold diluted with deionized water in a 96 well flat-bottom plate. The series of standard BSA solutions were then placed into the appropriate wells. After 50 μl of Bradford's reagent was added to each analyzed well, the plate was shaken with round orbit plate shaker (Biosan, OS-10, Latvia) at 190 rpm for 1 min and then left for 15 minutes. Finally, the plate was read at 595 nm using an ELISA plate reader (Biotek Synergy HT, Biotek instrument, USA). The OD of the obtained samples was used to calculate the protein concentration using the linear equation computed from the standard curve. During the column chromatographic separations, the elution profiles of proteins were determined by measuring the absorbance at 280 nm.

3.8 Laccase purification

Laccase from the fungal was purified from basal medium with the best carbon sources and nitrogen sources. The culture liquid from 12 days was separated from mycelia by centrifuged at 10,000*g* for 30 min (Beckman Coulter, USA). The supernatant was then fractionated with ammonium sulphate at 80% saturation and the supernatant containing the laccase activity was centrifuged at 10,000*g* for 30 min and dialysed (membrane molecular weight cut off 3,500 Da) at 4^{o}C. The crude enzyme was loaded onto a Q-sepharose fast flow column (1.6 cm×15 cm) previously equilibrated with 50mM sodium acetate, pH 5.0. The column washed with the sodium phosphate buffer until the A_{280} reading was less than 0.02. Bound protein was eluted with a linear salt gradient (0–1.0 M NaCl) at 2.0 ml/min., the eluted fractions were assayed for laccase activity and the A_{280} monitored. Active fractions were pooled and dialysed against the running buffer. The sample was loaded into a Sephadex G-75 column (1.6 cm × 60 cm). The column was equilibrated and eluted with 0.1M NaCl 50 mM sodium acetate buffer, pH 5.0. The sample was injected in each run and chromatographed at a flow rate of 1ml/min and collected in fractions of 5 ml. The fractions were assayed for laccase activity and the active fractions were pooled.

3.9 Determination of enzyme purity by native-PAGE and laccase activity staining

The enzyme from each step of purification was analyzed by its native protein pattern and its purity according to the method of Bollag *et al*. (1996). Electrophoresis conditions, protein and activity staining are described below.

3.9.1 Non-denaturating gel electrophoresis

Native PAGE was performed with 10% and 5% (w/v) acrylamide separating and stacking gels, respectively, with 100 mM Tris-glycine (pH 8.3) as the electrode buffer. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Hoefer model miniVE, Pharmacia Biotech, UK). After electrophoresis, proteins in the gel were visualized by Coomassie blue R-250 (Sigma) staining and activity staining.

3.9.2 Coomassie blue staining

Native (section 3.9.1) and reducing SDS-PAGE (section 3.10) gels were stained by immersion in 0.1% (w/v) Coomassie blue R-250 in1 0% (v/v) acetic acid / 45% (v/v) methanol for 45 min. Destaining was performed by immersing the gel in 10% (v/v) acetic acid / 45% (v/v) methanol, with several changes of this destaining solution until the background was clear.

3.9.3 Staining for laccase activity

After native-PAGE resolution the gel was directly immersed in sodium acetate buffer (pH 5.0) with 5 mM ABTS at room temperature.

3.10 Molecular weight determination by SDS PAGE

Discontinuous reducing 0.1% (w/v) SDS-PAGE was performed according to the procedure of Laemmli (1970) using 15% and 5% (w/v) acrylamide resolving and stacking gels, respectively. Samples were treated with reducing (2-mercaptoethanol containing) sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. High and low molecular weight standards were coresolved on each gel and used to determine the subunit molecular weight of the enriched lipase enzyme. After electrophoresis, proteins in the gel were visualized by staining with Coomassie blue R-250 as described in section 3.9.2

3.11 Laccase characterization

3.11.1 pH stability of laccase activity of isolated WR77

The pH stability for laccase was determined using 20 mM Glycine-HCl buffer (pH 2.0–4.0), 20 mM Sodium acetate buffer (pH 4.0–6.0), 20 mM phosphate buffer (pH 6.0–8.0), 20 mM Tris–HCl buffer (pH 8.0–10.0) and 20 mM Glycine-NaOH buffer (pH 10.0–12.0). The above laccase-buffer mixtures were left for 45-90 min at room temperature and then adjusted back to 100 mM sodium acetate buffer (pH 5.0) and assayed for laccase activity as in section 3.6. The control incubation was set at 100% activity and the activity of the samples from the different pH buffers were expressed relative to that of the control (100% activity).

3.11.2 Temperature stability of laccase activity of isolated WR77

The effect of temperature on laccase activity was determined by incubating the laccase fraction (post-superdex-75) in 100 mM sodium acetate buffer (pH 5.0) at various temperatures from -20 to 90 ^{o}C for 30 min. The temperature stability of the laccase after different preincubation times at 40, 50, 60 and 70^{o}C in 100 mM sodium acetate buffer (pH 5.0). and then assaying the residual lipase activity as described above.

3.11.3 Effect of various cation ions and reagents of laccase activity of isolated WR77

The effect of various metal ions and reagents on laccase activity was studied by incubating the enzyme in presence of 0.5,1.0, 5.0 and 10.0 mM of metal ions (Ca^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Hg^{2+}) and EDTA for 30 min. Then the enzyme activitywas tested in three replicates as described above. Relative laccase activity (%) was calculated taking that without metal ions/additives as 100%.

3.11.4 Enzyme kinetics of laccase activity of isolated WR77

Kinetic constants for ABTS was assayed at concentrations between 0.025 and 2.0 mM in 100 mM sodium acetate buffer (pH 5.0) . The rates of substrate oxidation were determined by measuring the absorbance increase at the respective wavelengths. Michaelis constants (Km) were calculated from Lineweaver-Burk plots.

3.12 Identification of white rot fungus

Identification of the selected strain was determined by using morphological characteristics and molecular characteristics. Morphological identification was done by examination of macroscopical and microscopical structure. Molecular identification, sequences of internal transcribe spacer (ITS) regions of rDNA from endophytic fungi was prepared as below. Genomic DNA was prepared from fresh mycelial cultures of the fungi and extracted with cetyltrimethylammonium bromide (CTAB) as described in Zhou *et al*. (1999). PCR amplification of the internal transcribed spacer (ITS) was performed in a total volume of 35 ul which comprised approx. 100 ng genomic DNA, 1×PCR master Mix (fermentas, Califonia, USA), the primer ITS1f and ITS4. The amplification was performed in a thermocycler with 94^{o}C

for 5 min, followed by 38 cycles of 94^{o}C for 1 min, 51^{o}C for 1 min and 72^{o}C for 1 min, with final extension of 72^{o}C for 5 min. The sequences of internal transcribe spacer (ITS) regions was sent for identification using the same primer as for amplification. Product was purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and sequenced externally by Macrogen (Seoul, Korea) using the same primer for amplification. The complete sequences were taken to compare with database of Genbank at NCBI and BLAST program at NCBI server for identification.

3.13 Dyes decolorization

3.13.1 Decolorization of synthetic dyes by isolated WR77

White rot fungas WR77 was precultured on 2% (w/v) malt extract agar for one week. The decolourization of Ambifix Blue H3R, Ambifix Yellow H3R, Ambifix Red HE3B and Malachite Green (MG) in liquid cultures was evaluated using 250-ml Erlenmeyer flasks containing 100 ml of the medium, with the initial concentration of each dye was 200 mg/L. Flasks were inoculated with three plugs (6 mm diameter) of pregrown mycelium and were incubated at 25°C for twenty days with rotary agitation (120 rpm). Each sample was taken and the absorbance of the residual dye was measured, by measuring the absorbance at 585 nm for Ambifix Blue H3R, 415 nm for Ambifix Yellow H3R, 535 nm for Ambifix Red HE3B and 617 nm for MG. The efficiency of dye removal was expressed as the percentration to initial dye concentration.

3.13.2 Decolorization of synthetic dyes by the crude enzymes

Reaction mixtures (200 µL) containing culture filtrate (final laccase activity 5 U mL^{-1}) and dye (200 mgL^{-1} Ambifix Blue H3R, Ambifix Yellow H3R, Ambifix Red HE3B or MG) were tested in 50 mM sodium acetate buffer, pH 5.0. Changes in absorbance were detected, using a Spectrophotometer (Synergy HT Biotek, USA).

CHAPTER IV

RESULT AND DISCUSSION

4.1 Selection of laccase-producing fungal stain

To select the laccase-producing strain, the 30 strains of write rot fungi were cultured on ABTS-agar medium. After 7 days of incubation, the colony diameter and the width of green zone around the colony were measured. Also, the ratio of green zone diameter to colony diameter was determined (Table 4.1). This colourless agar medium turns green due to the oxidation of ABTS to ABTS-azine in the presence of laccase (Niku-Paavola *et al*., 1990). The results revealed that all white rot isolates were found to secrete laccase. However, the white rot fungi WR77 showed the highest of the ratio of green zone diameter to colony diameter therefore this strain had the best for laccase production and provided for further study (Fig. 4.1).

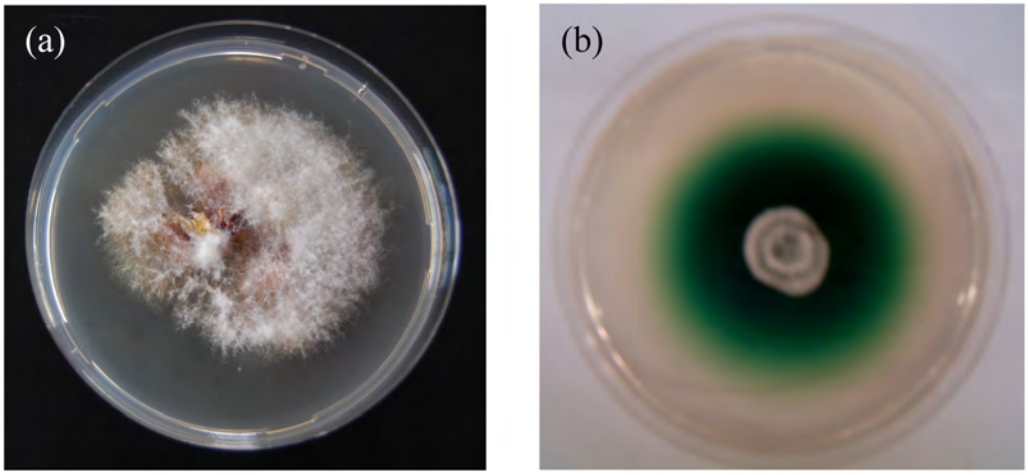


Fig. 4.1 Growth of white rot fungus WR77 on 2% (w/v) malt extract agar (a) and ABTS-agar medium (b).

Table 4.1 Screening of laccase producing strains on ABTS agar medium

White rot fungi No.	Diameter of colony (cm)	Diameter of green zone (cm)	Ratio of green zone diameter to colony diameter
WR5	1.43 ± 0.03	4.53 ± 0.03	3.16
WR6	2.67 ± 0.04	6.07 ± 0.05	2.28
WR9	5.45 ± 0.07	7.23 ± 0.12	1.32
WR12	4.03 ± 0.00	6.07 ± 0.05	1.50
WR17	8.50 ± 0.00	8.07 ± 0.03	0.95
WR19	8.50 ± 0.03	8.50 ± 0.00	1.00
WR24	3.83 ± 0.03	5.57 ± 0.03	1.45
WR25	4.20 ± 0.03	6.17 ± 0.03	1.47
WR26	8.03 ± 0.08	5.13 ± 0.03	0.64
WR28	3.07 ± 0.00	6.47 ± 0.03	2.11
WR33	8.50 ± 0.00	8.50 ± 0.00	1.00
WR35	8.50 ± 0.05	8.50 ± 0.00	1.00
WR43	4.50 ± 0.10	8.50 ± 0.00	1.89
WR48	2.53 ± 0.00	6.85 ± 0.03	2.70
WR50	8.50 ± 0.03	8.50 ± 0.03	1.00
WR51	2.57 ± 0.06	5.03 ± 0.03	1.96
WR54	5.83 ± 0.07	7.23 ± 0.03	1.24
WR57	6.37 ± 0.05	8.50 ± 0.00	1.34

Means of three replicates (cm) ± S.D.

Table 4.1 (continued) Screening of laccase producing strains on ABTS agar medium

White rot fungi No.	Diameter of colony (cm)	Diameter of green zone (cm)	Ratio of green zone diameter to colony diameter
WR58	3.43 ± 0.03	8.50 ± 0.00	2.48
WR60	2.77 ± 0.03	2.07 ± 0.05	0.75
WR61	7.03 ± 0.03	6.20 ± 0.00	0.88
WR63	3.83 ± 0.00	5.93 ± 0.03	1.55
WR66	8.50 ± 0.00	5.43 ± 0.05	0.64
WR67	6.60 ± 0.07	6.63 ± 0.03	1.00
WR73	4.27 ± 0.05	7.00 ± 0.05	1.64
WR75	4.73 ± 0.05	6.47 ± 0.03	1.37
WR76	1.73 ± 0.03	5.53 ± 0.03	3.19
WR77	1.87 ± 0.03	6.27 ± 0.03	3.36
WR80	5.85 ± 0.10	3.60 ± 0.05	0.62
WR81	1.95 ± 0.03	5.77 ± 0.03	2.96

Means of three replicates (cm) ± S.D.

4.2 Effect of various carbon sources on the laccase production

4.2.1 Effect of various carbon sources on the laccase production

The effect of different carbon in the growth medium on laccase production were determined. The maximum laccase activity (approx. 0.40 U/ml) was obtained after 16-day cultivation with rice bran as carbon source. Also, enzyme activities (approx. 0.37 U/ml) was obtained after 12-day cultivation with rice chaff as carbon source, while activity levels obtained from rice straw was 0.22 U/mL after 20 days of cultivation. Sugarcane bagasse and sawdust resulted in low laccase levels (fig. 4.2).

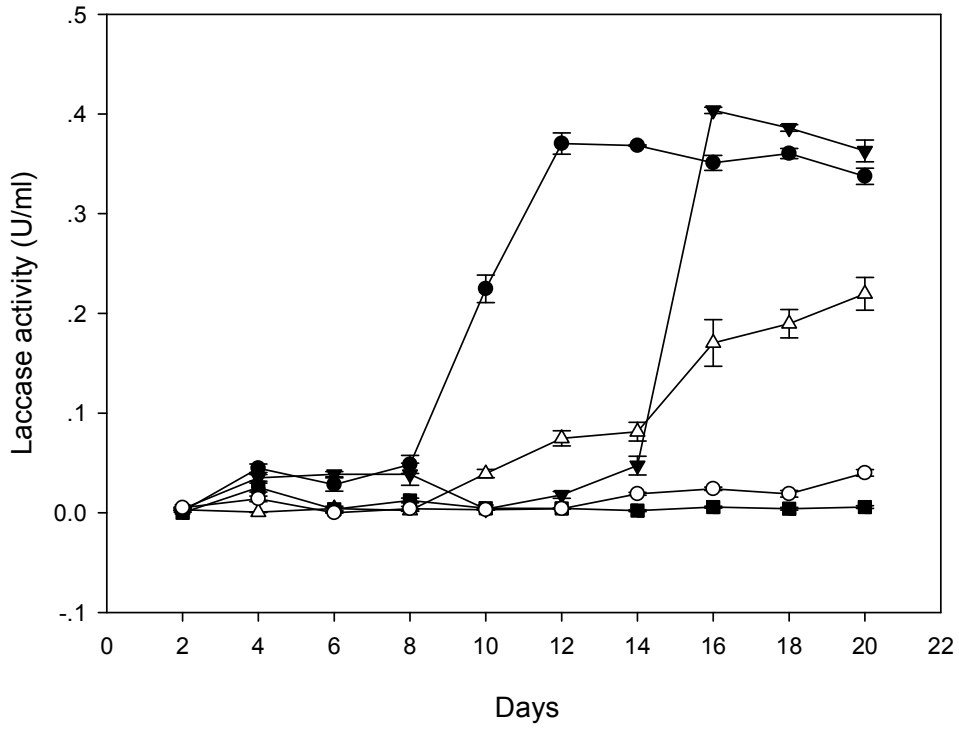


Fig. 4.2 Effects of various carbon sources on laccase production by white rot fungus WR77 when grown in the growth medium with various 1% carbon source. Means of three replicates (cm) ± S.E. chaff (●); baggasse (○); rice bran (▼); rice straw (Δ); and sawdust (■).

The result showed that when cultivated WR77 with rice bran as carbon source, laccase production rate was slower than when using rice chaff. It could be due to the fugal secrete other enzymes for hydrolysis of rice bran components such as cellulases, β-glucosidases and xylanases at early stages. Rice bran contains total carbohydrate 82% (w/w) approximately and the main composition (31%) was hemicellulose (Claye *et al*., 1996). The main component of hemicellulose was expected to be arabinoxylan as most sugars found are xylose and arabinose (Mod *et al*., 1978). Although, the main components of rice chaff are hemicelluloses, lignin (21–26%), cellulose and silicon dioxide (Yang *et al*., 2004). Therefore, laccase synthesis could be induced by lignin which is composition in rice chaff. It agrees with Moldes *et al*. (2003) research, in which grape seeds was the best support with higher content in lignin for laccase production by *Trametes hirsuta* in solid state cultures. From this result, rice chaff was selected as carbon source since laccase activity was comparable to that obtained with rice bran, but it is a cheap and easily available carbon source.

4.2.2 Effect of concentration of selected carbon sources on the laccase production

Effects of rice chaff concentration in liquid medium on laccase production is shown in Fig. 4.3. After 12 days, laccase production was 0.37 U/mL from 1.0% (w/v) rice chaff, while 0.32 and 0.21U/mL were obtained when using 2.0 and 0.5% (w/v) rice chaff, respectively. It was concluded that the optimum concentration of rice chaff was 1% (w/v). Rice chaff at 2% (w/v) might be caused the excess level of carbon and nitrogen, which directly affected on ligninolytic enzyme production. Many agricultural wastes have been investigated to use as substrates for laccase production by white rot fungi.

Stajic *et al*. (2006) report that the highest level of lacase activity in *Pleurotus eryngii* found in submerged culture with dry ground mandarine peels as the carbon source after 7 days of cultivation (999.5 U/l) and the highest level of laccase activity in *Pleurotus ostreatus,* strain No. 493 was observed under solid state fermentation (SSF) conditions of grapevine sawdust after 10 days of cultivation (2144.6±57.8 U/l).

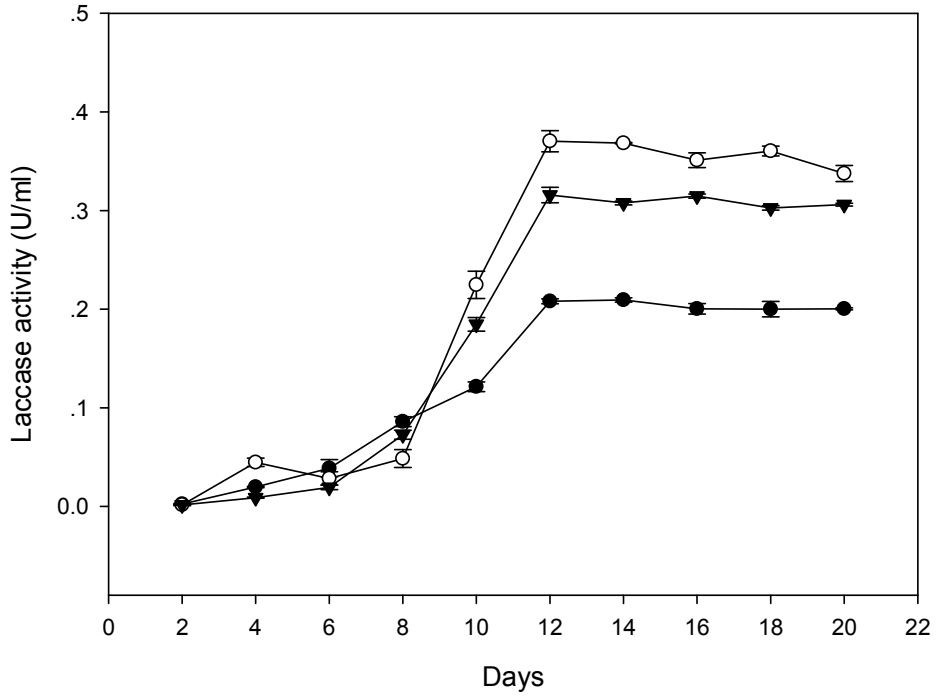


Fig.4.3 Effect of different concentrations of chaff on laccase activity of white rot fungus WR77. Means of three replicates (cm) ± S.E. 0.5% (●); 1.0% (o); and 2.0% (▼).

4.3 Effect of various nitrogen sources on the laccase production

The effect of inorganic nitrogen sources and different concentration (0.25, 0.5, 1.0 g/L) on laccase production was also studied. Among all, 0.5 g/L di-amonium tatrate gave maximum laccase production (0.37 U/ml), was found to be the best inorganic nitrogen source (Fig.4.4). Also, the effect organic nitrogen sources and various concentrations (0.005, 0.01, 0.02 g/L) for laccase production were studied. The maximum laccase activity (approx. 0.39 U/ml) was obtained with peptone as organic nitrogen sources with concentration of 0.01 g/L (Fig.4.5). Peptone has been shown to stimulate laccase production in the case of *Lentinus edodes* and *Pleurotus ostreatus* (Kaal *et al*.,1995), *Lentinus edodes* (Buswell et al.,1995) and *Phanerochaete chrysosporium* (Srinivasan et al.,1995). However, Stajic *et al*. (2006) investigated the effect of nitrogen sources on laccase production in *Pleurotus eryngii* and *Pleurotus ostreatus*, found that both *Pleurotus eryngii* and *Pleurotus ostreatus* strain no. 493, showed the highest laccase activity with $(NH_4)_2SO_4$, as a nitrogen source, with a nitrogen concentration of 20 and 30 mM, respectively.

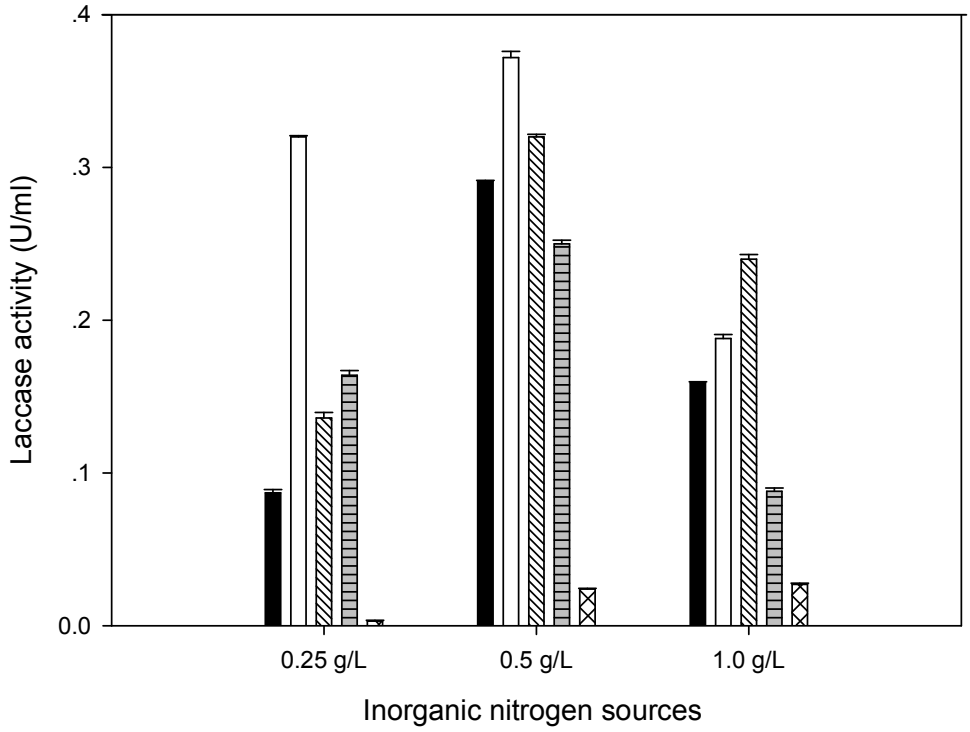


Fig. 4.4 Effects of various inorganic nitrogen sources and various concentration on laccase production by white rot fungus WR77 when grown in the growth medium. Means of three replicates (cm) ± S.E. sodium nitrate (■); di-amonium tatrate (❑); ammonium chloride (▨); ammonium hydrogen phosphate (▤); and ammonium persulphate (⊠).

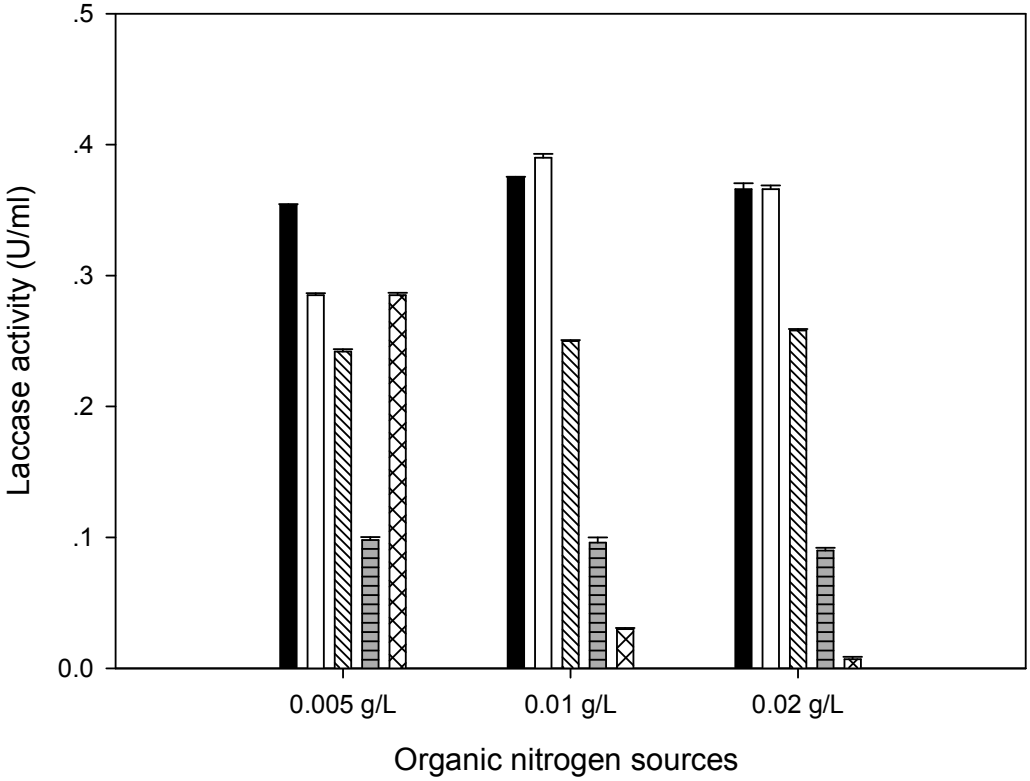


Fig.4.5 Effects of various organic nitrogen sources and various concentration on laccase production by white rot fungus WR77 when grown in the growth medium. Means of three replicates (cm) ± S.E. yeast extract (■); peptone (□);corn steep liquor (▨);soybean (▤); and urea (⊠).

4.4 Laccase purification

The culture supernatant had maximum laccase activity after 12 days of incubation. Laccase activity was purified from the culture supernatant by a Q-sapharose anion-exchange chromatography (Fig. 4.6). The gel filtration chromatography (Superdex 75), laccase activity was separated of other contaminant proteins (Fig. 4.7). The results of the purification of laccase are summarized in Table 4.2. At the end of the process, enzyme had been purified 60.09-fold, with a yield of 3.61% and a specific activity of 606.20 U/mg. The final specific activity obtained here was high compared to some fungi laccase, e.g. 32.9U/mg for *Pycnoporus sanguineus* (SCC 108) (Litthauer, 2007), 50.5 U/mg for *Daedalea quercina* (Baldrian, 2004) and 155 U/mg protein for *Lentinula edodes* (Nagai *et al.*, 2009)

Table 4.2 Purification table of laccase from WR77

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	133.20	1368.00	10.27	100.00	1.00
$(NH_4)_2SO_4$ precipitation	5.44	364.32	66.97	26.63	6.52
Q-sapharose	0.20	121.24	606.20	8.86	59.02
Sephadex-75	0.08	49.37	617.12	3.61	60.09

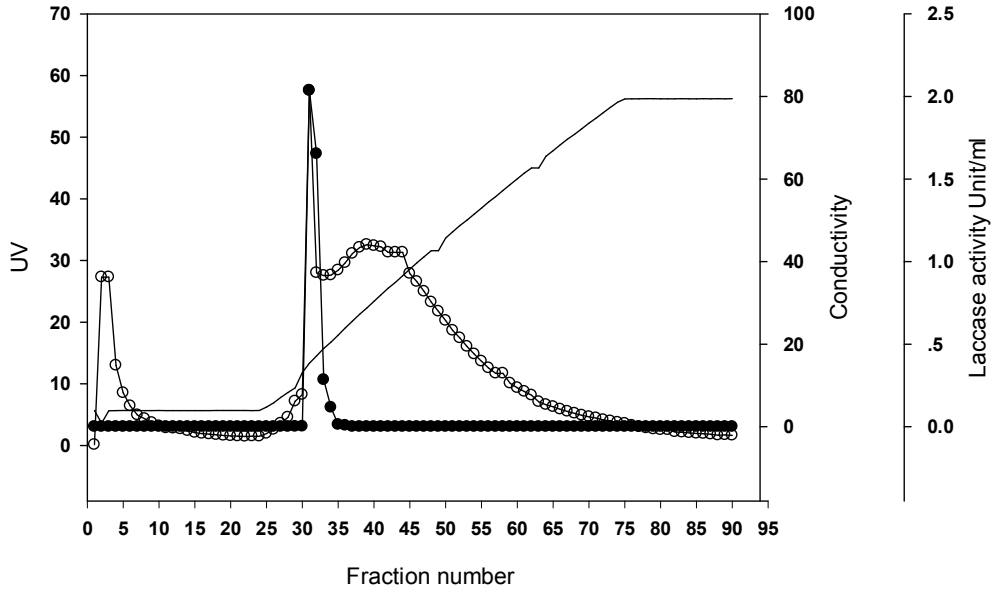


Fig.4.6 Q-sapharose anion exchange chromatography column, Absorbance at 280 nm (○); laccase activity(●), NaCl (-)

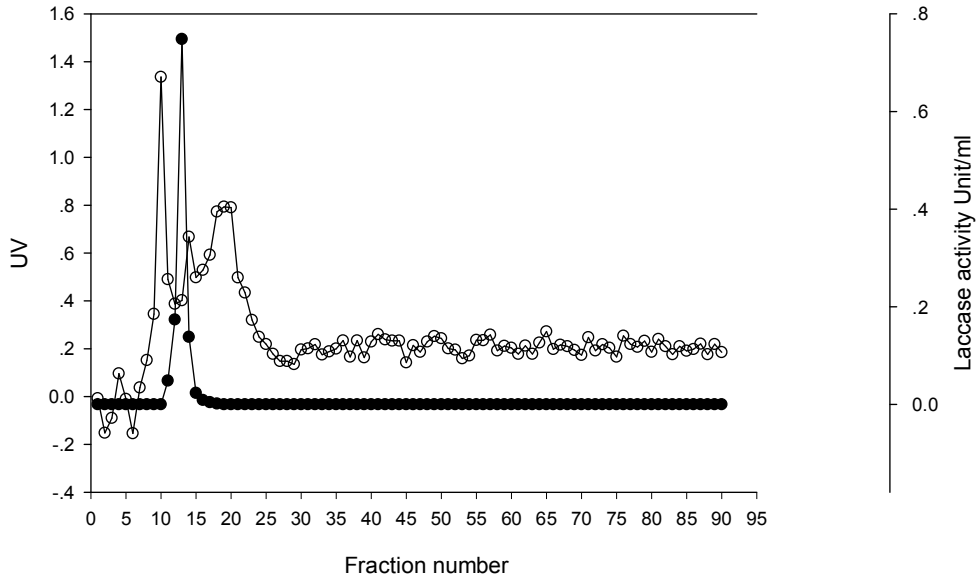


Fig.4.7 The gel filtration chromatography (Superdex 75), Absorbance at 280 nm (○); laccase activity(●)

4.5 Gel electrophoresis

The SDS-PAGE analysis of purified laccase in Fig. 4.8 displayed a single band with apparent molecular weight of 75.2 kDa. Activity staining of the laccase, with ABTS as substrate, revealed a single protein band corresponding with the position of the laccase activity (Fig. 4.9). The molecular masses of these laccases are consistent with the molecular masses of most other fungal laccases, which are between 60 and 80 kDa (Mayer & Staples 2002, Thurston 1994).

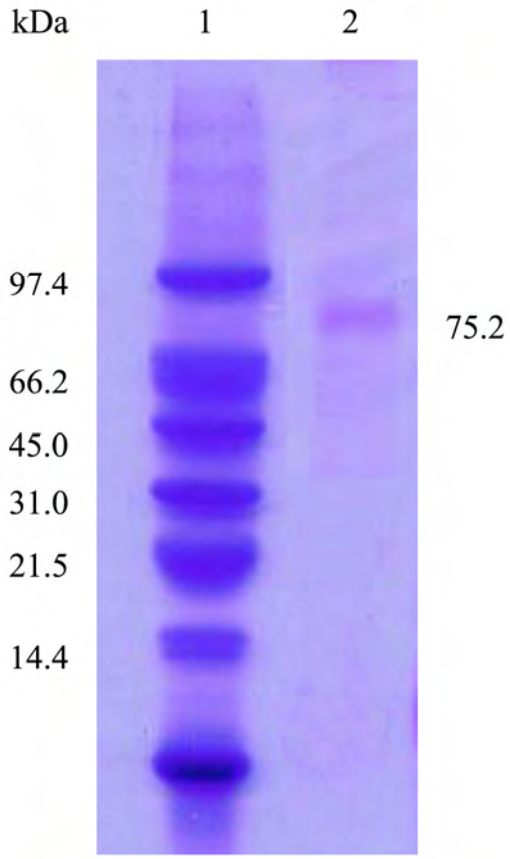


Fig. 4.8 The SDS-PAGE analysis of purified laccase of white rot fungus WR77: Lane 1, Low molecular weight protein markers; Lane 2, purified laccase (10 µg of protein).

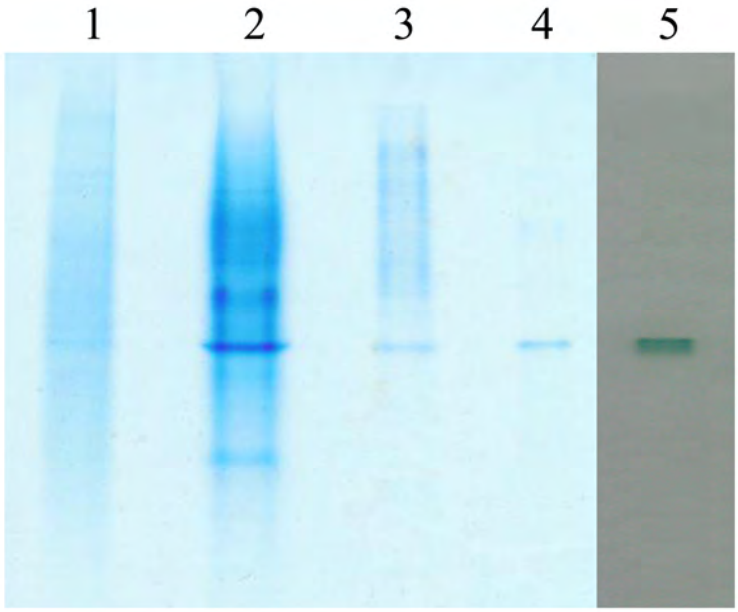


Fig. 4.9 Coomassie blue stained native-PAGE analysis of WR77 laccase fractions from each step of the enrichment and stained for protein by coomassie blue (Lanes 1 - 4) or for laccase enzyme activiy (Lane 5). Lane 1, crude enzyme (20 μg of protein); Lane 2, ammonium sulfate cut fraction (20μg of protein); Lane 3, post-DEAE-cellulose laccase fraction (10μg of protein); Lanes 4 & 5, post-Superdex-75 laccase fraction (10 μg of protein).

4.6 Laccase characterization

4.6.1 Thermostability of purified laccase of white rot fungus WR77

The effect of temperature on the activity of the purified laccase is shown in Fig. 4.10(a). The laccase was active over a wide range of temperatures, whereas at 80^{o}C, the laccase was completely inactivated. The thermal stability of the laccase is shown in Fig. 4.10(b). The enzyme was extremely stable at 40^{o}C. However, at 50°C, the enzyme was little lost activity after the allocated 30 min. Also, at 60 and 70°C, the enzyme activity was rapidly declined in 120 and 45 min respectively. The result of laccase thermal stability is in line with other fungal laccases that have been reported, such as *Marasmius quercophilus* strain 17 (Farnet *et al*., 2000) and *Trametes* sp. (Smirnov *et al*., 2001).

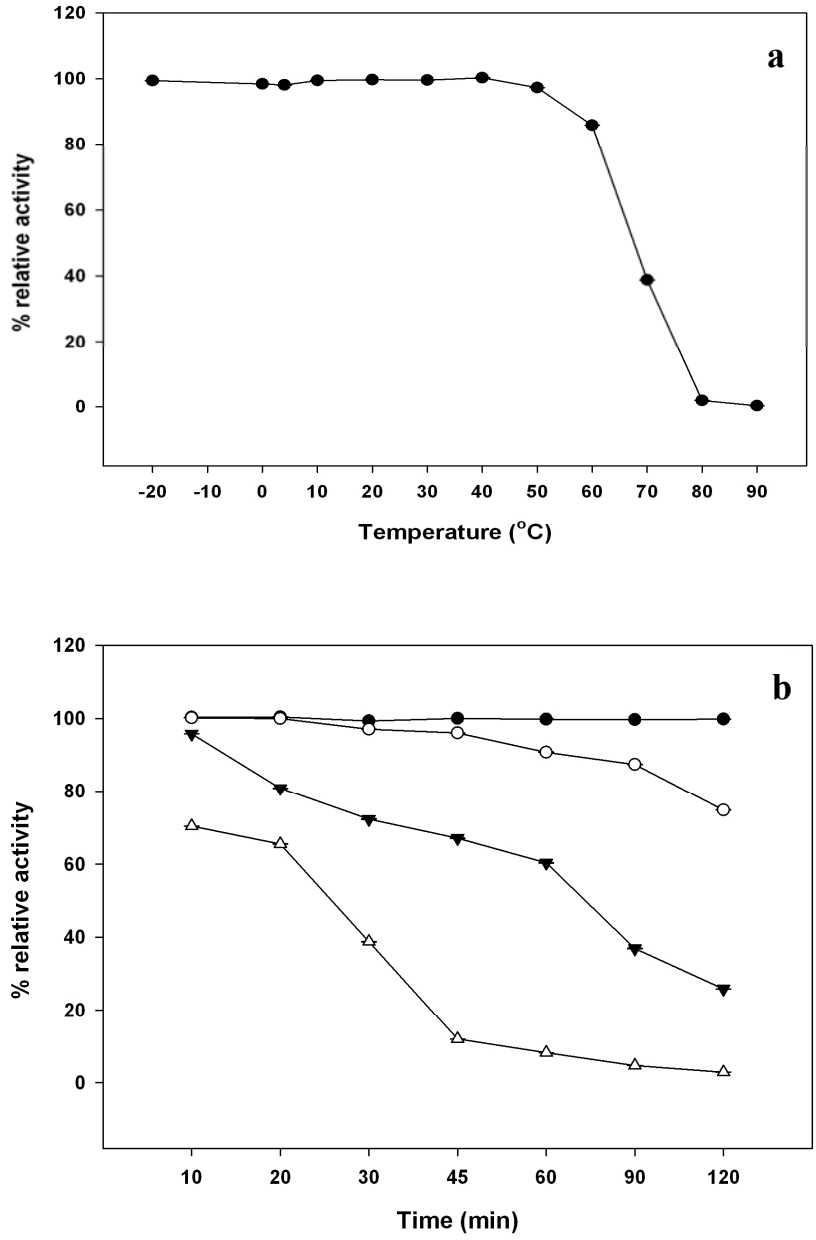


Fig. 4.10 (a) The effect of temperature and (b) thermostability of purified laccase of white rot fungus WR77. The following buffer systems were used 100 mM sodium acetate buffer pH 5.0: (●) 40°C; (○) 50°C; (▼) 60°C; (Δ) 70°C. Means of three replicates (cm) ± S.E.

4.6.2 pH stability of purified laccase of white rot fungus WR77

From the result, the laccase activity had stayed high at a pH range of 4.0 – 11.0 below and above which a sharp or steadily decline in activity was observed, although the stability of fungal laccases is generally higher at acidic pH (Leonowicz *et al*., 1984). Stability of the laccase of WR77 is quite high over wide range pH. Therefore, this could be very useful characteristic in various industrial applications.

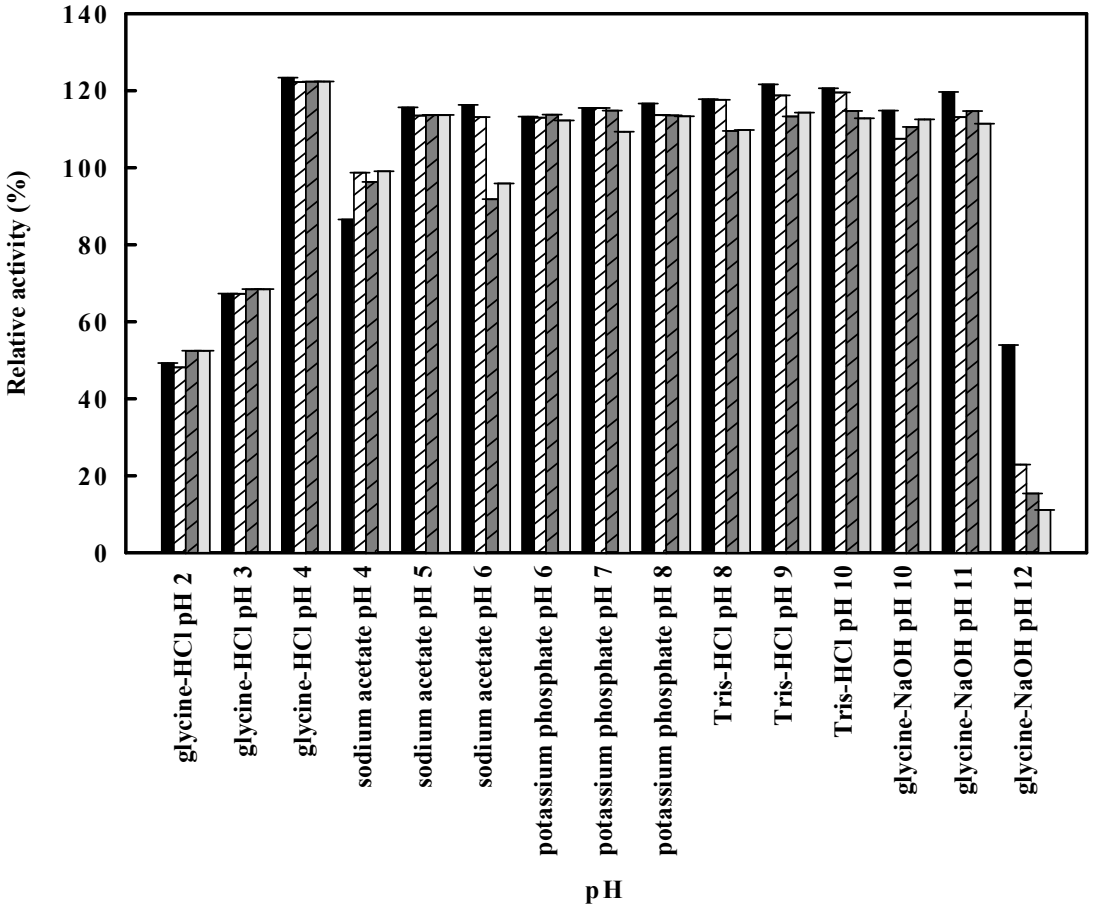


Fig. 4.11 pH stability of purified laccase from white rot fungus WR77. The 20 mM buffers were used as Glycine-HCl for pH 2.0, 3.0 and 4.0; Sodium acetate for pH 4.0, 5.0 and 6.0; Potassium phosphate for pH 6.0, 7.0 and 8.0; Tris–HCl for pH 8.0, 9.0 and 10.0 and Glycine–NaOH for pH 10.0, 11.0 and 12.0. Relative activity after incubation at various time: 45 min (■); 60 min (▨); 90 min (▩) and 120 min (□)

4.6.3 Effect of various cation ions and reagents of laccase activity of isolated WR77

Effect of various cations on the laccase activity are shown in Table 4.3. The purified enzyme was strongly inhibited by 5.0 mM Hg^{2+} and 1.0 mM Fe^{2+} (2.34% and 9.72% of control activity, respectively). The enzyme was also inhibited by 10 mM Zn^{2+} (77.8% of control activity) whereas EDTA showed only a slight inhibitory effect. Although, 10 mM Cu^{2+} ions stimulated laccase activity by 9.7%

Table 4.3 Effect of some cations on purified laccase activity from isolated WR77

Cation	Relative activity (%)[a] at various cation salt concentration			
	0.5 mM	1 mM	5 mM	10 mM
Cu^{2+}	98.8	100.0	100.4	109.7
Zn^{2+}	99.2	99.1	92.0	77.8
Ca^{2+}	100.1	100.4	100.8	100.6
Mn^{2+}	100.7	99.1	99.7	99.4
Hg^{2+}	42.8	21.8	2.3	2.6
Fe^{2+}	66.4	9.7	2.8	1.4

[a] The relative activity was determined by measuring laccase activity at 30^{o}C in 100 mM sodium acetate buffer pH 5.0 after pre-incubation at 30^{o}C for 30min with individual cations or reagents. Results are shown as the average three replicates. The activity assayed in the absence of cations or reagents was taken as 100%

Baldrian and Gabriel (2002) reported the highly negative effect of Hg^{+2} on laccase stability when added to a purified laccase from the white-rot fungus *Pleurotus ostreatus* at more than 0.1 mM. and it was increased in the presence of 0.05–50.0mM Cu^{+2}. Baldrian (2004) found that laccase from the white-rot fungus *Daedalea quercina* was inhibited by 1 mM Mn (9% of control activity) and 10 mM Hg^{2+} (7% of control activity), whereas 1 mM EDTA exhibited only a slight inhibitory effect (90% of control activity). Although, addition of 10 mM Cu increased enzyme activity by 17%.

In addition, Nagai et al. (2002) reported that a purified laccase from *Lentinula edodes* was inhibited by 55% in the presence of 1mM Hg^{+2}, and it was activated by 40% in the presence of 10mM Cu^{+2}. Couto *et al*. (2005) had been explained that the effect of metal ions on laccase stability depends on the laccase source, although, in general, Hg^{+2} strongly decrease stability of laccases. Metals can also influence the degradation reactions by the regulation of other factors affecting biodegradation. Thus, the activation or inhibition of proteolytic enzymes by metals can change the turnover rate of extracellular enzymes (Palmieri *et al*., 2001).

4.5.6 Determination of kinetic parameters

Laccase show a different kinetic behaviors depending on the substrate concentration, which the Lineweaver–Burk plot using ABTS as substrate (Fig. 4.12), the values of K_m and V_{max} for the purified laccase from WR77 were 447.93 mM and 104.17 µmol/min/mg protein, respectively. The laccase in *Daedalea quercina* had a Km value of 38 mM for ABTS (Baldrian, 2004). Laccases in *Lentinula edodes* Lcc1 showed Km values of 108 mM (Nagai *et al*., 2002). A Km value of 130 mM was observed in the laccase from *Pycnoporous sanguineus* (SCC 108) (Litthauer *et al*., 2007) and 207 mM was the Km in *Phellinus ribis* (Min *et al*., 2001).

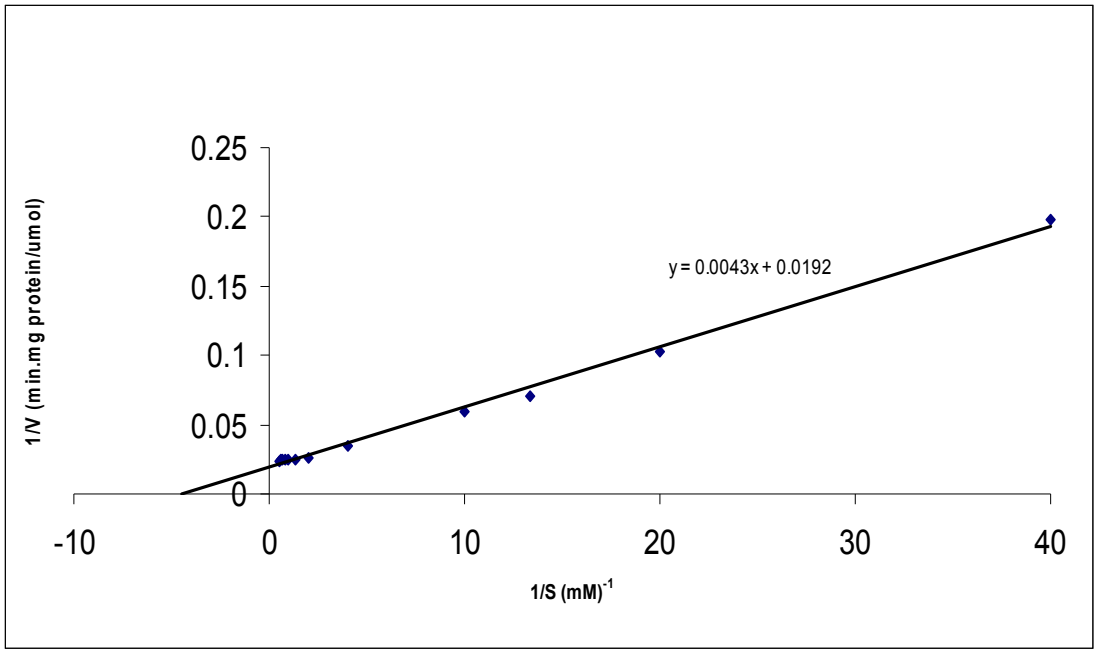


Fig.4.12 Lineweaver–Burk plot of the purified laccase of isolated WR77

4.7 Identification of white rot fungus

4.7.1 Morphological characteristics

Basidome of white rot fungus WR77 is shown in Fig. 4.13. Size of basidiodome was 7-9 cm broad, 0.5-1.0 cm thick. Shape of basidome is pileate to substipitate, rough surface with a thin cuticle on the *pileus*. Pileus with shell-shaped the fruit bodies usually had no stipe, shelf-like, and firmly attached the bark, cream-white color. Dimitic hyphal system with arboriform vegetative hyphae and cell content or wall that no reaction with Melzer's Reagent (nonamyloid).

Fig. 4.13 Basidome of white rot fungus WR77

4.7.2 Molecular identification

The ITS region of white rot fungus WR77 was amplified, sequenced, and submitted to GenBank. The obtained sequence was compared with those in the National Center for Biotechnology Information Nucleotide Sequence Database by using the Basic Local Alignment Search Tool (BLAST) algorithm. The sequence of the white rot fungus WR77 approximated size 532 bp exhibited the highest level of homology (86% identity) with *Polyporus pseudobetulinus* (accession number AF516571).

Identification base on morphological characteristics, and molecular techniques indicated that white rot fungus WR77 was similar to *Polyporus pseudobetulinus*. Species *Polyporus* showed laccase activity were as *Polyporus anceps* (Petroski *et al*.,1980), *Polyporus anisoporus* (Vaitkyavichyus *et al*.,1984), and *Polyporus pinsitus* (Heinzkill *et al*.,1998)

4.8 Dyes decolorization by isolated WR77 and the crude enzymes

The white rot fungi strain WR77 showed good ability for decolorization of Ambifix Blue H3R. The dye (200mg/L)was decolourised about 98% within 8 days. Whereas decolorilization of Ambifix Yellow H3R and Ambifix Red HE3B proceeded very slowly. Ambifix Yellow H3R (200mg/L) was decolorized by 24% in 10 days and Ambifix Red HE3B (200mg/L) was decolorized by 50% in 18 days (Fig. 4.14). This could be due to either enzyme inhibition by some products generated in the decolorilization process or substrate inhibition (Johann *et al*., 2007). Moreover, crude enzyme form the fungal (5 U/ml) had good ability for decolorization of Ambifix Blue H3R and MG (Fig 4.15). Ambifix Blue H3R (200mg/L) was decolourised about 65% within only 15min. Also, MG (200mg/L) was decolourised about 80% within 24hr. However, Ambifix Yellow H3R and Ambifix Red HE3B were no decolorization with crude enzyme (data not show). This is probably due to the complex structures of the dyes.

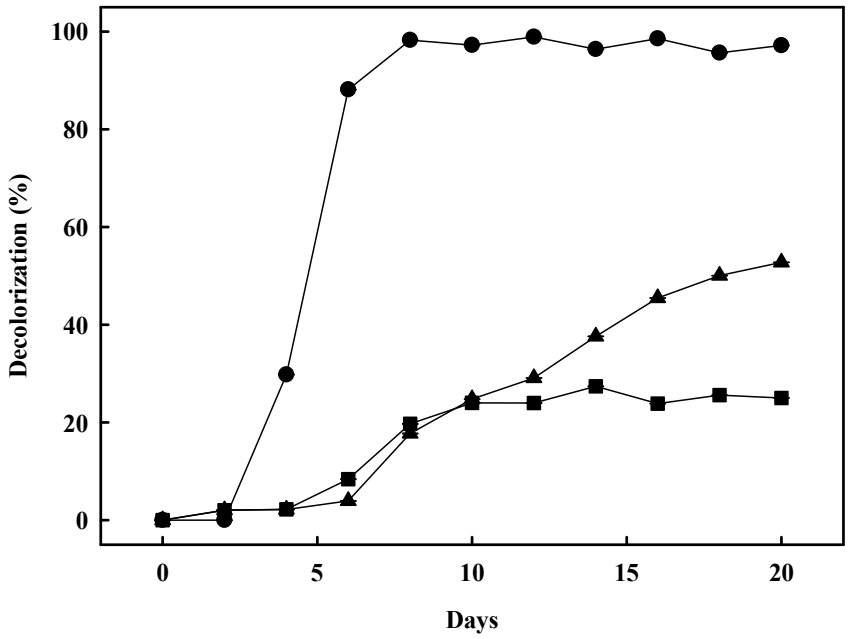


Fig. 4.14 Dye decolorization by white rot fungi WR77. Values represent the average of three replicate experiments; Ambifix Blue H3R (●); Ambifix Yellow H3R (▲); and Ambifix Red HE3B (■) (The initial concentration of each dye was 200 mg/L)

From this result showed that the fungal had ability for decolorization of Ambifix Yellow H3R and Ambifix Red HE3B while the crude enzyme form this fungal couldn't decolourise both of dyes. It could be possible to adsorption of dye on the fungal mycelium. Swamy and Ramsay (1999) suggested that mycelium-associated activities play an important role in dye decolorization by *Trametes versicolor*. It has also been shown that the decolorization of astrazone dye by isolated fungal pellets of *Funalia trogii* involves mechanisms other than biosorption and the degradation by enzymes secreted in the culture liquid (Cing and Yesilada, 2004; Yesilada *et al*., 2003).

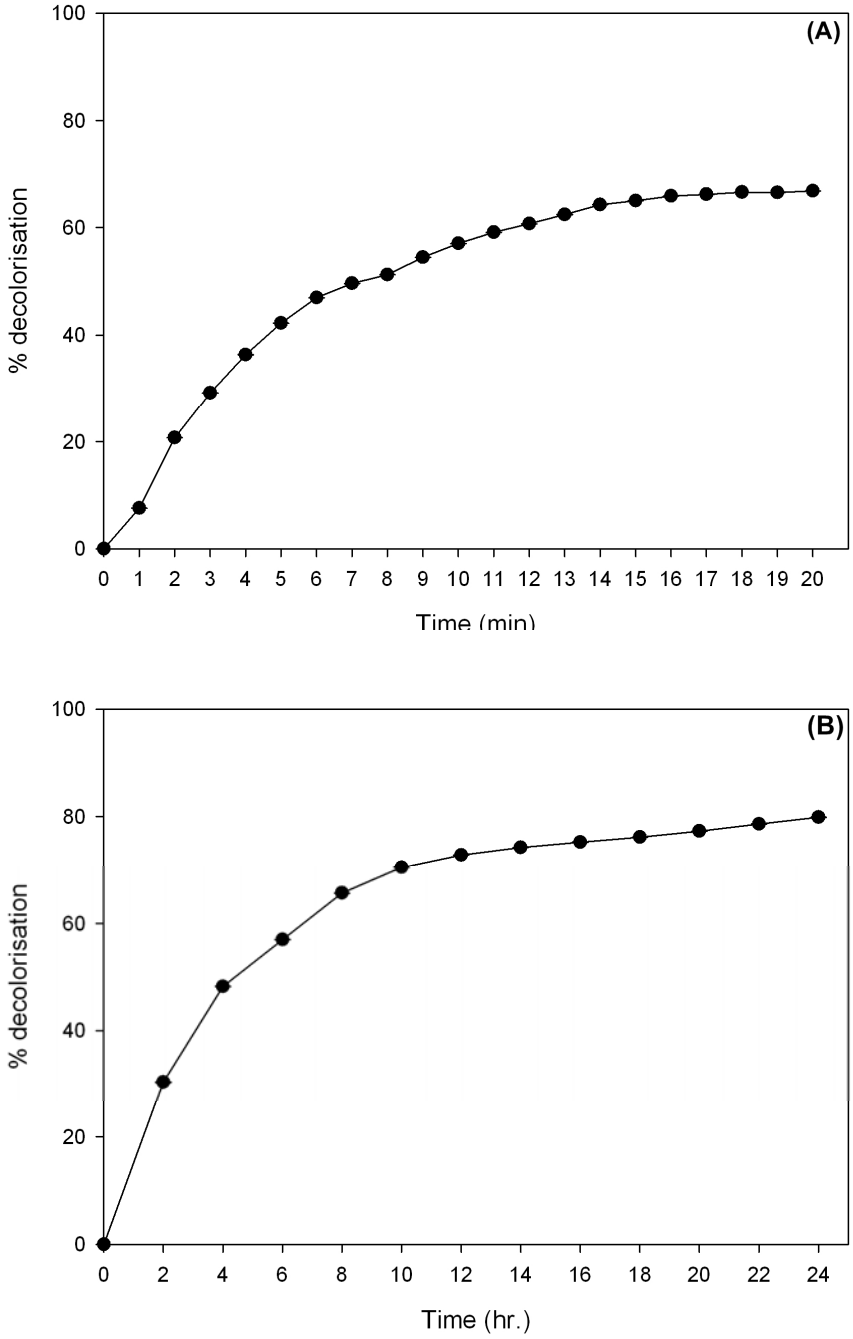


Fig. 4.15 Dye decolorization by crude laccase of white rot fungus WR77. a: Ambifix Blue H3R; b: MG; Values represent the average of three replicate experiments (The initial concentration of each dye was 200 mg/L)

Svobodova et al. 2008 demonstrated that *I. lacteus* cultivated in a liquid medium removed 88.5 % and 98.6 % of the initial amounts of the azo dye RO16 and RBBR (150 mg/l) within 24 h, respectively. Whereas no dye decolorization was detected by the crude culture filtrate, indicating that the dye decolorization observed in complete fungal cultures was not performed by enzymes secreted into the culture liquid. However, the crude enzyme from WR77 showed good ability for decolorization of MG of initial concentration of 200 mg/L but no dye decolorization was detected by the fungal cultures. This could be due to most of fungi cannot tolerate high concentrations of MG owing to its structural stability and toxicity (Cha *et al.*, 2001). Compared with laccases from other fungi, including *Rigidoporus lignosus* W1 (Li *et al*., 2008), *Phanerochaete chrysosporium* (Bumpus and Brock, 1998), *Cunninghamella elegans* (Cha *et al*., 2001), *Coriolus versicolor* (Levin *et al*.,2004) *Trametes trogii* (Levin *et al*.,2005), the MG decolorisation efficiency of the crude laccase from WR77 was very high, with about 160 mg L^{-1} MG being efficiently removed within 1 h at 30^{o}C and pH 5.0. From this result we found that write rot fungi strain WR77 showed good ability for laccase production and decolorize synthetic dyes therefore it would seem to be a good candidate for industrial application.

CHAPTER V

CONCLUSION

White rot fungus strain WR77 was effective in laccase production. Rice chaff (1% w/v) as a carbon source gave maximum laccase activity. The suitable inorganic and organic nitrogen sources for laccase activity were 0.5 g/L di-amonium tatrate and 0.01 g/L peptone, respectively. The laccase was purified by ammonium sulphate precipitation, Q-sapharose anion-exchange chromatography, and Sephadex G-75 gel filtration chromatography, which obtained a specific activity of 617.12 U/mg. The enzyme has a molecular mass of approximately 75.2 kDa as determined by SDS-PAGE, the optimum pH was 4 and had stayed high activity at a pH range of 4.0 – 11.0. Also, the laccase was most active and stable at 40°C. The Km value of the enzyme for substrate ABTS was 447.93 mM and its corresponding Vmax value of 104.17 μmol/min/mg protein. The purified enzyme was strongly inhibited by 5.0 mM Hg^{2+} and 1.0 mM Fe^{2+} whereas 10 mM Cu^{2+} ions stimulated laccase activity by 9.7%. Moreover, the ability of these strains and their crude laccase to decolorize synthetic dye Ambifix Blue H3R, Ambifix Yellow H3R and Ambifix Red HE3B was investigated with the initial concentration of each dye was 200 mg/L. The white rot fungi strain WR77 showed good ability for decolorization the dyes, Ambifix Blue H3R was decolorized by 98% in 8 days, Ambifix Yellow H3R was decolorized by 24% in 10 days and Ambifix Red HE3B was decolorized by 50% in 18 days. Also, the crude laccase (5 U/ml) could decolorized Ambifix Blue H3R by 65% within 15min and Malachite Green by 80% within 24hr. Therefore, it would seem to be a good candidate for industrial application.

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APPENDICES

APPENDIX A

MEDIA

1. Malt Extract Agar (2% MEA)

Malt extract	20	g
Agar	15	g
Distilled water	1,000	ml
pH 5.5 ± 0.01		

Dissolve with distilled water 900 ml thoroughly and adjust pH with HCl to pH 5.5. After that the distilled water was added to reach 1,000 ml. Sterillzation in a autoclave at 121^{o}C and pressure at 15 pounds/square inch for 15 minutes.

2. LME basal medium (LBM)

KH_2PO_4	1	g
$C_4H_{I2}N_2O_6$	0.5	g
$MgSO_4.7H_2O$	0.5	g
$CaC_{l2}\cdot 2H_2O$	0.01	g
yeast Extract	0.01	g
$CuSO_4.5H_2O$	0.001	g
$Fe(SO_4)_3$	0.001	g
$MnSO_4.H_2O$	0.001	g
Distilled water	1,000	ml

3. ABTS-agar medium

ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt)	10	g
Agar	15	g
LME basal medium (LBM)	1,000	ml

Dissolve with LBM 900 ml thoroughly and adjust pH with HCl to pH 5.5. After that the LBM was added to reach 1,000 ml. Sterilization in a autoclave at 121^{o}C and pressure at 15 pounds/square inch for 15 minutes. Then aseptically add 1 ml of a separately sterilized 20 % w/v aqueous glucose solution to each 100 ml of growth medium prepared.

APPENDIX B

Preparation for denaturing polyacrylamide gel electrophoresis

1. Stock solutions

2 M Tris-HCl (pH 8.8)

Tris (hydroxymethyl)-aminomethane 24.2 g

Adjusted pH to 8.8 with 1 M HCl and adjusted volume to 100 ml with distilled water

1 M Tris-HCl (pH 6.8)

Tris (hydroxymethyl)-aminomethane 12.1 g

Adjusted pH to 6.8 with 1 M HCl and adjusted volume to 100 ml with distilled water.

10% SDS (w/v)

Sodium dodecyl sulfate (SDS) 10 g

50% Glycerol (w/v)

100% Glycerol 50 ml

Added 50 ml of distilled water

1% Bromophenol blue (w/v)

Bromophenol blue 100 mg

Brought to 10 ml with distilled water and stirred until dissolved.

Filtration will remove aggregated dye.

2. Working solution

Solution A (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide)

Acrylamide	29.2 g
N,N,-methylene-bis-acrylamide	0.8 g
Adjust volume to 100 ml with distilled water	

Solution B (1.5 M Tris-HCl pH 8.8, 0.4% SDS)

2 M Tris-HCl (pH 8.8)	75 ml
10% SDS	4 ml
Distilled water	21 ml

Solution C (0.5 M Tris-HCl pH 6.8, 0.4% SDS)

1 M Tris-HCl (pH 6.8)	50 ml
10% SDS	4 ml
Distilled water	46 ml

10% Ammonium persulfate

Ammonium persulfate	0.5 g
Distilled water	5 ml

Electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)

Tris (hydroxymethyl)-aminomethane	3 g
Glycine	14.4 g
SDS	1 g
Dissolved in distilled water to 1 litre without pH adjustment (final pH should be 8.3)	

5x sample buffer

(60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 14.4 mM 2-mercaptoethanol)

1 M Tris-HCl (pH 6.8)	0.6	ml
Glycerol	5	ml
10% SDS	2	ml
1% Bromophenol blue	1	ml
2-mercaptoethanol	0.5	ml
Distilled water	0.9	ml

3. SDS-PAGE

15% Separating gel

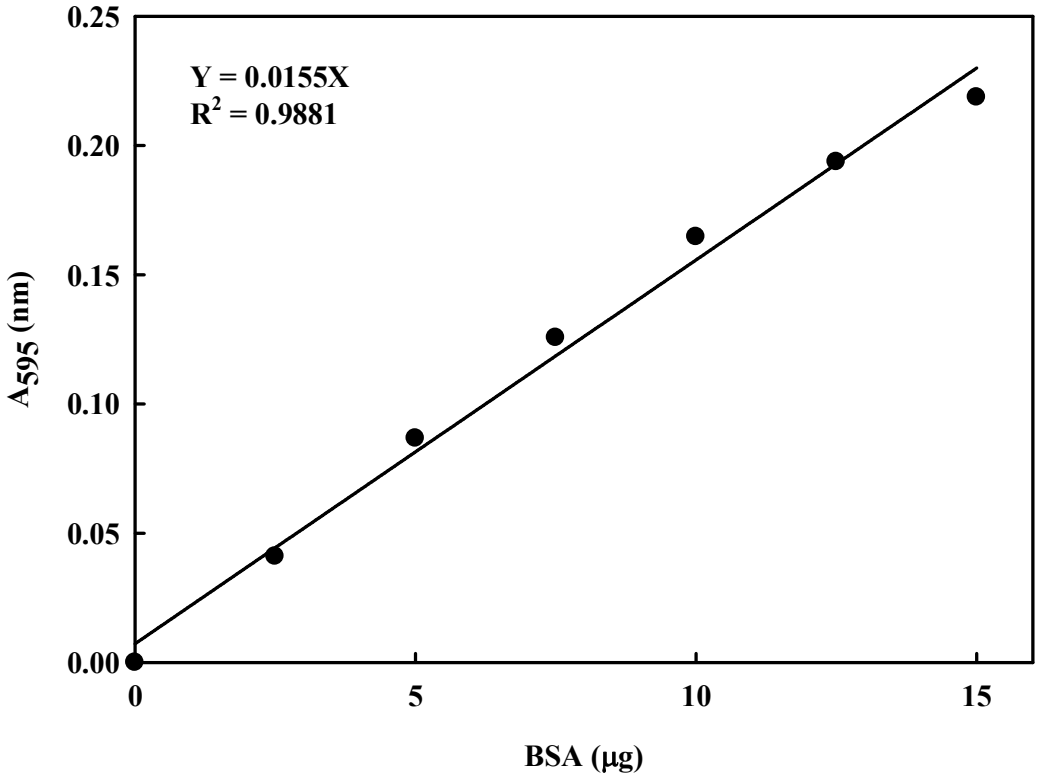
Solution A	10.0	ml
Solution B	5.0	ml
Distilled water	5.0	ml
10% Ammonium persulfate	100	ml
TEMED	10	ml

5.0% Stacking gel

Solution A	0.67	ml
Solution B	1.0	ml
Distilled water	2.3	ml
10% Ammonium persulfate	30	ml
TEMED	5.0	ml

APPENDIX C

Calibration curve for protein determination by Bradford method



APPENDIX D

WR77 nucleotide

ORIGIN

1 cgcgctacac tgacagagcc agcgagtttt ttttccttgg ccggaaggtc tgggtaatct
61 tgtgaaactc tgtcgtgctg gggatagagc attgcaatta ttgctcttca acgaggaatt
121 cctagtaagc gtgagtcatc agctcgcgtt gattacgtcc ctgccctttg tacacaccgc
181 ccgtcgctac taccgattga atggcttagt gaggtcttgg gattggcttt ggggtgccgg
241 caacggcgct tcgttgctga gaacttggtc aaacttggtc atttaaagga agtaaaagtc
301 gtaacaaggt ttccgtaggt gaacctgcgg aaggatcatt atcgagtttt tgaaaggagt
361 tgtagctggc ctcatggggc atgtgcacgc tctgcttcaa tccactctct acacctgtgc
421 acttactgtg ggctttccgg agggagggtt tgcaatccgt ttgtaaatct tttgggaggg
481 cctgcgttta ttcacaaaca cttataacag taatggaatg tatactatga tgtaacacat
541 ctataataca acttcagcaa cggatctctt ggctctcgca tcgatgaaga acgcagcgaa
601 atgcgataac taatg

Nucleotide sequence of isolate WR77

GTAGCTGGTCTTACGAGGCATTGTGCACGCCCCGCTCAATCCACTCTACACCTGTGAACTAACTGT
GGGTCTTTCTGGAGGGGTTTGCATTCTCTGTAAGCCCTTTTGGAAGCTCATGTTTACTTTACAAACAC
TTATAAAGTAATTGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCTCTT
GGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCACGCCTGTTTGAGTGTCAT
GTAATTCTCAACCTACAAAGTTCTTGTTAGCTTTGTTAGGCTTGGATATTGGAGGTCTAATTGTTGGC
TCGCATGAGTCGGCTCCTCTTAAATGCATTAGCTTTGGTCCTCTGTGGATCGGCTCTCGGTGTGATA
AGTTGTCTATGCCGTGGTCGTGAAGTGTTCCTTGGGAAAAGGGCTTCTAATCGTCTCTTAGC

BIOGRAPHY

Miss Pajareeya Songserm was born on October 04, 1985 in Udonthani province, Thailand. She graduated with a Bachelor Degree of Science from Faculty of Science, Department of Microbiology, Khon-Kean University in 2008. She had been studies for a Master Degree of Science in Microbiology, the Faculty of Science, Chulalongkorn University since 2009.

Academic presentation;

1) Songserm, P., Sihanonth, P., Piapukiew, J., and Karnchanatat, A. Decolorization of Synthetic Dyes by Selected White-rot Fungi, The 22nd Annual Meeting of the Thai Society for Biotechnology International Conference on Biotechnology for Healthy Living, 20-22 October 2010, Prince of Songkla University, Trang Campus, Thailand,
2) Songserm, P., Sihanonth, P., Piapukiew, J., and Karnchanatat, A. Decolorization of Synthetic Dyes by Selected White-rot Fungi, The 12th Graduate Research Conference Khon Kaen University 2011, 28 January 2011, Khon Kaen, Thailand.