

İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

**HETEROLOGOUS EXPRESSION OF AN ISOENZYME OF *PYCNOPORUS*
SANGUINEUS LACCASE IN YEAST *SACCHAROMYCES CEREVISIAE***

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JANUARY 2010

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Date of submission : 25 December 2009

Date of defence examination: 13 January 2010

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JANUARY 2010

***PYCNOPORUS SANGUINEUS* LAKKAZ İZOENZİMİNİN
SACCHAROMYCES CEREVISIAE MAYASINDA HETEROLOG
EKSPRESYONU**

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Tezin Enstitüye Verildiği Tarih : 25 Aralık 2009

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OCAK 2010

FOREWORD

First and foremost, I would like to express my thanks for my supervisor Prof. Candan Tamerler for her expertise, guidance and support throughout my study.

I would also like to thank Prof. Ayten Yazgan Karataş for her interest and guidance during my master education.

I am particularly grateful to Koray Yeşiladalı and Günseli Kurt Gür for their support for all laboratory studies.

I am also grateful to Hüseyin Tayran, Funda Özmen, Deniz Şahin, Ülkü Yılmaz and Berrak Gülçin Balaban for being such a great colleagues, and sharing their knowledge about experimental studies even in critical assays and stressful circumstances.

Finally, I feel deeply indebted to my parents, sister and brother for supporting my efforts for all these years with love and patience.

This study is supported by TUBITAK/ Italy Joint International Project, ‘Development and Characterization of New Biocatalists by Rational and Random Mutagenesis of Laccases from Different Sources’.

February 2010

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ABBREVIATIONS

AAD	: Aryl alcohol dehydrogenase
AAO	: Aryl alcohol oxidase
ABTS	: 2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid
AMFEP	: Association of Manufacturers and Formulators of Enzyme Products
BLAST	: Basic local alignment search tool
CBQ	: Cellobiose-quinone oxidoreductase
CDH	: Cellobiose dehydrogenase
cDNA	: Cloned DNA
CYC1	: Cytochrome C1
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide triphosphate
EBI	: European Bioinformatics Institute
EC	: Enzyme Commission
ER	: Endoplasmic reticulum
GLO	: Glyoxal oxidase
GOD	: Glucose 1-oxidase
GRAS	: Generally recognized as safe
HBA	: Hydroxybenzoic acid
IPTG	: Isopropyl β -D-1-thiogalactopyranoside
LAC	: Laccase
LB	: Luria-Bertani
LiP	: Lignin peroxidase
LMS	: Laccase mediator systems
MnP	: Manganese peroxidase
NCBI	: National Center for Biotechnology Information
OD	: Optical density
o/n	: Overnight
PAH	: Polycyclic aromatic hydrocarbon
PCR	: Polymerase chain reaction
PEG	: Poly(ethylene glycol)

SD	: Synthetic defined media
SDS	: Sodium dodecyl sulfate
SOD	: Superoxide dismutase
SS-DNA	: Salmon sperm DNA
TBE	: Tris/borate/EDTA
TEMPO	: 2,2,6,6-tetramethylpiperidine-1-oxyl
UV	: Ultra-violet
WRF	: White-rot fungi
VA	: Veratryl alcohol
YE	: Yeast extract
YNB	: Yeast nitrogen base

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HETEROLOGOUS EXPRESSION OF AN ISOENZYME OF *PYCNOPORUS SANGUINEUS* LACCASE IN YEAST *SACCHAROMYCES CEREVISIAE*

SUMMARY

White-rot fungi (WRF) are eukaryotic organisms which have an important role in global carbon cycle with the ability of lignin degradation. Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductases) are ligninolytic extracellular enzymes that may oxidise different aromatic compounds using molecular oxygen as acceptor.

Laccases have many applications in different biotechnological processes such as detoxification of waste-waters of paper and textile industries, bioremediation, and expression of textile dyes. Currently yeasts are useful in these applications for the purpose of producing high-yielded laccases from the organisms with recombinant methodology. But there are some important obstacles to obtain high-yielded enzyme with recombinant processes such as high cost, consumer acceptance and toxicology tests that should be overcome. Thus, enzymes which are produced with directed evolution will be used more extensively in industrial applications.

In this study, heterologous expression of *Pycnoporus sanguineus* laccase-2 cDNA in yeast *Saccharomyces cerevisiae* was performed. For this aim, a full length laccase-2 (*Lcc2*) cDNA, encodes for a mature laccase isoenzyme of ~1600 amino acid residues with a predicted molecular weight of ~60 kDa, was cloned into the pSAL4 expression vector which bears copper-inducible *CUP1* promoter. Recombinant plasmid was transferred into the *S. cerevisiae* W303-1A strain via using lithium acetate protocol and transformed colonies were selected in terms of the properties of oxidizing the substrate in selective medium supplemented with 0.2 mM ABTS and 0.6 mM CuSO₄. The colony which shows the highest activity was selected with screening assays between the positive transformant colonies, and then the effects of different media in terms of the carbon source and copper concentrations were determined for obtaining the optimum expression conditions.

PYCNOPORUS SANGUINEUS LAKKAZ İZOENZİMİNİN SACCHAROMYCES CEREVISIAE MAYASINDA HETEROLOG EKSPRESYONU

ÖZET

Beyaz küf mantarları lignini degrade edebilme özellikleri sayesinde global karbon döngüsünde önemli rol oynayan ökaryotik organizmalardır. Ligninolitik extraselüler enzimler olan lakkazlar (EC 1.10.3.2, p-difenol: dioksijen oksidoredüktazlar) moleküler oksijeni akseptör olarak kullanarak farklı aromatik bileşikler okside edebilirler.

Lakkazların kağıt ve tekstil endüstrilerinden gelen atık suların detoksifikasyonu, biyoremediasyon, tekstil boyalarının ekspresyonu gibi farklı biyoteknolojik proseslerde birçok uygulaması bulunmaktadır. Günümüzde bu uygulamalarda rekombinant teknoloji ile organizmalardan yüksek verimde lakkaz üretimi amacıyla mayalar kullanılmaktadır. Ancak rekombinant yöntemlerle yüksek verimde enzim eldesi için yüksek maliyet, tüketici kabulü ve toksikoloji testleri gibi aşılması gereken bazı önemli engeller bulunmaktadır. Böylece, yönlendirilmiş evrimle üretilen enzimler endüstriyel uygulamalarda daha geniş olarak kullanılabilecektir.

Yapılan çalışmada *Pycnopus sanguineus* lakkaz-2 cDNA 'sının *Saccharomyces cerevisiae* mayasında heterolog ekspresyonu gerçekleştirilmiştir. Bu amaçla, ~1600 amino asitlik ve ~60 kDa moleküler ağırlığında olan matür bir lakkaz izoenzimini kodlayan, tam boyda bir Lcc2 cDNA 'sı, bakırla indüklenebilen *CUP1* promotörü taşıyan pSAL4 ekspresyon vektörüne klonlanmıştır. Rekombinant plazmid, lityum asetat protokolü kullanılarak *S. cerevisiae* W303-1A suşuna aktarılmış ve transformant koloniler 0.2 mM ABTS ve 0.6 mM CuSO₄ içeren seçici besiyerinde substratı okside edebilme özelliklerine göre seçilmiştir. Pozitif transformant koloniler arasında yapılan tarama deneyleri ile en yüksek aktivite gösteren koloni seçilmiş ve sonrasında optimum ekspresyon koşullarının eldesi için karbon kaynağına ve bakır konsantrasyonlarına göre farklı besiyerlerinin etkileri tespit edilmiştir.

1. INTRODUCTION

1.1 White-rot Fungi

Soil is a complex ecosystem and it comprises all major groups of fungi, including Basidiomycetes and Ascomycetes. Fungal populations have been estimated which range from 10^4 to 10^6 /g of soil. Hyphal lengths range from 100 to 1000 meter/g, and biomass ranges from 37 to 184 g of dry weight/m² [1]. In many soil habitats fungi is dominant kingdom and there are over 1 million species of filamentous fungi populating every ecosystem on earth [2]. Soil contains not only fungal cells, but also their secretions have been detected in grassland and forest soil samples [3].

Saprophytic microorganisms have a fundamental role in the earth's carbon cycle. Best understood saprophytic fungi are those that cause wood decay. Studies show that white-rot, brown-rot, soft-rot, and bacterial erosion are the major types of this decay. The term "white-rot" refers to the characteristic color of the decayed wood with residual cellulose and it is off-white [4]. The white-rot basidiomycetes degrade lignin more rapidly than other studied microbial groups. Like the brown-rot fungi, they attack the lumen of wood cells to secrete enzymes that degrade lignin [5]. Also white-rot fungi (WRF) can degrade insecticides, herbicides, pentachlorophenol, creosote, coal tars, heavy fuels and turn them into carbon dioxide, water, and some basic elements [6].

1.1.1 Lignin Degradation

Enzymes are organic catalysts produced by living organisms that accelerate the transformation rate of substrate to the product by lowering the activation energy barrier of reaction. All enzymes are initially produced in the cell wall. There are two types of enzymes which differ in localization: intracellular enzymes (endozymes) that function in the cell, and extracellular enzymes (exozymes) that function outside the cell [7].

Extracellular phenoloxidizing activity for lignin degradation was discovered in WRF in the 1930s in the presence of ligninolytic enzymes[8]. Lignin is a chemical compound generally derived from wood of plants and some algae [9], [10]. It confers mechanical strength to the cell wall by filling the space between cellulose, hemicellulose and pectin components. Cellulose and hemicellulose are physically protected from enzymatic hydrolysis owing to lignin but some fungi and bacteria are able to secrete some specific enzymes which can biodegrade the polymer [11].

WRF may be divided into three groups with regard to their production patterns of extracellular ligninolytic enzymes. These groups are: LiP-MnP group, MnP-laccase group, and LiP-laccase group. Also several enzymes are essential for indirect lignin degrading activities, such as hydrogen peroxide (H_2O_2) production, in ligninolysis. All these enzymes are shown in Table 1.1 [12].

Table 1.1: Enzymes that function in ligninolysis

Enzyme	Numerical Classification
Lignin peroxidase (LiP)	EC 1.11.1.14
Manganese peroxidase (MnP)	EC 1.11.1.13
Other peroxidases	EC 1.11.1.7
Protocatechuate 3,4-dioxygenase	EC 1.13.11.3
Glucose 1-oxidase (GOD)	EC 1.1.3.4
Cellobiose-quinone oxidoreductase (CBQ)	EC 1.1.5.1
Cellobiose dehydrogenase (CDH)	EC 1.1.99.18
Aryl alcohol oxidases (AAOs)	EC 1.1.3.7
Aryl alcohol dehydrogenase (AAD)	EC 1.1.91
Glyoxal oxidase (GLO)	EC 1.2.3.5
Superoxide dismutases (SODs)	EC 1.15.1.1

According to hypothetical mechanisms of ligninocellulose transformation, GOD cooperates with LiP and MnP providing H_2O_2 together with laccase (LAC; EC 1.10.3.2), and reducing quinones yielded by laccase to phenols. In this mechanism LiP and MnP act as the first lignin-decomposing agent and LAC acts as the demethylating factor.

As a result of cellulose hydrolysis by the cellulases, glucose is produced and it becomes the substrate for GOD. Quinones produced by LAC from lignin oligomers can replace oxygen for GOD. After glucose oxidation, D-glucose-d-lactone is formed and it reinforces the fungus metabolism in the pentose-phosphate cycle or in glycolysis (Fig 1.1).

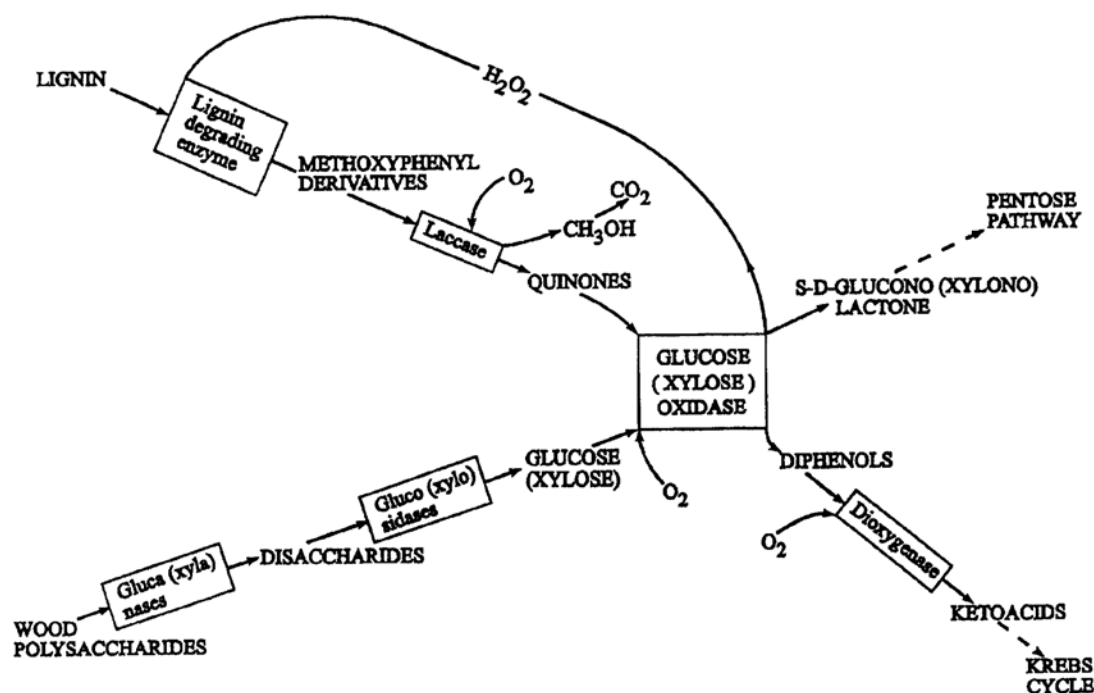


Fig 1.1: The hypothetical mechanisms of ligninocellulose transformation by WRF enzymes [13], [14].

Veratryl alcohol (VA, 3,4-dimethoxy- benzyl alcohol) is secondary metabolite of several WRF and it has a role in the mechanism of lignin degradation. LiP catalyzes the oxidation to the VA cation radical (VA⁺) and this radical acts as a mediator. If the radical complexes to the LiP this effect can enhance mediating properties of VA. Also somehow VA stimulates LiP but its mechanism is not properly explained by now [15].

The process of ligninocellulose transformation has been further rearranged by adding cellobiose-guinone oxidoreductase and mediating factor (Fig 1.2). Its functionality have been extended with feedback activity, veratryl alcohol and phenoxy radicals [12].

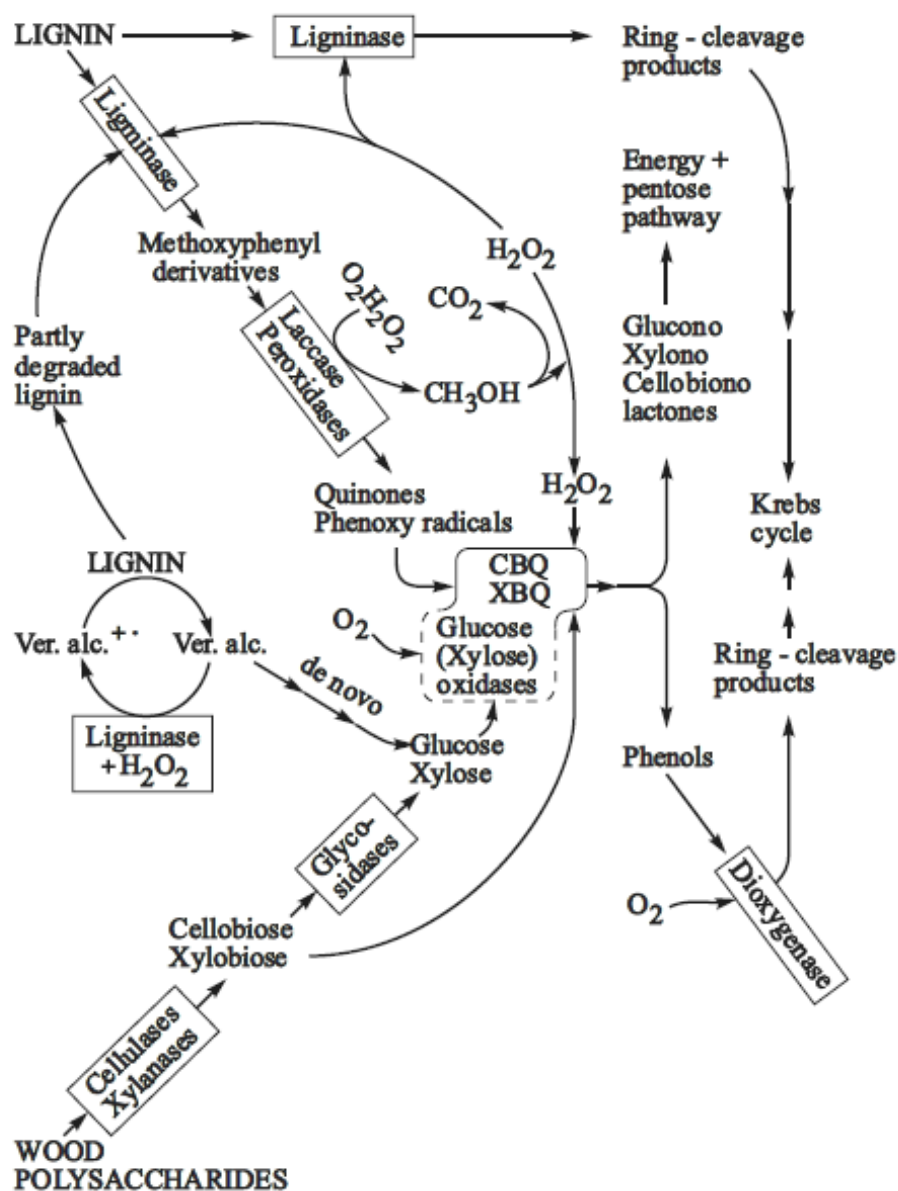


Fig 1.2: Hypothetical scheme for degradation of lignin, cellulose and xylan [8], [16].

1.1.2 *Pycnoporus sanguineus*

Pycnoporus sanguineus is biological name of “red fungus” and it has its name from red coloring. It is a saprophytic pathogen and belonging to the Basidiomycota phylum of the family Polyporaceae. It was firstly discovered on Guana Island, part of the Virgin Islands (Fig 1.3).



Fig 1.3: Photo of white-rot fungus, *P. sanguineus* [17].

This fungus was shown to synthesize some substances with medical applications. Also it produce some enzymes with industrial applications such as invertase, tyrosinase, α -amylase, xylanase, β -glucosidase and laccase. Although *P. sanguineus* produce several enzymes, laccase is the most important one currently because of its effectiveness in numerous biotechnological applications [18].

1.2 Laccase

Laccases (EC 1.10.3.2) are defined in the Enzyme Commission nomenclature as oxidoreductases acting on diphenols and related substances using molecular oxygen as acceptor. The first studied laccase was derived from *Rhus vernicifera*, a Japanese lacquer tree, in 1883 [19].

Laccases are capable of oxidising several phenolic and also non-phenolic compounds such as industrial dyes, polycyclic aromatic hydrocarbons (PAHs), pesticides and alkenes [20]. These enzymes have been identified in all groups of organisms and functions attributed to them include lignin biosynthesis in plants, melanin biosynthesis in bacterial spores, lignin degradation and pathogenesis in fungi, and esclerotization in insects [20]. In particular, fungal laccases are considered to take part in the production of dihydroxynaphthalene melanins, darkly pigmented polymers that protect organisms against stress, or in fungal morphogenesis by functioning in the formation of extracellular pigments [21].

The reactions that laccases catalyse are benzylic alcohol oxidation, rupturing of alkyl-aryl bonds, and also aromatic rings generating oxidised phenolic compounds. Moreover, in vitro studies have indicated that laccases can catalyse polymerisation, depolymerisation, methylation and demethylation reactions as well as oxidation of several phenolic compounds.

1.2.1 Structural Features

There are many studies related to fungal laccases and by the help of their three-dimensional structure monitored via X-ray crystallography, it was detected that they have a monophyletic branch of copper-binding oxidoreductases. The studies showed that there are four copper atoms that are essential for the enzymatic activity and fungal laccases have conserved regions in which histidine residues are located abundantly. The copper atoms are involved in the one-electron transfer from a reducing substrate to molecular oxygen with its final reduction to water [20].

All known laccases have a similar overall fold consist of cupredoxin-like domains and belong to an extended group of blue multicopper oxidases. Blue multicopper oxidases have at least one type 1 (T1) copper that forms the mononuclear center and one type 2 (T2) copper and two type 3 (T3, T3') copper forming the trinuclear center. Electrons are transferred from a substrate to the T1 copper as primary electron acceptor, and from T1 copper further to the trinuclear T2/T3 copper center, where molecular oxygen is the last electron acceptor [22]. T1 copper, provides the characteristic blue color of the enzyme and electron capture by this copper is increased by an available electronic environment, due to the amino acids involved in the copper binding. Thus phenolic compounds, like the polyphenols or methoxy-substituted monophenols found in paper pulp, are oxidized.

Besides, non-phenolic compounds can be oxidized in the presence of an adequate mediator, such as 2,2'-azino-bis-(3-ethylthiazoline-6-sulfonate) (ABTS) [23]. Due to be paramagnetic, T1 and T2 coppers can be detected by electron paramagnetic resonance spectroscopy, while T3 coppers form a diamagnetic spin-coupled copper pair and can not be detected by. T1 copper has an absorbance at 600-610 nm, whereas T3 copper has at 330 nm [22].

Organic molecules called mediators are often synthesized by fungi. Mediators help fungal laccases that oxidize substances. High oxidation potential prevents the oxidation of substances by the enzyme alone and mediators which are aromatic compounds, either natural or synthetic, function as diffusible oxidizing agents. They also provide an important research area and potential for the new biotechnological applications.

The delignification of pulp by laccase from *Trametes versicolor* was examined with several known mediators and also without mediators, and it was detected that there were differences between the reactivity of enzyme depending on the mediators used (Fig 1.4) [4].

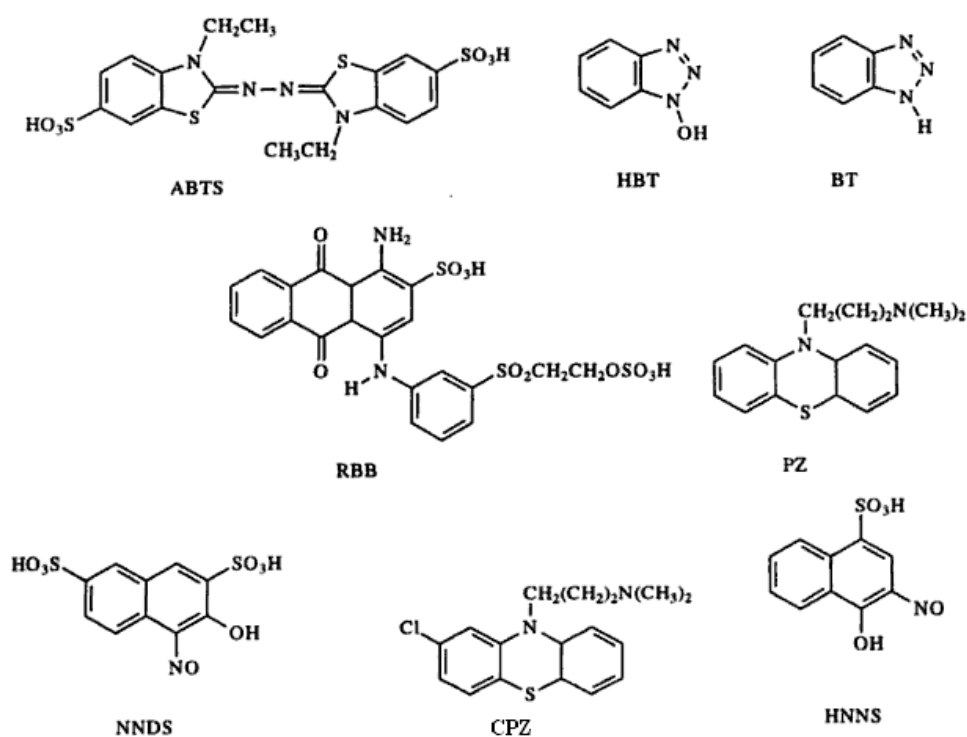


Fig 1.4: Several mediators that promote fungal laccase activity. ABTS = 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid; HBT = 1-hydroxybenzotriazole; BT = benzotriazole; RBB = Remazol Brilliant Blue; PZ = promazine; CPZ = chlorpromazine; HNNS = 2-nitroso-1-naphthol-4-sulfonic acid; NNDS = 1-nitroso-2-naphthol-3,6-disulfonic acid [4].

Another approach regarding laccase catalysed reaction has been performed with a synthetic mediator 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO). Enzyme catalyses the trimerisation of indole to 2,2-bis(3'-indolyl)- indoxyl in 50% yield in a controlled oxygen atmosphere of 2 bar pressure (Fig 1.5) [24].

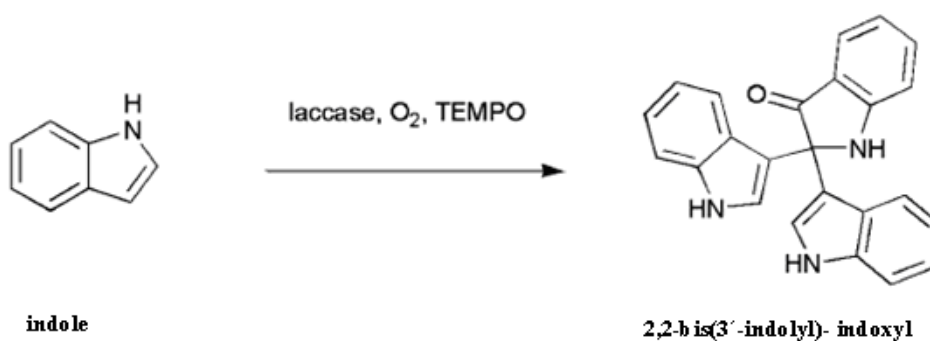


Fig 1.5: Laccase catalysed reaction with synthetic TEMPO as the mediator.

The natural mediators, especially hydroxybenzoic acid (HBA), 4-hydroxybenzyl alcohol, and their derivatives can be expected to be quite significant in the degradation of aromatic compounds by laccase-producing fungi. The oxidation of polycyclic aromatic hydrocarbons (PAHs) is carried out by the laccase mediator systems (LMS) via ABTS and 1-hydroxybenzotriazole by an indirect oxidation without direct contact of substrate and enzyme. Also oxidation of these two mediators by laccase via LMS provides free radicals and this hypothesis causes an assumption that typical laccase substrates which form radicals can act as mediator compounds, too. It was demonstrated that there are number of compounds which produced by fungi or already present during the degradation of substrates, mediate the oxidation of PAH by laccase from *Trametes versicolor* [25].

1.2.2 Laccase Glycosylation

It is known that almost 40% of the molecular weight of laccases corresponds to glycans. The term glycan refers to a polysaccharide or oligosaccharide. It may also be used to refer to the carbohydrate portion of a glycoconjugate, or proteoglycan as well. The most complete characterisation of the glycosylation profile of fungal laccase was reported from *Rigidoporus lignosus*., where rich mannose glycans were found. On the other hand although there are studies about the glycosylation profile of laccases from other basidiomycota, the reported data is only qualitative. Generally high mannose glycans were detected in fungal laccases except the laccase from *Botrytis cinerea*. A three-dimensional homology model (*P. sanguineus* laccase) was constructed upon the *T. versicolor* laccase structure (Fig 1.6). *N*-Glycosylation was modeled with the 2- mannose glycans, manually attached to the protein on the five predicted positions, linked to the pentasaccharide core.

T1 copper site is surrounded by nonpolar residues and with residue D226, a position equivalent to D206 of *T. versicolor* laccase. Hence it was assumed that localization stabilizes substrates by hydrogen bond interactions near alongside residue H477.



Fig 1.6: Three-dimensional model of the *P. sanguineus* laccase constructed over the *T. versicolor* crystallographic structure (PDB Q12718). Copper atoms are indicated by stars, residues D226 and H477 are depicted as white spheres, and *N*-glycans are depicted as black spheres [26].

Protein glycosylation is a major post-translational modification. In the cell, when growing polypeptide enters the endoplasmic reticulum (ER), *N*-glycans are often attached to secreted proteins. Glycosylation modifications are attained during their transport through the inner membrane system. There are two types of protein glycosylation. First one is *N*-glycosylation that occurs mainly in the ER and at asparagine residues within the peptides Asn-X-Ser/Thr [26]. This is a general characteristic of glycoproteins that the second amino acid following the asparagine residues is always serine or threonine. X represents any amino acid residue with the possible exception of proline. Second one is *O*-glycosylation that occurs mainly in the Golgi at hydroxyl groups of serine and threonine residues (Fig 1.7) [27].

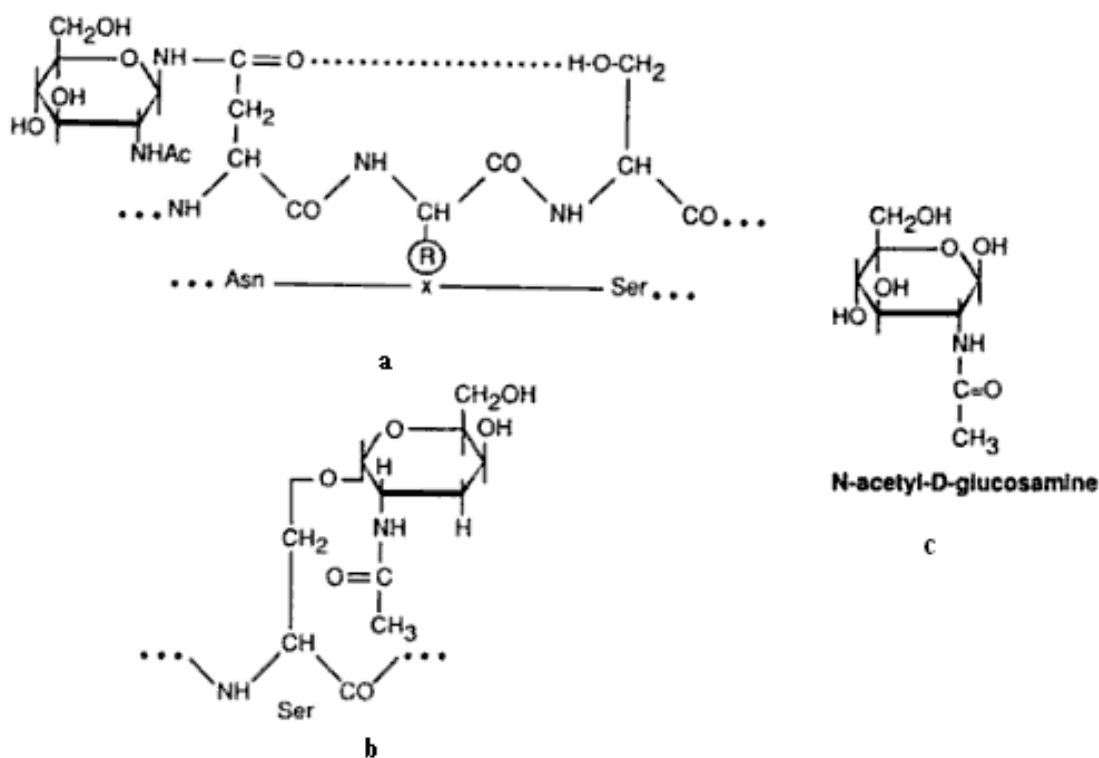


Fig 1.7: The *N*-glycosidic and *O*-glycosidic linkages. (a) The *N*-glycosidic bond between *N*-acetylglucosamine and asparagine. Asn-X-Ser(Thr) rule is also shown. (b) The *O*-glycosidic bond between *N*-acetylglucosamine and serine. (c) *N*-acetyl-*D*-glucosamine, a common membrane carbohydrate [27].

Protein glycosylation has been studied in several yeasts and also other fungi, especially in basidiomycetes. Extracellular enzymes from filamentous fungi (laccase i.e.) are often *N*-glycosylated with high mannose glycans. But few studies regarding to the glycosylation process of proteins have been performed so far[26].

1.2.3 Multifunctionality of Laccase

The most scientists have traditionally agreed about one enzyme – one physiological function idea. But studies showed that some enzymes moonlight and although they perform their primary catalytic function, they can continue to other functions, too. Moonlighting refers to the multiple functions of a protein but these functions are not because of gene fusion, splice variants or multiple proteolytic forms. There are few discovered moonlighting proteins such as enzymes, transcription factors, receptors and channels [28]. Photosynthetic proteins of cyanobacteria phages can be given as an example [29].

Laccase has multiple functions in different type of cells and intracellular or extracellular conditions which its izozymes get expressed (Table 1.2).

Table 1.2: Several multifunctional enzymes that have at least two functions [30].

Enzyme	First Function	Second Function	Third Function
Subtilisin	Peptidase	Esterase	-
Phytase	Phosphomonoesterase	Sulfoxidation	-
Hydroxynitrile lyase (HNL)	Oxynitrilase	Esterase	-
Rubisco	Carboxylase	Oxygenase	-
Decarboxylases (PDC, ADC)	Decarboxylase	Carboligation	-
Chymotrypsin	Amidation	Phosphotriesterase	-
O-Succinyl benzoate (OSBS)	Synthase	Racemization	-
Laccase	Iron oxidase	CotA oxidase	Polyphenol Oxidase (polymerization and depolymerisation)

In addition, multifunctional enzymes can act differently because of different conditions such as locations and binding sites (Fig 1.8) [30].

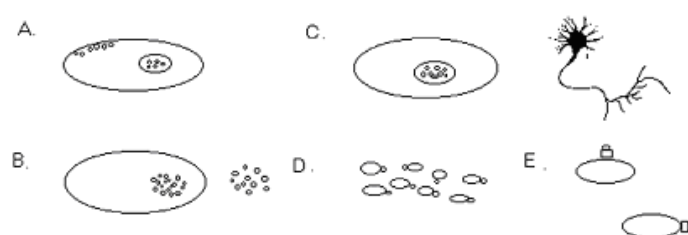


Fig 1.8: Different mechanisms of laccase as a multifunctional enzyme. (A) Different functionality at the same cell's different locations (B) Being inter- or intra cellular protein. (C) Different functionality of enzyme because of being expressed by different cell type. (D) Different activity of enzyme due to substrate, product or cofactor attachment. (E) Different binding sites of enzymes for different substrates [30].

While laccase is a preferred oxidoreductase because of being multi-functional enzyme, also it can be inhibited like the other enzymes as well. Different laccases have different tolerance toward inhibition by halides or heavy metals. Halides (except iodide), azide, cyanide and hydroxide can cause interruption of the internal electron transfer on the trinuclear Cu cluster. Dithiothreitol, thioglycolic acid, cysteine, diethyldithiocarbamic acid are some other laccase inhibitors. Likewise, substrates of laccase can competitively inhibit the transformation of other compounds and may decrease enzyme activity [31].

Under alkaline pH conditions, OH^- can inhibit catalysis, too. The other known inhibitors are several metal ions (Hg^{+2} i.e.), fatty acids, sulfhydryl reagents, hydroxyglycine, kojic acid, desferal and cationic quaternary ammonium detergents that may cause amino acid residue modification, Cu chelation, or conformational change [32].

1.3 Heterologous Laccase Expression

As it was mentioned before, laccases from WRF, are secreted glycoproteins. These glucans includes two disulphide bridges and four coppers located in two domains. Fungal laccases differ in their protein and polysaccharide moiety, notwithstanding all of them have a conserved active-site structure. So, the laccases which are produced mostly by fungi can be in different isoforms. Because of this phenomenon, there is not an effective enzyme transformation process for basidiomycetes to study enzyme's structure or function properties [33].

Another important point is purification difficulty, especially for high amount expression of enzymes, that naturally organisms express laccase as mixture of isoenzyme and analysis of this complex form is not preferred compared to the minor forms [34]. In order to achieve production of great quantities of proteins that have yield and purity grade at standard levels for commercial utilization, the directed evolution and expression of recombinant laccase genes has been currently applied in heterologous hosts, such as yeasts; including *Saccharomyces cerevisiae*, *Pichia pastoris*, *Pichia methanolica*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, and filamentous fungi; including *Trichoderma reesei*, *Aspergillus oryzae*, *Aspergillus sojae*, *Aspergillus niger* and *Aspergillus nidulans* [34, 35].

Studies showed that generally there are 50% or more levels of amino acid identity between the laccase sequences of members of a single fungal class, while this level is ~30% between sequences of organisms of different classes. For producing laccases heterologously in large volumes, various laccase genes have been chosen and cloned in experiments from different classes and species such as the fungal genes from basidiomycetes; including *Phlebia radiata*, *Coriolopsis Polyzona*, *Cryptococcus neoformans*, *Pleurotus ostreatus*, *Trametes versicolor*, *Trametes villosa*, *Pycnoporus cinnabarinus*, *Pycnoporus sanguineus* and *Coprinus cinereus*, and also from ascomycetes; including *Neurospora crassa*, *Aspergillus nidulans*, *Podospora anserina* and *Myceliophthora thermophila* [36].

Heterologous expression has some disadvantages as well. Production of a specific enzyme isoform is a preferred demand but when enzyme gets expressed heterologously, the product yield is less compared to the original host usage [34]. Another critical aspect is about the different enzyme activity ranges depending on the host organisms. Although the filamentous fungi were detected to be useful hosts for protein secretion and the best producers of laccases, with protein yields between 70 and 230 mg l⁻¹, genetic manipulations of them are complicated in comparison to the yeasts. Also it is a time consuming process with fungi. Contrarily, yeasts are useful hosts for creating new enzymes with demanded properties and cost-effective eukaryotes [35, 37]. Disadvantage for using yeasts is the difficulty of achieving reasonable expression yields. Especially in *S. cerevisiae*, highly optimized and sensitive process have to be carried out to measure enzyme activity. Remarkable enzyme activity levels are critical for using *S. cerevisiae* in recombinant expression studies. *P. pastoris* is a more advantageous yeast with its higher expression capability compared to *S. cerevisiae*. At the same time, *P. pastoris* is not advantageous for high-throughput screening because of reduced transformation frequency and lower integration of the expression vectors [36].

1.3.1 *Saccharomyces cerevisiae*

Yeast *Saccharomyces cerevisiae* is an eukaryotic member of Ascomycota phylum and identified as a unicellular fungus. This phylum members have a characteristic “ascus” structures functioning to produce and store spores.

S. cerevisiae has ~6270 genes [38] and it is an important budding yeast because its genome is the first eukaryotic genome that completely sequenced [39]. More than 60% of the genes have a determined function and more than 40% of genes contain conserved sequences with at least one assumed human gene [38]. *S. cerevisiae* (Fig 1.9) also called as “baker’s yeast” and its name originally means “sugar mold” or “sugar fungus”.



Fig 1.9: Nomarski (differential interference contrast microscopy) image of yeast *Saccharomyces cerevisiae* [40].

S. cerevisiae species is an appropriate model organism for genomics [41]. It is easily cultivated and fastly grown in the presence of oxygen and also without oxygen. It has been used for many years in brewing and baking. This GRAS (generally recognized as safe) organism is useful in genetic engineering especially as an host organism for recombinant technology and many heterologous expression studies are based on *S. cerevisiae* [42].

1.4 Applications of Industrial Enzymes

Enzymes are proteins, which catalyze chemical reactions with considerably specificity. The first enzyme produced industrially, was amylase takadiastase, functioned as pharmaceutical agent in the United States in 1894. Later on, more studies were performed on discovering the structural properties and function mechanisms of enzyme polypeptides. In 1965, The Enzyme Commission was set up by the International Union of Biochemists, and enzyme classification was prepared by this commission. Industrial enzyme production was increased in the long run, and enzymes such as proteases, lipases, amylases, pectinases and oxidoreductases were used especially in the detergent industry [43]. Table 3 presents the major industrial enzymes and their most preferred applications.

Table 1.3: Major industrial enzymes and their applications [7].

Enzyme	Applications
Alkaline protease	In laundry, dish washing, textile washing, food & dairy industries, etc.
Alpha amylases	In industries of food, detergent, pharmaceutical and paper
Glucose isomerase	Isomerise glucose to the sweetener molecule and high fructose, starch liquefaction, glucose-fructose sugar syrup and ethanol making industries
Penicillin acylases	In beta-lactam semi-synthetic antibiotics intermediates production and racemic mixture isolation
Cellulases	In food, feed and beverages, pulp & paper industries, detergent industries, bioethanol production
Xylanases	In industries of pulp & paper, food & feed and textile, bioethanol production
Pectinases	In food & feed production, fruit juice purification & stabilization, textile industries, retting and de-gumming of fibre crops & quality paper production
Lipases	In dairy and other food processes, detergents, pharmaceuticals, cosmetics, leather processing and production of aliphatic acids
Tannases	In hydrolyzing of tannins, leather processing, wine making by reducing the haze, preparation of cold water soluble instant tea, coffee, etc.
Phytases	In reducing phosphorus excretion of monogastric animals by replacing inorganic phosphates, animal nutrition, processing of human food and environmental protection
Laccases	Delignification of pulp and paper, fine paper making, fruit juice clarification and stabilization, bioremediation, xenobiotic substrate removal, detoxification of plant cell wall-derived sugar syrups.

The importance of enzymes in many processes has been discovered for many years. In history of Greece, some of the microbial enzymes were used in brewing, cheese making, baking and alcohol production. With the development of enzyme purification techniques, and the utilization from thermostable enzymes, which have been isolated majorly from thermophilic organisms, the number of industrial applications have fairly increased.

Enzyme technology is being used for the food, feed, agriculture, paper, leather and textile industries mostly because products are composed of biomolecules and biomolecules can be produced, degraded or modified by enzymatic reactions. For example, laccases can be used for bleaching to reduce the usage of raw material and the production of waste. Proteases are the dominant enzyme class, which can be mostly used in the detergent and baking processes. In the pulp and paper industries, enzymes are applied as well [43].

1.4.1 Heterologous Enzyme Applications

It was assumed that the world market for industrial enzymes is \$1.6 billion. Approximately 50% of the market enzymes are expressed in filamentous fungi because as it was explained before, industrially useful strains of fungi are GRAS and these strains can be cultivated in quite inexpensive media. Also they can both produce and secrete large amounts of recombinant enzyme.

Heterologous enzymes cover almost 1/3 of the all industrial usage. *Aspergillus* strains are the largest productive group, while *Saccharomyces* strains only accounts for 1% of the total products. According to the list of the Association of Manufacturers and Formulators of Enzyme Products (AMFEP), the distribution rates of industrial enzymes dependent on production strains were displayed on the Table 1.4.

Table 1.4: Range of industrial products and strains from AMFEP list (2004).

Total enzymes	186
Homologous product	64.5%
Heterologous product	35.5%
<i>Aspergillus</i> strains	36.6%
<i>Trichoderma</i> strains	10.8%
<i>Penicillium</i> strains	8.1%
<i>Kluyveromyces</i> strains	1.6%
<i>Saccharomyces</i> strains	1.1%
Prokaryotic strains	30.6%

The industrial market was ~\$1.6 billion in 1998 for some enzyme application sectors such as food (45%), detergents (34%), textiles (11%), leather 3%, pulp and paper (1.2%). For these non-pharmaceutical enzymes, that don't include diagnostic and therapeutic enzymes, the market was \$2 billion in 2000. More than 60% of the enzymes applied in the detergent, food, and starch-processing sectors are recombinant proteins but the number of heterologous fungal enzymes that are accepted in food applications is not very rich. Mostly recombinant food-grade enzymes are obtained from fungal origin because as it is known, non-fungal enzymes provide low yields. Table 1.5 shows some heterologous industrial enzymes from AMFEP [44].

Table 1.5: List of some heterologous industrial enzymes from AMFEP (2004)

Enzyme	Host Organism	Donor organism
Aminopeptidase	<i>Trichoderma reesei</i>	<i>Aspergillus sp.</i>
Catalase	<i>Aspergillus niger</i>	<i>Aspergillus sp.</i>
Cellulase	<i>Aspergillus oryzae</i>	<i>Humicola sp.</i>
Galactosidase (alpha)	<i>Saccharomyces cerevisiae</i>	Guar plant
Glucanase (beta)	<i>Trichoderma reesei</i>	<i>Trichoderma sp.</i>
Glucoamylase	<i>Aspergillus niger</i>	<i>Aspergillus sp.</i>
Glucose oxidase	<i>Aspergillus niger</i>	<i>Aspergillus sp.</i>
Laccase	<i>Aspergillus oryzae</i>	<i>Myceliophthora sp.</i>
Lactase or Galactosidase (beta)	<i>Aspergillus oryzae</i>	<i>Aspergillus sp.</i>
Lipase	<i>Aspergillus oryzae</i>	<i>Candida sp.</i>
Mannanase (endo-1,4-beta)	<i>Trichoderma reesei</i>	<i>Trichoderma sp.</i>
Pectin lyase	<i>Aspergillus niger var. awamori</i>	<i>Aspergillus niger</i>
Protease	<i>Aspergillus oryzae</i>	<i>Rhizomucor sp.</i>
Pullulanase	<i>Trichoderma longibrachiatum</i>	<i>Hormoconis sp.</i>

Another significant situation for heterolog enzyme expression is to optimize the reaction conditions to obtain the highest yield of protein.

So there are some methods discovered by the researchers that are useful for industrial enzyme production are, for example; using multicopy transformants to enhance gene dosage, and protease deficient mutants to repress proteolytic degradation of expressed enzyme. Also environmental conditions (temperature, pH i.e.) are changeable parameters for the prevention of enzyme degradation caused by proteases for industrial applications [45]. Laccase from *Trichophyton rubrum* LKY-7 is stable at below 50°C and pH 5-7, while laccase from *Pycnoporus coccineus* is stable at 65°C and pH 4-9 for an instance, and applications (bioremediation i.e.) are controlled by using these specific properties of laccases related to the strains [46].

1.4.2 Industrial Laccase Applications

Laccase is a highly useful enzyme in biotechnological applications, especially in waste-water detoxification or decolorization [47]. Also it has low substrate specificity and with its characteristic features it can be used in drug analysis, wine clarification, bioremediation, paper-pulp bleaching, decolorization of synthetic dyes, and biosensors. Additionally, it was detected that laccase is the inhibitor of HIV-1 reverse transcriptase enzyme. The industrial applications derived from laccase caused some requirements like new strains and isozymes with different catalytic properties. Although there are past studies with few organisms, only limited number of them were performed with the laccase expressed by *P. sanguineus* [18]. Moreover, dye decolorization is currently the most popular sector of heterologous laccase applications via using enzyme obtained from filamentous fungi and expressed into the yeast hosts.

1.4.2.1 Decolorization of Industrial Dyes

The production of synthetic compounds is an applied process but especially after the utilization as a dye, there is a high amount of waste that can not be biodegraded properly. Textile, dyeing, and printing are some of the industrial applications for synthetic dyes. There are ~800,000 tons of colorant production worldwide per a year. 1/8 of them are commercial products and unfortunately more than 10-15% of the total used dyes are released into the environment as wastes.

Dyes are resistant to light, water and many chemicals. They can be cationic, anionic or nonionic. Anionic dyes are the direct, acid and reactive compounds and reactive dyes comprise chromophoric groups, i.e., azo or anthraquinone, and reactive groups, i.e., vinyl sulfone, chlorotriazine.

The majority of the water-soluble synthetic dyes with the many different colors and structures are from the azo reactive group. These group members can not be aerobically biodegraded through simple methods. After the hydrolyzation of most of the dyes, these dyes are released into waters. Although they are not toxic at that time, they can react with the environmental compounds and be converted into potentially carcinogenic amines that are hazardous for the living organisms and also may cause to cancer.

Most recently there are comprehensive studies include physical, chemical and biological methods for decolorization of the industrial wastewater. Experiments showed that these methods alone can not be useful and there should be a complex process such as chemical-biological and physical-biological. Chemical oxidation, reverse osmosis, filtration and adsorption are some examples for physico-chemical methods for dye decolorization. They each have also some advantages and disadvantages but coagulation and adsorption are the most used techniques in spite of the fact that high amount of sludge occurs. This sludge is another problem for ecosystem. Table 1.6 displays some physical and chemical methods of dye decolorization and their disadvantages [15].

Table 1.6: Some physical and chemical methods/limitations of dye decolorization

Method	Disadvantages	Reference
Fenton's reagent	Excessive sludge generation	[48]
Ozonation	Short half-life (20 min)	[49], [50]
Photochemical	Formation of by-products, production of more toxic compounds, poor color removal, quite slow process	[51], [52]
NaOCl	Release of aromatic amines	[48]
Cucurbituril	Highly expensive	[53]
Reverse osmosis	Highly expensive, slow process	[54]
Electrochemical destruction	High cost of electricity, poor color removal	[55]
Activated carbon	Very expensive, excessive sludge generation	[54], [56]
Peat	Specific surface area for adsorption	[54], [48]
Wood chips	Requires long retention time	[56]
Membrane filtration	Concentrated sludge production	[48]
Ion Exchange	Not effective for all dyes, cost effective	[57], [48]
Irradiation	Requires a lot of dissolved O ₂	[58], [59]
Electro kinetic coagulation	High sludge production	[60]
Silica gel	Side reaction prevent commercial application	[48]
Ultrasound treatment	Production of toxic compounds, high energy input	[61]

Because of the limitations given above, there is a big demand for a different method that dye decolorization can be carried out more economically and effectively. The solution for the problem is considered to be biological methods.

Biotechnology has an increasing importance and many organisms i.e., bacteria and fungi, are being preferred for the biological methods for the dye waste degradation. Especially, the usage of lignin degrading WRF have a big impact on studies because of the ability of these organisms to degrade some organic pollutants such as azo and heterocyclic dyes. Also WRF is a useful group, just for the decoloring via adsorbing dye compound on fungal mycelia instead of a proper mineralization, too. Growth phase of the organisms, growth conditions, chemicals and inhibitors are the other countable factors which are effective in removing dyes from wastewaters.

Laccase is a major enzyme for decolorization of industrial dyes with WRF. This enzyme decolorizes some azo dyes with indirect cleavage of the azo bond. So it prevents the formation of toxic aromatic compounds. Furthermore, if it is combined with ultrasound treatment, this causes a synergistic effect and higher degradation for dyes. But still alternative procedures are being researched for decolorization with biotechnology. Because, although enzyme based applications are preferable for environment rather than chemicals, these biological procedures have major limitations such as low efficiency, high costs and enzyme deactivation possibility [15].

1.5 Aim of The Study

The aim of this study is to conduct heterologous expression of laccase coding *Lcc2* cDNA obtained from white-rot fungus *Pycnoporus sanguineus* in the yeast *Saccharomyces cerevisiae* by the help of recombinant enzyme methodology. The laccase gene was cloned into the cloning vector pDrive and then into the expression vector pSAL4, respectively. Each step was confirmed through the sequencing analysis. Next, pSAL4/*Lcc2* construct was introduced into the yeast to generate laccase enzyme. Subsequently, the produced enzyme was analysed with the laccase activity assay to confirm its oxidation with the mediator ABTS, and then the optimization experiments were performed with different nutrients and different molarities of CuSO₄.

In conclusion, heterologous laccase enzyme has been produced successfully by performing cloning and expression of a new gene from *P. sanguineus* and, optimization of expression studies were carried out to improve laccase activity.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial, Fungal and Yeast Strains

2.1.1.1 Bacterial *E. coli* Top10F' Electrocompetent Cells

F' {*proAB*, *lacI*^q, *lacZ*ΔM15, Tn10 (Tet^R)} *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), Φ80*lacZ*ΔM15, Δ*lacX74*, *deoR*, *recA1*, λ *araD139*, Δ(*ara-leu*)7697, *galU*, *galK*, *rpsL*(Str^R), *endA1*, *nupG* λ strain (Invitrogen, Catalog # C665-11).

2.1.1.2 Fungal Strain

Lcc2 laccase coding cDNA of white-rot fungus *Pycnoporus sanguineus* MUCL 38531 had been previously isolated by our group. Its sequence has not been submitted to GenBank, yet.

2.1.1.3 Yeast Strain

Yeast *Saccharomyces cerevisiae* W303-1A strain (*MAT ade2-1*, *his3-11, 15*, *leu2-3, 112*, *trp1-1*, *ura3-1*, *can1-100*) was kindly provided by Prof. Giovanni Sannia, Università di Napoli, Italy.

2.1.2 Cloning and Expression Vectors

2.1.2.1 pDrive Cloning Vector

pDrive cloning vector (given in Appendix A) was purchased from Qiagen (Catalog # 231122) for cloning of PCR products containing the single Adenine (A) overhang at each end.

2.1.2.2 pSAL4 Expression Vector

pSAL4 expression vector (Figure 2.1) was kindly provided by Giovanni Sannia, Università di Napoli, Italy. Known properties of vector were explained further in detail.

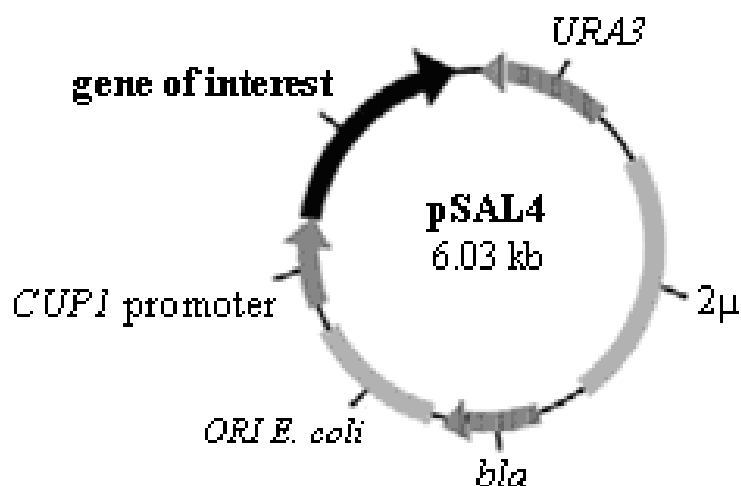


Fig 2.1: Map of pSAL4 expression vector.

2.1.3 Enzymes

2.1.3.1 Restriction Enzymes

EcoRI (G^AAATTC, Fermentas) and **BamHI** (G^AGATCC, Fermentas) restriction endonucleases and their reaction buffers were purchased from Fermentas (Catalog # FD0274 and Catalog # ER0051).

2.1.3.2 Pfu DNA Polymerase

The *Pfu* DNA Polymerase is a highly thermostable DNA polymerase from the hyperthermophilic archaeum *Pyrococcus furiosus*. The enzyme catalyzes the template-dependent polymerization of nucleotides into duplex DNA in the 5'⇒3' direction. The *Pfu* DNA Polymerase also exhibits 3'⇒5' exonuclease (proofreading) activity, that enables the polymerase to correct nucleotide incorporation errors. It has no 5'⇒3' exonuclease activity. It was purchased from Fermentas (Catalog # EP0502).

2.1.3.3 i-Taq DNA Polymerase

i-Taq DNA polymerase (recombinant) is DNA polymerase of *Thermus aquaticus*. The enzyme catalyses the incorporation of nucleotides into duplex DNA in the 5'→3' direction in the presence of Mg²⁺ at optimally 72°C. It was purchased from Intron Biotechnology (Catalog # 25021).

2.1.3.4 T4 DNA Ligase

T4 DNA ligase is useful for both sticky- or blunt-ended DNA fragments. The enzyme catalyzes the formation of phosphodiester bonds between neighboring 3'-hydroxyl and 5'-phosphate ends of double-stranded DNA. It also closes single-stranded nicks in double-stranded DNA. It was purchased from Roche (Catalog # 10481220001).

2.1.4 DNA Molecular Weight Markers

2.1.4.1 Lambda DNA/ *EcoRI*+*HindIII* Marker, 3

Lambda DNA/*EcoRI*+*HindIII* Marker, 3 (given in Appendix B), is premixed with a storage and loading buffer at a total DNA concentration of 0.1µg/µl and can be directly applied onto an agarose gel. The DNA Marker contains the following 13 discrete fragments (in base pairs): 21226*, 5148, 4973, 4268, 3530*, 2027, 1904, 1584, 1375, 947, 831, 564, 125. It was purchased from Fermentas (Catalog # SM0193).

2.1.5 Oligonucleotides

► Oligonucleotides given below were synthesised by Alpha DNA (Genova) company and were purchased as lyophilized form.

Lcc2 forward 5'-CCGGAATTCATGGAGGGATCGAGACCAACT-3'

Lcc2 reverse 5'-AACGCGGCCGCACTATCCACGAAATGACTG-3'

pSAL4 forward 5'-TAGATATTAAGAAAACAAACTG-3'

pSAL4 reverse 5'-TACCTACTTAACGTCG-3'

► Oligonucleotides given below were supplied by the kits purchased from the companies.

M13 forward –20 (pDrive) 5'-GTAAAACGACGGCCAGT-3'

M13 reverse (pDrive) 5'-AACAGCTATGACCATG-3'

2.1.6 Culture Media

2.1.6.1 Bacterial Culture Media

► LB (Luria-Bertani) Medium

10 g tryptone, 5 g yeast extract, 5 g NaCl were dissolved in distilled water up to 1 lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized by autoclaving for 15 minutes under 1.5 atm at 121°C. The medium was stored at room temperature.

► LB Agar Medium

10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g bactoagar were dissolved in distilled water and completed up to 1 lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized by autoclaving for 15 minutes under 1.5 atm at 121°C.

2.1.6.2 Yeast Culture Media

► YPD Agar Medium

10 g yeast extract, 20 g bacto-peptone and 20 gr glucose were dissolved in distilled water and completed up to 1 lt, and then sterilized by autoclaving for 15 minutes.

► SD Selective Medium

Medium composition for cultivation of *S. cerevisiae* is shown in Table 2.1.

Table 2.1 Medium composition for cultivation of *S. cerevisiae* [62].

pH:5.3	SD	Sd	SG	Sg
YNB - Yeast Nitrogen Base	0.67%	0.67%	0.67%	0.67%
Casamino acid	0.5%	0.5%	0.5%	0.5%
Glucose	2%	1%		
Galactose			2%	1%
Adenin	30 mg ^l ⁻¹	30 mg ^l ⁻¹	30 mg ^l ⁻¹	30 mg ^l ⁻¹
Tryptone	40 mg ^l ⁻¹	40 mg ^l ⁻¹	40 mg ^l ⁻¹	40 mg ^l ⁻¹
Succinate Buffer	50 mM	50 mM	50 mM	50 mM

15% bactoagar (Acumedia) was used for solid media.

2.1.7 Stock Solutions

2.1.7.1 Ampicillin Stock Solution

100mg/ml ampicillin sodium salt (Catalog # A0166, Sigma-Aldrich) was dissolved in deionized water, filter-sterilized and stored in dark at -20°C.

2.1.7.2 Salmon Sperm DNA Solution

UltraPure™ DNA solutions from Salmon Sperm DNA are prepared from highly pure, phenol/chloroform-extracted DNA and DNase-free and RNase-free (DEPC-treated) distilled, deionized water and are sheared to an average size of 2000 bp. The concentration of solution is adjusted to 10 mg/ml (Catalog # 15632-011, Invitrogen).

2.1.8 Buffers and Solutions

2.1.8.1 Na-Acetate Buffer (3 M, pH:4.6, 100 ml)

3 M of sodium acetate (Na-Ac) was dissolved in 75 ml distilled water (dH₂O). pH was adjusted to 4.6 and solution was completed to 100 ml with dH₂O.

2.1.8.2 Na-Citrate buffer (0.1 M, pH:3, 100ml)

0.1 M 100 ml citric acid (2.1 g citric acid dissolved in 100 ml dH₂O) and 0.1 M 20 ml tri-sodium citrate dihydrate (588 mg tri-sodium citrate dihydrate dissolved in 20 ml dH₂O) were prepared. Then 46.5 ml of 0.1 M citric acid was mixed with 3.5 ml of 0.1 M tri-sodium citrate dihydrate, and it was completed up to 100 ml with dH₂O.

2.1.8.3 Succinate Buffer (0.7 M, pH:5.3, 50 ml)

3.5 g of succinic anhydride was dissolved in 45 ml deionized water, its pH was titrated to 5.3 with NaOH, and the solution was completed up to 50 ml with deionized water. Then it was filter sterilized.

2.1.8.4 10X TBE (Tris/Borate/EDTA) Buffer (1000 ml)

108 g Of Tris base, 55 g boric acid and 20 ml 0.5 M EDTA at pH 8.0 were dissolved in 1 liter deionized water (dH₂O), its pH was titrated to 8.3 and sterilized for 5 min under 1.5 atm at 121°C.

2.1.8.5 Plasmid Isolation and Gel Extraction Buffers

► P1 - Resuspension Buffer (1000 ml)

6.06 g Tris base and 3.72 g EDTA-2H₂O were dissolved in 800 ml dH₂O. The pH was titrated to 8.0 with HCl and the volume was adjusted to 1 liter with dH₂O. 100 mg RNase A buffer (Catalog # 19101, Qiagen) was added per 1 liter and the buffer was stored at +4°C.

► P2 - Lysis Buffer (1000 ml)

8.09 g of NaOH was dissolved in 950 ml dH₂O and 50 ml 20% sodium dodecyl sulfate – SDS (w/v) solution was added. The final volume was 1 liter.

► P3 - Neutralization Buffer (1000 ml)

294.5 g potassium acetate was dissolved in 500 ml dH₂O. The pH was titrated to 5.5 with glacial acetic acid (about 110 ml). The volume was adjusted to 1 liter with dH₂O.

► PE - Wash Buffer (1000 ml)

6.09 g NaH₂PO₄·H₂O, 17.54 g NaCl and 1.36 g imidazole were dissolved in 1 liter deionized water. Its pH was titrated to 8.0 with NaOH.

► EB - Elution Buffer (1000 ml)

6.09 g NaH₂PO₄·H₂O, 17.54 g NaCl and 17 g imidazole were dissolved in 1 liter deionized water. Its pH was titrated to 8.0 with NaOH.

2.1.8.6 Other Chemical Solutions And Buffers

Table 2.2 shows the concentrations and molar ratios of other chemical solutions and buffers used for expression of *P. sanguineus* laccase.

Table 2.2 Other solution & buffer concentrations and molar ratios [37].

ABTS solution in Na-citrate buffer	2.5 mM
PEG 4000	40%
LiAc	0.1 M
Tris-HCl buffer (pH 7.5)	0.1 M
EDTA	1 mM
NaOH	10 M

2.1.9 Chemicals

Adenine was purchased from Merck (Germany).

Bactoagar was obtained from Difco Laboratories Inc (USA).

Boric acid was obtained from Merck (Germany).

Casein hydrolysate (casamino acid) was purchased from Sigma-Aldrich (Germany).

Citric acid trisodium salt was obtained from Sigma-Aldrich (Germany).

Copper sulphate pentahydrate was purchased from Merck (Germany).

Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) was obtained from Sigma-Aldrich (Germany).

Galactose was purchased from Riedel-de Haen (Germany).

Glucose was purchased from Riedel-de Haen (Germany).

Hydrochloric acid was obtained from Merck (Germany).

L-tryptophane was obtained from Merck (Germany).

Lithium acetate dihydrate was obtained from AppliChem (Germany).

Mycological peptone was purchased from Lab M (UK).

Poly(ethylene glycol) (PEG) 4000 was purchased from Sigma-Aldrich (Germany).

Sodium acetate was obtained from Riedel-de Haen (Germany).

Sodium chloride was purchased from Riedel-de Haen (Germany).

Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich (Germany).

Sodium hydroxide was purchased from Riedel-de Haen (Germany).

Succinic anhydride was obtained from Sigma-Aldrich (Germany).

Tri-sodium citrate dihydrate was obtained from Riedel-de Haen (Germany).

Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) was purchased from Merck (Germany).

Tryptone was purchased from Acumedia (USA).

Yeast extract (YE) was obtained from Lab M (UK).

Yeast nitrogen base (YNB) was purchased from Sigma-Aldrich (Germany).

2,2'-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) was purchased from Sigma-Aldrich (Germany).

2.1.10 Laboratory Equipments

Autoclaves	: Nüve OT 4060 Steam Sterilizer (Turkey) : Tuttnauer 2540 ML (Switzerland)
Centrifuges	: Microfuge 18, Beckman Coulter (Germany) : Allegra™ 25R Centrifuge, Beckman Coulter (Germany)
Deepfreezers and refrigerators	: 2021 D Deep Freezer, Arçelik (Turkey) : 1061 M Refrigerator, Arçelik (Turkey) : New Brunswick Scientific U410 Premium (England)
Electrophoresis equipment	: Bio-Rad (USA)
Gel documentation system	: UVIpro GAS7000, UVItec Limited
Ice machine	: AF 10, Scotsman (UK)
Incubators	: Memmert UM400 (Germany) : Nüve EN400 (Turkey)
Laminar flow cabinet	: Biolab Faster BH-EN2003 (Italy)
Magnetic stirrers	: AGE 10.0164, Velp Scientifica (Italy) : AGE 10.0162, Velp Scientifica (Italy)
Microplate reader	: Model 3559 UV Microplate, Bio-Rad (USA)
Micropipettes	: Eppendorf AG (Germany), 5000µl, 1000µl, 200µl, 100µl, 10µl
Microscope	: Olympus (USA)
Orbital shakers	: Shell lab 1575R-2E (USA) : Thermo Electron Corporation (USA)
pH-meter	: Mettler Toledo MP220 (Switzerland)
Pipettes	: Eppendorf AG (Germany) 10µl, 20µl, 200µl, 1000µl
Pure water systems	: USF Elga UHQ-PS-MK3, Elga Labwater
UV-visible spectrophotometers	: Perkin Elmer Inst. Lambda 25 (USA) : Shimadzu UV-Pharmaspec 1700 (Japan)
Vortex apparatus	: Heidolph Reax Top (Germany)

2.2. Methods

2.2.1 *Lcc2* cDNA/Vector Construction

2.2.1.1 Amplification of Double Stranded cDNA from pPICZB Vector

Laccase coding *Lcc2* cDNA was previously cloned into pPICZB (Invitrogen, Catalog # V190-20) vector by our group. Polymerase chain reaction (PCR) was set up with forward and reverse primers to amplify *Lcc2* cDNA. Reaction conditions were described below:

Polymerase Chain Reaction (PCR)

Template DNA (<i>Lcc2</i> cDNA)	2 µl
Forward primer (10 pmol)	2 µl
Reverse primer (10 pmol)	2 µl
dNTP mixture (10 mM each)	1 µl
10X Pfu buffer with MgSO ₄	5 µl
Pfu DNA polymerase	0.5 µl
Sterile dH ₂ O	37.5 µl
Total Volume	50 µl

Reaction Conditions

94°C 3 min

94 °C 1 min

55 °C 1 min	}	5 cycles
72 °C 2 min 30 sec		
94 °C 1 min		

60 °C 1 min	}	25 cycles
72 °C 2 min 30 sec		
72 °C 10 min		

While PCR was continuing, reaction was paused and 0.5 µl i-Taq DNA polymerase was added to reaction mix before final extension (72°C 10 minutes) step.

PCR product was run on 1.0% agarose gel and desired gene product was gel extracted.

► Gel Extraction Procedure

Gel extraction reactions in this study were performed with MinElute Gel Extraction Kit (Catalog # 28604, Qiagen).

1. DNA fragment was excised from agarose gel with a clean scalpel. Gel slices were put into microfuge tubes and their weight was calculated.
2. A 3-gel volume of QG (solubilization and binding) buffer was added to 1 volume of gel slice (600 µl for 100 mg gel). When gels were excised from a %2 or more concentrated gel, a 6-gel volume of QG buffer was applied.
3. Sample was incubated at 50°C for 10 minutes by vortexing every 2 minutes.
4. After the gel completely dissolved, 1 gel volume of room temperature %100 isopropanol was added and mixed by inverting the tube several times.
5. Sample was applied to the MinElute column* and centrifuged for 1 minute at 13000 rpm.
6. Flow-through was discarded from collection tube.
7. 500 µl QG buffer was applied to column and centrifuged for 1 minute at 13000 rpm.
8. Flow-through was discarded from collection tube.
9. In order to wash, 750 µl PE buffer was applied to the column and centrifuged for 1 minute at 13000 rpm.
10. Flow-through was discarded from collection tube and the MinElute column was placed back in the same tube. Then it was centrifuged for 1 minute at 13000 rpm.
11. Spin column was placed in a 1.5 ml graduated microcentrifuge tube and 10 µl** EB buffer was applied to elute the DNA. After 1 minute incubation the tube was centrifuged for 1 minute.

* Qiaquick column (Catalog # 28104, Invitrogen) was used for pSAL4 and pSAL4/*Lcc2* vector gel extractions.

** 10 µl EB buffer for *Lcc2* cDNA, and 30 µl EB buffer for pSAL4 expression vector were used.

Eluted DNA was stored at +4°C prior to usage or at -20°C for long term storage (several weeks).

2.2.1.2 Cloning into a TA vector

TA cloning vectors contain a U overhang at each ends providing easy and efficient ligation of PCR products with an A overhang. Unpaired A residues are added to PCR products during PCR reactions generated by *Taq* DNA polymerase. Hence the DNA obtained from PCR was extracted and used directly to be cloned into pDrive Cloning vector.

Ligation reaction was performed according to PCR Cloning Kit (Catalog # 231122, Qiagen).

Ligation Reaction

pDrive cloning vector (50ng/ µl)	1 µl
PCR product	4 µl
2X ligation master mix	5 µl
Total volume	10 µl

Reaction Conditions

The samples were incubated for 1 hour 30 minutes at 16°C and 10 minutes at 70°C for ligase inactivation.

► Preparation of Xgal/IPTG-Amp Plates

100 µl Xgal/IPTG solution and 50 µl ampicillin solution were put into 50 ml liquid warm LB agar and was poured into plastic petri dishes.

► Electrotransformation Procedure for Plasmids

1. 10 µl ligation product was mixed with 40 µl TOP10F' electrocompetent *E. coli* cell in a prechilled electroporation cuvette.

2. Cuvette was placed into electroporator and electrophoresis was applied at 1800 volts.
3. 1000 μ l LB medium was added to the cuvette and whole mixture was transferred into a microfuge tube. The tube was incubated at 37°C, 200-250 rpm for 1 hour on an orbital shaker.
4. After incubation, transformation mixture was centrifuged for 10 minutes at 5000 rpm.
5. 850 μ l of supernatant was removed and the pellet in the remaining supernatant was re-suspended by the help of pipette.
6. Mixture was spread onto LB agar plates containing Xgal/IPTG-ampicillin by using a spreader and the plate was incubated overnight (14-16 hours) at 37°C.

2.2.1.3 Plasmid DNA Purification

White colonies obtained subsequent to transformation were selected and incubated overnight in 5 ml LB containing 100 μ g/ml ampicillin. Plasmid DNA was purified from 2 ml bacterial culture by the help of Plasmid Mini Kit (Catalog # 12123, Qiagen) for sequencing and further applications Procedure is described in detail below.

1. 2 ml of the culture was centrifuged for 5 minutes at 13000 rpm.
2. The supernatant was discarded and the pellet re-suspended in 300 μ l of re-suspension buffer P1 by vortexing.
3. 300 μ l of lysis buffer P2 was added, mixed gently by inverting the tube 4-6 times and incubated at room temperature for 5 minutes.
4. 300 μ l of chilled neutralization buffer P3 was added, mixed immediately and gently by inverting 4-6 times and incubated on ice for 15 minutes.
5. The samples were centrifuged at 13000 rpm for 15 minutes.
6. 800 μ l of supernatant was transferred into a new 1.5 ml graduated microfuge and 560 μ l isopropanol was added to the tube, mixed gently by inverting the tube for several times.
7. The samples were centrifuged at 13000 rpm for 30 minutes and the supernatant was removed.

8. DNA pellet was washed with 1 ml of %70 ethanol and centrifuged at 13000 rpm for 5 minutes. The supernatant was decanted carefully without disturbing the pellet.
9. The pellet was air dried for 5-10 minutes and the DNA was re-dissolved in 10 μ l of elution buffer EB.
10. The samples were kept on thermomixer for 15 minutes at 350 rpm 37°C.

The microcentrifuge tube containing the eluted plasmid DNA was either used directly in applications such as cloning or sequencing or stored at -20°C for the further analyses.

2.2.1.4 Plasmid DNA Sequencing

Sequence PCR reactions were set up with Big Dye® Terminator v 3.1 Cycle Sequencing Kit (Catalog # 4337455, Applied Biosystems).

Plasmid DNA	1 μ l
M13 reverse primer	1 μ l
5X sequencing buffer	2 μ l
Big dye terminator	2 μ l
Sterile dH ₂ O	4 μ l
Total volume	10 μ l

Reaction Conditions

95°C 5 min

95°C 1 min	} 30 cycles
50°C 30 sec	
60°C 4 min	

► NaAc-Ethanol Precipitation (Purification) of PCR Products

1. 1 μ l 3 M pH 4.6 sodium acetate (cold) and 25 μ l 95% ethanol (cold) were mixed for each sample.

2. 26 μ l mixture was added into each PCR product and samples were incubated on ice for 15 minutes.
3. After incubations, samples were centrifuged for 15 minutes at 14000 rpm.
4. Supernatant was discarded and DNA pellet was washed with 250 μ l cold 70% ethanol.
5. Samples were centrifuged for 15 minutes at 14000 rpm.
6. Ethanol was discarded, the residual ethanol was evaporated by incubating at 94°C for ~1 minute.
7. DNA pellet was dissolved in 20 μ l Hi-Di formamide* and samples were denatured by putting at 94°C for 3 minutes and then at -20°C for 5 minutes.

* Formamide was purchased from Applied Biosystems (Catalog # 4311320), USA.

ABI 3100 Avant (Applied Biosystems Inc, USA) automated sequencer was used for DNA sequencing.

2.2.1.5 Cloning of *Lcc2* cDNA into pSAL4 Expression Vector

pDrive cloning vector and pSAL4 expression vector were digested with restriction enzyme and the DNA fragments were extracted from the gel. Cleaned and concentrated insert DNA was ligated into cleaned pSAL4 expression vector. Procedure is described in detail below.

► Restriction Enzyme Digestion

Lcc2 cDNA was digested from pDrive cloning vector with *EcoRI* restriction enzyme. Also pSAL4 expression vector was digested with same enzyme. The digested sequences were extracted from the gel as previously described.

Digestion of pDrive/*Lcc2* and pSAL4 Vectors

pDrive/ <i>Lcc2</i>	10 μ l	pYG132	10 μ l
10X buffer	4 μ l	10X buffer	4 μ l
<i>EcoRI</i>	1 μ l	<i>EcoRI</i>	1 μ l
Sterile dH ₂ O	25 μ l	Sterile dH ₂ O	25 μ l
Total volume	40 μ l	Total volume	40 μ l

Reaction Conditions

The mixtures were incubated at 37°C for 30 minutes. Digestion reactions were run on 1% agarose gel electrophoresis. The DNA band corresponding to our vector DNA fragment (~1.7 kb) and pSAL4 expression vector (~6.03 kb) were gel extracted.

► Ligation into pSAL4 Expression Vector

Ligation reaction was set up with T4 DNA Ligase kit (Catalog # 10481220001, Roche).

1. 0.5 µl pSAL4 vector was combined with 9.5 µl of insert into the 0.2 ml graduated PCR tube.
2. It was kept at 65°C for 5 minutes for denaturation and 1 minute on ice.
3. 10 µl reaction mixture was prepared into a new 0.2 µl graduated PCR tube.

Poly(ethylene glycol)	2 µl
10X T4 ligase buffer	2 µl
T4 ligase	2 µl
Sterile dH ₂ O	4 µl
Total volume	10 µl

4. After a short spin in centrifuge, two PCR tubes were mixed together.
5. The 20 µl mixture was centrifuged for a short spin.

Reaction Conditions

Ligation mixture was incubated for 16 hours at 16°C and 10 minutes at 65°C for ligase inactivation.

The ligation product was transformed into the TOP10 electrocompetent *E. coli* cells. Plates were incubated overnight at 37°C. Plasmid DNA purification, plasmid DNA sequencing and NaAc-ethanol precipitation (Purification) of PCR products were performed respectively, as previously described. After the DNA sequencing analyses, a plasmid bearing the desired *Lcc2* cDNA was chosen for transformation into the yeast.

2.2.2 Transformation of pSAL4/*Lcc2* Vector into the host *S. cerevisiae*

After the positive DNA sequencing result, the plasmid bearing the desired *Lcc2* cDNA was cut with an appropriate restriction enzyme and then the linear pSAL4 plasmid was introduced into the yeast. Procedure is described in detail below.

► Restriction Enzyme Digestion

pSAL4/*Lcc2* vector was digested with *Bam*HI restriction enzyme and the digested sequence was gel extracted as previously described.

Digestion of pSAL4 Vector

pSAL4	10 µl
10X buffer	4 µl
<i>Bam</i> HI	1 µl
Sterile dH ₂ O	25 µl
Total volume	40 µl

Reaction Conditions

The reaction was incubated at 37°C for 30 minutes and then run on 1% agarose gel. The DNA band corresponding to pSAL4/*Lcc2* vector (~7.60 kb) was gel extracted.

► Transformation of Yeast *S.cerevisiae*

The yeast *S. cerevisiae* cells had been provided on a YPD (1% yeast extract, 2% bacto-peptone, %2 Glucose) agar plate. Yeast cells were scraped from the YPD plate and washed with 1 ml of H₂O. The cell pellet was then incubated in 1 ml of 0.1 M LiAc for 5 min at 30°C. Cells were sedimented by centrifuging at 12000 rpm for 2 minutes, resuspended in a mixture containing;

- 350 µl tamponne plate*
- 100 µl SS-DNA (2.0 mg ml⁻¹)
- transforming DNA plasmid (up to 5 µg)

and incubated at 42° C for 20 minutes. Cells were then centrifuged, resuspended in 200 µl H₂O, and spread on selective medium.

Plates were incubated inverted for 4 days at 28°C and checked for the development of green or orange colour which indicates the laccase oxidation.

***Tampone Plate**

PEG 4000	40% w/v
LiAc	0.1 M
TrisHCl (pH:7.5)	0.1 M
EDTA	1 mM

2.2.3 Enzyme Expression Analyses

Laccase production by the positive transformants first assayed on SD plates supplemented with 0.6 mM CuSO₄ and 0.2 mM ABTS. After the green or orange zone determination around the colonies, these colonies were chosen for liquid culture assays.

2.2.3.1 Yeast Cultivation

S. cerevisiae colonies were grown in 15 ml flasks containing 3 ml of selective SD medium for 4 days. Culture aliquots (250 µl) were collected daily and cells were pelleted by centrifugation (12000 rpm for 2 minutes at 4°C) for laccase activity assay to chose the best laccase producer colony.

2.2.3.2 Laccase Activity Assay

Laccase activities in the culture supernatants were routinely determined by measuring the oxidation of ABTS (2.5 mM) in the sodium-citrate buffer (0.1 M, pH:3.0) at 414 nm ($\epsilon = 36 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit (U) of laccase activity was defined as 1.0 µmol of product formed per minute under the assay conditions. Laccase activities were monitored on the Perkin-Elmer Lambda 25 UV-Vis Spectrophotometer (Shelton CT, USA).

The reaction mixture which contained 160 µl sodium citrate buffer, 34 µl substrate (ABTS) and 8.5 µl enzyme loaded into the spectrophotometer and measured at 414 nm for 3 minutes of reaction time at 25°C [63]. The unit enzyme (U/l) activity was calculated by the formula given below using the slope of the spectrogram (Table 2.3).

Table 2.3: Formula for laccase activity measurement

$\text{Laccase (U/l)} = ((\Delta A/t) / \epsilon \cdot d) \cdot (1 \times 10^6 \mu\text{mol/mol}) \cdot (V/v)$	
ΔA	= absorbance change at 414 nm (dA/dt)
ϵ	= extinction coefficient of ABTS at 414 nm = 36000 M ⁻¹ cm ⁻¹
d	= light path of the cuvette container cell (cm)
V	= total reaction volume (ml)
v	= enzyme volume (ml)

2.2.4 Optimization of Expression

For optimization assays *S. cerevisiae* cultures were used to improve laccase production under varying growth conditions, with a particular attention to the effect of nutrients and copper concentration. The selected transformants were first grown in selective SD medium, started from 0.5 OD₆₀₀, in the presence of CuSO₄ with different molar ratios. Then colonies were cultivated in different selective media containing glucose or galactose in the presence of optimal CuSO₄. Also after the best condition was determined for colonies to produce laccase in terms of CuSO₄ concentrations, optimization assays were continued to determine an optimal molar ratio for the best nutrient.

2.2.4.1 CuSO₄ Optimization

Copper is one of the most strong inducers of laccase activity assays among the putative inducers tested. So CuSO₄ was selected as the inducer for the expression optimization experiments. *S. cerevisiae* culture was grown in 15 ml flasks containing 3 ml of selective SD media for 7 days with different CuSO₄ concentrations; 0.3 mM, 0.6 mM, 1.2 mM, and 1.8 mM. Culture aliquots (250µl) were collected daily for OD₆₀₀ measurements. Also pellets of these aliquots were used for activity assays. OD₆₀₀ and activity measurements daily determined between 4th and 7th days.

2.2.4.2 Nutrient Optimization

The selected colony was used for nutrient optimization. It was cultivated for 7 days in different SD media supplemented with 0.6 mM CuSO₄, including glucose or galactose with different molar ratios; 1% and 2%. OD₆₀₀ and activity measurements daily assayed between 4th and 7th days as previously described in CuSO₄ optimization.

3. RESULTS AND DISCUSSION

3.1 Construction of the pSAL4/*Lcc2* Vector

In order to obtain pSAL4/*Lcc2* construct, first the cloning of the *Lcc2* cDNA into the pDrive cloning vector was accomplished. For this purpose, *Lcc2* cDNA was amplified with *Lcc2* forward and *Lcc2* reverse primers and pPICZB/*Lcc2* construct was used as template. After amplified sequence was cloned into the cloning vector, it was cut off and integrated into the pSAL4 expression vector.

3.1.1 Cloning of *Lcc2* cDNA sequence into pDrive cloning vector

For the amplification of laccase coding *Lcc2* cDNA, PCR was set up with pFU and i-taq DNA polymerase enzymes. I-Taq adds a single adenine residue at 3'-end of amplified DNA sequence with a longer final extension step (72°C 10 min) during PCR. Amplified PCR product was loaded into the gel with DNA molecular weight marker, 3, for electrophoresis and it was run for approximately 25 minutes at 120 volt.

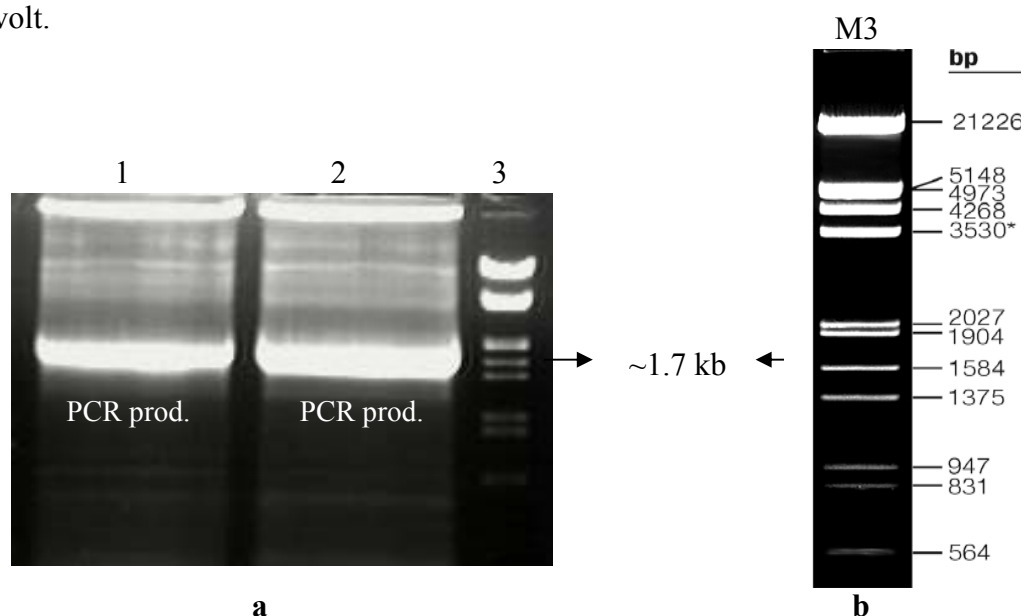


Fig 3.1: The photos of 1% agarose gel electrophoresis of *lcc2* fragments. **a.** Lane 1, 2: PCR products, Lane 3: Marker 3, Lambda DNA/ *EcoRI*+*HindIII*. **b.** Marker 3 (M3).

Agarose gel electrophoresis is used to run the molecules through the gel matrix related to the size of them by applying an electric current at different rates. These molecules are stained to make them visible under ultraviolet light. So their photographs can be taken for size and intensity comparisons between bands of interest and selected marker which has a mixture of molecules of known sizes [64].

DNA is uniformly negatively charged because of phosphate groups of sugar-phosphate backbones and caused to migrate toward the positive electrode. As it was depicted in Fig 3.1, two PCR products were run on the gel in different lanes (Lane 1 and Lane 2) and both of them were approximately ended up at the same distance from the top contain molecules that passed through the gel with the same speed. This usually means that they are the same sized bands. Another aspect about the image is smearing of the products which reflect to multiple unresolved components in addition to desired bands. An assumption about this undesired profile is the improper annealing of the primers because of the temperature. It can be overcome by examining different PCR conditions. So for annealing step of PCR, initially 5 cycles at 55°C and then 25 cycles at 60°C was applied and the best UV image was obtained with this conditions.

A ~1.7 kb band was gel extracted in order to get rid of nonspecific sequences. By obtaining the desired DNA fragment on agarose gel, the extracted PCR product was cloned into pDrive which is a linearized cloning vector carrying the single U overhangs at each 3'-ends. Therefore it allows PCR product containing 3'-end adenine overhangs to be directly ligated and cloned with high efficiency. Besides, the location that was designed for PCR product is between two *EcoRI* restriction sites on the cloning vector at its multiple cloning site. This makes the vector suitable for PCR product to be cut properly with this enzyme. As it will be explained further, *EcoRI* restriction site is an important region for all cloning studies including both cloning vector and the expression vector pSAL4.

pDrive cloning vector which carries PCR product was then transformed into TOP10F' electrocompetent *E. coli* cells. After an o/n incubation on LB-ampicillin plate at 37°C, 48 white colonies were obtained and their plasmids were isolated. In pursuit of isolation, plasmids were cut with *EcoRI* enzyme to observe the insert on gel. A plasmid which carries desired insert was detected with agarose gel electrophoresis (Figure 3.2).

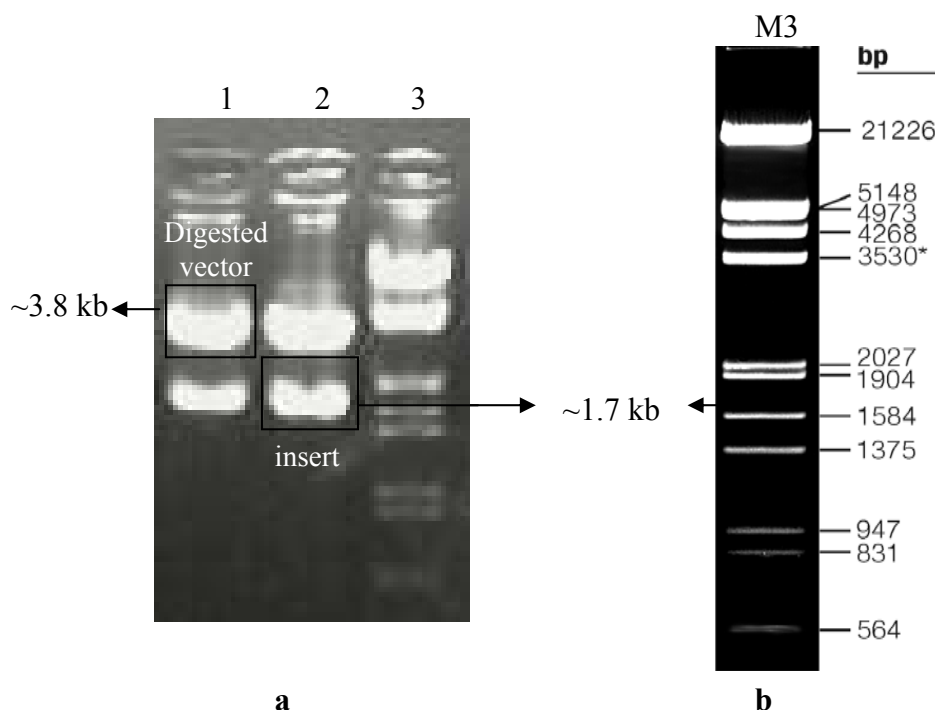


Fig 3.2: The photos of 1% agarose gel electrophoresis DNA fragments. **a.** Lane 1, 2: DNA fragments obtained from *EcoRI* digestion of pDrive/*Lcc2* constructs, Lane 3: M3. **b.** M3.

In the Fig 3.2, it was depicted that there are two bands after restriction enzyme digestion; a ~3.8 kb linearized vector band and a ~1.7 kb insert band. So it was concluded that pDrive/*Lcc2* construct had been integrated into the *E. coli* electrocompetent cell and it could be selected for sequence confirmation to detect if the amplified PCR product has a homology with the other known laccase sequences.

The DNA sequence of chosen recombinant pDrive/*Lcc2* plasmid was verified by sequence analysis following sequence PCR and purification assays. Homology of *Lcc2* cDNA with some laccases were observed via using BLAST (basic local alignment search tool) program by NCBI (National Center for Biotechnology Information) database. In this program the sequences of interest are aligned locally and its very useful for dissimilar sequences that are predicted to contain regions of similarity.

Moreover similar sequence motifs within their larger sequence context can be detected via using this program. This motifs may be caused by the conservation of base pairs. When similar regions are identified, then functional or evolutionary relationships between the sequences can be detected. The sequence alignment is useful for the analyses of DNA, RNA and also protein sequences [65].

In the study, aligned sequences of nucleotide residues represented as rows within a matrix, were compared to the similar database sequences via using BLAST program. After the comparison of the nucleotide query sequence (*Lcc2*) against the nucleotide sequence database, laccases with maximum identity were selected for depicting Table 3.1.

Table 3.1: Homology of *Lcc2* cDNA with several laccases on NCBI blast data. Accession numbers obtained from GenBank [66].

Accession No	Description	Max. Score	Query Coverage	Max Identity
AF548035.1	<i>Trametes</i> sp. I-62 laccase (pox3) mRNA, comp. cds	443	98%	76%
AF152170.1	<i>Pycnoporus cinnabarinus</i> laccase (lac1) mRNA, comp. cds	345	82%	75%
AY693776.1	<i>Trametes versicolor</i> laccase 1 (lcc1) mRNA, comp. cds	327	77%	75%
FJ513077.1	<i>Pycnoporus sanguineus</i> laccase mRNA, complete cds	313	79%	74%
FJ598130.1	<i>Coriolopsis gallica</i> laccase mRNA, comp. cds	306	86%	73%

When a record is submitted to GenBank, a unique identifier is assigned to the entire sequence and it is called the “accession number” which was symbolized in table. This term was also used in the next table to represent the same descriptions. “Maximum score” is the highest alignment score of a set of aligned segments from the same database sequence while the “maximum identity” is the highest percent identity for a set of aligned segments to the same database sequence. In addition, “query coverage” is the percent of the query length that is contained in the aligned segments.

Multiple sequence alignment of *Lcc2* cDNA and several laccases were observed via using ClustelW2 program by EBI (European Bioinformatics Institute). This program is an extension of pairwise alignment and provides comparison of more than two sequences at a time. It is tried to align all of the sequences in a given query segment by the program. Additionally, it is useful for identifying conserved sequence regions across a group of sequences assumed to be evolutionarily related [67].

In the study the multiple alignment program is used to align the identical nucleotides from *Lcc2* cDNA, and the other laccases that were mentioned in previous table with the high scores for query coverage in *Lcc2* comparison. The program calculated the best match for the selected sequences, and lined them up via using different colors for each nucleotide (Table 3.2).

Table 3.2: Multiple sequence alignment of *Lcc2* cDNA and several laccases on EBI ClustelW2 program. Identical amino acids were marked by asterisks. [68].

AF152170.1	AGCCCCGATGGCTTCGCTCGCGAGGCCGTCGTGGTGAACGGTATCACCCCTGCCCTCTC	168
AY693776.1	TCGCCCCGACGGCTTCCTTCGGGATGCCATCGTGGTCAACGGCGTGGTCCCTTCCCCGCTC	165
AF548035.1	TCCCCCGACGGTTTCCAGCGTGCGGCGGTGTCGCGAACGGCGGGGTCCCTGGCCGCTC	180
<i>Lcc2</i>	TCTCCAGATGGCTACAGCGAGCAGCGGTAGTGGCAAACGGTGTCATGCCCGCACTC	166
	* * * * *	
AF152170.1	ATCACAGGCAATAAGGGCGATCGATTCCAGCTCAATGTCATCGACCAATTGACAAATCAT	228
AY693776.1	ATCACTGGGAAGAAGGGAGACCGCTTCCAGCTCAACGTCGTGCGACACCTTAACCAACCAC	225
AF548035.1	ATTAACGGCCAGAAGGGTGACCATTTCCAGATCAATGTGGTCAACCAAGCTTACGAACCAC	240
<i>Lcc2</i>	ATTACGGGCACGAAGGGCGACGAGTTCAAGATCAATGTCATCAACAACCTGACAAATCAT	226
	* * * * *	
AF152170.1	ACCATGTTGAAAACATCTAGTATTTCATTGGCACGGCTTCTTCCAGCAAGGCACGAACTGG	288
AY693776.1	TCCATGCTCAAGTCCACTAGTATCCACTGGCACGGCTTCTTCCAGGCAGGCACCAACTGG	285
AF548035.1	ACCATGCTCAAGTCCACCAGTATCCACTGGCACGGTTTCTTCCAGGAAGGGCACGAACTGG	300
<i>Lcc2</i>	ACCATGCTCAAGTCTACTAGTATTCACTGGCACGGACTATTCCAAAGGTCACGAACTGG	286
	* * * * *	
AF152170.1	GCCGATGGTCCCGCTTCGTGAACCAAGTGTCCCATCGCTTCGGGCCACTCGTTCTTGTAT	348
AY693776.1	GCAGACGGAACCGCGTTCGTCAACCAAGTGCCTATTGCTTCGGGCAATTCATTCTGTAC	345
AF548035.1	GCGGACGGTCTGCTTCGTGAACCAAGTGTCTATTGCAACCGGCCATTCGTTCTGTAT	360
<i>Lcc2</i>	GCGGATGGCCCTGCTTCGTGAATCAATGTCCATTGCGACTGGGCAATTCATTCTTTAT	346
	* * * * *	
AF152170.1	GACTTTCAAGTTCCCGACCAAGCAGGGACTTTCTGGTACCATAGCCATCTCTCCACGCAA	408
AY693776.1	GACTTCCATGTGCCCGACCAAGGCAGGGACGTTCTGGTACCACAGTCATCTGTCTACGCAA	405
AF548035.1	GACTTCCAGGTCCCCGATCAGGCTGGTACTTTCTGGTATCACAGCCACTTGTGACGCA	420
<i>Lcc2</i>	GACTTCCAGTCCCAGACCAAGGCCGGTACCTTCTGGTATCACAGTCATCTGTCCACGCA	406
	* * * * *	

In the Table 3.2 gaps are inserted between the residues so that identical nucleotides are aligned in successive columns. The table was depicted to show the homology between *Lcc2* cDNA and other laccases obviously. The conservation of base pairs can indicate a similar functional or structural role. This table supports that *Lcc2* cDNA has a significant homology with other known laccases.

After this confirmation, the plasmid which bear full-length *Lcc2* sequence was used to amplify the insert to have a required amount for ligation into expression vector.

3.1.2 Cloning of *Lcc2* cDNA sequence into pSAL4 expression vector

The Top10F' *E. coli* which was verified to bear pDrive/*Lcc2* sequence was amplified by the help of streak plate method. After an o/n incubation on an LB-ampicillin plate, plasmid isolation of bacteria was performed following o/n incubation in LB-ampicillin broth.

Then digestion reactions of both pDrive/*Lcc2* and pSAL4 vectors were set up simultaneously with *EcoRI* restriction enzyme in order to reobtain the insert and linearize the expression vector. Reaction mixtures was run on gel and desired ~1.7 kb fragment and linear pSAL4 vector were gel extracted (Figure 3).

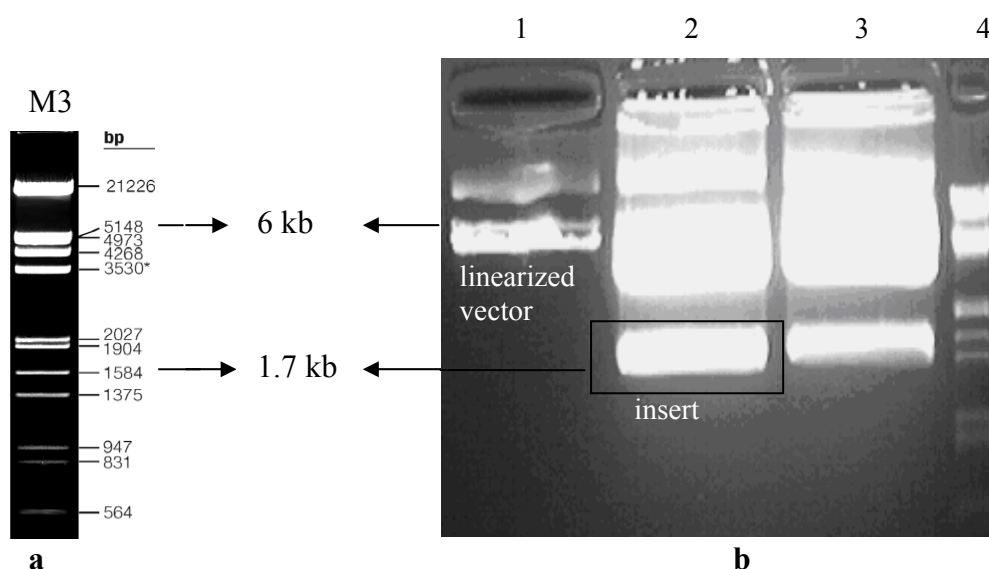


Fig 3.3: The photos of 1% agarose gel electrophoresis of digested vectors. **a.** M3. **b.** Lane 1: DNA fragment obtained from *EcoRI* digestion of pSAL4, Lane 2, 3: *Lcc2* fragments obtained from *EcoRI* digestion of pDrive/*Lcc2*, Lane 4: M3.

In Fig 3.3, the bands are highly dense after digestion reaction. pSAL4 vector is ~6 kb and this value is more than 3 times compared to the insert. The amounts of linearized vector and the insert were calculated consistent to this data for ligation reaction.

Following the extraction of the digestion products which had the same sticky ends, the *Lcc2* DNA fragment was ligated into the pSAL4 vector. The ligation product was transformed into TOP10F' electrocompetent *E. coli* cells. White colonies on LB-ampicillin plate were detected, grown in LB broth medium o/n and plasmid DNA isolation steps were performed, respectively. Then all plasmids were cut with *EcoRI* enzyme to control on gel (Figure 3.4) if they bear insert or not.

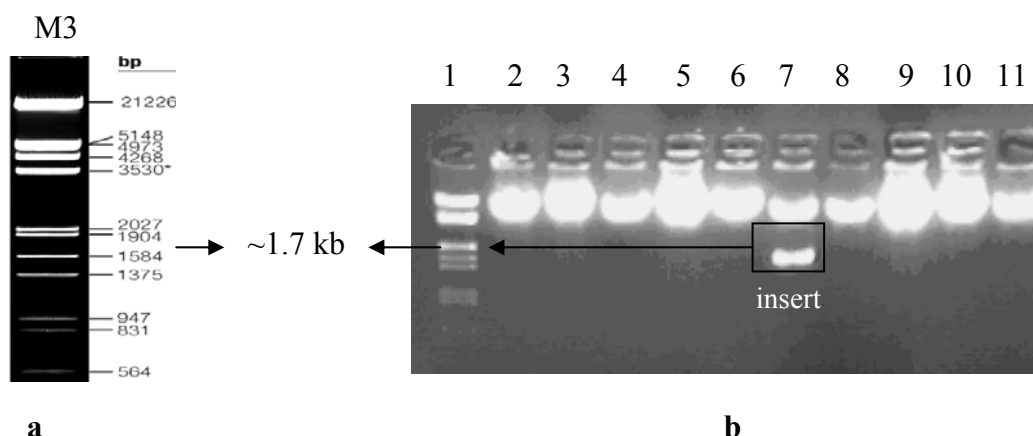


Fig 3.4: The photos of 1% agarose gel electrophoresis of digested pSAL4/*lcc2* constructs. **a.** M3. **b.** Lane 1: M3, Lane 2,3,4,5,6,8,9,10,11: pSAL4 vectors digested with *EcoRI*. Lane 7: pSAL4 vector digested with *EcoRI* and bear ~1.7 kb insert.

After gel electrophoresis the plasmid which include the desired insert was chosen for the future experiments. Previous to send for DNA sequencing, firstly sequence PCR was set up with chosen plasmid and secondly the reaction product was purified. Results of analyses confirmed that pSAL4/*Lcc2* vector was including a full-length *Lcc2* sequence in frame with the C terminal. So, with this verification, construction of pSAL4/*Lcc2* vector was successfully accomplished.

3.2 Introduction of pSAL4/*Lcc2* expression vector into the *S. cerevisiae*

After verifying the construction of the expression vector with the sequencing results, expression studies were performed. The expression vector used in this study is pSAL4 as it was mentioned before. This vector had been designed with the characteristic properties and was kindly provided by our TUBITAK-Italy joint project partner Prof. Giovanni Sannia, Università di Napoli, Italy.

pSAL4 vector has the copper-inducible promoter sequence from metallothionein gene (*CUP1*), the terminator sequence from the *CYC1* gene which is coding for cythochrome C, *URA3* gene which provides the auxotrophic selection, and the native laccase leader sequence. Also there is a unique *EcoRI* site which is used to clone laccase cDNA into pSAL4 plasmid. As protein secretion in yeast is a complex process, there is no generally considered signal sequence which directs secretion. The *EcoRI* flanked laccase encoding sequences, containing their natural signal sequences, are ligated into pSAL4 under the control of the *CUP1* promoter sequence [54]. A second important restriction enzyme for this study is *BamHI* and there is a *BamHI* site on the pSAL4 vector right before the *CUP1* promoter. So this enzyme is appropriate for digestion reactions to obtain a linearized pSAL4 vector to introduce into the *S. cerevisiae*.

Transformation of yeast cells were done with pSAL4/*Lcc2* vector which was linearized with *BamHI* before the application of lithium acetate protocol. Then the cells were spread on the selective SD medium. Plates were left upside down for 4 days at 28°C. Colonies were first apparent after 3 days on the plate surface and full development was observed by the 4th day of transformation, when colonies numbered up to 60-70 per plate (Figure 3.5).

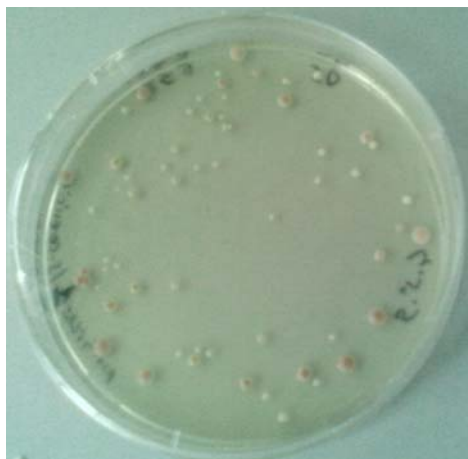


Fig 3.5: Transformed *S. cerevisiae* colonies on the 4th day of incubation.

3.3 Expression of the laccase enzyme

Expression assays were performed with both solid and broth medium, respectively. Many colonies were screened to observe enzyme expression. Experiments were repeated at least three times for the broth culture activity assays.

3.3.1 Plate activity assay

For plate activity assay, fresh SD plates were supplemented with inducer (0.6 mM CuSO_4) and substrate (0.2 mM ABTS). Then transformed yeast colonies were transferred onto these plates as ~10 colonies per a plate. Each single plate was including colonies separately patched on the surface of the agar to clearly observe the zone of extracellular enzyme around colonies. Totally 7 expression plates were used for patching all colonies and in two of these plates positive transformed colonies surrounded by a green zone were detected on the 4th day of incubation (Figure 3.6).

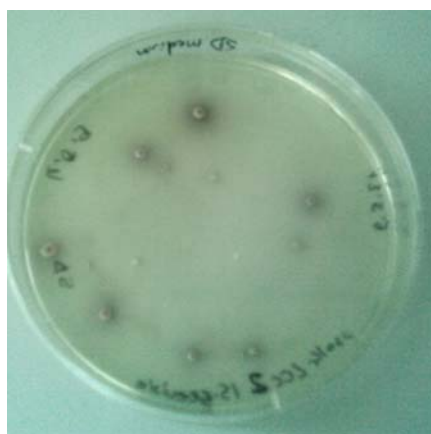


Fig 3.6: Positive *S. cerevisiae* colonies that include pSAL4/*Lcc2* constructs.

The occurrence of mentioned green zone indicated the substrate oxidation caused by expressed laccase which was directly visible because of being an extracellular enzyme.

3.3.2 Broth culture activity assay

Following the plate assay, broth culture experiments were carried out. 19 positive transformant colonies which can oxidize ABTS were grown in SD broth medium supplemented with 0.6 mM CuSO_4 for 6 days. After 3th day, OD_{600} measurements and laccase activity assays were done daily. It was observed that maximum activity was performed on the 5th day by 10th numbered colony (Figure 3.7).

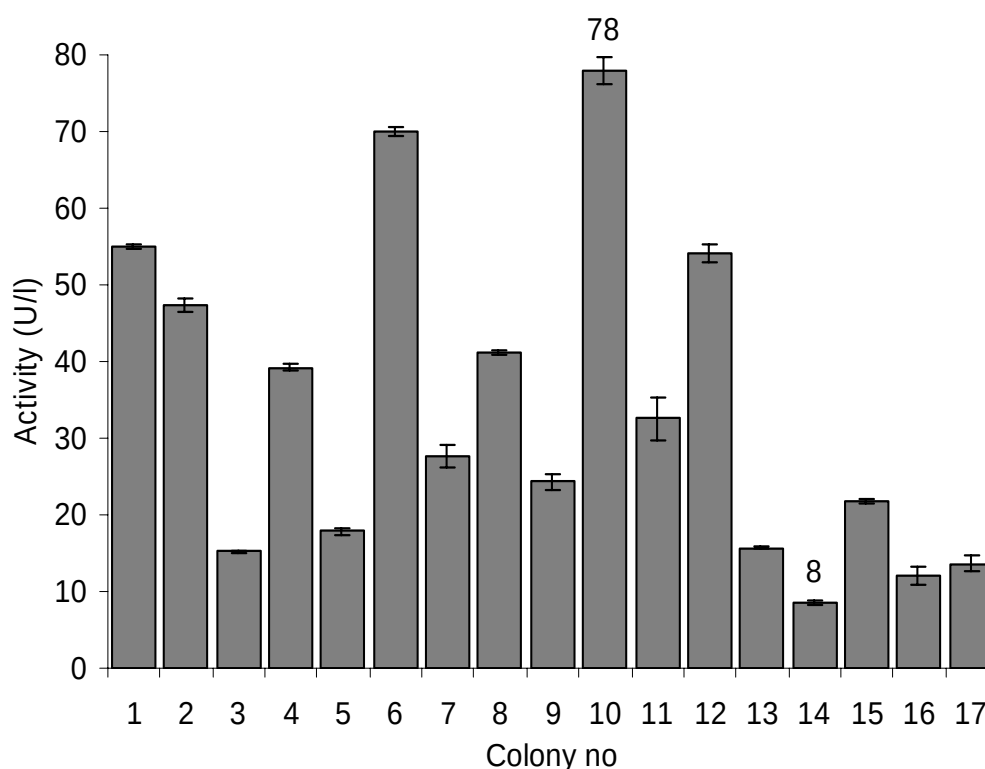


Fig 3.7: Comparison of activities of positive transformant colonies on the 5th day. The mean value of at least three experiments is shown. Bars represent $\pm$ standard deviation of triplicate experiments.

It was depicted in figure 3.7 that the highest laccase activity (78 U l^{-1}) was obtained from 10th numbered colony in contrast with 14th numbered colony which showed the lowest value (8 U l^{-1}). In the figure each bar represents the mean $\pm$ standard deviation of at least three experiments which was lower than 10% of the mean value in all the measurements.

3.4 Optimization of the laccase expression

10th colony was chosen for future optimization of expression studies. This colony was used to improve laccase production in the different growth conditions. The particular effects of nutrients and inducer was analysed for optimization. All measurements for optimization experiments were repeated at least three times.

3.4.1 CuSO₄ optimization

The promoter of expression vector, *CUP1*, is inducible by copper. Copper also works as laccase cofactor and because of this property variable inducer concentrations were tested for optimization. Selected transformant colony was grown in SD medium with different CuSO₄ concentrations for 7 days. OD₆₀₀ and activity measurements daily assayed between 4th and 7th days.

Measurements showed that the cell growth was not affected by copper as the biomass yield was almost identical at all copper concentrations (Figure 3.8). But, enzyme activities were affected. It was observed that the transformed yeast produced the highest laccase activity in the presence of 0.6 mM CuSO₄ (Figure 3.9).

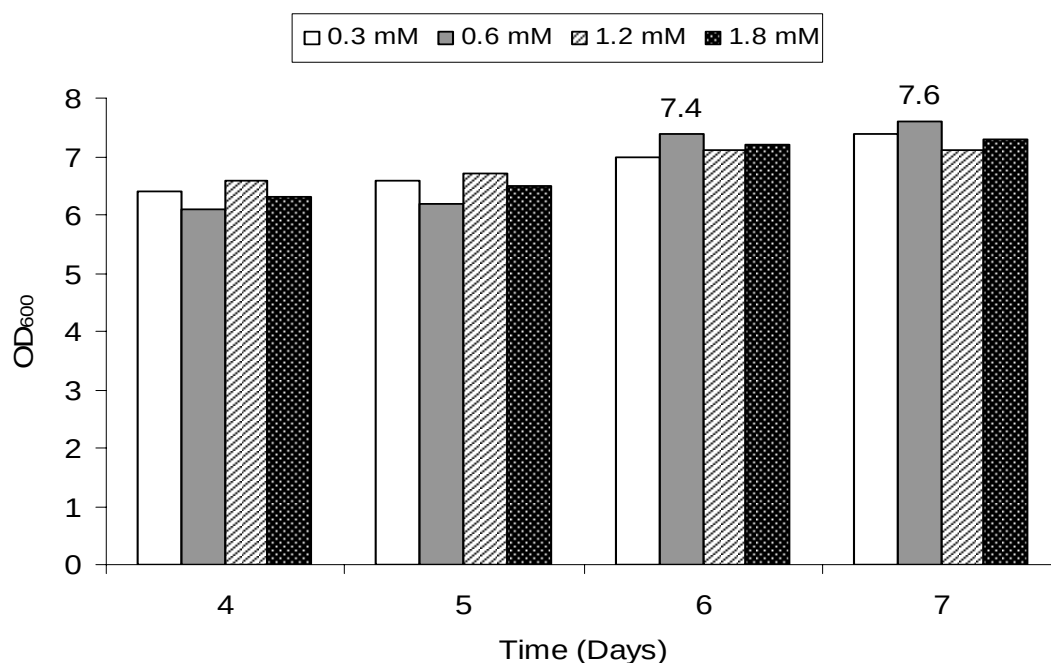


Fig 3.8: Inducer (CuSO₄) effect on biomass yield. Cultures were grown in SD medium including different copper concentration; 0.3 mM CuSO₄, 0.6 mM CuSO₄, 1.2 mM CuSO₄, 1.8 mM CuSO₄.

In fig 3.8 it is understood that on the 4th and 5th day of cell growth, the OD₆₀₀ values are very close in all copper concentrations. Although same situation is acceptable for 6th and 7th days, it was observed that the cells grown with 0.6 mM CuSO₄ had top values on these days with OD₆₀₀ 7.4 and OD₆₀₀ 7.6, respectively. So 0.6 mM CuSO₄ was selected for nutrient optimization experiments.

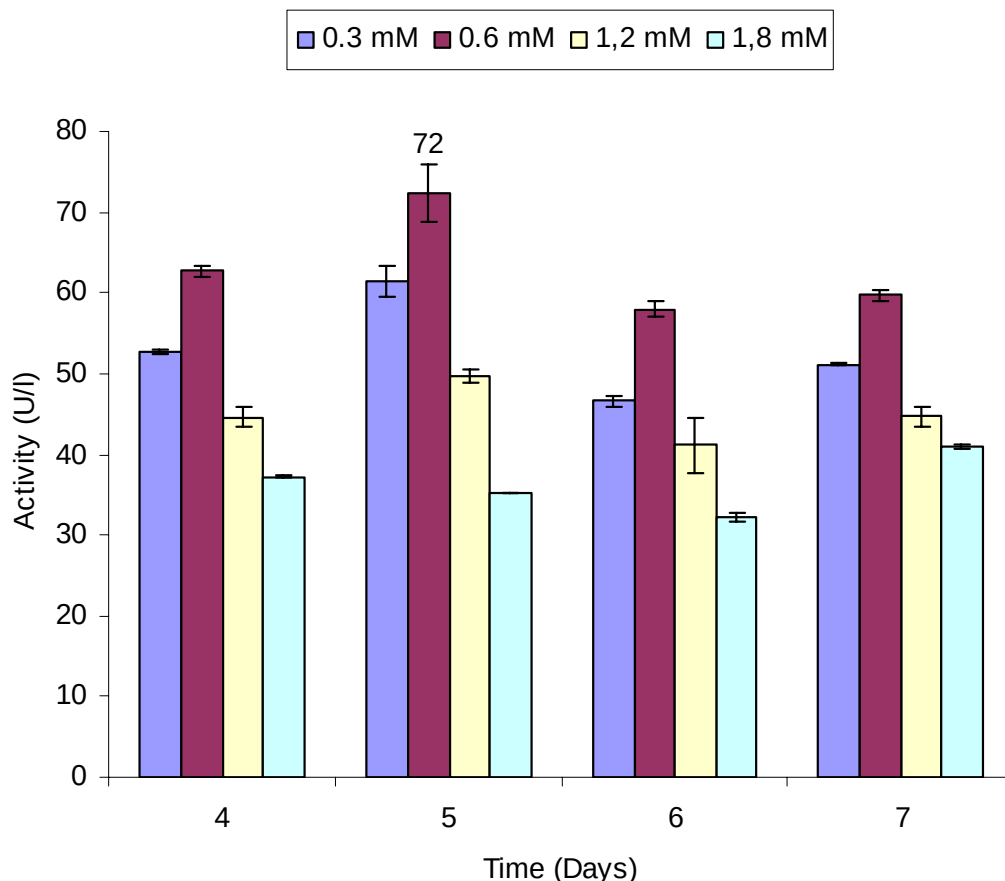


Fig 3.9: Inducer (CuSO₄) effect on laccase activity. Cultures were grown in SD medium including different copper concentration; 0.3 mM CuSO₄, 0.6 mM CuSO₄, 1.2 mM CuSO₄, 1.8 mM CuSO₄. Bars represent $\pm$ standard deviation of triplicate experiments.

It is observed from the figure 3.9 that inducer effect on laccase activity is a quite important parameter for expression studies. Especially on the 5th day of growth, the highest enzyme activity (72 U l⁻¹) was obtained from the cells in SD media supplemented with 0.6 mM CuSO₄. Activities increased as copper concentration increased but after the 0.6 mM concentration, activity values contrarily decreased. Minimum activities were observed with the 1.8 mM concentration among all days. In the figure each bar represents the mean $\pm$ standard deviation of at least three experiments and it was lower than 10% of the mean value in all the analytical assays.

3.4.2 Nutrient optimization

The influence of media composition on laccase production was then enhanced with nutrient assays. Carbon source is the primary parameter in affecting growth and consequently the laccase activity. The selected transformed colony was cultivated in different selective media including glucose or galactose in the presence of 0.6 mM CuSO_4 for 7 days. OD_{600} and activity measurements daily assayed between 4th and 7th days.

The maximum biomass yield was obtained in SG medium on the 6th day (Figure 3.10). Also the highest activity of expressed recombinant laccase was detected in SG medium on 5th day (Figure 3.11). Therefore, SG medium was chosen as the best media for recombinant laccase expression for selected colony of yeast *S. cerevisiae* with optimum galactose concentration (2%).

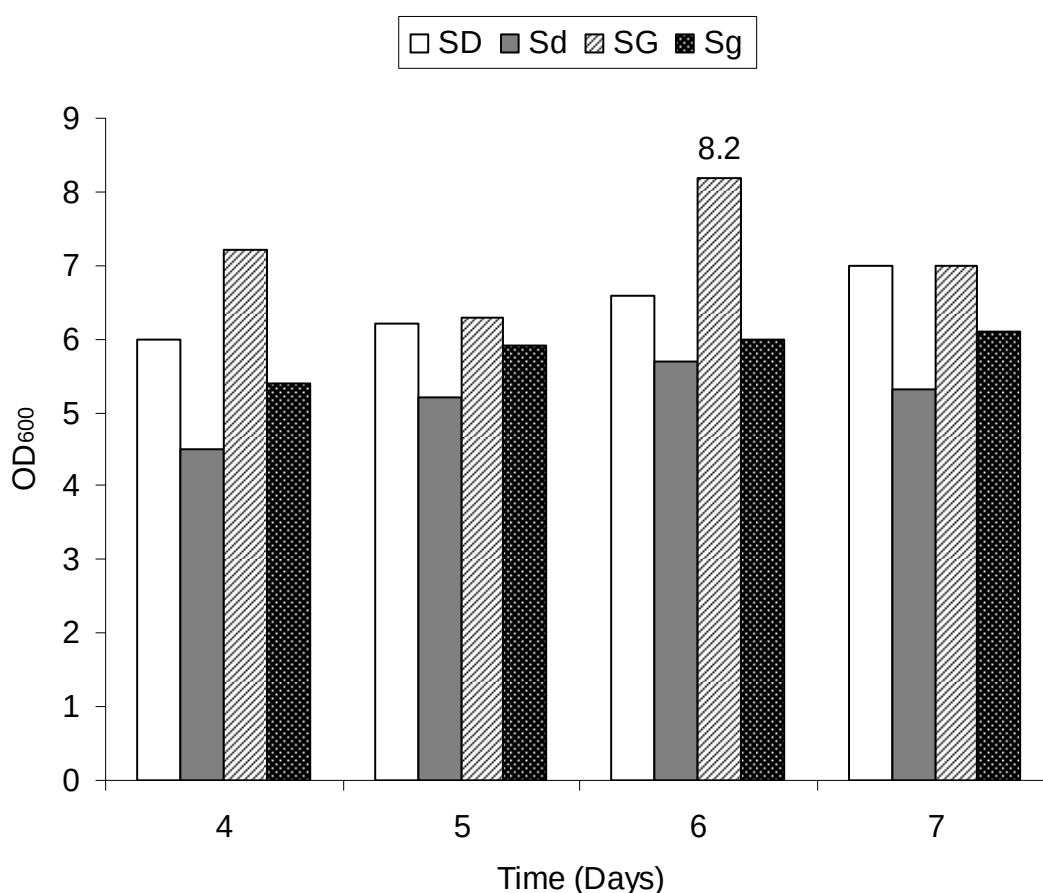


Fig 3.10: Nutrient effect on biomass yield. Cultures were grown in the presence of 0.6mM CuSO_4 , and different selective media; SD (selective medium containing 20 g/l glucose), Sd (selective medium containing 10 g/l glucose), SG (selective medium containing 20 g/l galactose), Sg (selective medium containing 10 g/l galactose).

As it was shown in figure 3.10, by the 4th day the cell density was maximum in SG medium, and also SD medium was the secondary favoured medium for the cell growth in terms of nutrient effects. On the 5th day value of cell growth in SG and SD media were fairly close to each other but OD₆₀₀ values of cells in SG medium were the highest in all daily measurements. Even though OD₆₀₀ values were getting increased in SD medium by the 7th day, they were still less than SG medium. Maximum OD₆₀₀ value (8.2) was obtained in SG medium on the 6th day.

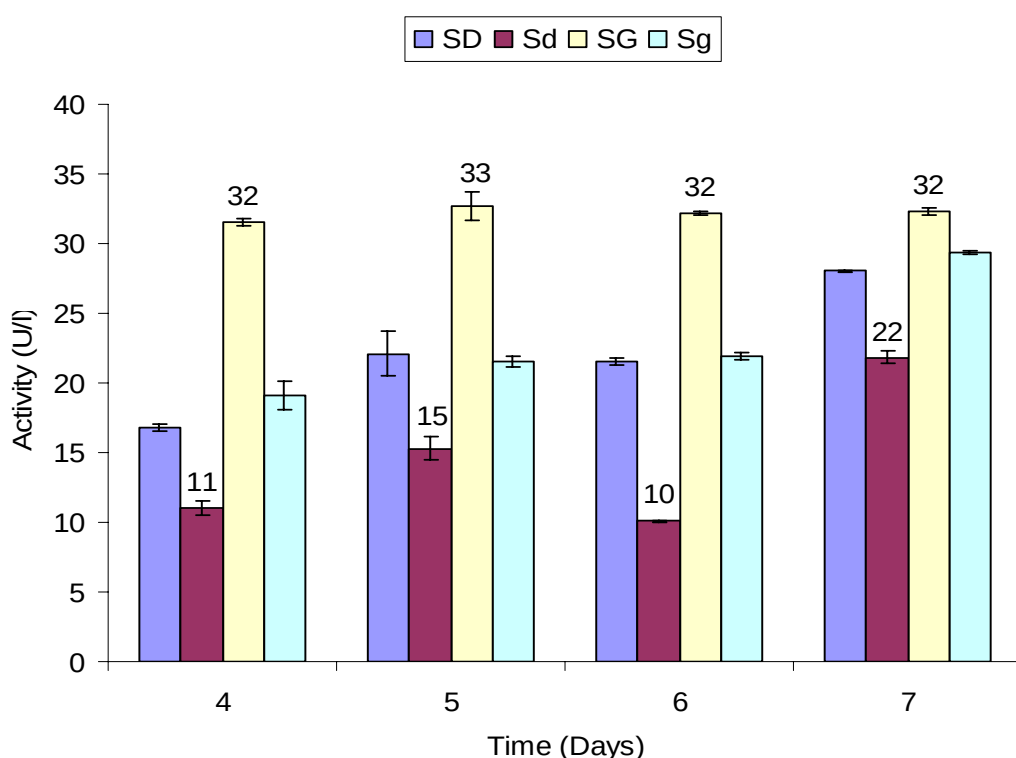


Fig 3.11: Nutrient effect on laccase activity. Cultures were grown in the presence of 0.6mM CuSO₄, and different selective media; SD (selective medium containing 20 g/l glucose), Sd (selective medium containing 10 g/l glucose), SG (selective medium containing 20 g/l galactose), Sg (selective medium containing 10 g/l galactose). Bars represent $\pm$ standard deviation of triplicate experiments.

Fig 3.11 shows that the highest laccase activity (33 U l⁻¹) was obtained on the 5th day from the cells grown in SG medium, and the lowest one (10 U l⁻¹) was obtained on the 6th day from the Sd medium. Activities were getting increased by the 7th day for the cells grown in all media except SG medium. Surprisingly the mean values for SG medium were almost same in daily measurements between 4th and 7th days. Also in the figure $\pm$ standard deviation is lower than 10% of the mean value in all the analytical assays.

Optimization studies were successfully accomplished with the production of highest yielded laccase enzyme. The selected *S. cerevisiae* colony bearing pSAL4/*Lcc2* vector was transferred to the fresh SD plate by the help of streak plate method for preservation at +4°C for the future experiments.

4. CONCLUSION

White-rot fungi secrete some enzymes and these enzymes are capable of degrading lignin. Laccase is one of the extracellular lignin degrading enzymes and it reduces methoxyphenyl derivatives to quinones as demethylating factor. More recently this enzyme is very popular because it is a natural pigment source as oxidising several non-phenolic compounds such as textile dyes. Moreover, biotechnological production of this enzyme with the existing easy and cheap cultivation techniques is very preferable compared to synthetic dyes.

The production of synthetic compounds is an applied chemical process but more than 10% of the total used these dyes are released into the environment as wastes. So there is a huge demand for non-toxic and biodegradable dyes and laccase can be an applicable fungal enzyme for dye decolorization and waste-water detoxification. However, there are some important obstacles such as toxicology tests, manufacturing costs and consumer acceptance that should be overcome for industrial production of recombinant enzymes.

In this study, it was aimed to obtain highly active heterologous laccase production. *P. sanguineus* laccase cDNA was used initially and it was transformed into the host *S. cerevisiae* strain for high yielded and easy production. Different media compositions were tested according to the literature and it was detected that recombinant *S. cerevisiae* strains show higher laccase activity when galactose (2%) is the sole carbon source in comparison to glucose. Subsequently copper existence was assayed with different amounts in SD media and it was concluded that optimum molar ratio of CuSO_4 for highest activity is 0.6 mM in comparison to 0.3 mM, 1.2 mM and 1.8 mM CuSO_4 ratios.

Thus, heterologous laccase was produced and optimized successfully via using recombinant methodology.

► **Future Studies**

- Further studies can be extended to purification and characterization of the expressed laccase enzyme.
- Rational and random mutagenesis experiments may be carried out to obtain increased enzyme activities.
- Expressed laccase can be used in different industrial applications such as decolorization or enzyme based textile dye production.

REFERENCES

- [1] **Thorn, G.** (1997). The fungi in soil. In: van Elsas, J. D., Trevors, J. T., Wellington, E. M. H., eds. *Modern Soil Microbiology*. Marcel Dekker, 63-127
- [2] **de Moura Sarquis, M. I., de Oliveria, P. C.** (1996). Diversity of microfungi in the sandy soil in Ipanema Beach. *J Basic Microbiol*, **36**, 51-58
- [3] **Gramss G.** (1997). Activity of oxidative enzymes in fungal mycelia from grassland and forest soils. *J Basic Microbiol*, **37**, 407-423.
- [4] **Bollag, J-M., Stotzky, G.** (2000). *Soil Biochemistry*. Marcel Dekker, **10**(9), 379-380.
- [5] **Kirk, T. K., Farrell, R. L.** (1987). Enzymatic “combustion”: The microbial degradation of lignin. *Annu Rev Microbiol*, **41**, 465-505.
- [6] **Christian, V., Shrivastava, R., Shukla, D., Modi, H. A., Vyas, B. R.** (2005). Degradation of xenobiotic compounds by lignin-degrading white-rot fungi: enzymology and mechanisms involved. *Indian Journal of Experimental Biology*, **43**(4), 301–12
- [7] **Chandel, A. K., Rudravaram, R., Rao, L. V., Ravindra, P., Narasu, M. L.** (2007). Industrial enzymes in bioindustrial sector development: An Indian perspective. *Journal of Commercial Biotechnology*, August, **13**(4), 283-291.
- [8] **Eriksson, K-E, Blanchette, R. A., Ander, P.** (1990). *Biodegradation of lignin*. In: Timell, T. E. (ed.): Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, 225-397.
- [9] **Lebo, S. E. J., Gargulak, J. D., McNally, T. J.** (2001). *Lignin*. Kirk-Othmer Encyclopedia of Chemical Technology, 4th Edition. John Wiley & Sons, Inc.
- [10] **Martone, P. T., Estevez, J. M., Lu, F., Ruel, K., Denny, M. W., Somerville, C., Ralph, J.** (2009). Discovery of Lignin in Seaweed Reveals Convergent Evolution of Cell-Wall Architecture. *Current Biology*, **19**(2), 169-175.
- [11] **Carlile, M. J., Watkinson, S. C.** (1994). *The Fungi*, 482. London etc., Academic Press.
- [12] **Leonowicz, A., Choi, N-S., Luterek, J., Wilkolazka, A., Wojtas-Wasilewska, M., Matuszewska, A., Hofichter, M., Wesenberg, D., Rogalski, J.** (2001). Fungal laccase: properties and activity on lignin. *J. Basic Microbiol* **41**(3–4), 185–227
- [13] **Leonowicz, A., Rogalski, J., Szczodrak, J., Fiedurek, J.** (1986). The possible key role of glucose oxidase in transformation of lignocellulose. In: *Proc. 3rd Int. Conf. Biotechnol. Pulp Paper Ind.*, 160–162.

- [14] **Rogalski, J.** (1986). Cooperation of selected hydrolases and phenol oxidases in the degradation of ligninocellulose. Ph. D. thesis, Maria Curie-Skłodowska University, Lublin, 1–215.
- [15] **Husain, Q.** (2006). Potential Applications of the Oxidoreductive Enzymes in the Decolorization and Detoxification of Textile and Other Synthetic Dyes from Polluted Water: A Review. *Critical Reviews in Biotechnology*, **26**, 201–221.
- [16] **Eriksson, K-E.** (1993). Concluding remarks: Where do we stand and where are we going? Lignin biodegradation and practical utilization. *J. Biotechnol*, **30**, 149–158.
- [17] **Url-1**
<<http://www.forestry.sarawak.gov.my/forweb/wildlife/sanc/lanjak/psangu.htm>>, accessed at 23.10.2009.
- [18] **Lu, L., Zhao, M., Zhang, B., Yu, S., Bian, X., Wang, W., Wang, Y.** (2007). Purification and characterization of laccase from *Pycnoporus sanguineus* and decolorization of an anthraquinone dye. *Appl Microbiol Biotechnol*, **74**, 1232–1239.
- [19] **Dubé, E., Shareck, F., Hurtubise, Y., Daneault, C., Beauregard, M.** (2008). Homologous cloning, expression, and characterisation of a laccase from *Streptomyces coelicolor* and enzymatic decolourisation of an indigo dye. *Appl Microbiol Biotechnol*, **79**, 597–603.
- [20] **Necochea, R., Valderrama, B., Di'az-Sandoval, S., Folch-Mallol, J. L., Va'zquez-Duhalt, R., Iturriaga, G.** (2005). Phylogenetic and biochemical characterisation of a recombinant laccase from *Trametes versicolor*. *FEMS Microbiology Letters*, **244**, 235–241.
- [21] **Galhaup, C., Haltrich, D.** (2001). Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Appl Microbiol Biotechnol*, **56**, 225–232.
- [22] **Kallio, J. P., Auer, S., Jänis, J., Andberg, M., Kruus, K., Rouvinen, J., Koivula, A., Hakulinen, N.** (2009). Structure–Function Studies of a *Melanocarpus albomyces* Laccase Suggest a Pathway for Oxidation of Phenolic Compounds. *J. Mol. Biol*, **392**, 895–909.
- [23] **Sigoillot, C., Record, E., Belle, V., Robert, J. L., Levasseur, A., Punt, P. J., van den Hondel, C. A. M. J. J., Fournel, A., Sigoillot, J. C., Asther, M.** (2004). Natural and recombinant fungal laccases for paper pulp bleaching. *Appl Microbiol Biotechnol*, **64**, 346–352.
- [24] **Grogan, G.** (2009). Biotransformations. *Annu. Rep. Prog. Chem, Sect. B*, **105**, 206–231.
- [25] **Johannes, C., Majcherczyk, A.** (2000). Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. *Applied and Environmental Microbiology*, **66**(2), 524–528.
- [26] **Vallejoa, O. V., Palomaresb, L. A., González, E. D., Castro, H. G. A., Anayaa, C. M. A., Valderramab, B., Mallola, J. F.** (2009). The role of *N*-glycosylation on the enzymatic activity of a *Pycnoporus sanguineus*. *Laccase Enzyme and Microbial Technology*, **45**, 233–239.

- [27] **Petty, H. R.** (1993). *Molecular biology of membranes : structure and function*. Plenum Press, 52-54.
- [28] **Jeffery C. J.**, (2003). Moonlighting proteins: old proteins learning new tricks. *Trends Genet*, **19**, 415–417.
- [29] **Lindell D., Jaffe J. D, Johnson Z. I, Church G. M., Chisholm S. W.** (2005). Photosynthesis genes in marine viruses yield proteins during host infection. *Nature*, **438**(3), 86–89.
- [30] **Sharma, K. K., Kuhad, R. C.** (2008). Laccase: enzyme revisited and function redefined. *Indian J. Microbiol*, September, **48**, 309–316.
- [31] **Baldrian, P.** (2006). Fungal laccases-occurrence and properties. *FEMS Microbiol Rev*, **30**, 215-242.
- [32] **Gianfreda, L., Xu, F., Bollag. J.** (1999). Laccases: A useful Group of Oxidoreductive Enzymes. *Bioremediation J*, **3**, 4-25.
- [33] **Madzak, C., Otterbein, L., Chamkha, M., Moukha, S., Asther, M., Gaillardin, C., Beckerich, J. M.** (2005). Heterologous production of a laccase from the basidiomycete *Pycnoporus cinnabarinus* in the dimorphic yeast *Yarrowia lipolytica*. *FEMS Yeast Research*, **5**, 635–646.
- [34] **Ranieri, D., Colao, M. C., Ruzzi, M., Romagnoli, G., Bianchi, M. M.** (2009). Optimization of recombinant fungal laccase production with strains of the yeast *Kluyveromyces lactis* from the pyruvate decarboxylase. *FEMS Yeast Res.*, **9**, 892-902.
- [35] **Bleve, G., Lezzi, C., Mita, G., Rampino, P., Perrotta, C., Villanova, L., Grieco, F.** (2008). Molecular cloning and heterologous expression of a laccase gene from *Pleurotus eryngii* in free and immobilized *Saccharomyces cerevisiae* cells. *Appl Microbiol Biotechnol*, **79**, 731–741.
- [36] **Kiiskinen, L-L., Saloheimo, M.** (2004). Molecular Cloning and Expression in *Saccharomyces cerevisiae* of a Laccase Gene from the Ascomycete *Melanocarpus albomyces*”, *Applied and Environmental Microbiology*, January, 137–144.
- [37] **Piscitelli, A., Giardina, P., Mazzoni, C., Sannia, G.** (2005). Recombinant expression of *Pleurotus ostreatus* laccases in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*, **69**, 428–439.
- [38] **Alves-Rodrigues, I., Galˆao, R. P., Meyerhans, A., D’iez, J.** (2006). *Saccharomyces cerevisiae*: A useful model host to study. *Virus Research*, **120**, 49–56
- [39] **Goffeau A, Barrell B. G., Bussey H, Davis R. W., Dujon B, Feldmann H, Galibert F, Hoheisel J. D., Jacq C, Johnston M, Louis E. J., Mewes H. W., Murakami Y, Philippsen P, Tettelin H, Oliver S. G.** (Oct 1996). Life with 6000 genes. *Science*, **274**(5287), 546, 563–567.
- [40] **Stolc, V., Reinsch, S.** (2007). *Saccharomyces Cerevisiae Yeast Strain. National Aeronautics and Space Administration. Url-2*

<http://www.nasa.gov/centers/ames/news/releases/2004/yeast/yeast_prt.htm>, accessed 28.Oct.2009.

- [41] **Kumar, A., Cheung, K-H., Ross-Macdonald, P., Coelho, P. S. R., Miller, P., Snyder, M.** (2000). Triples: a database of gene function in *Saccharomyces cerevisiae*. *Nucleic Acids Research*, **28**(1), 81-84
- [42] **Buckholz, R. G., Gleeson, M. A. G.** (1991). Yeast systems for the commercial production of heterologous proteins. *Bio/Technology*, **9**, 1067-1072.
- [43] **Soccol, C. R., Vandenberghe, P. S., Woiciechowski, A. L., Babitha, S.** (2006). Applications of industrial enzymes. in Pandey, A., Webb, C., Soccol, C. R., Larroche, C. *Enzyme technology*. Springer, 533-536.
- [44] **Sharma, R., Katoch, M., Srivastava, P. S., Qazi, G. N.** (2009). Approaches for refining heterologous protein production in filamentous fungi. *World J Microbiol Biotechnol*.
- [45] **O'Callaghan, J., O'Brien, M. M., McClean, K., Dobson, A. D. W.** (2002). Optimisation of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris*. *Journal of Industrial Microbiology & Biotechnology*, **29**, 55–59.
- [46] **Munusamy, U., Sabaratnam, V., Muniandy, S., Abdullah, N., Pandey, A., Jones, E. B. G.** (2008). Characterisation of laccase from *Pycnoporus sanguineus* KUM 60953 and KUM 60954. *Journal of Biological Sciences*, **8**(5), 866-873.
- [47] **Lomascolo, A., Record, E., Herpoe'l-Gimbert, I., Delattre1, M., Robert, J. L., Georis, J., Dauvrin, T., Sigoillot, J-C., Asther, M.** (2003). Overproduction of laccase by a monokaryotic strain of *Pycnoporus cinnabarinus* using ethanol as inducer. *Journal of Applied Microbiology*, **94**, 618–624.
- [48] **Robinson, T., McMullan, G., Marchant, R., Nigam, P.** (2001). Remediation of dyes in textile effluents: A critical review on current treatment technologies with a proposed alternative. *Biores. Technol*, **77**, 247–255.
- [49] **Xu, Y., Lebrun, R. E.** (1999). Treatment of textile plant effluent by nanofiltration membrane. *Separ. Sci. Technol*, **34**, 2501–2519.
- [50] **Koch, M., Yediler, A., Lienert, D., Insel, G., Kettrup, A.** (2002). Ozonation of hydrolyzed azo dye reactive yellow 84 (CI). *Chemosphere*, **46**(1), 109–113.
- [51] **Yang, Y. Q., Wyatt, D. T., Bahorsky, M.** (1998). Decolorization of dyes using UV/H₂O₂ photochemical oxidation. *Text. Chem. Color*, **30**(4), 27–35.
- [52] **Coute, S. R., Sanroman, D. A.** (2002). Photocatalytic degradation of dyes in aqueous solution operating in a fluidized bed reactor. *Chemosphere*, **46**, 83–86.
- [53] **Karcher, S., Kornmuller, A., Jekel, M.** (1999). Removal of reactive dyes by sorption/complexion with cucurbituril. *Water Sci. Technol*, **40**, 425–433.

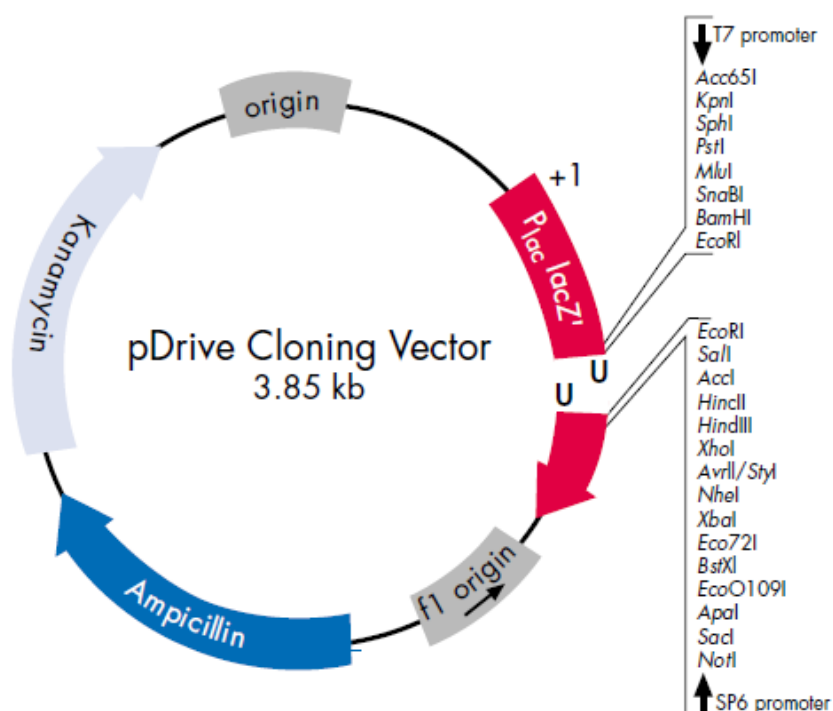
- [54] **Bhunia, A., Durani, S., Wangikar, P. P.** (2001). Horseradish peroxidase catalyzed degradation of industrially important dyes. *Biotechnol. dye Bioeng*, **72**, 562–567.
- [55] **Pelegri, R., Peralta-Zamora, P., de Andrade, A. R., Reyes, J., Duran, N.** (1999). Electrochemically assisted photocatalytic degradation of reactive dyes. *Appl. Catal. B: Environ*, **22**, 83–90.
- [56] **Nigam, P., Armour, G., Banat, I. M., Singh, D., Marchant, R.** (2000). Physical removal of textile dyes and solid state fermentation of dye adsorbed agricultural residue. *Biores. Technol*, **72**, 219–226.
- [57] **Mishra, G., Tripathy, M.** (1993). A critical review of the treatments for decolorization of textile effluent. *Colourage*, **40**, 35–38.
- [58] **Keharia, H., Madamvar, D.** (2003). Bioremediation concept for treatment of containing wastewater: A review. *I. J. Exp. Biol*, **41**, 1068–1075.
- [59] **Liu, S-S., Liang, T-T.** (2004). Return sludge employed in enhancement of color removal in the integrally industrial wastewater treatment plant. *Water Res*, **38**, 103–110.
- [60] **Gahr, F., Harmanutz, F., Opperman, W.** (1994). Ozonation-an important technique to comply with new German law for textile wastewater treatment. *Water Sci. Technol*, **30**, 255–263.
- [61] **Tauber, M. M., Guebitz, G. M., Rehorek, A.** (2005). Degradation of azo dyes by laccase and ultrasound treatment. *Appl. Environ. Microbio*, **71**, 2600–2607.
- [62] **Piscitelli, A.** (2005). Recombinant expression of fungal oxidases for industrial application, PhD thesis, Università di Napoli Federico II & Université Paul Cézanne Aix-Marseille III, Scienze Biotechnologiche. **Url-3** <<http://de.scientificcommons.org/22102835>>, accessed 05.Nov.2009.
- [63] **Eggert, C., Temp U., Dean, J. F. D., Errikson, K. E. L.** (1996). A fungal metabolite mediates the degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Letters*, **391**, 144–148.
- [64] **Roby J. F., White B. J.** (1990). *Biochemical techniques: theory and practice*. Waveland Press Inc, 291–320
- [65] **Altschul S. F., Gish W, Miller W, Myers E. W., Lipman D. J.** (1990). Basic local alignment search tool. *J Mol Biol*, **215**(3), 403–410.
- [66] **Url-4** <<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>>, accessed 08.Nov.2009.
- [67] **Wang L, Jiang T.** (1994). On the complexity of multiple sequence alignment. *J Comput Biol*, **1**, 337–48.
- [68] **Url-5** <<http://www.ebi.ac.uk/Tools/clustalw2/index.html>>, accessed 25.Dec.2009.

APPENDICES

Appendix A.

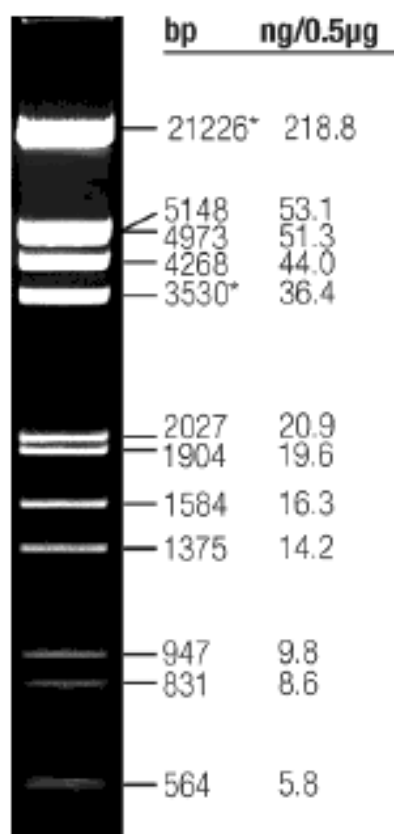
Vector Map of pDrive

<u>Element</u>	<u>Position (bp)</u>
Multiple cloning site	266–393
LacZ α -peptide	216–593
T7 RNA polymerase promoter	239–258
T7 transcription start	256
SP6 RNA polymerase promoter	398–417
SP6 transcription start	400
Ampicillin resistance gene	1175–2032
Kanamycin resistance gene	2181–2993
pUC origin	3668
Phage f1 origin	588–1043



Appendix B.

Molecular Weight Marker



Lambda (λ) DNA/*EcoRI*+*HindIII* digested marker-3, 1.0% agarose, 0.5µg lane, 8 cm length gel, 1X TAE, 17V/cm.

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