วิศวกรรมของแลกเคสจาก *Trametes versicolor* เพื่อปรับปรุงประสิทธิภาพการเร่งปฏิกิริยาในการฟอกสีสังเคราะห์

นางสาวมนต์ณัฐ ธีระชาติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ENGINEERING OF LACCASE FROM *Trametes versicolor* TO IMPROVE THE CATALYTIC EFFICIENCY IN SYNTHETIC DYES DECOLORIZATION

Miss Monnat Theerachat

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biological Sciences
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มนต์ณัฐ ธีระชาติ : วิศวกรรมของแลกเคสจาก *Trametes versicolor* เพื่อปรับปรุงประสิทธิภาพการเร่งปฏิกิริยาในการฟอกสีสังเคราะห์. (ENGINEERING OF LACCASE FROM *Trametes versicolor* TO IMPROVE THE CATALYTIC EFFICIENCY IN SYNTHETIC DYES DECOLORIZATION). อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร.วรวุฒิ จุฬาลักษณานุกูล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : Prof. Magali Remaud-Simeon, Ph. D., Asst. Prof. Sandrine Morel, Ph. D., 118 หน้า.

สีสังเคราะห์ถูกนำมาใช้ในหลากหลายอุตสาหกรรม เมื่อสีเหล่านี้ปนเปื้อนสู่แหล่งน้ำจะกลายเป็นมลภาวะสำคัญ วิธีทางฟิสิกส์และเคมีได้ถูกนำมาใช้เพื่อกำจัดสีสังเคราะห์ อย่างไรก็ตามวิธีเหล่านี้มีราคาสูงและเกิดสารพิษเป็นผลิตภัณฑ์ร่วม จากการศึกษาการย่อยสลายสีสังเคราะห์เจ็ดชนิดในกลุ่มโครงสร้างต่างๆกัน (azo, anthraquinone และ indigo) พบว่าสารสกัดเอนไซม์แลกเคสจากรา *Trametes versicolor* สายพันธุ์ดีเอสเอ็ม 11269 สามารถย่อยสลายสีในกลุ่ม anthraquinone ได้แก่ Alizarin Red S และ Remazol Brilliant Blue R คิดเป็น 55 และ 70 เปอร์เซ็นตามลำดับภายในสามชั่วโมง ในขณะที่สีในกลุ่ม azo (Amaranth, Cibacron Brilliant Red 3B-A, Direct Blue 71และ Reactive Black 5) และ indigo (Indigo Carmine) ย่อยสลายได้น้อยกว่า 10 เปอร์เซ็นต์ในสามชั่วโมง อย่างไรก็ตามการผลิตเอนไซม์จากราต้องการ สารในกลุ่มฟีนอลิกซึ่งเป็นพิษในการเหนี่ยวนำการผลิตเอนไซม์ และใช้เวลานาน การแสดงออกของแลกเคสในสิ่งมีชีวิตอื่นจึงเป็นวิธีที่น่าสนใจอีกวิธีหนึ่งในการผลิตเอนไซม์ การศึกษานี้ต้องการนำยีนแลกเคสจากรา *T. versicolor* มาแสดงออกในยีสต์ *Yarrowia lipolytica* ซึ่งเป็นยีสต์ที่มีความสามารถในการแสดงออกของโปรตีนและประสิทธิภาพการทรานฟอร์เมชันสูง โดยซีดีเอน เอของยีนแลกเคส (*lcc1*) จากรา *T. versicolor* ได้มาโดยโดยเทคนิคอาร์ที-พีซีอาร์ แลกเคส (LCC1) ประกอบด้วยกรดอะมิโน 498 เรซิดิวซ์ และ สัญญานเพปไทด์อีก 22 เรซิดิวซ์ ยีน *lcc1* จะถูกแสดงออกในยีสต์ *Y. lipolytica* ภายใต้การควบคุมของโปรโมเตอร์ที่มีการแสดงออกอย่างต่อเนื่องชื่อ pTEF เมื่อเอนไซม์แลกเคสถูกทำให้บริสุทธิ์โดยเทคนิคโครมาโตกราฟีแบบจำเพาะ พบว่ามีน้ำหนักโมเลกุลประมาณ 75 กิโลดาลตัน ซึ่งประกอบด้วย เอนลิงค์ไกลแคน ประมาณ 20 เปอร์เซ็นต์ หลังจากการชักนำการกลายพันธุ์โดยเทคนิค วิวัฒนาการระดับโมเลกุล พบว่ามิวแทนต์ rL185P/Q214K (rM-4A)มีแอกทิวิตีรวมเพิ่มขึ้นประมาณ 6 เท่า หลังจากการทำเอนไซม์ให้บริสุทธิ์พบว่าแอกทิวิตีจำเพาะของมิวแทนต์เพิ่มขึ้น 2.1 เท่าเมื่อเทียบกับสายพันธุ์ดั้งเดิม เมื่อใช้ สารเอบีทีเอสเป็นสารตั้งต้นพบว่า ค่า k_{cat} and k_{cat}/K_m เพิ่มขึ้น 1.7 และ 2.4 เท่าตามลำดับ และเมื่อใช้ 2, 6 ไดเมทอกซีฟีนอล เป็นสารตั้งต้น ค่า k_{cat} และ k_{cat}/K_m เพิ่มขึ้นเป็น 2.5 และ 2.8 เท่าตามลำดับ แลกเคสดั้งเดิมและแลกเคสมิวแทนต์มีความเสถียรสูงที่พีเอชในช่วงกว้าง(3.6-7.6) และที่อุณหภูมิปกติ โดยหลังจาก 1 ชั่ วโมง มีแอกทิวิ ตีสูงกว่า 88 เปอร์เซ็น ต์ที่อุณหภูมิ 25-35 องศาเซลเซียส เมื่อใช้แลกเคสดั้งเดิมและแลกเคสมิวแทนต์ ทำปฏิกิริยาการย่อยสลายสีสังเคราะห์ร่วมกับรีดอกซ์เมดิเอเตอร์ชื่ออะซิโตไซลิงโกน พบว่าสามารถย่อยสลาย Amaranth ที่ความเข้มข้นเริ่มต้น 74.6 ไมโครโมลาร์ ด้วยอัตราการย่อยสลายประมาณ 70 นาโนโมลาร์ต่อวินาที

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4973837023 : MAJOR BIOLOGICAL SCIENCES
KEYWORDS : ENGINEERING, LACCASE, *Trametes versicolor*, DECOLORIZATION
MONNAT THEERACHAT : ENGINEERING OF LACCASE FROM *Trametes versicolor* TO IMPROVE THE CATALYTIC EFFICIENCY IN SYNTHETIC DYES DECOLORIZATION. ADVISOR : ASSOC. PROF. WARAWUT CHULALAKSANANUKUL, Ph.D, CO-ADVISOR : PROF. MAGALI REMAUD-SIMEON, Ph. D., ASST. PROF. SANDRINE MOREL, Ph. D.,118 pp.

Synthetic dyes are extensively used in many industries. The effluents from these factories represent a major source of water pollution. Actually, physical and chemical methods are used for the treatment of these effluents but they are expensive and produce hazardous by-products. First, the decolorization of seven dyes with diverse chemical structure (azo, anthraquinone and indigo) was studied. An enzyme extract enriched in laccase from the fungus *Trametes versicolor* strain DSM11269 was found to decolorized the anthraquinone derivative dyes, Alizarin Red S and Remazol Brilliant Blue R, in three hours by 55% and 70%, respectively. The azo compounds (Amaranth, Cibacron Brilliant Red 3B-A, Direct Blue 71 and Reactive Black 5), and the indigo molecule (Indigo Carmine), showed a higher resistance to decolorization (<10% in 3 hours). Biotechnological and environmental applications require large amounts of enzymes and the secretion of laccase by fungal organism may not be suitable due to undesirable preparation procedure (such as presence of toxic inducers) and time consuming. So, heterologous expression of laccase in optimized hosts is a considerable way to produce the recombinant enzyme. The yeast *Yarrowia lipolytica* has been choosen as an efficient system for recombinant protein expression with high transformation efficiency. A cDNA encoding laccase (*lcc1*) was isolated from the white-rot fungus *T. versicolor* by RT-PCR. The cDNA encodes a laccase isoenzyme of 498 amino-acid residues preceded by a 22-residue signal peptide. The *lcc1* was expressed in *Y. lipolytica* under the control of the pTEF constitutive promoter. After purification by affinity chromatography, the recombinant laccase has an apparent molecular weight of ~75 kDa, comprised of ~20% N-linked glycans. The activity produced for the mutant L185P/Q214K (rM-4A) generated by molecular evolution was by six-fold higher than that obtained with the wild type enzyme. After purification, rM-4A mutant showed a 2.1-fold higher specific activity and a 1.7- and 2.4- fold enhancement of the k_{cat} and k_{cat}/K_m values, respectively, with ABTS substrate and a 2.5- and 2.8-fold increase, respectively, with 2, 6-dimethoxyphenol substrate, compared to the wild type (rWT) laccase. Both the rWT and the rM-4A Lcc1 enzymes were stable over a broad pH range (3.6-7.6) and active at normal temperatures (>88% activity at 25-35^{o}C after one hour). The rWT and mutant laccase preparation decolorize 95 and 97% of 74.6 µM Amaranth solution. The results revealed that decolorization rate is about 70 nM.s^{-1} in the presence of acetosyringone redox mediator.

Field of Study : Biological Sciences ..... Student's Signature .....

Academic Year : 2011 ..... Advisor's Signature .....

Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

2,6-DMP	2-6-dimethoxyphenol
ABTS	2'2-azino-bis(3-ethylbenzothiazoline-6-sulphic acid)
BSA	Bovine Serum Albumine
cDNA	complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
IgG	Immunoglobulin G
IMAC	Immobilized metal affinity chromatography
kDa	Kilodalton
LB	Luria-Bertani culture media
LiAc	Lithium acetate
NCBI	National center for biotechnology information
OD	Optical density
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
SD	Standard deviation
SDS-PAGE	S odium dodecyl sulfate polyacrylamide gel electrophoresis
T	Temperature
Tris	Trishydroxymethylaminomethane

CHAPTER I

INTRODUCTION

Synthetic dyes are extensively used in the textile, dyeing, printing and leather industries and often their contamination in the water effluents from these industries represents a major source of water pollution, affecting the water quality, transparency and oxygen content. The removal of such dyes from wastewater is of prime concern for environment protection but it remains a difficult task due to the structural complexity, toxicity and high stability of these molecules. Physical and chemical methods, such as adsorption, coagulation-flocculation and filtration, are commonly used for the treatment of these effluents. However, these methods are expensive, not always efficient and sometimes produce hazardous by-products.

Green oxidation technologies by using the enzymes to replace the conventional non-biological methods are attractive for biotechnological applications. Among the oxidant enzymes, laccases (EC 1.10.3.2) have been the subject of intensive studies in the last decades. Laccase is a type of lignin-modifying enzyme able to oxidize a wide range of molecules, which can serve in bioremediation and many industrial purposes. The simple requirements of laccase catalysis (only substrate and O_2), as well as its apparent stability, make this enzyme attractive for biotechnological applications. Fungal laccases are the most studied group. Their presence have been reported in almost fungi, especially *Trametes versicolor, Trametes hirsuta* and *Aspergillus niger* (Majeau et al., 2010).

However, the production of laccase by the fungal is time consuming and required the induction by toxic phenolic compound. Thus, effort have to be made in order to achieve cheap overproduction of laccase in safe heterologous hosts which can produce the enzyme in a short time and also their modification by protein engineering

to obtain more robust and active enzyme. Laccase genes have been expressed in fungi and yeast such as *Aspergillus oryzae*, *Trichoderma reesei*, *Aspergillus niger*, *Saccharomyces cerevisiase*, *Pichia pastoris* and *Pichia methanolica*. However, several problems such as low secretion efficiency in *S. cerevisiae*, low transformation efficiency and time consuming in *Pichia pastoris* and *P. methanolica* have limited the genetic engineering of recombinant laccase. The yeast *Yarrowia lipolytica* has been reported as an efficient system for recombinant protein expression with its low cost, post translational modification efficiency and high transformation efficiency (~8000 transformant/μg of DNA). Moreover, this yeast is considered as nonpathogenic and several processes based on this organism were classified as generally regarded as safe (GRAS) by the Food and Drug Administration (FDA, USA). For these reasons, *Y. lipolytica* is a very interesting host for laccase expression.

Objectives

1. To determine the ability of laccases from white-rot fungus *Trametes versicolor* to decolorize several synthetic dyes.
2. To express laccase gene (*lcc1*) from *T. versicolor* in *Y. lipolytica* strain zeta and MTLY60 with an expression integrative vector based on a constitutive promoter (p*TEF* promoter). The secretion signal peptide was a native laccase *lcc1* signal peptide from *T. versicolor*.
3. To perform directed evolution to increase the activity of the enzyme. The laccase was purified and characterized.
4. To decolorize synthetic dyes by recombinant enzymes.

CHAPTER II

LITERATURE REVIEW

PART I : Synthetic dyes decolorization

1. Synthetic dyes

Synthetic dyes are widely used in a number of industries. Among industrial wastewater, dye waste water from textile and dyestuff industries is one of the most difficult to treat because of complex structure of the dyes (Table II-1) that make them more stable and more difficult to be biodegraded (Fu and Viraraghavan, 2001). Vaidya and Datye (1982) and Zollinger (2002) reported that there are over 1,000,000 commercially dyes with an annual production over $7x10^5$ metric tonnes worldwide are available and 5-10% of the dyestuffs are lost in the industrial effluent. Several dyes are visible in water at concentration as low as 1 mg/L and reducing light penetration in water systems, thus causing a negative effect on photosynthesis. Government legislation is stringent, especially in the more developed countries, regarding the removal of dyes from industrial effluents. Concern arises, as several dyes are made from carcinogens such as benzidine and other aromatic compounds. Moreover, their discharge into surface water leads to aesthetic problems and obstructs light penetration and oxygen transfer into waterbodies which affect aquatic life (Levine, 1991; Wong and Yu, 1999; Poots and McKay, 1976).

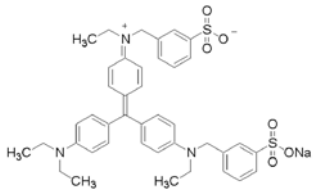
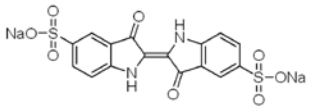
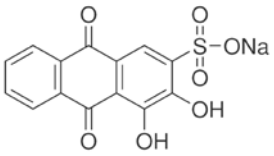
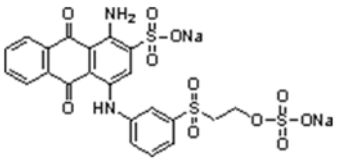
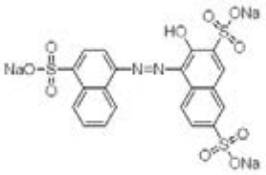
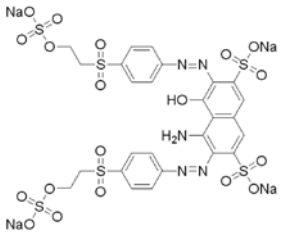
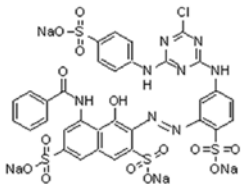
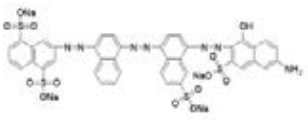
Mishra and Tripathy (1993) classified the dyes as follows

1. Anionic : direct, reactive and acid dyes
2. Cationic : basic dyes
3. Non-ionic: disperse dyes

The chromophores in anionic and non-ionic dyes are azo and anthraquinone types. Azo dyes (Table II-1), which are aromatic compounds with at least one -N=N-

group, constitute the largest class of synthetic dyes used (>50% of dye production worldwide) (Pandey et al., 2007). Decomposition of azo dye may lead to the formation of carcinogenic amines under anaerobic conditions in the environment (Chung and Stevens, 1993). Fernández (2011) classified the azo dyes base on the principal characteristics and substrate to be dyed (Table II-2). Anthraquinone dyes are more difficult for degradation because of their fused aromatic structures. These dyes can remain colored for a longer time in the wastewater. Basic dyes have high intensity and brilliance of colors and highly visible at low concentration. The metal dyes are mostly base on chromium, which is carcinogenic (Mishra and Tripathy, 1993).

Table II-1 Molecular structure of synthetic dyes used in this study.

Dye (classification)	Structure	Dye (classification)	structure
Acid Violet 17 (Triarylmethane)		Indigo Carmine (indigo)	
Alizarin Red S (anthraquinone)		Remazol Brilliant Blue R (RBBR) (anthraquinone)	
Amaranth (azo)		Reactive Black 5 (azo)	
Cibacron Brilliant Red 3B-A (azo)		Direct Blue 71 (azo)	

The toxicity of dyes were also test by ETAD (Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry). They found that over 90% of 4000 dyes tested had LD50 values greater than 2 $x10^3$ mg/kg. The highest rates of toxicity were found in diazo direct and basic dyes (Shore, 1996). Thus, the removal of color from wastewater has been a major concern.

Table II-2. Classification of the azo dyes based on their principal characteristics and nature of the substrate to be dyed (Fernández, 2011).

Azo dye	Characteristic/property	Substrate
Anionic monoazo dye	Soluble in water due to the presence of sulfonate groups	Paper and leather
Acid dye	Anionic monoazodye with 1 to 3 sulfonate groups. It requires acidic conditions during the dyeing of materials	Wool and silk
Azoic dyes	Water-insoluble monoazo dyes	Cellulosic fibers
Disperse dyes	Partly soluble in water, and thus suspended in aqueous solutions	Polyester fibers
Cationic azo dyes	Need mordant compounds	Paper
Metal-complex azo dye	Metal complexed dye	Ink-jet printing
Direct dyes	Do not need mordant compounds	Cellulosic fibers
Reactive azo dyes	Covalent attachment between dye and substrate	Protein fibers

2. Degradation of synthetic dyes

Many studies focus on the use of physicochemical methods for color removal form dyes containing in effluents. Among of these methods, coagulation and precipitation are the most commonly used. However, these techniques produce huge amounts of sludge, which requires safe disposal. The other popular methods,

adsorption, lead to secondary waste streams which require further treatment (Pandey et al., 2007). Advantage and disadvantage of the transformation of synthetic dyes by physical, chemical and biological methods are described in Table II-3.

2.1 Physical treatments

2.1.1 Adsorption

Adsorption techniques is one of the most popular techniques due to their economically feasible and efficiency for the removal of the pollutants. This method is influenced by several factors such as dye/sorbent interaction, particle size, sorbent surface area, contact time, temperature and pH (Kumar et al., 1998). Many wastes and/or low cost materials can be used as adsorbents for dye removal such as bark, rice husk, coal, clay, cotton waste, maize, bagasse, peat and wood chips.

2.1.1.1 Activated carbon

Activated carbon is the most popular method for dye removal by adsorption technique (Nasser and El-Geundi, 1991). This method is very effective for adsorbing cationic, acid and mordant dye. The removal is moderate for direct, dispersed, vat, and reactive dyes (Raghavacharya, 1997; Rao et al., 1994). Activated carbon is well suited for one particular waste system and ineffective in the other. Activated carbon is also expensive. Furthermore, carbon has to be reactivated otherwise disposal of the waste has to be considered. Normally, about 10-15% of the sorbent is loss during reactivation process and hence there is a need for regeneration to make it cost effective (Robinson et al., 2001).

2.1.1.2 Bagasse pith

Bagasse pith is an agricultural or industrial by-products which can be used for economically cheap adsorbent. Bagasse was found to be an effective adsorbent for removal of basic dyes from aqueous solution (Anjaneyulu et al., 2005).

2.1.1.3 Peat

Peat has the ability to adsorb polar organic compounds from wastewater containing dyes. The cost of peat is cheap. Moreover, peats do not need activation unlike activated carbon. However, the capacity for adsorption of peat is lower than activated carbon because activated carbon's powdered has much larger surface area (Anjaneyulu et al., 2005).

2.1.1.4 Wood chip

Wood chip has a good adsorption capacity for acid dyes. However, due to their hardness, they require longer contact time (Anjaneyulu et al., 2005).

2.1.2 Irradiation

The treatment by irradiation such as electron beams and gamma rays are efficient technique which can eliminate an organic contaminants and disinfect harmful microorganism. However the application of these technologies is limited due to the cost economics and maintenances.

2.1.3 Ion exchange

Ion exchange technique has not been widely used for the treatment of dye-containing effuents due to the opinion that this technique cannot apply for a wide range of dyes (Slokar and Le Marechal, 1997). Dye containing wastewaters is passed over the ion exchange resin until the available exchange sites are saturated. This technique successfully removed anionic and cationic dyes from effluents. Advantages of this method include no loss of adsorbent on regeneration. The disadvantage is a high operation cost. Moreover, this technique is not effective for disperse dyes (Mishra and Tripathy, 1993).

2.2 Chemical treatment

2.2.1 Oxidative processes

This is the most commonly chemical method used of decolourisation due to its simple handing. Hydrogen peroxide (H_2O_2) is one of the widely used agent that needs to be activated by some means, for example, ultra violet light. Many methods of chemical decolourisation vary depending on the way in which the H_2O_2 is activated (Slokar and Le Marechal, 1997). Chemical oxidation removes dye from the wastewater containing dye by oxidation resulting in aromatic ring cleavage of the dye molecules (Raghavacharya, 1997).

2.2.1.1 H_2O_2-Fe(II) salts (Fenton's reagent)

Fenton's reagent (hydrogen peroxide, activated with Fe(II) salts) is a suitable chemical means of treating wastewaters which are resistant to biological treatment. This method is effective in decolorizing both soluble and insoluble dyes (Pak and Chang, 1999). Advantages of this technique include toxicity and color reduction. One major problem of this technique is the generation of sludge through the flocculation of the reagent and the dye molecules. The sludge containing the concentrated impurities, still requires disposal.

2.2.1.2 Ozonation

Ozone is very good oxidizing agent which can selectively oxidize aromatic structures and unsaturated bonds (–C=C– or –N=N–) (Adams and Gorg, 2002). Chromophore groups in the dyes are generally organic compounds with conjugated double bonds that can be broken down forming smaller molecules, resulting in reduced coloration. Oxidation by ozone is capable of degrading chlorinated hydrocarbons, pesticides, phenols and aromatic hydrocarbons (Xu and Lebrun, 1999). A disadvantage of ozonation is its short half-life, typically being 20 min or less with stability being affected by the presence of salts, pH, and temperature. Moreover, the operation cost is expensive

2.2.1.3 Sodium hypochloride (NaOCl)

The reagent react with the amino group of the dye molecule. It initiates and accelerates azo bond cleavage. An increase in decolouration is seen with an increase in chlorine concentration and decreasing pH of the medium (Robinson et al., 2001). The use of chlorine for dye removal is becoming less frequent due to the release of aromatic amines or toxic molecules into waterways (Slokar and Le Marechal, 1997).

2.2.1.4 Electro chemical oxidation

This method is cheap and efficient for dye removal. Electrochemical processes usually have low temperature requirement and do not require any additional chemicals (Kim et al., 2002). The process of electrochemical oxidation is relatively nonspecific which is, applicable to a large variety of contaminants. The breakdown metabolites are not hazardous. So, this method is safe for treated effuent to be released back into environment.

2.2.2 Coagulation and precipitation

The coagulants such as hydrolyzing metal salts of iron and aluminium are usually used to promote the formation of aggregates in effluent and reduce the concentration of colorants. The advantage of this technique is short detention time. Major disadvantage of coagulation and precipitation are high cost of chemicals, disposing problem of generated sludge and high concentration of residual cation that remains in the supernatant (Kartikeyan, 1990). The color removal was accomplished by aggregation/precipitation and adsorption of coloring substances onto the coagulant species and onto hydrated flocks (Kartikeyan, 1990; Alinsafi et al., 2005).

2.2.3 Cucurbituril

Cucurbituril is a cyclic polymer of glycoluril and formaldehyde. Cucurbituril can be used as an aqueous and solid state. This method can be applied with all the dye classes (disperse, reactive, basic and acid,). However, cost is the limitations of this method. (Robinson et al., 2001)

Table II-3. Advantage and limitations of various decolorization methods for industrial effuents (Anjaneyulu et al., 2005)

Treatment methodology	Stage of treatment	Type of industry	Advantages	Limitations
Available methods				
I. Physical methods				
1. Adsorption				
a. Activated carbon	Pre/post treatment	Textile/tannery/brewery	Economically attractive. Good removal efficiency	Cost intensive regeneration process
b. Baggasse	Pre treatment	Sugar/brewary	Waste to treat another waste	Post treatment disposal
c. Peat	Pre treatment	Any industry as post or pretreatment	Effective adsorbent due to cellular structure. No activation required	Surface area is lower than activated carbon
d. Wood chips	Pre treatment	Any industry as post or pretreatment	Good sorption for specific colourant	Larger contact times and huge quantities are required
2. Irradiation	Post treatment	Kraftmill/Tannery/distillary/pulp and paper	Effective removal for a wide range of colourants at low volumes	Dissolved oxygen requirement is high. Ineffective for light resistant colourants
3. Ion-exchange	Main treatment	Any industry	Regeneration with low loss of adsorbents	Specific application
II. Chemical methods				
1. Oxidation			Effective for both soluble and insoluble colourants	Problem with sludge disposal
a. Fenton's reagent	Pre/main treatment	Textile/tannery/pulp and paper	Capable of decolourizing wide variety of wastes. No alternation in volume	Prohibitively expensive
b. Ozonation	Main treatmeat	Textile/tannery/brewary/distillary	Effective for azodye removal	Not suitable for dispersed dyes. Releases aromatic amines
c. Sodium hypochlorite	Post treatment	Brewary/distillary	Low temperature requirement.	Cost intensive process
d. Electrochemical oxidation	Pre treatment	Kraftmill/distillary	No additional chemicals required and end products are non-hazardous	
2. Coagulation and precipitation	Pre/main treatment	Any industry	Short detention time and low capital costs. Good removal efficiencies	High cost of chemicals for pH adjustment. Dewatering and sludge handling problems
3. Cucurbituril	Post treatment	Sugar/pulp and paper/kraftmill	Complete decolourization for all class of dyes	Expensive

2.3 Biological treatment

2.3.1 Decolorization by fungi

The ability of fungi to degrade a wide range of dyes is widely documented. Although early studies focused on the fungus *Phanerochaete*, in recent years fungi such as *T. versicolor, Bjerkandera, Pleurotus eryngii* and *Clitocybula dusenii* have been tested for the decolorization of wastewaters (Heinfling et al., 1998, Robinnson et al., 2001). A list of the decolorization studied by *T. versicolor* is shown in Table II-4.

Table II-4 Decolorization of dyes by *Trametes versicolor* strains in liquid solution.

Strain	Dyes	Culture time	Decolorization mode	condition	Percent decolorization	References
T. versicolor (MTCC 138)	Reactive Orange-16 (660 mg/L) Reactive Red-35 (680 g/L)	4 days 4 days	Whole fungal Whole fungal	25-30°C, 180 rpm, pH4.5 25-30°C, 180 rpm, pH4.5	94.5 (7 days) 90.7 (7 days)	Srinivasan and Murthy, 2009
T. versicolor (CBS100.29)	Indigo Carmine (60 μM)	22-29 days	purify isoenzyme	30°C, pH 4.5	~65% by LccI ~15% by LccII (90 min)	Lorenzo et al., 2006
T. versicolor (ATCC 20869)	Amaranth (50 ppm) Tropaeolin O (20 ppm) Remazol Black B (60 ppm) Remazol Orange (60 ppm) Reactive Blue (20 ppm)	9 days 9 days 9 days 9 days 9 days	Whole fungal Whole fungal Whole fungal Whole fungal Whole fungal	30°C, 200 rpm, pH5 30°C, 200 rpm, pH5 30°C, 200 rpm, pH5 30°C, 200 rpm, pH5 30°C, 200 rpm, pH5	100% (24 h) 100% (24 h) 100% (24 h) 100% (24 h) 100% (24 h)	Swamy and Ramsay, 1999
*T. versicolor (*CCT-4521)	RBBR (50mg/L) Reactive Red 251 (50mg/L) Reactive Orange 122 (50mg/L) Reactive Black B (50mg/L)	20 days 20 days 20 days 20 days 20 days	immobilized laccase immobilized laccase immobilized laccase immobilized laccase	28°C 28°C 28°C 28°C	45% (30 min) 30% (30 min) 45% (30 min) 10% (30 min)	Peralta-Zamora et al., 2003
*T. versicolor (*Institut of Forestbotanisches, Gottingen, Germany)	Reactive Black (40ppm) Reactive Red152 (80ppm) Reactive Golden Yellow R (80ppm) Procion Red (80ppm) Reactive Violet 5 (80ppm) Reactive Blue 28 (80ppm) Ponceau Red 4R	0 day 0 day 0 day 0 day 0 day 0 day	Whole fungal Whole fungal Whole fungal Whole fungal Whole fungal Whole fungal	26°C, 150 rpm 26°C, 150 rpm 26°C, 150 rpm 26°C, 150 rpm 26°C, 150 rpm 26°C, 150 rpm	100% (5days) 14% (6 days) 100% (5 days) 100% (4 days) ~15% (6 days)	Keharia and Maramwar, 2002

	(80ppm) Novatic Blue BC S/D (80ppm)	0 day 0 day 0 day	Whole fungal Whole fungal Whole fungal	26°C, 150 rpm 26°C, 150 rpm 26°C, 150 rpm	100%, (4 days) 100% (4 days) ~5% (6 days)	
T. versicolor (ATCC 20869)	Amaranth (33mg/L) Tropaeolin O (53mg/L) Congo Red (30.5mg/L) Reactive Blue 15 (29.5mg/L) Reactive Black 5 (23.5mg/L) Cibacron Brilliant Red 3G-P (48mg/L) Cibacron Brilliant Yellow 3BA (34.5mg/L) Remazol Brilliant Blue R (30mg/L)	7 days 7 days 7 days 7 days 7 days 7 days 7 dqys 7 dqys	Fungal pellets Fungal pellets Fungal pellets Fungal pellets Fungal pellets Fungal pellets Fungal pellets Fungal pellets	22°C, 200 rpm 22°C, 200 rpm 22°C, 200 rpm 22°C, 200 rpm 22°C, 200 rpm 22°C, 200 rpm 22°C, 200 rpm 22°C, 200 rpm	100% (3.5 h) 100% (24.8 h) 100% (22 h) 100% (20 h) 100% (48 h) 89% (32.5h) 69% (97h) 30% (8h)	Ramsay and Nguyen, 2002
T. versicolor (provided by Dr. A. Mswaka, University of Zimbabwe, Zimbabwe)	Acid Blue 74 (31.25 mg/L) Acid Blue 225 (31.25 mg/L) Acid Violet17 (31.25 mg/L) Basic Red 9 base (31.25 mg/L) Direct Blue 71 (31.25 mg/L) Reactive Black 5 (31.25 mg/L) Reactive Blue 19 (31.25 mg/L) Reactive Blue 221(31.25 mg/L)	9 days. 9 days 9 days 9 days 9 days 9 days 9 days 9days	Crude enzmyme Crude enzmyme Crude enzmyme Crude enzmyme Crude enzmyme Crude enzmyme Crude enzmyme Crude enzmyme	pH4.5 , 90 rpm, 50°C pH4.5 , 90 rpm, 50°C pH4 , 90 rpm, 50°C pH4.5 , 90 rpm, 50°C pH4 , 90 rpm, 50°C pH4.5 , 90 rpm, 50°C pH4.5 , 90 rpm, 50°C pH4.5 , 90 rpm, 50°C	58% (3 h) 48% (6 h) 54% (6 h) 65% (6 h) 52% (6 h) 18% (6 h) 98% (6 h) 100% (3 h)	Nyanhongo et al., 2001
T. versicolor (DSM 11309)	Reactive Violet 5 Reactive Blue 38 (200mg/L)	2 days 2 days	Dye mycelial Dye mycelial	28°C, 70rpm 28°C, 70rpm	95% (6 days) 95% (4 days)	Heinfling et al., 1997
T. versicolor (CNPR 8107)	Remazol Blue RR (150mg/L) Remazol Red RR (150mg/L) Remazol Yellowi RRi (150mg/L)	3 days 3 days 3 days	Whole fungal Whole fungal Whole fungal	25°C, 60rpm 25°C, 60rpm 25°C, 60rpm	95% (5 days) 50% (5 days) 50% (5 days)	Toh et al., 2003

Removal of dyes by fungi occurs as a result of both adsorption by biomass pellets and enzymatic degradation. Adsorption studies were conducted on malachite green, basic blue and fast red by using biomass of the fungus *A. niger*. The result showed that 98% of malachite green, basic blue and fast red was removed (Anjaneyulu and Bindu 1997, 2001a). However, treatments with whole cells, either alive or dead, also show some drawbacks and in particular in the scale-up process and fungal biomass treatment (Zeng et al., 2011). Also, biosorption by yeast can be occured. Aksu and Dönmez (2003) found that dried yeast (*S. cerevisiae, Schizosaccharomyces pombe, Kluyveromyces marxianus*, *Candida* spp., *C. tropicalis, C. lipolytica, C. utilis, C. quilliermendii and C. membranaefaciens*) showed very high dye (Remazol Blue) sorption at 100 mg/l initial dye concentration. Among these, *C. lipolytica* showed the maximum biosorption capacity at pH 2 binding 173.1 mg dye/g dry biomass. Unfortually, biosorption of the dye was maximum at pH 2 and then declined sharply with further increase in pH for all yeast species which limit the decolorization of effluents at pH higher than 2.

For enzymatic degradation, the low specificity of the lignin-degrading enzymes (lignin peroxidase, Mn peroxidase and laccase) also suggests that they may be suitable for treating the range of dyes in textile effluents. The disadvantage of this method is culture maintenance cost (see herafter).

2.3.2 Decolorization by bacteria

Numerous bacteria capable of dye decolorization have been reported such as *Bacillus subtilis, Bacillus cereus, Aeromonas hydrophilia, Klebsiella pneumonia* and *Acetobacter liquefaciens*. These bacteria were considered as suitable for future application in azo dye decolorization. Drawback of requiring a fermentation process limits the applicability of this technique to cope with large volumn of effuents. Moreover, under anaerobic condition, bacteria reduce azo dyes generates amines which are mutagenic or carcinogenic (Kulla et al., 1983 and Zimmerman et al., 1982).

2.3.3 Decolorization by enzyme

Phenoloxidase-lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase, which act unspecifically on aromatic rings, showed high potential to degrade a wide range of aromatic ring including dyes. These enzymes are produced by white-rot fungi and involved in the degradation of lignin (Thurston, 1994). Abadulla et al. (2000) reported that only laccase or the mixture of enzyme (laccase, Lip and MnP) preparation from *Pleurotus ostreatus*, *Schizophyllum commune*, *Neurospora crassa*, *Trametes villosa* and *Myceliophtora thermophila* can efficiently decolorize several classes of dye including azo, triarylmethane, anthraquinone and indigoid dyes.

The efficiency of LiP from another specie, *Phanerochaete chrysosporum, have been* report to decolorize 80% for Porocion Brilliant Blue HGR and 83% for Ranocid Fast Blue after 1 hour in the presence of hydrogen peroxide and veratryl alcohol(Verma and Madamwar, 2002). More recently, Champagne et al (2005) reported that purified MnP from *T. versicolor* culture able to decolorize azo dyes (Amaranth, Reactive black 5 and Cibacron brilliant yellow) in the presence of manganese. The decolorization rate can increase by addition of hydrogen peroxide. However, the enzyme cannot decolorize anthraquinone dye (RBBR).

2.4 Transformation of synthetic dyes by laccase

In most countries, environmental regulations require that wastewater must be decolorized before its discharge. This has led to the necessity of finding a friendly treatment technologies to complement the conventional ones. Most currently existing processes to treat dye containing wastewater are ineffective and not economical. Therefore, the development of processes based on laccases either crude or purified extracellular enzyme preparations seems an attractive solution due to their potential in degrading dyes of diverse chemical structure (Hou et al., 2004). In particular, decolorization by crude enzyme filtrates has many advantages. The production process is not expensive and allows a separate dye decolorization step from fungal growth and enzyme production, thus eliminating the problem of any fungal growth inhibition by the dye molecules (Papinutti et al., 2008; Zeng et al., 2011). In particular, laccases that catalyze the four-electron reduction of O_2 to water coupled

with the oxidation of phenolic compounds have been shown to be efficient in degrading various dye molecules (Lu et al., 2007; Yang et al., 2009).

The mechanism of decoloration of azo dyes by laccase is not yet completely understood. Soares et al. (2001) proposed hypothetical mechanism for the biotransformation of phenolic di-azo dyes (dye IV) (Figure II-1). The biodegradation of dye IV was studied using purified laccase from *A. niger* (Novo Nordisk A/S). Dye IV which had a relatively weak electron-donating carboxylic group in the *meta* position of the phenolic moiety was totally decolorized by laccase. They are suggested that decolorization proceeds via a one-electron oxidation. This leads to the formation of a phenoxy radical which is in turn oxidized by laccase to produce a carbonium ion in which the charge is localized on the phenolic ring carbon with the azo linkage.

Abadulla et al. (2000) reported that purified laccase from *T. versicolor* was able to degrade triarylmethane, indigoid, azo and anthraquinonic dyes used in dyeing textiles. However, the problems to commercialise the use of laccase are the cost of enzyme and the lack of sufficient enzyme stocks. The production of laccase by the fungal is also time consuming and required the induction by toxic phenolic compound. Thus, effort have to be made in order to achieve cheap overproduction of laccase in safe heterologous hosts which can produce the enzyme in a short time and also their modification by protein engineering to obtain more robust and active enzyme.

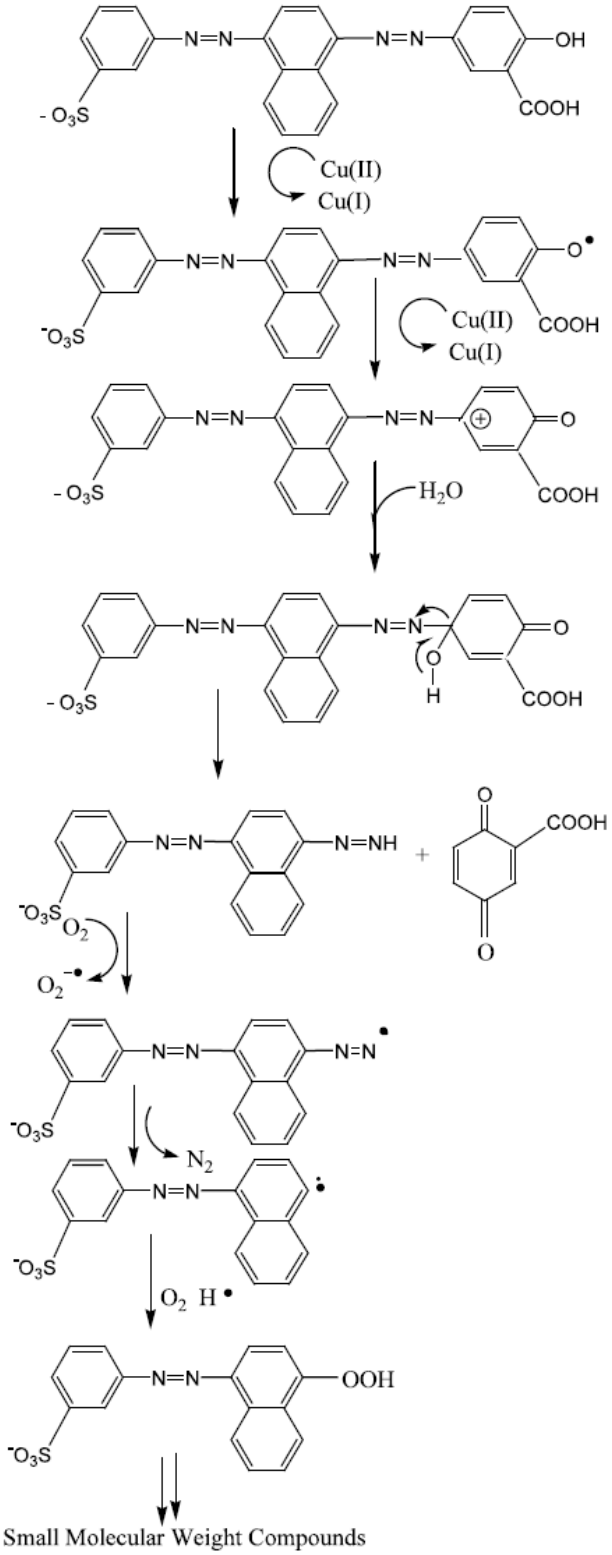


Figure II-1 Hypothetical mechanism for the biotransformation of dye IV (azo dye) (Soares et al., 2001).

PART II : Laccases –powerful enzymes for industrial application

1. Origin and general properties of laccase

Laccase (benzenediol: oxygen oxidoreductase; p-diphenol oxidase EC 1.10.3.2), a blue copper containing oxidase, was first discovered by Yoshida (1883) as a component of the resin ducts of the Chinese or Japanese lacquer trees (*Rhus vernicifera*). Data on other higher plant laccase are scarce. To the present, more than a hundred laccases have been found and characterized. The majority of laccases were isolated from white rot fungi, whereas plant laccase are much less studied. Fungal laccases were discovered by Bertrand and Laborde in 1896 (Kunamneni et al., 2007). Laccases are thought to be nearly ubiquitous among fungi, actually the presence of laccases have been documented in virtually every fungus examined for it.

Laccases are produced in multiple isoforms depending on the fungal species and environmental conditions (conditions of cultivation and the presence of an inducer in the medium). Fungal laccases often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular mass of the monomer ranges from about 50 to 130 kDa. Some characteristic of laccases isolated from different sources are showed in Table II-5. Laccases are glycoproteins. The carbohydrate moiety of the majority of laccases consists of mannose, galactose and *N*-acetylglucosamine, and constitutes about 45% of the protein mass in laccases of plant origin. Fungal laccases have lower carbohydrate contents (10-20%). The carbohydrate moiety may contribute to the high stability of the enzymes. Moreover, it has also been supposed to protect the enzyme against proteolysis and inactivation by free radicals (Morozova et al., 2007a, 2007b). Laccases generally are more stable at alkaline pH than at acidic pH, probably due to the OH^- inhibition of autooxidation (Stoilova et al., 2010).

Table II-5 Characteristic of some laccases isolated from different sources (Morozova et al., 2007a).

Laccase	M_r, kD	Carbohydrates, %	p*I*	t_{opt}, °C	$T^{*}_{1/2}$, h
1	2	3	4	5	6
Plant laccases					
Rhus vernicifera [8]	110	45			
Rhus succedanea [8]	130				
Acer pseudoplatanus [10]	97	40-45			
Pinus taeda [8]	90	22			
Liriodendron tulipifera [14]	61		9.3-9.5		
Fungal laccases					
Agaricus bisporus [45]	65	15			
Agaricus blazei [46]	66		4.0	20	
Basidiomycete PM1 [47]	64	6.5	3.6	80	
Botrytis cinerea [48]	74	49	4.0	60	
Ceriporiopsis subvermispora [49] L1	71	15	3.4		$T^{60}_{1/2} = 2$
L2	68	10	4.8		$T^{60}_{1/2} = 1$
Cerrena maxima [50]	67	13	3.5		$T^{50}_{1/2} = 52$
Cerrena maxima [51]	57	13	3.5	50	$T^{40}_{1/2} = 12$
Cerrena unicolor [52]	66		3.95		$T^{60}_{1/2} = 2.5$
Cerrena unicolor 0784 [53]	56	23	3.75		
Cerrena unicolor 137 [54] LaccI	64		3.6	60	
LaccII	57		3.7	60	
Chaetomium thermophilum [19]	77		5.1	60	$T^{50}_{1/2} = 12$
Coprinus cinereus [55]	58		4.0	60	
Coriolopsis fulvocinerea [56]	54	32	3.5		
Coriolopsis fulvocinerea [50]	65	32	3.5		$T^{50}_{1/2} = 64$
Coriolopsis gallica [57]	84	21	4.3	70	$T^{60}_{1/2} = 192$
Coriolopsis rigida [58]	66	9	3.9		
Coriolus hirsutus [59]	73	11	7.4	45	
Coriolus hirsutus [56]	55	12	4.0		$T^{50}_{1/2} = 72$
Coriolus zonatus [60]	60	10	4.6	55	
Daedalea quercina [61]	69		3.0	60	$T^{65}_{1/2} = 0.5$
Gaeumannomyces graminis [22]	190	12	5.6		
Magnaporthe grisea [62]	70		6.0	30	
Marasmius quercophilus [63]	63	12	3.6	75	
Mauginiella sp. [64]	63	5-7	4.8-6.4		$T^{60}_{1/2} = 0.7$
Melanocarpus albomyces [65]	80		4.0	60-70	$T^{60}_{1/2} = 5$
Neurospora crassa [66]	65	11			$T^{50}_{1/2} = 72$
Panus rudis [67]	58	8	3.5	60	
Panus tigrinus [68]	69.1	6.9	3.15	55	$T^{60}_{1/2} = 0.4$
Phellinus ribis [69]	152	28			
Pleurotus eryngii [70] I	65	7	4.1	65	$T^{50}_{1/2} = 0.5$
II	61	1	4.2	55	$T^{55}_{1/2} = 0.5$

1	2	3	4	5	6
Pleurotus ostreatus [71] POXA1b	62	3	6.9	20-50	$T_{12}^{60} = 3$
Pleurotus ostreatus [72] POXA1w	61	9	6.7	45-65	$T_{12}^{60} = 3.3$
Pleurotus ostreatus [72] POXA2	67		4.0	25-35	$T_{12}^{60} = 0.2$
Pleurotus ostreatus [73] POXA3a	83-85		4.1	35	$T_{12}^{40} = 6$
Pleurotus ostreatus [73] POXA3b	83-85		4.3	35	$T_{12}^{40} = 14$
Pleurotus ostreatus [72] POXC	59	5	3.3	50-60	$T_{12}^{60} = 0.5$
Polyporus versicolor [1] A	64.4	14	3.0-3.2		
B	64.7	10	4.6-6.7		
Pycnoporus cinnabarinus [74]	81	9	3.7		$T_{12}^{70} = 1$
Pycnoporus cinnabarinus [75]	63	11	3.0		$T_{12}^{80} = 2$
Pycnoporus sanguineus [76]	58		6.7	55	$T_{12}^{75} = 3$
Rhizoctonia solani [77]	130	10	7.5		
Rigidoporus lignosus [78]	54		3.2	40	$T_{12}^{65} = 0.5$
Sclerotium rolfsii [79]	55		5.2	62	$T_{12}^{60} = 1$
Trametes gallica [80] Lac I	60	3.6	3.1	70	
Lac II	60	4	3.0	70	
Trametes hirsuta [50]	70	12	4.2		$T_{12}^{50} = 65$
Trametes ochracea [50]	64	10	4.7		$T_{12}^{50} = 56$
Trametes pubescens [81] Lc1	67	13	5.1		
Lc2	67	13	5.3		
Trametes pubescens [82]	65	18	2.6		
Trametes versicolor [8] I	67	10-12			
II	70				
Trametes villosa [83] 1	130	14	3.5		
3	126	7	6.5		
Trametes sp. C30 [84]	65		3.2		
Trametes sp. AH28-2 [85]	62	11-12	4.2		$T_{12}^{75} = 0.5$
Trichophyton rubrum [86]	65		4.0		$T_{12}^{60} = 0.25$

* Half-inactivation time of enzyme at the temperature indicated.

2. Structure of laccase

For the catalytic activity, a minimum of four copper atoms per active protein unit is needed. These copper sites in laccases are categorized into three groups, Type-1 or blue copper center, Type-2 or normal copper and Type-3 or coupled binuclear copper centers (Figure II-2). The four copper atoms are differing in their characteristic electronic paramagnetic resonance (EPR) signals (Bertrand et al., 2002, Dwivedi et al., 2011).

Type 1 copper has a trigonal coordination, with two histines and a cysteine as conserved equatorial ligands and one position usually variable. This axial ligand is methionine in the bacterial (CotA) and phenylalanine or leucine in fungal laccases.

This possibly axial position ligand provides the mechanism for regulating its activity. Modifications in the axial ligand of T1 (replace methionine by phenylalanine or leucine) led to an increase of redox potential 0.06-0.1 V compared to the wild type (Durão et al., 2006). The redox potential is directly related to how good a laccase will catalyze the reactions. Type 1 copper is the site where substrate oxidation takes place. Base on the T1 site redox potential, laccases are subdivided onto high, medium and low oxidation potential enzyme. Fungal laccases have higher reduction potentials for the copper sites compared with those from other organisms. The T1 site potential is known for many laccases (Table II-6). A higher redox potential of laccase at type 1 site or a lower oxidation potential of substrate often results in a higher rate for substrate oxidation (Gianfreda et al., 1999). The intense electronic absorption band around 600 nm caused by the covalent copper–cysteine bond of type 1 copper gives its typical blue color to the enzyme (Claus, 2004; Morozova et al., 2007).

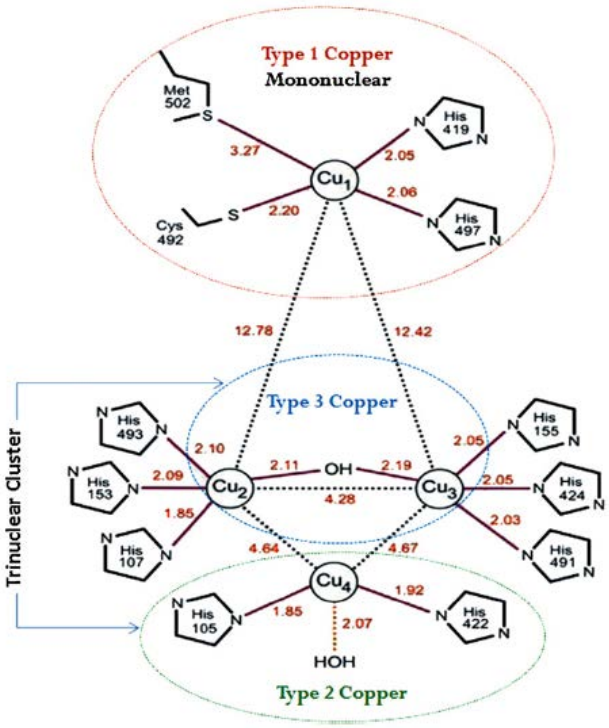


Figure II-2. Schematic representation of copper coordination centers, including interatomic distances among all relevant ligands of the laccase from *B. subtilis* (Claus, 2004)

Table II-6 Potentials of the T1 site of some laccases (Morozova et al., 2007a)

Laccase	Potential of the T1 site, mV (vs. NHE)
High-potential	
Trametes trogii [135]	790
Trametes ochracea [50]	790
Trametes hirsuta [50]	780
Trametes villosa [136]	780
Trametes versicolor [137]	780
Coriolopsis fulvocinerea [50]	780
Cerrena maxima [60]	750
Trametes pubescens LAC1 [138]	746
Pleurotus ostreatus POXC [135]	740
Trametes pubescens LAC1 [138]	738
Basidiomycete C30 Lac1 [84]	730
Rigidoporus lignosus B [135]	730
Medium-potential	
Rhizoctonia solani [136]	710
Rigidoporus lignosus D [135]	700
Pleurotus ostreatus POXA1b [135]	650
Basidiomycete C30 Lac2 [84]	560
Coprinus cinereus [55]	550
Trichophyton rubrum [86]	540
Basidiomycete C30 Lac [139]	530
Scytalidium thermophilum [140]	510
Myceliophthora thermophila [136]	470
Low-potential	
Rhus vernicifera [137]	430
Ascorbate oxidase [140]	

Type 2 copper has a characteristic electron paramagnetic resonance (EPR). This copper has two histidines and water as ligands. Type-2 copper shows no absorption in the visible spectrum and is positioned close to the Type-3 copper. The pair of strongly coupled type 3 coppers is EPR-silent in the presence of dioxygen as the result of the strongly anti-ferromagnetic coupling of the copper pair bridged by a hydroxide. Each of the Type-3 copper is coordinates with three histidines. Type-3 copper shows an electron absorption at 330 nm. The mononuclear T1 site extracts electrons from the reducing substrate and mediates their transfer to the trinuclear T2/T3 center where molecular oxygen is reduced (Bertrand et al., 2002, Dwivedi et al., 2011). In the enzymes, the twelve amino acid residues serving as the copper

ligands are housed within these conserved regions. The amino acid ligands of the trinuclear cluster are eight histines. These histidines occur in a conserved pattern of four HXH motifs. One of this motifs, X is the cysteine bound to the T1 copper while each of the histines is bound to one of the two type 3 coppers (Claus, 2004).

3. Reaction mechanism

To perform catalytic function, laccase depends on Cu atoms which are distributed at the three copper centers as described above. Laccase withdraws the electron from the substrates and converts them in free radicals that can be polymerized. After receiving four electrons, the laccase donates them to molecular oxygen to form water molecule (Figure II-3 – II-4). The reaction did not produce any peroxide intermediates. Overall, there are three major steps (Dwivedi et al., 2011; Giardina et al., 2010).

1 Type-1 Cu is reduced by reducing substrate.

2 The electrons are transferred through the highly conserved His–Cys–His tripeptide to Type-2 and Type-3 Cu trinuclear cluster.

3 O_2 is reduced to water at Type-2 and Type-3 Cu (Dwivedi et al., 2011).

The overall reaction and the catalytic cycle are as follows:

$$4RH + O_2 \rightarrow 4R\bullet + 2H_2O$$

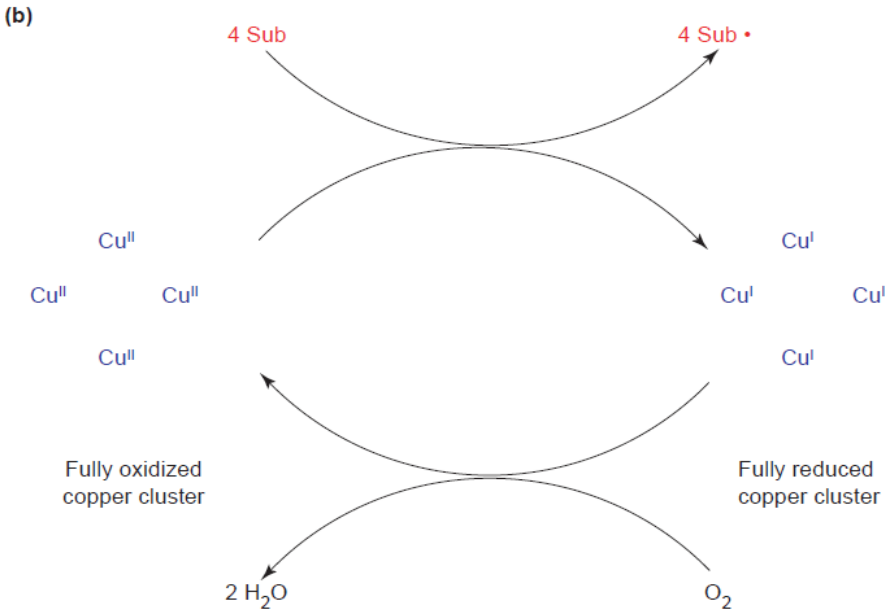


Figure II-3. Schematic representation of a laccase catalytic cycle. The reaction produces two molecules of water from the reduction of one molecule of molecular oxygen and the concomitant oxidation of four substrate molecules to the corresponding radicals. Sub: substrate molecule; Sub•: oxidized substrate radicals (Riva, 2006).

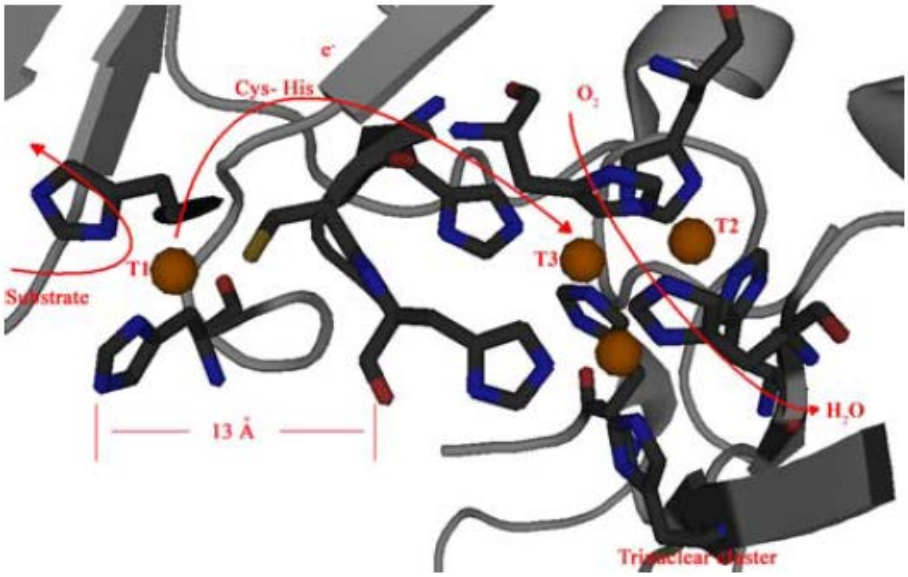


Figure II-4. The structure of the laccase active site with arrows marking the flow of substrates, electrons (e^-) and O_2 (Giardina et al., 2010).

In brief, laccase use oxygen to oxidize aromatic and non-aromatic hydrogen donor via a mechanism involving radical that can undergo further laccase catalyzed reactions or non-enzymatic reactions. Oxidation by laccase of phenolic substrates leads to the formation of an aryloxyradical, an active species that can convert to quinone. The quinone intermediates can spontaneously react with each other to form oligomers. Many non-enzymatic reactions can happen after the oxidation by laccase as describe below.

3.1. Cross-linking of monomers

Phenolic compounds oxidation of by laccases generate radicals which react with each other to form oligomers, polymers or dimers, covalently coupled by C–C, C–N and C–O bonds. Phenolics compounds or aromatic amines in soil can thus be bound to the organic humic matrix. For substituted compounds, the reaction can be accompanied by partial demethylations and dehalogenations (Claus and Filip, 1998; Durán and Esposito, 2000).

3.2. Degradation of polymers

Laccases are involved in the degradation of complex natural polymers, such as humic acids and lignin. The reactive radicals generated, lead to the cleavage of covalent bonds and to the release of monomers (Claus and Filip, 1998).

3.3. Ring cleavage of aromatics

Laccase catalyzed ring-cleavage of aromatic compounds has been reported in many cases. This reaction is very useful for degrade xenobiotics compounds such as synthetic dyes and nitroaromatics (Durán and Esposito, 2000; Claus et al., 2002).

4. Laccase substrate

Natural substrates of laccase are phenolic compounds such as *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines and aryl diamines. The efficiency of oxidation by a particular laccase is influenced by the nature and position of substituents on the phenolic ring. These phenolic compounds are typical substrates for laccase due to their low redox potential (0.5-1 V). The oxidation of these molecules is represented in Figure II-5 and Figure II-6. Here, laccase oxidizes the molecule with a simultaneous radical formation, which can spontaneously rearrange to cleave the aromatic rings or promote their polymerization (Fernández, 2011). The other nonphenolic and some phenolic compounds might have a higher redox potential, which determines the low efficiency of laccase towards the substrate. Laccase can also oxidize lignins, some inorganic ions, some polycyclic aromatic hydrocarbons (PAHs), organophosphorus pesticides and dyes (Couto and Herrera, 2006) (Figure II-6).

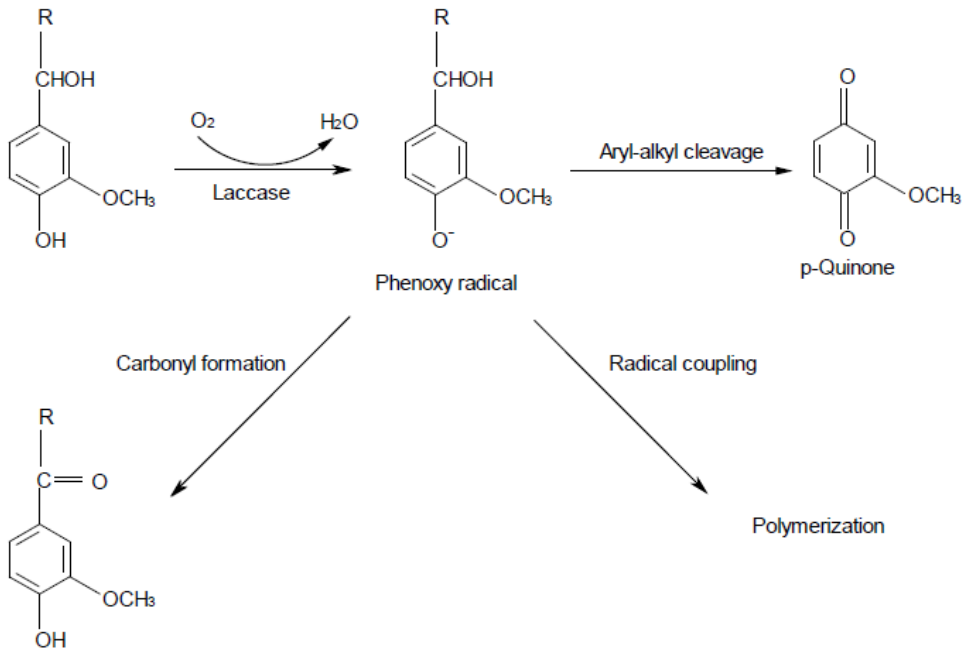


Figure II-5. Oxidation of a phenolic compound (natural substrate) by laccase (Fernández, 2011).

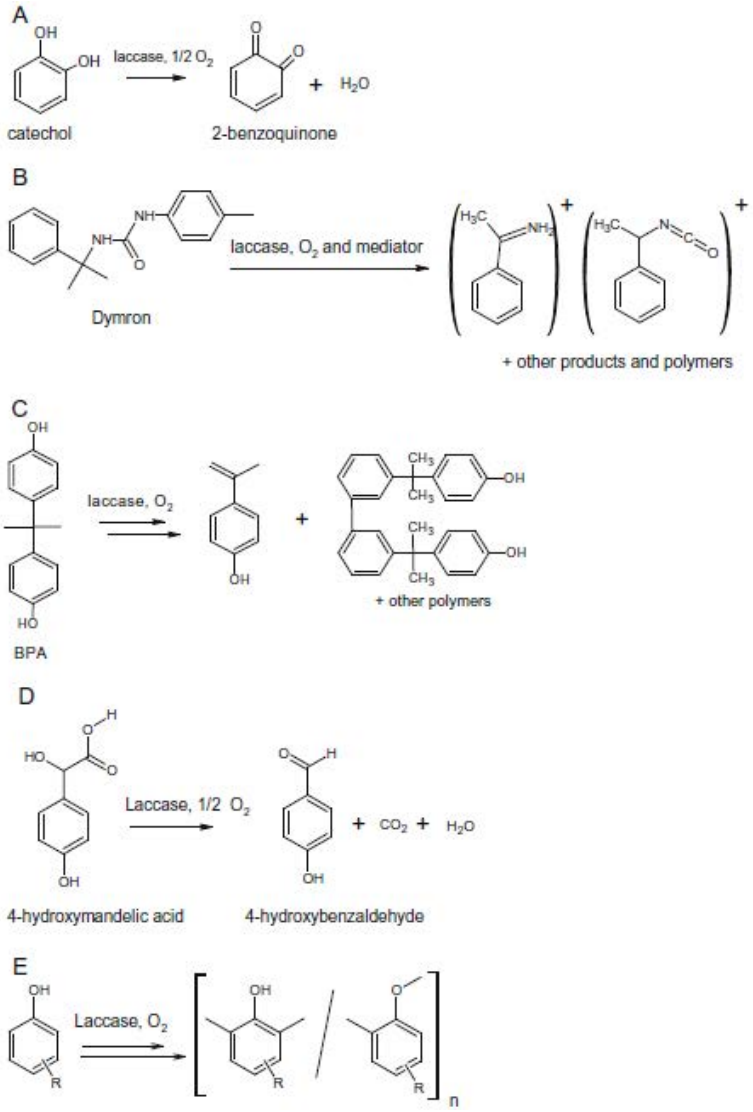


Figure II-6. Example of laccase-catalysed oxidation. (A) Catechol, a typical substrate; (B) oxidation products from the herbicide Dymron; (C) Major by-products of bisphenol A (BPA) oxidation; (D) Example of oxidative decarboxylation; (E) Oxidative polymerization of phenol derivatives in aqueous organic solvents forms polymers of phenylene and oxyphenylene units (Majeau et al., 2010).

5. Laccase mediator system

Although, laccase can oxidase broad substrates range, however some substrates cannot be oxidized directly by this enzyme, either because they are too big

to penetrate into the active site of the enzyme or because they have a high redox potential. In this case, it is possible to add the mediators to overcome the limitation.

Mediators are the compounds which act as intermediate substrates for laccase, whose oxidized radical forms are able to interact with the bulky or high redox-potential substrate (Riva, 2006). Mediators have the capacity to change the redox potential during the oxidation i.e., from 0.78 V to 1.084 V (Zille et al., 2004; Bourbonnais et al., 1998). Furthermore, the mediator acts as a diffusible electron carrier enabling the oxidation of high molecular weight polymers. The steric issues that hinder the direct interaction between laccase and polymer are overcome by the action of the redox mediator. Consequently, the oxidation of non-natural substrates and/or with high redox potentials is possible (Kunamneni et al., 2008b).

Now, the mechanism by which redox mediators play a role in the reactions catalyzed by the enzyme is well characterized. Laccase oxidizes the redox mediator, which in turn either does a one-electron oxidation of the substrate to a radical cation or it abstracts an H-atom from the substrate converting it into a radical (Fabbrini et al., 2002 a&b). A schematic representation of laccase-catalyzed redox cycles for substrate oxidation in the absence (a) and presence (b) is shown in Figure II-7. Several organic and inorganic compounds have been reported as effective mediators (Table II-7). The use of naturally-occurring mediators would present economic and environmental advantages. Camarero (2005) reported natural mediators such as acetosyringone and syringaldehyde represented high efficiency and ecofriendly alternative to synthetic mediator to degrade different type of dyes and some recalcitrant compounds by laccase.

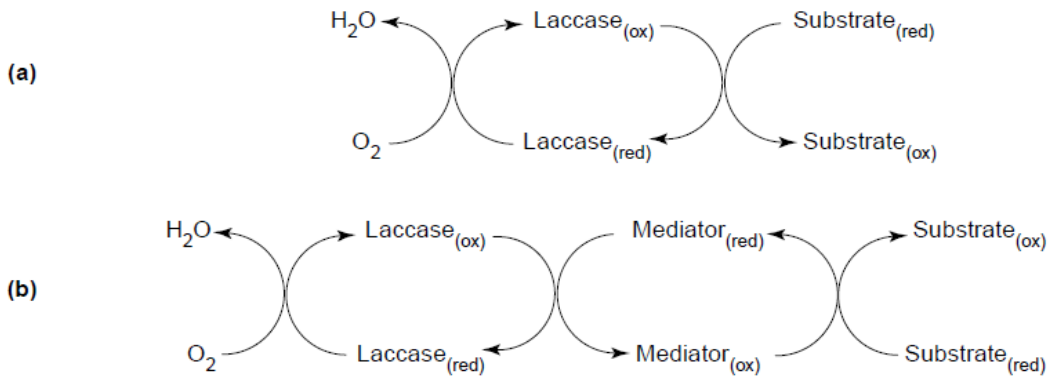
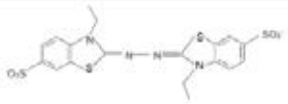
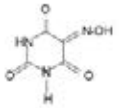
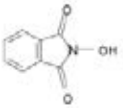
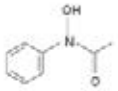
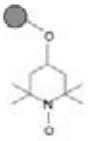
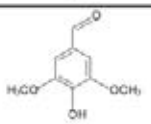
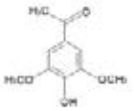
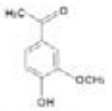
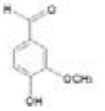
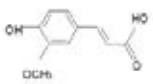
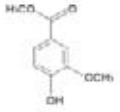


Figure II-7. Schematic representation of laccase-catalyzed redox cycles for substrates oxidation in the absence (a) or in the presence of mediator (b) (Riva, 2006).

Table II-7. Mediators used in the laccase oxidation system (Fernández, 2011)

	Mediator	Structure
Synthetic mediators	ABTS	
	HBT	
	Viorulic acid	
	HPT	
	TEMPO	
	NHA	
	PEG-TEMPO	
Natural mediators	Syringaldehyde	
	Acetosyringone	
	Acetovanillone	
	Vanillin	
	p-Coumaric acid	
	Methylvanillate	

6. Production of fungal laccase

Several fungal strains have been reported for their ability to produce laccase. The production of laccase by fungi is influenced by several parameters: the strain of the fungal, growth conditions, inducer, induction time, and composition of the medium, type of cultivation, agitation (stationary or agitated culture mode), aeration, cultivation time and genetic manipulation. However, the most critical factors are the concentration of glucose and nitrogen, and the type and concentration of the inducer (Gianfreda et al., 1999; Majeau et al., 2010). The fungi producers' laccase are showed in Table II-8. Aromatic compounds structurally related to lignin, such as xylidine, ferulic acid or veratric acid can be added to fungal cultures to increase laccase production. However, the induction level being highly sensitive to small differences in the chemical structures (Vanhulle et al., 2007).

Xiao et al., 2006 also showed that, copper ion is necessary for laccase synthesis in *Trametes* sp. AH28-2. More interestingly, they also found that different aromatic compounds can selectively induce the production of distinct laccase isozymes. For example, *o*-toluidine inducing the expression of laccase A (LacA) while 3,5-dihydroxytoluene mainly stimulating the production of laccase B (LacB).

Among basidiomycete species, *Trametes* spp, is assumed to be one of the main producers of laccases. Maximum laccase activity and parameters for laccase production by *Trametes* sp. are shown in Table II-9.

Table II-8. Fungi producers of laccase (Gianfreda et al., 1999)

.

Fungus	Class
Abortiporus biennis	Basidiomycete
Agaricus bisporus	Basidiomycete
Agaricus brunnescens	Basidiomycete
Armillaria mellea	Basidiomycete
Aspergillus nidulans	Ascomycete
Botryosphaeria sp.	Ascomycete
Botrytis cinerea	Deuteromycete
Ceriporiopsis subvermispora	Basidiomycete
Cerrena maxima	Basidiomycete
Cerrena unicolor	Basidiomycete
Chaetomium thermophile	Ascomycete
Coriolopsis occidentalis	Basidiomycete
Coriolus consicolor	Basidiomycete
Coriolus hirsutus	Basidiomycete
Coriolus verllereus	Basidiomycete
Cryphonectria parasitica	Enterobacteriaceae
Curvularia sp.	Deuteromycete
Cyathus bulleri	Basidiomycete
Daedalea flavida	Basidiomycete
Flammulina velutipes	Basidiomycete
Fomes annosus	Basidiomycete
Ganoderma lucidum PTK3	Basidiomycete
Inonus hispidus	Basidiomycete
Junghuhnia separabilima	Basidiomycete
Lentinus edodes	Basidiomycete
Lentinus tigrinus	Basidiomycete
Marine fungi	Several strains
Monocillium indicum saxena	Ascomycete
Myceliophthora thermophila	Deuteromycete
Neurospora crassa	Ascomycete
Ophiostoma novo-ulmi	Ascomycete
Panellus stipticus	Basidiomycete
Panus tigrinus	Basidiomycete
Penicillium crysogenum	Imperfect fungus
Phanerochaete chrysosporium	Basidiomycete
Phanerochaete flavido-alba	Basidiomycete
Phellinus igninarius	Basidiomycete
Phellinus torulosus	Basidiomycete
Phlebia brevispora	Basidiomycete
Phlebia ochraceofulva	Basidiomycete
Phlebia radiata	Basidiomycete
Phlebia tremellosa	Basidiomycete
Pholiota aegerita	Basidiomycete
Pholiota mutabilis	Basidiomycete
Pleurotus eryngii	Basidiomycete
Pleurotus spp.	Basidiomycete
Pleurotus ostreatus	Basidiomycete
Pleurotus pulmonarius	Basidiomycete
Pleurotus sajor-caju	Basidiomycete
Pleurotus tigrinus	Basidiomycete
Polyporus anceps	Basidiomycete
Polyporus anisoporus	Basidiomycete
Polyporus brumalis	Basidiomycete
Polyporus ciliatus	Basidiomycete
Polyporus hirsutus	Basidiomycete
Polyporus pinsitus (Trametes villosa)	Basidiomycete
Podospora anserina	Ascomycete
Pycnoporus cinnabarinus	Basidiomycete
Pycnoporus coccineus	Basidiomycete
Pycnoporus sanguineus	Basidiomycete
Rhizoctonia praticola	Deuteromycete
Rhizoctonia solani	Deuteromycete
Rigidoporus lignosus	Deuteromycete
Schizophyllum commune dikaryon	Basidiomycete
Scytalidium thermophilum	Basidiomycete
Trametes hirsuta	Basidiomycete
Trametes sanguinea	Basidiomycete
Trametes versicolor	Basidiomycete
Trichocladium canadense	Deuteromycete
Trichoderma	Deuteromycete
Tyromyces incarnatus	Basidiomycete

Table II-9. Maximum laccase activity and parameters for laccase production by *Trametes* sp. (Majeau et al., 2010)

Fungus	Culture mode	Carbon and nitrogen concentration (g/l)	Volume (L)	Inducer	Production time (h)	Maximum laccase activity obtained (U/l)
Trametes versicolor	SmF shake flask	Glucose, 20; malt extract, 5; peptone,10	0.05	2,5-xylidine 1 mM $CuSO_4 \cdot 5H_2O$ 0.4 mM	400	12,756
Trametes versicolor	SmF Stationary flask	Glucose, 5; malt extract, 3.5	0.015	2,5-xylidine 0,5 mM	NR	±5200
Trametes versicolor	SmF Airlift reactor	Glucose, 10; basal medium iii	2	2,5-xylidine 1 mM	960	1670
Trametes versicolor	SmF Shake flask	Glucose, 15; starch, 15; ye, 2.5	0.05	$CuSO_4$ 1 mM 2,5-xylidine 1 mM	240	820,000 (460,000 WX)
Trametes versicolor	SmF Airlift reactor (alginate beads)	Glucose, 10; basal medium iii	2	$CuSO_4$ 3 mM Veratryl alcohol 20 mM	NR	1500
Trametes pubescens	SmF, Fed-batch (continuous glucose fed of <0.25 g/l)	Glucose, 40; meat peptone, 10	15	$CuSO_4$ 2 mM	300	740,000
Trametes pubescens	SmF Stirred tank	Glucose, 20; ye, 5; peptone, 1	15	$CuSO_4$ 2 mM	150	61,900
Trametes trogii	SmF Static flask	Glucose, 10; asparagine, 3	0.25	Tryptophan 2 mM	960	6550
Trametes trogii	SmF Shake flask	Glucose, 10; peptone, 5; ye, 1 ammonium tartrate, 2	0.3	$CuSO_4$ 0.15 mM	120	13,000
Trametes hirsuta	Air-lift Fed-batch with glycerol 1 time	Glucose, 10; ye, 0.9	6	$CuSO_4$ 1 mM	NR	19,400

7. *T. versicolor* laccase

Trametes (Coriolus, Polyporus) versicolor belongs to the white-rot fungi and is a lignin-degrading basidiomycetes. *T. versicolor* is one of the best studied white-rot fungi. This fungus can be found in temperate and sub-tropical forests all over the world (Eriksson et al., 1990). When the fungal decays wood, it first attacks the cell wall and removes lignin from the secondary wall. Then, cellulose is rapidly degraded. Laccase and Manganese peroxidase are the major ligninolytic enzymes produced by this fungal (Stoilova et al., 2010). *T. versicolor* laccase is a secreted glycosylated enzyme with four copper ions and two disulphide bridges. Several isoforms of the laccase are secreted by *T. versicolor*. *Lcc1* (EMBL Data Library accession number X84683) is a laccase gene which has been isolated from a genomic library of *T. versicolor*. This gene contains ten introns. (Jönsson et al., 1995; Jönsson et al., 1997 and Reinhammar, 1984). *T. versicolor* also secretes a multitude of peroxidase

isoenzymes. Many peroxidase genes have recently been characterized from strain PRL572 (Jönsson and Nyman 1994; Johansson and Nyman 1996).

The crystal structure of the fungal laccase from *T. versicolor* is available. Bertrand et al (2002) and Piontek et al (2002) studied the crystal structure of a four-copper laccase from *T. versicolor* (LacIIIb) complexed with a xylidine. The crystal structure showed the Cu1 cavity is fairly wide, and it can accommodate a large variety of substrates that are not tightly buried in it. This structure represents the first high resolution of a laccase structure with an 2,5-xylidine, in the substrate binding cavity (Figure II-8 – II-9). The residues His458 and Asp206 have also been shown to be important for the interaction between the amino group of the reducing substrate and the enzyme. The ring of the His458, coordinated to the Cu1 and well conserved in all multicopper oxidases, is close to the amino group of 2,5-xylidine at a hydrogen bond distance (3.2 A°), suggesting that His458 is the entrance door of the electron during its transfer to Cu1. They also found that the amino group of 2,5-xylidine is also hydrogen bound to a terminal oxygen of theAsp206 side chain, that is located at the rear wall of the binding site. The Asp206 is highly conserved among fungal laccases from basidiomycetes, whereas glutamate can be found among ascomycetes (Giardina et al., 2010). The interaction of Asp206 with the reducing substrate has been shown to have an important role in determining the pH dependence of laccase activity.

Two types of culture techniques can be used to produce laccase from this fungus: Solid-State Fermentation (SSF) and Submerged Fermentation (SMF). The laccase produced by this fungal has been reported to be involved in the degradation of aromatic compounds. The transformation of these compounds occurs at an extracellular environment and hence do not require internalization prior to degradation (Mohamad et al., 2008).

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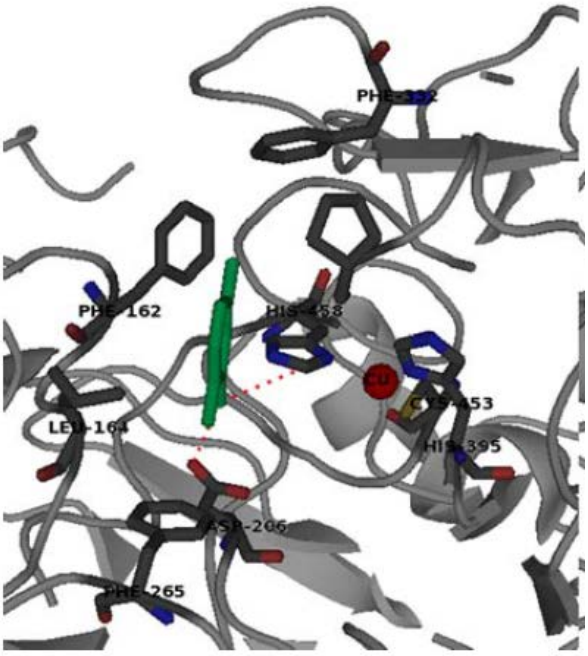


Figure II-8. Stereo view of the active site of *Trametes versicolor* laccase (TvL1KYA), which binds substrate 2,5-xylidine (in green), elaborated with PyMol from the crystallographic structure (Giardina et al., 2010).

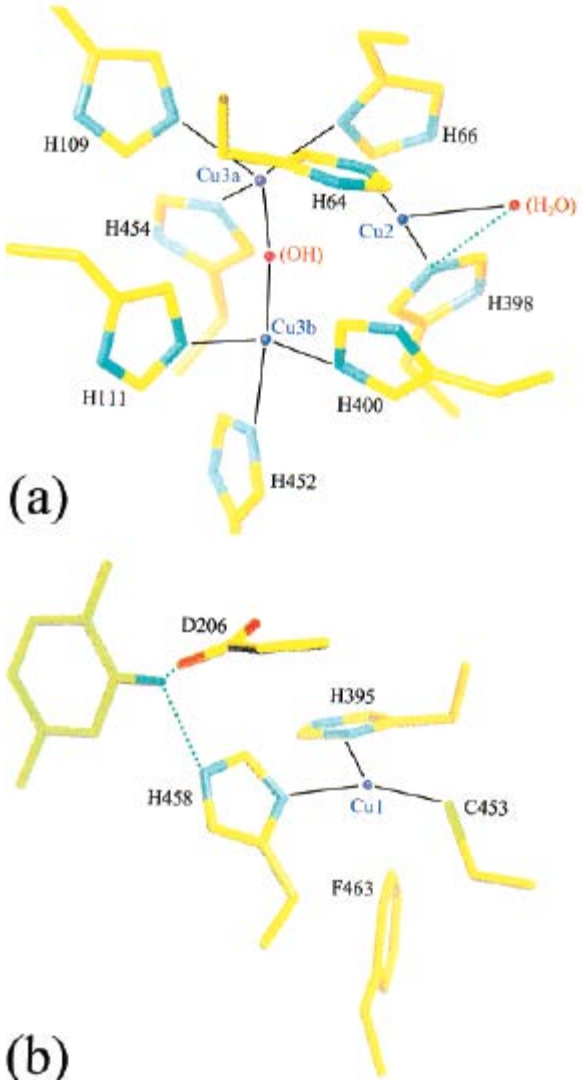


Figure II-9. The copper sites of LacIIIb and their environment. (a) The trinuclear T2/T3 site. Histidine side chains involved in copper binding (copper atoms are blue) are in sticks (C atoms are yellow, N atoms are cyan), and labeled. Oxygen atoms involved in copper cordination are red. Copper coordinations are indicated with dark lines. Dotted lines indicate interacting atoms distant from less than 3.2 Å. (b) The T1 site and the neighboring 2,5-xylidine ligand. The side chains of the copper-coordinating histidines and cysteine are drawn. Also indicated are the side chains of the noncoordinating Phe 463, and of the Asp 206 residue that interacts with 2,5-xylidine (Bertrand et al., 2002).

7.1 some properties of T. versicolor laccase

Stoilova et al (2010) studied the Properties of crude laccase from *T. versicolor* produced by solid-substrate fermentation. They found that the crude laccase established maximal activity at 45ºC. Moreover, the enzyme retained over 90% of its activity in the temperature range 40-47ºC and pH 4.5. The enzyme showed high stability in the presence of KCl, NaCl, $CaCl_2$, $MnSO_4$ and $MgSO_4$. These properties make the laccase from this fungus very attractive for genetic manipulation for further investigations as well as for its application to different biotechnology areas (Bertrand et al., 2002; Giardina et al., 2010).

The *T. versicolor lcc1* cDNA was subcloned into the *Pichia methanolica* expression vector pMETαA and transformed into the *Pichia methanolica*. The result from DNA sequencing revealed the cDNA contains 1563-bp. This gene encodes a laccase isozyme of 498 amino acid residues preceded by a 22-residue signal peptide. The molecular mass of mature laccase was 64 kDa by Western Blot analysis. The extracellular laccase activity with α-factor signal peptide was 9.79 Uml^{-1}. The optimum laccase activity was obtained in the presence of 0.2mM copper (Guo et al., 2005). The recombinant laccase was further purified. The purified recombinant laccase decolorized more than 90% of 80 mgl^{-1} Ramezol Brillint Blue R (RBBR) after 16 hours at 45°C and pH5 when 25U laccase ml^{-1} was used (Guo et al., 2008).

8. application of laccase

8.1 Environmental Applications

Recently, increasing interest has been focused on the application of laccases in bioremediation. Laccases can apply for degradation of many substances such as phenolic compounds, plastic waste having olefin units, undesirable contaminants, polyurethanes, and fluorescent brighteners. (Kunamneni et al., 2008a; Majeau et al., 2010). Moreover, polycyclic aromatic hydrocarbon (PAHs), which arise from natural oil deposits and utilisation of fossil fuels, were also found to be degraded by laccases (Osma et al., 2010, Couto and Herrera, 2006).

Another environmental application of laccases is the bioremediation of contaminated soils such as some xenobiotics, chlorophenols, and other contaminants. Laccase can be also used to reduce the concentration of synthetic heterocyclic compound such as halogenated organic pesticides in the soil (Kunamneni et al., 2008a). Collins et al. (1996) provided evidence that a crude laccase as well as purified enzyme from *T. versicolor* were able to degrade PAHs, anthracene and benzo (*a*) pyrene.

8.2 Food Industry

Laccases can be applied to modify or enhance the color appearance of beverage and food. Musts and wines are complex mixtures of different chemical compounds such as ethanol, phenolic compounds, salts and organic acids. Alcohol and organic acids are responsible for the wine aroma while the color and the taste are depend on type of phenolic compounds present in different types of wines (Brenna and Bianchi, 1994, Minussi et al., 2002). The application of laccases include the elimination of undesirable phenolics, responsible for the browning and haze formation development in beer, wine and juice. Furthermore, in baking industry, laccase from the fungus *Trametes hirsuta* can increase the resistance of dough and decreased the dough extensibility in both gluten dough and flour (Selinheimo et al., 2006).

8.3 Pulp and paper industry

The separation and degradation of lignin from woody tissues is a process that has attracted in the pulp and paper industry. Environmental concerns urge to replace conventional and polluting chlorine-based delignification/bleaching procedures. Camarero et al. (2004) explored the potential of laccase mediator system (LMS) to remove lignin-derived products responsible for color from a high-quality flax pulp. They showed the feasibility of LMS to substitute chlorine-containing reagents in manufacturing of these paper pulps. Laccase from *T. versicolor* has been reported it efficiently demethylated and delignified kraft pulp. The efficiency of enzymatic action was monitored by methanol release and the decrease in the kappa number (Bourbonnais and Paice, 1992). Moreover, laccase-mediated processes may

be regarded as much safer and less contaminating methods for cleaning up waste from the pulp and paper industry. (Gianfreda et al., 1999).

8.4 Nanobiotechnology

Due to the ability of laccases that can catalyse electron transfer reactions without additional cofactors, their use has also been studied in biosensors to detect various phenolic compounds, oxygen, morphine, codeine, catecholamines and plant flavonoids (Couto and Herrera, 2006). DeniLite® has been applied to assemble amine-derivatized platinum electrodes for phenol detection. This sensor is very fast response and high stability toword p-phenylenediamine, catecholamine and catechol (Quan and Shin, 2004). Similary, Kulys and Vidziunaite (2003) developed graphite electrode base biosensors for phenol determiation by immobilization of two recombinant fungal laccase from *Polyporus pinsitus* and *M. thermophile*, commercially available from Novozymes.

8.5 Synthetic chemistry

Laccase might be useful in synthetic chemistry such as for vinblastin synthesis from vindolin, oxidation of hydroxyl groups of sugar derivatives and free radical polymerization on polysulfostyrene matrix resulting in electroconducting water-soluble polyaniline (Morozova et al., 2007b). Moreover, this enzyme can be used for the polymerization of catechol monomers for polycatechol synthesis (Aktaş et al., 2003), or for the production of inert phenolic polymers, such as poly (1-napthol) (Ceylan et al., 2008). These polymers have application in wood composites, laminates, coatings, fiber bonding and adhesives.

PART III Heterologous expression of fungal laccase

The production of laccase from native host cannot meet the increasing market demand due to long cultivation time and low yield. Recombinant protein expression in handling host and easily cultivable can reduces costs and time for production with higher productivity. Furthermore, the production of enzyme from toxin-production species or pathogen can take advantage of safer or even generally recognized as safe (GRAS) hosts such as *Yarrowia lipolytica*. Moreover, enzyme engineering can be employed to improve the activity, specificity and stability of the enzyme, thus made them can be produced to suit the requirement of the application. Recombinant laccase expression is interested by many researcher groups since the beginning of nineties (Kunamneni et al., 2008a).

Heterologous expression of laccase in bacteria, yeast and fungi has been reported. However, laccase heterologous expression in bacteria usually gives low production yield and recombinant enzymes form aggregates difficult to purify (Suzuki et al., 2003). The attraction of fungi host is based on their ability to secrete large amounts of proteins into the medium. The yields of heterologous laccase production by the fungi are considerably higher than those obtained in bacteria and yeast. Most commercial laccases are produced in *Aspergillus* hosts (Stoilova et al., 2010). The production levels of *Melanocarpus albomyces* laccase expressed in *Trichoderma reesei* are the highest heterologous laccase expression levels reported so far. The production level reach 230 mg l^{-1} in flask cultures (Kiiskinen et al., 2004). Nevertheless, the production by filamentous fungal is usually time consuming and required toxic expensive inducer. By contrast, yeast offer the quickness of growth and ease of microbial and gene manipulation of bacteria along with the ability to perform eukaryote specific post translational modifications. The manipulations of yeast are not expensive and usually give high yield (Piscitelli et al., 2010).

1. Fungal laccase expression in yeast

The expression of laccase in yeasts has been investigated taking into account an array of several parameters with controversial results. For example, both native and

yeast secretion signal peptides gave different results for various expressed enzymes. So, the best signal peptide to drive the secretion of recombinant laccases in yeasts seems not to be a priori predictable (Piscitelli et al., 2010). *Pichia pastoris*, *Pichia methanolica and Saccharomyces cerevisiae* are the most popular yeast for laccase expression. The engineering of *Myceliophthora thermophila* laccase in *S. cerevisiae* by directed molecular evolution has been reported (Bulter et al., 2003). Further, the production of fuel ethanol has been obtained using *T. versicolor* laccase *(lcc2)* expressed in *S. cerevisiae* with an increased resistance of the yeast to phenolic inhibitors (Larsson et al., 2001). Laccase from *T. versicolor* (LCC1) is actively expressed in *P. pastoris* and *P. methanolica*, but not in *S. serevisiae* (Table II-10) (Jönsson et al., 1997; Guo et al., 2006; Cassland and Jönsson, 1999). A list of heterologous laccase expression is showed in Table II-10 (Kunamneni et al., 2008b).

However, several problems such as low secretion efficiency in *S. cerevisiae*, low transformation efficiency and time consuming in *P. pastoris* and *P. methanolica* have limit the genetic engineering of recombinant laccase. *Yarrowia lipolytica* is a non-conventional yeast with has limited extent of glycosylation. The yeast *Y. lipolytica* has been report as an efficient system for recombinant protein expression with its low cost, post translational modification efficiency and high transformation efficiency (~8000 transformant/µg of DNA). Moreover, this yeast is considered as nonpathogenic and several processes based on this organism were classified as generally regarded as safe (GRAS) by the Food and Drug Administration (FDA, USA).

Interestingly, the comparison between different host systems, *P. pastoris*, *A. niger* and *Y. lipolytica*, confirmed that *Y. lipolitica* is the best host for random or directed mutagenesis of the heterologous gene. *P. pastoris* did not appear competitive in the particular case of *P. cinnabarinus* laccase: transformation efficiency was very low (about 25 transformants per µg DNA (Otterbein et al., 2000), recombinant laccase was hyperglycosylated and maximum activity in flask cultures was more than three folds lower than observed in *Yarrowia* system. By contract, the *Aspergillus* system gives high laccase production level (70mg/L) with a molecular mass similar to that of

the native protein. The weak side of *Aspergillus* system is the complicate transformation system and the low transformation efficiency (~100 transformants per μg DNA). These characteristics should not be a problem for obtaining a production strain, but would be a major drawback for engineering the enzyme by directed evolution or random mutagenesis of the heterologous gene. Muller et al. (1998) also compared various alternate yeasts with the yeast *S. cerevisiae*. The comparison was carried out on 6 different enzymes and 5 expression hosts (*Hansenula polymorpha, Y. lipolytica, Klyveromyces lactis, Schizosaccharomyces pombe* and *S. cerevisiae*). The authors studied various parameters: quantity of secreted enzyme, glycosylation profile, effectiveness of transformation, and plasmid stability. Amongst the different systems studied, *Y. lipolytica* was shown to be the most effective as it exhibited a good transformation yield, stable plasmids and correct growth. These characters make it become a good alternative host over the such conventional yeast which enables this system to be tuned up for new and challenging applications.

2. Fungal laccase expression in *Y. lipolytica*

There are a few reports about the expression of laccase in *Y. lipolytica*. Jolivalt and co-worker (2005) reported the production level of 2.5 mg/L (0.23 U/ml) from the expression of laccaseIIIb from *T. versicolor* in *Y. lipolytica* (strain Po1g), which is suitable for their engineering purpose. They found native secretion signal gave higher enzyme activity than yeast secretion signal. The recombinant laccase was partly purified and some kinetic data were acquired. The K_m value being ten-fold higher for 2,6-DMP than for ABTS substrate.

More recently, Madzak et al. (2006) studied the influence of the residue at position on the catalytic properties of *T. versicolor* laccaseIIIb expressed in *Y. lipolytica* (strain Po1g). The laccase production level of the mutants was rather low (~ 1 mg/L). The quantity of enzyme available was not sufficient to undergo an extended purification process. The influence of the mutation on the optimal pH with 2,6 DMP substrate showed the Asn mutation led to a significant shift (pH=1.4) of the optimum towards higher pH).

Another example was found in the expression of fungal *Pycnoporus cinnabarinus* laccase gene (*lac1*) in *Y. lipolytica* stain Po1g with *Y. lipolytica* signal peptide (Madzak et al., 2005). Production was estimated to 20 mg/L in a bioreactor.

Table II-10 List of heterologously expressed laccases (Kunamneni et al., 2008b).

Laccase	Source	Host	Comments
PO1	*Coriolus hirsutus*	*Saccharomyces cerevisiae*	Active laccase secreted in the medium.
PO2			Active laccase secreted in the medium.
PrL	*Phlebia radiata*	*Trichoderma reesei*	Laccase secreted activity of 7.7 nkat ml^{-1} (ABTS). The enzyme was purified and partially characterized.
LCC1, LCC4	*Rhizoctonia solani*	*Aspergillus oryzae*	Laccase activity secreted in the medium. The enzyme was purified and partially characterized.
LCC2			Active laccase secreted in the medium.
LCC1	*Trametes villosa*	*Aspergillus oryzae*	Active laccase secreted in the medium. The enzyme was purified and partially characterized.
MtL	*Myceliophtora thermophila*	*Aspergillus oryzae*	Laccase secreted activity of 0.85 U ml^{-1} (SGZ). The enzyme was purified and partially characterized.
		Saccharomyces cerevisiae	Laccase secreted activity of 0.6 U l^{-1} (ABTS). Total activity was enhanced 170-fold by directed evolution (18 mg l^{-1}).
LCC1	*Trametes versicolor*	*Pichia pastoris*	Active laccase secreted in the medium. Production yield was further optimised.
LCC1		*Saccharomyces cerevisiae*	Undetectable laccase activity in the medium.
LCC2		*Saccharomyces cerevisiae*	Active laccase secreted in the medium. Production of ethanol from raw materials (0.12 U l^{-1}).
LCCI		*Pichia pastoris*	Active laccase secreted in the medium. The enzyme and a truncated version (LCCIa) were purified and partially characterized.
LCCIV		*Pichia pastoris*	Laccase secreted activity of 0.15 U ml^{-1} (ABTS). The enzyme was purified and partially characterized.
LCCI		*Zea mays L*	Laccase activity was found in the seed, and variability in the amount was seen. The highest level was 0.55% TSP (respect to total soluble protein).
LCC1		*Pichia methalonica*	9.79 U ml^{-1} of laccase acivity in recombinant with the α-factor signal peptide.
LACIIIb		*Yarrowia lipolytica*	2.5 mg l^{-1} (0.23 U ml^{-1}) of active enzyme with limited excess of glycosylation.
LCCα		*Saccharomyces cerevisiae*	0.035 U l^{-1} of laccase activity produced by *S. cerevisiae*.
LCC1, LCC2		*Pichia pastoris Aspergillus niger*	2.8 U l^{-1} of laccase activity produced by *P. pastoris* and up to 2700 U l^{-1} by *A. niger*.
Gene IV		*Aspergillus niger*	592 U l^{-1} of enzyme activity in solid-state fermentation produced by *A. niger*.

LAC	*Schizophyllum commune*	*Aspergillus sojae*	Laccase secreted activity of 774 U ml^{-1} (Gallic acid).
LCC1	*Coprinus cinereus*	*Aspergillus oryzae*	Transformants secreted from 8.0 to 135 mg of active laccase per liter. The enzyme was purified and partially characterized.
LCC1	*Coprinopsis cinerea*	*Coprinopsis cinerea*	Maximal activity (3 U ml^{-1}) reached with the *gpdII* promoter and 0. 1 µM $CuSO_4$ (homologous expression).
LtLACC2	*Liriodendron tulipifera*	*Tobacco cells*	Protoplasts retained laccase activity which could be measured once the protoplasts were lysed.
LAC1	*Pycnoporus cinnabarinus*	*Pichia pastoris*	Transformants secreted 8.0 mg l^{-1} of hyperglycosylated active laccase.
LAC1		*Aspergillus niger*	70 mgl^{-1} of active laccase using the A. *niger* signal peptide which represent a 77-fold increased activity (7000 U ml^{-1}) (ABTS). The enzyme was purified and partially characterized.
LAC 1		*Aspergillus oryzae*	80 mgl^{-1} of active laccase.
LAC 1		*Pycnoporus cinnabarinus*	Laccase secreted activity of 1200 mg l^{-1} (homologous expression)
LAC 1		*Yarrowia lipolytica*	20 mg l^{-1} of active enzyme in bioreactor.
LAC2	*Loblolly pine (Pinus taeda)*	*Saccharomyces cerevisiae*	Yeast cells accumulated the expected fusion protein in insoluble fractions without degradation of products, but no laccase activity was detected.
PPOA	*Marinomonas mediterranea*	*Escherichia coli*	Production of recombinant protein, with the most of activity, located in the membrane fraction rather than in the soluble one.
LAC4	*Pleurous sajor-caju*	*Pichia pastoris*	Transformants produced 4.85 mg l^{-1} of active laccase. The enzyme was purified and partially characterized.
PPO	*Solanum tuberosum L.*	*Lycopersicon esculentum*	Active laccases secreted in the medium conferring resistance to pathogen *Pseudomonas syringae* pv *tomato*.
LAC 1	*Melanocarpus albomyces*	*Trichoderma reesei*	920 mg L l^{-1} of active laccase
LAC 1		*Saccharomyces cerevisiae*	168 U l^{-1} of laccase activity produced (around 3 mg l^{-1})
LAC3	*Trametes sp. strain C30*	*Saccharomyces cerevisiae*	2 mg l^{-1} of rLAC3 produced in bioreactor.
POXA1b, POXC	*Pleurotus ostreatus*	*Kluyveromyces lactis* *Saccharomyces cerevisiae*	*K. lactis* was more effective host (1.1 of POXA1b and 1.4 mg l^{-1} of POXC laccase) than *S. cerevisiae*.
3M7C mutant		*Saccharomyces cerevisiae*	~30 mU OD600 l^{-1} after 6 days of incubation in shaken flask.
POXA3		*Kluyveromyces lactis*	80 U l^{-1} after 10 days of incubation.

LCC1	*Pycnoporus coccineus*	*Aspergillus oryzae* *Saccahromyces cerevisiae*	High copper concentrations are required for the production of active laccase.
LCC1	*Coprinopsis cinerea*	*Coprinopsis cinerea*	Maximal activity (3 U ml^{-1}) reached with the *gpdII* promoter and 0. 1 µM $CuSO_4$
LCC	*Tametes trogii*	*Pichia pastoris*	17 mg l^{-1} of active enzyme, reaching up to 2520 U l^{-1} in fed-batch culture.
LCC1		*Kluyveromyces lactis*	6.6 U l^{-1} of bioactive molecule produced by *K. lactis*.
LACB	*Trametes sp.*	*Pichia pastoris*	Overexpression (1.01 U/mg) of active laccase (32000 U ml^{-1}).
LACD	*Trametes sp 420*	Pichia pastoris	8.3×10^4 U l^{-1}+of active laccase.
Ery3	*Pleurotus eryngii*	*Aspergillus niger*	Partially characterization of recombinant laccase.
Pel3		*Saccharomyces cerevisiae*	139 mU ml^{-1} of laccase in alginate immobilized cells and 18°C.
LCC	*Fome lignosus*	*Pichia pastoris*	3.7-fold expression improvement (up to 144 mg l^{-1}) with EMS random mutagenesis.

3. *Y. lipolytica* expression system

As shown previously, *Y. lipolytica* offers a number of advantages as expression system. Thus, this yeast has become a reliable and a popular system for the expression of heterologous proteins. *Y. lipolytica* retains the advantages of microbial expression systems in its ease of manipulation and growth capacity. In contrast to these latter, it has an ability to perform post-translational processing of eukaryotic proteins. Furthermore, it was shown to be a very attractive alternative host, especially in terms of performance reproducibility and high transformation efficiency. Moreover, a *Y. lipolytica* strain specifically dedicated to high throughput screening was recently developed (Bordes et al*.,* 2007) enabling the reproducible 96-well plate production of protein and activity assays.

3.1 Main characteristics

The hemiascomycetous yeast *Y. lipolytica* (formerly, *Candida, Endomycopsis* or *Saccharomycopsis lipolytica*) is a non conventional and non pathogenic yeast, and several processes based on this organism were classified as generally regarded as safe (GRAS) by the Food and Drug Administration (FDA, USA).

It is naturally found in high lipid containing products such as cheeses, dairy products, plant oils and sewages (Madzak et al., 2004). *Y. lipolytica* is heterothallic dimorphic yeast that grows as budding cells, hyphae or pseudohyphae depending on growth conditions. It is strictly aerobic and grows at temperatures ranging from 5°C to 30°C. This yeast can use many carbon sources such as glucose, acetate, alcohols, and hydrophobic substrates (Barth and Gaillardin 1997). This yeast is attractive in the industry because of its capacity to produce high amounts of organic acids (Tsugawa et al., 1969). The genome of *Y. lipolytica* is organized in 6 chromosomes, representing a total of 20.5 Mb (much more than *S. cerevisiae* and *S. pombe*), with size varying between 2.6 and 4.9 Mb. No plasmidic DNA was identified (Casaregola et al*.,* 2000; Dujon et al*.,* 2004).

3.2 Secretion of proteins

The early steps of protein secretion can follow two pathways: co-translational or post-translational translocation. Co-translational was shown to be predominant in *Y. lipolytica*. This feature constitutes an advantage for the efficient production of complex heterologous proteins of this yeast over *S. cerevisiae*, in which the latter pathway is predominant. Moreover, high secretion efficiency, low overglycosylation, good product yield, and performance reproducibility are additional features of *Y. lipolytica* (Coelho et al., 2010). Until now, More than forty proteins of various origins were successfully expressed in this yeast (Madzak et al., 2004).

3.3 Expression vectors

The transforming vectors for expression in *Y. lipolytica* are shuttles vectors which allowing at the same time replication in bacterial hosts and expression in yeast. The yeast part of the vector contains a selection marker, the expression cassette (promoter – signal sequence - gene - termination), and the elements necessary for maintenance into yeast cell. The list of these elements is showed in Table II-11 (Madzak et al., 2004). The vectors designed for integration into yeast chromosome can be used. Homologous integration can be carried out by simple crossing-over (within the site LEU2, URA3, in the ribosomal DNA or the platform of chromosomal integration). This integration is efficiency directed by linearization, which results in

very high transformation frequencies (up to 10^6 transformants/μg of DNA). In addition non-homologous can also be carried out due zeta zones (the long terminal repeat zeta of the *Y. lipolytica* retrotransposon Ylt1) flanking the expression cassette. The use of these vectors makes it possible to get rid of the bacterial part of the plasmid (Nicaud et al*.,* 1998; Pignède et al*.,* 2000).

Furthermore, the integrative *Y. lipolytica* vectors offer the possibility of multiple integrations that correlated increase in gene expression. The multicopy shuttle vectors can integrate into the recipient genome. Their amplification is selected for using a defective selection marker (Madzak et al., 2004), a mutated allele of the *URA*3 gene in which part of the promoter was deleted: ura3d4 (Le Dall et al*.,* 1994). For auxotroph complementation, the gene thus has to be present in several copies. Zeta-based auto-cloning vectors have been used successfully to overexpress *Y. lipolytica* lipase (Pignède et al., 2000). In comparison with monocopy vectors that integrate at a precisely identified genomic site, the multicopy vectors generate integrants which have greatly heterologous production depending on the copy number. Although, the transformation efficiency of the later is lower than a classical integrative vector, the high-copy-number integrants has high stability (Nicaud et al., 1998). A technique of transformation by lithium acetate method is possible to obtain a good effectiveness of transformation (Xuan et al*.,* 1988).

Table II-11 Components available for *Y. lipolytica* expression/secretion vectors (most commonly used and most interesting items are underlined) (Madzak et al., 2004).

Component[a]	Characteristics
Marker genes	
LEU2, *URA3*, *LYS5*, *ADE1*	Auxotrophy complementation
ura3d4	Item + copy number amplification (cf. text)
Phleo^R, *hph* (*E. coli*)	Antibiotic resistance (respectively, to phleomycin and hygromycin B)
SUC2 (*S. cerevisiae*)	Sugar utilization
Promoters (source)	
p*LEU2* (β-isopropylmalate dehydrogenase)	Inducible by leucine precursor
p*XPR2* (alkaline extracellular protease)	Inducible by peptones
p*POX2*, p*POT1* (respectively, acyl-CoA oxidase 2, 3-oxo-acyl-CoA thiolase)	Inducible by fatty acids and derivatives, and alkanes
p*ICL1* (isocitrate lyase)	Inducible by fatty acids and derivatives, alkanes, ethanol and acetate
p*POX1*, p*POX5* (acyl-CoA oxidases 1 and 5)	Weakly inducible by alkanes
p*G3P* (glycerol-3-phosphate dehydrogenase)	Inducible by glycerol
p*MTP* (bidirectional: metallothioneins 1 and 2)	Inducible by metallic salts
hp4d (hybrid promoter derived from p*XPR2*)	Growth-phase-dependent
p*TEF*, p*RPS7* (respectively, translation elongation factor-1α, ribosomal protein S7)	Constitutive
Secretion signals[b]	
Native	Frequently efficient in *Y. lipolytica*
XPR2 prepro	13 aa pre/10 XA or XP dipeptides/122 aa pro/KR cleavage site
XPR2 pre + dipeptide	13 aa pre/5–10 XA or XP dipeptides
XPR2 pre	13 aa pre/LA cleavage site
LIP2 prepro	13 aa pre/4 XA or XP dipeptides/10 aa pro/KR cleavage site
Terminators	
XPR2t, *LIP2t*, *PHO5t*	Respectively, 430, 150 and 320 bp fragments
Minimal *XPR2t*	PCR-synthesized 100 bp fragment with added restriction sites
Elements for maintenance in yeast cells	
ARS18, *ARS68*	Autonomously replicative vectors can maintain only 1–3 copies/cell[c]
Homology to genome	Homologous integration (in *LEU2*, *URA3*, *XPR2* terminator, rDNA, or, when present, in zeta or pBR322 docking platform)
Zeta (Ylt1 LTR)	Non-homologous integration in Ylt1-devoid strains (cf. text)

[a] References: Barth and Gaillardin (1996), or references indicated for the corresponding works in Table 3.

[b] The pre-region corresponds to the signal sequence; the dipeptides XA and XP are substrates for diamino-peptidase; the dibasic KR cleavage site is substrate for Xpr6p endoproteinase (Matoba and Ogrydziak, 1989). Abbreviations: aa, amino acids; X, any aa; A, alanine; P, proline; K, lysine; R, arginine; L, leucine.

[c] Due to the obligate association of replication origins with centromeres in *Y. lipolytica* (Vernis et al., 1997; Madzak et al., 2000).

3.4 Selection markers

A gene of resistance to kanamycine, ampicilline or tetracycline enables bacterial selection. *Y. lipolytica* is found to be sensitive to only the bleomycin/phleomycin group of antibiotic (Gaillardin and Ribet, 1987), and hygromycine B (Cordero Otero and Gaillardin, 1996). Thus, the selection by autotrophy markers remains the good choice. The *LEU2* and *URA3* are the most commonly used (Barth and Gaillardin, 1997).

3.5 Promoters

The strong *XPR2* promoter was undoubtedly the mostly used promoter. Unfortunately, its complex regulation (pH> 6, obligatory presence of peptones in the

medium) hindered its industrial use. Then, the upstream activating sequences (UAS1) was used to design a hybrid promoter which composed of four tandem UAS1 copies upstream from a minimal LEU2 promoter. This new develop promoter, termed hp4d, is poorly affected by environmental condition and is able to drive a strong expression in virtually any medium (Blanchin-Roland et al*.,* 1994, Madzak et al., 1995, 2000). This promoter is considered as semi-constitutive, because its induction is dependent on growth phase. However, the elements controlling its regulation are still unclear. The two other strong constitutive promoters were thus afforded. TEF (translation elongation Factor-1 alpha) and RPS7 (ribosomal protein S7) were described by Muller et al. (1998). Others inducible promoters were also described such as the promoter from acyl-CoA oxidases (*POX*) and 3-oxo-acyl-CoA thiolase (*POT*1). These promoters were highly inducible by alkanes and fatty-acids (Madzak et al., 2004).

3.6 Secretion signals

Secretion signal is needed to target the heterologous protein to the secretion pathway. The most commonly used secretion signal peptide are the prepro sequence of the XPR2 gene, that encodes for the extracellular alkaline protease and the prepro sequence the LIP2 gene. However, the signal sequences of the interest proteins were also used successfully (Muller et al., 1998)

PART IV Laccase Engineering and screening method

The availability of established recombinant expression system for laccase has allowed their engineering and improving enzymatic features for specific industrial needs. Rational and random mutagenesis has often been used to generate laccase variants.

1. Site directed mutagenesis

Site-directed mutagenesis is a molecular biology technique in which a mutation is created at a defined site in a DNA molecule. In vitro site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships and gene expression, and for carrying out vector modification. The growing library of laccase structures helps inform rational choice of candidate residues for site directed mutagenesis. Madzak and co-workers (2006) reported significant changes in pH optimum for mutated laccase IIIb from *T. versicolor* expressed in the yeast *Y. lipolytica*. The single residue modification (Asp→Asn) at the hydrophobic substrate binding pocket led to a significant raise of pH optimum by 1.4 units, which suggests significant alterations to interactions between the reducing substrate and the binding pocket. The changing of pH optimum was also observed in *Trametes villosa* laccase by Xu et al. (1999). The mutation F463M which close to the type 1 site resulted in a more basic optimal pH as well as an increase in K_{cat} and K_m.

The role of the C-terminus in fungal laccase has been evaluated using site directed mutagenesis. Gelo-Pujic et al. (1999) reported that the reduction potential of the active site of *T. versicolor* LCC1 is shift to a lower value when the C-terminus is truncated. In addition, the result of amino acid deletion obtained with *Melanocarpus albomyces* laccase clearly confirmed the critical role of the last amino acids in its C-terminal. Andberg and co-worker (2009) found that the deletion of the last four amino acids dramatically affected the activity and TNC geometry of the enzyme.

2. Errorprone PCR

Error prone PCR is a random mutagenesis technique for generating amino acid substitutions in proteins by introducing mutations into a gene during PCR. Error-prone PCR introduces random copying errors by imposing imperfect, and thus mutagenic reaction conditions (e.g. by adding Mg^{2+} or Mn^{2+}) to the reaction mixture. The mutated PCR products are then cloned into an expression vector. The mutant library can be screened for changes in protein activity. This technique is useful for generation of randomised libraries of nucleotide sequences, and also for the introduction of mutations during the expression and screening process in a mutagenesis step (Pritchard et al., 2005).

3. Saturation mutagenesis

Saturation mutagesis is a semi-rational mutagenesis method. This technique involves the mutation of any single amino acid codon to all the other codons. This mutation will generate the twenty naturally occurring amino acids coupled to screen for desire function. This approach is employed to improve the characteristics of enzyme at hot spot residues which already identified by random mutagenesis (Kunamneni et al., 2008b). Alcalde et al (2006) has performed saturation mutagenesis to the residues S510 of *M. thermophila* laccase (MtLT2) expressed in *S. cerevisiae*. This codon belongs to the tripeptide $_{509}VSG_{511}$ that is common to low-medium E^0 laccases. They found one positive mutation $_{TCG}S510G_{GGG}$ that showed 3-fold higher turnover rates than the parent type. This mutation could not be achieved by conventional error-prone PCR approach because it was dependent on the two consecutive nucleptide changes.

4. Directed evolution

Directed evolution techniques and applications have widely expanded in the past few years. This technique becomes a successful approach for tailoring protein properties to industrial demand and for understanding of structure-function

relationships in biocatalysts (Shivange et al., 2009). Directed evolution is a process that directs molecular evolution in the test tube to produce the desired protein. This has resulted in the development of enzymes with improved desired properties. The first step is the isolation of the wild type gene responsible for encoding the desired protein. Then, this DNA fragment is subjected to random mutagenesis. After that, the mutant genes library is inserted into an expression system. The improved variants are identified by suitable screening method. Next, the improved enzymes are used as a parents for the next round of evolution (Kaur and Sharma, 2006; Bloom and Arnold, 2009) (Figure II-10).

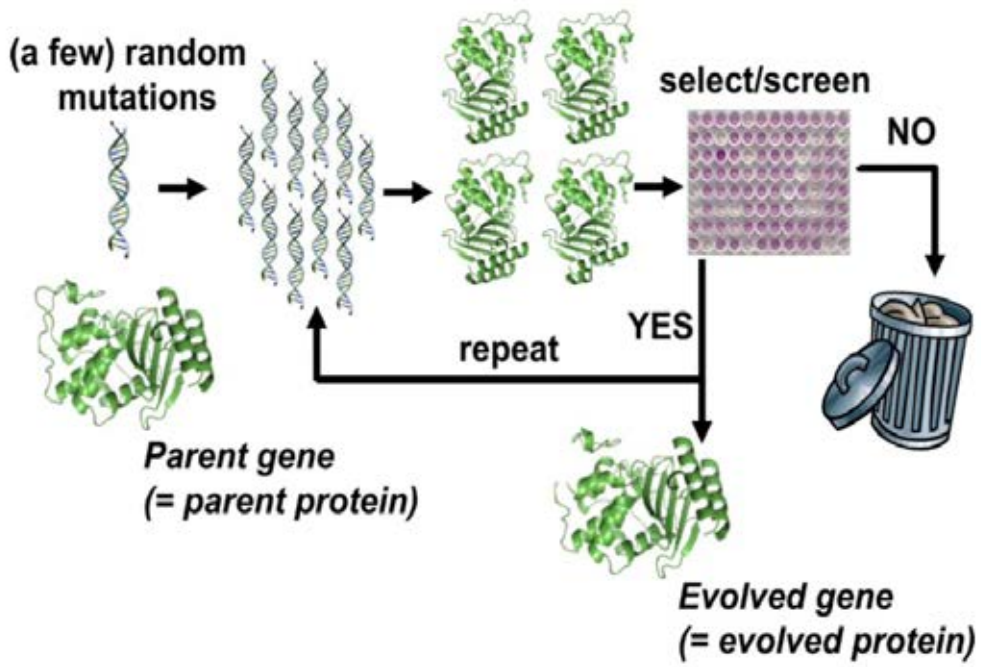


Figure II-10. Schematic outline of a typical directed evolution experiment. The parent gene is randomly mutagenized by using error-prone PCR or some similar technique. The library of mutant genes is then used to produce mutant proteins, which are screened or selected for the desired target property (e.g., improved enzymatic activity). Mutants that fail to show improvements in the screening/selection are typically discarded, while the genes for the improved mutants are used as the parents for the next round of mutagenesis and screening. This procedure is repeated until the evolved protein exhibits the desired level of the target property (Bloom and Arnold, 2009)

Laccase is a good target for directed evolution approaches because knowledge of structure-function relations underlying their key properties is limited. Functional expression of a laccase from *Myceliophthora thermophile* (MtL) in the yeast *S. cerevisiae* by ten rounds of directed evolution giving a 170-fold increase of total activity and 22-fold increase in K_{cat}. This observation lead to the highest production yet reported (18 mg/L) for a laccase in a yeast (Bulter et al., 2003).

More recently, directed evolution has been exploited to improve laccase stability and activity. For example, Zumarraga et al. (2007, 2008) improved variant *M. thermophila* laccase expressed in *S. cerevisiae* by five rounds of directed evolution. The variant tolerates high concentrations of co-solvents which known to promote the loss of enzyme activity and unfolding of the native enzyme. The mutant is capable of resisting a wide array of biotechnologically relevant miscible co-solvents at concentrations as high as 50% (v/v). In 40% (v/v) ethanol or 30% (v/v) acetonitrile, the performance of the mutant laccase was comparable to the parental enzyme. This property is very useful for the application in organic syntheses that must be carried out in the presence of organic solvents.

In addition, a basidiomycete PM1 laccase has undergone directed evolution and rational approaches. After eight rounds of molecular evolution, the total activity with ABTS was enhanced 34,000-fold, improving its activity to about 3200 U/L (production level 8mg/L, 400 U/mg) (Maté et al., 2010).

5 Selection and screening

Screening methods which are reliable and short time requirement are being demanded by biocatalysts'engineers. Screening of mutant laccase library base on High-throughput screening (HTS) is very popular method due to its high efficiency to screen a high number of the mutant in a short time. After the transformed cells were plated on appropriate medium, all individual colonies were picked into 96-well plates containing minimal starter medium by the help of a picking robot. The plates were

incubated in suitable condition for 24-48 hours. Then, the expression medium was added to each well (Alcalde et al., 2006; Bulter et al., 2003; Maté et al., 2010) or the cell from starter plates were used for inoculation into the new 96-well plates containing the expression medium (Koschorreck et al., 2009). The cultures plates were incubated for an additional 24-48 h for laccase production. After that, the plates were then centrifuged and the supernatant was then transferred into another plate (usually by liquid handler) for activity determination.

The popular substrate for activity assay is 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 6 Dimethoxyphenol. Detection of laccase activity may be difficult in some libraries due to their low secretion levels, and because of very small scale production in 96-well plates. In this case, the screening had to be performed in an end-point mode and may obtain reliable responses after incubating the supernatants up to 24 h in the presence of the substrates (Maté et al., 2010). Moreover, pre-screenings in ABTS solid plate can be combined. Those colonies that developed a green halo were then examined in the liquid screen described above (Maté et al., 2010).

Although the High-throughput screening is very efficience method for laccase library screening, this equipments are very expensive. Furthermore, small scale production in the 96-well plate lead to the low enzyme production that is difficult to determine. Also, many factors (agitation, medium composition, time for incubation, etc.) need a long time to optimize before screening.

In this study, *Y. lipolytica* mutants that secreted a high laccase activity were initially selected on ABTS agar plates in terms of selecting those colonies which gave rise to a dark green zone around them, These selected colonies were then further evaluated for their secretion of laccase (in terms of total enzyme activity) in narmal liquid expression culture in Erlenmeyer flask to confirm their high laccase activity level. This is a very fast method because only mutants which secreted a high laccase activity on ABTS plate were selected for further determination in liquid culture. Also,

it’s useful in order to screen the library which produce low amount of enzyme and doesn’t require any expensive equipments.

CHAPTER III

MATERIAL AND METHODS

Part I Comparison of synthetic dye decolorization by whole cells and a laccase enriched extract from *T. versicolor* DSM11269

1. Microorganism and chemicals

The fungal strain *T. versicolor* DSM11269 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany. Guaiacol, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) and Amaranth were obtained from Fluka. Direct Blue 71, Reactive Black 5, Cibacron Brilliant Red 3B-A, Alizarin Red S, Remazol Brilliant Blue R (RBBR) and Indigo Carmine were obtained from Sigma-Aldrich.

2. Growth conditions and enzyme production

Five pieces (1 cm^2) of *T. versicolor* DSM 11269, collected after seven days of growth on malt extract peptone agar plates (MEPA: 30 g malt extract l^{-1}, 3 g peptone l^{-1} and 15 g agar l^{-1}) were used to inoculate 75 ml of basal fermentation medium (XH medium) (Xiao et al., 2006) in 250 ml Erlenmeyer flasks. The fungus was then grown at 28 °C with continuous agitation at 100 rpm for 144 hours before the addition of 2 mM guaiacol to induce laccase production and 0.5 mM $CuSO_4$. After five days, the culture broth was filtered and centrifuged at 10 000 rpm for 15 min. The supernatant was frozen and then defrosted to precipitate the remaining polysaccharides, which were removed by centrifugation. The resulting clear supernatant was used directly without additional purification for enzyme activity assays and dye decolorization experiments.

3. Enzyme activity assays

Laccase activity was determined by measuring the oxidation of 2, 2'-azino-bis (3-ethylthiazoline-6-sulfonate) (ABTS). The assay mixture contained 1.5 ml sodium acetate buffer (1 mM, pH 5.0), 1.5 ml ABTS (0.5 mM) and 1.5 ml culture medium. Oxidation of ABTS was monitored by measuring the absorbance at 420 nm (ε_{420}= 3.6 x 10^4 M^{-1} cm^{-1}), as described by Li et al. (2008). One Unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of ABTS per minute at 30 °C.

4. Dye decolorization on solid plates

Dye agar plates were prepared using MEPA containing 0.2 mM of each individual dye. Plates were inoculated with a 1 cm^2 piece of *T. versicolor* fungus isolated from a seven day MEPA plate growth, cut out from the actively growing fungal culture. Dye agar plates that were not inoculated served as controls. All plates were incubated at 30 °C. After seven days, the size of the decolorization halo was measured.

5. Dye decolorization by the crude enzyme preparation

Dye solutions were prepared by the method of Nyanhongo et al. (2002). The reaction mixture for decolorization experiments contained 0.5 ml of 250 mg dye l^{-1}, 0.5 ml of enzyme preparation at 1 U ml^{-1} and 3 ml of 1 mM sodium acetate buffer (pH 5). The reaction mixture was incubated at 40, 50 or 60 °C, to determine the effect of temperature on decolorization, with shaking at 90 rpm. The absorbance of the mixture at the appropriate maximum wavelength (Table III-1) was recorded with a Zenyth 200-Anthos Microplate-Spectrophotometer. Decolorization was calculated by measuring the decrease in the absorbance according to the following expression: decolorization (%) = $(A_0 - A)/A_0$ x 100%, where A_0 was the initial absorbance and A was the final absorbance. Enzymatic activity and decolorization assays were performed in triplicate.

Part II Engineering and production of laccase from *Trametes versicolor* in the yeast *Yarrowia lipolytica:* application to the decolorization of synthetic dyes

1. Microorganism, chemicals and media

The fungal strain *T. versicolor* DSM 11269 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany. *Escherichia coli* strain Top10 was used for DNA propagation. The *Y. lipolytica* strains, zeta and MTLY60 (Fickers et al., 2005) and the plasmid JMP6 (Pignède et al., 2000) were generously provided by Dr J-M Nicaud and Pr A. Marty (INSA, Toulouse, France). The enzymes used in recombinant DNA techniques were purchased from New England Biolab Inc. (Berverly, Ma., USA). DNA purification was performed using QIAQuick (PCR purification and gel extraction) and QIASpin (miniprep) purification kits from Qiagen (Basingstoke, United Kingdom), as per the manufacturers' instructions. DNA sequencing was carried out commercially by Cogenics (Grenoble, France) using the dideoxy chain termination procedure. PCR was carried out using Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). Peptone, tryptone, malt extract and yeast extract were purchased from Difco (Paris, France). All other chemicals were of commercial quality and were purchased from Sigma–Aldrich (Saint Louis, MO). YPD medium (1% (w/v) yeast extract, 1% (w/v) Bacto Peptone, 1% (w/v) glucose) and YPD agar (YPD plus 1.5% (w/v) agar) were used for the growth and storage media of the yeast.

2. Preparation of RNA and cDNA

For RNA extraction, the *T. versicolor* DSM 11269 was grown as reported by Theerachat et al. (2012). The mycelium was then harvested and total RNA was prepared from them using an Epicenter master pure purification kit (USA), as per the manufacturer's instructions. Then, cDNA was synthesized by RT-PCR using the one-step RT-PCR kit (Invitrogen) and the primer pair Lcc1F 5'-*CGC*<u>*GGATCC*</u>*CACA*ATGGGCAGGTTCTCATCTC -3' and Lcc1R 5'-*TCCA*<u>*CCTAGG*</u>*TC*GGATGAGTCAAGAGCGTT-3', which contain 5' flanking regions (italic) that encode *Bam*H1 and *Avr*II restriction sites (underlined), respectively, for directional in frame cloning.

3. Cloning of cDNA of the laccase gene from Trametes versicolor

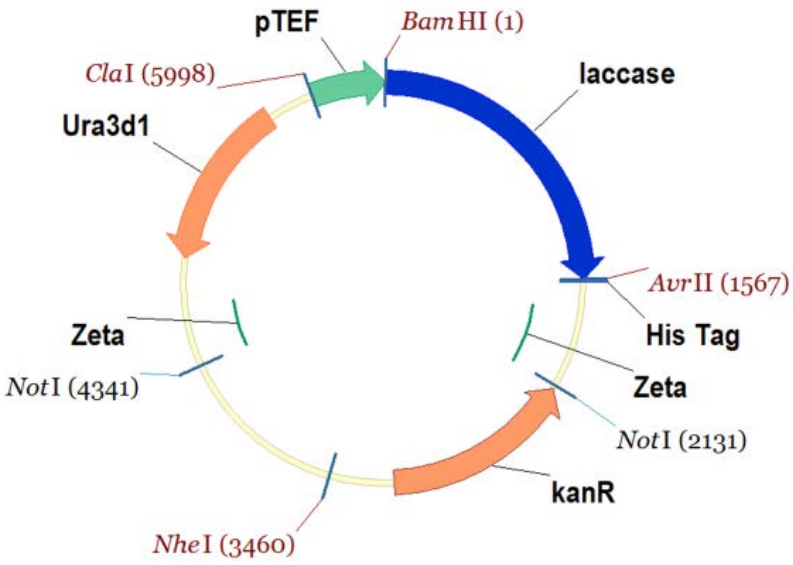
For the expression of *lcc1* in *Y. lipolytica*, the cDNA fragment that includes the secretion signal peptide was amplified by RT-PCR with the Lcc1F/R primers and then purified and recovered from agarose gel electrophoresis using the QiaQuick gel extraction kit. This laccase encoding fragment and the expression vector JMP62Ura3d1ExpTEFHis were digested with *Avr*II and *Bam*H1, purified and the two digested fragments were then ligated and subsequentially cloned into the *E. coli* strain TOP10. Transformants were selected in Luria-Bertani (LB) agar plates containing kanamycin (100 μg ml^{-1}), and the selected clones were confirmed for expression of the correct construct by DNA sequencing of the plasmid.

4. Heterologous expression of Lcc1 gene in Y. lipolytica

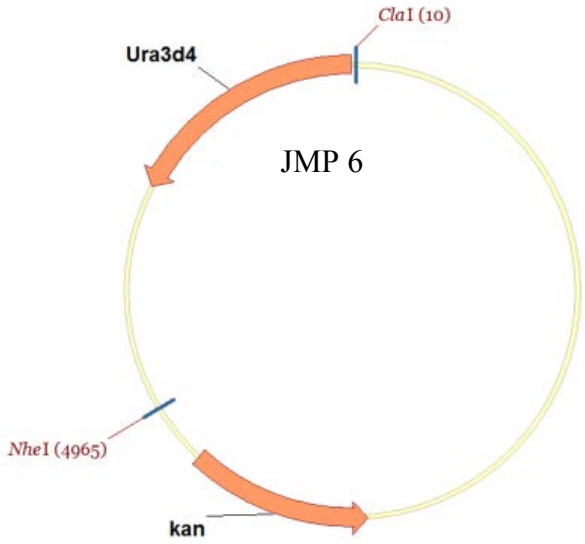
The laccase expression plasmid (JMP62Ura3d1ExpTEFHis/lcc1) is schematically shown in Figure III-1A. The plasmids were extracted and digested by *Not*I in order to obtain a linearized product and to discard the prokaryotic DNA sequence. The expression cassette, flanked by the zeta regions and containing the URA3d1 selection marker, the strong constitutive p*TEF* promoter and the *lcc1* gene was then used for transformation into the *Y. lipolytica* zeta strain for single copy genome integration. This strain enables single integration of the expression cassette into the genome at a defined locus: the ‘‘zeta docking platform’’ (Bordes et al., 2007). Transformants were spread on minimal medium YNB agar (YNB (0.17% (w/v) of yeast nitrogen base (without ammonium and without amino acids), 0.5% (w/v) of ammonium chloride, 1% (w/v) glucose, 0.2% (w/v) Casamino Acids, 50 mM phosphate buffer pH 6.8) and incubated at 28 °C for 72 h in order to select the Ura^+ transformants. The Ura-positive colonies were randomly selected and grown in 20 ml-flask YTD medium (1% (w/v) yeast extract, 2% (w/v) tryptone, 10% (w/v) glucose and 100 mM phosphate buffer pH 6.8) for 24 h. Then, these cultures were used to inoculate a fresh YTD medium (20 ml) supplemented with 1.2 mM $CuSO_4$ to an OD_{600} of 0.2 and then grown under agitation (135 rpm) at 28 °C for 30 h. The cultures were then centrifuged and the supernatants were used for enzymatic assay.

The expression of the rWT laccase in the multicopy MTLY60 strain was achieved using the same vector as described above and replacing the URA3d1 selection marker by URA3d4 marker. This MTLY60 strain doesn't contain zeta docking platform like zeta strain. To select multicopy transformant, a defective allele of ura3 gene (ura3d4) was used (Fickers et al., 2005). A single copy of this defective gene is not able to restore the growing of *Y. lipolytica* on selective medium. So, only transformants that have inserted more than one copy of this gene are able to grow on YNB medium (minimum medium). The method used to change URA3d1 marker to the URA3d4 marker is described below. The vector JMP6 (Figure III-1B), which contains the URA3d4 marker, and the vector JMP62Ura3d1ExpTEFHis/lcc1, were cut by *Nhe*1 and *Cla*1. The vector JMP62Ura3d1ExpTEFHis/lcc1 deleted of the URA3d1 marker was then ligated with the URA3d4 marker resulting in JMP62Ura3d4TEFHis/lcc1 (Figure III-1C). The transformants were then spread on YNB agar and incubated at 28 °C for three weeks. The Ura-positive colonies were analyzed for laccase activity as described above.

(**A**)



(**B**)



(C)

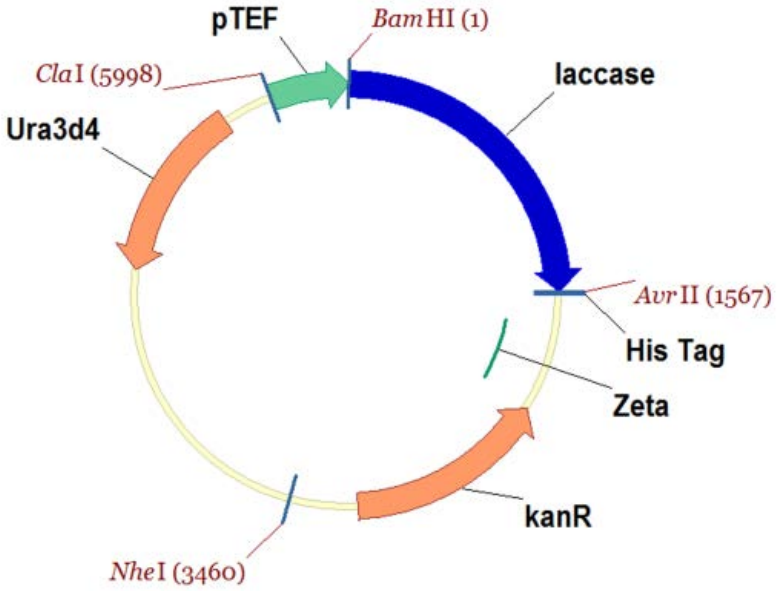


Figure III-1. The vectors employed to express the *T. versicolor* DSM 11269 *lcc1* gene including the signal peptide in *Y. lipolytica*. (**A**) JMP62Ura3d1ExpTEFHis/lcc1 used

for the transformation of the *Y. lipolytica* zeta strain. (**B**) Schematic diagram of JMP6 expression vector. To construct the JMP62Ura3d4TEFHis/lcc1 vector, the Ura3d4 selection marker was excised from the vector JMP6. (**C**) JMP62Ura3d4TEFHis/lcc1 was used to transform the *Y. lipolytica* MTLY60 strain. Note that both (A) and (C) result in the *lcc1*gene being under the strong constitutive p*TEF* promoter with the native *T. versicolor* signal peptide and C-terminal $(His)_6$ tag.

5. Effect of copper ions (Cu^{2+}) on the laccase activity

Transformed *Y. lipolytica* cells, pregrown in YTD medium for 24 h, were inoculated into fresh YTD medium (20 ml) supplemented with $CuSO_4$ (0-2 mM) to an OD_{600} of 0.2 and then grown under agitation (135 rpm) at 28 °C for 39 h.

6. Deglycosylation

N-linked glycans on rWT and rM-4A laccase were removed using endoglycosidase H (New England Biolab Inc.) and monitored by western blotting. To this end the supernatants from yeast cultures were centrifugally concentrated through an Amicon 30 kDa cutoff filter and then denatured by heating at 95 °C for 5 min. Then 1 U of Endoglycosidase H per 10 μg of denatured protein was added and the sample was incubated at 37 °C for 4 h. The deglycosylated protein isoforms were then separated by SDS-PAGE (10% (w/v) acrylamide resolving gel) and electrotransferred to a nitrocellulose membrane using the XCell II™ Blot Module, (Invitrogen). Laccase was detected using unconjugated anti-His(C-term) primary antibody (Invitrogen) and anti-mouse IgG secondary antibody conjugated to alkaline phosphatase (Invitrogen) as per the manufacturer's instructions. The substrate of alkaline phosphatase, BCIP/NBT Liquid Substrate System (Biorad), was used for detection of the protein.

7. Laboratory Evolution of Lcc1 laccase from Trametes versicolor

First generation

The first mutagenic library (~9000 mutants) was constructed using the Genemorph II kit (Stratagene) with a mutation rate of 0-4.5 mutations per kb. Random mutation was performed over the entire zeta cassette. Error-prone PCR was performed on a gradient thermocycler using the following parameters: 95 °C for 2 min (1 cycle); 95 °C for 30

sec, 56 °C for 30 sec and 72 °C for 6 min (30 cycles); and 72 °C for 10 min (1 cycle), using the primer pair zetaF (5'-GCGGCCGCCTGTCGGGAACCGCGTTCAGGTGGAACAGGAC 3') and zetaR (5'-GCGGCCGCACTGAAGGGCTTTGTGAGAGAGGTAACGCCG-3'). The PCR products were purified and then cut by *Not*I before being transformed into the *Y. lipolytica* strain zeta and plating in a Q-tray plate with YNB agar supplemented with 0.6 mM 2'2-azino-bis(3-ethylbenzothiazoline-6-sulphic acid (ABTS) and 0.4 mM $CuSO_4$, and incubated for 4 days at 28 °C. The colonies that developed a dark green halo on the solid plate were picked and cultured in a 250 ml Erlenmeyer flask containing 20 ml YTD medium for 24 h at 28 °C and 135 rpm, followed by seeding at an OD_{600} of 0.2 in fresh YTD medium supplemented with 1.2 mM $CuSO_4$ and grown under agitation (135 rpm.) at 28 °C for 30 h before determination of the culture laccase activity. From this first mutagenic library (~9000 mutants), three clones (mutants rM-1A, rM-2A and rM-3A) were subjected to DNA sequencing.

Site-directed mutagenesis was used to recreate the same point substitutions from the WT-*lcc1* template using PfuTurbo® DNA Polymerase (Stratagene, CA) as follows.

Mutant rM-1AC was engineered by introducing the L185P mutation into the WT-*lcc1* plasmid with the L185PF (5'-GTGCTGCCTTCCCGGTCGGCTCGGAC-3') and L185PR (5'-GTCCGAGCCGACCGGGAAGGCAGCAC-3') primer pair. Mutant rM-2AC was engineered by introducing the Q214K mutation into the WT-*lcc1* plasmid with the Q214KF (5'- CATCACTGTTGAGAAGGGCAAGCGCTACC-3') and Q214KR (5'- GGTAGCGCTTGCCCTTCTCAACAGTGATG-3') primer pair. Mutant rM-3AC was engineered by introducing the D494N mutation into the WT-*lcc1* plasmid with the D494NF (5'-GAGGACACCGCCAACGTCTCGAACACGAC-3') and D494NR (5'-GTCGTGTTCGAGACGTTGGCGGTGTCCTC-3') primers.

The mutation PCR was performed with the following parameters: 95 °C for 2 min (1 cycle); 95 °C for 30 sec, 59-64 °C for 30 sec (L185P: 64 °C, Q214K: 59 °C,

D494N: 62 °C), and 72 °C for 5 min (20 cycles); and 72 °C for 10 min (1 cycle). PCR products were purified and digested by *Dpn*I, and then transformed into *E. coli* stain JM109 and plated on LB-agar-kanamycin plates. The plasmids were extracted from the positive clones, linearized by *Not*1 and transformed into the *Y. lipolytica* zeta strain. The transformants were cultured in YTD medium and screened for extracellular laccase activity.

Second round evolution

Three mutants (mutants rM-4A, rM-5A and rM-6A) were constructed and the extracellular laccase activity level obtained with zeta strain (rM-4A, rM-5A and rM-6A laccases, respectively) grown in the YTD expression medium was compared between them and the rWT laccase. Mutant rM-4A, containing the two substitutions L185P and Q214K, was engineered by introducing the Q214K change into the L185P encoding *lcc1* plasmid (mutant rM-1AC) derived from the first round, using the Q214KF/Q214KR primers for the site directed mutagenesis. Likewise mutant rM-5A, containing the two substitutions L185P and D494N, was engineered by introducing the D494N change into the L185P encoding *lcc1* plasmid, using the D494F/494NR primers. Mutant rM-6A, containing the three substitutions L185P, Q214K and D494N, was engineered by introducing the D494N change into the above derived (L185P/Q214K) mutant rM-4A plasmid, using the D494NF/D494NR primers.

8. Enzymatic assays

Assays for laccase activity were performed using ABTS as the substrate, with the oxidation of the ABTS being monitored by measuring the absorbance at 420 nm (ε_{420}= 3.6 x 10^4 M^{-1} cm^{-1}). The reaction mixture contained 30 µl of the appropriately diluted test enzyme preparation and 270 µl of 1 mM ABTS in 0.1 M sodium acetate buffer pH 3.0 at 30 °C. One unit (U) of enzyme activity was defined as the amount of enzyme that oxidizes 1 µmol of ABTS per min, and the activity was expressed in U ml^{-1}.

9. Purification and characterization of recombinant laccase

9.1 Purification of recombinant laccase produced by Y. lipolytica

The $(His)_6$-tagged rWT and rM-4A laccases were obtained from the YTD medium supplemented with 1.2 mM $CuSO_4$ as follows. The 150 ml culture broth, after 30 h of culture, was centrifuged at 10 000 rpm for 10 min at 4 °C to pellet the yeast cells. The supernatant was then filtered (0.2 µm Acrodisc Gelman) and concentrated 10-fold by ultrafiltration with an Amicon 30-kDa cutoff membrane centrifugal filter (Millipore), and the copper containing medium was exchanged to Talon buffer (50 mM phosphate buffer, pH 8.0, 300 mM NaCl) by centrifugation. The 10-fold concentrated sample (15 ml) in Talon buffer was then purified by immobilized metal affinity chromatography (IMAC) (Clontech, Mountain View, CA). The sample was first adjusted to pH 8.0 by NaOH and then filtered (0.22 µm Acrodisc Gelman) prior to loading on a Fast flow column (Clontech) containing a 2 ml bed volume of Talon^{TM} metal affinity resin. The column was equilibrated with 20 ml of Talon buffer. After binding at 4 °C for 30 minutes, the resin was washed with 20 ml of Talon buffer under gravity feed and then the bound proteins were eluted by increasing the concentration of imidazole up to 200 mM in the Talon buffer. Each eluted fraction (5ml) was analyzed by SDS-PAGE and those that contained a large amount of the required protein of sufficient purity were dialyzed against 20 mM sodium acetate buffer containing 0.1 mM $CuSO_4$ (pH 6.0) at 4 °C for 48 h, and then the same buffer but at pH 4.5 and without any copper ions at 4 °C for 24 h, as modified from Liu et al. (2011). The protein concentration was determined using the method of Bradford (1976). The enriched enzyme was then concentrated by centrifugation through an Amicon (30 kDa cutoff) filter and stored at -20 °C prior to evaluation of its kinetics. The purity and molecular mass of the purified rLcc1 laccases were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% (w/v) polyacrylamide resolving gel. For estimation of the protein's molecular mass, prestained precision standards (10–250 kDa; Bio-Rad) were used and their migration distances (Rf) compared to that of the Coomassie brilliant blue R250 stained protein bands.

9.2 Effect of pH and temperature on laccase activity and stability

The effect of pH on the laccase activity was determined within the pH range of 2-6.8 using ABTS as the substrate in 0.1 M citrate-phosphate buffer at 30 ^{o}C, whilst

the optimum temperature of the purified laccase was assessed at 25-60 °C with ABTS as the substrate at the determined pH optimum. The effect of pH on the enzyme stability was investigated by measurement of the residual activity after 1 h preincubation at 30 °C in 0.1 M citrate-phosphate buffer between pH 2.8-9, whilst the thermal stability was tested after preincubation in 0.1 M citrate-phosphate buffer (pH 5.2) for 1 h at 25-45 °C.

9.3 Kinetic parameters determination

The K_m and k_{cat} value of the enzymes were determined using ABTS (ε_{420}= 3.6 x 10^4 M^{-1} cm^{-1}) or 2,6-dimethoxyphenol (2,6-DMP) (ε_{468}= 4.96 x 10^4 M^{-1} cm^{-1}) as the substrate in 0.1 M citrate-phosphate buffer at pH 3 and 4.8 for ABTS and 2,6-DMP, respectively. ABTS was assayed at concentrations between 10 to 1600 µM and 2,6-DMP at 50 to 2000 µM. Spectrophotometric measurements of substrate oxidation by purified rLcc1 laccases were carried out at 30 °C. The apparent K_m value of the enzyme for ABTS and 2,6-DMP were determined from the Lineweaver-Burk plots. All assays were performed in triplicate, and independent values differed by less than 5% from the mean.

10. Dye decolorization

The culture supernatant prepared from *Y. lipolytica* transformed with the WT and rM-4A *lcc1* laccase genes were investigated for their ability to decolorize five synthetic dyes that represent the three main dye groups of azo (Amaranth, Direct Blue 71 and Reactive Black 5), anthraquinone derivative (RBBR) and triarylmethane (Acid Violet 17) dyes. Stock solutions of the synthetic dyes were prepared in sterilized distilled water and diluted to the required concentration. The initial concentration of each of the dyes was selected to obtain ~1.0 absorbance unit at the maximum wavelength in the visible spectrum of each dye. Each 1 ml reaction assay contained the dye (at 50 mg/l for Amaranth, Direct Blue 71 and Reactive Black5, 20 mg/l for Acid Violet 17 and 100 mg/l for RBBR) in 50 mM citrate-phosphate buffer (pH 3-9) with acetosyringone (50-1500 µM) and the crude laccase preparation (0.5 U). The reactions were initiated with the addition of the enzyme and incubated at 30 °C, 90 rpm in the dark for 1 h. Control reactions were carried out in parallel without the

addition of the enzyme. The dye decolorization efficiency of the crude rWT and rM-4A laccase preparations were determined by monitoring the decrease in absorbance on a spectrophotometer at the appropriate maximum absorbance wavelength of each dye (Table IV-5). Decolorization was calculated from:

$$\text{decolorization (\%)} = (A_0 - A)/A_0 \times 100\%$$

where A_0 and A are the initial and final absorbance, respectively. The assays were performed in triplicate and the results are shown as the mean $\pm$ one standard deviation (1 SD).

11. Bioinformatics analysis: Sequence alignment and homology modeling of laccase

Analysis of the homology between the *lcc1* nucleotide and other known laccases was performed using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The isoelectric point and theoretical molecular mass (without post-translational modifications) of the protein was derived from the predicted amino acid sequence using the Computed pI/MW tool (http://web.expasy.org/compute_pi/).

To construct the 3D model, the amino acid sequences from *T. versicolor* DSM11269 was aligned with 5 crystal structures of laccases from *Trametes* spp. The MultiAlignment was performed using CLUSTALW2 (http://www.ebi.ac.uk/clustalw/) to determine percentage identity and similarity. Finally, to select the template for building the target protein, the criterion was to use high percentage sequence identity, and a high crystallographic resolution of the structure (see supplementary data). Crystal structure of laccase from *Tramestes* sp. AH28-2 was selected as the templates to model the three dimensional structure (PDB ID: 3KW7). The 3D model was generated using the software tool ESyPred3D (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred). Once the 3D model was prepared, hydrogen atoms were added and side chain orientations optimized through energy minimization. All calculations were done within the InsightII environment (Accelrys, San Diego, California) using the Discover package. Potential

energy was expressed with the CFF91 force field implemented in Discover (version 2.97). Minimisation (10.000 cycles) were performed with the steepest descent algorithm.

CHAPTER IV

RESULTS AND DISCUSSION

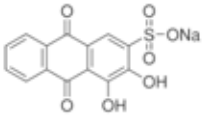
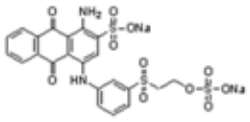
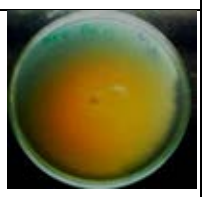
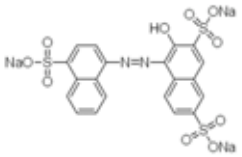
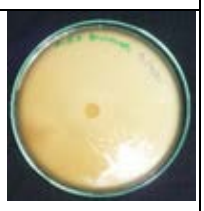
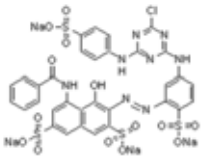
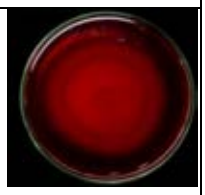
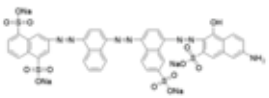
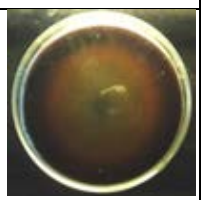
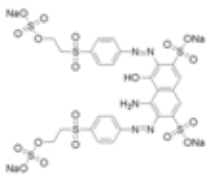
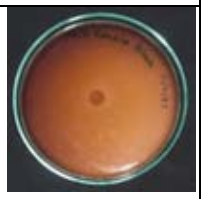
Part I Comparison of synthetic dye decolorization by whole cells and a laccase enriched extract from *T. versicolor* DSM11269

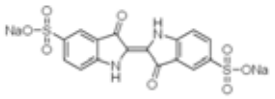
1. Decolorization of dyes on solid plates

The ability of *T. versicolor* to decolorize seven selected dyes was first investigated using solid plate assays (Table IV-1). After seven days, Amaranth and Reactive Black 5 were completely decolorized, as revealed by the > 80 mm diameter of the decolorized zone. Decolorization of Remazol Brilliant Blue R (RBBR) was less extensive with a decolorized zone diameter of between 60 - 79 mm. The other four selected dyes (Alizarin Red S, Indigo Carmine, Direct Blue 71 and Cibacron Brilliant Red 3B-A) have never been tested before with *T. versicolor* on solid medium. Alizarin Red S and Indigo Carmine were completely decolorized whereas partial decolorization was obtained for Direct Blue 71. Finally, no decolorization was observed for Cibacron Brilliant Red 3B-A.

T. versicolor showed different potential to decolorize seven dyes. The ability to decolorize Amaranth, Reactive Black 5 and Remazol Brilliant Blue R on solid plate are in agreement with the observations reported by Swamy and Ramsay (1999) and Junghanns et al. (2008) for *T. versicolor* strains ATCC 20869 and DSM 11269, respectively.

Table IV-1. Characteristics, molecular structure and decolorization studies of the selected dyes during solid plate growth of *T. versicolor* strain DSM11269.

Dye (classification)	λ_{max} (nm)	Structure	Diameter of decolorized zone on solid media after seven days (mm)	Photograph
1. Alizarin Red S (anthraquinone)	423		>80 mm	
2. Remazol Brilliant Blue R (RBBR) (anthraquinone)	595		60 - 79 mm	
3. Amaranth (azo)	521		>80 mm	
4. Cibacron Brilliant Red 3B-A (azo)	517		0 - 10 mm	
5. Direct Blue 71 (azo)	594		60 - 79 mm	
6. Reactive Black 5 (azo)	597		>80 mm	

7. Indigo Carmine (indigo)	602		>80 mm	

2. Laccase activity and dye decolorization by the crude enzyme preparation

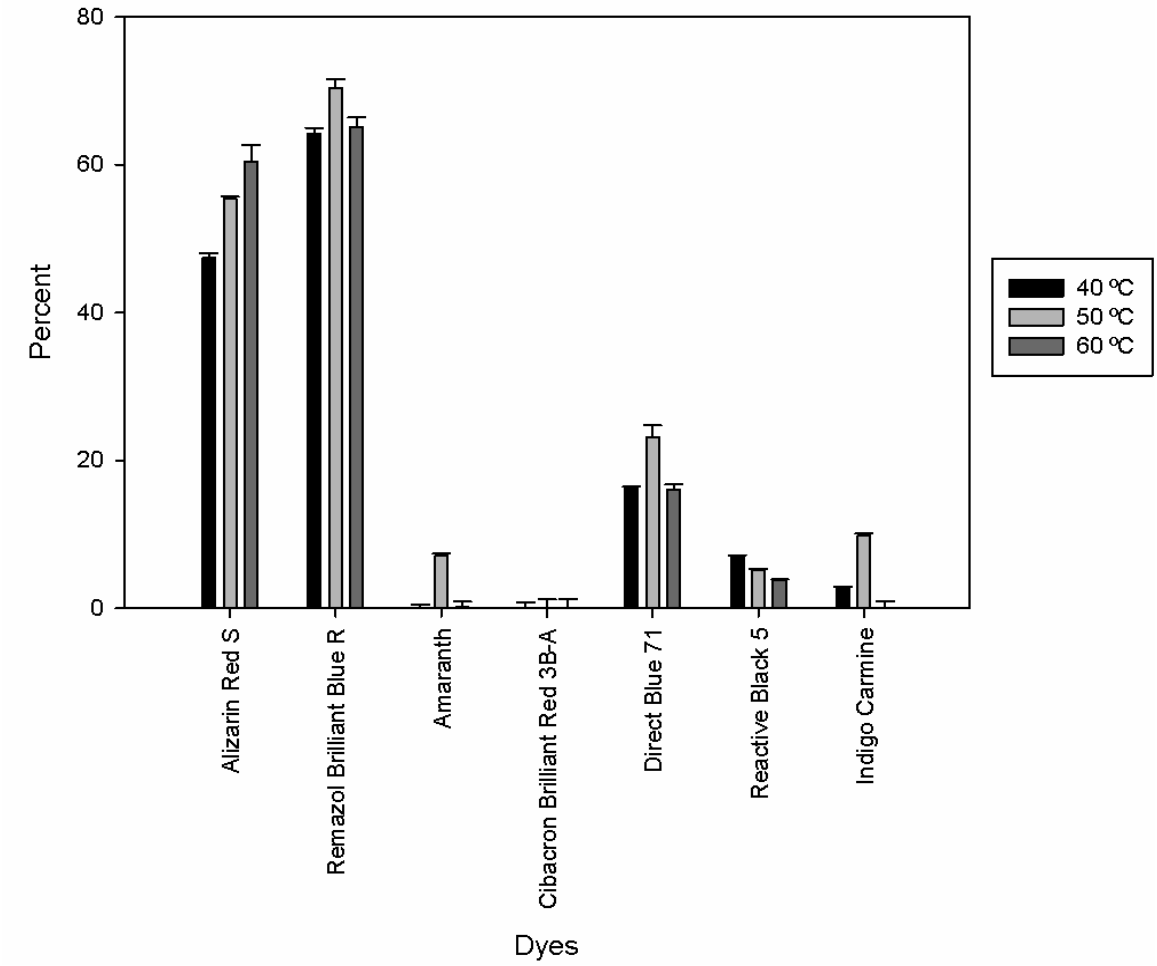
The treatment of dyes by white rot fungi has been extensively studied, but the evaluation of the use of their enzymes is more limited. The enzyme extract enriched in laccase activity was first prepared and tested for its ability to decolorize the seven selected dyes. Following induction with Guaiacol, the level of laccase production reached 3.23 U ml^{-1} of culture medium. The clear supernatant was diluted to 1 U ml^{-1} and incubated with the dyes at 40, 50 or 60 °C. The decrease in the absorbance of the respective maximum absorption wavelength (λ_{max}) (Table IV-1) was determined after three and six hours of incubation (Figure IV-1). The anthraquinone derivatives, Alizarin Red S and RBBR, were relatively strongly decolorized, reaching 60% and 70% decolorization, respectively, after three hours at 60 °C or 50 °C (Figure IV-1).
The azo and indigo dyes were more resistant to decolorization. The best result for azo dye decolorization was obtained with Direct Blue 71, where after three hours at 50 °C, 23% decolorization was observed (Figure IV-1 and Table IV-2). By contrast, another two azo dyes (Amaranth and Reactive Black 5), and the indigo dye (Indigo Carmine), showed a very low level of decolorization at less than 10% during the same 3-6 hours incubation time (Table IV-2).

The strongly decolorized of anthraquinone derivatives, Alizarin Red S and RBBR, by crude enzyme preparation were correlated with those obtained on solid medium. RBBR decolorization is efficient and comparable to that obtained with laccase enriched preparations from *Trametes hirsuta* and *Cerrena unicolor* (Moilanen et al., 2010) and this is the first report of Alizarin Red S decolorization by laccase enriched enzyme extracts.

Regarding the Indigo and three azo dyes (Direct Blue 71, Amaranth and Reactive Black 5), there is no correlation between the results obtained on solid medium and those obtained with the enzyme extract that is enriched in laccase. The decolorization observed on solid medium is, therefore, likely to be due to other oxidizing activities, that could be better excreted when growing the fungi on agar plates. Moreover, we can suggest that laccase may not be highly active on these substrates.

The azo dye Cibacron Brilliant Red 3B-A is highly recalcitrant to degradation. Indeed, no decolorization was observed either on solid medium or in liquid assay. This could be attributed to the complex structure of this dye. Indeed, similar results were reported by Wong and Yu (1999) with the laccase from *T. versicolor* ATCC 48424, where the azo (Acid Violet 7) and indigo (Indigo Carmine) dyes were not decolorized in contrast to the anthraquinone dye (Acid Green 27) which was more easily decolorized. Additionally, Champagne and Ramsay (2005) reported that the purified laccase from *T. versicolor* ATTC 20869 did not efficiently degrade azo compounds compared to the MnP produced by the same strain. Our results are in agreement with these observations. Regarding indigo and by analogy with the results obtained with azo compounds, we can suggest that laccase is not responsible for the high level of degradation that is observed on the solid medium. For all dyes tested, the decolorization rates were optimal at 50 ^{o}C, except for Alizarin Red S and Reactive Black 5 that were slightly faster at 60 °C and 40 ^{o}C, respectively. Previously, Nyanhongo et al. (2002) reported an optimal temperature of 50 °C for the decolorization of Indigo Carmine using an enzyme extract from *T. modesta* that was enriched in laccase activity. In addition, the decolorization was enhanced by the addition of redox mediators (Nyanhongo et al., 2002; Moilanen et al., 2010).

A)


80
60
40
20
0
Percent
Alizarin Red S
Remazol Brilliant Blue R
Amaranth
Cibacron Brilliant Red 3B-A
Direct Blue 71
Reactive Black 5
Indigo Carmine
Dyes
40 ºC
50 ºC
60 ºC

B)

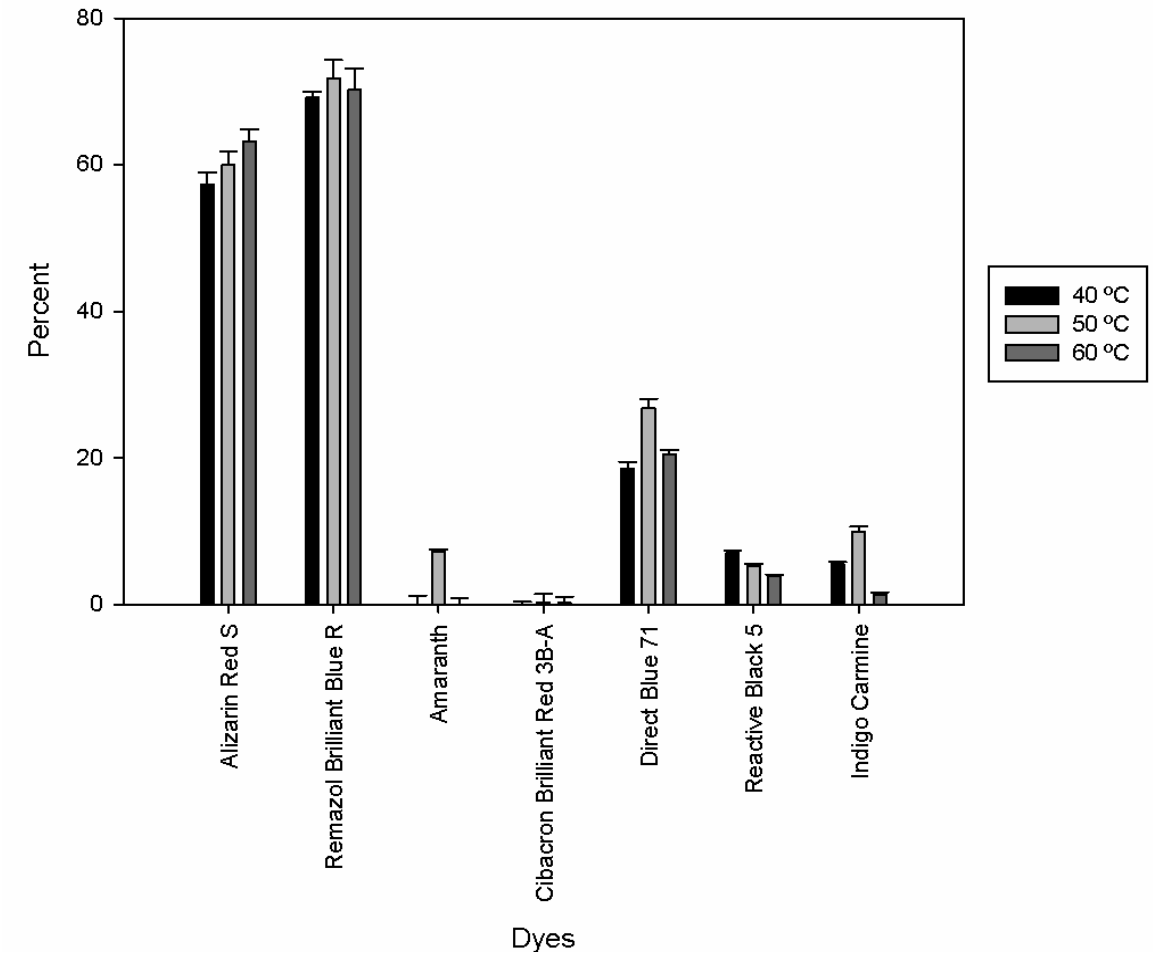


Figure IV-1. Effect of temperature on the decolorization of each dye by the crude laccase enzyme preparation from *Trametes versicolor* DSM 11269 after (A) three and (B) six hours. Results are expressed as the mean $\pm$ 1 S.D. and are derived from triplicate experiments.

Table IV-2. Percentage of dye decolorization obtained by the crude enzyme preparation from *T. versicolour* DSM 11269 after three and six hours at 50 °C.

Dye	Alizarin Red S (anthraquinone)	Remazol Brilliant Blue R (RBBR) (anthraquinone)	Amaranth (azo)	Cibacron Brilliant Red 3B-A (azo)	Direct Blue 71 (azo)	Reactive Black 5 (azo)	Indigo Carmine (indigo)
3 hours	55.4 $\pm$ 0.3	70.4 $\pm$ 1.2	7.1 $\pm$ 0.3	0.1 $\pm$ 1.1	23.2 $\pm$ 1.6	5.2 $\pm$ 0.3	9.9 $\pm$ 0.2
6 hours	60.0 $\pm$ 1.9	71.8 $\pm$ 2.5	7.2 $\pm$ 0.3	0.3 $\pm$ 1.2	26.8 $\pm$ 1.3	5.2 $\pm$ 0.3	9.9 $\pm$ 0.7

Data are shown as the mean $\pm$ 1 S.D. and are derived from 3 independent repeats.

Part 2 Engineering and production of laccase from *T. versicolor* in the yeast *Y. lipolytica:* application to the decolorization of synthetic dyes

1. Cloning of cDNA of the laccase gene from T. versicolor

A cDNA fragment was generated by RT-PCR using the Lcc1F/R primers to amplify the cDNA, derived from a total RNA extraction from *T. versicolor* DSM 11269. The amplicon was inserted into JMP 62Ura3d1ExpTEFHis/lcc1 vector as described in materials and methods section. The resulting plasmid was used to transform *E. coli* and this recombinant plasmid was then submitted for DNA sequencing. The derived 1563-bp cDNA is predicted to encode a Lcc1 laccase of 498 amino acid residues preceded by a 22-residue signal peptide, with a calculated molecular mass of 56 kDa (excluding any post-translational modifications) and isoelectric point of 4.3. BLASTn searching of the NCBI database revealed that this cDNA shows 97% nucleotide sequence identity and 99 % protein identity to the *T. versicolor lcc1* laccase of *T. versicolor* PRL 572 described by Jönsson et al. (1995) (accession number X84683) (Figure IV-2).

Nucleotide sequence of *lcc1* from *Trametes versicolor* DSM11269

atgggcaggttctcatctctctgcgcgctcaccgccgtcatccactcttttggtcgtgtctctgccgctatcgggcctgtgacc
gacctcaccatctctaatgcggacgtttctcccgacggcttcactcgtgccgcagtgcttgcaaacggtgtcttcccgggtcc
tctcatcacgggaaacaagggcgacaacttccagatcaatgttgtcgacaacctctctaacgaaacgatgtcgaagtcgac
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ccgacc

Protein sequence of LCC1 from *Trametes versicolor* DSM11269

MGRFSSLCALTAVIHSFGRVSAAIGPVTDLTISNADVSPDGFTRAAVLANGVFPGPLITG
NKGDNFQINVVDNLSNETMSKSTSIHWHGFFQKGTNWADGAAFVNQCPIATGNSFLYDFT
ATDQAGTFWYHSHLSTQYCDGLRGPMVVYDPSDPHADLYDVDDETTIITLSDWYHTAASL
GAAFLVGSDSTLINGLGRFAGGDSTDLAVITVEQGKRYRMRLLSLSCDPNYVFSIDGHNM
TIIEADAVNHEPLTVDSIQIYAGQRYSFVLTADQDIDNYFIRALPSAGTTSFDGGINSAI
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INDVSFVSPTVPVLLQILSGTTSAADLLPSGSLFALPSNSTIEISFPITATNAPGAPHPF
HLHGHTFSIVRTAGSTDTNFVNPVRRDVVNTGTAGDNVTIRFTTDNPGPWFLHCHIDFHL
EAGFAIVFSEDTADVSNTTTPSTAWEDLCPTYNALDSSD

```
CLUSTAL 2.0.12 multiple sequence alignment


1KYA              1   MSRFHSLF--AFVVASLTAVAHAGIGPVADLTITNAAVSPDGFSRQAVVVNGGTPGPLIT  58
3FPX              1   MSRFQSLL--TFINISLVAVAHAAVGPVADLTITDAAVSPDGFSRQAVVVNGVTPGPLVA  58
2HRG              1   MARFQSLL--TFITLSLVASVYAAIGPVADLTISNGAVSPDGFSRQAILVNDVFPSPLIT  58
1GYC              1   MG-LQRFS--FFVTLALVARSLAAIGPVASLVVANAPVSPDGFLRDAIVVNGVVPSPLIT  57
3KW7              1   MGKLQSFVNVVALSLSLSRGVLGAIGPVTDLTISNADVTPDGFTRAAVVANGVFPGPLIT  60
T.v.DSM11269      1   MGRFSSLCALTAVIHSFGR-VSAAIGPVTDLTISNADVSPDGFTRAAVLANGVFPGPLIT  59
                  *. :  :     :  ::     ..:***:.*.:::. *:**** * *::.*.  *.**::

1KYA              59  GNMGDRFQLNVIDNLTNHTMLKSTSIHWHGFFQKGTNWADGPAFINQCPISSGHSFLYDF  118
3FPX              59  GNIGDRFQLNVIDNLTNHTMLKSTSIHWHGFFQHGTNWADGPAFINQCPISPGHSFLYDF  118
2HRG              59  GNKGDRFQLNVIDNMTNHTMLKSTSIHWHGFFQHGTNWADGPAFVNQCPISTGHAFLYDF  118
1GYC              58  GKKGDRFQLNVDDTLTNHSMLKSTSIHWHGFFQAGTNWADGPAFVNQCPIASGHSFLYDF  117
3KW7              61  GNKGDNFQINVIDNLTNATMLKTTTIHWHGLFQHGTNWADGPAFVNQCPIASGNSFLYDF  120
T.v.DSM11269      60  GNKGDNFQINVVDNLSNETMSKSTSIHWHGFFQKGTNWADGAAFVNQCPIATGNSFLYDF  119
                  *: **.**:** *.::* :* *:*:*****:** *******.**:*****:.*::*****

1KYA              119 QVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPAADLYDVDNDDTVITLVDWYHVAAK 178
3FPX              119 QVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPHASRYDVDNDDTVITLADWYHTAAK 178
2HRG              119 QVPDQAGTFWYHSHLSTQYCDGLRGPIVVYDPQDPHKSLYDVDDDSTVITLADWYHLAAK 178
1GYC              118 HVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPKDPHASRYDVDNESTVITLTDWYHTAAR 177
3KW7              121 TVPDQAGTFWYHSHLSTQYCDGLRGPLVVYDPSDPYASMYDVDDDTTVITLSDWYHTAAK 180
T.v.DSM11269      120 TATDQAGTFWYHSHLSTQYCDGLRGPMVVYDPSDPHADLYDVDDETTIITLSDWYHTAAS 179
                   ..**********************:*****.**  . ****:: *:*** **** **

1KYA              179 LGPAFPLGADATLINGKGRSPSTTTADLSVISVTPGKRYRFRLVSLSCDPNYTFSIDGHN 238
3FPX              179 LGPRFPGGADATLINGKGRAPSDSPAELSVIKVTKGKRYRFRLVSLSCNPNHTFSIDGHN 238
2HRG              179 VGSPVPT-ADATLINGLGRSIDTLNADLAVITVTKGKRYRFRLVSLSCDPNHVFSIDGHS 238
1GYC              178 LGPRFPLGADATLINGLGRSASTPTAALAVINVQHGKRYRFRLVSISCDPNYTFSIDGHN 237
3KW7              181 LGPAFPPNADSVLINGLGRFAGGNASDLAVITVEQNKRYRFRLVSLSCDPNFTFSIDGHN 240
T.v.DSM11269      180 LGAAFLVGSDSTLINGLGRFAGGDSTDLAVITVEQGKRYRMRLLSLSCDPNYVFSIDGHN 239
                  :*. .   :*:.**** **  .   : *:**.*  .****:**:*:**:**..******.

1KYA              239 MTIIETDSINTAPLVVDSIQIFAAQRYSFVLEANQAVDNYWIRANPNFGNVGFTGGINSA 298
3FPX              239 LTIIEVDSVNSQPLEVDSIQIFAAQRYSFVLDANQAVDNYWIRANPNFGNVGFDGGINSA 298
2HRG              239 LTVIEADSVNLKPQTVDSIQIFAAQRYSFVLNADQDVGNYWIRALPNSGTRNFDGGVNSA 298
1GYC              238 LTVIEVDGINSQPLLVDSIQIFAAQRYSFVLNANQTVGNYWVRANPNFGTVGFAGGINSA 297
3KW7              241 MTIIEVDGVNHEPLEVDSIQIFASQRYSFVLNATQSVDNYWIRAIPNTGTIDTTGGLNSA 300
T.v.DSM11269      240 MTIIEADAVNHEPLTVDSIQIYAGQRYSFVLTADQDIDNYFIRALPSAGTTSFDGGINSA 299
                  :*:**.*.:*  *  ******:*.******* * * :.**::** *. *. .  **:***

1KYA              299 ILRYDGAAAVEPTTTQTTSTAPLNEVNLHPLVATAVPGSPVAGGVDLAINMAFNFNGTNF 358
3FPX              299 ILRYDGAPAVEPTTNQTTSVKPLNEVDLHPLVSTPVPGSPSSGGVDKAINMAFNFNGSNF 358
2HRG              299 ILRYDGAAPVEPTTSQTPSTNPLVESALTTLEGTAAPGSPAPGGVDLALNMAFGFAGGKF 358
1GYC              298 ILRYQGAPVAEPTTTQTTSVIPLIETNLHPLARMPVPGSPTPGGVDKALNLAFNFNGTNF 357
3KW7              301 ILRYSGADIVDPTANATTSVIPLVETDLVPLDSPAAPGDPVVGGVDLAMNLDFSFNGTNF 360
T.v.DSM11269      300 ILRYSGASEVDPTTTETTSVLPLDEANLVPLDSPAAPGDPNIGGVDYALNLDFNFDGTNF 359
                  ****.**  .:**:. *.*. ** *  * .*   ..**.*  **** *:*: *.* * :*

1KYA              359 FINGASFTPPTVPVLLQIISGAQNAQDLLPSGSVYSLPSNADIEISFPATAAA-----PG 413
3FPX              359 FINGASFVPPTVPVLLQILSGAQTAQDLLPSGSVYVLPSNASIEISFPATAAA-----PG 413
2HRG              359 TINGASFTPPTVPVLLQILSGAQSAQDLLPSGSVYSLPANADIEISLPATAAA-----PG 413
1GYC              358 FINNATFTPPTVPVLLQILSGAQTAQDLLPAGSVYPLPAHSTIEITLPATALA-----PG 412
3KW7              361 FINNETLIPPTVPVLLQILSGAQSASDLLPTGSVYTLPLNSTIELSFPITTVNGVTNAPG 420
T.v.DSM11269      360 FINDVSFVSPTVPVLLQILSGTTSAADLLPSGSLFALPSNSTIEISFPITATN----APG 415
                   **. :: .*********:**: .* ****:**:: ** :: **:::* *:       **

1KYA              414 APHPFHLHGHAFAVVRSAGSTVYNYDNPIFRDVVSTGTPAAGDNVTIRFRTDNPGPWFLH 473
3FPX              414 APHPFHLHGHTFAVVRSAGSTVYNYDNPIFRDVVSTGTPAAGDNVTIRFDTNNPGPWFLH 473
2HRG              414 FPHPFHLHGHTFAVVRSAGSSTYNYENPVYRDVVSTGSP--GDNVTIRFRTDNPGPWFLH 471
1GYC              413 APHPFHLHGHAFAVVRSAGSTTYNYNDPIFRDVVSTGTPAAGDNVTIRFQTDNPGPWFLH 472
3KW7              421 APHPFHLHGHAFSVVRSAGSSDYNYVNPVRRDTVSTGNP--GDNVTIRFTTDNAGPWFLH 478
T.v.DSM11269      416 APHPFHLHGHTFSIVRTAGSTDTNFVNPVRRDVVNTGTA--GDNVTIRFTTDNPGPWFLH 473
                   *********:*::**:***:  *: :*: **.*.**..  ******** *:*.******

1KYA              474 CHIDFHLEAGFAVVFAEDIPDVASANPVPQAWSDLCPTYDARDPSDQ 520
3FPX              474 CHIDFHLEGGFAVVMAEDTPDVKAVNPVPQAWSDLCPTYDALDPNDQ 520
2HRG              472 CHIDFHLEAGFAVVMAEDIPEVAATNPVPQAWSDLCPTYDALSPDDQ 518
1GYC              473 CHIDFHLDAGFAIVFAEDVADVKAANPVPKAWSDLCPIYDGLSEANQ 519
3KW7              479 CHIDFHLEAGFAIVFAEDTPDTASVNPVPTAWSDLCPTYDALDPSDH 525
T.v.DSM11269      474 CHIDFHLEAGFAIVFSEDTADVSNTTTPSTAWEDLCPTYNALDSSD- 519
                  *******:.***:*::** .:.  ... . **.**** *:. .  :
```

Figure IV-2. Alignment between laccase of *Trametes versicolor* DSM11269 and 5 crystal structures of laccases from *Trametes* sp.

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CLUSTAL 2.0.12 Multiple Sequence Alignments

Sequence format is Pearson
Sequence 1: 1GYC          519 aa
Sequence 2: 1KYA          520 aa
Sequence 3: 2HRG          517 aa
Sequence 4: 3KW7          525 aa
Sequence 5: 3FPX          520 aa
Sequence 6: T.v.DSM11269   519 aa
Start of Pairwise alignments
Aligning...

Sequences (1:2) Aligned. Score:  77
Sequences (1:3) Aligned. Score:  73
Sequences (1:4) Aligned. Score:  70
Sequences (1:5) Aligned. Score:  77
Sequences (1:6) Aligned. Score:  66
Sequences (2:3) Aligned. Score:  78
Sequences (2:4) Aligned. Score:  72
Sequences (2:5) Aligned. Score:  87
Sequences (2:6) Aligned. Score:  68
Sequences (3:4) Aligned. Score:  70
Sequences (3:5) Aligned. Score:  78
Sequences (3:6) Aligned. Score:  67
Sequences (4:5) Aligned. Score:  71
Sequences (4:6) Aligned. Score:  76
Sequences (5:6) Aligned. Score:  67
Guide tree file created:   [infile.dnd]

There are 5 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences:   2     Score:10708
Group 2: Sequences:   3     Score:10150
Group 3: Sequences:   4     Score:10089
Group 4: Sequences:   2     Score:10114
Group 5: Sequences:   6     Score:9453
Alignment Score 37507

CLUSTAL-Alignment file created  [infile.aln]
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Figure IV-2. Alignment between laccase of *Trametes versicolor* DSM11269 and 5 crystal structures of laccases from *Trametes* sp. (continue)

2. Heterologous expression of the laccase gene in Y. lipolytica

The heterologous expression of the *lcc1* gene from *T. versicolor* in *Y. lipolytica* gave significant levels of extracellular laccase activity (0.25 U ml^{-1}) from the transformed zeta strain (monocopy) in liquid culture after 30 h. Thus, *Y. lipolytica*

can express and secrete the heterologous rWTLcc1 laccase in an active and stable form and so is a suitable host for such expression. In addition, the native signal peptide from *T. versicolor* was clearly effective for directing the expression and secretion of Lcc1 in *Y. lipolytica*. The level of activity obtained from the transformed zeta strain was broadly equivalent to that reported by Jolivalt et al. (2005), who expressed laccaseIIIb in *Y. lipolytica* Po1g (monocopy), at 0.23 U ml^{-1}. However, in this study a shorter culture time (30 h compared to 6 days) was required. Moreover, a four-fold higher total activity level of the rWT laccase in the culture medium (1 U ml^{-1}) within 30 hours was obtained using multicopy expression in the MTLY60 strain.

To the best of our knowledge, no data on the expression of laccase gene from *T. versicolor* in multicopy strain has been reported prior to this study. Our results, show that *Y. lipolytica* could be an efficient strain for laccase production. Although a very high laccase activity (12 $U.ml^{-1}$) has been reported for *lcc1* expression in *P. methanolica* after 5 days under optimized conditions (Guo et al., 2006), but the expression required induction by toxic methanol in contrast to that reported here for the *Y. lipolytica* system. Moreover, the optimization and use of larger fermentation cultivations may well lead to further enhanced expression levels in the *Y. lipolytica* system comparable to that obtained for recombinant production of the lipases from *C. antarctica* (Emond et al., 2010).

Laccases are multicopper containing enzymes that require copper ions for their activity. Over the tested concentration range (0.4-2.0 mM) in zeta strain, the addition of $CuSO_4$ to the *lcc1*-transformed *Y. lipolytica* culture medium significantly increased the total extracellular rWT laccase activity (Figure IV-3). Essentially no laccase activity was detected in the absence of $CuSO_4$, whilst the total laccase activity increased with increasing $CuSO_4$ levels to a maximum level of 1.2 mM $CuSO_4$ (0.25 U ml^{-1}). Increasing the copper concentration above 1.2 mM then led to an almost 50% reduction in the total laccase activity levels. Previously, Jönsson et al. (1997) expressed the *T. versicolor lcc1* gene in *P. pastoris*. The culture was performed at 0.1mM $CuSO_4$. They found the expression level obtained was too low for any subsequent characterization of the enzyme. The optimum concentration observed in

this study is higher than the optimal level of 0.2 mM $CuSO_4$ reported by O' Callaghan et al. (2002) and Guo et al. (2006) for *P. pastoris* and *P. methanolica*, but somewhat similar to that for stimulating laccase synthesis (1.0 mM) observed by Xiao et al (2006) and Fan et al (2011). At 1 mM $CuSO_4$ with *Trametes* sp. AH28-2, Xiao et al (2006) found about 0.18 Uml^{-1} of laccase activity. At the same copper concentration, laccase activity was observed at 15.27 Uml^{-1} with *Trametes* sp. 48424 (Fan et al., 2011). In contrast, a higher $CuSO_4$ concentration (3.5 mM) yielded about 8 U ml^{-1} of *T. versicolor* CBS100.29 laccase activity (Lorenzo et al., 2006). The correlation between laccase activity and copper availability is due to the copper requirement of the enzyme and appears to be host dependent (Guo et al., 2006).

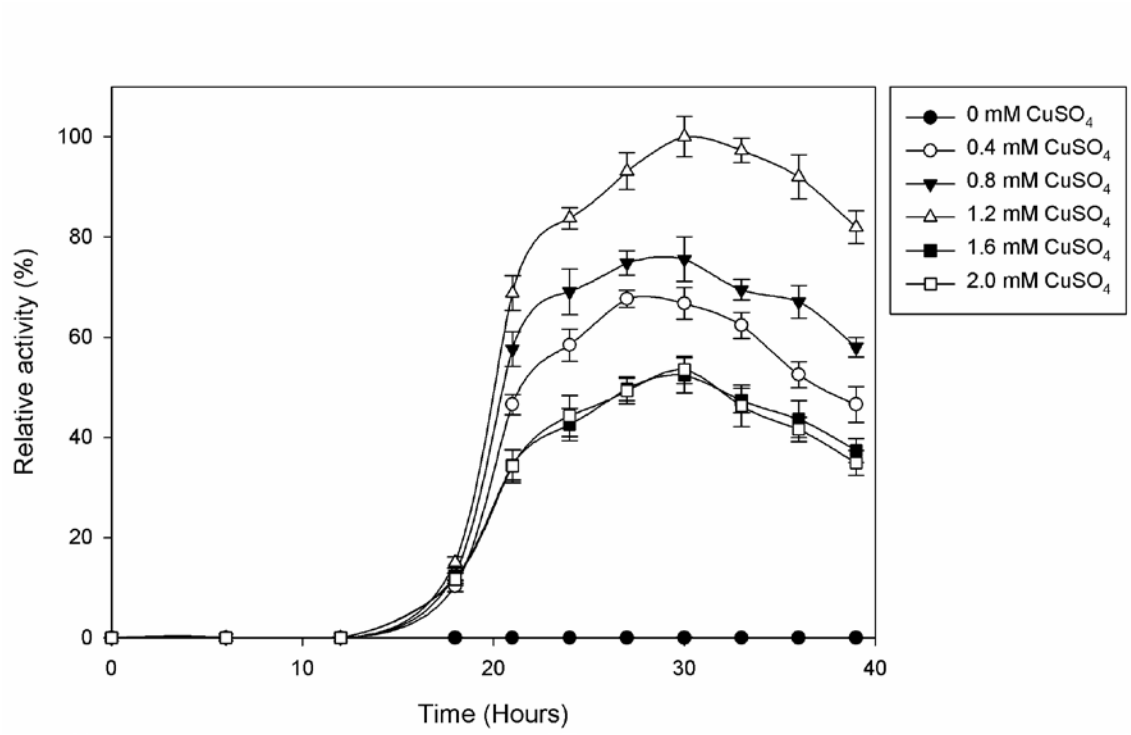


Figure IV-3. Effect of different copper sulfate concentrations in the transformed *Y. lipolytica* culture media on the total extracellular rWT laccase activity after 39 h culture. Data are shown as the mean $\pm$ 1 SD relative activity, where 100% is set as the highest observed activity, and are derived from three independent repeats.

3. Laboratory evolution of Lcc1 laccase from Trametes versicolor

The first mutagenic library (~9000 mutants) was constructed by error-prone PCR. The PCR products were used to transform into *Y. lipolytica* and plating in ABTS agar plate. The *Y. lipolytica* mutants that secreted a high laccase activity were selected on ABTS agar plates in terms of selecting those colonies which gave rise to a dark green zone around them, which represents the secretion of an active laccase. Note that no green zone was observed around control colonies transformed without the *lcc1* gene. These selected colonies (~70 colonies) were then further evaluated for laccase secretion (in terms of total enzyme activity) in liquid culture medium as described in materials and methods. The three mutants (mutants rM-1A, rM-2A and rM-3A) from the first round of evolution were isolated and zeta cassette subjected to DNA sequencing. Each of the three mutants revealed between 4 to 16 mutations in the zeta cassette region, but only one non-synonymous substitution (different one for each mutant) in the *lcc1* gene was found, namely L185P, Q214K and D494N for mutants rM-1A, rM-2A and rM-3A, respectively. *De novo* construction of the L185P, Q214K and D494N point substitutions by site-directed mutagenesis to yield mutants rM-1AC, rM-2AC and rM-3AC, respectively, was used to confirm that the effect was only due to mutation in the gene. The result revealed the equivalent total laccase activity as that seen in the corresponding mutants rM-1A, rM-2A and rM-3A. Thus, the L185P, Q214K and D494N substitution mutations are responsible for the observed 4.7, 2.0 and 1.8-fold increase in the total extracellular laccase activity compared to that seen with the rWT (Figure IV-4).

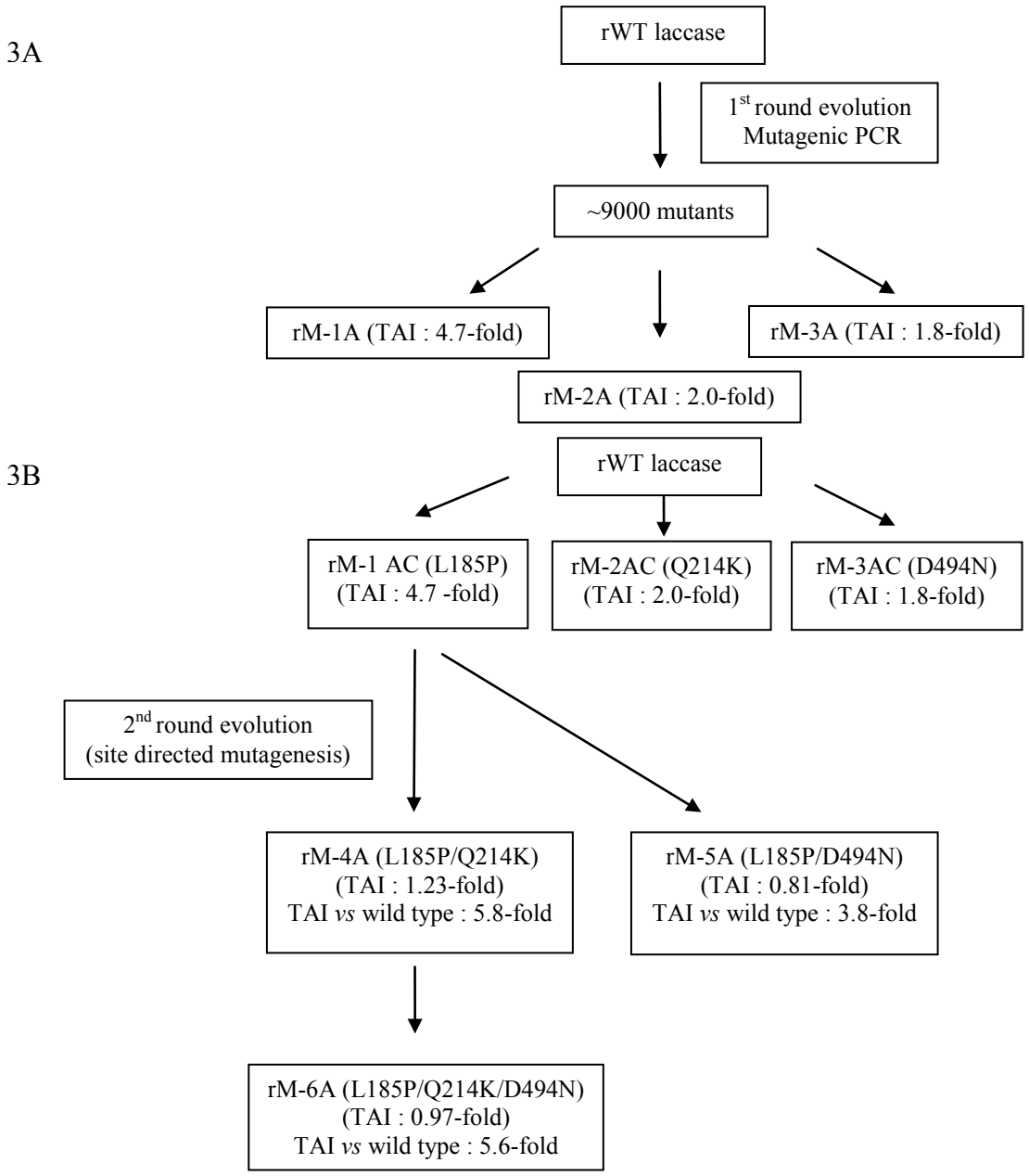


Figure IV-4. Artificial evolution pathway. 3A : First round of evolution. EP PCR carried out using lcc1 gene as matrix. 3B : Construction of single, double and triple mutants containing mutations at positions identified from the first round. TAI (total activity improvement) value indicates the improvement in laccase activity detected in *Y. lipolytica* strain zeta for each mutant when compared with the parental type of the corresponding generation.

The L185P mutation was used as a parental template to construct the two double mutant (rM-4A, L185P/Q214K and rM-5A, L185P/D494N) and one triple mutant (rM6A, L1895P/Q214K/D494N). The selected clone for mutant rM-4A (L185P/Q214K) was found to yield a 5.8-fold higher total extracellular laccase activity while the mutant rM-5A (L185P/D494N) and rM-6A (L1895P/Q214K/D494N) were found to yield 3.8 and 5.6 -fold higher total extracellular laccase activity improvement which corresponded to 1.5, 1 and 1.4 Uml^{-1}, respectively.

4. Purification and characterization of recombinant laccase

4.1 Purification of recombinant laccase produced by Yarrowia lipolytica

Due to the highest total activity improvement observed in the mutant rM-4A (in the result part 3.3), this mutant was undertaken for purification and comparison with the wild type laccase. The supernatant of the rWT (produced by MTLY60 strain) and mutant rM-4A (rL185P/Q214K) were used for purification by ultrafiltration and metal affinity chromatography (Table IV-3).

Table IV-3 Purification of the recombinant wild type (rWT) and L185P/Q214K mutant-4A (rM-4A) laccases

Purification step	Total protein (mg)		Total activity (U)		Specific activity (U mg^{-1})		Purification (fold)		Yield (%)	
	rWT	rM-4A	rWT	rM-4A	rWT	rM-4A	rWT	rM-4A	rWT	rM-4A
1.Culture filtrate	38.95	36.09	226.1	230.4	5.8	6.4	1.0	1.0	100.0	100.0
2. Ultrafiltration	11.81	5.36	196.8	196.6	16.7	36.7	2.9	5.7	87.0	86.1
3. Affinity chromatography (IMAC)	0.06	0.03	9.0	9.3	147.6	305.9	25.4	47.9	4.0	4.0

Purification factor 25.4-fold and 47.9-fold, respectively were obtained with a laccase activity yield of 4% in both cases. Both laccase isoforms appeared as a single

protein band on a Coomasie-blue stained SDS-PAGE gel with an estimated mass of 75 kDa (Figure IV-5A, IV-5B). This is significantly higher than the theoretically expected 54.1 kDa derived from the deduced amino acid sequence. However, after endoglycosidase H treatment to remove N-linked glycans the apparent MW of the rWT and rM-4A isoforms were both reduced to ~60 kDa (Figure IV-5C), which suggests it is N-glycosylated to ~20% by weight. The molecular mass obtained is 14.7% difference in mass between the recombinant *T. versicolor* Lcc1 heterologously produced in *Y. lipolytica* (75 kDa) and in *P. methanolica* (64 kDa) (Guo et al., 2006) which is likely to reflect host-dependent differences in glycosylation patterns. Indeed, the molecular mass obtained is consistent with the reported molecular mass of other *T. versicolor* laccases (Cassland and Jönsson, 1999), but lower than the presumably hyperglycosylated laccaseIV (85 KDa) from *T. versicolor* when expressed in *P. pastoris* (Brown et al., 2002).

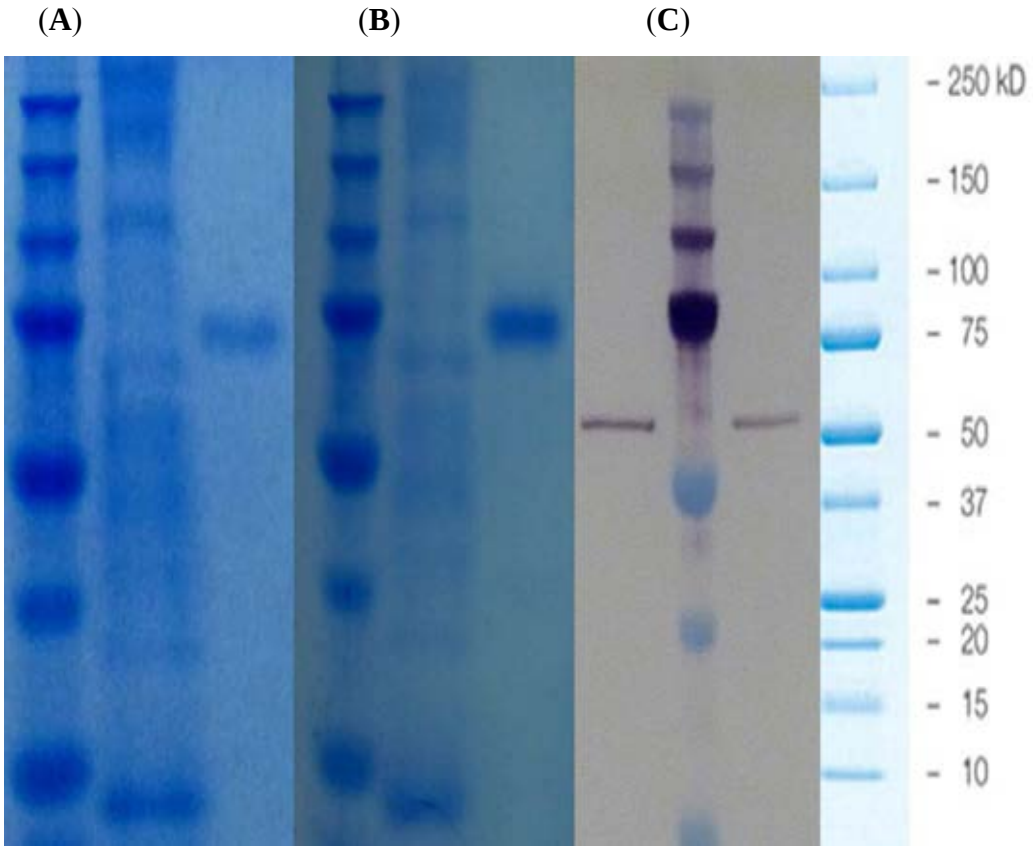


Figure IV-5. Coomassie blue stained SDS-PAGE resolution of the crude and purified (**A**) rWT and (**B**) rM-4A laccases produced in the *Y. lipolytica* strains MTLY60 and zeta, respectively. Lane 1: molecular mass markers, Lane 2: crude culture supernatant, Lane 3: after purification. (**C**) Western blot analysis of the marker (lane 2), rWT (lane 1), and rM-4A (lane 3) laccase after endoglycosidase H deglycosylation showing the molecular mass decrease from ~75 kDa to ~60 kDa for both rWT and rM-4A.

The effect of pH and temperature on the activity and stability of the purified rWT and rM-4A laccases were nearly identical. (Figure IV-6)

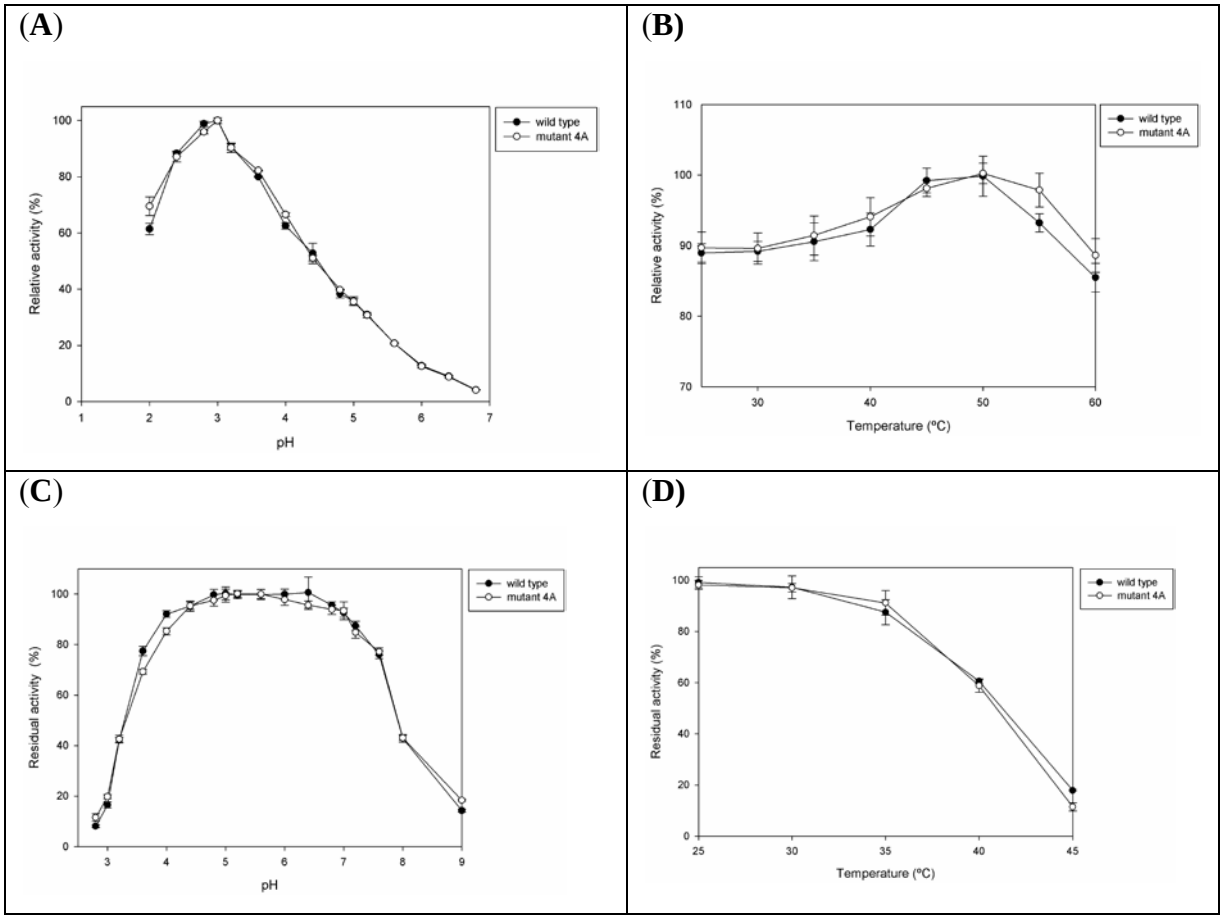


Figure IV-6. Effect of varying the (**A**) pH and (**B**) temperature on the purified rWT and rM-4A laccase activity when incubated at 30 °C in (**A**) 0.1 M sodium acetate buffer at different pH values or (**B**) at different temperatures in 0.1 M sodium acetate

buffer pH 3.0. Effect of varying the (**C**) pH and (**D**) temperature on the stability of the purified rWT and rM-4A laccase activity. ABTS was used as the test substrate. Data are shown as the mean ± 1 SD relative activity, where 100% is set as the highest observed (optimal) activity, and are derived from three independent repeats.

4.2 Kinetic parameters determination

The kinetic parameters of the purified rWT and rM-4A laccases were determined using a spectrophotometric method for the two substrates, ABTS or 2,6-DMP. The K_m value for ABTS are lower than that determined for 2,6-DMP indicating a higher affinity of both enzymes for ABTS (Table IV-4). Besides, the kcat values of rM-4A mutant were 1.7 and 2.5 fold higher than that of the wild type for ABTS and 2,6-DMP, respectively. This results in an increase of the catalytic efficiency k_{cat}/K_m of rM-4A mutant compared with rWT (2.4-fold on ABTS and 2.8-fold on 2,6-DMP). These values confirm that rM-4A mutant is more efficient than the rWT. However, improvement is more modest than that observed on crude preparation indicating that the double mutant is also better expressed by the zeta strain than the wt enzyme.

Table IV-4 Comparison of the kinetic parameters of the purified recombinant wild type (rWT) and L185P/Q214K mutant-4A (rM-4A) laccases

Laccase	Substrate	K_m(µM)	k_{cat} (S^{-1})	k_{cat}/K_m ($mM^{-1}S^{-1}$)
rWT	ABTS	169 ± 7.5	125 ± 6.1	738
	2,6-DMP	249 ± 8.4	49 ±3	197
rM-4A	ABTS	120 ± 6.1	208 ± 9.5	1732
	2,6-DMP	225 ±6.9	124 ±5.8	553

Data are shown as the mean ± 1 SD and are derived from three independent repeats

A structural alignment was performed with all the crystallographic laccase available from *Trametes* species and the best alignment was found with *Trametes sp*. AH 28-2 (PDB ID: 3KW7). 76% identity with *Trametes sp.* AH 28-2 which was subsequently selected to generate a 3D model of *lcc1* from *Trametes versiclor* DSM 11269. As shown in the model, the mutations introduced are not directly found in the active site, they are located in loops at the surface of the protein. These observations suggest that the increase in the catalytic efficiency can be attributed to long range effect (Figure IV-7).

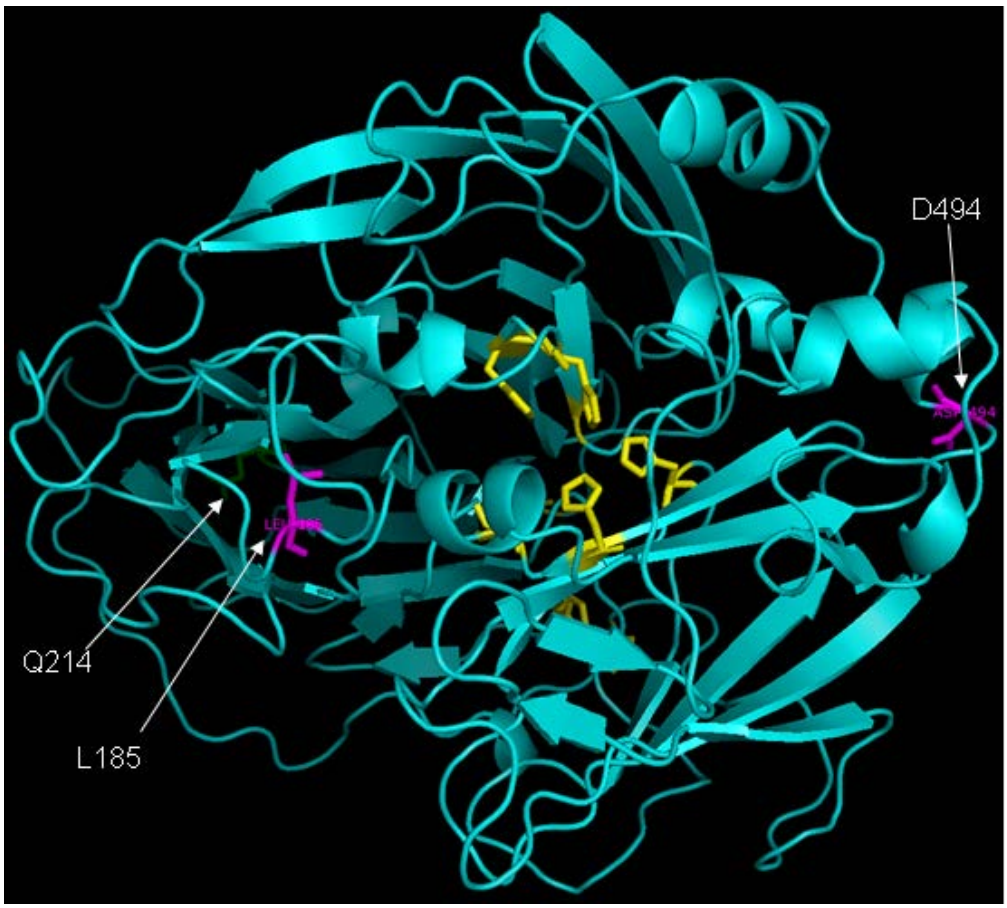


Figure IV-7. 3D model of laccase from *T. versicolor* DSM 11269 predicted by ESyPred3D using the crystallized laccase B from *Trametes. sp* AH28-2 (3KW7) as a template. Spatial location of L185, Q214 and D494 residues are indicated by arrow. Histidines of the coppers sites are drawn in yellow.

5. Enzymatic dye decolorization

The crude rLcc1 laccase from the liquid culture of *Y. lipolytica* was tested in the presence of the natural mediator acetosyringone for its ability to decolorize five dyes, whose structures cover the three main dye structures (azo, anthraquinone and triphenylmethane). As shown in Figure IV-8A, the required acetosyringone concentration for the optimal efficiency of decolorisation varied with each dye, the values ranging from 100 µM for Reactive Black 5, through 250 µM for Acid Violet 17, 500 µM for Amaranth and RBBR up to 1000 µM for Direct Blue 71 (Figure IV-8A). Excess levels of acetosyringone tended to be inhibitory for the oxidation of RBBR and Acid Violet 17 and in the absence of the mediator no decolorisation is observed. In accord with this notion, Soares et al. (2001) reported that laccase alone could not decolorize RBBR, but rather its decolorization was only observed when the redox mediator 1-hydroxybenzotriazole was present. Several other reports have suggested that a redox mediator can increase the laccase-mediated decolorization efficiency (Moilanen et al., 2010).

The crude rWT and rM-4A laccase preparations could efficiently decolorize all five dyes tested over the pH range 4-6, From the data obtained (Figure IV-8B), optimal final pH used in all subsequent decolorization assays was, therefore, 4, 4, 5, 6 and 7 for the Acid Violet 17, Amaranth, RBBR, Direct Blue71 and Reactive Black 5 dyes, respectively. The visible absorbance spectra for each of the five dyes tested before and after treatment with 0.5 U ml^{-1} of rM-4A laccase at the optimum pH and acetosyringone concentration for each dye are shown in Figure IV-9. The absorbance maximum of all five dyes clearly decreased after 1 h of enzyme incubation, which is associated with the oxidation of the dye. The optimum pH for the decolorization of RBBR is similar to that reported for the purified rWTLcc1 of *T. versicolor* expressed in *P. methanolica* (Guo et al., 2008). For the five dyes, the decolorization rates were comprised between 1.5 $nM.s^{-1}$ and 70 $nM.s^{-1}$. The highest value being observed for Amaranth decolorisation. These results reflect the efficiency of decolorisation and can be favourably compared to the values reported by Champagne and Ramsay for Amaranth decolorisation rate (0.75 $nM.s^{-1}$) in the presence of ABTS mediator (Table IV-5). The rWT and rM-4A laccases showed essentially the same decolorization

efficiency over time when at an equal dose in the reaction (Table IV-5, Figure IV-8C, IV-8D). This indicates that the screening procedure based on ABTS oxidation allowed isolation of mutants improved for ABTS transformation but not adapted to the natural substrate, acetosyringone, which is the most attractive mediator that could be used for dye decolorization.

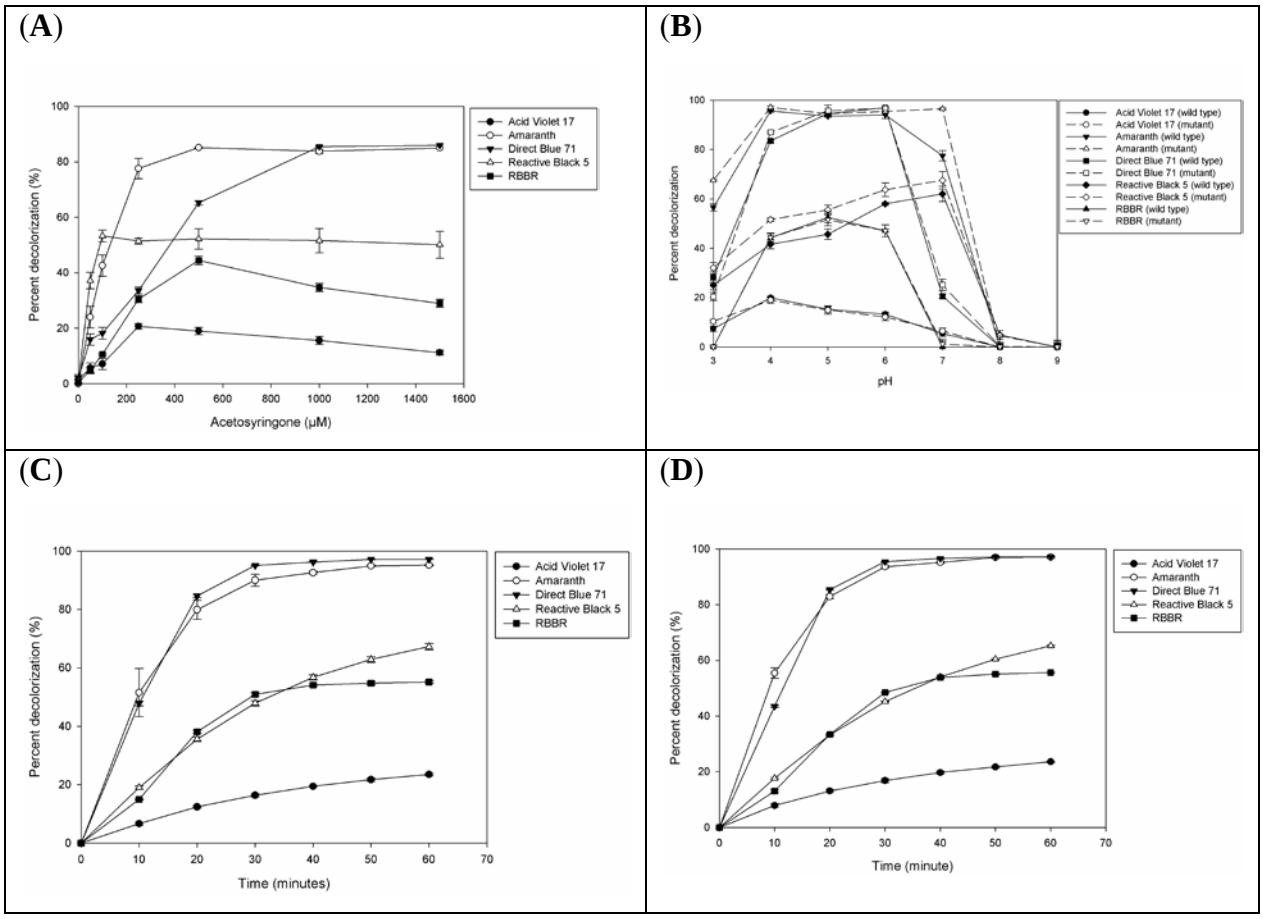


Figure IV-8. (**A**) Effect of varying the concentration of the natural redox mediator, acetosyringone, on the decolorization efficiency by the crude rWT laccase preparations. Reactions were performed with 0.5 U ml^{-1} laccase at 30 °C, 90 rpm, pH 4.5 for 1 h. (**B**) Effect of varying the pH on the decolorization efficiency of the crude rWT and r-M4A laccase preparations. Reactions were performed with 0.5 U ml^{-1} laccase at 30 °C, 90 rpm, and acetosyringone at the optimal concentration. (**C**)

Decolorization of the five synthetic dyes by 0.5 U ml^{-1} of the rWT and (**D**) rM-4A laccases at 30 °C, 90 rpm and the optimum pH and acetosyringone concentration for each dye. Data are shown as the mean ± 1 SD % decolorization, and are derived from three independent repeats.

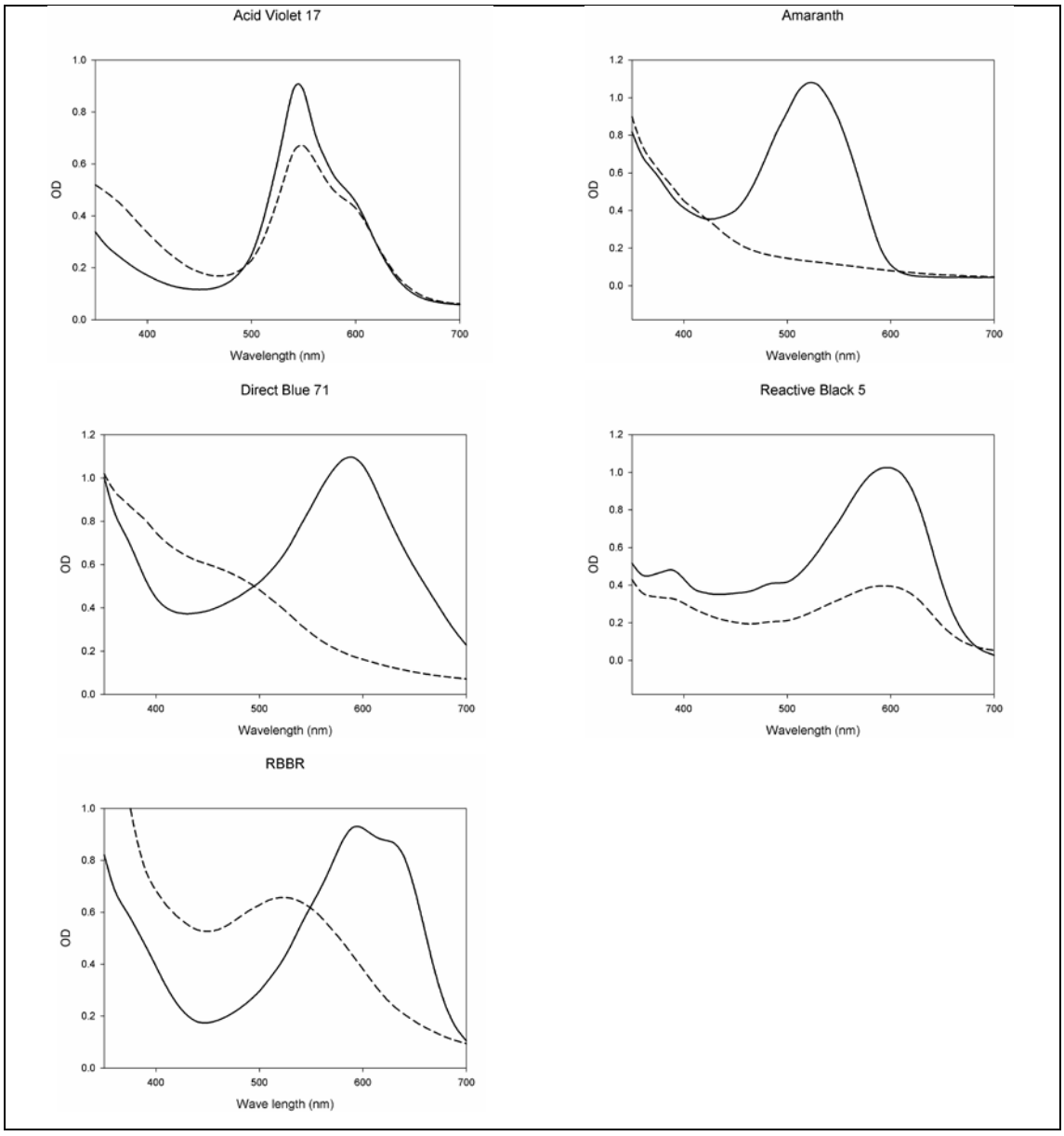
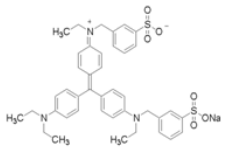
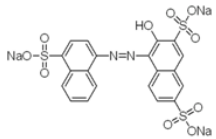
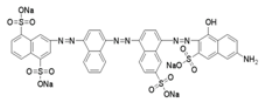
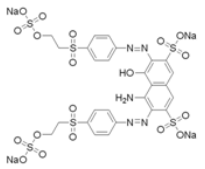
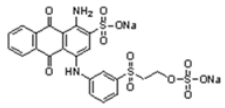


Figure IV-9. Visible absorbance spectrum for the synthetic dyes tested (solid line) 0 h and (dotted line) 1 h after treatment with 0.5 U ml^{-1} of crude rM-4A laccase at 30 °C, 90 rpm and with the optimum pH and acetosyringone concentration for each dye. Spectra shown are representative of those seen from three independent repeats.

Table IV-5 Characteristics and molecular structure of the selected dyes. Dye decolorization rates of the crude recombinant wild type (rWT) and L185P/Q214K mutant-4A (rM-4A) laccases (0.5 U ml^{-1}) at the optimum reaction conditions for each dye

Dye	Acid Violet 17		Amaranth		Direct Blue 71		Reactive Black 5		RBBR	
	Triarylmethane		Azo		Azo		Azo		Anthraquinone	
Dye class										
	λ_{max} (nm) : 545		λ_{max} (nm) : 521		λ_{max} (nm) : 594		λ_{max} (nm) : 597		λ_{max} (nm) : 595	
Conc. (μM)	13		74.6		25.9		27.8		80	
Decolorization	rWT	rM-4A	rWT	rM-4A	rWT	rM-4A	rWT	rM-4A	rWT	rM-4A
rates (nMs^{-1})	1.5 ± 0.1	1.7 ± 0.1	69.2 ± 0.9	67.8 ± 0.9	20.8 ± 0.2	18.7 ± 0.1	8.9 ± 0.2	8.1 ± 0.1	20.0 ± 0.1	17.7 ± 0.4

Data are shown as the mean ± 1 SD and are derived from three independent repeats

CHAPTER V

CONCLUSION

Synthetic dyes are extensively used in the textile, dyeing, printing and leather industries and often their contamination in the water effluents from these industries represents a major source of water pollution. The removal of such dyes from wastewater is of prime concern for environment protection but it remains a difficult task due to the structural complexity, toxicity and high stability of these molecules. Enzymatic treatment by laccase is gaining in interest due to its ability to oxidize a wide range of molecules and its apparent stability but laccases secreted from native source are usually not suitable for large-scale-production due to undesirable preparation procedure (such as presence of toxic inducers) and time consuming. The production of laccase in safe and short time production heterologous host and also laccase engineering are the good way to obtain more robust enzyme for the decolorization. The enzyme productivity can be increased by the use of multiple copies, strong promoter and efficient signal sequences (designed to address proteins to the extracellular medium). In this study, firstly, the ability to biodegrade various types of dyes by enzyme extract enriched in laccase from the fungal *T. versicolor* liquid culture supernatant was evaluated. Secondary, laccase gene (*lcc1*) from the fungus was expressed in *Y. lipolytica* under the control of the pTEF constitutive promoter. The secretion signal peptide is a native laccase *lcc1* signal peptide from *T. versicolor*. Then, directed evolution was performed to increase the activity of the enzyme. The laccase was purified and characterized. Finally, recombinant enzymes were used for decolorization of synthetic dyes.

1. **Synthetic dye decolorization by *T. versicolor* DSM11269**

Before expression of laccase in another host, the ability of the fungus to decolorize synthetic dyes was studied. Enzymatic extract enriched in laccase produced by *T. versicolor* strain DSM11269 can efficiently decolorize two anthraquinones (Alizarin Red S, RBBR) and one azo dye (Direct Blue 71) without any addition of redox mediators, demonstrating the potential interest in such crude enzyme extracts for the removal of dyes issued from industrial effluents. However, the other azo derivatives (Amaranth, Reactive Black5 and Cibacron Brilliant Red 3B-A) resist to the action of the enzymatic extract. The results show that decolorization may be due to the activity of laccase but also from other oxidizing activities produced by the fungus. The addition of redox mediators and/or engineering of laccase should be investigated to improve the decolorization process.

2. **Cloning and expression of *lcc1* from the white-rot fungus *Trametes versicolor* in the yeast *Yarrowia lipolytica***

Enzyme evolution strategies need an efficient and reproducible expression host allowing to rapidly compared enzyme variants. Therefore, we first tested an expression of laccase gene (*lcc1*) in *Y. lipolytica* strain zeta. This strain enables the integration of the expression cassette to a given locus of the *Y. lipolytica* genome. The expression integrative vector is based on a constitutive promoter (pTEF promoter). The secretion signal peptide is a native laccase *lcc1* signal peptide from *T. versicolor*. This is the first report of production of heterologous *lcc1* from *T. versicolor* in this yeast. Significant levels of laccase activity (0.25 U ml^{-1}) were measured after 30 hours, showing that *Y. lipolytica* can express laccase in an active and stable form. However, the expression in this strain is not sufficient for further purification step, so a multicopy strain *Y. lipolytica* strain MTLY60 was used and the level of expression of LCC1 was increased to 1 U ml^{-1} after 30 hours. Laccase produced by MTLY60 strain was purified and characterized biochemically.

3. Laboratory evolution and laccase characterization

To improve enzymatic activity of laccase lcc1, directed evolution was undertaken to select a better mutant with better activity. After two rounds of molecular evolution, a screening test permit to select the mutant (rM-4A) which contain two mutations (L185P and Q214K) can enhance ~6-fold of total laccase. The purified rM-4A laccase showed 1.7- and 2.4- fold enhancement of the k_{cat} and k_{cat}/K_m values, respectively, with 2'2-azino-bis(3-ethylbenzothiazoline-6-sulphic acid) (ABTS) as the substrate and a 2.5- and 2.8-fold increase, respectively, with 2, 6-dimethoxyphenol substrate, compared to the wild type (rWT) LCC1. Kinetic parameter determination indicated that both wild type and mutant rM-4A laccase have higher affinity for ABTS substrate. The effect of pH and temperature on enzyme activity and stability were nearly identical for wild type and mutant rM-4A laccase. Both enzymes were very stable at temperature ranging from 25-35°C. The broad range of pH stability (3.6-7.6) and the ability to work at natural temperature are encouraging for potential environmental and industrial application of the enzyme.

4. Decolorization of synthetic dyes by recombinant enzyme

Decolorization of synthetic dyes by white rot fungi has been extensively studied. However, the production of the enzyme by fungi is time consuming and need induction by toxic phenoxic compounds. This study showed that laccase from *Trametes versicolor* could be expressed in *Y. lipolytica*. The decolorization capacity of the laccase for five dyes in the presence of natural mediator acetosyringone which cover three main structures of dye; azo (Amaranth, Direct Blue71, Reactive Black 5), anthraquinone (RBBR) and triphenylmethane (Acid Violet 17) dye was studied. The rWT and mutant rM-4A laccase produced by *Y. lipolytica* could efficiently decolorize the dyes at acidic and neutral pH. The highest decolorization level was found for Amaranth and Direct Blue 71. The rWT and mutant laccase preparation decolorize 95 and 97% of 74.6 µM Amaranth solution with a decolorization rate of about 70 $nM.s^{-1}$ in the presence of acetosyringone redox mediator.

The study thus demonstrates the potential of the *Y. lipolytica* to be used as host for laccase molecular evolution and open new perspectives for additional rounds of evolution that can be performed using a screening procedure allowing isolation of positive clones in the presence of dye and acetosyringone mediator.

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Outcome from this study

Theerachat M, Morel S, Guieysse D, Remaud-Simeon M, Chulalaksananukul W. 2011. Comparison of synthetic dye decolorization by whole cells and a laccase enriched extract from *Trametesversicolor*DSM11269. *Afr. J. biotechnol* 11(8) 1964-1969.

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