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M.S. Thesis of Genetics and Bioengineering

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MOLECULER CLONING, EXPRESSION AND PURIFICATION OF LACCASE ENZYME FROM *SHEWANELLA PUTREFACIENS*

by

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Zeynep AYDIN

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

_____

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January 2012

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Supervisor: Assist. Prof. Dr. Fahri AKBAŞ

ABSTRACT

Shewanella putrefaciens is a Gram-negative bacterium. *S. putrefaciens* is also a facultative anaerobe with the ability to reduce iron and manganese metabolically; that is, it can use iron and manganese as the terminal electron acceptor in the electron transport chain in both solid and liquid media, *S. putrefaciens* is often recognizable by its bright pink color. They are resistant to changeable environmental conditions. Laccases are also found to be widespread among bacteria, based on homology searches in protein databases and bacterial genomes. Laccase genes have been cloned from fungi and bacteria and then have been searched enzyme activity. In this study, we preferred *S. putrepaciens* ATCC 8071 because its laccase enzyme was not characterized before. Laccase genes in Shawenalla putrefaciens were transferred into *E. coli* with molecular cloning techniques. Also, laccase enzyme was purified and characterized.

Keywords: *Shewenalla putrefaciens,* laccase, cloning, gene expression.

SHEWANELLA PUTRAFACİENS' DEN LAKKAZ ENZİMİNİN MOLEKÜLER KLONLANMASI, GENİN İFADE EDİLMESİ VE SAFLAŞTIRILIMASI

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ÖZ

Shawenalla putrefaciens topraktan ve denizden izole edilebilen, lakkaz aktivitesi içeren gram negatif bir bakteridir. Metabolik olarak manganez kullanırlar ve demiri indirgeyebilen fakültatif anerobtur. Hem katı hem de sıvı ortamda parlak pembe renkleriyle ayrılırlar. Ortam koşullarının değişmesine çok dayanıklı bir mikroorganizmadır. Lakkazların mantar ve gelişmiş bitkilerin yani sıra çoğu bakteri türünde de yaygın olarak bulunduğu, homolojik olarak yapılan protein bilgi bankası ve bakteri genom araştırmaları ile tespit edilmiştir. Lakkaz geni hem fungal sistemlerden hem de çeşitli bakterilerden klonlanıp fonksiyonel analizleri yapılmıştır. Bu çalışmada, daha önce lakkaz geninin varlığı bilinen ancak karakterizasyonu yapılmamış olan *S. Putrefaciens* ATCC 8071 seçildi. *S. putrefaciens*'teki lakkaz geni indüklenebilir bir ekspresyon vektörüne klonlandı ve *E.coli bakterisine* aktarıldı. Aynı zamanda, lakkaz enzimi saflaştırılarak karakterizasyonu yapıldı.

Anahtar Kelimeler: *Shewenalla putrefaciens*, lakkaz, klonlama, genin ifade edilmesi.

DEDICATION

This thesis is dedicated to

my father Niyazi, my mother Fatma, my uncle Fevzi, my sister Merve

and my engaged Deniz

for their endless love and support.

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LIST OF SYMBOLS AND ABBREVATIONS

DOE	Department of Energy
S. putrefaciens	*Shewanella putrefaciens*
ATCC	American Type Culture Collection
E. Coli	Escherichia
GC	Guanine plus Cytosine
DNA	Deoxyribonucleicacid
rRNA	ribozomalribonucleicacid
DMSO	Dimethyl sulfoxide
CDC	Centers for Disease Control and Prevention
MFCs	Microbial fuel cells
EDTA	Ethylenediamminetetraacetic acid
MCOs	Multicopper oxidases
EPR	Electron Paramagnetic Resonance
UV	Ultraviolet
AIDS	Acquired Immune Deficiency Syndrome
LMCO	Like Multicopper oxidases
SDS-PAGE	Sodium Dodecyl Suphate-Polyacrylamide Gel-Electrophoresis
ABTS	2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
HAA	3-hydroxyanthranilic acid
HBT	N-hydroxybenzotriazole
HPI	N-hydroxyphtaimide
VLA	Violuric acid
GRAS	Generally Recognized as Safe
SLAC	Streptomyces Coelicolor Laccase
PPO	potato laccase
LCCI	London Chamber of Commerce and Industry
EMS	Ethyl methane sulfonate-based

BCBD	Blue Copper Binding Domain
PCBs	Polychlorinated biphenyls
PAHs	Polyhydroxyalkonoates
HO-PCB	hydroxyl-polychlorinated biphenyl
CBM	Carbohydrate binding module
HIV	Human Immuno Defiency Virus
APS	Ammonium persulphate
BSA	Bovine serum albumine
TEMED	Tetramethylethylenediammine
LB	Luria- Bertani
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
TAE	Trisacetic acid-Ethylenediammineaceticacid
PCR	Polymerase Chain Reaction
CIAP	Calf intestinal alkaline phosphatase
dNTPs	Deoxydiribonucleotide triphosphate
Blast	Basis Local Alignment Search Tool

CHAPTER 1

INTRODUCTION

Genus *Shewanella* are a type of gram-negative, facultative anaerobic bacteria mostly found in aquatic and marine environments and can often be isolated from spoiling fish. Usually, Shewanella are known to be the members of the γ-subclass of the Proteobacteria that are gram-negative rods, 0.4 – 0.7 μm in diameter, 2–3 μm in length, and motile by a single polar flagellum [1]. Many types of Shewanella are grown in the laboratory with general growth media following enhancement from environmental samples [2] with a range of salt concentrations, temperatures, and barometric pressures and have distinct roles from food spoilage organisms to symbionts, epibionts, and opportunistic pathogens [1]. In the absence of oxygen, Shewanella is able to carry out anaerobic respiration by using a broad range of final electron acceptors. This feature not only enables the members of the genus Shewanella to survive in various environments with the capacity of growing naturally almost anywhere, but also to be utilized for bioremediation of contaminated environments by reducing some certain metals and compounds in an altered state [4]. For example, Shewanella can convert uranium dissolved in contaminated groundwater to a form unable to dissolve in water, so that the uranium will not spread as the groundwater flows. Hence, areas such as DOE sites contaminated during the manufacture of nuclear weapons can be cleaned and confined. Shewanella has benefits that make it easier for researchers to study. Escherichia and Shewanella are well known to be related. Tools and techniques developed since 1970s for Escherichia works with Shewanella, which can tolerate oxygen a useful ability that makes it easier to work with in the laboratory and is missing in other groups of metal metabolizing bacteria.

S. putrefaciens is also a facultative anaerobe with the ability to reduce iron and manganese metabolically; that is, it can use iron and manganese as the terminal electron acceptor in the electron transport chain in both solid and liquid media, *S. putrefaciens* is

often recognizable by its bright pink color. They are resistant to changeable environmental conditions. Laccase in *S. putrefaciens* likely to be resistant to hard conditions [5-8].

Laccase catalyze reduction of molecular oxygen to water with the oxidation of a wide variety of phenolic and nonphenolic compounds. They are considered to be industrially relevant enzymes for a variety of applications, including decolorization of different types of recalcitrant dyes, bioremediation of soils and water, and kraft pulp biobleaching, the synthesis of natural products like pigments and antioxidants through dimerization of phenolic and nonphenolic acids, as well as in other biotechnological applications. Laccases are also used as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics. Recently, the utility of laccases has also been applied to nanobiotechnology [5-8].

Laccase is widely distributed in higher plants, fungi and has been found also in insects and bacteria. Currently, laccases are also found to be widespread among bacteria, based on homology searches in protein databases and bacterial genomes. Laccase genes have been cloned from fungi and bacteria and then have been searched enzyme activity. Bacterial laccases may have advantageous properties compared to classical fungi laccases. The highly efficient expression of fungal laccases is often much more difficult than that of bacterial enzymes [5-8].

In this study, we preferred S. putrepaciens ATCC 8071 because of its laccase enzyme have not been characterized before. Laccase genes in Shawenalla putrefaciens were transferred into E. coli with molecular cloning techniques. Also, laccase enzyme was purified and characterized.

CHAPTER 2

2.1 SHEWANELLA AS A VERSATILE ORGANISM

2.1.1 A Brief History of The Isolation of *Shewanella*

The isolation of the first Shewanella was performed in 1931 as one of various contaminating microorganisms in charge of butter putrefaction [9]. Derby and Hammer were hesitant to identify the microorganism as *Achromobacter putrefaciens* [9], although the taxon was renamed to Pseudomonas (*Pseudomonas putrefaciens*) following further growth and biochemical characterizations in 1941 [10]. In 1960, Shewan *et al.* suggested a classification scheme [11] based on the oxidase positive reaction and motility by means of polar flagella reinforced reclassification into the genus Pseudomonas. On the other hand, Baumann and coworkers introduced a new scheme in 1972 primarily based on moles percent guanine plus cytosine (mol % GC) content of DNA. A numerous strains of nonfermentative marine bacteria previously classified as Pseudomonas was placed into a newly created genus, Alteromonas [12].

The type species isolated in 1931 [9] was changed from *Pseudomonas putrefaciens* to *Alteromonas putrefaciens* [13] in 1977. Finally, based on 5S rRNA sequence data, MacDonell and Colwell [14] proposed reclassification into a new genus, Shewanella, in honor of the late Dr. James M. Shewan and in recognition of his contributions to fisheries microbiology. In 1985, *Shewanella putrefaciens* was born, and no more reclassifications at the genus level have been made up to date. Approximately 40 species are assigned to the genus Shewanella based primarily on DNA at present: DNA hybridization and 16S rRNA sequences.

2.1.2 Species Characterization

Although the first Shewanella were characterized based on phenotypic characteristics like morphology, standard biochemical reactions, gram stain, and growth at different physiological conditions, molecular analyses have enabled evaluation, comparison, and classification of phenotypically distinct bacteria into the genus Shewanella based primarily on DNA hybridization and 16S rRNA gene sequences. Thus, several phenotypically distinct bacteria have been reclassified under the genus Shewanella, and existing Shewanella species have been assigned new species names or better characterized. Phylogenetic associations based on 16S rRNA gene sequences are presented in Figure 2.1 for well-characterized type strains of Shewanella. Most new Shewanella strains were then classified as *S. putrefaciens* despite indications that the group was more diverse [15]. The study of Owen and coworkers [15] showed that the species *S. putrefaciens* included at least four clearly different DNA homology groups (I-IV) based on DNA-DNA hybridization experiments that included several *P. putrefaciens* strains. While the number of species increases, the phenotypic characteristics of the Shewanella are as diverse as the environments in which they live [1,2]. The distribution of Shewanella on the earth appears to be rooted in two basic physiological observations: (*a*) their incomparable ability to respire compounds found in the environment and (*b*) their ability to survive at low temperatures.

Some groups of Shewanella are grouped together (*different colors*) that were isolated from similar environments. For instance, several fish intestinal isolates cluster together (*S. pneumatophori, S. schlegeliana, S. marinintestina,* and *S. sairae*). Nevertheless, other examples such as the clustering of the butter surface taint isolate *S. putrefaciens* with the deep-ocean sediment isolate *S. profunda* indicate that 16S rRNA gene comparisons have restricted predictive power in terms of ecology. *S. abyssi* was not taken into account in this analysis because of some difficulties determining the appropriate 16S rRNA sequence for this type strain [16]. Sequences were trimmed to ~1200 bp to smooth the progress of making comparisons between species with incomplete 16S rRNA gene sequences.

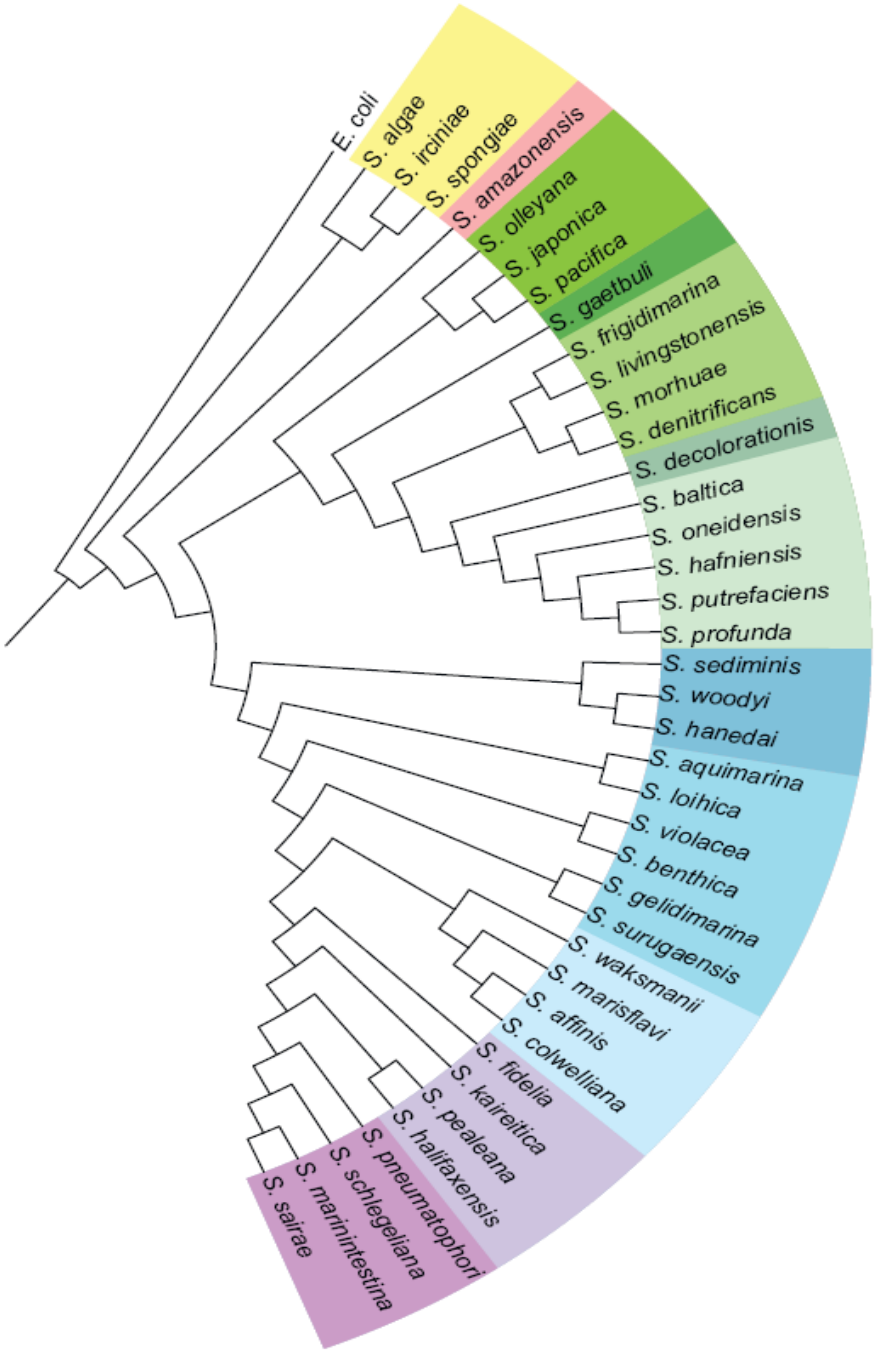

Figure 2.1 Phylogenetic analysis of *Shewanella* type strains.

2.1.2.1 Respiratory Diversity

As a genus, Shewanella are the most diverse respiratory organisms described so far. There are roughly twenty inorganic and organic compounds that can be respired by Shewanella and these have several insoluble metals and toxic elements. A partial list are as follows:

Trimethylamine-*N*-oxide (TMAO) → Trimethylamine (Me_3N),

Fe (III) Chelate and Fe (III) Oxide → Soluble Fe (II),

Mn (III and IV) Chelates and Mn (III and IV) oxides → Soluble Mn (II),

Sulfur/polysulfide → H_2S,

Sulfite → H_2S,

Thiosulfate→ H_2S,

Dimethyl sulfoxide (DMSO) → Dimethylsulfide,

Arsenate → Arsenite, Fumarate, and Succinate.

A complete list and vivid illustrations can be found in the literature [2]. For some compounds reduction has been exhibited but growth has not (e.g., selenite). The respiratory diversity of Shewanella is one of their greatest benefits in terms of survival in the environment. The fact that all isolates seem to be facultative anaerobes and the anaerobic electron acceptors are various, suggests these organisms are normally localized in both oxic and anoxic environments. Oxygen can be limiting in sediments, in intestinal tracts of higher organisms, and in organic-rich flocculates such as marine snow and fecal pellets. Some aquatic systems are permanently or temporarily stratified, allowing the formation of large anoxic zones. The respiratory diversity of Shewanella allows them to breath almost anywhere. In anoxic environments, Shewanella are likely to respire one if not several compounds. In organic-rich flocculates the electron acceptor may be DMSO. In sedimentary environments, the electron acceptors may be insoluble iron or manganese oxide minerals. The mechanism of anaerobic respiration in these organisms is implicit at the genetic level for some compounds (Fumarate, DMSO, As, V, Fe, and Mn). Many of these compounds are reduced by terminal reductases located outside of the cell, and reviews have recently concentrated on the molecular details of this process [17,18].

2.1.2.2 Low- Temperature Growth

Growth at low temperatures (4^{o}C) appears to be a hallmark of the Shewanella genus. Most Shewanella strains are psychrotolerant, meaning that they have the capacity to grow at low temperatures (<5^{o}C), but their optimum temperature is above 16^{o}C. Though some isolates do not demonstrate robust growth at low temperatures, many behave as true psychrophiles with temperature optima below 16^{o}C [1,19,20]. The capacity to thrive at low temperatures gives these organisms an advantage in permanently cold environments such as the ocean and the Polar Regions. This ability is also useful in environments with large temperature fluctuations. For example, the temperature of Oneida Lake drops dramatically in the winter months, with the lake freezing over completely for several months [21]. The physiological changes that take place to allow low-temperature growth in the Shewanella are unknown.

2.1.3 Ecology

2.1.3.1 Syntrophy

The diverse distribution of Shewanella is explained by their potential to build syntrophic affiliations with fermentative microbes or those microbes that can use Shewanella's by-products. Most Shewanella strains are nonfermenters, but colonization with fermenters would allow them to employ products of fermentation (lactate, formate, hydrogen, and some amino acids) for anaerobic respiration. By-products produced during anaerobic respiration such as acetate, ammonia, and alanine could be further consumed by acetogens, methanogens, organotrophs, or lithotrophs, depending on the environment [2].

2.1.3.2 Pathogenicity

Shewanella most commonly known as secondary or opportunistic pathogens, and the possibility of infections caused by them are very rare; nevertheless, infections are being reported with higher frequency because of better diagnostics in the clinical microbiology laboratory [2,22]. *S. putrefaciens* and *S. algae* are most commonly isolated though human clinical specimens examined thus far are very narrow. The

reason for this is a short of criteria for good speciation as an example molecular characterizations to differentiate between *S. putrefaciens*, *S. algae*, and other Shewanella species. Additionaly, during isolation of the Shewanella, other bacterial pathogens are also isolated beside the Shewanella. Because of this fact that, the function of the Shewanella in pathogenesis and their clinical importance have not been understood yet.

On the other side, there are reports of monomicrobial Shewanella infections; patients present clinically with cellulitis in the context of other skin and soft tissue manifestations, bacteremia/septicemia, otitis media or otitis externa, respiratory distress, intra-abdominal infection, pneumonia, and empyema [22,23]. Beside there are some exceptions, *S. putrefaciens* and *S. algae* are vulnerable to common antibiotics used to treat bacterial infections [22,23] though drug-resistant strains have been documented to emerge during the course of patient treatment [24].

Since earlier isolates have been classified under another genus, in 1963 the first *S. putrefaciens* was isolated from human clinical specimen. From 1963 to 1997, about 75 case of human infection caused by *S. putrefaciens* had been reported [25]. There is a strong relation between *S. putrefaciens* infection and an immunocompromised state, and liver disease is a strong risk factor [25]. In 1985, three different biovars were before construction of the species *S. algae* in 1990 commonly, but incorrectly, there were reports which identified many human isolates as *S. putrefaciens* rather than *S. algae* [22]. After understanding of the fact that *S. algae was* different from *S. putrefaciens*, Gilardi biovar 2 was used to illustrate *S. algae* [27,28].

Because of the fact that the possibility of Shewanella infections are very low, *S. algae* become the most common human pathogen. In addition to this, *S. putrefaciens* are responsible for nonhuman pathogenicity [22,26]. CDC biotype 1 (*S. putrefaciens*) and CDC biotype 2 (*S. algae*) were recognized as two types of clinical isolates in 1995 by Centers for Disease Control and Prevention [26]. But there was need for more detailed molecular characterizations to differentiate into subgroups other than *putrefaciens* and *algae.* A review by Holt *et al.* that was recently published shows important points in differentiating to the clinical microbiologist. On the other hand, DNA:DNA

hybridization or 16S rRNA sequences that are examples of molecular characterizations can only distinguish *S. putrefaciens* and *S. algae* from other members of the genus Shewanella.

There has not been information that describe the virulence factors for *Shewanella* clinical isolates yet but according to one study, after comparision many human and nonhuman Shewanella clinical isolates, it was found that *S. algae* was more pathogenic than *S. putrefaciens* on the strength of resistance to antimicrobials, production of hemolysin, and pathogenicity in mice [26]. According to the study that examined pathogenicity of various *S. putrefaciens* isolates in mice, it was found that doses ≥1 × 109 organisms per mouse were only lethal [29]. Extracellular virulence factors for instance siderophores, exoenzymes, and tetrodotoxin, a potent marine neurotoxin produced by *S. algae* have been determined and by some investigators and may be a part of in pathogenesis [22].

2.1.4 Applications in Biotechnology

With a diverse group of electron acceptors, genus Shewanella have able to respire and have shown an adaption to the life in extreme and different environments. Shewanella can easily grow in the lab conditions and are open to genetic manipulation. So, Shewanella have potential to remediate environmental pollutants and in microbial fuel cells (MFCs), where their metabolism have capacity to produce electricity [30-33].

2.1.4.1 Bioremediation of Radionuclides and Toxıc Elemental Waste

Contaminated environments can be effectively and inexpensively cleaned up by the help of microorganisms which prevent difficulties in efficient bioremediation strategies [34]. Shewanella are superior candidates for potential use in pollutant bioremediation among dissimilatory metal-reducing bacteria due to their intrinsic ability to respire using a wide range of electron acceptors [2]. The solubility and mobility of elements in soils, sediments, and water can be influenced with their oxidation states. Microorganisms display one means by which changes in oxidation states are catalyzed so that transport into rivers and groundwater can be blocked and cleanup facilitated [2,35]. Applications might contain ex situ remediation strategies and in situ

bioremediation in storage tanks or areas of environmental contamination [2]. For instance, in manufacturing of nuclear weapons and as a main fuel for nuclear reactors, uranium (U) is used. Some Shewanella can able to play a role in the reduction of U (VI) to the insoluble U (IV) form [2] and this would cause to precipitation and prevent further spread in groundwater at the contaminated site. There is another radionuclide, Technetium (^{99}Tc VII), which is obtain as an end product of nuclear reactor operations and fallout from nuclear weapons testing. Many oxidations states of Technetium (^{99}Tc VII) can be observed but the most reduced form is largely immobile [36]. ^{99}Tc can be reduced by *S. putrefaciens* [2,36] *S. oneidensis* MR-1 [37], and *S. algae* [36] and they can be used in remediation of Tc-contaminated environments and waste streams. Similar to Technetium (^{99}Tc VII), Cobalt (^{60}Co) is a radionuclide and formed after weapons operations. Co(III)EDTA^{-} is the mostly found form of cobalt at contaminated sites and in groundwater, and some Shewanella can use this form of cobalt as an electron acceptor[38,39]. ^{60}Co(III)EDTA is reduced to ^{60}Co(II)EDTA^{2-} which causes reduction of ^{60}Co(III)EDTA^{-} to ^{60}Co(II)EDTA^{2-} leads to immobilization and therefore restricted transport in subsurface environments.^{60}Co(II)EDTA^{2-} that is the reduced form is not stable sorbs to mineral surfaces facilitating elimination [38,39]. Chromium (Cr), mercury (Hg), and arsenic (As) can be reduced by some type of Shewanella but there can be advantages and disadvantages of these reductions. Cleanup efforts can be made possible by the formation of solid oxides as a result of reduction of soluble Cr(VI) to Cr(III) by some Shewanella[2,40]. But *S. oneidensis* MR-1 reduce ionic mercury [Hg(II)] to elemental mercury [Hg(0)] and this cause an increase in mobility and an oxidation state readily available to form methylmercury, a bioaccumulative environmental toxin. In addition to these examples, reduction of arsenate [As(V)] to arsenite by Shewanella cause big problems. Drinking waters are contaminated and poisoned by As (III) since this form of arsenate is mobile.

As a result, understanding the role of Shewanella, as well as other microorganisms in the oxidation of target compounds and the results in varying these oxidation reactions are precursor factors to optimize cleanup strategies. Bioremediation strategies consisting Shewanella can be only applied in the laboratory and have not been used outside the lab yet. The future applications of Shewanella in bioremediation strategies consist of cleanup of contaminated global environments and groundwater. Since

Shewanella mainly lives in aqua, further studies will be focused on how Shewanella behave in soil and contaminated groundwater environments [3].

2.2 MULTICOPPER OXIDASES

2.2.1 Enzymology of Multicopper Oxidases

Multicopper oxidases (MCOs) are a family of enzymes belonging to the highly diverse group of blue copper proteins which contain from one to six copper atoms per molecule [41]. The currently well-defined multicopper oxidases are laccase, ascorbate oxidase, and ceruloplasmin. Recently several new additional ones have been isolated and characterized: phenoxazinone synthase, bilirubin oxidase, dihydrogeodin oxidase, sulochrin oxidase, and FET3 [42]. Historically, copper atoms coordinated in proteins have been classified into three types (type 1, type 2 and type 3) based on their spectroscopic properties. In the UV-visible spectrum, type 1 copper (blue copper) shows maximum absorption around 610 nm, and type 3 copper (coupled binuclear copper) shows maximum absorption around 330 nm. Type 1 and type 2 (normal copper) coppers are EPR (electron paramagnetic resonance) detectable, while the binuclear type 3 coppers are EPR silent [43].

Laccase, ascorbate oxidase, and ceruloplasmin exhibit EPR features, indicating the presence of a combination of type 1 and type 2 centers, and have an additional EPR inactive, antiferromagnetically coupled type 3 center. The type 2 plus type 3 centers were originally shown on laccase to form a trinuclear copper cluster (Figure 2.2a), and this has also been determined by crystallography for ascorbate oxidase and ceruloplasmin. The function of the type 1 copper site within the enzyme is longrange intramolecular electron transfer, shuttling electrons from the substrate to the trinuclear cluster. The trinuclear cluster is the site of dioxygen binding and reduction (Figure 2.2b) [42].

The type 1 copper-binding site consists of two histidines, one cysteine, and one methionine (Figure 2.2b, Figure 2.3). The first three residues are essential for the blue copper-binding site and form a tight trigonal coordination with the copper ion, while the coordination of the fourth residue, an axial methionine, is rather distant and weaker, and this residue can be replaced with different amino acids, such as leucine or phenylalanine [43]. The type 2 and type 3 copper sites form a trinuclear cluster the peptide ligands of which are eight histidines that occur in a highly conserved pattern of four HXH motifs in Figure 2.3. In one, X is the cysteine bound to the type 1 copper and each of the histidines binds to one of the type 3 coppers. About 35-75 residues upstream of this is another HXH motif. Close to the N-terminal end are two more HXHs separated by about 35-60 residues [42].

Suresh Kumar et al.[44] developed 4 signature sequences for laccases corresponding to the 4 copper binding regions shown in Figure 2.3:

1. H-W-H-G-X9-D-G-X5-Q-C-P-I
2. G-T-X-W-Y-H-S-H-X3-Q-X-C-D-G-L-X-G-X-(F/L/I/M)
3. H-P-X-H-L-H-G-H
4. G-(P/A)-W-X-(L/F/V)-H-C-H-I-(D/A/E)-X-H-X3-G-(L/M/F)-X3-(L/F/M)

Although the sequences were designed specifically for laccases, they show significant similarities to other multi-copper oxidases containing 4 copper atoms.

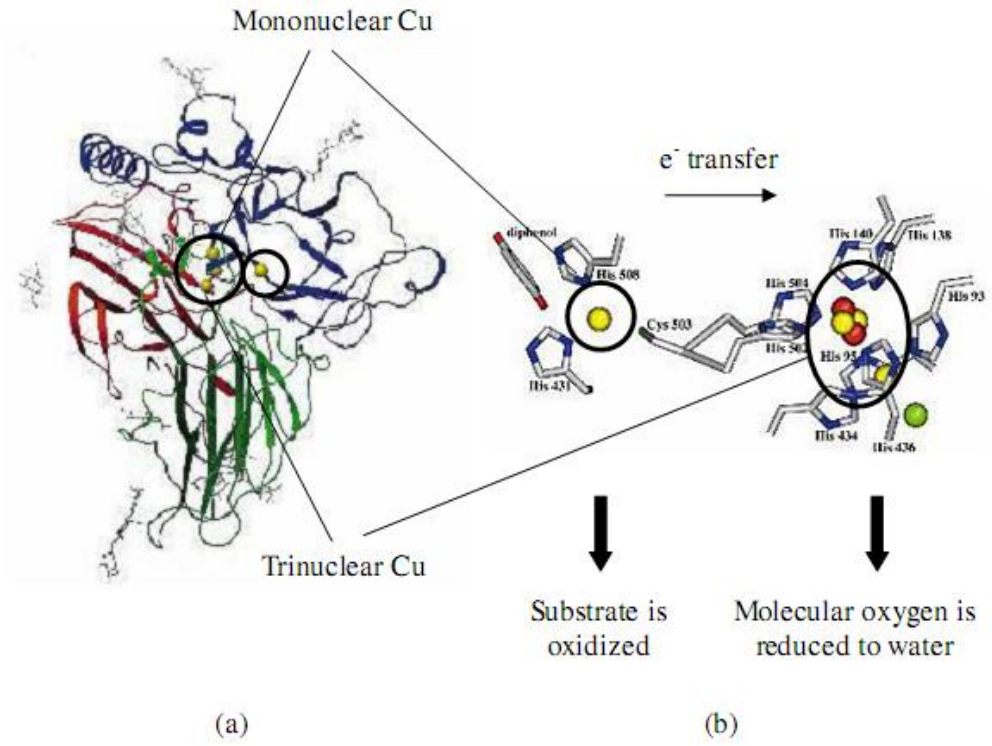

Figure 2.2 (a) Three-dimensional structure, **(b)** copper binding sites of Melanocarpus albomyces laccase [45].

(a) Domain A (red) includes residues that participate in the binding of coppers at the thinuclear site. Domain B (green) contains residues that take part in the substrate binding. Domain C (blue) contains residues that participate in the binding of coppers at the mononuclear and the trinuclear site, as well as in substrate binding. The mononuclear site is located entirely in domain C, and the trinuclear site is located at the interface between domains A and C. The diphenolic substrate-binding site is located in the cleft between domains B and C. (b) The mononuclear site is on the left and the trinuclear site is on the right. Oxygen atoms are represented by red balls; a chlorine atom, by a green ball.

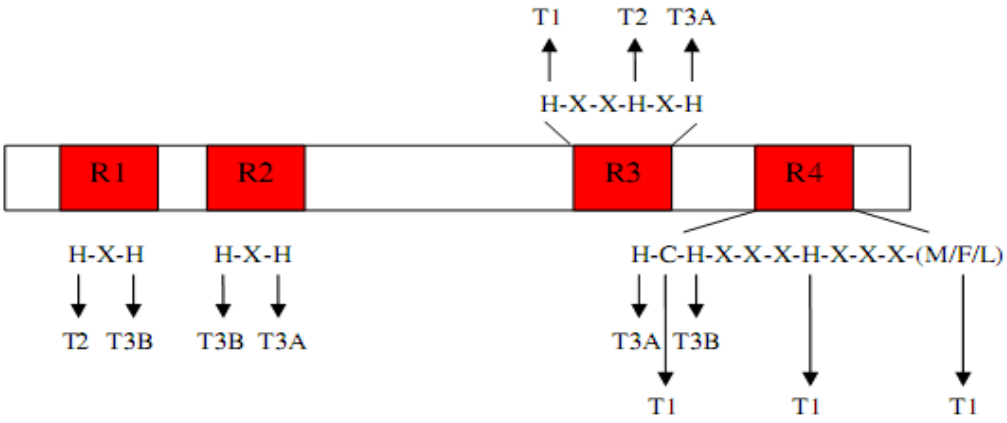

Figure 2.3 Copper binding regions of multicopper oxidases showing high degree of homology. R and T denotes for region and copper type, respectively. T3A: type 3 copper A, T3B: type 3 copper B.

The interactions of the multicopper oxidases with substrates can be broadly divided into two categories: enzymes with low substrate specificity and enzymes with high specificity. The plant and fungal laccases fall into the former category. They can oxidize diphenols, aryl diamines, and aminophenols, indicating that there is probably no binding pocket for substrate and that the oxidation is strictly outersphere. The other multicopper oxidases possess a significant degree of substrate specificity, implying a substrate binding pocket. Ascorbate oxidase shows a high degree of specificity for L-ascorbate and analogs, and ceruloplasmin shows a high specificity for Fe(II). Sulochrin oxidase and dihydrogeodin oxidase also have very high substrate specificities. In addition, the reactions these enzymes catalyze are stereospecific [42].

2.3 LACCASE

Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) belong to the super family of multicopper oxidases [46]. Laccases are widely distributed inmany eukaryotes e.g. fungi, plants [47] as well as in prokaryotes e.g. bacteria [48] and exhibit various functions, depending on their source organism, physiological and pathological conditions. Range of functions exhibited by laccases is broadly divided into three categories: (1) cross-linking of monomers, (2) degradation of polymers, and (3) ring

cleavage of aromatic compounds [49]. Thus, the various functions carried out by laccases include lignification, wound healing and iron oxidation (in plants), delignification, pigmentation, fruiting body formation as well as pathogenesis (infungi) andmelaninformation, endospore coatprotein synthesis (in bacteria) [50-55]. The localization of plant and fungal laccase is extracellular, while in bacteriamost of the laccases are intracellularly localized [56]. Laccases are dimeric or tetrameric glycoproteins. To perform their catalytic function, laccases depend on Cu atoms that are distributed at the three different copper centers Type-1 or blue copper center, Type-2 or normal copper and Type-3 or coupled binuclear copper centers, differing in their characteristic electronic paramagnetic resonance (EPR) signals [57,58]. At three dimensional structure level, laccases (bacterial, fungal and plant) have been suggested to have three sequentially arranged cupredoxin-like domains. From wet lab analysis as well as from in silico studies, it is evident that despite their wide taxonomic distribution and substrate diversity, molecular architecture of catalytic site of laccases exhibits commonalitywith that ofmulticopper oxidases. The ability of laccases to catalyze the oxidation of various phenolic aswell as non-phenolic compounds, coupled to the reduction of molecular oxygen to water, makes them valuable from the standpoint of their commercial application [59].

2.3.1 Classification of Laccases

2.3.1.1 Bacterial laccases

The first bacterial laccase was found in plant-root associated bacterium *Azospirrullum lipoferum*, which was involved in melanin formation. Azospirillum bacteria were prevalently found in soil and in the rhizosphere of a variety of grasses and cereals. Cultivated plant inoculated with these bacteria shows significant growth improvements. Laccase activity was also reported in a heterocystous cyanobacterium, *Anabaena azollae*. In *Bacillus subtilis* a thermostable cot A laccase, involved in production of brown spore pigment in endospore coat, was reported. These laccases could help in the protection of spore coat against UV light and hydrogen peroxide. Laccases in *Streptomyces cyaneus* and *Streptomyces lavendulae* were also reported. Most of the bacterial laccases are intracellular for example, *A. Lipoferum, Marinomonas*

mediterranea and *B. subtilis laccases*. In contrast to fungal laccases, bacterial laccases are highly active and much more stable at high temperatures, at high pH as well as high concentrations of chloride and copper ions and the immobilized spore laccases are more compatible with almost all industrial processes [60].

2.3.1.2 Fungal laccases

In fungi, laccases are widely distributed in ascomycetes, duteromycetes, and basidiomycetes. These laccase producing fungi (called as wood-degrading fungi) include *Trametes (Coriolus) versicolor, Trametes hirsute, Trametes ochracea, Trametes villosa, Trametes gallica, Cerrena maxima, Phlebia radiata, Coriolopsis polyzona, Lentinus tigrinus, Plreurotus eryngii*, etc. Laccases are also reported in saprophytic ascomycetes of composts (Myceliophthora thermophila, Aspergillus, Curvularia, Penicillium and Chaetomium thermophile) and in the soil hyphomycete Mycelia sterlia INBI 2-26. Laccases have also been purified and characterized from a few fungi-forming ectomycorrhiza e.g. *Cantharellus cibarius, Lactarius piperatus, Russula delica* and *Thelephora terestris* or orchideoidmycorrhiza such as Armillariamellea as well as from the species of genera that contain both saprotrophic andmycorrhizal fungi e.g. Agaricus, Marasmius, Tricholoma and Volvariella. Better penetrative ability, due to extensive hyphal organization, has been suggested to be the reason for efficientwood degradation by fungi in nature. Furthermore, the high activity of laccases in wood-rotting basidiomycetes fungi suggests that the main role of fungal laccases is to depolymerize the complex cell-wall constituents such as lignin. This degradation process also involves the synergistic effects of some other enzymes and non-enzymatic components that help to establish equilibriumbetween enzymatic polymerization and depolymerization [60]. In addition to laccases, the other enzymes implicated in lignin degradation are:

1. lignin peroxidase,which catalyzes the oxidation of both phenolic and non-phenolic units,
2. manganese-dependent peroxidase,
3. glucose oxidase and glyoxal oxidase for H_2O_2 production,
4. cellobiose-quinone oxidoreductase for quinone reduction.

White-rot fungi, most efficient lignin degraders, are characterized by high laccase activity. It has been postulated that almost all white-rot fungi produce laccase except for Phanerochaete chrysosporium, which is reported to produce a range of isoenzymes of lignin peroxidase and manganese peroxidase. In white-rot fungi, manganese peroxidase in combination with either laccase or lignin peroxidase may be the minimum necessary enzymatic component for the lignin biodegradation. Based on the enzyme production patterns followed, three categories of fungimay be postulated:
(i) lignin–manganese peroxidase group (e.g. *P. chrysosporium*),
(ii) manganese peroxidase–laccase group (e.g. *Dichomitus squalens*),
(iii) lignin peroxidase–laccase group (e.g. *Phlebia ochraceofulva*).

Besides degradation of biopolymers, fungal laccases are also reported to perform several other functions, such as development associated pigmentation (dihydroxynaphthalene melanins, that are produced against environmental stress), fruiting body formation, fungal morphogenesis, detoxification, sporulation, and pathogenesis. Plant-pathogenic fungi are reported to produce laccases that are proposed to detoxify the toxic components generated by the plant defence systems. *Botytis cinerea* that causes soft rot infections in many crop plants such as carrot, cucumbers as well as the noble rot and grey rot in grapes produces extracellular laccases, which are involved inthe pathogenesis. In *Cryptococcus neoformans*, laccases are expressed as virulence factors, thus they are considered as a major fungal pathogen in immuno-compromised individuals such as AIDS patients, organ transplant recipients and high doses corticosteroid treated patients. This fungal laccase is thought to convert host catecholamines into melanin, which protects *C. neoformans*, allowing it to cause more damage to the host [60].

Laccase production in fungi is sensitive to the nitrogen concentration. Usually high nitrogen concentration is required to obtain greater amounts of laccases. For example, when *Lentinula edodes*, and *Rigdoporus lignonus* were grown in a high nitrogen (24–26mM) containing medium, laccase production becomes highest. In contrary to this, enhanced production of the laccase in nitrogen-limitedmedia is also reported in *Pycnoporus cinnabarinus* and *Phlebia radiate* [60].

2.3.1.3 Plant laccases

Laccases are member of multigene family in plants. The first laccase was identified in sap from *Rhus vernicifera*, the Japanese lacquer tree. Subsequently, laccases were reported from variety of plants such as lacquer, mango, mung bean, peach, pine, prune, and sycamore. Evenmultiple forms of laccaseswere reported from some plants. Thus, eight laccases were reported in loblolly pine (*Pinus taeda*), five distinct laccaseswere shown to be expressed in the xylemtissues of poplar (*Populus trichocarpa*). In addition, cell suspension culture of sycamore maple (*Acer pseudoplatanus*) was reported to produce and secrete laccases like multicopper oxidases (LMCO). Four closely related LMCOs were identified in xylemtissues of yellow-poplar (Liriodendron tulipifera). LMCOs have also been reported in other species, including *Zinnia elegans*, tobacco (*Nicotiana tabacum*), and Zeamays. Monocot laccase fromryegrass (Loliumperenne) was also cloned and characterized. Plant laccases perform varieties of functions, such as lignin polymerization through dehydrogenative mechanism, wound healing and iron oxidation by converting Fe(II) to Fe(III). Transgenic approaches, using laccase genes, for over expression as well as down regulation, have also been used in past ten years or so for utilization of plant biomass for various purposes such as energy production, phytoremediation and alteration in phenolic metabolism. Both plant and fungal laccases are glycosylated enzymes. Plant laccases are showing a higher extent of glycosylation (22-45%) than the fungal laccases (10-25%). The carbohydrate moiety of the majority of laccases consists of mannose, N-acetyl glucosamine, and galactose. Fungal laccases often have lower molecular mass than the plant laccases. On SDS-PAGE about 10–50% of molecular weight was reported to be attributed to glycosylation. The glycosylation is useful for the secretion, copper retention, thermal stability, activity of the enzyme [60].

2.4 STRUCTURE OF COPPER CENTER/ACTIVE SITE OF LACCASES

Laccases are dimeric or tetrameric glycoproteins, containing four copper atoms per monomer. These copper sites in laccases are categorized into three groups (Fig. 2.4), Type-1 or blue copper center, Type-2 or normal copper and Type-3 or coupled binuclear copper centers [60-62]. The four copper atoms are differing in their characteristic electronic paramagnetic resonance (EPR) signals. Type-1 copper shows coordination with two histidines, one cysteine and one methionine as ligands. The Type-1 copper center shows an intense electronic absorption band near 600 nm (ε = 5000 M^{-1} cm^{-1}), which is responsible for their deep blue color. The laccases which lack the typical absorption around 600 nm have also been reported. For example, a "white laccase" (containing 1Cu, 1Fe, 2Zn atoms) in *Pleurotus ostreatus* while "yellow laccases" (containing copper but in an altered oxidation state) in *Panus tirinus*. The Type-2 copper has two histidine and water as ligands. The Type-3 copper coordinates with three histidines and a hydroxyl bridge, which maintains the strong anti-ferromagnetic coupling between the Type-3 copper atoms. Type-2 copper shows no absorption in the visible spectrum and is positioned close to the Type-3 copper which shows an electron adsorption at 330 nm. Depending on the structure and properties of the copper centers laccases are also divided into low-redox potential and high-redox potential laccases. Laccases from basidiomycetes (especially white-rot fungi) are high-redox potential laccases whereas, bacterial and plant laccases are the examples of low-redox potential laccases [60-64].

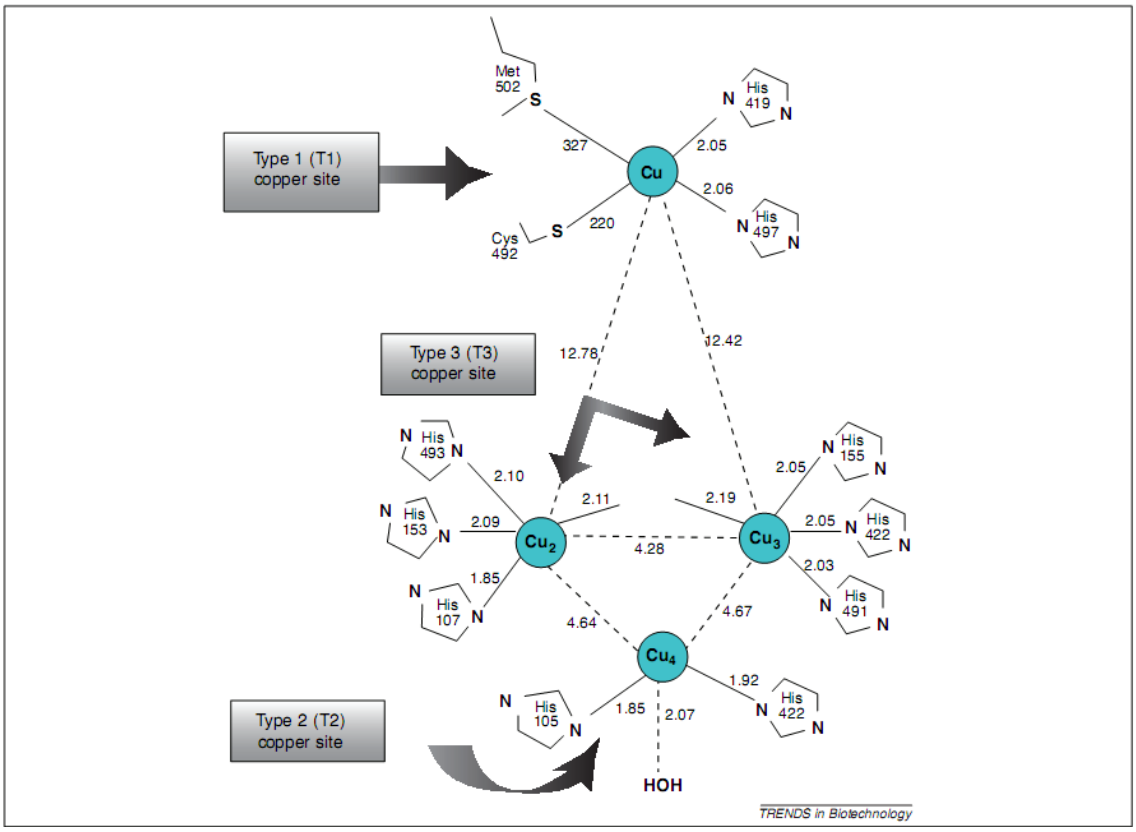

Figure 2.4 Schematic representation of the copper centers in CotA from Bacillus subtilis.

Gray arrows point to the copper atoms in each site. The type 1 (T1) site has a mononuclear, paramagnetic, 'blue' copper ion that imparts a characteristic light blue color to the laccase. It has a pronounced electronic absorption at a wavelength of 600 nm and also displays a weak parallel superfine splitting in the electron paramagnetic resonance (EPR) spectrum. The type 2 (T2) site is a mononuclear paramagnetic, 'non-blue' copper, which is invisible in the electronic absorption spectrum but displays an ultrafine splitting in the EPR spectrum [65]. The type 3 (T3) site consists of a binuclear, diamagnetic spin-coupled pair of copper ions, which makes this site invisible in the EPR spectrum. A shoulder at 330 nm in the UV region reveals the presence of this site. The T2 and T3 sites together form a trinuclear cluster in which the T2 copper is coordinated by two histidine residues and the T3 copper atoms by six histidine residues [65]. A hydroxide bridge maintains the strong anti-ferromagnetic coupling between the two T3 copper atoms [66].

2.5 MECHANISM OF ACTION OF LACCASES

In contrast to peroxidases, laccases consume O_2 instead of H_2O_2 to oxidize the monolignols. To perform catalytic function, laccase depends on Cu atoms that are distributed at the three different copper centers asmentioned above in Section 2.4. The laccase enzyme withdraws the electron from the substrates and converts them in free radicals, which can be polymerized. After receiving four electrons, the enzyme donates them to molecular oxygen to form water molecule (Eq. (2.1)) [67]. Overall, there are three major steps in laccase catalysis: a) Type-1 Cu reduction by reducing substrate. b) Internal electron transfer from Type-1 Cu to Type-2 and Type-3 Cu trinuclear cluster. c) Reduction of oxygen (to water) at Type-2 and Type-3 Cu.

The overall reaction is as follows:

$$4RH + O_2 \rightarrow 4R\bullet + 2H_2O \qquad \text{(Eq. 2.1)}$$

In vitro studies on small lignin model compounds suggested that the first step of laccase mediated lignin degradation is an oxidative reaction with the loss of one electron from phenolic hydroxyl groups of lignin to produce phenoxy radicals. The radicals may spontaneously reorganize and give rise to the cleavage of alkyl side chains of the polymer. Laccase degrades both β-1 and β-O-4 dimers via C_β–C_β cleavage, Cα oxidation and alkyl-aryl cleavage. Since laccases show activity in the absence of toxic H_2O_2, they could play a role in the early stages of lignification inliving cells. Thus, it has also been postulated that laccases might be the principal lignification enzymes under conditions where lignin concentration has reached a level where the middle lamella has become so hydrophobic that most of the water and H_2O_2 is excluded, whereas O_2 is still available [60].

It was also found that polymerizing activity of laccases on kraft lignin was prevented when compounds such as 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were present [68-70]. ABTS acts as a mediator and allows laccase to oxidize and cleave non-phenolic lignin substrates (Fig. 2.5) [60]. In other words, ABTS functions as a diffusible electron carrier and the action of the laccase-ABTS couple proceeds via carbon-hydrogen abstraction, with a consequent C-C bond cleavage in condensed lignins [70].

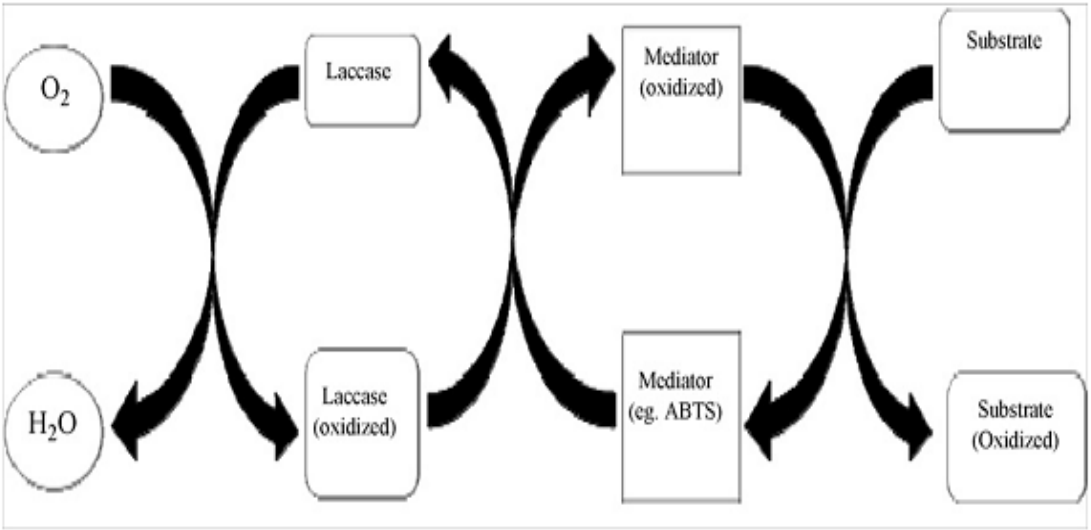

Figure 2.5 Schematic Representation of laccase enzyme catalysis in presence of mediator.

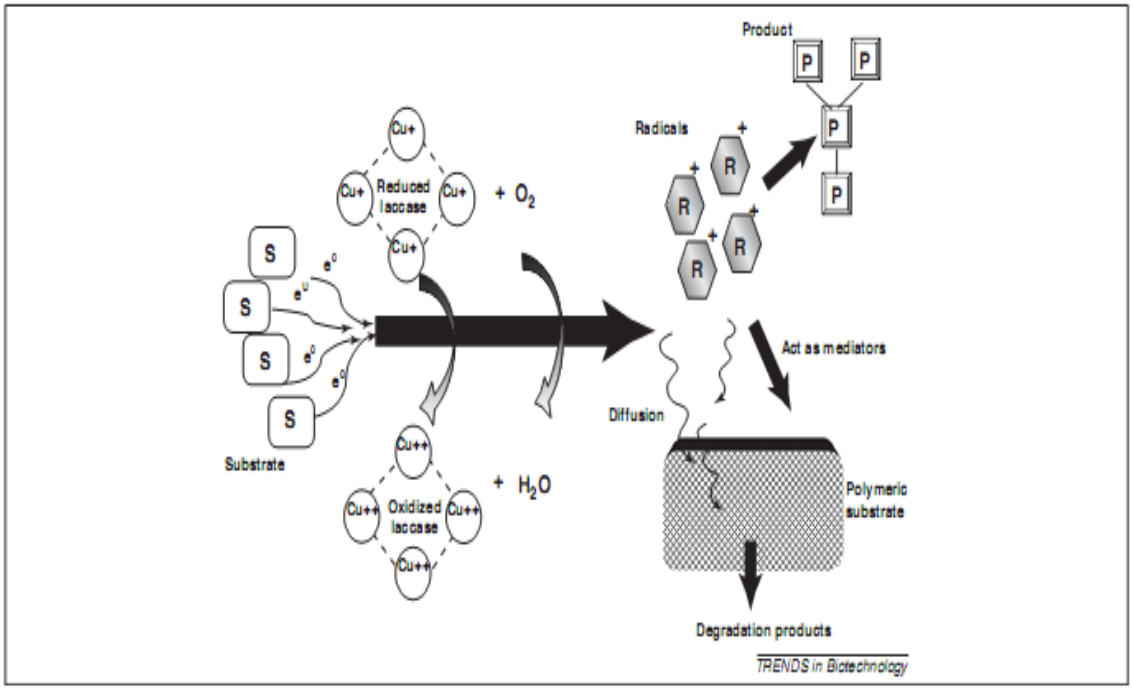

Figure 2.6 Schematic representation of the catalytic reaction.

Initiation of the catalytic cycle is thought to take place at the T1 site where the oxidation of the substrate takes place. This step is conjectured to be the rate-limiting step [72]. Four electrons produced by the oxidation of four substrate

molecules are then transferred to the trinuclear cluster where the co-substrate, dioxygen, is reduced to two water molecules [65]. Electrons are thus driven from the reducing substrate to molecular oxygen without the release of any toxic peroxide intermediates. The reactive radicals created from the oxidized substrate may undergo further non-enzymatic reactions [73].

ABTS is a specific substrate for laccase in the absence of hydrogen peroxide, and a substrate for peroxidase in presence of hydrogen peroxide [71]. ABTS in solution, in reduced form, has a very faint green color, while, in oxidized form, it turns dark green so it was used to detect laccase activity. Pycnoporus cinnabarinus laccase efficiently degrades the lignin in the presence of 3-hydroxyanthranilate, an endogenous fungal metabolite that mediates the oxidation of non-phenolic components of lignin and thereby acts like a mediator [68-71]. More than 100 possible mediator compounds (e.g. 3-hydroxyanthranilic acid (HAA), N-hydroxybenzotriazole (HBT), N-hydroxyphtaimide (HPI), methyl ester of 4-hydroxy-3,5-dimethoxy-benzoic acid (syringic acid), violuric acid (VLA)) have been investigated for their capability to oxidize lignin or lignin model compounds through the selective oxidation of their benzylic hydroxyl groups [67].

2.6 PHYSICOCHEMICAL PROPERTIES OF LACCASES

Laccases are usually present as several isoenzymes having their own unique substrate specificity [60]. In addition to mono and polyphenols, laccases have capability to oxidize various aromatic compounds, such as substituted phenols, diamines, aromatic amines and thiols, and even some inorganic compounds such as iodine, $Mo(CN)_8^{4-}$, and $Fe(CN)_6^{4-}$ [60]. The organic substrates of laccases can be divided into three groups: ortho- (e.g.guaiacol, o-phenylenediamine, pyrocatechol, dihydroxyphenylalanine, pyrogallol, caffeic acid, gallic acid, and protocatechuic acid), meta-(m-phenylenediamine, orcinol, resorcinol, and phloroglucin) and para-(p-phenylenediamine, p-cresol, and hydroquinone) substituted compounds with a lone electron pair. Ortho-substituted compounds are the best substrates for most laccases [60,61]. Syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) is typically referred to as a specific substrate for laccase. Furthermore, in presence of mediators, substrate specificity of laccases can be further broadened leading to oxidation of more

complex substrates. Substrate specificity and affinity of laccase vary with changes in pH. For substrates whose oxidation does not involve proton exchange (such as ferrocyanide), the laccase activity often decreases as pH increases, whereas for substrates whose oxidation involves proton exchange (such as phenol), the pH–activity profile of laccase can exhibit an optimal pH whose value depends on source of laccase rather than substrate [60]. For phenols, the optimal pH range is between 3 and 7 for fungal laccases as well as for bacterial laccases and may increase to 9 for plant laccases. The lower pH optima for fungal laccases may be due to the adoptability of the fungi to grow well in acidic condition, but the plant laccases exhibited their optimal pH nearer to the physiological range due to intracellular nature. The difference in pH optima suggests to be linked to their physiological functions [74]. Temperature optima of laccase activity range from 50 $^{\circ}$C to 70 $^{\circ}$C but few enzymes showing temperature optima below 35 $^{\circ}$C have also been reported. Thermal stability of laccases varies significantly with the temperature range of the growth of the source organism. The fungal laccases usually have lower thermal stability than bacterial laccases [60]. The thermal stability of laccases has been suggested to be linked to the interaction between the copper ions of copper centers and salt bridges as well as hydrogen bonding network in the internal protein structures [75]. The more acidic isozymes of laccase were reported to have more thermostability [76].

Laccases have been reported to be inhibited by various reagents such as small anions as halides (excluding iodide), azide, cyanide, and hydroxide. These inhibitors have been suggested to bind to the Type-2 and Type-3 Cu, resulting in an interruption of the internal electron transfer and subsequent inhibition of the activity. Other inhibitors of laccases include, metal ions (e.g. Hg^{2+},Mg^{2+}, Ca^{2+}, Sn^{2+}, Ba^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, and Zn^{2+}), fatty acids, sulfydryl reagents, hydroxyglycine, kojic acid, EDTA, l-cysteine, dithiothreitol, glutathione, thiourea, and cationic quaternary ammonium detergents [60]. These agents are suggested to affect the laccase activity by chelating the Cu(II) atoms or by modifying amino acid residues or by causing conformational change in the glycoprotein [60].

2.7 COMPARATIVE 3-D STRUCTURE ANALYSIS OF BACTERIAL, FUNGAL AND PLANT LACCASES

From previous experimental evidences and from modeled structure, it has been shown that despite their wide taxonomic distributionand diversity of substrates, molecular architecture of laccases is common for all multicopper oxidases. A comparative modeling study was conducted by us to provisionally explain profound differences among bacterial, fungal and plant laccases. One of the purposes in building a model was also to have a better understanding of the features that are important for catalytic activity. The three dimensional structure of bacteria (*B. subtilis*), fungi (*T. versicolor*) and plant (*P. trichocarpa*) laccases was predicted by homology modeling approach using template 1HL0, 1KYA and 1AOZ, respectively, by modeling 9v6 in Figure 2.7 [60].

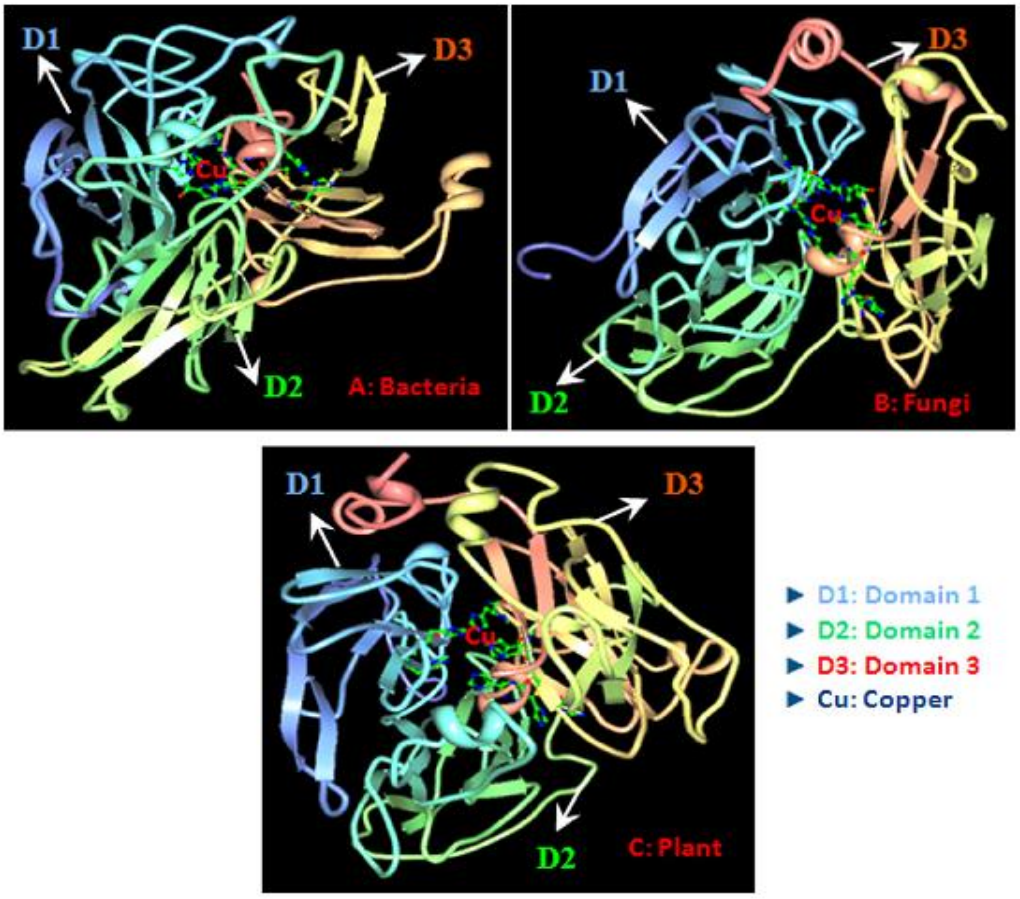

Figure 2.7 Three dimensional structure of (A) bacterial laccase (*Bacillus subtilis*), (B) fungi laccase (*Tramates versicolor*), and (C) plant laccase (*Populus trichocarpa*).

The qualities of each modeled structure for laccases are carried out, using PROCHECK, ProSA and PROQ. The PROCHECK analysis for modeled laccases shows that 90.1% (bacteria), 89.5% (fungi) and 86.5% (plant) residues were in favored and allowed regions in Ramachandran plot. These values match well with those for experimentally determinedmodels. Results fromProSA gave a z-score of −8.28, −6.22 and −5.79 and PROQ analysis gave a LG score value of 6.006, 5.293 and 4.874 for *B. subtilis, T. versicolor* and *P. trichocarpa*, respectively. These results taken together suggest that the values for homology model built for bacteria, fungi and plant fall within the range of values observed for experimentally determined structures and the built models are very reliable for interpretation of structure–function relationships. Three dimensional structure predictions, at monomeric level, for all laccases (bacteria, fungi and plant) suggest that they are composed of three sequentially arranged cupredoxin-like domains as presented in Fig. 2.7. These cupredoxin domains mainly formed by β-barrels (Greek key motif) consisting β-sheets and β-strands, arranged in sandwich conformation [60]. Comparative analysis of predicted models shows, first domain present at N-terminal region (blue color) in bacteria (Fig. 2.7.(A)) is somewhat distorted conformation in comparison with the equivalent domain in fungi (Fig. 2.7.(B)) and plant (Fig. 2.7.(C)). (Fig. 2.7.(A)) depicts, presence of a coiled section, which connects Domain 1 and Domain 2 in bacteria, is absent in fungi and plant. This coiled section also helps in packaging between Domain 1 and Domain 2 in bacteria [60]. In fungi and plant, short α-helical regions connect Domain 1 to Domain 2 and Domain 2 to Domain 3 (Fig. 2.7.B and C). These helices also connect β-strands in structure topology. By comparing models, it is observed that, in bacteria a large loop segment link Domains 2 and 3 through external connection (Fig. 2.7.A), whereas, in fungal and plant laccases, the corresponding link is made through internal connections (Fig. 2.7.B and C). The Domain 2 (green color) acts as bridging element between Domain 1 and Domain 3 [60]. The structure analysis revealed, tri-nuclear copper cluster (T2/T3) embedded between Domains 1 and 3with both domains providing residues for the coordination of the coppers. The copper interacting residue is highlighted in allmodeled structures (Fig.2.7). Finally, Domain 3 (red/yellow) in all modeled structures not only contains the mononuclear copper center, but also contributes to the formation of the binding site of the trinuclear copper center, which is located in the interface between Domains 1 and 3. Moreover, in all multicopper oxidases Domain 3 includes the putative substrate binding

site, located at the surface of the protein, close to the Type-1 mononuclear copper center. A protruding section, formed by a loop and a short α-helix, forms a lid-like structure over the substrate binding site in bacteria.No similar element has been found in the previously analyzed 3-D structure of plant and fungal laccases. Therefore, this structural element may represent a distinctive feature of bacterial laccase. The overall structure analysis shows that, it shares a common β-barrel motif in all domains. In all laccases, the C-terminal portion is characterized by short (13 residues) α-helix stretch, stabilized by two disulfide bridges, the first bridge (e.g. in fungi -Cys-106–Cys-509) connects Domains 1–3 and second disulfide bridge (in fungi -Cys-138–Cys-226) connects Domains 1 and 2 [60].

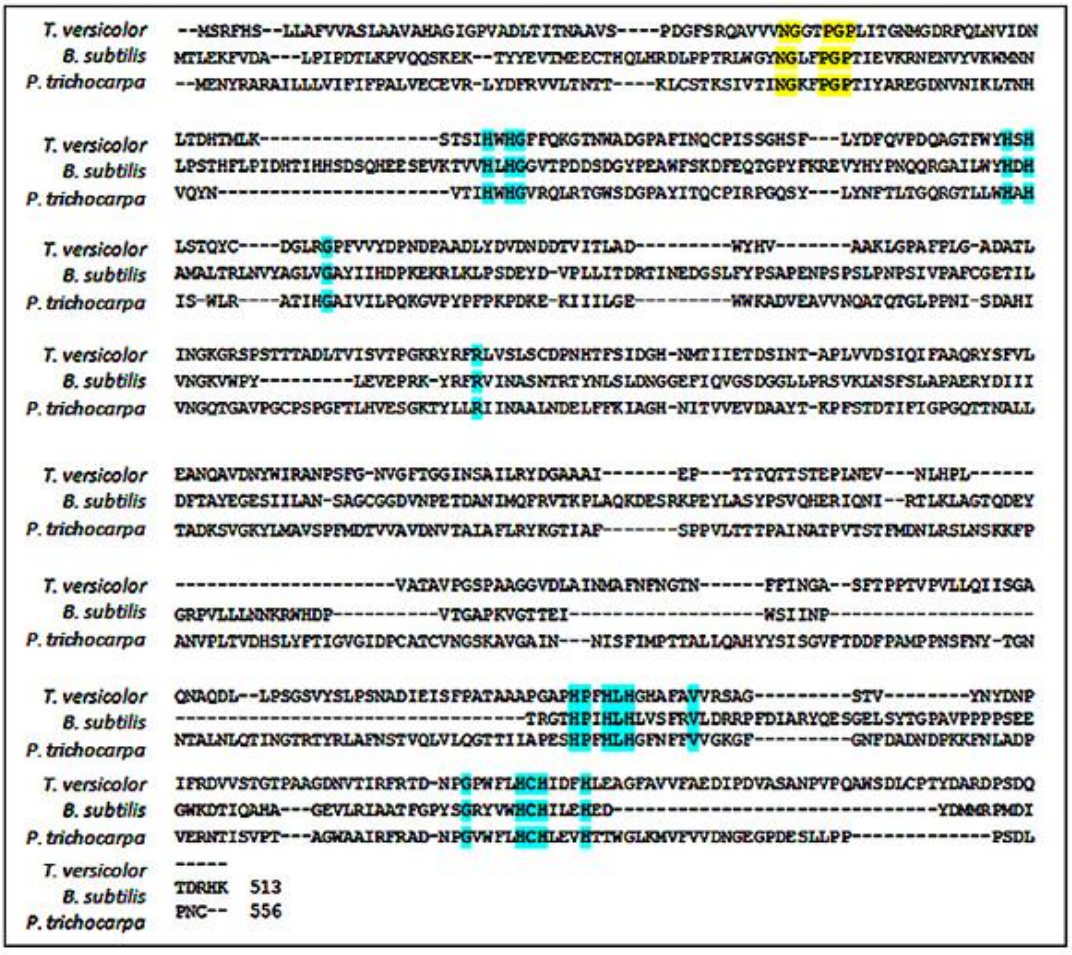

Figure 2.8 Structure based sequence alignment of laccase, by COBALT multiple alignment tool.

Invariant residues are highlighted in cyan and yellow color. The alignment shows highest conservation at copper interacting sites present in Domain 1 and Domain 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Multiple alignment of primary sequences of all three modeled laccases shows that, the copper binding motives are highly conserved in all sequences (Fig. 2.8). The similarities aremore significant in the N- and C-terminal regions, corresponding to Domains 1 and 3, as the copper interacting motif is present in Domains 1 and 3 not in Domain 2. This structural conservation reflects a common reaction mechanism for the copper oxidation and the O_2 reduction in these enzymes [60].

2.8 HETEROLOGOUS LACCASE PRODUCTION

Heterologous expression may provide higher enzyme yields and may permit to produce laccases with desired properties (such as different substrate specificities, or improved stabilities) for industrial applications. This surveys researches on heterologous laccase expression focusing on the pivotal role played by recombinant systems towards the development of robust tools for greening modern industry.

2.8.1 Laccase Recombinant Expression

Laccase production from native sources cannot meet the increasing market demand due to low yields incompatibility of the standard industrial fermentation processes with the conditions required for the growth of many microorganisms. Recombinant protein expression in easily cultivable and handling hosts can allow higher productivity in shorter time and reduces the costs of production. The versatility and scaling-up possibilities of the recombinant protein production opened up new commercial opportunities for their industrial uses [77]. Enzyme productivity can be increased by the use of multiple gene copies, strong promoters and efficient signal sequences, properly designed to address proteins to the extracellular medium, thus simplifying down-stream processing. Moreover, protein production from pathogenic or toxin-producing species can take advantage of safer or even GRAS (generally recognized as safe) microbial hosts. In addition, protein engineering can be employed to

improve the stability, activity and/or specificity of an enzyme, thus tailor made enzymes can be produced to suit the requirement of the users or of the process. Enzymes of superior quality have been obtained by site-directed or random mutagenesis, where single changes in aminoacidic sequences yield improvement in biochemical (pH optimum, thermostability, substrate specificity) and catalytic parameters (Vmax, K_M and Ki). Also, pooling and recombining parts of similar genes from different species or strains by "DNA shuffling" methods, yields remarkable improvements in enzymes in a very short amount of time [78]. Since the beginning of nineties, recombinant expression of laccases has been field of research and matter of debate of many researcher groups. Heterologous expression of laccases in bacteria, yeasts, filamentous fungi and plants has been reported, along with examples of homologous expression [79-84].

Laccase heterologous expression in *Escherichia coli* has been often used as a strategy to get around the problem of obtaining laccases not easily producible in natural hosts. The recombinant expression of *Bacillus subtilis* CotA in E. coli has allowed its deep characterization, structure solving, and functional evolution [85,86]. However, very often the production yield is low, and recombinant enzymes form aggregates difficult to purify [87]. On the other hand, recombinant production of *Streptomyces coelicolor* laccase (SLAC) in *Streptomyces lividans* has yielded considerable large amount of laccase (350 mg/ l) with high purity [88].

Plants have been successfully used as hosts for the recombinant expression of fungal and plant laccases. Expression of secreted recombinant laccases can result, for example, in phytoremediation systems [89,90]. Furthermore, the over expression of a potato laccase (PPO) in tomato conferred to the transgenic plant an enhanced resistance against a bacterial pathogen [91].

2.8.2 Laccase Engineering

The availability of established recombinant expression systems for laccase isoenzymes has allowed their engineering with the aim of deepening knowledge of structure function relationships and of improving several enzymatic features for specific industrial needs. As a fact, (rational or random) mutagenesis has often been used to

generate laccase variants, either in yeasts and in filamentous fungi. In their pioneering work, Xu and co-workers [92,93] reported significant changes in pH optimum K_M and k_{cat} for mutated fungal laccases. New insights into the binding of the reducing substrate into the active T1 site of a laccase produced by *T. versicolor* have been provided by site directed mutagenesis along with induced modifications in catalytic properties of the enzyme [94].

The role of the C-terminus in basidiomycetous and ascomycetous laccases has been evaluated using site directed and random mutagenesis. Gelo-Pujic and co-workers [95] found that the barrier to heterogeneous electron transfer is reduced when the C-terminus of LCCI from *T. versicolor* is truncated. An additional consequence of truncating the C-terminus of LCCI is a shift in the reduction potential of the active site to a lower value. When Kiiskinen and Saloheimo [96] studied the expression of *M. albomyces* in *S. cerevisiae*, they found that the introduction of a stop codon after the native processing site at the C-terminus gives rise to a sixfold increase of laccase activity. A role for the C-terminal tail of P.ostreatus POXA1b in affecting both catalytic performance and stability properties of the enzyme has been inferred by Autore and colleagues[97]. More recently, results obtained with *M. albomyces* laccase clearly confirmed the critical role of the last amino acids in its C-terminus [98].

The deletion of the last four amino acids dramatically affected the activity of the enzyme. Moreover, the crystal structure of the mutant expressed in *S. cerevisiae* showed that the C-terminal mutation had clearly affected the TNC geometry. Further insights in the significance of the laccase C-terminal tail have also been provided through random mutagenesis. Functional expression of a laccase from *Myceliophthora thermophila* by directed evolution has been first reported by Bulter and co-workers [99] giving a 170-fold increase of total activity, thus leading to the highest production yet reported for a laccase in a yeast (18 mg l^{-1}). A 22-fold increase in kcat has also been observed. The most effective mutation (10-fold increase in total activity) adjusts the protein sequence to the different protease specificities of the heterologous host, thus confirming the role played by C-terminus processing in acquiring functional structures. AP. *ostreatus* POXA1b has undergone directed evolution through random mutagenesis, and one of the selected mutant has been found mutated in a variable and mobile loop at the C-terminus

[100]. Molecular dynamic simulations on 3D model structure of this mutant have shown the mutation affects flexibility of some regions of the protein, thus leading to an improved stability and activity of the enzyme. Other techniques to randomly mutate laccases have been employed. Directed evolution followed by saturation mutagenesis rendered laccases able to tolerate high concentration of organic cosolvents [101-105]. A higher laccase production and an increased thermal stability have been obtained in *P. pastoris* through mutagenesis with low-energy nitrogen ion implantation [106]. Random mutagenesis through ethyl methane sulfonate-based (EMS) technique has improved laccase production up to 144 mg l^{-1} in *P. Pastoris* [107]. Taking inspiration from evolutionary pathways within the blue copper binding domain (BCBD) protein family, laccase chimeras through yeast mediated homologous recombination of *Trametes sp*. strain C30 laccase cDNAs have been constructed [108]. The catalytic efficiency of the best-performing hybrid (LAC131) is 12-fold higher than that of the parental enzyme (LAC3). Compared to studies involving mutagenesis, this increase is one of the highest ever observed in a single mutational step, thus confirming how homologous recombination constitute a valuable tool set to study the plasticity of the enzyme.

2.8.3 Recombinant Laccases as Tools for Greening Industry

Laccases have shown a great potential within a variety of industrial applications, where they represent an attractive route for "greening" chemical processes. Owing to the heterogeneous properties observed among laccases from various sources [109] an ever-increasing suite of native laccases has been applied to different biotechnological processes, with the aim to find the most suitable enzyme for a specific application [110,111]. However, only few examples of industrial uses of laccases currently exist. The major obstacle to their practical use is the large amount of enzymes required to meet industrial targets. Important break-throughs towards an industrial use of laccases have been made by their recombinant expression in optimized hosts and production of genetically modified tailored biocatalysts [112].

Most of biotechnological applications with recombinant laccases are based on the same commercial preparation. DeniLite® has been applied to assemble amine derivatized platinum electrodes for phenol detection. This sensor has shown a very fast response and a remarkable long-term stability towards p-phenylenediamine [113] catechol and catecholamines, with submicromolar detection limits [114]. The same laccase preparation has been employed in the construction of stable and high-sensitive ionic liquids-based biosensors, for the detection of rosmarinic acid in plant extracts [115], rutin [116] and luteolin [117] resulting in a low cost, reproducible and stable analytical method. Similarly, Kulys and co-workers developed graphite- or printed graphite- electrode based biosensors for environmental surveillance of phenolic compounds, by covalent immobilization of two recombinant fungal laccases from *Polyporus pinsitus* (*T. villosa, Coriolus pinsitus*) and *M. thermophila*, commercially available from Novozymes. Sincethe balance between optimal pHs for laccase function and substrate reversibility has been shown to be responsible for pH profile of catechol and catecholamines biosensors [114], it can be expected that modifications of enzyme properties by recombinant expression of mutated variants may result in fine-tuning of biosensor performances.

Recombinant laccases have been widely applied in bioremediation purposes, especially for the treatment of synthetic dyes [118-120] and, more recently, of toxic polychlorinated biphenyls (PCBs) [121], and PAHs [122]. The purified recombinant Lcc1 from *Trametes trogii*, expressed in *P. pastoris* differently decolorizes several synthetic textile dyes, depending on their chemical structures. The extent of decolorisation is enhanced by the addition of synthetic mediators. Moreover, this enzyme has proved to be stable and active in the presence of moderate amounts of organic solvents [120]. A potential use of a recombinant *Pycnoporus sanguineus* laccase expressed in *P. pastoris* for the treatment of dye-containing effluents has also been suggested by Lu and co-workers in light of its remarkable ability to degrade, at different extents, four synthetic dyes belonging to different classes (azo, anthraquinone, triphenylmethane and indigo) [118]. Finally, a POXA1b laccase mutant selected for its improved stability in a wider pH range, has been successfully applied to dye decolorisation. This variant shows a further enlargement of dye degradation ability with

respect to the wild-type, being also able to decolorize a recalcitrant dye with a complex stilbene type structure [119].

In view of an application to real colored wastewaters, laccase treatment of a synthetic dye house effluent, containing various reactive dyestuffs and auxiliary chemicals, has been studied in a batch reactor using the commercial preparation DeniLite® Significant correction of some water quality parameters has been achieved and a reliable kinetic model has been developed to simulate the decolorisation process [123]. As far as PCBs degradation is concerned, two laccase isoenzymes from T. versicolor produced in A. oryzae are effective towards all tested hydroxyl-PCBs, with higher chlorinated hydroxy-PCBs (HO-PCB) being less susceptible to laccase treatment than lower chlorinated HO-PCBs. Interestingly, these isoforms show different specificities in oxidation of HO-PCB congeners[121]. In a similar report, four *T. versicolor* laccase isoenzymes, expressed in *P.pastoris*, exhibit different efficiencies towards PAHs oxidation [111].

Laccases have also found interesting applications in biopulping and biobleaching of lignocellulosic materials for paper manufacturing[124,125]. Sigoillot and co-workers investigated the pulp bleaching efficiency of *P. cinnabarinus* laccase expressed in two distinct Aspergilli hosts, in comparison with the native enzyme. The results obtained, together with the observed differences in redox potentials of the recombinant laccases, have been ascribed to the host-specific processing[124]. In order to improve laccase treatments of pulp, Ravalson and co-workers synthesized a chimeric laccase by fusing *P. cinnabarinus* laccase lac1 to the carbohydrate binding module (CBM) of A. niger cellobiohydrolase B [125]. The chimeric protein was investigated for its softwood kraft pulp biobleaching potential in comparison with the native counter part. By conferring to the chimeric protein the ability to bind to a cellulosic substrate, CBM addition greatly improves laccase delignification properties. In a similar approach, laccases for bleaching carotenoid-containing stains on fabrics, have been engineered. Peptide sequences, selected for their ability to specifically bind to carotenoid stains, have been linked to C-terminus of *Stachybotrys chartarum* laccase. The targeted peptide-laccase fusion demonstrated enhanced catalytic properties on stained fabrics [126].

Fungal laccases are ideal green catalysts for many transformations in organic synthesis, spanning from oxidation of functional groups and coupling of phenols and steroids, to construction of carbon-nitrogen bonds and synthesis of complex polymers. In an interesting example, a recombinant laccase from *M. thermophyla*, supplied by Novozymes A/S, has been applied to the synthesis of a resveratrol dimer, a compound exhibiting promising antioxidant activity. The reaction has been carried out on a preparative scale, in very mild conditions resulting in improved yields in comparison with the analogous chemically catalyzed reaction. Improved tolerance to high concentrations of organic solvents is an enviable quality for laccase application in organic chemistry, since most of the transformations are carried out at high concentrations of organic solvents in which laccases may undergo unfolding, thereby losing their activity. Zumarraga et al. addressed this target by selecting an enzyme able to tolerate high concentrations of cosolvents after five rounds of directed evolution of a laccase from *M. thermophila*. Regarding immobilization, the recombinant expression of an histidine-tagged *Trametes sp*. strain C30 laccase has allowed the oriented binding of a fully active monolayer of laccases on a chemically modified gold electrode. Such an immobilization strategy may be useful to modulate the electrical communication between an electrode and a redox protein site bond on its surface, as well as to improve ligand detection in solution. Biocatalytical production of elemental iodine (I_2)-an attractive antimicrobial molecule-by oxidation of iodide, has been investigated using the recombinant laccases from *P. pinsitus* (rPpL), *M. thermophila* (rMtL), *C. cinereus* (rCcL), and *Rhizoctonia solani* (rRsL) in presence of methyl syringate as mediator. Tested enzymes show different kinetic behavior during the reaction. As a fact, the fitting kinetic data have revealed that the reversibility of the reaction increases for laccases with lower redox potential copper type I [127].

Recombinant laccases have also been employed as important bio-control measures to safe-guard or improve the quality and acceptability of food and beverages. For example, a *T. versicolor* UAMH 8272 laccase, has been successfully used for eliminating the highly toxic and mutagenic toxin AflatoxinB (AFB1) in food sources. The degradation has also shown to coincide with a significant and typical dose response loss of mutagenicity of the AFB1 molecule [128]. In another interesting report, a spectrophotometric method has been developed for antioxidant activity determination in

"rich with antioxidant" food samples, by using *P. pinsitus* and *M. thermophila* laccases expressed in *A. oryzae*. The method, based on simultaneous oxidation of the antioxidant and an highly reactive laccase substrate producing chromophoric radical cation, allows the detection of submicromolar concentration of an antioxidant.

The recent research on heterologous laccase expression focusing on the pivotal role played by recombinant systems towards the development of robust tools for greening modern industry as depicted in Figure 2.9. Enhanced protein production, and genetic tailoring of the enzyme profiles have been carried out successfully to construct novel recombinant enzymes for industry. When producing recombinant biocatalyst to address a specific industrial need, the choice of expression host strain cannot be made solely on the basis of production yields. Other aspects, such as regulatory issues, play a very important role in this choice. Moreover, patents and intellectual property rights call for searching for expression hosts other than the species traditionally used.

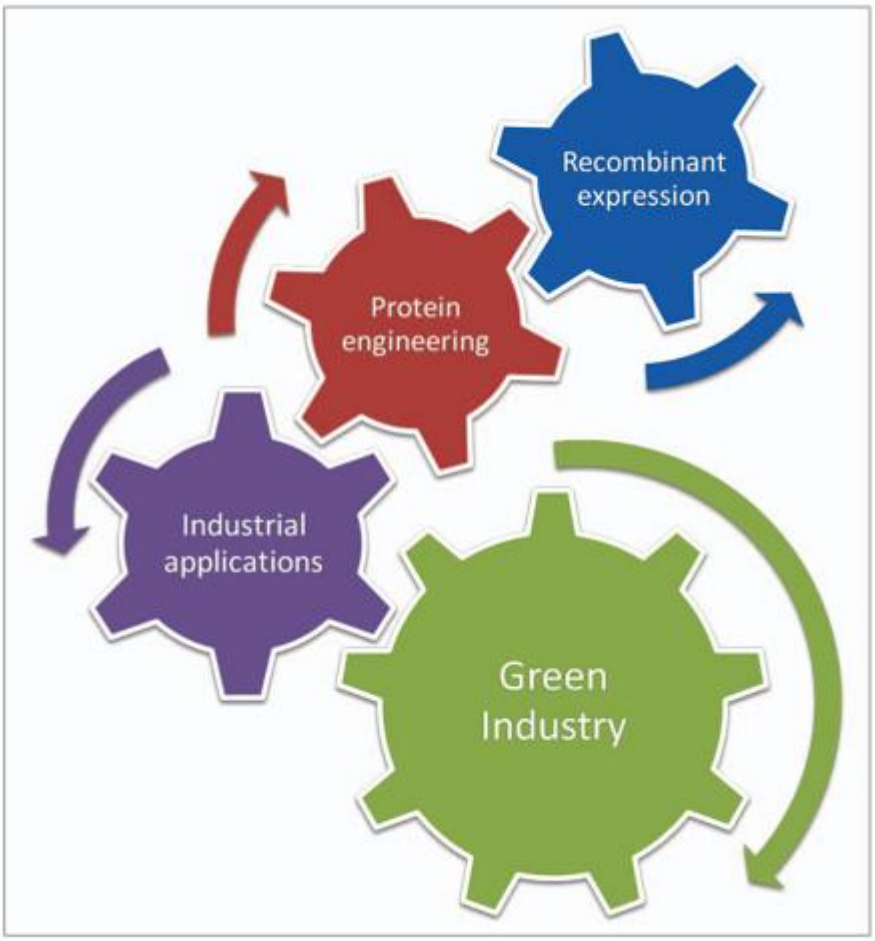

Figure 2.9 Schematic representation of the steps towards "greening" chemical industry.

2.9 APPLICATIONS OF LACCASES

A few laccases are at present in market for textile, food and other industries, and more candidates are being actively developed for future commercialization. A vast amount of industrial applications for laccases have been proposed and they include pulp and paper, textile, organic synthesis, environmental, food, pharmaceuticals and nanobiotechnology. Being specific, energy-saving, and biodegradable, laccase-based biocatalysts fit well with the development of highly efficient, sustainable, and eco-friendly industries.

2.9.1 Pulp and Paper Industry

In the industrial preparation of paper the separation and degradation of lignin in wood pulp are conventionally obtained using chlorine- or oxygen-based chemical oxidants. Non-chlorine bleaching of pulp with laccase was first patented in 1994 using an enzyme treatment to obtain a brighter pulp with low lignin content [129]. Oxygen delignification process has been industrially introduced in the last years to replace conventional and polluting chlorine-based methods. In spite of this new method, the pre-treatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also respect the integrity of cellulose [129-131]. Laccases are able to delignify pulp when they are used together with mediators [130]. Small natural low-molecular weight compounds with high redox potential (>900 mV) called mediators may be used to oxidize the non-phenolic residues from the oxygen delignification [131]. The mediator is oxidized by laccase and the oxidized mediator molecule further oxidizes subunits of lignin that otherwise would not be laccase substrates [132]. Although the LMS has been studied extensively, there are still unresolved problems concerning with mediator recycling, cost and toxicity. However, some environmental benefits are envisaged and the fact that LMS could be easily implemented in the existing bleaching sequences is seen as a major advantage that could possibly lead to a partial replacement of ClO_2 in pulp mills. Furthermore, the application of laccases in pulp-kraft bleaching may result in higher pulp yields and energy savings. Most of studies have been patented about the use of LMS in the pulp-kraft bleaching processes [132,133]. More recently, the potential of this enzyme for cross-linking and

functionalizing ligninaceous compounds was discovered [133]. In another related application, laccases can be even used for deinking and decolorizing a printed paper. Finally, laccases can be used for binding fiber-, particle- and paper-boards [134].

2.9.2 Textile Industry

Laccase is used in commercial textile applications to improve the whiteness in conventional bleaching of cotton and recently biostoning. Potential benefits of the application include chemicals, energy, and water saving. Cellulases were used to partially replace the load of pumice stones and laccases could bleach indigo-dyed denim fabrics to lighter shades [134]. Laccase also can be used in situ to convert dye precursors for better, more efficient fabric dyeing. In the last few years, various patents reported on coloration achieved with laccase. Laccases find potential applications for cleansing, such as cloth- and dishwashing [135]. Laccase may be included in a cleansing formulation to eliminate the odor on fabrics, including cloth, sofa surface, and curtain, or in a detergent to eliminate the odor generated during cloth washing.

2.9.3 Food Industry

Many laccase substrates, such as carbohydrates, unsaturated fatty acids, phenols, and thiol-containing proteins, are important components of various foods and beverages. Their modification by laccase may lead to new functionality, quality improvement, or cost reduction [135]. Sometimes O_2 is detrimental to the quality or storage of food/beverage because of unwanted oxidation. Laccases may be used as O_2-scavengers for better food packing. The flavor quality of vegetable oils can be improved with laccase by eliminating dissolved oxygen. Laccase can also deoxygenate food items derived partly or entirely from extracts of plant materials. Cacao was soaked in solutions containing laccase, dried and roasted in order to improve the flavor and taste of cacao and its products. The reduction of odors with laccase is documented in the patent literature. Treatment with a fungal laccase can also be performed to enhance the color of a tea-based product. It is also used to perform the cross-link of ferulic acid and sugar beet pectin through oxidative coupling to form gels for food ingredients. Various enzymatic treatments have been proposed for fruit juice stabilization, among which it

can be found the use of laccase [136]. Laccases are added to the dough used for producing baked products, to exert an oxidizing effect on the dough constituents and to improve the strength of gluten structures in dough and/or baked products [136].

Wine stabilization is one of the main applications of laccase in the food industry as alternative to physical-chemical adsorbents [137]. Musts and wines are complex mixtures of different chemical compounds, such as ethanol, organic acids (aroma), salts and phenolic compounds (color and taste). Polyphenol removal must be selective to avoid an undesirable alteration in the wine's organoleptic characteristics. Laccase presents some important requirements when used for the treatment of polyphenol elimination in wines, such as stability in acid medium and reversible inhibition with sulphite [138]. Laccases are also used to improve storage life of beer. Haze formation in beers is a persistent problem in the brewing industry. Nucleophilic substitution of phenolic rings by protein sulphydryl groups may lead to a permanent haze that does not re-dissolve when warmed. As an alternative to the traditional treatment to remove the excess of polyphenols, laccase could be added to the wort [137]. Other studies of laccase application for phenolic compounds removal have also been patented. A laccase has recently been commercialized (Suberzyme®) for preparing cork stoppers for wine bottles.

2.9.4 Bioremediation

Laccases have many possible applications in bioremediation [139]. Laccases may be applied to degrade various substances such as undesirable contaminants, by products, or discarded materials. Laccase may be applied to degrade plastic waste having olefin units. Likely, an oxidation of the olefin units by the LMS, could initiate a radical chain reaction, leading to the disintegration of the plastic. Also this LMS can be used to degrade polyurethanes. LMS facilitated the degradation of phenolic compounds (environmental hormones) from biphenol and alkylphenol derivatives and also the decomposition of fluorescent brighteners. Laccase may also be used to eliminate odor emitted from places such as garbage disposal sites, livestock farms, or pulp mills. Also, they could be used for decolorizing dye house effluents that are hardly decolorized by conventional sewage treatment plants [140,141]. In addition to dye house effluents,

laccases can decolorize waste waters from olive oil mills and pulp mills by removing colored phenolic compounds [142].

Another potential environmental application for laccases is the bioremediation of contaminated soils, as laccases and LMS are able to oxidize toxic organic pollutants, such as various xenobiotics, PAHs, chlorophenols, and other contaminants [143]. Phenolic compounds are present in wastes from several industrial processes, as coal conversion, petroleum refining, production of organic chemicals and olive oil production among others [143]. Immobilized laccase was found to be useful to remove phenolic and chlorinated phenolic pollutants [144]. Laccase was found to be responsible for the transformation of 2,4,6-trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone. LMSs have been also used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene. Isoxaflutole is an herbicide activated in soils and plants to its diketonitrile derivative, the active form of the herbicide: laccases are able to convert the diketonitrile into the acid. Laccase can be also used to reduce the concentration of synthetic heterocyclic compound such as halogenated organic pesticides in the soil. LMS has been extensively study in the oxidation of recalcitrant PAHs, main components of several ship spills. In this sense, LMS is being included in several enzymatic bioremediation programs [145].

2.9.5 Organic Synthesis

Recently, increasing interest has been focused on the application of laccase as a new biocatalyst in organic synthesis. Laccase provided an environmentally benign process of polymer production in air without the use of H_2O_2 [146,147]. Laccase-catalyzed cross-linking reaction of new urushiol analogues for the preparation of “artificial urushi” polymeric films (Japanese traditional coating) was demonstrated. It is also mentioned that laccase induced radical polymerization of acrylamide with or without mediator. Laccases are also known to polymerize various amino and phenolic compounds [148]. Recently, to improve the production of fuel ethanol from renewable raw materials, laccase from T. versicolor was expressed in S. cerevisiae to increase its resistance to (phenolic) fermentation inhibitors in lignocellulose hydrolyzates. The preparation of crosslinked enzyme aggregrates with aldehydes and amines had

improved stability and was used in starch oxidation. Selective oxidation of the primary hydroxyl groups of sugars using the LMS is described in patent literature dealing with the partial oxidation of cellulose and other polysaccharides. Also, LMS was used for the determination of monoclonal antibody of azelaic acid from oleic acid. The enzymatic preparation of polymeric polyphenols by the action of laccases has been investigated extensively in the past decades as a viable and non-toxic alternative to the usual formaldehyde-based chemical production of these compounds. Laccase can also be used to synthesize various functional organic compounds including polymers with specific mechanical/electrical/optical properties, textile dyes, cosmetic pigments, flavor agents, and pesticides [149].

2.9.6 Pharmaceutical Sector

Many products generated by laccases are antimicrobial, detoxifying, or active personal-care agents. Due to their specificity and bio-based nature, potential applications of laccases in the field are attracting active research efforts. Laccase can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory, antibiotics, sedatives, etc. [149] including triazolo(benzo)cycloalkyl thiadiazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins, and dimerized vindoline [139,150]. One potential application is laccase-based in situ generation of iodine, a reagent widely used as disinfectant [139]. Also, laccase has been reported to possess significant HIV-1 reverse transcriptase inhibitory activity. Another laccase has been shown capable of fighting aceruloplasminemia (a medical condition of lacking ceruloplasmin, a multi-Cu serum oxidase whose ferroxidase activity regulates iron homeostasis) [151]. Some years ago, a new enzymatic method based on laccase was developed to distinguish simultaneously morphine and codeine in drug samples injected into a flow detection system. A novel application field for laccases is in cosmetics. For example, laccase-based hair dyes could be less irritant and easier to handle than current hair dyes [139] More recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed. Laccases may find use as deodorants for personal-hygiene products, including toothpaste, mouthwash, detergent, soap, and diapers. Protein engineered laccase may be used to reduce allergenicity [139].

2.9.7 Nanobiotechnology

Nanoscience has grown rapidly in the last decade. Recently, more attention is focused on the applications of nanotechnologies. The high potential impacts of nanotechnology almost cover all fields of human activity (environmental, economy, industrial, clinical, health-related, etc). Nanostructured materials (nanoparticles, nanotubes, and nanofibers) have been used extensively as carrying materials for biosensoring, and biofuel cells.

A biosensor is an integrated biological-component probe with an electronic transducer, thereby converting a biochemical signal into a quantifiable electrical response that detects, transmits and records information regarding a physiological or biochemical change. Some of the major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability, real-time analysis and operation simplicity. Thus laccases can be applied as biosensors or bioreporters. A number of biosensors containing laccase have been developed for immunoassays, and for determination of glucose, aromatic amines and phenolic compounds [139]. Laccase catalysis can be used to assay other enzymes [139]. Laccase covalently conjugated to a bio-binding molecule can be used as a reporter for immunochemical (ELISA, Western blotting), histochemical, cytochemical, or nucleic acid-detection assays. The bioreporter applications are of interest for the high-sensitivity diagnostic field.

In addition to biosensors, laccases could be immobilized on the cathode of biofuel cells that could provide power, for example, for small transmitter systems [152]. Fuel cells are very attractive energy sources, particularly at micro-, mini-, portable-, or mobile-scale, that potentially have higher energy conversion/usage efficiency and lower pollution effect than any of the existing/emerging energy sources.

For example, a bio-implantable electrochemical cell system for active implantable medical devices is described by Choi. In one embodiment, the fuel cell includes an electrode structure consisting of immobilized anode and cathode enzymes deposited on nanostructured high-surface-area metal nanowires or carbon nanotube electrodes as depicted in Figure 2.10. The anode enzyme comprises immobilized glucose oxidase and

the cathode enzyme comprises immobilized laccase. Glucose is oxidized at the surface of the anode and oxygen is reduced at the surface of the cathode. The coupled glucose oxidation/oxygen reduction reactions provide a self-generating current source. In another embodiment, the nanowires or carbon nanotubes, along with the adjacent surface anode and cathode electrodes, are coated with immobilized glucose oxidase and immobilized laccase containing biocolloidal substrates, respectively. This results in the precise construction of enzyme architecture with control at the molecular level, while increasing the reactive surface area and corresponding output power by at least two orders of magnitude [152].

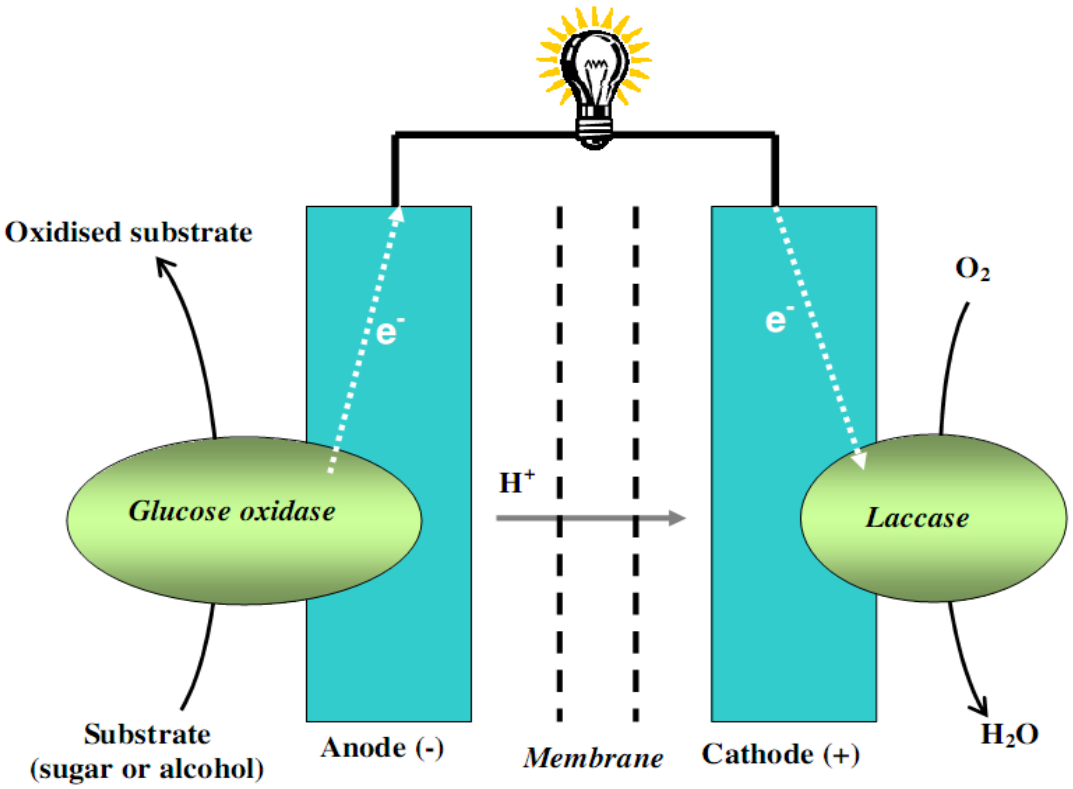

Figure 2.10 Schematic representation of a biofuel cell involving glucose oxidase and laccase enzymes.

Laccase may be applied as a biocatalyst for the electrode reactions. Laccase-based miniature biological fuel cell is of particular interest for many medical applications calling for a power source implanted in a human body [153].

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

All laboratory chemicals were analytical grade from Sigma –Aldrich (Germany), Merck (Germany), Riedel-de Haen (Germany), Fluka (Switzerland), Bio-Rad (USA), Fermentas (USA).

Table 3.1 List of Chemicals

Acetic acid	Merck (Germany)
Acrylamide	Sigma –Aldrich (Germany)
Agarose	Sigma –Aldrich (Germany
Ammonium persulphate (APS)	Merck (Germany)
Ammonium sulfate	Riedel-de Haen (Germany)
2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS)	Sigma- Aldrich (Germany)
B-Mercaptoethanol	Merck (Germany)
Bovine serum albumine (BSA)	Sigma-Aldrich (Germany)
Bromophenol Blue	Sigma-Aldrich (Germany)
Bisacrylamide	Sigma-Aldrich (Germany)
Boric acid	Merck (Germany)
Calcium chloride dihydrate	Merck (Germany)
Cobalt chloride hexahydrate	Riedel-de Haen (Germany)
Coomassie Brilliant Blue	Bio-Rad (USA)
Copper sulfate pentahydrate	Merck (Germany)
Glucose	Sigma-Aldrich (Germany)
Glycine	Merck (Germany)
Glycerol	Fluka (Switzerland)

Hydrochloric acid	Merck (Germany)
Manganese chloride tetrahydrate	Merck (Germany)
Manganese sulfate monohydrate	Merck (Germany)
Methanol	Merck (Germany)
Protein molecular weight marker	Fermentas (USA)
Sodium acetate	Merck (Germany)
Sodium bicarbonate	Riedel-de Haen (Germany)
Sodium carbonate	Merck (Germany)
Sodium dodecyl sulphate	Sigma-Aldrich (Germany)
Sodium fluoride	Merck (Germany)
Sodium hydroxide	Riedel-de Haen (Germany)
Sodium dihydrogen phosphate	Riedel-de Haen (Germany)
Sodium tartarate dihydrate	Merck (Germany)
Tartaric acid	Riedel-de Haen (Germany)
TEMED	Sigma-Aldrich (Germany).
Tris-HCl	Merck (Germany)
Yeast extract	Merck (Germany)

3.1.2 Laboratory Equipments

Table 3.2 List of Equipments

Autoclaves	Nüve OT 4060 Steam Sterilizer (Turkey)
Balances	Precisa XB620C (Germany)
Cellulose nitrate fitler 0.20 µm cellulose nitrate membrane filter	Sartorius (USA)
Centrifuges	Beckman Coulter (Germany)
Deep Freezers	- 20 ºC Arçelik (Turkey) - 80 ºC New Brunswick Scientific U410 Premium (England)
Electrophoresis equipment	Bio-Rad (USA)
Ice machine AF 10	Scotsman (UK)
Incubators	Nüve EN400 (Turkey)

Magnetic stirrers	Labworld (Germany)
Microplate reader	Model 3559 UV Microplate, Bio-Rad
Micropipettes	Thermo (USA), 1000µl, 200µl, 100µl, 10µl
Microwave Oven	Beko,(Turkey)
Orbital Shakers	Thermo (USA)
pH-meter	Mettler Toledo MP220 (Switzerland)
Power Supplies	Bio-Rad Power PAC (USA)
Thermocycler	Techne(UK)
Transilluminator	Bio-Rad GelDoc 2000 (USA)
UV-Visible Spectrophotometers	NanoDrop, Thermo (USA)
Vortex apparatus	Heidolph Reax top (Germany)
Water Purification System	Millipore(Germany)
Waterbath	Nüve (Turkey)

3.1.3 Preparatıon of Media and Reagents

3.1.3.1 Shewanella Culture Medium

Shewanella putrefaciens was provided from ATCC and number was 8071. Luria-Bertani (LB) agar and broth (Merck) were used for the cultivation of *Shewanella putrefaciens.* The composition of the growth media for *S. putrefaicens* was as follows:

Luria-Bertani (LB) broth medium: (per litre)

Tryptone (pancreatic digest of casein)	10 g
Yeast extract	5 g
NaCl	5 g

For the preparation of 500 mL LB broth medium, 10 g powder was suspended in 500 mL of disstilled water. The media were autoclaved at 121^{o}C for 15 minutes, then cooled to 50^{o}C, and stored at 4^{o}C until use.

Luria-Bertani (LB) agar medium: (per litre)

Tryptone (pancreatic digest of casein)	10 g
Yeast Exract	5 g
NaCI	5 g
Agar	15 g

For the preparation of 500 mL LB agar medium, 17.5 g powder was suspended in 500 mL of disstilled water. The suspention was heated to boiling while stirring to dissolve all ingredients completely. The media were autoclaved at 121^{o}C for 15 minutes, then cooled to 50^{o}C. Afterwards, the sterile media were poured into sterile Petri dishes. Petri dishes were cooled in order to solidify the media under aseptic conditions, and then sealed with stretch film and stored at 4^{o}C until use.

3.1.3.2 Media for The Growth of E. Coli

LB is the most commonly used medium for the growth and expression of *E. coli*. After sterilization of LB broth, the medium was allowed to cool to ~55°C before adding antibiotic (either ampicillin, 100 μg/ml final concentration; or chloramphenicol, 100 μg/ml final concentration).

For preparation of LB plates plus antibiotic; after autoclaving, the medium was allowed to cool to 50°C before adding ampicillin to a final concentration of 100 μg/ml and poured the plates. 30–35ml of medium was poured into 100 mm petri dishes. The agar was let to harden. All plates were stored at 4°C for up to 1 month or at room temperature for up to 1 week.

3.1.4 Molecular Biology Kits

All of the molecular biology kits used for DNA isolation, plasmid isolation, and PCR purification are listed below;

Table 3.3 List of Molecular Biology Kits

High Pure PCR Template Preparation kit	Roche (Germany)
High Pure PCR Template Purification Kit	Roche (Germany)
High Pure Plasmid Isolation Kit	Roche (Germany)
Rapid DNA Ligation kit	Fermentas (USA)
Transform Aid Bacterial Transformation Kit	Fermentas (USA)

3.1.5 Buffers and Solutions

3.1.5.1 Ampicillin Stock Solution

100 mg/ml of ampicillin was dissolved in water, filter sterilized, stored in aliquots at -20°C.

3.1.5.2 Chloramphenicol Stock Solution

100 mg/ml of chloramphenicol was dissolved in alcohol, filter sterilized, stored in aliquots at –20°C.

3.1.5.3 IPTG stock Solution

1.2g of IPTG was added deionized water to a final volume of 50ml. Filter-sterilize and store at 4°C.

3.1.5.4 ABTS Stock Solution

The ABTS test was performed in 50mM acetate buffer pH 5.0 with 2mM final concentration of the substrate.

3.1.5.5 SDS-PAGE Solutions and Buffers

Monomer solution for SDS-PAGE: 58.4 g acrylamide, 1.6 g bisacrylamide was dissolved in 200 ml of distilled water and stored at 40^0C in the dark.

4X Running (Separating) gel buffer for SDS-PAGE: 1.5 M Tris-HCl (pH 8.8) was dissolved in 200 ml of distilled water.

4X stacking gel buffer for SDS-PAGE: 0.5 M Tris-HCl (pH 6.8) was dissolved in 50 ml of distilled water.

2X sample buffer for SDS-PAGE: 2.5 ml 4X Stacking buffer, 4 ml SDS solution (10%), 2 ml Glycerol, 1 ml β-Mercaptoethanol and 0.05 % (w/v) Bromophenol blue were dissolved in 10 ml of distilled water.

Running buffer for SDS-PAGE: 3 g Tris, 14.4 g Glycine, 10 ml SDS (0.1%) were dissolved in 1 L of distilled water.

Coomassie Blue Staining Solution for SDS-PAGE: 0.1 g Coomassie brillant blue, 50 ml Methanol and 10 ml Acetic acid were dissolved in 100 ml of distilled water.

Gel Destain Solution for SDS-PAGE: 5 ml Methanol, 10 ml Acetic acid were dissolved in 100 ml of distilled water.

Ammonium persulphate (APS) concentration 10% (w/v)

Sodium dodecyl sulphate (SDS) concentration 10% (w/v)

3.1.6 Bacterial Strains

Shewanella putrefaciens were purchased from ATCC (USA) number of 8071.For sub cloning purposes *Escherichia coli* XL1 Blue cells (Invirtogen, USA) are used.During expression studies, *Escherichia coli* BL21(DE3)pLysS cells (Invirtogen, USA) are used.

3.1.7 Plasmids and Vectors

pet14B vectors are purchased from Novagen(Germany).

3.1.8 Primers

All primers were designed by primer3 software and purchased from Gen Ova Medical (Istanbul,TR). The primers were diluted using PCR grade water according to the manufacturer’s instructions.

3.1.9 Enzymes

Table 3.4 List of Enzymes

Restriction enzymes	Nde1, BamH1 (NEB enzyme, UK)
PCR amplification	Primestar HS DNA polymerase (Takara, Japan) Taq DNA Polymerase(Takara, Japan)
Ligation	T4 DNA ligase (Fermentas,USA)
Dephosphorylation	Calf Intestinal Alkaline Phosphatase (CIAP) (Fermentas,USA)

3.2 METHODS

3.2.1 Cultıvatıon of Microorganısms

3.2.1.1 Aerobıc Growth of Shewanella putrefaciens ATCC 8071

S. putrefacience was grown on LB agar at 25°C. For aerobic growth, fresh cultures of 200 ml in 1000 ml flasks were shaken continuously on a rotary shaker (Sartorious Certomat IS) at 160 rpm and 25°C. After 36 h of growth, the suspention were pipetted into a 50 ml sterile Falcon tubes and centrifuged at 13,000 X g, for 15 min at 4°C. After centrifugation, the supernatant was discarded and the pellet was washed three times with 10 ml of 50 mM Na-phosphate buffer, pH 7.0, containing 0.1 M NaCl.

After sub-culturing of *Shewanella putrefaciens*, 0.5 ml of fresh culture was aliquoted into 5 sterile 1.5 ml Ependorf tube and stored, following the addition of sterile 50% glycerol in each tube, at -70°C for up to 1 year.

3.2.1.2 Growth of E. coli

Escherichia coli XL-1 Blue and BL21 (DE3) pLysS served as host for DNA manipulation. *E. coli* transformants were grown at 37°C in the Luria- Bertani (LB) broth and on LB agar plates containing 100 µg of ampicillin and 50 µg of chloramphenicol per ml with shaking.

3.2.2 Cloning of Laccase Gene into pET14-B Vector

3.2.2.1 Isolatıon of Genomıc DNA from Shewanella putrefaciens ATCC 8071

Genomic DNA isolation was performed using High Pure PCR Template preparation kit (Roche, Germany) according to the manufacturer's instructions. Quantification of total DNA was carried out by absorbance at 260 and 280 nm using aNanoDrop. The absorbance at 260 and 280 nm ratio (260/280) was used to assess the purity of the DNA.

3.2.2.2 Amplification of Laccase Gene

One of the most important cloning strategy is the addition of Restriction Endonuclease Sites into PCR product if necessary. For that reason, the oligonucleotide primers were designed to include restriction enzyme recognition sites near their 5' termini.

Suitable forward and reverse primers including the appropriate restriction-enzyme site at the 5' end were designed using a websites that were useful in aiding primer design. A particularly good one used in this study was Primer 3 (www-genome.wi.mit.edu/cgi-bin/primer/ primer3_ www.cgi). In order to amplify the laccase gene of *Shewanella putrefaciens* nested PCR strategy was used. Both first and second PCR primers listed on Table 2.1. Primers, F1 and R1 were used to first amplify the laccase gene. At the end of the first PCR, amplicon expected size was ~1200 bp. A second set of primers, F2 and R2 used for the second PCR was designed in this study. The second protocol (nested PCR) was predicted to amplify a 754 bp DNA fragment.

Primer F2 (5'-TCCATATGTTTAATCACGATTGGC-3'), containing a *NdeI* restriction site at 3' end was used as the forward primer for the second PCR. The reverse primer, R2 (5'- GGATCCAAGCTGTAAGGGAATTAG -3'), contained a *BamHI* restriction site. Both primers were obtained from Gen Ova Medical (Istanbul, TR).

3.2.2.3 Nested PCR Conditions

PCR was carried out in a Thermocycler Techne TC512 Gradient Termal Cycler in thin-walled 0.2 ml PCR tubes. The first PCR protocol was as follows: 2 µl (~200-300 ng) of genomic DNA was added to a 25 µl reaction containing 4 µl dNTPs (10µM) (Fermentas), 1.5 µl (0.5 µM) forward (F1) and reverse primers (R1) (Genova, Turkey), 0.5 µl Primestar HS DNA polymerase (Takara), and 10µl 10X Primestar HS DNA polymerase buffer with Mg^{++} (Takara), 32.5 µl PCR graded H_2O. The reaction mixture was spin down for 10 s and performed for the first PCR reaction. The PCR conditions were as follows; initial denaturation at 94ºC for 2 minutes followed by 30 cycles of denaturation step (at 98ºC for 10 seconds), annealing step (at 58ºC for 30 seconds) and an extension step (at 72ºC for 50 seconds). These cycles were then followed by a final extesion step at 72ºC for 5 minutes.

Table 3.5 Oligonucleotide primers used for PCR of laccase gene of *Shewanella putrefaciens*

Primer	T_M (^oC)	Length of primers (bp)	Oligonucleotide Sequences
F1	61	23	5'- GCCACGTATTAGTGGGCGATCC
R1	57	24	5'- TGAACGGCTTTGAATTAGCGTGTC
F2	62	24	5'- TCCATATGTTTAATCACGATTGGC
R2	62	24	5'- GGATCCAAGCTGTAAGGGAATTAG

The expected PCR product size was 1200 bp. First PCR product was run on a 1,5% TAE gel stained with ethidium bromide (5 mg/ml) to confirm successful amplification. 1: 100 dilution of first round PCR reaction was made to be used as template for the second round of PCR. This reaction mixture contains 10 µl 10 X buffer (Takara), 4 µl 0,2 µM of all four dNTPs, 1,5 µl (10 µM) second set of primers, F2 and R2, 0,5 µl Prime Star DNA polymerase, 1 µl of template DNA and 32,5 µl PCR graded

H_2O in a 50 µl reaction volume. A second round was performed for 30 cycles and all conditions were same first round PCR. The reaction was ended and cooled to 4^oC for storage. Amplification products were fractionated by electrophoresis in 1,5% agarosa / TAE gels and appropriate bands (about 754 bp) were seen.

3.2.2.4 Gel Electrophoresis

The PCR products were run at 80V for 30 minutes using 1.5% agarose gels. Agarose gels were prepare using 1XTAEthathad been prepared according to the protocol in Molecular Cloning: A Laboratory Manual. The gel was examined under UV light in order to determine whether DNA band of interest was present on the appropriate place on the gel according to molecular size marker or not. Only a band was detected about 754bp. This band was purification with the High Pure PCR Purification Kit (Roche, Germany) as per the manufacturer's instructions. Concentration of DNA in the samples was measured by spectrophotometry by taking the OD_{260} reading and using the NanoDrop.

3.2.2.5 Restriction Enzyme Digestion of PCR Product and Vector

Restriction of the PCR product and vector were done according to the protocols provided by NEB. We used Nde1 and BamH1 restriction endonucleases.

PCR product was digested with Nde1 and BamH1. The reaction mixture was pipetted and spinned to ensure that the contents was at the bottom of the microcentrifuge tube. The tube was incubated 1 hour at 37°C. Following the double digestion of insert, mixture was purified to remove the enzyme with High Pure PCR Purification Kit (Roche, Germany).

The following were added to a 0,6 mL tube:

Restriction Enzyme Buffer	10 µl
Insert DNA	80 µl
BamH1	1 µl
Nde1	1 µl
Sterile dd H_2O	8 µl
Final Volume	**100 µl**

Pet14b vector was digested with Nde1 and BamH1.

The following was added to a 0.6 mL tube on ice:

10X Restriction Buffer	5 µl
Vector DNA (~1 µg)	5 µl
BamH1 (10u/µl)	1 µl
Nde1 (10u/µl)	1 µl
Sterile ddH_2O	38 µl
Final Volume	**50 µl**

The suspention was mixed by pipetting and spinned to ensure that the contents was at the bottom of the microcentrifuge tube. The tube was incubated 1 hour at 37°C. Dephosphorylation of digested vector was performed with CIAP (calf intestinal alkaline phosphatase) enzyme immediately. For that purpose, 2 µl of CIAP (1u/ µl) was added directly to the digested vector DNA sample to prevent the self- ligation of the cut plasmid. The reaction mixture was incubated at 37°C for 1 hour. Afterwards, CIAP was inactivated by heating at 70 °C for 15 minutes.

3.2.2.6 Gel Extraction of The Vectors

The cut vectors were run at 85V for 45 minutes using 1% agarose gels. Agarose gels were prepared using 1XTAEthat had been prepared according to the protocol in Molecular Cloning: A Laboratory Manual, Sambrook et al., 2001. High Pure PCR Template Purification Kit (Roche, Germany), then used to extract the vectors.

3.2.2.7 Ligation

Plasmid DNA and purified PCR fragment were digested with the appropriate restriction enzyme for ligation. Then, plasmid DNA was dephosphorylated with calf intestine alkaline phosphatase (Fermentas). Dephosphorylation reaction was done according to the protocols provided by Fermentas. Digested and dephosphorylated plasmid DNA and digested PCR fragment were then purified the High Pure PCR Purification Kit (Roche, Germany). Ligation reaction was performed with a vector-insert molar ratio of 1:3 using vector and insert using Rapid DNA Ligation kit from Fermentas. The reaction were done according to the protocols provided by Fermentas. Then 5 µl aliquots of the ligation reaction were used to transform E. coli XL1 Blue.

3.2.2.8 Transformation into Competent XL-1 Blue Cells

Competent cells were prepared using Transform Aid Bacterial Transformation Kit(Fermentas) according to the manufacturer's instructions.

The transformation was carried out as follows: One tube containing 250 µL of *E. coli* XL-1 Blue competent cells was removed from -80^{o}C and thawad on ice. For each transformation, 75 µL of bacterial suspension was aliquoted into a sterile Eppendorf tube. Approximately 1 ng of plasmid DNA (control) was added into the tube containing competent cell for the control of transformation, and 5-10 µL ligation products were added into the other tube and mixed gently. Pipet up and down or vortex the tube was not done. For each set of transformations, a negative control that consists of competent cells without DNA, and a positive control using a standard plasmid were prepared. The transformation mix was placed on ice for 20 min. The transformation mix was transferred to a 42°C water bath and incubated for exactly 45 s. The temperature and

time was crucial in this step. The tubes were not shaked. The transformation mix was immediately placed on ice for 2 min. 900 µL of room temperature LB broth was added. The tubes were incubated at 37°C for 30 minutes, shaking vigorously (approx 250 rpm) to ensure good aeration. A range of serial dilutions of the bacteria was prepared from the transformation mix (50 µL and 100 µL from each transformation) and spreaded on to the prewarmed LB agar plates containing 50 µg of ampicillin per ml. The agar plates were incubated overnight at 37°C.

After incubation, at least five colonies were selected and each colony was inoculated into 3 ml of LB broth medium containing ampicillin (50 µg /ml). Then each tube was incubated at 37°C until reached with a density of 1.5-5.0 A_{600} units per ml. Arterwards, plasmid DNA was extracted from each transformants using High Pure Plasmid Isolation kit (Roche) and analyzed by restriction digestion.

3.2.2.9 Screening of Clones by PCR, Restriction Enzyme Digestion and Sequence analysis

This technique was used to screen for containing of a laccase gene insert into the pet14B vector. Colony PCR was performed with the insert-specific forward and reverse PCR primer. Ten colonies were chosen from the cloning experiment for analysis. Colony preparation for PCR was as the following: The transformation plates were examined and ten well-isolated colonies were picked, then transferred to 3 ml sterile LB broth containing appropriate ampicillin for overnight culture and miniprep. Each colony to be screened was labelled. When the colonies incubated at least 3 hours, 10 µl of culture from each tube was taken for performing colony PCR. 10 µl of grown culture from each colony was diluted in 90 µl sterile water, respectively. Each suspention were boiled for 5 min at 95°C in a thermal cycler. 2 µl of the boiled culture was used in each amplification as a template. Positive clones with a suitable size DNA fragment insert were screened by PCR. A master PCR reaction protocol was; 2 µl of µl of bacterial cell suspension was added to a 25 µl reaction containing 4 µl dNTPs (10µM) (Fermentas), 1.5 µl (0.5 µM) forward (F2) and reverse primers (R2), 0.5 µl Primestar HS DNA polymerase (Takara), and 10µl 10X Primestar HS DNA polymerase buffer with Mg^{++} (Takara), 32.5 µl PCR graded H_2O. PCR conditions were as follows: 1 cycle of 94ºC for

2 min for initial denaturation; 30 cycles of 98ºC for 10 sec, 58ºC for 10 sec and 72ºC for 50 sec; then a final extention at 72ºC for 10 min before storage at 4ºC. Amplification products (10 µl) were analyzed on a 1% agarose gel. Positive clones with insert laccase gene were stored at -70ºC in 50 % glycerol. As an important note here, the amount of bacteria needed for PCR screening is usually very small. Too many bacteria in the mix will inhibit the PCR reaction.

In addition to screen transformants by PCR, a plasmid miniprep followed by restriction digestion was performed. Well-isolated colonies were picked from a plate and transferred to LB broth culture medium containing the appropriate antibiotic for selection. All cultures were incubated over night with shaking (~250 rpm). The plasmid isolation experiment was performed using a High Pure Plasmid Isolation (miniprep) kit (Roche).

Once the DNA was purified, a portion of plasmid was screened by restriction digestion. 1 µg of plasmid was used in our digest. BamH1 and Nde1 were used for screening the orientation of the insert.

The following components were assembled to a 1,5 mL Eppendorf tube:

Restriction Enzyme Buffer	5 µl
Plasmid DNA (~ 1,5 µg)	30 µl
BamH1	1 µl
Nde1	1 µl
Sterile dd H_2O	13 µl
Final Volume	**50 µl**

Reaction mixture was incubated 1hour at 37ºC.Then, all tubes were analysed on 1% agarose gel and positive clones were sequenced by IONTEC.

3.2.3 Heterologous Expression of Laccase Gene form pet14-b/laccase plasmide in *E. coli* BL21 (DE3) pLysS

The PCR product was digested with Nde1 and BamH1 and then ligated into Nde1 and BamH1–treated expression vector pET-14b and transformed into E.coli BL21(DE3)Plyss. The E.coli cells transformed with this plasmid were plated on LB agar containing 100 μg/ml ampicillin.The transformant was grown in a 10 ml flask containing 5 ml LBmedium supplemented with 100 μg/ml ampicillin and 100 μg/ml chloramphenicol at 37°C shaking 250 rpm overnight. Next day we used 1:100 dilution overnight culture into 50 ml flask containing 20 ml LB and no antibiotics.The optical density at 600 nm reached to 0.6–1.0, and then 1 Mm IPTG and 0.2 mM $CuSO_4$ of the final concentration were added to induce target protein expression. We collected expressed protein each hour by centrifugation and were stored at -20°C until further use.

3.2.4 Characterızatıon of Laccase

3.2.4.1 SDS-PAGE

SDS-PAGE was carried on a 10 % separating gel and a 5 % stacking gel. When all the samples had been collected from protein induction step, each cell pellet was resuspended in 100 μl of 2X SDS-PAGE sample buffer and was vortexed. The cell suspention was boiled for 5 minutes and incubated on ice for 2 minutes. Then, all tubes were centrifuged at 11,000 rpm for 2 minutes. The gel apparatus was assembled and filled with 1X SDS-PAGE running buffer. 15 μl of each sample was loaded on an SDS-PAGE gel and electrophoresed. The samples were saved by storing them at -20°C. When the dye front of the sample buffer reached the bottom of the plate, the gel was removed. In order to analyze the samples, the polyacrylamide gel was stained with Coomassie blue for 1 h with slow shaking. The gel was incubated in destain solution with slow shaking, until the protein bands were clearly visible. The gel was looked for a band of increasing intensity in the expected size range for the recombinant protein. The uninduced culture was used as a negative control. Molecular Weight Standard (Fermentas), was loaded in the same gel for control identification. Samples were seperated by electrophoresis for 1 hour at 120 volts.

3.2.4.2 Laccase Enzyme Assay

The non-phenolic dye 2,2'-azinobis-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) is oxidized by laccase to a cation radical. The concentration of the cation radical responsible for the intense blue-green colour can be correlated to the enzyme activity and is most often read 420 nm After the cell culture was removed by centrifugation (20 min at 13,500 x g), laccase activities in the culture pellet were routinely determined by measuring the oxidation of ABTS (2.5 mM) in a sodium acetate buffer (0.1 M, pH 5.0) at 420 nm (ε = 36 mM-1cm-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 UV-Vis Spectrophotometer. The reaction mixture which contained 950 µl sodium acetate buffer, 200 µl substrate and 50 µl enzyme, loaded into spectrophotometer and measured at 420 nm for 5 minutes of reaction time at 28°C.

3.2.4.3 Effect of Temperature on The Enzyme Activity

The effect of temperature on laccase acitivity was determined by incubating the laccase in Na-acetate buffer (0.1 M, pH 5.0) for 2 minutes at various temperatures ranging 20^{o}C to 80^{o}C and then determining the laccase activity with the ABTS assay method.

3.2.4.4 Effect of pH on The Enzyme Activity

The influence of pH on laccase activity was studied spectrophotometrically. The pH dependence was determined with ABTS (2.5 mM) as the substrate in 50 mM glycine-HCl buffers (pH 2.0 to 3.0), 50 mM sodium acetate buffers (pH 4.0 to 5.0) and 50 mM sodium phosphate buffers (pH 6.0 to 7.0).

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 MOLECULAR CLONING OF LACCASE GENE INTO pET14-b VECTOR AND HETEROLOGOUS EXPRESSION OF LACCASE GENE

In this study, in order to express *Shewanella putrefaciens laccase* gene in, pet14b (as depicted in Figure 4.1) vectors were used to construct and transcript a recombinant laccase enzyme in prokaryotic host cells. After performing of the plasmid constraction, laccase gene was tried to express into *E. coli* host cells.

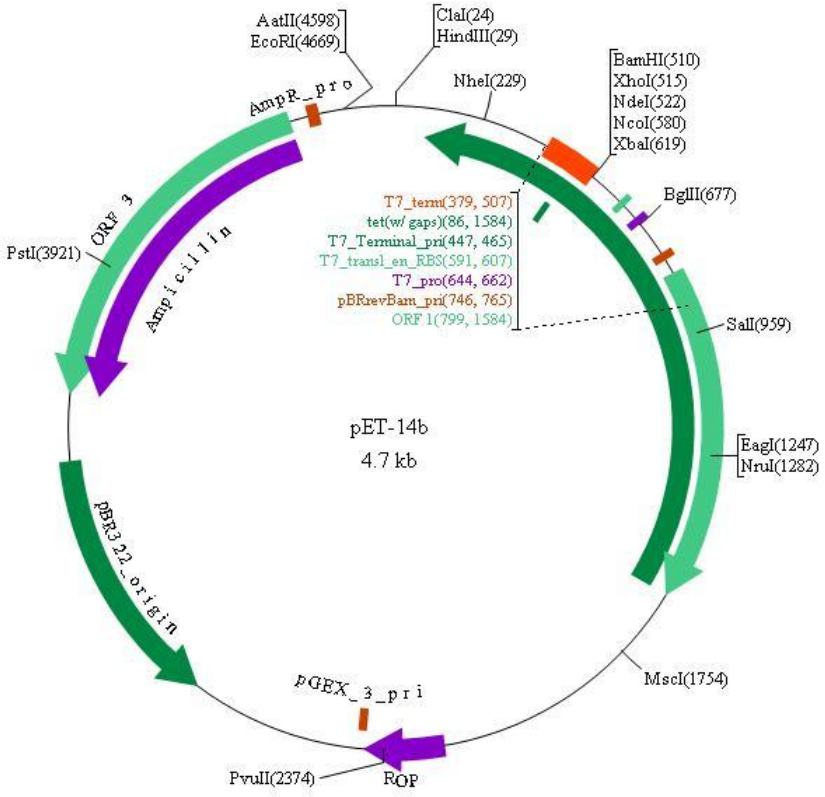


Figure 4.1 Schematic represantation pET14b expression vector map.

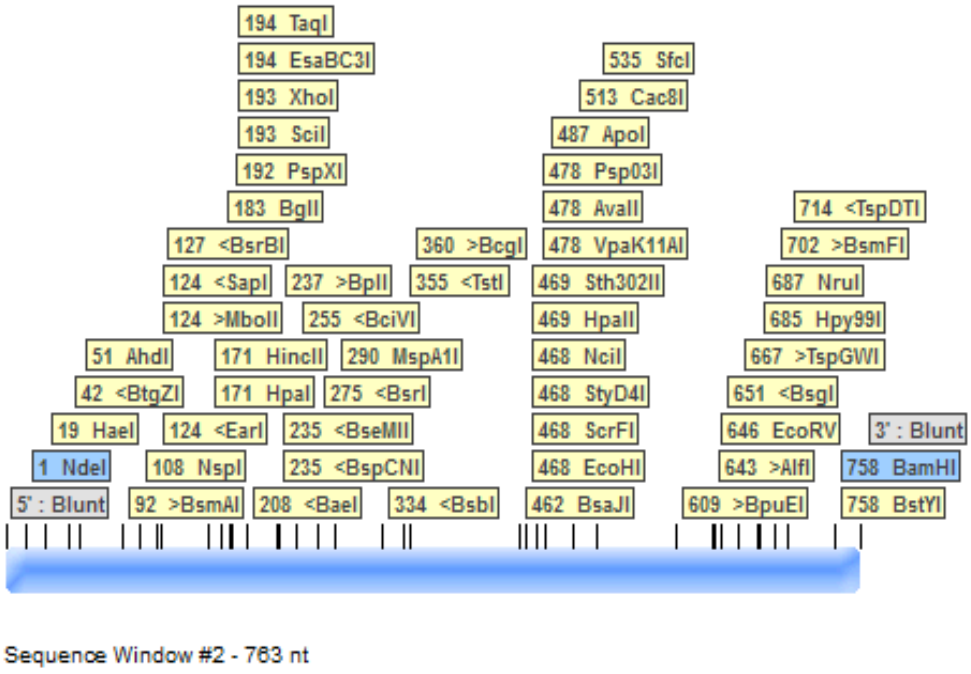


Figure 4.2 Schematic represantation of laccase gene and restriction enzyme site.

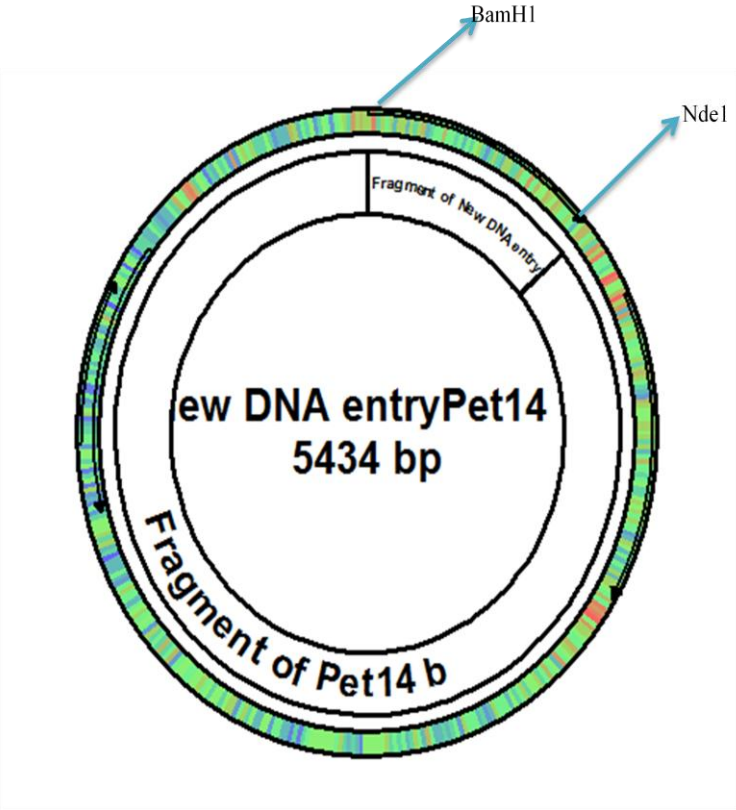


Figure 4.3 Schematic represantation of pet14b and laccase gene ligation.

The general outline of the cloning strategy in order to constract pet14b/laccase is presented above in Figure 4.3. The laccase insert DNA (754 bp) lies between the *BamH1* and *Nde1* restriction site. Laccase was cloned into the pet14 b vector using standart cloning techniques, incubating the digested PCR product with the pet14b expression vector in a ligation reaction for and transforming the ligation mixture into competent cells of *E. coli.* The genomic DNA isolated from *Shewanella putrafaciens* was quantified spectrophotometrically by absorbance at 260 nm and 280 nm. The concentration of genomic DNA of *Shewanella putrafaciens* according to the value of 260 nm absorbance. This total DNA solution had a concentration of 2 µg/ µL and OD 260/ 280 =1.82. This demonstrates that the total DNA extracted was high quality to perform the subsequent analysis. This genomic DNA from *Shewanella putrafaciens* was used as template to amplify the laccase gene by PCR with Prime Star Taq DNA Polymerase. The laccase gene fragment synthesized by the nested polymerase chain reaction (PCR) was analyzed by agarose gel electrophoresis. Figure 4.4 shows only one band that belongs to laccase gene and was obtained from total DNA isolated from *Shewanella putrafaciens.* The expected size of the first PCR product was 1200 bp, as demonstrated by its migration on the agarose gel. After performing the nested PCR, 754 bp was obtained.

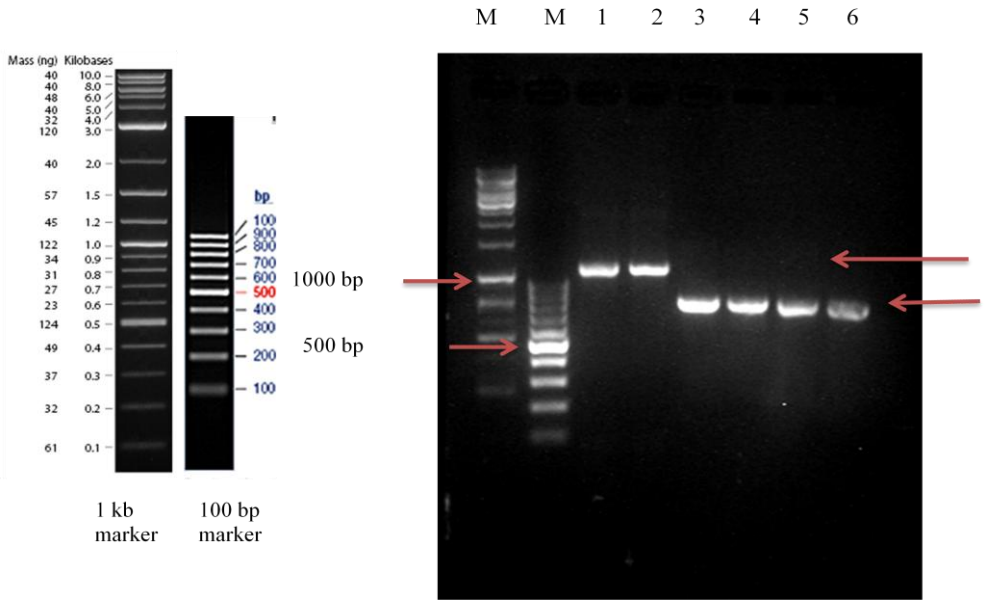


Figure 4.4 Shows that first PCR and second PCR. First and second well shows marker and first is 1 kb and second is 100 bp marker. 1 and 2 wells show first PCR ;3,4,5 and 6 wells show second PCR.

After obtaining PCR product in correct size, PCR product of laccase gene and expression vector pet14b were digested by *BamH1 and Nde1* restriction enzymes and both digested products were analysed on a 1% agarose-Tris/Acetate/EDTA gel and also was purificated. The concentrations of the both digested DNA insert and plasmid vector were calculated after measurement optical density at 260 nm. This PCR product had a concentration of 500 ng/ µL and vector concentration had 300 ng/ µL.

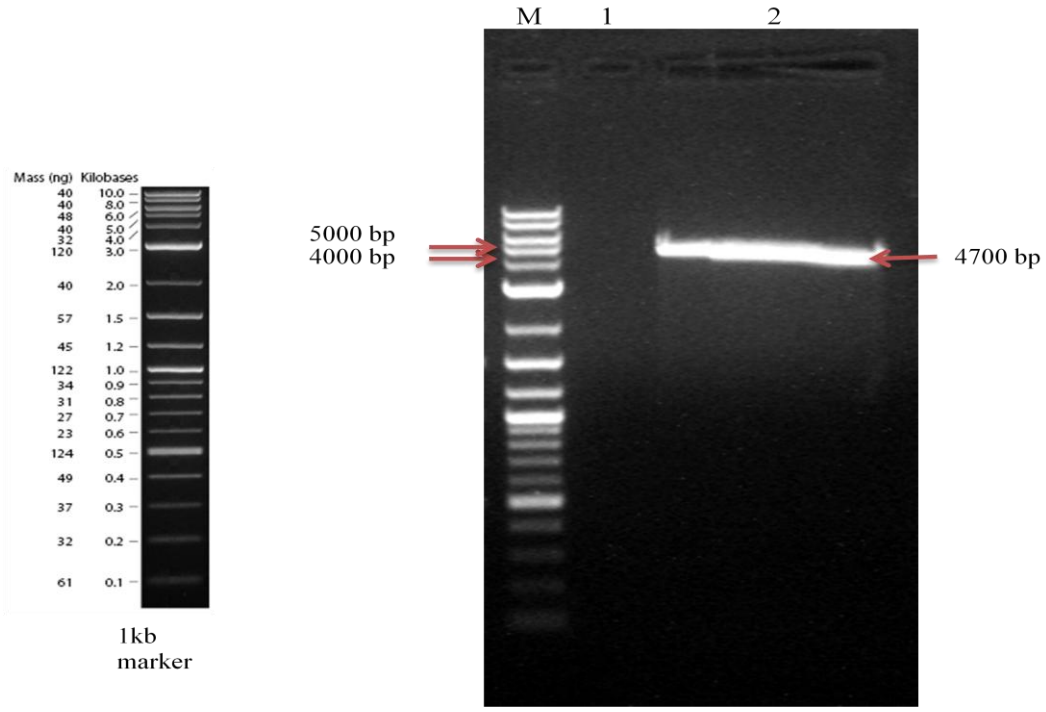


Figure 4.5 Schematic represanrtation of pet14b vector.

It is necessary to estimate the concentration of both vector and insert DNA before ligation. This may be estimated by agarose gel electrophoresis when run against molecular-weight markers of known concentration or by using a spectrophotometer. After performing ligation step with Rapid DNA Ligation Kit, all ligation products were transformed into *E. coli* host cell, and then incubated at 37ºC for overnight period. The next day, ten transformants were chosen and mini-preparations of plasmids encoding the laccase were done for screening positive clones that contain the proper orientation of insert DNA by PCR using the F2 and R2 primers as depicted in Figure 4.6.

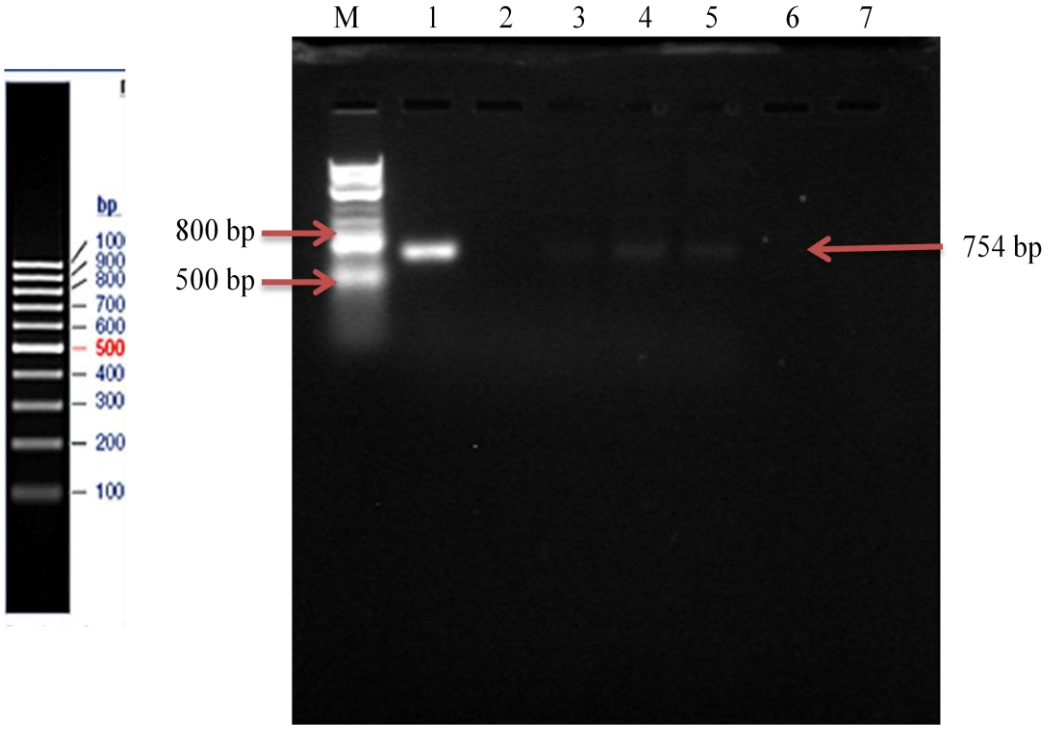


Figure 4.6 Schematic represantation of colony PCR and it shows pozitive clone.

As shown in Figure 4.7, only one constract contains 754 bp laccase gene insert. No insert of interest was seen within the other clones. The positive clone containing the insert laccase gene was sequenced by cycle sequencing.

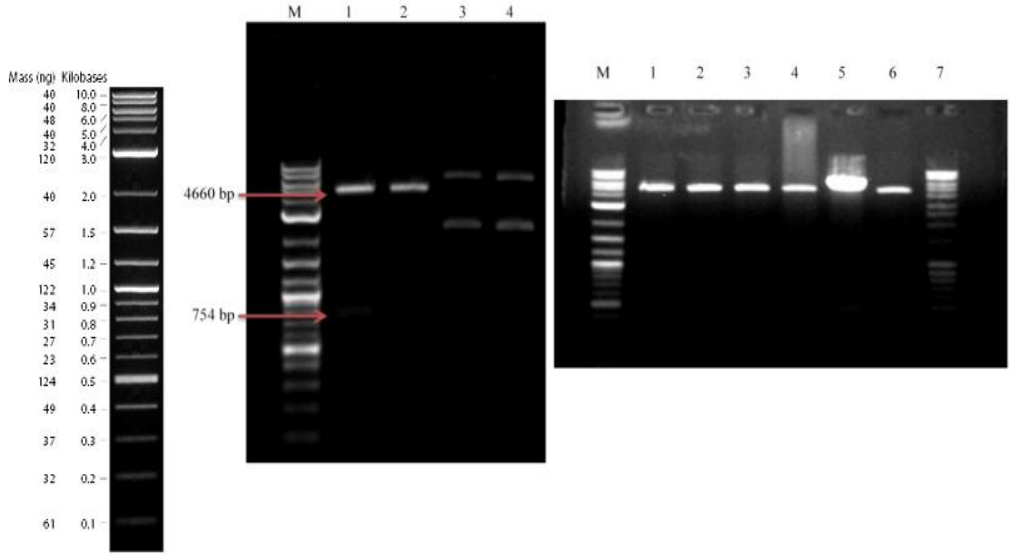


Figure 4.7 Pozitive clone after digestion with BamH1 and Nde1.

After performing cycle sequencing, only one clone was selected for the expression studies. Once sequenced plasmid selected for the protein expression was denominated pet14b/laccase. After this clone was transformed into strain BL21 (DE3) pLysS hostcell. Sequence analysis was performed using the computer program pDRAW32 and the sequences of laccase gene was compared to GenBank database. The most effective pet14b/laccase expression transformant was selected by protein expression studies. Recombinant laccase expression was confirmed by SDS-PAGE analysis. Additionally, for the control of the IPTG expression system, pet14b original vector was induced. Recombinant laccase was secreted into the bacterial cell almost as a single band on SDS-PAGE gel. The expression vector pet14b/laccase was transformed into *E. coli* BL21 (DE3) pLysS host cell. Five colonies were isolated and the expression of the recombinant laccase was determined.

Heterologous expression of laccase from pet14b/laccase was achieved within 4 h of induction with 1 mM IPTG at 37^{o}C in *E. coli* strain BL21 (DE3) pLysS. The choice of such a bacterial host strain for protein expression was dictated by the observation that, due to its high GC content. Expression of recombinant laccase was observed by SDS-PAGE analysis.

SDS-PAGE of bacterial cell *E. coli* strain BL21 (DE3) pLysS containing recombinant pet14b/laccase vector in Figure 4.8. Lane 1 shows bacterial cell containing pet14b/laccase plasmid before IPTG induction. Lane 2 shows bacterial cell containing pet14b/laccase plasmid after 1 h of 1 mM IPTG induction. Lane 3 contains bacterial cell containing pet14b/laccase plasmid after 2 h of 1 mM IPTG induction. Lane 3 contains bacterial cell containing pet14b/laccase plasmid after 3 h of 1 mM IPTG induction It was used as negative control sample (zero time point). Lane Mr: Prestained Protein Molecular Weight Marker (Fermentas). The white arrows show the laccase gene (28 kDa) expression.

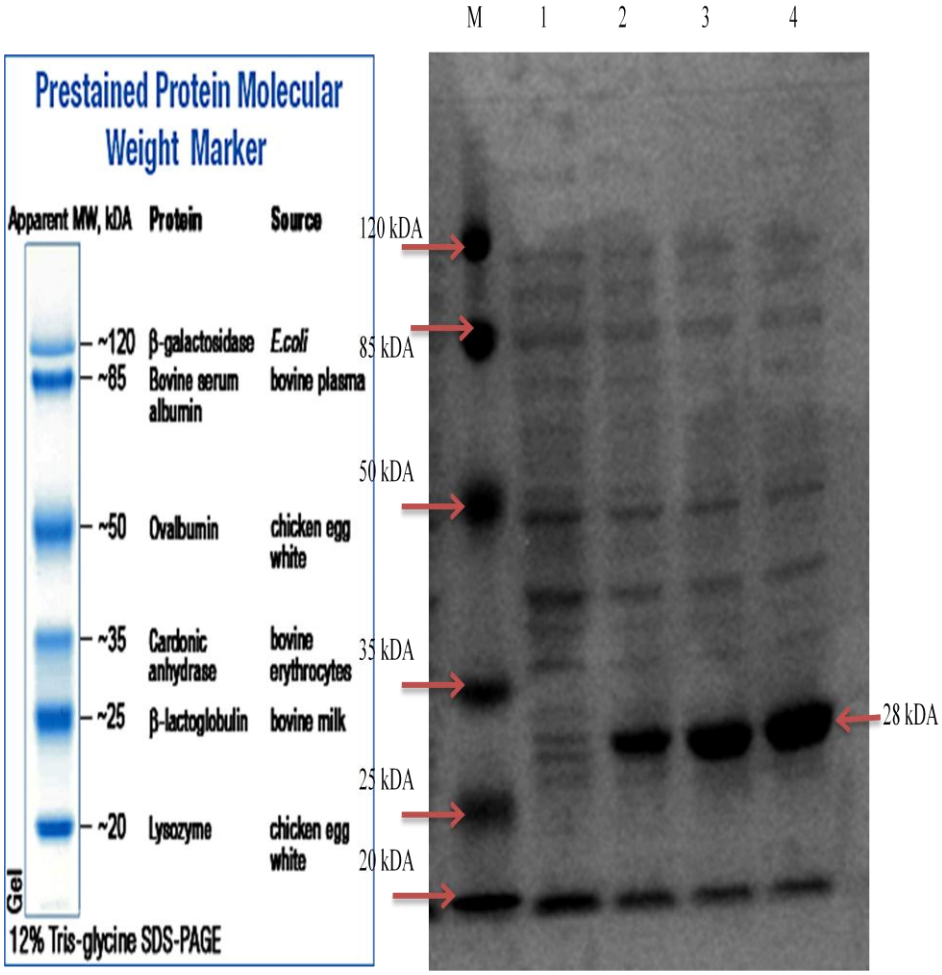


Figure 4.8 SDS- PAGE analysis. Marker is prestained protein molrcular weight marker (Fermentas), First lane shows before IPTG induction. Second line shows after 1 hour IPTG induction, Third line shows after 2 hours IPTG induction and fourt lane shows after 3 hours IPTG induction.

4.2 CHARACTERIZATION OF LACCASE ENZYME

4.2.1 Enzyme Assay Analysis

For the enzyme assay, ABTS was used**.** The non-phenolic dye 2,2'-azinobis-bis-(3-ethylbenzthiazolinesulphonate) (ABTS) is oxidized by laccase to a cation radical. The concentration of the cation radical responsible for the intense blue-green colour can be correlated to the enzyme activity and is most often read 420 nm After the cell culture was removed by centrifugation (20 min at 13,500 x g), laccase activities in the culture

pellet were routinely determined by measuring the oxidation of ABTS (2.5 mM) in a sodium acetate buffer (0.1 M, pH 5.0) at 420 nm (ε = 36 mM-1cm-1). Laccase activities were monitored UV-Vis Spectrophotometer. The reaction mixture which contained 950 µl sodium acetate buffer, 200 µl substrate and 50 µl enzyme, loaded into spectrophotometer and measured at 420 nm for 5 minutes of reaction time at 28°C.

Then we searched effect of temparature and pH on the enzyme activity. It was determined by spectrophotometer. 5 different 96 well-plates were designed and determined the enzyme activity.

The laccase enzyme was tested for its characteristics under different pH conditions such as 3 to 12.The results demonstrated that the enzyme had an optimal pH value at pH 5.0 with ABTS as the substrate. It did not show any activity after pH 7.0.

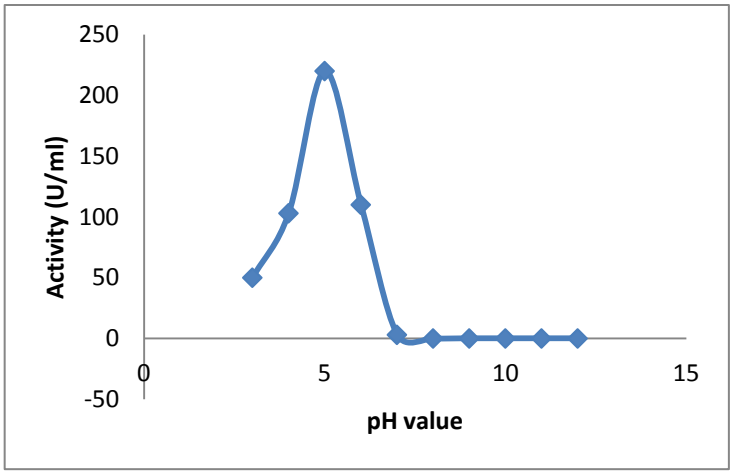


Figure 4.9 Effect of ph on the enzyme activity

The results showed that the optimum temperature for laccase was 40C. The enzyme rapidly started to lose its activity after 55 C. It did not show any activity at 75 C and was completely inactivated.

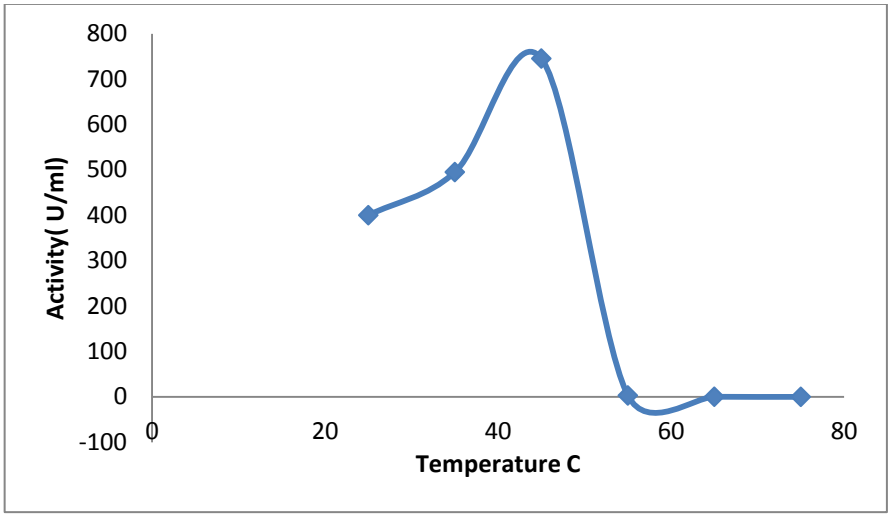


Figure 4.10 Effect of temparature on the enzyme activity

4.3 BIOINFORMATIC ANALYSIS

Sequence analysis was performed using the computer program pDRAW32 and the sequences of laccase gene was compared to GenBank database. We converted to recombinant laccase gene to protein seguance. After BLASTp program was used from NCBI. Result of BLASTp was compered various organism and was found complementary domain and superfamily.

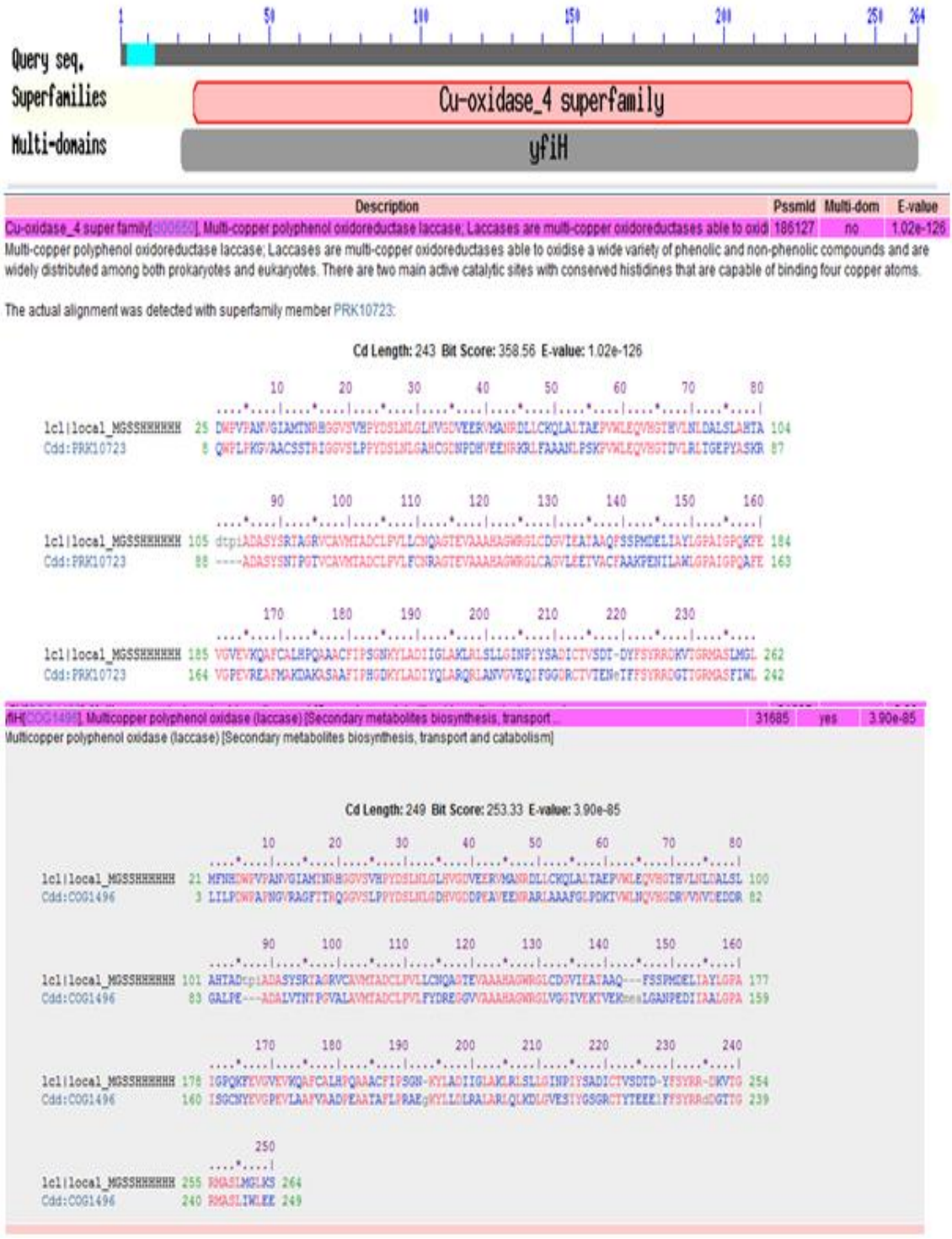


Figure 4.11 Blastp results and its domain study.

We compared Wild type S.putrafaciens laccase gene and recombinant laccase gene with CLC-BIO main workbench software and was done alignment study.

ATGTTTAATCACGATTGGCCTGTTCCTGCTAATGTTGGCATCGCTATGACAAATCGTCATGGCGGTGTGAGCGTACATCCCTATGATAGTCTCAATCTTGGGCTG
CATGTCGGTGATGTTGAAGAGCGGGTGATGGCTAATCGTGACTTACTCTGCAAACAATTAGCGTTAACCGCCGAGCCTGTCTGGCTCGAGCAAGTGCATGGTAC
TCATGTGCTTAATTTAGACGCGCTGAGCTTAGCTCATGCTGCGGATACACCGATTGCCGATGCCAGTTACAGCCGAACCGCTGGACGAGTTTGCGCTGTGATGA
CCGCCGATTGTTTGCCTGTGTTGCTGTGTAATCAAGCGGGAACCGAAGTGGCTGCGGCTCATGCTGGTTGGCGTGGTTTGTGTGATGGCGTGATTGAGGCAACT
GCGGCGCAGTTTAGTTCACCTATGGATGAGTTGATTGCCTACCTCGGCCCGGCTATTGGTCCACAAAAATTTGAAGTCGGCGTTGAAGTACAGCAAGCCTTTTGT
ACTTTACATCTACAGTCGGCAGCGTGTTTTATTCCAAGTGGTAACAAGTATCTTGCCGATATTATTGGCTTGGCTAAATTACGCTTGAGTTTACTCGGGATAAAC
CCTATTTATAGCGCAGATATCTGCACAGTCAGCGACACGGATTACTTCTCTTATCGTCGCGATAAGGTGACGGGACGCATGGCTTCATTGATCTGGCTTAAGTCT
TAA

Figure 4.12 Wild type *S.putrafaciens* laccase gene

CATATGTTTAATCACGATTGGCCTGTTCCTGCTAATGTTGGCATCGCAATGACAAATCGTCATGGCGGTGTGAGCGTACATCCCTATGATAGTCTCAATCTTGGGCTG
CATGTCGGTGATGTTGAAGAGCGGGTGATGGCTAATCGTGACTTACTCTGCAAACAATTAGCGTTAACCGCCGAGCCTGTCTGGCTCGAGCAAGTGCATGGTACTCA
TGTGCTTAATTTAGACGCGCTGAGCTTAGCTCATACTGCGGATACACCGATTGCCGATGCTAGTTACAGCCGAACCGCTGGACGAGTTTGCGCAGTGATGACCGCCG
ATTGTTTGCCTGTGTTGCTGTGTAATCAAGCGGGAACCGAAGTGGCTGCGGCTCATGCTGGTTGGCGTGGTTTGTGTGATGGCGTGATTGAGGCCACTGCGGCGCAGT
TTAGTTCACCTATGGATGAGTTGATTGCCTACCTCGGCCCGGCTATTGGTCCACAAAAATTTGAAGTCGGTGTTGAAGTAAAGCAAGCATTCTGTGCTTTGCATCCAC
AGGCGGCAGCGTGTTTTATTCCAAGTGGTAACAAGTATCTTGCCGATATTATTGGCTTGGCTAAATTACGCTTGAGTTTACTCGGGATAAACCCTATTTATAGCGCAG
ATATCTGCACAGTCAGCGACACGGATTACTTTTCTTATCGTCGCGATAAGGTGACGGGACGCATGGCTTCATTAATGGGGCTTAAGTCTTAAGCTAATTCCCTTACAG
CTTGGATCC

Figure 4.13 Recombinant *S.putrafaciens* laccase gene

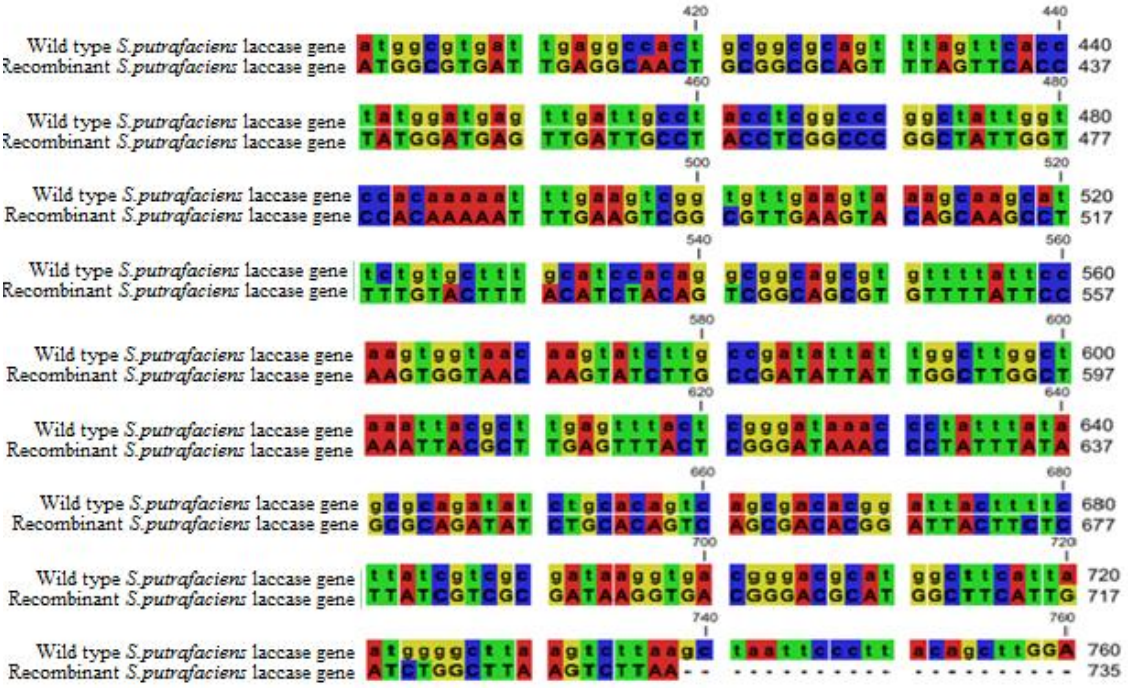


Figure 4.14 Alignment of wild type *S.putrafaciens* laccase gene and recombinant *S.putrafaciens* laccase gene

Protein alignment has been performed and protein modeling has been performed with swiss-model programming and 3D structure of protein was compared with each other.

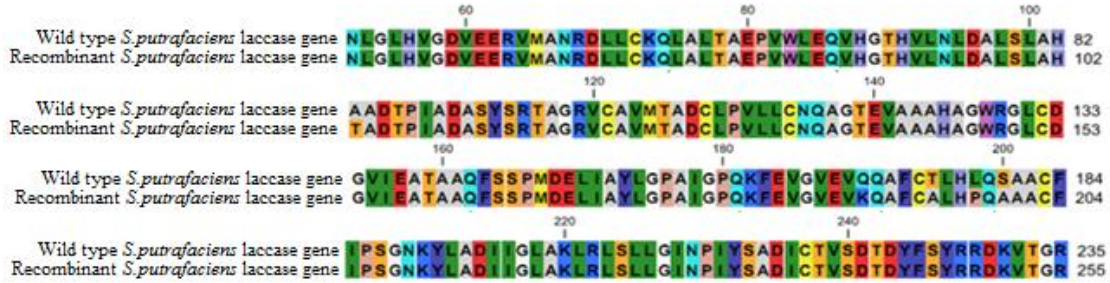
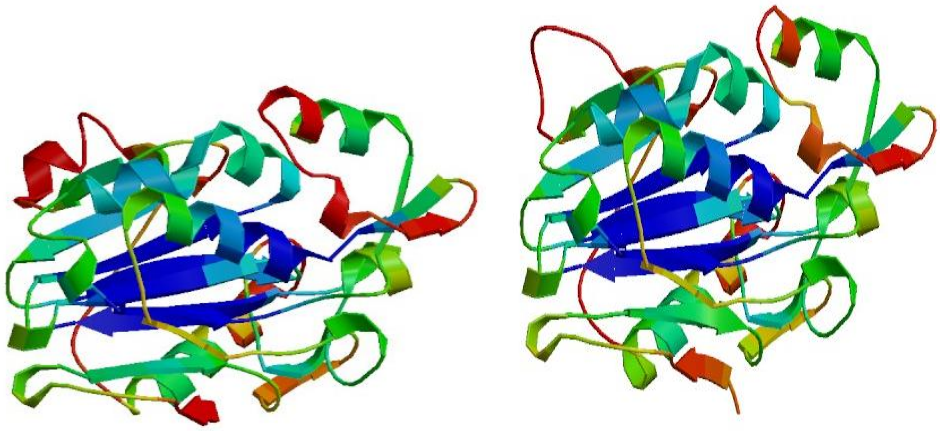


Figure 4.15 Alignment of wild type *S.putrafaciens* laccase gene and recombinant *S.putrafaciens* laccase gene protein sequences.



Wild type *S.putrafaciens* laccase Recombinant *S.putrafaciens* laccase

Figure 4.16 3D modeling of wild type *S.putrafaciens* laccase and recombinant *S.putrafaciens* laccase

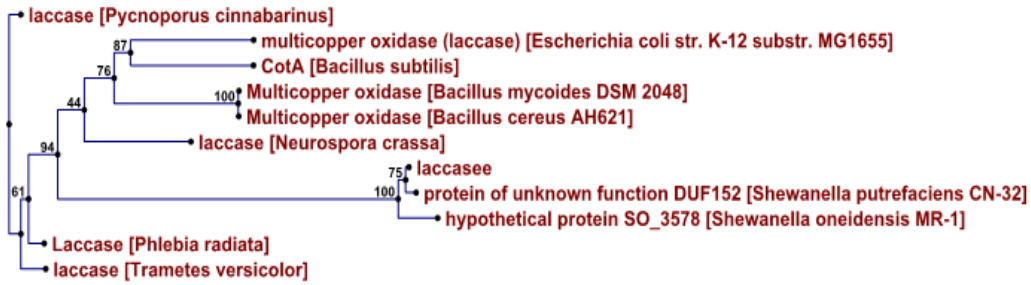


Figure 4.17 Phlygenetic tree of laccase.

After all of them, active site alignment another organism with CLC-BIO mainworkbench programing has been performed.

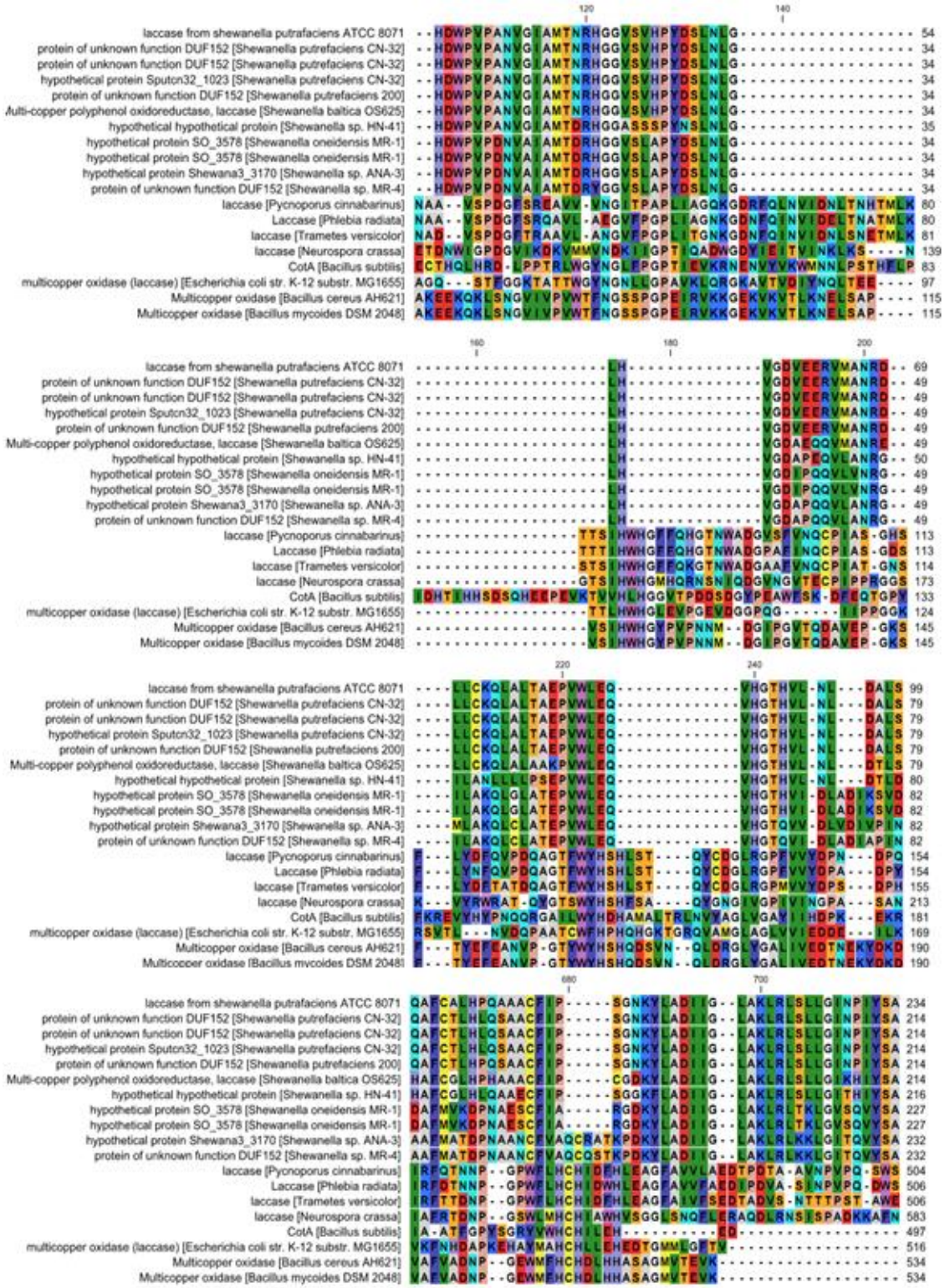


Figure 4.18 Alignment of the four copper binding domain of recombinant laccase several bacterial laccase and fungal laccase.

CHAPTER 5

CONCLUSION

In this study, we prefered *S. putrepaciens* ATCC 8071 because its laccase enzyme was not characterized before. Laccase genes in *Shawenalla putrefaciens* ATCC 8071 was amplified and transferred into pET14b expression vector and E.coli (DE3)pLysS competent cell. Our laccase gene was expressed, then was analysed by SDS- PAGE and bioinformatic study.

According to the bioinformatic study our recombinant laccase has 264 aa, 28.4 kd and its isoelectric point is 6.45. SDS-PAGE results are the same as bioinformatic analysis. These results show that our laccase gene has high kd and isoelectric point value. Also, this recombinant laccase gene has more histidine aminoacid which Cu binds. This shows that our recombinant protein may be more active than others. There are some differences between 3D modeling recombinant laccase and wild type laccase. That is why some of characteristic of recombinant laccase may be different from wild type laccase.

This is the first record in the literature in which laccase enzyme from *S. putrepaciens* ATCC 8071 was isolated, cloned, purified and characterized. We think that our recombinant laccase has different characteristic from another laccase enzyme.

In future we will continue to characterized our enzyme and will develop our enzyme with protein engineering techniques. Also we will use this enzyme in order to develop some biosensors and biofuel cell.

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