

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

CHARACTERIZATION AND REDESIGN OF A NEW LACCASE
FOR INDUSTRIAL BIOTECHNOLOGICAL
APPLICATIONS

AHMET TÜLEK
A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

GEBZE
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THESIS SUPERVISOR
ASSOC. PROF. DR. BARIŞ BİNAY

GEBZE

2020

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GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

ENDÜSTRİYEL BİYOTEKNOLOJİK
UYGULAMALAR İÇİN YENİ BİR LAKKAZ
ENZİMİNİN KARAKTERİZASYONU VE
YENİDEN TASARLANMASI

AHMET TÜLEK
DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. BARIŞ BİNAY

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2020

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GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 06/11/2020 tarih ve 2020/53 sayılı kararıyla oluşturulan jüri tarafından 27/11/2020 tarihinde tez savunma sınavı yapılan Ahmet TÜLEK'in tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında DOKTORA tezi olarak kabul edilmiştir.

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SUMMARY

Laccases are enzymes that oxidize a wide variety of aromatic and non-aromatic compounds. Their wide substrate selectivity is effective in their use in many industrial biotechnological fields, hence, the laccase gene from *Madurella mycetomatis* was selected and transferred to the heterologous expression system *Pichia pastoris* X-33 host cell using pPICZ α A and pPICZA plasmids. Following transformation, the genomic integration was confirmed by colony PCR and laccase enzyme activity assessment using MM plates containing ABTS medium. The optimal growth conditions for *P. pastoris* are 1% methanol, 0.2 mM CuSO₄ at 18 °C. Recombinant laccase (MmLac) was produced and purified under these optimal conditions as 54.4 mgL⁻¹. MmLac demonstrates good activity in low pH (4.0–6.0); it's stable in the presence of cationic metals, organic solvents and under high temperatures (50–60 °C); also stable for long-term storage at –20 °C and –80 °C for up to eight weeks. Kinetic properties of MmLac were calculated for ABTS, 2,6–DMP and SGZ substrates. While the molecular weight of the MmLac enzyme was 67.4 kDa, the deglycosylation form was 62.0 kDa. Additionally, it was analyzed by docking 2,6–DMP substrate to the active site of laccase enzyme with the bioinformatics AutoDock program.

Detailed structural properties of the recombinant laccase were determined, and its utility in decolorization and textile bleaching applications was examined. *MmLac*, besides showing high efficiency and stability, it increased textile whiteness reducing chemical input. The enzyme's performance makes it worthy of investigation within textile applications, environmental and food technologies.

Keywords: Laccase, *Madurella mycetomatis*, heterologous expression, textile bleaching, *Pichia pastoris*.

ÖZET

Lakkazlar çok çeşitli aromatik ve aromatik olmayan bileşikleri oksitleyen çok bakırlı enzimlerdir. Birçok endüstriyel biyoteknolojik alanda kullanılmalarında geniş substrat seçiciliği etkilidir. Bu nedenle *Madurella mycetomatis*'in lakkaz geni seçilmiş, pPICZ α A ve pPICZA plazmitleri aracılığıyla heterolog ekspresyon sistemi *Pichia pastoris* X-33 konak hücresine aktarılmıştır. Transformasyondan sonra, genomik entegrasyon koloni PCR ile doğrulanmış ve lakkaz enzim aktivitesi ABTS içeren MM plate ortamında değerlendirilmiştir. *P. pastoris* için optimal büyüme koşulları, 18 °C, % 1 metanol, 0.2 mM CuSO₄ olarak belirlenmiştir. Rekombinant lakkaz (MmLac) saflaştırılmış ve optimal biyokimyasal koşullar altında 54.4 mgL⁻¹ olarak üretilmiştir. MmLac'ın, asidik pH aralığında (4.0–6.0) iyi aktivite gösterdiği; katyonik metaller, organik çözücüler varlığında ve yüksek sıcaklıklarda (50 °C–60 °C) stabil olduğu; –20 °C ve –80 °C'de sekiz haftaya kadar uzun süreli saklama için stabil olduğu gösterilmiştir. Ayrıca kinetik özellikleri ABTS, 2,6–DMP, SGZ substratları için hesaplanmıştır. MmLac enziminin moleküler ağırlığı 67.4 kDa iken, deglikolize formu 62.0 kDa olarak hesaplanmıştır. Biyoinformatik AutoDock programı kullanılarak lakkaz enziminin aktif bölgesine 2,6–DMP substratı bağlanarak analiz edilmiştir.

Rekombinant lakkazın ayrıntılı yapısal özellikleri belirlenmiş, dekolorizasyon ve tekstil ağartma uygulamalarındaki kullanımı incelenmiştir. MmLac yüksek verimlilik ve stabilite sergilemesinin yanında daha az kimyasal madde girişi gerektiren tekstil beyazlığında bir artışa neden olmuştur. Enzimin bu performansı, onu tekstil biyoteknolojisi uygulamalarında ayrıca çevre ve gıda teknolojilerinde de kullanımı için araştırmaya değer kılmaktadır.

Anahtar Kelimeler: Lakkaz, *Madurella mycetomatis*, heterolog ifade, *Pichia pastoris*, tekstil ağartma.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
aa	: Amino acid
ABTS	: 2–2’–azinobis–(3–ethylbenzthiazoline–6–sulphonate)
BLAST	: Basic Local Alignment Search Tool
BMGY	: Buffered Glycerol Complex Medium
BMMY	: Buffered Methanol Complex Medium
bp	: Base pair
BRENDA	: Braunschweig Enzyme Database
DMP	: 2,6–dimethoxyphenol
DNA	: Deoxyribonucleic acid
<i>EcoRI</i>	: <i>Escherichia coli</i> RY13 I
IMAC	: Immobilized Metal Affinity Chromatography
k_{cat}	: Turnover number
kDa	: Kilo Dalton
K_m	: Michaelis–Menten constant
LB	: Luria–Bertani
M	: Molar
MALDI-TOF	: Matrix–Assisted Laser Desorption/Ionization–Time of Flight
MM	: Minimal Methanol
mM	: Millimolar
min	: Minute
mL	: Milliliter
<i>mmlac</i>	: <i>Madurella mycetomatis</i> laccase gene
MS	: Mass Spectrophotometer
ng	: Nanogram
nm	: Nanometer
NCBI	: National Center for Biotechnology Information Database
Ni ²⁺ –NTA	: Nickel–Nitrilotriacetic Acid
PCR	: Polymerase chain reaction

<i>PmeI</i>	: <i>Pseudomonas mendocina</i> I
RNA	: Ribonucleic acid
rpm	: Rounds per minute
<i>SalI</i>	: <i>Streptomyces albus</i> I
sec	: Second
SDS–PAGE	: Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
SGZ	: Syringaldazine
SOC	Super Optimal Broth
TAE	: Tris–Acetate–EDTA
U	: Units
UniProt	: Universal Protein Resource
V	: Volt
YNB	: Yeast Nitrogen Base
YPD	: Yeast Nitrogen Dextrose
YPDS	: Yeast Nitrogen Dextrose Sorbitol
μg	: Microgram
μL	: Microliter

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1. INTRODUCTION

Enzymes are useful biocatalysts that are used in a variety of industrial processes and perform many chemical reactions. Therefore, some or all of the commercial and environmental disadvantages of many chemical conversion processes used in the industries can be practically eliminated by using enzymes classified as “Green Biotechnology” [1]. Green solution is one of the popular ways to eliminate environmental problems in today’s world. There are many industrial enzymes produced and used for this purpose. Their applicability and benefits as pharmaceutical, food, technical, detergent, biofuel, and feed enzymes have influenced the global market scenario of these areas [2, 3]. Commercial enzyme production has increased in volume and number of products in recent years in response to the growing demand for expanding markets and new biocatalysts, particularly for heavy environmental burden. Value of the enzyme market was about \$7082 million in 2017 and it is expected to reach \$10.519 million by 2024 [4]. Therefore, the development of new enzymes and technologies to meet the growing demand in the global enzyme market is crucial. There are various organisms in nature that can be used as an enzyme source for these purposes in different habitats. However, most of the enzymes are generally unsuitable for industrial use. Enzymes with the desired activity for use in industrial conditions can be obtained by protein engineering using regulated process conditions and directed evolution [5].

There are many oxidoreductase enzyme classes which are dispersed in various categories. In the areas of the biotechnology industry potential applications of enzymes have been increased in recent years. The laccases have great potential, with a wide range of substrate specificity, and well-studied ligninolytic enzymatic activities, which is highly versatile with a wide variety of industrial processes. To date, a significant number of reports have been published focusing on the biochemical properties of these enzymes, their use in chemical reactions, and their application in technological and bioremediation processes. In the thesis study, a new laccase enzyme was produced from a fungus using a heterologous expression system and its biochemical characterization was performed. It has also been tested for its applicability in some industrial applications.

There is still a lack of laccase enzymes suitable for different industrial application environments. Laccases obtained from *Ascomycetes* group organisms suitable for extreme environmental conditions (such as high temperature, wide pH spectrum, salt tolerance) have been reported previously. For this reason, the organism selected from this group has the potential of succeeding under extreme conditions, for this proposed study *M. mycetomatis* was selected and testing the extraction, characterization and industrial applications of laccase in a laboratory environment was aimed as main goals of this thesis study.

2. LITERATURE SURVEY

2.1. General Characteristics and Global Market of Enzymes

Since ancient times, microorganisms have been used to produce many products such as bread, yoghurt, wine, vinegar for centuries without understanding their biotechnological potential and biochemical dynamics in their metabolism. As the mechanisms and dynamics of biological processes begin to be discovered over time, the structure of enzymes and their functions in the processes have begun to be explored. These molecules are biocatalysts that accelerate biochemical reactions by decreasing activation energy in complex metabolic pathways and other biological processes. They constitute the most important functional unit of metabolism in all biological processes. Although most of them are structural proteins, there are also enzymes in RNA structure (ribozyme). They are divided into 6 classes (Table 2.1) according to the type of reaction catalyzed by the Enzyme Commission (EC) [6].

Table 2.1: Classification of enzymes according to the reaction type.

Class (EC)	Group of Enzyme	Reaction Catalyzed	Example
1	Oxidoreductases	Oxidation–reduction reactions	Laccase
2	Transferase	Transfer of functional groups	Transaminases
3	Hydrolase	Hydrolysis reactions	Lipases
4	Lyases	Group elimination (forming double bonds)	Aldolase
5	Isomerase	Isomerization reactions	Glucose isomerase
6	Ligases	Bond formation coupled with a triphosphate cleavage	DNA ligase

Nowadays, enzymes are used in many industrial applications because of their advantages such as the ability to catalyze very specific reactions, being a green alternative and reducing the costs with low energy requirements when compared with the traditional catalysers [7]. For this purpose, the literature and database has been defined and characterized according to many enzyme classes (Figure 2.1).

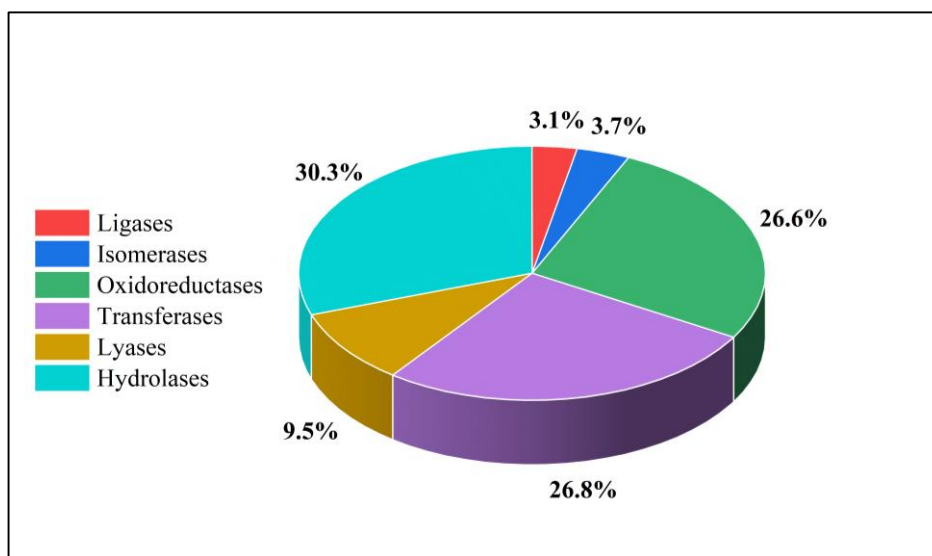


Figure 2.1: Distribution of six enzyme classes according to the BRENDA database.

The global economic development in the last half-century, the rapidly increasing population density, and the rapidly increasing demand for food and energy due to global urbanization, environmental pollution are the main factors driving the increasing market demand for industrial enzymes. As a result, the increasing demand for industrial enzymes creates a large “Global Enzyme Industry”. Many commercial enzymes are produced and used in different fields of the industry since the 1800s. These biocatalysts constitute a global market due to their widespread use in different sectors such as food, chemicals, agriculture, medicine, and energy. Many companies provide services in these fields and increasing demand and new market expectations continue today (Figure 2.2) [8]. However, breakthroughs are needed for new industrial enzymes that have superior properties and have adapted to harsh conditions, and have a wide range of uses [8].

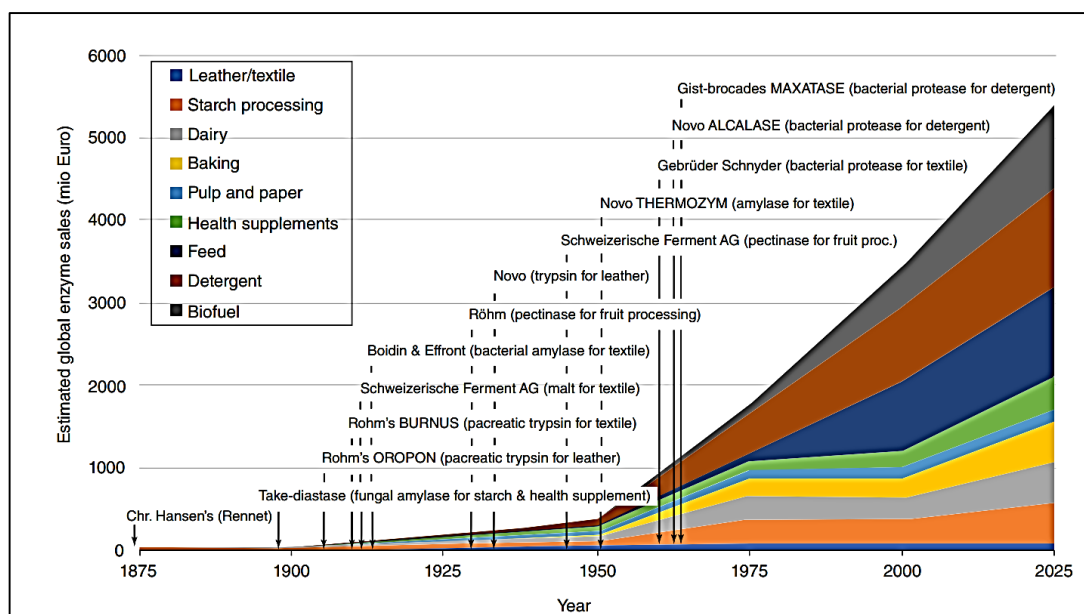


Figure 2.2: Historical events and growth forecast in the commercial industrial enzyme market from 1875 to 2025.

Enzymes naturally obtained from microorganisms are inadequate for industrial applications due to different reasons such as low catalytic efficiency, stability, low production. Various innovative modifications (rational design as site directed mutagenesis, directed evolution) and optimizations are required in different processes to meet the increasing demand at the industrial level. In this way, catalytic efficiency, amount and applicability of the enzymes can be increased for low-cost production of value-added products [9].

This thesis focuses on a new laccase, a member of the oxidoreductase family, which has a different application potential considering industrial demands. Identification, biochemical characterization and production of new laccases are essential for a variety of biotechnological applications, due to the increasing demand for environmentally friendly, highly efficient and low-cost industrial processes with different physiological and biochemical properties.

2.2. Laccases

Laccase (EC 1.10.3.2), *p*-diphenol oxidase (synonym; benzenediol: oxygen oxidoreductase) are multi-copper enzymes first obtained by Yoshida in 1883 from the resin of the *Rhus vernicifera* tree [10]. It is widely used in industrial

biotechnological applications since it has a high catalytic efficiency to wide range of aromatic, non-aromatic substrates. In particular, laccases have been extensively studied over the past 20 years due to their low substrate specificity, and new laccase enzymes are characterized [11]. Compared to other enzymes, laccase has great potential due to different biotechnological and environmentally friendly applications.

These multicopper enzymes are divided into 11 superfamilies for their structural and phylogenetic properties [12]:

- Plant Laccases
- Insect Laccases
- Fungal Ferroxidases
- *Ascomycete* Laccases
- Fungal Pigment MCOs (multi-copper oxidases)
- *Basidiomycete* Laccases
- Bacterial Bilirubin Oxidases
- Fungal and plant AOs
- Bacterial CopA Proteins (copper resistance proteins)
- SLAC homologs (small laccase family)
- Bacterial CueO Proteins (copper efflux)

2.2.1. Sources of Laccases

The main source of laccases is fungi while these enzymes are also found in higher plants, bacteria, and insects [13] (Figure 2.3). Therefore, they are ubiquitous biocatalysts and show different structural and biochemical properties depending on the source of laccases [14].

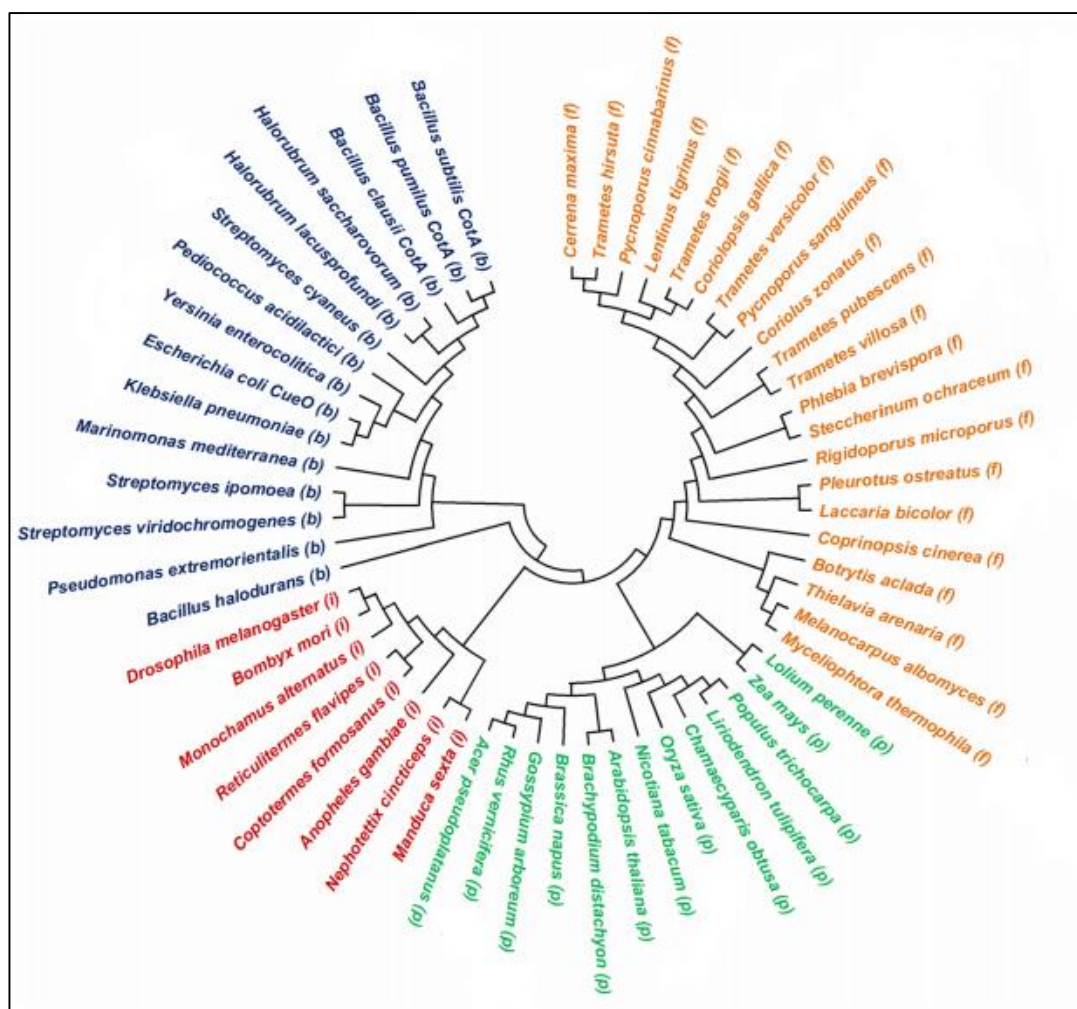


Figure 2.3: Phylogenetic tree created with some laccases from different organism sources. Laccases of bacterial (b), insect (i), plant (p), and fungal (f) origin are colored in blue, red, green, or orange, respectively.

Generally, laccases derived from plants, insects and bacteria have a low redox potential, while those in fungi have high redox potential. Some conserved domains related to laccases with low, medium and high redox potential have been identified. Although the laccase amino acid sequence homology differs between organisms, the catalytic region is highly conserved. In these organisms, laccases are involved in many cellular processes, both anabolic and catabolic reactions [15] (Figure 2.4).

These enzymes are involved in many cellular processes such as morphogenesis, pigmentation, lignification and delignification, virulence pathogenesis, detoxification, sporulation in the cells, growth, and development of rhizomorphs, sports jacket resistance and polymerization of melanin precursors.

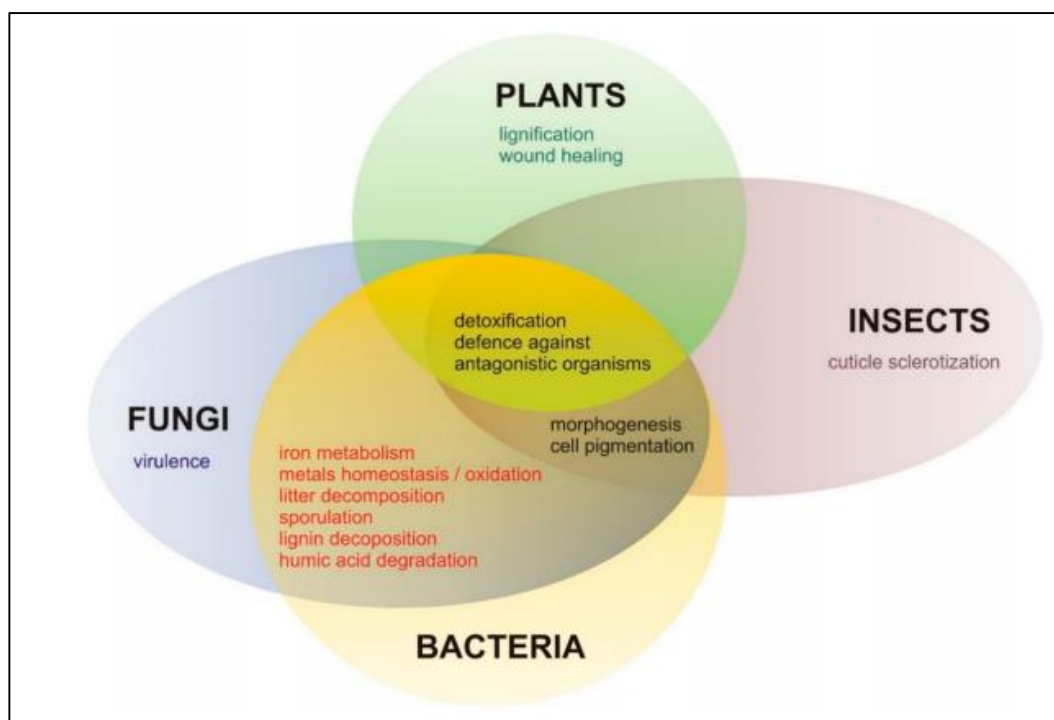


Figure 2.4: Functional comparison of plant, fungus, insect, and bacterial laccase.

Fungal laccases play a role in sporulation, pigment production, fruit stem formation, and plant pathogenesis. White rotten *Basidiomycetes*, one of the fungal laccases, functions in the processes of efficient lignin, cellulose, and hemicellulose decomposition and their conversion to carbon dioxide. This group is the most studied and characterized fungal laccases [16, 17].

Laccases function during the sclerotization process for epidermal cuticle synthesis, such as other processes like wound healing and immune system development and preservation [18–20]. They also oxidize catechols in the cuticle to the corresponding quinones which catalyze protein crosslinking reactions. In some holometabolous insects, laccase has been identified as the main enzyme associated with cuticular hardening [21, 22].

Bacterial laccases on the other hand have various functions such as morphogenesis processes, pigment biosynthesis such as melanin and brown spore pigment. Furthermore, they function in copper homeostasis and spore formation [23, 24].

Studies on plant-derived laccases are lesser than fungal and bacterial laccase studies. The first identified and reported plant originated laccase is from the Japanese

lacquer tree *Toxicodendron vernicifluum* (*Rhus vernicifera*) [10]. Their molecular structures and reaction mechanism are similar to fungal laccases. In general, they have a lower E° (redox potential) level like bacterial laccases (0.41 V for *R. vernicifera* and a pI between 7.0 and 9.6) [25]. These laccases exhibit a higher glycosylation pattern (22–45%) and also contain 500–600 amino acids and molecular weight about 60–130 kDa [26, 27]. The laccase enzyme in plants catalyzes the polymerization reaction of lignin, elongation, and stress response [28, 29]. While plant laccases function in lignin polymerization, fungal laccases function in biodegradation of lignin. After the laccases were first obtained from *Rhus vernicifera*, the Japanese lacquer tree, they were also obtained from lacquer, mango, mung bean, peach, pine, prune, and sycamore plants [30]. Also, the laccase enzyme was obtained in the embryo of maize (*Zea mays*) seeds [30, 31].

2.2.2. Physico–Chemical Properties and Structure of Laccases

As a glycoprotein structure commonly found in nature, laccases are multi–copper enzymes that oxidize aromatic and non–aromatic compounds using molecular oxygen as the electron acceptor and form the only by-product water as a result of the reaction. These enzymes catalyze different free radical reactions depending on their structural properties and reaction conditions. The most common of these is the coupling of free radicals producing dimeric, polymeric, and oxidative decarboxylation. Oxidation of the substrate is carried out using molecular oxygen. In each reaction, 2 H_2O molecules are produced. For each reduced oxygen, 4 substrate molecules are oxidized without hydrogen peroxide is formed (Figure 2.5). It is defined as “green catalysts” or “eco-friendly” because laccases use molecular oxygen in the reaction and form H_2O [32].

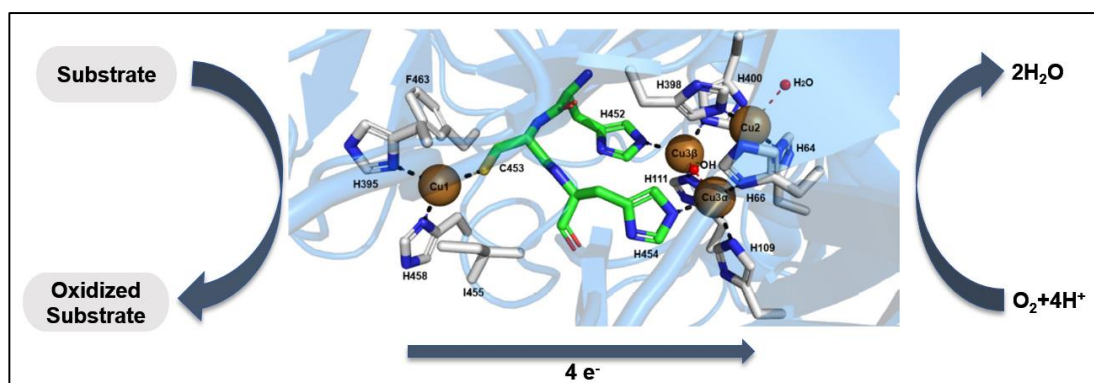


Figure 2.5: The catalytic site of the *Trametes versicolor* laccase enzyme (PDB 1KYA). The histidine–cysteine pathway is shown in green, Cu^{2+} ions in brown.

Laccases can exist in monomeric, homotetrameric, heterodimeric, or multimeric form. Molecular weights can vary between 50–130 kDa according to the organism they are originated from [33]. Laccases are usually in the glycoprotein structure. The carbohydrate content in their structure varies according to the organism. While this rate is 45% for plant laccases, it varies between 10–30% for fungal laccases. The carbohydrate region enhances the stability of the enzyme and protects it from proteolysis and inhibition [17, 34]. The Cu^{2+} atoms in the catalytic region of laccases divide it into 3 regions, defined as Type I, Type II, and Type III (Figure 2.6).

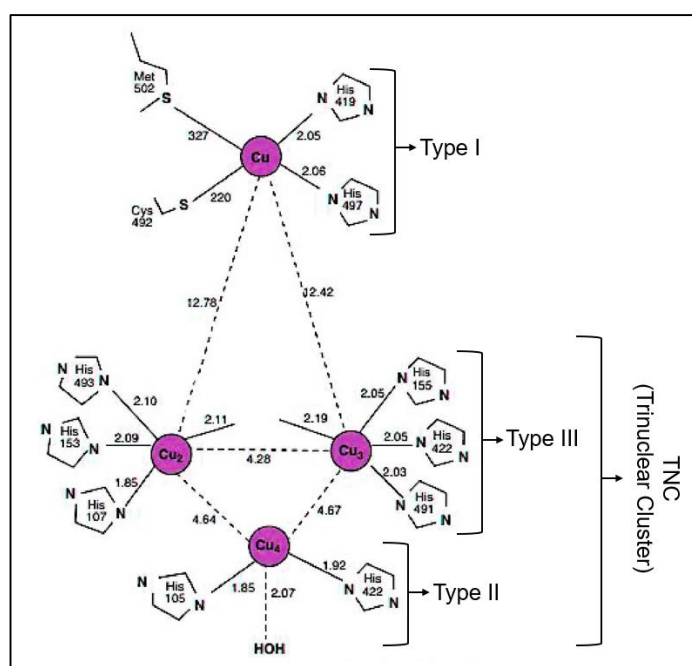


Figure 2.6: Copper centers of *Bacillus subtilis* laccase (CotA) enzyme.

The type I copper (paramagnetic ‘blue’) region composes blue color due to the intense electronic absorption of the covalent copper–cysteine bond. Substrate oxidation occurs in this region due to its high redox potential (+790 mV). The absorbance value of this region is 610 nm [35]. The Type II Cu^{2+} (paramagnetic “normal/non-blue”) region does not exhibit intense absorption in the visible region. It also reveals normal electron paramagnetic resonance (EPR) spectra. Type II copper is coordinated by two histidine residues and is strategically located near Type III copper [36]. The Type III Cu^{2+} (diamagnetic spin-coupled copper–copper pair) region is a binuclear center regulated by 6 Histidine. It gives absorbance at 330 nm. The Type II Cu^{2+} and Type III Cu^{2+} regions form a tri–nuclear cluster (TNC) where molecular oxygen is reduced and water is released [37]. In the catalytic mechanism of the laccase, an electron is first donations into the substrate by the Type I copper region. Thus, it starts with an internal electron transfer from the Type I region to Type II and Type III copper regions. Type III copper functions as a two–electron receiver in the aerobic oxidation process where the presence of Type II copper is required. Finally, the reduction of oxygen to water takes place in the TNC (Type II and Type III) region (Figure 2.7).

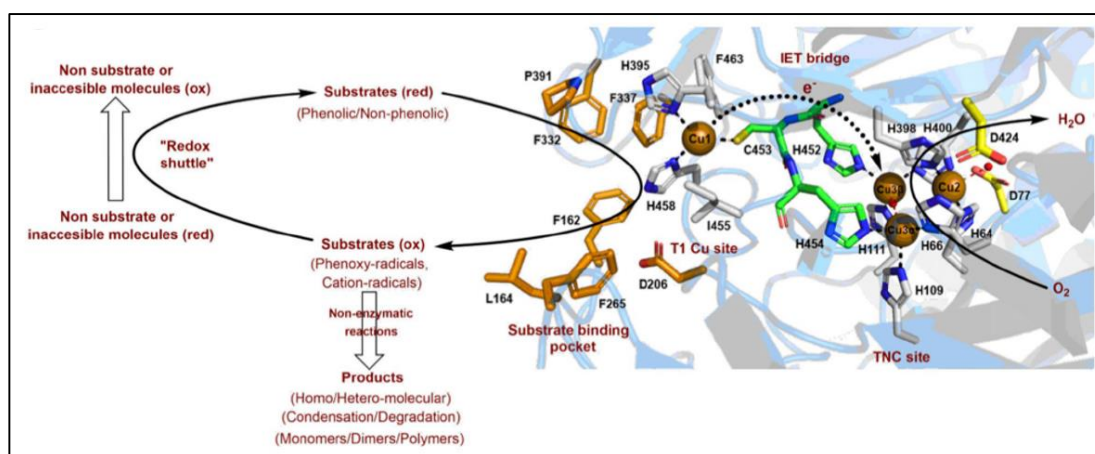


Figure 2.7: *Trametes versicolor* laccase reaction mechanism and functional properties (PDB 1KYA).

Laccases catalyze two types of basic reactions: direct oxidation and indirect oxidation. Direct oxidation involves oxidation of the substrate to the corresponding radical as a result of direct interaction with the copper cluster. However, in some reactions, direct oxidation is not possible because laccase can only oxidize

compounds whose ionization potential does not exceed the redox potential of the T1 copper ion. This can be overcome with the use of the mediator, a two-step process [38, 39] (Figure 2.8).

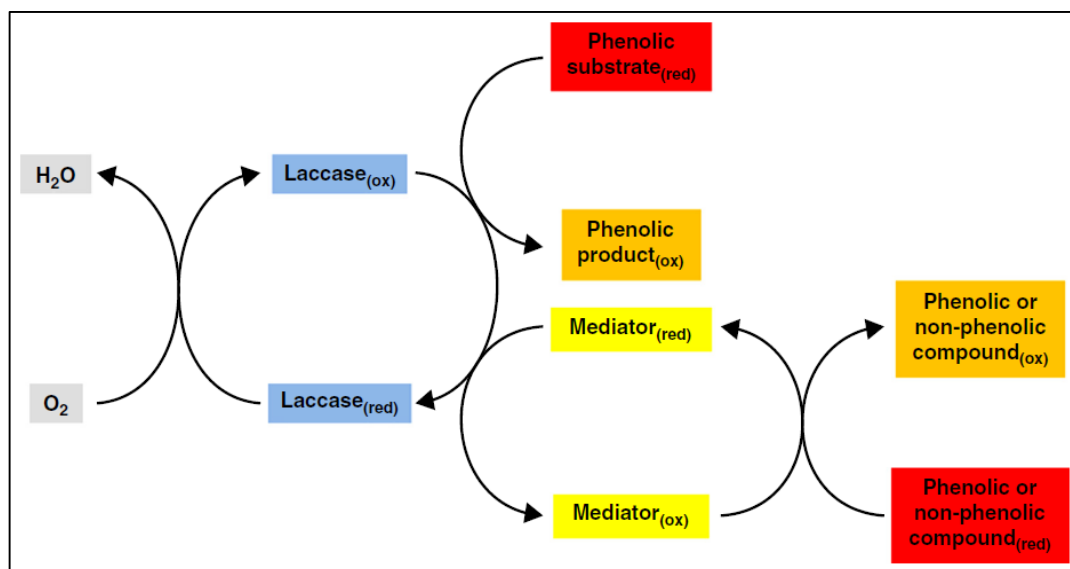


Figure 2.8: Laccase substrate oxidation types. Direct oxidation and indirect oxidation through a laccase mediator system (LMS).

The redox potential in the T1 copper region varies according to the laccase enzyme. This value is between 0.4 and 0.8 V against the normal hydrogen electrode (NHE). Accordingly, laccases are divided into three groups as low (0.4–0.5 V), medium (0.5–0.6 V), and high (0.7–0.8 V) redox potential laccases [40, 41]. Generally, *Basidiomycete* fungi have high redox potential, *Ascomycete* fungi medium, plant and bacterial laccase have low redox potential [42].

2.3. Multifaceted Applications of Laccases

Laccases are commercially used in many industrial areas due to their wide substrate selectivity and different redox potentials. For this reason, the patents of laccase enzymes, which have different biochemical and physiological properties, have been increasingly registered until today (Figure 2.9) Also, these enzymes have large biotechnological potential and high market expectations in different areas due to their wide substrate selectivity [43].

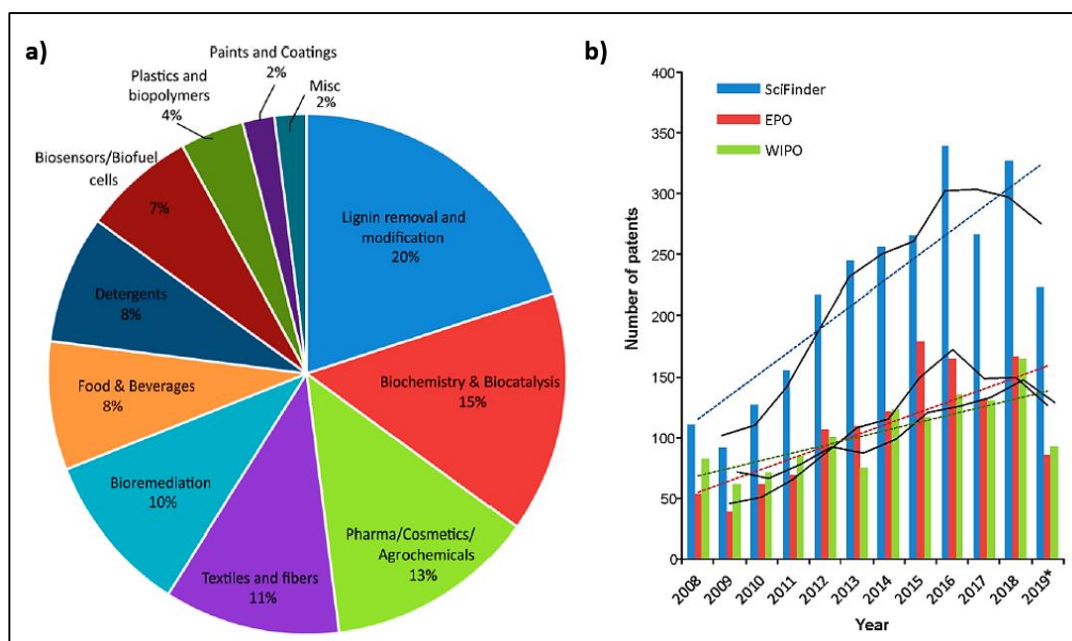


Figure 2.9: Patent distribution of laccases in different industrial areas (a) and yearly trend analysis according to three different databases (SciFinder, EPO, WIPO) (b) between 2008-2019.

Laccases with activity in different pH and temperature ranges have been defined in many industrial areas. In addition, while most of these enzymes include fungal laccases, they have different molecular weights (Table 2.2). Depending on their physiological and biochemical properties, they are used in textile, biofuel, food, biosensor and diagnostics, organic synthesis, pulp and paper, bioremediation, pharmaceutical, and cosmetic industries.

Table 2.2: Some laccases used in different industrial areas and their biochemical properties.

Source	Origin	Mw (kDa)	pH range	Temperature range (°C)	Industrial Application	Reference
<i>Myceliophthora thermophila</i>	Asco-Fungus	85	3.0–8.0	20–80	Paper coating	[44]
<i>Aspergillus flavus</i>	Asco-Fungus	60	2.0–9.0	25–60	Textile wastewater	[45]
<i>Penicillium chrysogenum</i>	Asco-Fungus	67	4.5–6.5	20–80	Degradation of syntans	[46]
<i>Cyathus bulleri</i>	Basidio-Fungus	60	2.0–6.0	25–70	Dye degradation	[47]
<i>Trametes versicolor</i>	Basidio-Fungus	97	2.5–7.0	0–60	Baking	[48]
<i>Pleurotus ostreatus</i>	Basidio-Fungus	67	2.5–7.0	25–65	Clarification of fruit juice	[49]
<i>Pycnoporus sanguineus</i> RVAN5	Basidio-Fungus	50–70	3.0–6.0	25–75	Denim bleaching	[50]
<i>Coriolopsis gallica</i>	Basidio-Fungus	66	2.5–6.0	25–80	Beer factory wastewater	[51]
<i>Rhizoctonia praticola</i>	Basidio-Fungus	78	4.0–9.0	4–90	Phenolic compound removal	[52]
<i>Lentinula edodes</i>	Basidio-Fungus	72	3.0–7.0	20–80	Effluent treatment	[53]
<i>Bacillus tequilensis</i>	Bacteria	32	5.5–8.0	55–100	Pulp bio-bleaching	[54]

2.3.1. Textile Industry

Enzymes are being used increasingly to reduce pollution and save energy due to their non-toxic eco-friendly properties in various industries. The textile industry is one of the most important industry which used laccase enzyme in their processes. The laccase enzyme is used to bleach textiles and is an enzyme with the potential to replace traditional chemical processes in the textile industry. Laccases are managed in the textile industry mainly in dye transformation and biosynthesis, waste effluent treatment, dye bleaching applications. These enzymes are used in commercial textile applications to improve whiteness and biostoning in traditional bleaching of cotton. With these applications, chemical, energy, and water savings are achieved. Every year, approximately 800 thousand tons of paint is produced in the world, 10% of the produced paint is disposed of as waste to the environment [55]. Physical and chemical methods are used to clean these wastes, but they are expensive and have limited functionality. Laccase enzymes are used in textiles for decolorization of these dyes. For this reason, various laccase processes have been developed [56, 57].

Trametes versicolor laccase enzyme is used effectively in the denim industry. In denim finishing, the laccase enzyme is used for indigo degradation [58, 59]. Also, it is used to increase the fixation of the dye on wool and to decolor the wool from azo dyes. Thus, the amount of paint is reduced, and the cost is reduced [60]. The laccase enzyme can be used in the roving process to increase yarn regularity. The benefit of using laccase enzyme in rove scrubbing is that the process is carried out under mild reaction conditions, thus leading to an ecologically friendly process [61]. The traditional chlorination method is a process that is applied against preventing the shrinkage of the wool and harms the environment. Therefore, the laccase enzyme is used in this process as an eco-friendly method [62]. These enzymes are also used to produce new textile dyes developed from phenolic and polyphenolic compounds.

2.3.2. Biofuels

Lignocellulosic materials are an important source for bioethanol production. Many laccases play a role in the breakdown of these complex molecules with pretreatment of biomass and take part in the process of bioethanol production. Lignin

biodegradation pretreatment with laccase enzymes is carried out with a cost-effective process [63]. The laccase enzyme produced by heterologous expression from *Trametes versicolor* has been used in the process of bioethanol production by eliminating phenolic compounds [64]. However, the laccase from *Ganoderma lucidum* removed the phenolic content by 84%, increasing bioethanol production by 10% [65].

2.3.3. Food and Baking Industry

Molecules found in many foods and beverages are recognized by the laccase as a substrate. With the laccase enzyme applications in the food industry, new functionality not only improves quality but also reduces cost [66, 67]. These enzymes are used as additives in food and beverage processing. Modify, change color and appearance of the drink and food are such processes that laccases used in. For example, it is used as an oxidative agent in wine production to stabilize the drink and prevent cork stoppers from leaving stains [68, 69]. It is used to stabilize the beer, remove oxygen during the production process, thereby extending the storage life [70]. It is used for clarifying and stabilizing the color of fruit juice processing [71, 72]. Because of their ability to cross-link the biopolymers in the baking process, laccases function in increasing the workability of the dough, mainly by increasing the strength of the dough, reducing its stickiness. It is also used to increase the volume of the baked product, to improve crumb structure and softness [73]. To improve sensory parameters in foods, laccases are used in various food products to control odor, increase taste or reduce unwanted products [74].

Laccases are used as oxidizing agents in baking. It can increase the strength of some dough components such as gluten because of cross-linking biopolymers. For this reason, it increases the strength, stability and volume of the dough and also decreases the stickiness. Thus, it offers the quality workability of the dough, a more appropriate crumb structure and a more ideal baked product structure. *Trametes hirsuta* is used for this purpose [75, 76].

2.3.4. Biosensor and Diagnostic

Laccases are used to identify and measure polyphenols in different food products such as wine, beer and tea. Biosensors have been produced for this purpose [77]. Polyphenols found in wines are also detected through an amperometric biosensor consisting of tyrosinase and laccase from *Trametes versicolor* [78].

2.3.5. Organic Synthesis

New bioactive compounds with therapeutic value are required in many developing industries such as medicine, agriculture, pharmaceutical, and food. The production of these molecules enabled the use of enzymes in organic synthesis in terms of cost and efficiency. Laccase enzymes are also used in many industries to produce different products in organic synthesis reactions [79, 80] (Table 2.3).

Table 2.3: Laccase reaction using for organic synthesis.

Transformation of Phenolics	Oxidative Decomposition
	Detoxification of Phenolic Pollutants
	Detoxification of Azo Dyes
	Monolignol Oxidation
	Dimerization
	Oxidative Coupling
Synthesis via Quinone Formation	C–N, C–C, C–S and C–O Bond Formation / C–S and C–N, C–O and C–C Bond Formation
Important Pharmaceutical Compounds	Dimerization
	Antibiotic Derivatization
	Amino Acid Derivatization
Cross–Coupling Reactions	
Deprotection Reactions	
Decarboxylation Reactions	
Dye synthesis	

2.3.6. Pulp and Paper Industry

Laccases are widely used in paper industry and pulp industry. Also, it is chloride-free in biopolypation process and is used to remove various paints and adhesives from wood materials [81].

The laccase enzyme is used for the delignification and polishing of pulp, as well as for the removal of lipophilic extracts responsible for the accumulation of pitch from both wood and non-wood pulp. These enzymes can improve different features of the pulp by forming reactive radicals with lignin or by functionalizing lignocellulosic fibers. It can also make colored and toxic compounds released from various industries as wastewater non-toxic through polymerization and depolymerization reactions.

2.3.7. Removal of Pollutants (Bioremediation)

One of the most important problems of today is environmental pollution. Drugs, cosmetics, many chemical products, different industrial paint wastes are emerging contaminants that can seriously affect the environment and organisms. Some of these molecules can cause nervous, hormonal and reproductive system disorders and even cancer. Therefore, their removal from the environment is important for both living systems and the environment. Due to the reaction catalyzed by laccase enzymes, it has many applications and potential in biological treatment and removal of environmental pollutants. These enzymes can catalyze oxidation-reduction reactions to biodegrade some of these pollutants very efficiently and can also be used to improve biodegradation of pharmaceutical pollutants [82, 83].

2.3.8. Pharmaceutical and Cosmetic Industry

In the cosmetic industry, laccases are used as oxidizing agents instead of hydrogen peroxide. Laccase-based hair dyes are less irritating than hydrogen peroxide-based hair dyes and are easier to use. Furthermore, it has been done in cosmetic and dermatological studies, which are used as skin whiteners and contain various proteins [84]. New laccases, especially from *Actinomyces Thermobifida*

fusca and basidiomycete *Flammulina velutipes*, have recently been used in the oxidation of dye intermediates commonly used in hair coloring [85, 86]. Moreover, different hair dye colors have been developed recently using laccase.

2.4. *Madurella mycetomatis*

Madurella mycetomatis is a fungus belonging to the *Ascomycete* group, a granulomatous inflammatory disease pathogen known as black-grain mycetoma (tropical eumycetoma) in humans. This fungus progresses initially to the skin and subcutaneous tissues and then to other deep structures by slow-growing and disease is especially common in South America, Africa, and India [87]. *M. mycetomatis* is morphologically reddish-brown or black with a diameter of 0.5–5 mm. They have hyphae in a grey or brown matrix and slow growth (Figure 2.10). The fact that *Madurella* species are usually dark color is caused by melanin pigmentation [88]. Growth temperatures can be up to 40 °C.

There are no reported any industrial enzymes isolated from this organism. This study is the first study reported that an enzyme isolated from *M. mycetomatis* for industrial application.

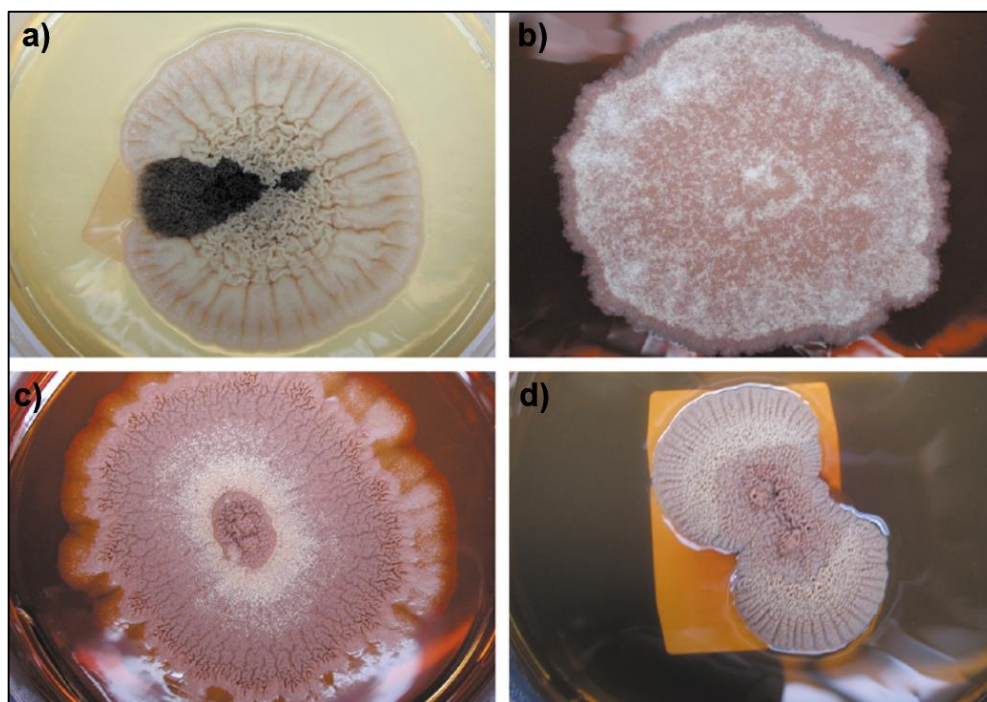


Figure 2.10: *Madurella mycetomatis* clinical isolates: (a), (b), (c) and (d).

The *Madurella mycetomatis* mm55 strain (Sudan) genome used in the thesis was determined in 2016. DDBJ/EMBL/GenBank under the accession number [LCTW000000000](#), BioProject PRJNA267680 [89].

3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Laccase Gene (*mmlac*) Structures and Plasmids

Direct laccase gene manipulations from *M. mycetomatis* were not performed due to the high cost of the organism and cloning studies, the difficulty of codon optimization. Instead, services were purchased from synthetic gene technology. Two different constructs from source of *M. mycetomatis* were synthesized commercially (GenScript, USA) for the *mmlac* gene. *P. pastoris* expression plasmids pPICZ α A (3.6 kb) and pPICZA (3.3 kb) were obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA).

The pPICZA and pPICZ α A plasmids allow gene insertion with multiple cloning regions (10 unique restriction sites). Two plasmids containing a strong, highly inducible P_{AOX1} promoter were used for the expression of the gene. The pPICZA plasmid was then used for secretion with the native signal sequence, and the other plasmid pPICZ α A was used for secretion by the alpha signal. Each plasmid has a polyhistidine tag (6xHistag). This tag is required for the purification of protein affinity chromatography. All plasmids contain the Zeocin™ resistance gene (*Sh ble* gene, *Streptoalloteichus hindustanus ble* gene) for positive selection in *E. coli* and *P. pastoris* and, pUC origin allows the replication of the plasmid in *E. coli*. Also, these plasmids contain regions (from *PmeI*, *BstXI* or *SacI* restriction digestion sites) that allow linearization of plasmids at the AOX1 location for efficient integration into the *P. pastoris* genome (Figure 3.1).

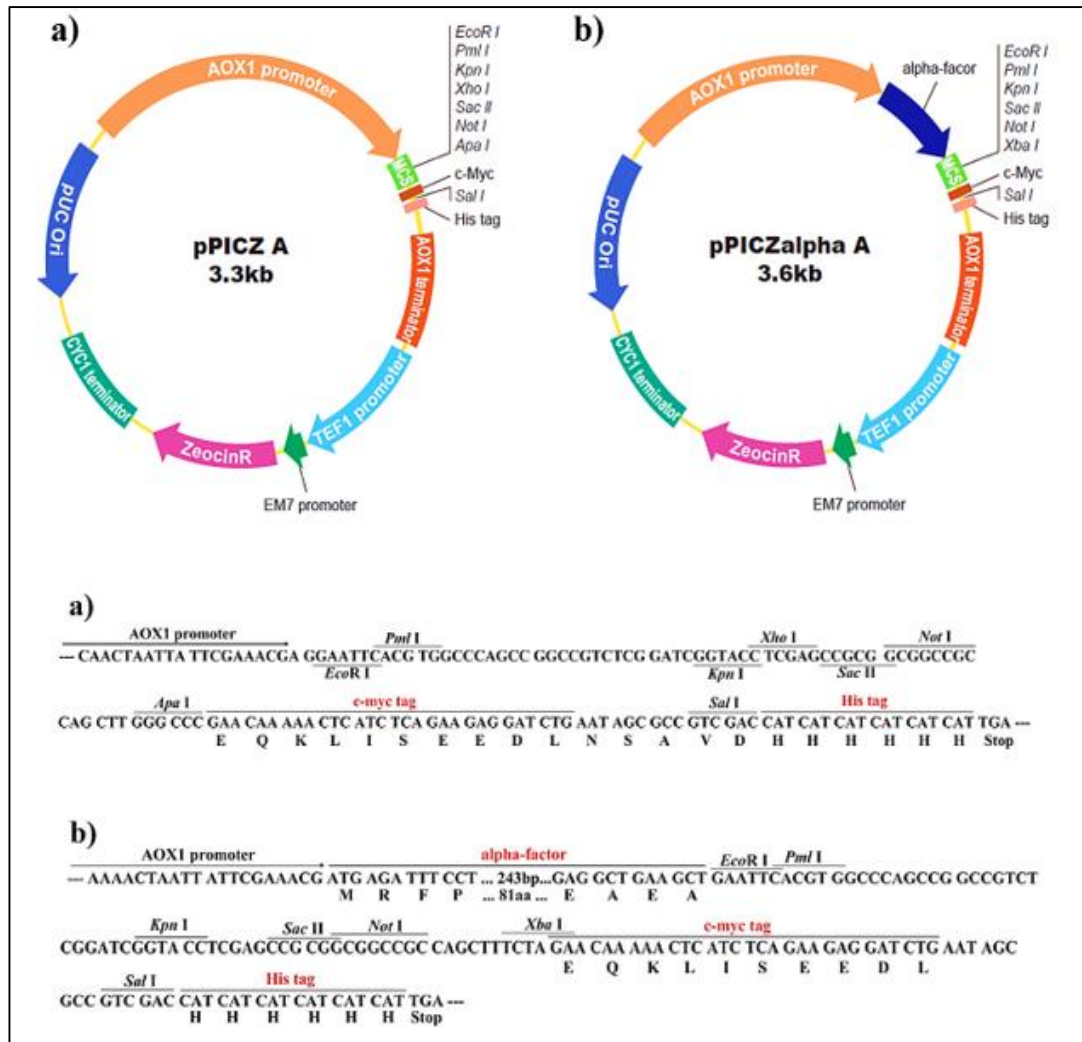


Figure 3.1: Maps (a) and multiple cloning sites (b) of pPICZA and pPICZαA plasmids.

3.1.2. Bacteria and Yeast Strains

Escherichia coli TOP10 (Genotype; F' {*proAB*, *lacI* *q*, *lacZ*Δ*M15*, *Tn10* (*TetR*)} *mcrA*, Δ(*mrrhsdRMS*–*mcrBC*), *φ80lacZ*Δ*M15*, Δ*lacX74*, *deoR*, *recA1*, λ–*araD139*, Δ(*ara*–*leu*)7697, *galU*, *galK*, *rpsL*(*StrR*), *endA1*, *nupG*) and *Pichia pastoris* X–33 (wild type, Mut⁺) cells were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). All media used for *E. coli* and *P. pastoris* were prepared according to the EasySelect *Pichia* Expression Kit manual (Invitrogen).

E. coli TOP 10 cells were used to amplify and purify recombinant plasmids. *P. pastoris* is a commonly used heterologous eukaryotic expression system. The advantages of the *Pichia* expression system are protein folding, post–translational

modifications, the presence of processes in high eukaryotic cells such as protein processing, easy molecular manipulations, and inexpensive, rapid and high level of protein expression [90]. In this expression system, *P. pastoris*, a methylotrophic yeast, uses methanol as the sole carbon source. Methanol is oxidized to formaldehyde using molecular oxygen with alcohol oxidase enzyme encoded by alcohol oxidase (AOX1 and AOX2) genes. Besides, hydrogen peroxide, a toxic product, is formed in this reaction. To protect against this effect of hydrogen peroxide, *P. pastoris* performs the metabolism of methanol in an organelle called the peroxisome. To compensate for the weak affinity of alcohol oxidase to oxygen, it produces a large amount of this enzyme with the AOX, a powerful promoter. Thus, the product that is intended to be produced as heterologous is produced through this promoter [91].

P. pastoris is manipulated so that it can produce foreign proteins intracellularly or extracellularly (secreted). A secretion sequence (signal) is required for foreign proteins to be secreted extracellularly. This signal sequence can be found naturally in some proteins or it can be designed as a fusion protein. However, the secretion efficiency may vary for a different protein and signal sequence [90]. Since natural proteins secreted extracellularly are low levels in *P. pastoris*, heterologous extracellular protein is an advantage for protein production. The medium is minimal in the *Pichia* growth medium. The most of protein in the medium is heterologously expressed protein. This provides a great advantage in the protein to be purified [92, 93]. However, *P. pastoris* is a suitable expression system for post-translational modifications. In the presence of Asn-X-Ser/Thr in proteins, it can produce glycosylation. The absence of hyperglycosylated in proteins secreted extracellularly in *P. pastoris* is an advantage over *Saccharomyces cerevisiae* [94, 95].

To express a gene in *P. pastoris*, it must first be cloned into a suitable plasmid and then inserted into the host cell genome. Integration into the genome occurs by crossover recombination (Figure 3.2). In the final stage, different strains and culture optimization are required for the expression of the recombinant integrated gene.

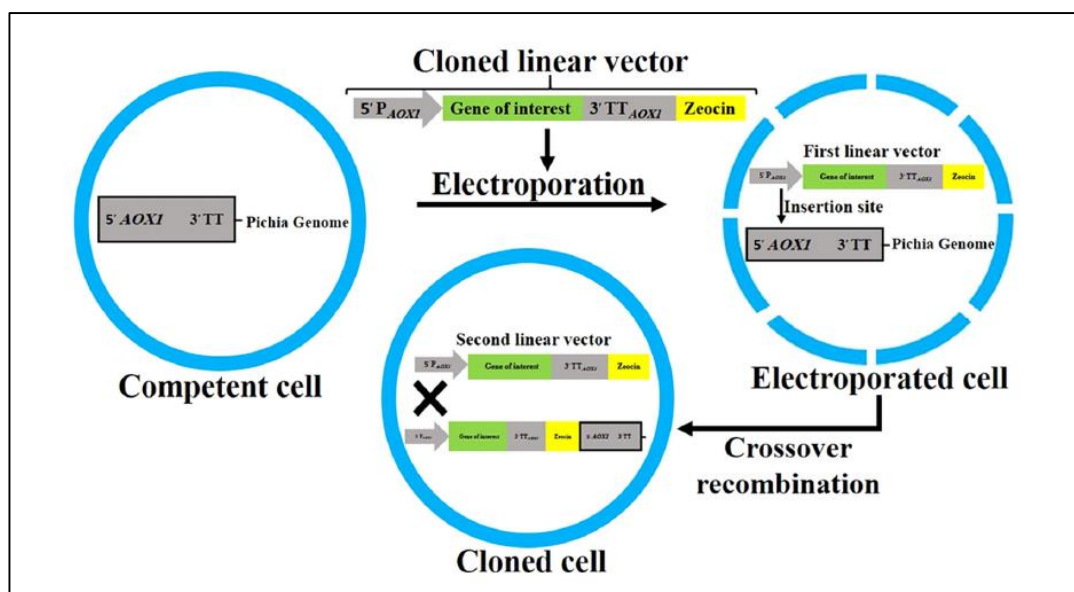


Figure 3.2: Integration of a foreign gene into the *P. pastoris* host cell genome by crossover recombination. P_{AOX1} : alcohol oxidase 1 promoter; TT_{AOX1} : alcohol oxidase 1 transcription termination.

To increase transformation efficiency to competent *Pichia* host cells, plasmids should be linearized. Integration into the host cell genome occurs by cross-recombination between the 5' promoter region of the plasmid, P_{AOX1} , and the yeast AOX1 region. If the loss of the AOX1 gene occurs as a result of crossover recombination, the strains formed are expressed as Mut^- or Mut^s (Methanol utilization slow). This results in the loss of alcohol oxidase activity of the cells and a decrease in the ability to metabolize methanol. Therefore, cells grow poorly in the methanol medium. Cells showing the Mut^+ (Methanol use plus) phenotype are strains that metabolize methanol as a sole carbon source. These two phenotypes are used when evaluating *Pichia* transformants for the integration of genes.

3.1.3. Chemicals, Enzymes, and Kits

Cationic metals ($MgSO_4$, $CuSO_4$, $FeSO_4$, $AgSO_4$, $HgSO_4$, $ZnSO_4$, $CoSO_4$, $LiSO_4$, $MnSO_4$, $Ca(NO_3)_2$ and laccase enzyme substrates (ABTS, SGZ, 2,6-DMP) were purchased from Sigma. Restriction enzymes (*PmeI*, *Sall*, and *EcoRI*), DNA ligase, EndoH, *Taq* DNA polymerase was purchased from (Nebbiolabs). 2-Propanol (Sigma), acetic acid (Merck), agar (Biolife), agarose (Sigma), D (-) sorbitol (Merck), DMSO (Sigma), EDTA (Merck), ethanol (Merck), glycerol (Sigma) hydrochloric

acid (Sigma), LB-broth (Acumedia), (Sigma), methanol (Merck), peptone (Fischer Scientific), PEG 8000 (Bio Froxx), potassium chloride (Sigma), sodium chloride (Sigma), sodium hydroxide (Sigma), sucrose (Caissonlab), Tris (Appllichem), Triton X-100 (Bio Froxx), yeast extract (Biolife), zinc chloride (Sigma), Zeocin (Invitrogen), LB-agar (Caisson), citric acid (Merck), biotin (Sigma), YNB (BD), D(+) glucose (Neofroxx), DNA ladder (1 kb, Thermo), BCA Kit, (Thermo). Protein ladder (PI26620), Plasmid Isolation Kit, and BCA Protein Assay Kit were obtained from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA).

3.1.4. Laboratory Equipment

Refrigerated centrifuge (Awel-CF48R and Nüve-NF400), ultracentrifuge (Beckman Coulter, Optima MAX-XP), plate centrifuge (Sigma), autoclave (Hirayama-HV110L), ultrapure water system (Gel-SpA), micropipettes (Axygen), dry air sterilizer oven (VWR-Dry-Line), deep freezers (-80 °C) (VWR-Symphony), ice machine (Bar Line-BF85), gel imaging system (VilberLourmat), SDS-PAGE electrophoresis system (Biorad), spectrophotometer (Biorad-Smartspec 3000), nanodrop (Shimadzu-Biospecnano), sonicator (Diagenode-Biorupter Pico), shaking incubator (Inforce AG-CH4103 and Wisc, IS-20), precision weighing scale (Sartorius, BL-3100 and BL-210-S), magnetic stirrer (SCILOGX-MSPA), hotplate&magnetic stirrer (Daihan Scientific, MSH-20D), pH meter (HannaHI-2211), electroporator (BIO-RAD, Gene Pulser x-cell), vortex (Daihan Scientific, VM-10), water bath (Nüve, NB20), thermal cycler (Biometra, TRIO), incubator (Nüve, ES120), sterile cabinet (Nüve, MN090), plate reader spectrophotometer (Thermo, Varioskan), mini spin (Nüve, NF024).

3.1.5. Culture Mediums

Low Salt LB (Luria-Bertani) Medium (1 L):

- 10 g tryptone, 5 g yeast extract, 5 g NaCl are mixed in 950 mL deionized water. The pH is adjusted to 7 with 10 N NaOH and the final volume is completed to 1 L. The autoclave is done at 121 °C for 20 minutes and

Zeocin™ to 25 µg/mL final concentration is added when it cools to ~55 °C. If Zeocin is added to the medium, it should be stored in the dark at 4 °C.

Low Salt LB Agar Plates (1 L):

- 15 g/L agar is added to the Low Salt LB medium before autoclaving. When the medium cools to ~55 °C after autoclave, Zeocin is added to 25 µg/mL final concentrations. Medium is poured into Petri plates. After the plates have hardened, they are inverted and stored at 4 °C. If Zeocin™ is added, petri plates can be stored in the dark at 4 °C for 1–2 weeks.

YPD (Yeast Extract Peptone Dextrose) Medium (1 liter):

- 20 g peptone and 10 g yeast extract are mixed in 900 mL of deionized water. (If YPD agar plates are to be made, add 20 g of agar). The autoclave is done for 20 minutes. When medium cools to ~60 °C, 100 mL of 10X Dextrose is added. If desired, add 1 mL of 100 µg/mL of Zeocin™ into the medium. Liquid medium or agar plates added with Zeocin can be stored at 4 °C for 1–2 weeks.

YPDS (Yeast Extract Peptone Dextrose Sorbitol) Zeocin™ Agar (1 liter):

- 10 g yeast extract, 182.2 g sorbitol, 20 g peptone, 10 g agar are mixed in 900 mL of deionized water and autoclaved for 20 minutes. Autoclave for 20 minutes on the liquid cycle. When medium cools to ~60 °C, 100 mL of 10X Dextrose and 1 mL of 100 µg/mL Zeocin™ are added in medium. Liquid medium or agar plates added with Zeocin can be stored at 4 °C for 1–2 weeks.

MM (Minimal Methanol) Agar ABTS Medium:

- 15 g of agar is mixed in 800 mL of deionized water and autoclaved for 20 minutes. When medium cools to 60 °C, add 100 mL of 10X YNB, 2 mL of

500X Biotin, 0.5 mM ABTS, 0.2 mM CuSO₄, 100 mL of 10X methanol, 1 ml of 100 µg/mL Zeocin™. Medium is poured into petri plates. After the plates have hardened, they are inverted and stored at 4 °C. If Zeocin™ is added, petri plates can be stored in the dark at 4 °C for 1–2 weeks.

BMGY (Buffered Glycerol–Complex Medium) and BMMY Buffered Methanol–complex Medium (1 L):

- 10 g of yeast extract and 20 g peptone are mixed in 700 mL of deionized water and autoclaved for 20 minutes. When medium cools to 60 °C, add 100 ml 1 M potassium phosphate buffer (pH 6.0), 100 ml 10X YNB, 2 ml 500X Biotin, 100 ml 10X glycerol. For BMMY, add 100 ml 10X M instead of glycerol. The medium can be stored at 4 °C for 2 months.

3.1.6. Buffers and Solutions

Protein Purification Buffers:

Proteins purification from the supernatant was done by using HisTrap protein purification columns. Purification buffers are given below:

- Buffer 1: 1.38 g NaH₂PO₄·H₂O (20 mM), 14.61 g NaCl (500 mM), and 1.02 g imidazole (30 mM) were dissolved in 500 mL dH₂O and the pH was fixed to 7.4 using NaOH.
- Buffer 2: 1.38 g NaH₂PO₄·H₂O (20 mM), 14.61 g NaCl (500 mM), and 17.02 g imidazole (500 mM) were dissolved in 500 mL dH₂O and the pH was fixed to 7.4 using NaOH.
- Buffer 1 with 100 mM imidazole (50 mL): 42.50 mL Buffer 1 and 7.50 mL Buffer 2 were mixed.
- Buffer 1 with 200 mM imidazole (50 mL): 32 mL Buffer 1 and 18 mL Buffer 2 were mixed.
- Buffer 1 with 400 mM imidazole (50 mL): 10.50 mL Buffer 1 and 39.50 mL Buffer 2 were mixed.

Zeocin (Powder) solution:

- 100 mg/mL concentration of Zeocin is dissolved in HEPES (5 g/L, pH: 7.2) buffer and filtered through a 0.22 μm filter. It is stored at $-20\text{ }^{\circ}\text{C}$ in the dark for 18 months.

Enzyme Activity Assay Buffers:

- Sodium acetate buffer (0.1 M, pH: 5.0): 5.772 g sodium acetate and 1.778 g acetic acid are mixed in 800 mL deionized water. 10 N HCl and 10 N NaOH are used to set the pH to 5. The final volume is completed to 1 L with deionized water.
- Britton Robinson buffer (0.1 M pH: 2.0–3.0 and 8.0–11.0): 0.1 M acetic, boric and phosphoric acid mixture. The desired pH value is set at 10N HCl or 10N NaOH.

0.02% Biotin (500X):

- Filter sterilized solution which contains 40 mg of biotin dissolved in 200 mL of water can be stored at $2\text{--}8\text{ }^{\circ}\text{C}$. The solution can be stored about 1 year.

YNB (Yeast Nitrogen Base with ammonium sulfate without amino acids, 10X):

- 134 g of YNB is dissolved in 1 L of deionized water and filtered through a 0.22 μm filters. It is stored at $4\text{ }^{\circ}\text{C}$.

20% Dextrose (10X):

- 200 g dextrose is dissolved in 1 L deionized water and autoclaved for 15 minutes. The solution can be stored for 1 year at room temperature.

5% Methanol (10X):

- 50 mL of methanol is mixed with 950 mL of deionized water. It is passed through 0.22 μ m filters. It can be stored at 4 °C for 2 months.

10% Glycerol (10X):

- 100 mL of glycerol is mixed with 900 mL of deionized water and autoclaved for 15 minutes. The solution can be stored for 1 year at room temperature.

Potassium phosphate buffer, 100 mM pH 6.0:

- Potassium phosphate buffer (0.1 M, pH: 6.0): 2.405 g K_2HPO_4 and 11.73 g KH_2PO_4 are mixed in 800 mL deionized water. 10 N HCl and 10 N NaOH are used to set the pH to 6. The final volume is completed to 1 L with deionized water.

SOB (Super Optimal Broth, 1 L) and SOC (1 L) medium:

- 2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ were measured. Medium completed to 1 liter with ddH₂O and autoclaved. For SOC medium, glucose is added to the SOB medium with a final concentration of 20 mM and autoclaved.

TSS Buffer (Transformation and Storage Solution, pH: 6.5):

- 10% PEG (6000), 5% DMSO, 50 mM $MgCl_2$ is measured and autoclaved.

Chloroform–Isoamylalcohol (CIA, 50 ml):

- 48 mL chloroform, 2 mL Isoamylalcohol

Solutions Used for Agarose Gel Electrophoresis:

- 50X TAE Buffer (1 L): Measure 242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH: 8.0), and complete to 1 L with the final volume ddH₂O.
- 0.8% Agarose gel (40 ml): 0.32 g agarose, 3 μ L Serva DNA Stain Clear-G, 40 mL 1X TAE.

Solutions Used for SDS-PAGE:

- 4X Sample Buffer (pH: 6.8): 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl.
- 10X SDS Running Buffer (1 L): 151.5 g of Tris, 720 g of glycine, and 50 g of SDS are dissolved in 1 L of ddH₂O.
- 30% Acrylamide-Bisacrylamide Solution (pH: 7.0): 290 g of acrylamide and 10 g of N, N'-Methylenebisacrylamide is dissolved in 1 L of ddH₂O and storage at 4 °C.
- Coomassie Staining Solution (2 Liter) : 2 g Coomassie Blue (R-250) 900 mL methanol, 200 mL glacial acetic acid and 900 mL ddH₂O.
- Destaining Solution: 400 mL methanol, 100 mL glacial acetic acid, and 500 mL ddH₂O.

Solutions Used for MALDI-TOF-MS: Matrix Solutions (20 mg/mL):

- Sinapinic acid: acetonitrile, 0.1% trifluoroacetic acid (70:30, v/v).
- α -Cyano-4-hydroxycinnamic acid (CHCA): acetonitrile and 5% formic acid (70:30, v/v)
- 2,5-dihydroxybenzoic acid (DHB): acetonitrile and 0.1% trifluoroacetic acid (TFA) (70:30, v/v)
- CHCA-DHB: Mix α -Cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid solution in 40:60 ratio (v/v)

3.2. Methods

3.2.1. Nucleic Acid and Protein Manipulation Techniques

3.2.1.1. Agarose Gel Electrophoresis

1 g of agarose is dissolved by boiling in 100 mL of 1X TBE buffer. After cooling, 3 μ L of Serva DNA Stain Clear-G was added to the agarose gel. The agarose gel tank was then filled with this solution. After the gel was frozen, the tank was filled with 1X TBE buffer. DNA samples were then loaded onto the gel along with the DNA marker and the gel was run at 90 V. DNA bands were displayed under UV light.

3.2.1.2. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on the 12% separation gel defined by Laemmli [96] and 5% loading gel in the presence of 0.1% anionic detergent SDS. Bio–Rad Mini–Slab Cell CT was used for vertical gel electrophoresis. 20 μ L enzyme samples were mixed with 4X sample buffer containing 1% SDS and kept in boiling water for 20 minutes. Protein samples were then loaded onto the gel along with the molecular weight marker and the gel was run at 90 V. After the electrophoresis was completed, the gel was stained with Coomassie Brilliant Blue–R250 and then washed several times with the dye–removing solution and removed from the dye. The bands formed by the proteins were compared with the protein bands whose weight in the molecular weight marker was known and the molecular weights of the proteins were determined.

3.2.1.3. Determination of Protein Amount by BCA Method

The amount of protein purified to be used in activity experiments was determined by BCA (Bicinchoninic acid) method. Of the protein samples obtained, 10 μ L of 96 wells were placed on the microplate plate. 200 μ L of the BCA solution

created by mixing freshly prepared 50 volumes of solution A and 1 volume of solution B was added to each sample. Bovine serum albumin (BSA) was used as the standard in different concentrations (1– 20 μ g). Samples that were incubated at 37 °C for 45 minutes were measured with 490 nm absorbance Elisa reader (Elx800, Universal Microplate Reader, Biotek. Ins.). Protein amounts in the samples were calculated based on the standard curve drawn according to the values obtained from BS standards.

3.2.1.4. MALDI–TOF–MS Analysis

Matrix–assisted laser desorption/ionization–time of flight (MALDI–TOF) was performed to ascertain the accurate molecular weight of purified *MmLac*. Mass spectrometry of the recombinant *MmLac* was performed by MicroFlex instruments (Bruker Daltonics, Billerica, MA). The MS was calibrated with broad range molecular weight markers (BioRad), which contained the proteins phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa).

The previously reported protocol used for MALDITOF MS analysis is set out below [97, 98].

- Buffer Exchange for *MmLac* protein sample preparation: Buffer exchange was performed because the purification and storage buffers used for *MmLac* were not suitable for the study. This step especially affects the spectrum quality. 20 μ L of micromolar concentrations of *MmLac* (15 μ M) were used for buffer exchange. Enzyme solution for buffer exchange carried out using centrifugal ultrafiltration device (Vivaspin) and was repeated 3x. For this, aqueous NH_4Ac solution at a concentration between 5 mM–1 M, pH 6–8 was used.
- Recrystallization of Matrices: 10 mL of 40% (EtOH) was poured into an Erlenmeyer flask and 600 mg of matrix was added. The solution was dissolved by mixing with a magnetic stirrer. The solution was thoroughly mixed then allowed to cool slowly. Matrix crystals were collected by filtration and allowed to dry.

- **Cleaning of Target for MALDI (Stainless Steel):** The MALDI plate was rinsed with MeOH and wiped gently with a cleaning tissue. The target was rinsed with H₂O and wiped again with a cleaning tissue. The MALDI plate was placed in a 600 ml beaker and covered with 50% EtOH. The target was sonicated in EtOH solution for 10 minutes in an ultrasonic bath. This step has been repeated several times. Finally, the target was allowed to dry at room temperature after rinsing with MeOH and wiping with a cleaning tissue.
- **Preparation–Deposition on the MALDI Target for α -CHCA Thin Layer:** A saturated solution was obtained by dissolving α -Cyano-4-hydroxycinnamic in acetone. By hand, a tip of 10 μ L was taken and immersed in α -Cyano-4-hydroxycinnamic acid saturated solution. The solution remaining on the pipette tip due to capillarity was touched very quickly to the MALDI target with the pipette tip and the α -Cyano-4-hydroxycinnamic acid–acetone solution was placed on the MALDI target. This process has formed the matrix thin layer where the *MmLac* sample will be deposited.
- ***MmLac* Deposition on the MALDI Target:** 0.5 μ L of *MmLac* sample was placed on the α -Cyano-4-hydroxycinnamic acid thin layer and 0.5 μ L of matrix solution was added immediately after. Thus, the *MmLac* sample and the matrix were mixed on the target. The *MmLac* sample was mixed to the matrix in a mixed ratio using acetonitrile–formic acid solution. However, MALDI spectra gave the best quality results at the ratio of 1:1.
- **Calibrant Deposition:** Matrix solution (0.5 μ L) was added immediately after 0.5 μ L of calibrant standard (next to the *MmLac* sample spots) was added.
- **Determination of MALDI spectra for Calibrant standard and *MmLac* Samples:** The target was placed in the MALDI–TOF instrument to obtain sample spectra, and the parameters were adjusted according to the manufacturer’s recommendations. For this, the device is used in linear mode. A 500 ns “pulsed ion extraction” was used to analyze the *MmLac* samples.

3.2.2. Design of *mmlac* Gene Constructs and Bioinformatic Analysis

Before the commercially *mmlac* gene, its putative form (GenBank accession number: KXX78172.1) was analyzed [99]. Conserved domains were determined with

the Conserved Domain Database [100]. Predicted signal sequences were identified using SignalP 4.1 [101] and MitoProt II–v1.101 [102]. The gene sequence was optimized for the codon usage preference of *P. pastoris* expression [103]. The codon–optimized gene was submitted to GenBank (Accession number: MT450838). N–glycolysis sites, pI (isoelectric point), and Mw (molecular weight) of the *Mmlac* enzyme coded by the engineered *mmlac* gene were estimated Expasy Proteomics server [104].

Two gene sequences for commercial synthesis were constructed based on the *mmlac* gene sequence analysis. The first construct (*mmlac1*) was a codon–optimized *mmlac* gene with its native signal peptide present that was cloned into pPICZA. The identified native and pro–signal in the N–terminal and pro–signal peptide after coding Asp–Ser–Gly–Leu (DSGL) amino acid sequence patterns in the C–terminal [105, 106] were removed in the second construct (*mmlac2*). Furthermore, 6 amino acids (ETEAEF) and polyhistidine tag sequence coding regions have been added to the N–terminal *EcoRI* region of *mmlac2*.

3.2.3. Restriction Digestion Reactions

Double digest reactions using *SalI* and *EcoRI* restriction enzymes were prepared to obtain adhesive ends from each gene structure and plasmids (Table 3.1).

Table 3.1: Restriction endonuclease reactions prepared for gene constructs and plasmids.

Restriction Components	Final Concentration	Restriction Components	Final Concentration
Gene construct	~5000 ng	Plasmid	~6700 ng
<i>EcoRI</i>	20 U	<i>EcoRI</i>	40 U
<i>SalI</i>	20 U	<i>SalI</i>	40 U
Buffer 4	1X	Buffer 4	1X
dH ₂ O	1.5 µL	dH ₂ O	2.5 µL
Total	75 µL	Total	75 µL

After the incubation process was applied at 37 °C in a water bath for 2.5 hours, synthetic gene fragments (*mmlac1* and *mmlac2*) and plasmids (pPICZA and pPICZαA) digest by *EcoRI* and *Sall* restriction enzymes were loaded into a 1% agarose gel. The fragments in agarose gel were run by electrophoresis at 90 V for about an hour. After electrophoresis, the size of the DNA fragments was verified by comparison with the 1 kb molecular DNA marker. The appropriate bands displayed with a gel imaging device (under UV light) were cut with a sterile scalpel and transferred to Eppendorf tubes. The DNA Purification Kit from the Gel was used to purify the DNA fragment contained in the cut gel particles (GeneJET™ PCR Purification Kit, Fermentas). Gene fragments and plasmids, purified as recommended by the manufacturer, were stored at –20 °C after concentration.

3.2.4. Ligation Reaction

The *mmlac1* and *mmlac2* gene construct was digested with *EcoRI* and *Sall* restriction endonucleases was transferred to the pPICZA and pPICZαA plasmids respectively, which was cut and linearized with the same restriction enzymes. Reaction mixtures containing 100 ng plasmid DNA, 300 ng gene fragment, and 3 U T4 DNA Ligase were incubated overnight at 16 °C in 2X ligation buffer. While preparing the ligation reaction, insert and plasmid ratios were calculated as 1:3, 1:5, and 1:8, and their calculations were made based on the formula given below (3.1):

$$ng(insert) = \frac{ng(vector) \times insert(bp)}{vector(bp)} \quad (3.1)$$

3.2.5. Preparation of Competent *Escherichia coli* TOP10 Cells

The method proposed by Chung et al. [107] was used to make cells competent and transformed. According to this procedure, the *E. coli* TOP10 cells to be competent were inoculated into 5 mL of liquid LB and incubated overnight. The next day, 100 µL of pre-culture was added to 10 mL of fresh liquid LB. The growth amounts of the cells were measured with a spectrophotometer at 600 nm and expected to reach the desired OD value (0.4–0.5). When the culture reached the desired OD, it was centrifuged at 1500×*g* for 5 minutes at 4 °C and the supernatant

was discarded. After OD measurements, 10% of the remaining culture volume TSS was added and the cells were used for transformation after the pellet was thawed in ice.

3.2.6. Transformation into *Escherichia coli* TOP10 Competent Cells

10 µL of the ligation reaction was added to the Eppendorf tubes previously kept on ice and cooled. 50 µL of newly prepared competent *E. coli* TOP10 cells were added in Eppendorf tubes. After the tubes were mixed slowly, they were kept in ice for 30 minutes, and immediately afterwards a 45-second heat shock was applied to the cells. It was then incubated in ice for 2 minutes. 950 µL SOC medium was added to each tube and incubated at 37 °C, 150 rpm for 80 minutes. Cells diluted after transformation were seeded into LB agar (25 µg/mL Zeocin) medium plates to obtain single colonies. After overnight incubation at 37 °C, possible recombinant cells growing on the medium were collected and pure culture was prepared by inoculating the new LB agar medium plates. Plasmid isolation was performed from these cultures by miniprep method [108]. The recombinant plasmids obtained were prepared for transforming into competent *P. pastoris* cells by cutting with *PmeI* restriction endonuclease.

3.2.7. DNA Sequencing of *mmlac* Gene

The accuracy of gene structures in recombinant plasmids obtained as a result of plasmid isolation was demonstrated as a result of sequence analysis. All sequencing was done by Eurofins (Germany).

3.2.8. Linearizing Recombinant Plasmids

Recombinant plasmids were digested with *PmeI* restriction enzyme reaction to increase transformation efficiency (Table 3.2). After determining the amount of linear recombinant plasmids (Nanodrop), they were visualized in 1% agarose gel.

Table 3.2: Restriction endonuclease digestion reaction prepared to linearize the recombinant plasmids.

Restriction Components	Final Concentration
Recombinant Plasmid	~6500–7000 ng
<i>PmeI</i>	40 U
Buffer B	1X
dH ₂ O	5 µL
Total	100 µL

3.2.9. Phenol–Chloroform Extraction and Ethanol Precipitation of Recombinant Linear Plasmids

At the end of the cut reaction with *PmeI*, the restriction enzyme was treated for 20 minutes at 95 °C for inhibition of restriction enzyme inhibition. Recombinant plasmids digest and linear with *PmeI* restriction enzyme were first treated with phenol/chloroform isoamyl alcohol (CIA) for use in electroporation. With this method, restriction enzymes are removed. Ethanol precipitation was used to concentrate linear recombinant plasmids. The Phenol chloroform extraction and ethanol precipitation method used is summarized below:

- For the 300 µL sample, 150 µL CIA and 150 µL phenol were added.
- Centrifuged for 5 min at 11.000×*g*. The supernatant was carefully collected with the pipette.
- Added 150 µL CIA and 150 µL phenol to the supernatant. Centrifuged for 5 min at 11.000×*g*. The supernatant was carefully collected with the pipette. Added 300 µL CIA to the supernatant. Centrifuged for 5 min at 11.000×*g*.
- The supernatant was carefully collected with the pipette. Added 1/10 volume of sodium acetate (3 M, pH 5.2). Added 2.5–3.0X volume (calculated after addition of sodium acetate) of at least 95% ethanol. Incubated at room temperature or on ice for at least 15 minutes. Centrifuged at 13.000×*g* for 30 minutes at –4 °C. Discarded supernatant and added 70% ethanol. Centrifuged again for 15 minutes and discarded supernatant. The pellet was dissolved in ddH₂O.

3.2.10. Preparation of Competent *Pichia pastoris* X–33 Cells

A modified protocol was used to generate competent yeast cells [109]. Single colony *P. pastoris* X–33 is inoculated as 2x1 cm on a YPD agar medium plate. It is incubated for 18 hours at 30 °C. Cells from the YPD agar medium plate are transferred to Eppendorf containing 1 mL of YPD broth. Cells are dissolved by making vortex. Centrifuge at room temperature for 3 minutes at 1500×*g* and discard the supernatant. 1 mL sterile ddH₂O is added to the pellet and dissolved by pipette. The supernatant is discarded by centrifugation at 1500×*g* for 3 minutes at room temperature. This step is repeated 3 times. Add 1 mL of the following solution (Table 3.3) to the pellet and incubate at 100 rpm for 20 minutes at 30 °C. The supernatant is discarded by centrifugation at 1500×*g* for 3 minutes at room temperature.

Table 3.3: The culture medium prepared for the competent cell.

HEPES–NaOH (pH: 8.0)	40 µL (1 M stock)
DTT	40 µL (1 M stock)
LiCl	100 µL (1 M stock)
Sorbitol	600 µL (1 M stock)
ddH ₂ O	220 µL

1 mL of cold 1M sorbitol is added to the pellet and the pellet is dissolved by pipette. The supernatant is discarded by centrifugation at 1500×*g* for 3 minutes at 4 °C. This step is repeated for 750, 500, 250, 100 µL sorbitol (1 M), respectively. At the last stage, 70 µL of 1 M sorbitol is added to the pellet and dissolved by pipette. The competent cells obtained are used in electroporation.

3.2.11. Transformation of *Pichia pastoris* X–33 Competent Cells

Linearized 5 µg plasmid mixed with 80 µL competent *P. pastoris* X–33 was placed in a cold 0.2 mm electroporation cuvette and kept in ice for 5 minutes. Electroporation was performed at 1500 V, 25 µF, 200 Ω. Cold 1 M sorbitol was added into the electroporation cuvette and taken into 15 mL falcon. Cells are

incubated for 2 hours at 100 rpm at 30 °C. After incubation, cells were spread as 10, 25, 50, 100 and 200 µL into YPDS medium (Zeocin 100 µg/mL). The plates were incubated for 3–10 days at 28 °C until colonies were seen.

3.2.12. Colony Polymerase Chain Reaction (cPCR)

The presence of transformed *mmlac* gene constructs in *P. pastoris* X-33 cells was performed following the colony PCR protocol mentioned below:

Each colony taken with a sterile pipette tip was dissolved in 10 µL of 0.02 M NaOH and kept in the microwave oven at the highest temperature for 3 minutes. Colonies were centrifuged at 3000×*g* for 1 minute. 1 µL was taken from the supernatant and added as template DNA in the PCR reaction (Table 3.4). After colony PCR, samples were visualized on a 1% agarose gel.

Table 3.4: Primers used for colony PCR and reaction conditions

MF-α Forward primer	5'-TACTATTGCCAGCATTGCTGC-3'	
AOX1 Forward primer	5'-GACTGGTTCCAATTGACAAGC-3'	
AOX1 Reverse primer	5'-GCAAATGGCATTCTGACATCC-3'	
<i>mmlac</i> Reverse primer	5'-GACACCAATTCTTCTAACAGCAGG-3'	
Cycle	Temperature (°C)	Time (min)
1	95	1
35	94	1
	58	1
	72	1:30
1	72	10

3.2.13. Culture Optimization and Heterologous Expression of the *mmlac* Gene in *Pichia pastoris* X-33

An increasing concentration of Zeocin⁺ (100–2000 µg/mL) was used to identify the multi-copy transformant to be used in *mmlac* expression. Also, the high expression transformants which showing a deeper green color in the MM agar plate were selected for *mmlac* expression experiments. The transformed *P. pastoris* cells

were grown in 250 mL flasks containing 50 mL BMGY medium at 250 rpm and 30 °C. When the cell density was reached to 2–6 at OD₆₀₀ nm, the cells were harvested by centrifugation 2000×*g* for 15 min and inoculated into 250 mL BMMY medium in 2 L Erlenmeyer flask with an initial OD₆₀₀ value of ~1.0. BMMY medium was enriched with CuSO₄ at concentrations ranging from 0.2–2.5 mM to analyze the effect of copper ions on laccase activity. Different temperatures (18, 24, and 30 °C) and methanol concentrations (0.1–2 mM, every 24 hours) were tested separately for determining the optimal temperature and methanol concentrations on *mmlac* expression. *P. pastoris* cells were growing at optimized temperature and methanol (v/v) concentration, the production of laccase was induced by adding various CuSO₄ concentration. During the optimization of process parameters (temperature, methanol, CuSO₄), the supernatant of recombinant *P. pastoris* cell cultures were collected periodically to analyze the level and ensure *mmlac* expression on SDS–PAGE. All activities were measured against the ABTS substrate during parameter optimization studies.

3.2.14. Purification of the Recombinant *MmLac*

Recombinant *MmLac* was purified by IMAC, using a HisTrap FF column and was produced under optimal biochemical conditions. For this, supernatants of recombinant *P. pastoris* X–33 cultures in BMMY were harvested after 7 days by centrifugation (14000×*g*, 4 °C, 25 min) and was filtered through a 0.45 µm filter then applied to a HisTrap FF column which was pre–equilibrated with the Buffer 1 (20 mM NaPi, 500 mM NaCl, 30 mM imidazole pH 7.4). The column was washed with 5 mL of Buffer 1 and the *MmLac* was eluted with Buffer 1 containing different imidazole concentrations (100, 200 and 400 mM). Collected fractions were analyzed by SDS–PAGE to identify fractions containing pure protein, which were then combined and diluted to 50 mL by addition of 100 mM of NaPi pH 7.4. Proteins were concentrated with Amicon Millipore centrifugal concentrators. The concentration of the purified *MmLac* was determined by BCA Assay Kit. Purified *MmLac* was stored at 4 °C until required. The expression and purification efficiency were monitored by %12 SDS–PAGE. Samples were prepared by mixing 15 µL sample and 5 µL 4X sample loading buffer. Samples were heated at 95 °C for 10

minutes then centrifuged at $14,000\times g$ for 10 minutes. The SDS gel was placed in the tank than the tank was filled with running buffer. 5 μL protein standard was loaded into the first well and 5 μL of sample is loaded into the next wells. Then the running was started with 80 V for 10 minutes and it was continued 100 V for 1 hour.

3.2.15. Effect of pH, Temperature, Metal Ions and Halides on Enzyme Activity

Different buffers have been tested to specify the optimal pH values of the *MmLac* enzyme. In the reactions for ABTS, SGZ, and 2,6-DMP as substrate, 100 mM sodium acetate buffer for pH: 4.0–6.0, 100 mM Britton Robinson buffer for pH: 2.0–3.0 and 7.0–11.0 were used. The thermal stability of *MmLac* enzyme was estimated via 400 $\mu\text{g mL}^{-1}$ enzyme and 100 mM sodium acetate buffer. The reactions were carried out in microplate (96-well) at 300 μL volume. To determine the optimal temperature values of 3 substrates, reactions were applied in 5 $^{\circ}\text{C}$ increments between 25–90 $^{\circ}\text{C}$. ABTS was used as a substrate to analyze the effect of metal ions, organic solvents, NaCl on enzyme activity. Reactions were carried out in triplicates at 55 $^{\circ}\text{C}$, pH 4.0, 420 nm. Furthermore, the same reaction conditions were used to define the storage stability of *MmLac* enzyme. The effect of Mg^{2+} , Cu^{2+} , Fe^{2+} , Ag^{2+} , Hg^{2+} , Zn^{2+} , Ca^{2+} , Co^{2+} , Li^{2+} , Mn^{2+} cationic metal ions on *MmLac* enzyme activity was investigated by using 3 different concentrations (1, 10 and 100 mM). Sulfated forms were used to show the effect of cationic metal ions. $\text{Ca}(\text{NO}_3)_2$ form was used only for Ca^{2+} due to low solubility. *MmLac* enzyme inhibition with chlorine ions was detected with different concentrations of NaCl (0 to 1000 mM). For this purpose, the reaction was incubated at 4 $^{\circ}\text{C}$ for 2 hours at a reaction volume of 300 μL . Moreover, the effects of organic solvents such as DMSO, isopropanol, methanol, ethanol, acetone, acetonitrile, cyclohexane, ethyl acetate at different concentrations such as 10, 25 and 50% (v/v) were examined.

3.2.16. Enzyme Activity Determination and Kinetic Parameters

Substrate activity of *MmLac* was followed against ABTS (420 nm; $\epsilon=36,000 \text{ M}^{-1} \text{ cm}^{-1}$), SGZ (530 nm; $\epsilon=65,000 \text{ M}^{-1} \text{ cm}^{-1}$) and 2,6-DMP (468 nm; $\epsilon=49,600 \text{ M}^{-1} \text{ cm}^{-1}$) substrates in 100 mM sodium acetate buffer spectrophotometrically in

optimized pH and temperature [110]. Specific activity and kinetic parameters (K_m , k_{cat} , k_{cat}/K_m) of *MmLac* were calculated for 0.5 mM ABTS, 1 mM 2,6–DMP, 0.1 mM SGZ and against various substrates concentrations (0.1–2 mM) respectively. The curves formed for all 3 substrates were adapted to the Michaelis–Menten equation with GraphPad Prism 8.0 (GraphPad Software, San Diego, USA) by the nonlinear least–squares regression. One unit was described as the amount of enzyme that transforms 1 μ mol of substrate per minute to the product. All experiments were carried out in triplicates.

$$U/L = (\Delta E \times V_t) / (\epsilon \times d \times V_s) \quad (3.2)$$

ΔE ; being the change in the extinction of light [min^{-1}] at 420 nm

V_t ; is the total volume you measured

ϵ ; being the molar absorption coefficient of ABTS [$\text{M}^{-1} \text{cm}^{-1}$]

d ; being the layer thickness [cm] in your cell that the light has to pass

V_s ; is the volume of the enzyme stock solution

3.2.17. Storage Stability of *MmLac*

The evaluation of the storage stability of the various laccase preparation for four different temperatures (20, 4, –20, and –80 °C) were used to determine the storage stability of purified *MmLac* enzyme. The measurement of enzyme activity was carried out once a week and followed by 8 weeks. The enzymatic assay was performed with a defined optimum pH, 100 mM sodium acetate buffer, and ABTS substrate.

3.2.18. Deglycosylation of *MmLac*

Recombinant laccase was deglycosylated by treatment with EndoH. 5 μ g glycoprotein, 1 μ L 10X Glycoprotein Denatured Buffer and H_2O were added in a total reaction volume of 10 μ L and then incubated at 100 °C, 10 min. A total of 20 μ L of reaction volume was made by adding 2 μ L of 10X GlycoBuffer 3, H_2O and 2

μ L EndoH and then incubated at 37 °C for 1 hour. Deglycosylation was monitored by SDS–PAGE.

3.2.19. Homology Modelling and Molecular Docking of *MmLac*

The 3D homology model of *MmLac* was generated through I-TASSER [111] using the published structure of *Melanocarpus albomyces* laccase (*MaLac*, PDBID: 3FU8) [112] as a template. 2,6–DMP substrate was docked into *MmLac* active site pocket *in silico* via the AutoDock platform [113]. Drawings were done by PyMOL (Molecular Graphics System, Version 2.0 Schrodinger, LLC). Amino acid sequences of characterized laccases from PDB were aligned with Clustal Omega and the figure was created with Geneious software. (*Mm*: *Madurella mycetomatis*; *Ma*: *Melanocarpus albomyces* (PDBID: 3FU7); *Ta*: *Thielavia arenaria* (PDBID: 3PPS), *Ba*: *Botrytis aclada* (PDBID: 3SQR); *An*: *Aspergillus niger* (PDBID: 5LM8) and *Tp*: *Trametes pubescens* (PDBID: 2HZH) [114]. Protein secondary structure predicted with EMBOSS tool *garnier* and the figure was created with Geneious software [115, 116].

3.2.20. Industrial Applications of *MmLac*

3.2.20.1. Dye Decolorization

To show the decolorization effect of *MmLac* enzyme on synthetic dyes, 3 different dyes were used: Amido Black 10 B (λ_{max} =620 nm), Rhodamine 6 G (λ_{max} =547 nm), Crystal violet (λ_{max} =590 nm) and Remazol Brilliant Yellow (λ_{max} =595 nm). 0.25 mg of synthetic dye, 4.5 mL of 100 mM sodium acetate buffer, 0.05 mM ABTS and 0.5 mL of purified enzyme solution (5.25 U/L) were added in a 5 mL reaction mixture. In all test tubes, reactions were carried out at 55 °C, 120 rpm for 120 hours. *MmLac* enzyme was not added to the reactions designed as a control group. Samples taken every 24 hours were measured spectrophotometrically. To measure the amount of decolorization (%), the following formulation based on measuring the initial (A_{ini}) and final (A_{fin}) absorbance were used [117]:

$$\text{Decolorization (\%)} = 100 \times [A_{\text{ini}} - A_{\text{fin}}] / A_{\text{ini}} \quad (3.3)$$

3.2.20.2. Textile Cotton Bleaching

The bleaching performance of *MmLac* in cotton fabrics was investigated by a modified procedure from [118]. The woven raw fabrics (100% cotton) were scoured using the exhaust method, before bleaching with *MmLac*. All fabrics were exhausted with 1 g/L wetting agent (mixture of nonionic surfactants), 0.2 g/L stabiliser (blend of anionic phosphonates) and 0.05 g/L ABTS (20 mM). NaOH was at 45% wt. and H₂O₂ at 50% wt. All procedures were run at pH 5.0, at either 55 °C or 80 °C, for 45 mins in a Gyrowash. Treatments were as follows (Table 3.5):

Table 3.5: Laccase cotton bleaching treatments.

Compositions (g/L)	TREATMENTS											
	1*	2	3	4	5	6	7	8	9	10	11	12
	Control				50 °C				80 °C			
<i>MmLac</i>	–	2	–	–	3	6	6	12	3	6	6	12
H ₂ O ₂ (50%)	–	–	–	2	2	2	3	2	2	3	2	2
NaOH (45%)	–	–	5	5	3	3	5	3	3	5	5	3

*100% raw cotton fabric only

Various concentrations of H₂O₂ (1 g/L to 5 g/L increments) were used as control under same the conditions. Fabrics were then dried at 130 °C for 4 mins in a rapid steamer (Rapid Ht Steamer Model H-TS-3, China). To soften, wet fabrics and enabling chemical penetration of chemicals to fabrics [119], a constant amount of wetting agent was used in all treatments. The stabiliser is included to prevent the immediate breaking down of H₂O₂ to have a sustained bleaching effect in the system [120]. Twelve experimental treatments were carried out in the textile laboratory; detailed experimental parameters are given for 55 °C and 80 °C. After drying, fabric bleaching performance was detected using a colour spectrophotometer (HunterLab Colorquest XE, USA) to evaluate the Berger whiteness index (WI) of the fabrics [121]. The WI is specified for illuminant C and 2-degree observer functions only.

However, the equation is commonly used with other illuminants and observer functions, therefore the values will depend on the primary illuminant and observer function. This calculation was done using the spectrophotometer (Hunterlab Colourquest XE, USA) and the formula for calculating WI was as follows:

$$WI = Y + 0.3018Z - 3.831X \quad (3.4)$$

Where X, Y, and Z are the tri-stimulus value for the sample. Berger whiteness was calculated by spectrophotometer for each fabric treated with the recipe. Values greater than 33.33 indicate bluish-white, while smaller values indicate yellowish-white. Berger whiteness was increased as the bleaching performance and whiteness increases.

4. RESULTS

4.1. Bioinformatics Analysis of *mmlac* gene

The analyses of the putative *mmlac* gene of 1833 bp encoding (Figure 4.1) of 611 amino acids (GenBank accession number: KXX78172.1) showed that amino acid sequence patterns were native (Figure 4.2) and pro-signal peptide (Figure 4.3) (1–25 and 1–49 aa) in N-terminal. (592–611 aa) the peptide is pro-signal sequence patterns after coding Asp–Ser–Gly–Leu (DSGL) in the C-terminal [105, 106] (Figure 4.4). Both native and pro-signal peptides have mainly hydrophobic amino acids that are well-known characteristic of signal peptides in eukaryotic cells.

```
5' ATGTATTGGTTGTTGAATAGATCCGCTGCTGCTTGTCTGCTGTTTTGTTGACTTTGTTGAAG
TCTACTTTGGGTTCTCCAGCTAAACCTGATCCAGTTGTTGGTTTGTGTCCTAGACAACTATTGT
TCCAGGTGGTAGACCTTGTGGTCAACACGGTCCAACATAAGAGGTTGTTGGAAGAACAATTGGA
ACATTTCTACTGATTACGAACATGATACTCCACCTGCTTTCAATACTCACGTTTTTGATTTCCAT
ATCATAACGTTACTGATTGGTTGGGTCCAGATGGTATTAGAAAAGCCTGCTATGTTGATTAACAA
CCAATTTCTGGTCCAACCTTTGTGGGTGATTGGGGAGATTACATCGTTATTAATGTTTACAACG
ATATGCAAGATAACGGTACTTCTATTTCATTGGCACGGTATTAGACAATTCGGTGAATCTAATCAA
GATGGTGCTAACGGTGTTACTGAGTGTTCCAATTCACCTGGTTCCTTAAGACTTATGATTTCCA
TGTTACTCAATACGGTACTTCTTGGTATCATTTCTCACTACTCTAACCAATACGGTAACGGTGTTG
TTGGTGCTTTGATTGCTAGAGGTCCATCTTCTGCTGATTATGATATTGATTTGGGTCTTTACATG
ATTACTGATTACTATCACGAACTGCTGATAGATTGCATTTGCAAGCTGAGTTGGTTAGAAATGG
TCCACCTCCAGATTCTGATAACATCTTGTTTCAGAGGTAAAAACATCAACCCTAACGGTCCAGGTG
GTTCTTATGATAGATTGACTTTGACTCCAGGTAAAAAGCACTTGATCAGATTGATTAATGCTTCT
GTTGATAACTCTTTTCGTTGTTTCTTTGGTTGGTGCATAACTTCACTGTTGTTACTACTGATTTGGT
TCCTATTACTCCAGTTGTTAGATCCTCTTTGTTTTTGGGTGTTGGTCAAAGATACAACGTTATCG
TTGAGGCTAACCAAGCTGTTGATAATTACTGGTTCAACGCTACTTTGGAACTAATGGTAACGTGT
GGTCATTCTCACAATCCTTATCCAGCTGCTATTTTTCAATACGAGGGTGCTTCTTCTACTGCTTT
GCCAACTAACAGAGGTACTCCTTTGGTTGCTACTTGTGATGGTGAAAAGGGTTTTTCTCCAATTG
TTCAAAGATCCGTTCCACCATCTCAATTCAGACCATCTACTTTGCCTATTACTTTGGAATTTCTCT
ACTCACGATAGAGGTCAAGTTTTTCGAGTGGAGAGTTAAAAATACTCCAATTAACGTTGAATGGGG
TCATCCTGTTTTGGAATACGTTTTTGGAGAACAATGATACTTTCCCAGGTTCTTTGAATTTGATTG
AGGTTTCTGAACCTGAGGTTTGGACTTTTTTGGGTATTTCAAAACGATTTTCGCTTTGCCTCACCCA
ATTCATTTGCACGGTCATGATTTCTTGGTTTTTGGGTGTTGGTAATGGTACTTTTCGATCCAGCTAC
TATGGCTGGTTTTGTTGAACCTTCAACAACCCAATCAGAAAGAGATGTTGAACAAATGCCTGGTGCTG
GTTGGTTGGTTATTGCTTTCAAGACTGATAATCCTGGTTGTTGGTTGATGCACTGTCATATTGGT
TGGCATGTTGCTATGGGTTTGGGTATTCAATTTTTTGGAGAGAAAGTCTGATATTAGAGCTTTGAT
GGCTTTGGATCAAATGGTTCCAACTGTGATGCTTGGAGAGCTTATGAACCTACTTCTCCATACT
TGCCTAAGATTGATTCTGGTTTGAAAAGAGGTGTTGGTGTTGATATGGAGGGTATGGAGCCTGCT
GTTAGAAGAATTGGTTAA3'
```

Figure 4.1: Nucleotide sequence of the codon-optimized *mmlac* gene (1833 bp).

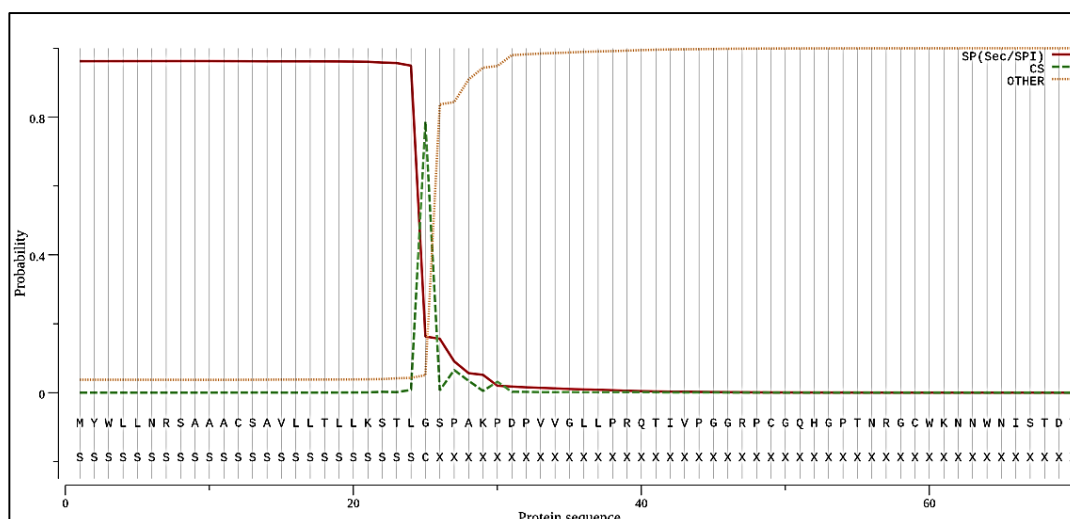


Figure 4.2: Signal sequence analysis (SignalP-5.0) of the *mmlac* gene.

Input sequence length : 611 aa					
VALUES OF COMPUTED PARAMETERS					
Net charge of query sequence	:	-14			
Analysed region	:	68			
Number of basic residues in targeting sequence	:	7			
Number of acidic residues in targeting sequence	:	1			
Cleavage site	:	49			
Cleaved sequence	:	MYWLLNRSAAACSAVLLTLLKSTLGSPAKPDPVVGLLPQRTIVPGGR			
HYDROPHOBIC SCALE USED					
		GES	KD	GVH1	ECS
H17	:	1.329	1.553	0.204	0.597
MesoH	:	-0.241	0.490	-0.321	0.300
MuHd_075	:	48.582	24.045	16.759	9.564
MuHd_095	:	32.186	20.112	9.665	7.041
MuHd_100	:	27.756	19.319	9.209	6.366
MuHd_105	:	30.721	16.135	9.552	6.147
Hmax_075	:	8.167	2.567	4.248	2.870
Hmax_095	:	10.412	21.787	3.747	3.762
Hmax_100	:	15.400	19.100	4.900	5.130
Hmax_105	:	3.617	8.050	1.247	3.465
PROBABILITY					
of export to mitochondria: 0.9114					

Figure 4.3: Mitochondrial signal sequence analysis (MitoProt II – v1.101) of the *mmlac* gene.

In addition to the signal peptide, the *mmlac* gene has been determined to have a mitochondrial signal sequence. This data aided in the cloning of the *mmlac* gene into the pPICZαA plasmid by removing the propeptide region alongside the signal peptide.

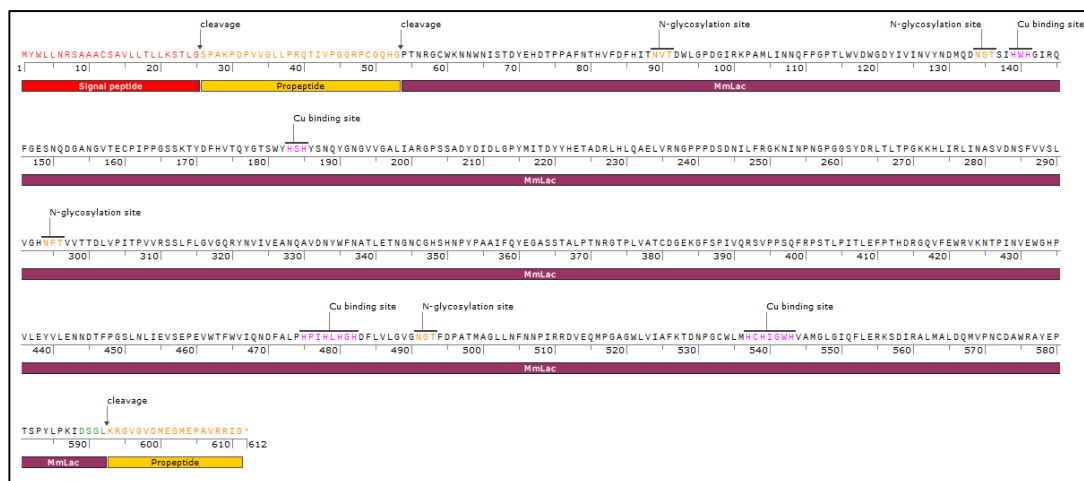


Figure 4.4: Amino acid sequence and functional regions of fusion *MmLac*. Propeptides are shown in yellow, native signal in red, N–glycosylation site in yellow, Cu^{2+} –binding site in purple, other residual *MmLac* sequence in claret red.

The amino acid sequence of the *MmLac* enzyme has four conserved motifs (HWHG, HSH, HPIHLH, and HCHIGWHVANGM/L/F) identified in laccases (Figure 4.4). These conserved regions form the catalytic region by doing the T1 and T2/T3 copper–binding function in laccase enzymes. It also has four predicted N–glycosylation sites according to conserved sequence Asn–X–Ser/Thr (NXT). These N–glycosylation sites were elucidated as Asn⁸⁹, Asn¹³⁴, Asn²⁹⁴, Asn⁴⁹⁰. Confirmation of glycosylation sites in *MmLac* was analyzed in MALDI–TOF–MS by deglycosylation with the EndoH enzyme.

Theoretically, Mw and pI values of *MmLac* enzyme were calculated as 61 kDa and 5.64. *MmLac* exhibits sequence similarities with *Asco*–laccases from *Thielavia arenaria* (50%), *Melanocarpus albomyces* (49%), *Myceliophthora thermophila* (48%), *Botrytis aclada* (38%), *Aspergillus niger* (35%) (Figure 4.5). Despite the differences in amino acid sequence homology, it was identified by multiple amino acid sequence alignments that the consensus sequences in the catalytic region are similar to other laccases.

The active site pocket of *MmLac* enzymes shows homology to multiple aligned laccases. Conserved amino acids in these regions are defined as Pro²²³, Asp²⁸³, Phe⁴¹⁹, Trp⁴²¹, Leu⁴⁷³.

“ACGATGT”, upstream of the native signal within the *mmlac1* construct. pPICZαA plasmid readily contains a Kozak sequence located upstream of the α-signal peptide (89 aa) followed by secretory signal peptides and His-tag respectively within *mmlac2* construct. *mmlac2* encodes a protein of 552 amino acids including 6 amino acids (ETEAEF) extending peptide for the original mature protein and 6xHis for His-tag (Figure 4.6 and Figure 4.7).

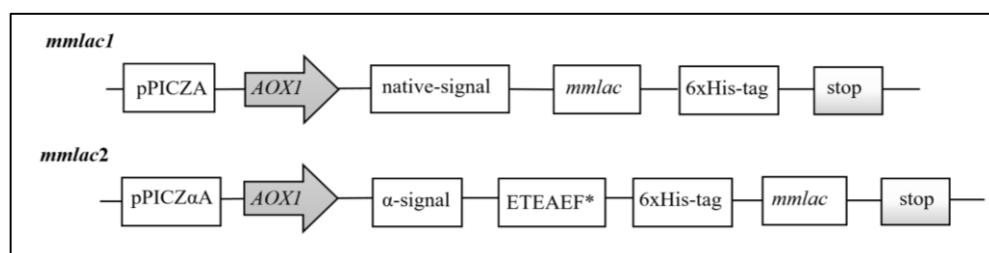


Figure 4.6: Schematic representation of recombinant *mmlac1* and *mmlac2* plasmid constructs. * N-terminal extension sequence for mature *mmlac*; E: Glutamic acid, T: Threonine, A: Alanine, F: Phenylalanine



Figure 4.7: Amino acid sequence and functional regions of fusion MmLac. Native signal peptide and native propeptide are shown in yellow, MF-α signal in grey, N-terminal extension sequence (6 amino acids) in red, polyhistidine tag (6xHis-tag) in purple, MmLac in claret red.

4.2.1. Restriction Digestion

The commercially synthesized *mmlac1* and *mmlac2* structures were cut with the *EcoRI* and *SalI* restriction enzymes for the ligation reaction and purified using the Agarose Gel Extraction kit. Each two *mmlac* DNA fragment structure was visualized on a 1% agarose gel (Figure 4.8).

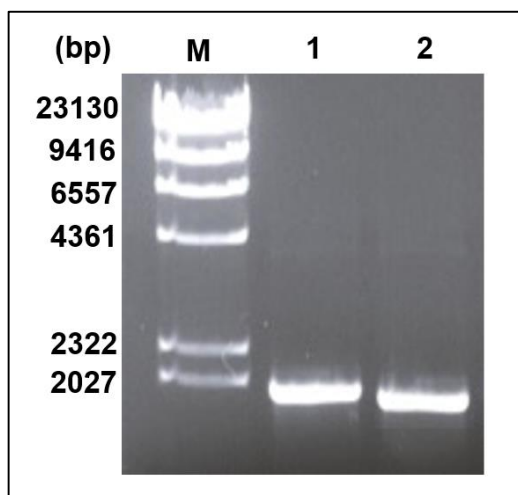


Figure 4.8: *EcoRI* and *SalI* double digestion of *mmlac* gene constructions. M: Marker, Lane 1: *mmlac1* (~1865 bp), Lane 2: *mmlac2* (~1788 bp).

pPICZA and pPICZ α A plasmids were double digest by *EcoRI* and *SalI* enzymes for ligation reaction and visualized in 1% agarose gel (Figure 4.9).

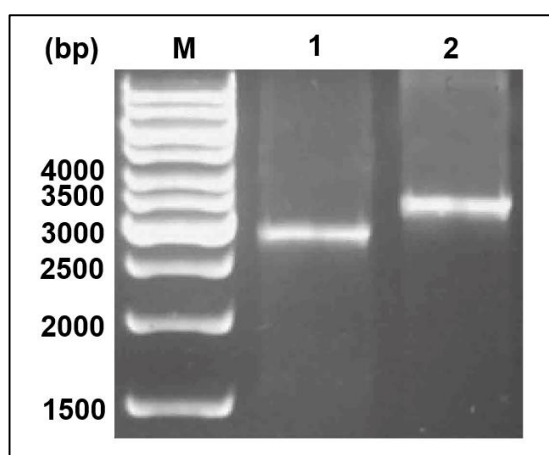


Figure 4.9: *EcoRI* and *SalI* double digestion of pPICZA and pPICZ α A plasmids. M: Marker, Lane 1: pPICZA (~3195 bp), Lane 2: pPICZ α A (~3481 bp).

4.2.2. Ligation of *mmlac* Gene Construction and Plasmids

The double digest *mmlac1* and *mmlac2* constructs were ligated to the pPICZA and pPICZ α A plasmids digested by the same enzymes, respectively, by the ligation reaction. Thus, recombinant plasmids (pPICZA–*mmlac1* and pPICZ α A–*mmlac2*) were prepared for competent *E. coli* TOP10 host cells.

4.3. Transformation of Recombinant Plasmids into Competent *Escherichia coli* TOP10 Cells

Recombinant plasmids obtained after the ligation reaction were transformed into *E. coli* TOP 10 cells by heat shock. Recombinant cells were visualized on the plate with LB agar medium containing 25 μ g/mL Zeocin. *E. coli* TOP 10 without recombinant plasmids was used as the control group (Figure 4.10). Cells growing on the plate were used as a recombinant plasmid source containing *mmlac* gene constructs for later studies.

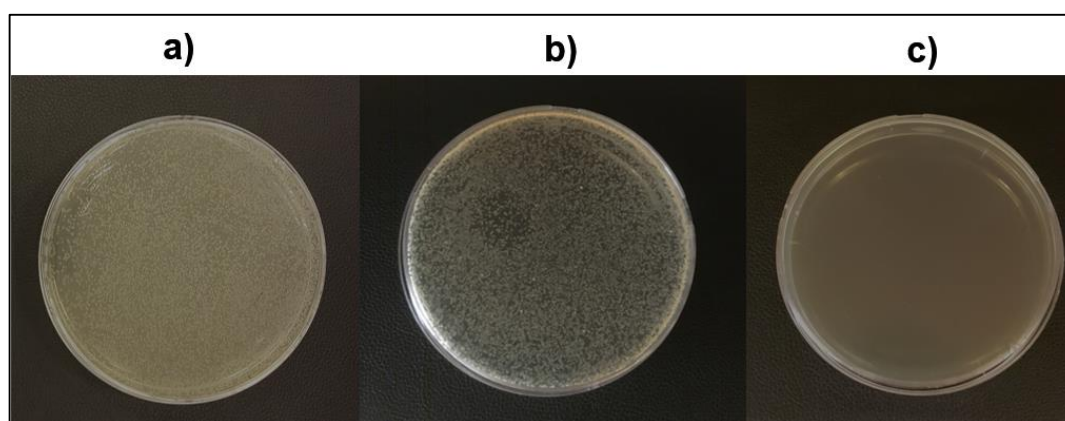


Figure 4.10: *E. coli* TOP10 colonies developing on LB agar medium as a result of transformation.

4.4. Confirmation of *mmlac* Gene Constructions in *Escherichia coli* TOP 10 Host Cells

Control of *mmlac* gene structures transformed into *E. coli* TOP10 cells after plasmid isolation was confirmed by sequence analysis and restriction enzyme digestions.

4.4.1. Digestion of the Recombinant Plasmids by Restriction Endonucleases

The presence of recombinant plasmids in *E. coli* TOP10 host cells was performed by *Eco*RI and *Sal*I restriction endonuclease double digestion enzyme reactions and visualized in 1% agarose gel (Figure 4.11). In-gel images, the *mmlac1* and *mmlac2* gene constructs were also determined by the pPICZA and pPICZ α A plasmids they were ligated. Thus, the accuracy of plasmid isolated recombinant plasmids and *mmlac* gene constructs have been proven. These plasmids were re-isolated and prepared for sequence analysis to determine that the *mmlac* gene constructs are properly ligated into the recombinant plasmids.

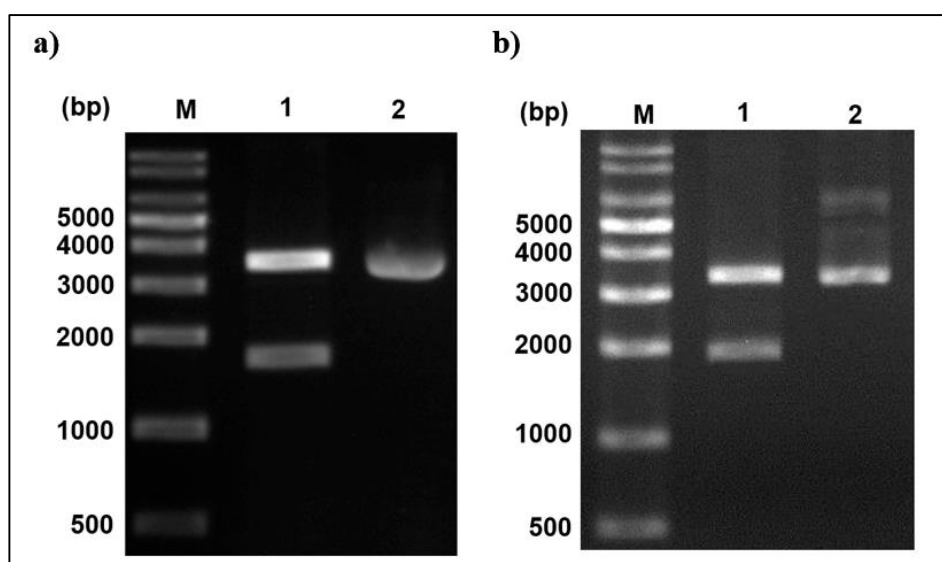


Figure 4.11: Image of recombinant plasmids in agarose gel after restriction enzyme digestions. a) M: marker, Lane 1: *mmlac1* (~1865 bp) and pPICZA (~3195 bp), Lane 2: circular pPICZA-*mmlac1*. b) M: marker, Lane 1: *mmlac2* (~1788 bp) and pPICZ α A (~3481 bp), Lane 2: circular pPICZ α A-*mmlac2*.

4.4.2. DNA Sequence Analysis and Comparison of *mmlac* Gene with the Hypothetical Gene

The presence of *mmlac* gene constructs ligated into recombinant plasmids and their status within the plasmid (whether they are ligated towards the plasmid) were compared with the hypothetical gene (GenBank accession number: KXX78172.1) by DNA sequence analysis. As a result of sequence analysis, the presence, and correct ligation of both gene constructs in plasmids were determined.

4.5. Transformation of the Recombinant Plasmids into Competent *Pichia pastoris* X–33 Cells

After recombinant plasmids were transferred to *Pichia pastoris* X–33 cells by electroporation, colony development was observed 7 days later in YPDS agar medium containing Zeocin (100 µg/mL). During this period, while colony development was not observed in the control group, colonies containing *mmlac* gene structures were identified (Figure 4.12).

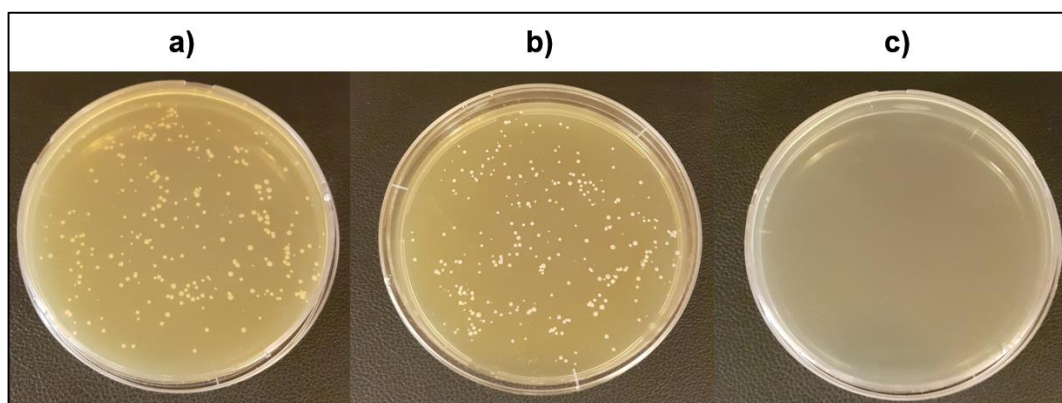


Figure 4.12: *P. pastoris* X–33 colonies developing on YPDS agar medium as a result of transformation. (a) Colonies containing the *mmlac1* gene constructs (b) Colonies containing the *mmlac2* gene constructs (c) Control: wild type *Pichia pastoris* X–33

4.6. Colony PCR of Recombinant *Pichia pastoris* X–33 Cells

The presence of *mmlac* gene structures in *P. pastoris* X–33 colonies was determined by colony PCR. For the *mmlac1* gene construct, AOX1 promoter forward and terminator reverse primers were used, while for the *mmlac2* gene construct, MF– α forward and AOX1 reverse terminator primers were used. Results are displayed on a 1% agarose gel (Figure 4.13). According to these results, the transformation of recombinant plasmids and the presence of *mmlac* gene structures in colonies are shown.

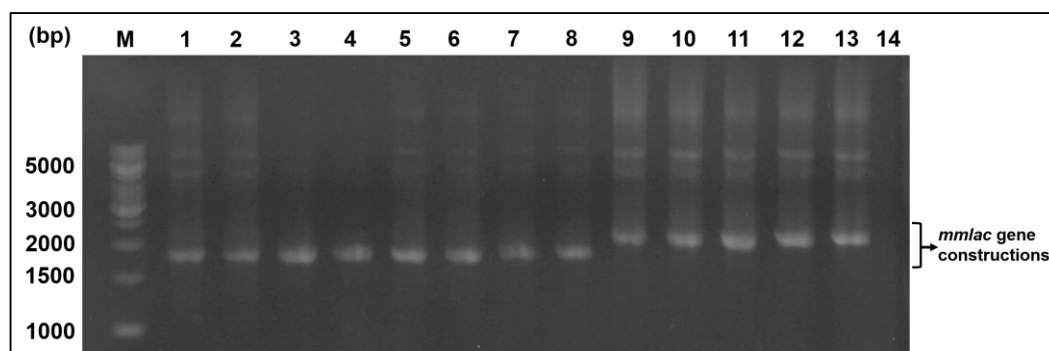


Figure 4.13: Colony PCR results. M: marker, Lane 1–8: *mmlac2* construct (~1950 bp), Lane 9–14: *mmlac1* construct (~1948 bp), Lane 14: Control (wild type *P. pastoris* X–33).

4.7. Determination of *mmlac* Activity on MM Agar Medium

The commercially synthesized and codon–optimized *mmlac1* and *mmlac2* gene construct coding *MmLac* in *P. pastoris* was successfully ligated into pPICZA and pPICZαA respectively by confirming sequence analysis. The observed recombinant *P. pastoris* X–33 colonies on YPDS Agar plate demonstrate efficient transformation for both constructs *mmlac1* and *mmlac2*. However, the green color formation was seen only around colonies having *mmlac2* construct after screening recombinant *P. pastoris* X–33 colonies on ABTS–containing MM Agar medium plate (Figure 4.14). The green color formation verifies the efficient secretion of *mmlac2* coding active enzyme against ABTS. Therefore, further studies were continued with colonies having *mmlac2* construct.

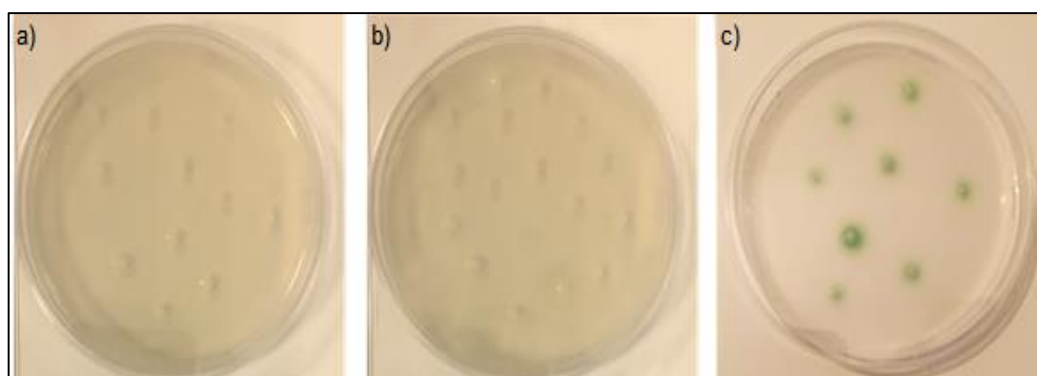


Figure 4.14: Screening and production of extracellular *MmLac* in MM agar plate. (a) Control plate, (b) *Pichia pastoris* X–33 containing *mmlac1* construct (c) *P. pastoris* X–33 containing *mmlac2* construct.

4.8. Heterologous Expression of *mmlac* in *Pichia pastoris* X-33 Cells

To produce active recombinant protein in *P. pastoris* with high efficiency, the highest level of *mmlac* expression was determined on the seventh day by 12% SDS analysis. In subsequent studies, induction was performed 7 days for *mmlac* expression (Figure 4.15).

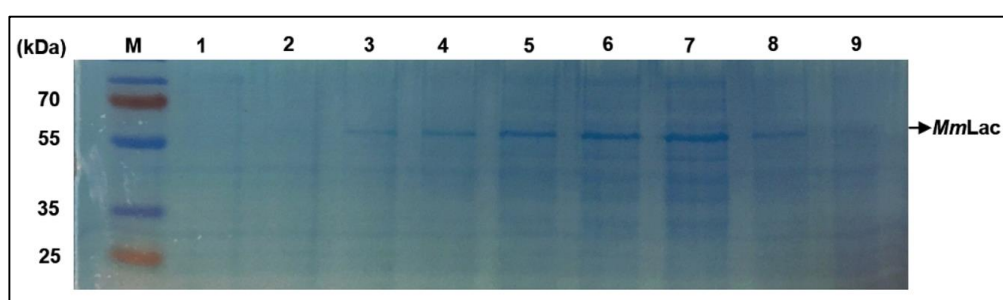


Figure 4.15: Analysis of *mmlac* expression in the SDS-PAGE. M: marker, Lane 1–9: days.

4.8.1. Determination of the Effect of Temperature on *mmlac* Expression

In *mmlac* expression experiments using 3 different temperatures, expression at only 18 °C temperature was observed, while *mmlac* expression at 24 and 30 °C was not detected in SDS analysis (Figure 4.16). According to these results, temperature increases above 18 °C to prevent *mmlac* expression by decreasing.

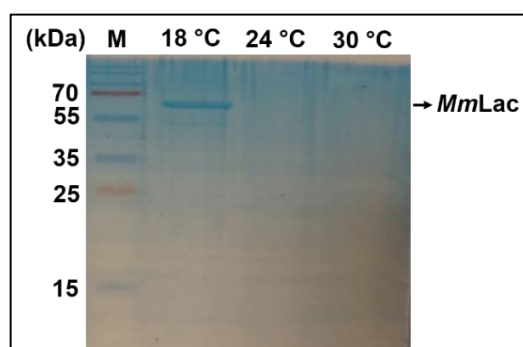


Figure 4.16: SDS-PAGE analysis of culture supernatant from *P. pastoris* X-33 for production levels of *MmLac* enzyme during the growth time at different temperatures for 7 days. M: marker, Lane 1: expression of *mmlac* at 18 °C, Lane 2: expression of *mmlac* at 24 °C, Lane 3: expression of *mmlac* at 30 °C.

4.8.2. Determination of the Effect of Methanol Concentration on *mmlac* Expression

Induction conditions for the best recombinant *P. pastoris* X-33 determined as 1% methanol. While the effect of methanol concentration on *mmlac* gene expression was 1.0>0.5>1.5>0.1 (%), *mmlac* expression at 3% methanol concentration was not detected in SDS-PAGE analysis (Figure 4.17).

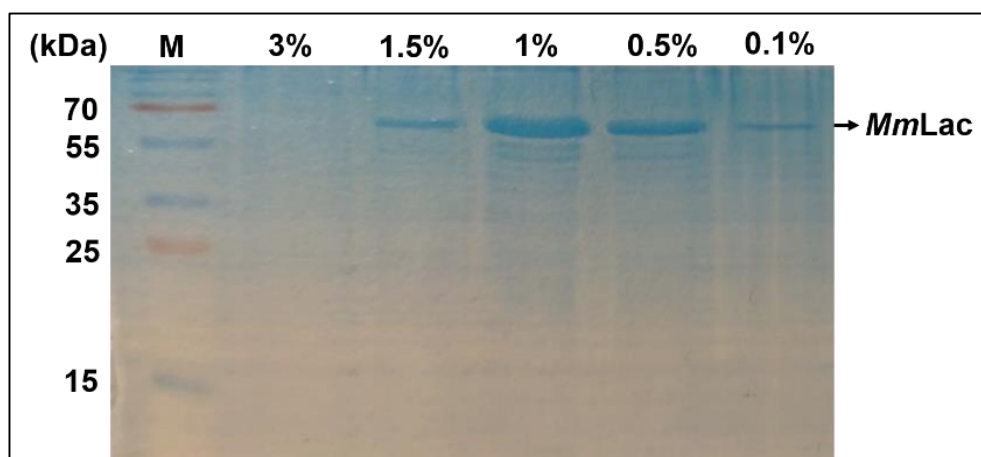


Figure 4.17: Effect of methanol concentrations on the expression of *mmlac2*.

4.8.3. Determination of the Effect of CuSO_4 Concentration on *mmlac* Expression

The addition of CuSO_4 from 0.1 to 0.2 mM to recombinant *P. pastoris* cell culture enhanced *Mmlac* activity. The laccase activity decreased in the presence of 0.4 mM Cu^{2+} and above (Figure 4.18). Therefore, the recombinant *P. pastoris* cell culture including 0.2 mM CuSO_4 was used as a source of pure *MmLac*. The activity of purified *MmLac* was assayed for the optimization of the different parameters.

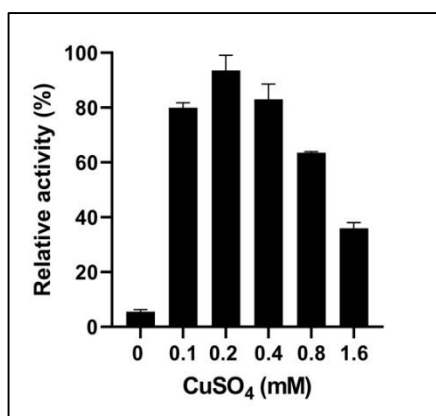


Figure 4.18: Effect of CuSO₄ on *MmLac* activity.

4.9. Purification of *Mmlac* Enzyme by Affinity Chromatography

The recombinant *MmLac* was purified from recombinant *P. pastoris* X-33 cultures grown in BMMY medium under the optimized induction conditions. SDS-PAGE shows that the amount and level of purity of the *MmLac* were enough for kinetic studies (Figure 4.19). Change of total and specific laccase activity, level of refinement during the purification process were outlined in Table 4.1. The results suggested that Ni-NTA chromatography of the supernatant led to 14.2-fold purification and 54.4 mg/L *MmLac* enzyme in a laboratory scale culture level. The specific activity of the purified *MmLac* enzyme was calculated as 143.7 U/mg.

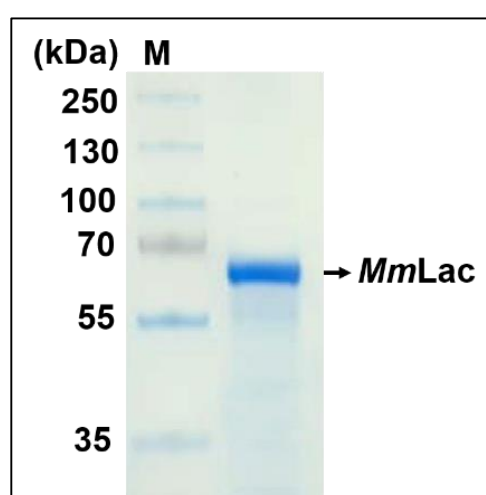


Figure 4.19: SDS-PAGE analysis of *Mmlac* purified and concentrated. M: Marker.

Table 4.1: Purification steps of the *MmLac*

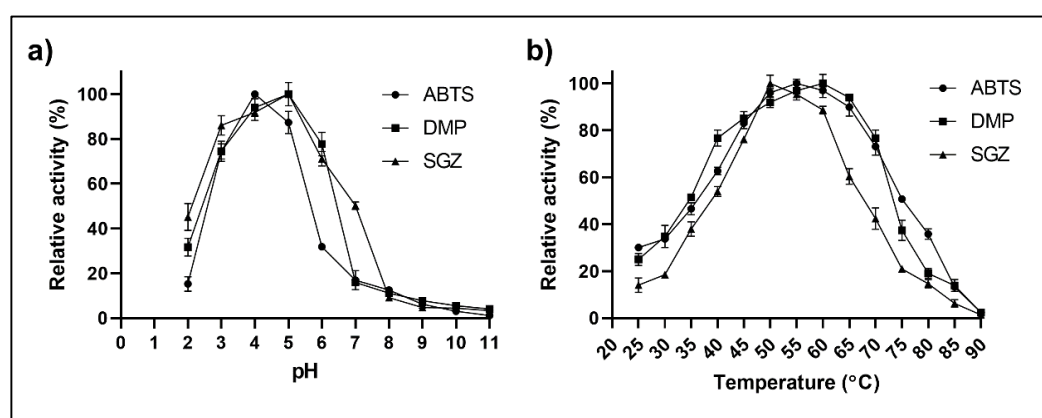
Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold Purification	Activity yield (%)
Supernatant	254.8	2573	10.1	1.0	100
Ultrafiltration	79.4	2279	28.7	2.8	88.5
Ni ²⁺ -NTA	13.6	1954	143.7	14.2	75.9

4.10. Biochemical Characterization of the Recombinant *MmLac*

4.10.1. Determination of Optimum pH and Temperature

The pH optimum of 4–5 *MmLac* was detected against three commonly used laccase substrates: ABTS, SGZ, and 2,6-DMP. *MmLac* was more active under acidic conditions and it started to lose activity quickly upon further increasing the pH. The enzyme had activity less than 20% between pH 5.0 and 8.0, but it did not show any substantial activity above pH 9.0. Consequently, the optimum pH was observed as 4.0 for ABTS and 5 for both SGZ and DMP (Figure 4.20a).

The optimum temperature on the laccase activity was determined for ABTS, SGZ, and DMP as 55, 50, and 60 °C (Figure 4.20b). However, 15–20% remaining activity was measured even above 80 °C. The enzyme lost activity completely at 90 °C.

Figure 4.20: Effect of pH (a) and temperature (b) on *mmlac* relative activity.

4.10.2. Enzyme Kinetics of *MmLac*

MmLac catalytic efficiency (k_{cat}/K_m) for ABTS, SGZ, and DMP substrates was found 4.7, 2.2, and 1.5 $\text{s}^{-1} \mu\text{M}$ respectively (Figure 4.21). *MmLac* activity tests showed that it has the best catalytic efficiency against ABTS. However, this number is not comparable to the reported data relating to ABTS catalytic efficiency of laccases. *MmLac* catalytic efficiency against 2,6–DMP is noticeable and against SGZ partially better compared to the other reported *Asco*–laccases activity (Table 4.2).

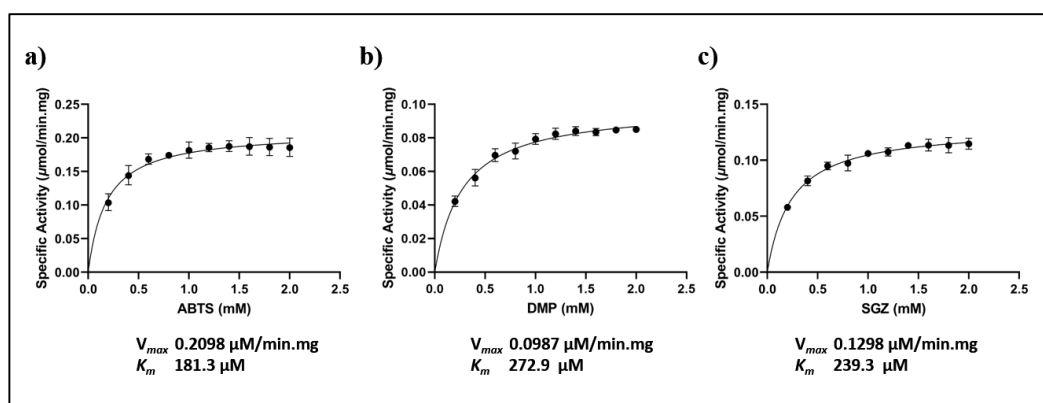


Figure 4.21: Michaelis–Menten plot of *MmLac* in the presence of ABTS (a), DMP (b), and SGZ (c).

Table 4.2: Kinetic parameters of *Mm*Lac and the selected *Asco*-laccases

Organism	Origin	ABTS			SGZ			2,6-DMP			Ref.
		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	
<i>M. mycetomatis</i>	Asco-fungus	181.3	848.7	4.7	239.3	525.1	2.2	273.0	399.3	1.5	This study
<i>Thielavia sp.</i>	Asco-fungus	23.7	4.1	0.2	n.r. *	n.r.	n.r.	24.3	3.46	0.1	[122]
<i>Y. lipolytica</i>	Asco-fungus	114	1990	17.5	n.r.	n.r.	n.r.	75	1210	16.1	[123]
<i>Shiraia sp.</i>	Asco-fungus	190	7109	37.4	400	938.1	23.5	370	1476	39.9	[124]
<i>S. thermophilum</i>	Asco-fungus	260	1431	5.5	n.r.	n.r.	n.r.	360	3495	9.7	[125]
<i>F. solani MAS2</i>	Asco-fungus	79.4	107	1.3	n.r.	n.r.	n.r.	8.6	121	1.4	[126]
<i>M. albomyces</i>	Asco-fungus	400	28.3	0.07	37	40.17	1.085	11	10.1	0.9	[112]
<i>Phoma sp.</i>	Asco-fungus	8	10.6	1.3	20	1.5	0.075	266	4.3	0.01	[127]
<i>B. aclada</i>	Asco-fungus	2.9	108	37.2	n.r.	n.r.	n.r.	19.2	45.6	2.4	[128]
<i>M. thermophila</i>	Asco-fungus	290	13.1	0.04	1.6	35	21.9	n.r.	n.r.	n.r.	[129]

*n.r., not reported

4.10.3. Effect of Cationic Metal Ions on *MmLac* Activity

Cationic metals exhibited different effects on *MmLac* activity depending on the concentration. 100 mM Zn^{2+} , Co^{2+} , Mn^{2+} , Mg^{2+} , Cu^{2+} do not exhibit a serious effect on the of *MmLac* activity. However, the presence of Ag^{2+} , Ca^{2+} , Hg^{2+} , and Fe^{2+} , Li^{2+} at different percentages led to a 20–60% decreasing of the *MmLac* activity (Table 4.3).

Table 4.3: Metal ion effects on the *MmLac* activity.

Metals	Relative Activity (%)			Metals	Relative Activity (%)		
	1 mM	10 mM	100 mM		1 mM	10 mM	100 mM
Control	100	100	100	Control	100	100	100
Zn^{2+}	99.2±1.6	109.0±1.2	94.3±1.4	Co^{2+}	93.3±1.9	92.0±2.3	87.0±1.9
Ag^{2+}	38.0±2.3	15.0±1.4	2.1±0.6	Mn^{2+}	94.4±1.2	103.0±0.5	91.2±1.5
Ca^{2+}	95.2±3.0	74.0±0.6	60.9±0.8	Fe^{2+}	13.4±1.3	5.2±1.3	1.0±0.1
Hg^{2+}	61.5±1.9	23.1±0.8	1.2±0.1	Mg^{2+}	101.2±0.5	106.0±2.0	95.3±1.3
Li^{2+}	81.4±1.3	85.0±2.0	66.0±2.8	Cu^{2+}	113.3±3.0	111.0±2.1	96.3±1.8

4.10.4. Effect of Various Organic Solvents on *MmLac* Activity

10% of DMSO, methanol, ethanol, ethyl acetate slightly enhanced the activity of *MmLac*, but 50% concentration of them inactivated *MmLac* ranged from 4–32%. 50% of acetonitrile and acetone inhibited 70% activity of *MmLac*. It has been seen that 10% acetonitrile, isopropanol, and acetone almost inhibited the enzyme (Table 4.4).

Table 4.4: Effect of various solvent on the *MmLac* activity.

Solvents	Relative Activity (%)			Solvents	Relative Activity (%)		
	10%	25%	50%		10%	25%	50%
Control	100	100	100	Control	100	100	100
DMSO	117.0±2.2	103.3±2.3	93.0±2.0	Isopropanol	89.8±2.4	76.3±1.4	59.8±2.5
Ethanol	121.3±1.4	106.1±1.3	96.0±2.4	Acetonitrile	87.6±0.7	75.3±2.7	29.5±0.9
Acetone	87.6±1.7	45.1±1.7	28.0±2.6	Ethylacetate	108.8±1.3	93.6±1.9	87.9±1.9
Methanol	103.4±2.2	79.3±1.6	67.7±2.7	Cyclohexane	93.5±1.4	75.3±1.6	63.2±1.1

4.10.5. Effect of Halides on *Mm*Lac activity

*Mm*Lac catalytic activity was strongly inhibited in the presence of NaCl at different concentrations as halides. More than 90% of the enzyme activity was inhibited at a concentration of 1 M NaCl (Figure 4.22).

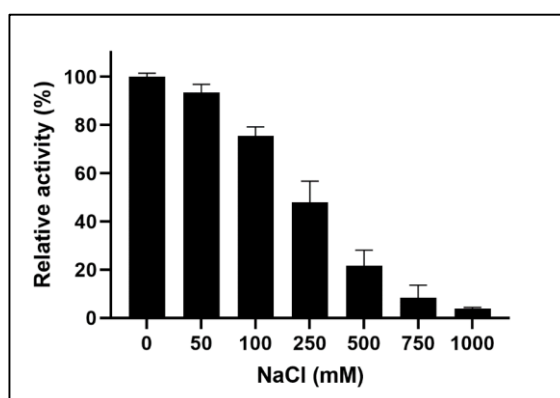


Figure 4.22: Effect of NaCl on relative *Mm*Lac activity.

4.10.6. Determination of *Mm*Lac Storage Stability

The storage stability of the purified laccase enzyme at different temperatures is represented in Figure 4.23. It can be seen that the stability of *Mm*Lac at -20 and -80 °C was higher than 4 and 20 °C. The optimum storage temperature is -20 °C.

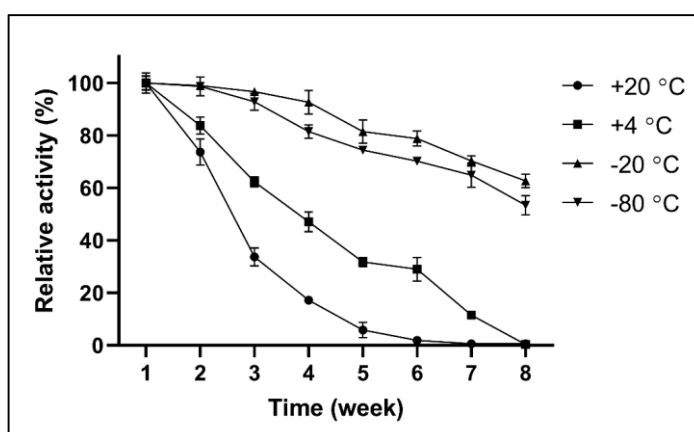


Figure 4.23: Time-dependent *Mm*Lac storage stability at different temperatures.

4.11. Deglycosylation and MALDI–TOF–MS Analysis of *MmLac*

The enzyme structure was experimentally and computationally analyzed. The purified enzyme was seen as a single band between 55–70 kDa in the SDS–PAGE (Figure 4.24). *MmLac* molecular weight was indicated as 62 kDa without glycosylation site by MALDI–TOF MS chromatogram correlated well with the theoretically calculated Mw (61 kDa) and apparent approximately molecular mass between 55–70 kDa. Two peaks of the MALDI–TOF MS also proved *MmLac* digestion with EndoH resulted in releasing the glycosylation site (Figure 4.25).

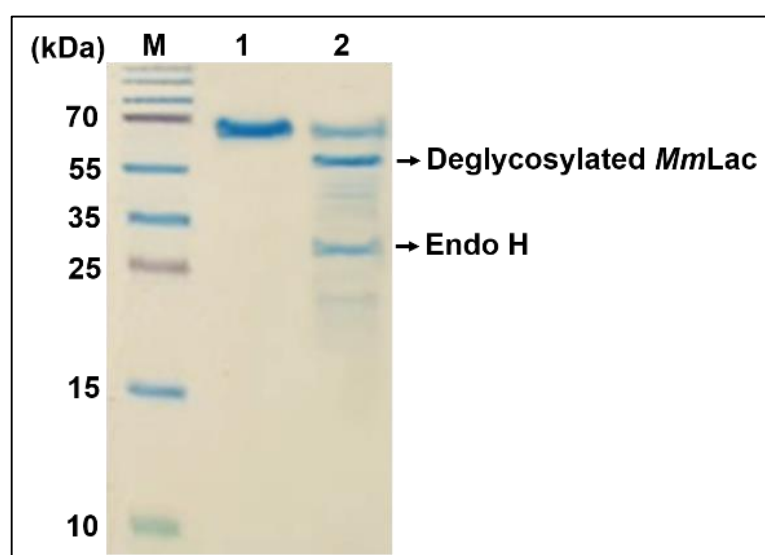


Figure 4.24: Deglycosylation of purified *MmLac*. M: Marker, Lane 1: Purified laccase, Lane 2: deglycosylated laccase with EndoH treatment, Lane

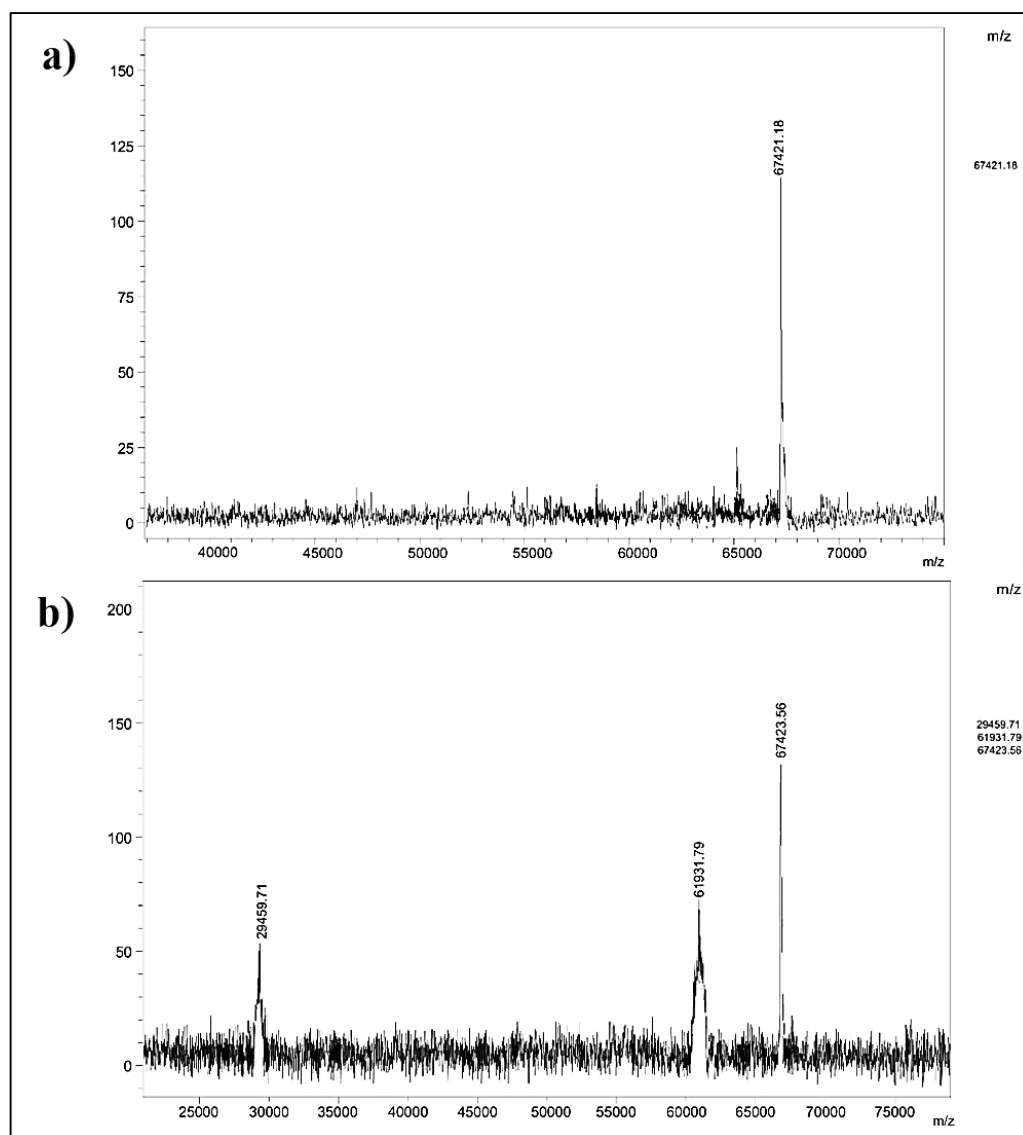


Figure 4.25: MALDI-TOF mass spectrum of the purified *MmLac* laccase. a) Purified *MmLac* with glycosylation site (67.4 kDa), b) Deglycosylated laccase (62.0 kDa).

4.12. Homology Modelling and Docking Analyzing

Homology modeling results confirm that the substrate-binding pocket of *MmLac* is formed by residues Gly¹⁸⁵, Pro¹⁸⁶, Asp²³⁰, Leu³⁵⁵, Phe³⁶⁶, Trp³⁶⁸, Phe⁴¹⁸, Leu⁴²⁰, Trp⁴⁸⁹, and His⁴⁹⁰ (Figure 4.26a). The overall shape of the substrate-binding pocket and active site residues of the two enzymes (*MaLac*) was shown Figure 4.26b.

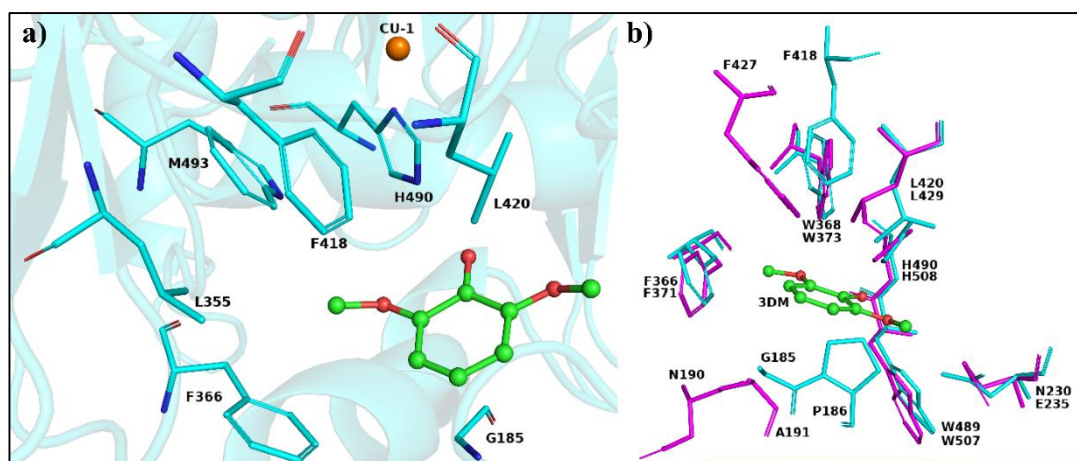


Figure 4.26: The homology model of *MmLac* superimposed with the structure of *MaLac* (PDBID: 3FU8). (a) The active site of *MmLac* enzyme docked 2,6-DMP substrate (Type-1 Cu ion in orange), and *MmLac* homology model shown in cyan. (b) Active site comparison of *MmLac* (cyan) and *MaLac* (magenta), showing highly similar active site architecture and identical amino acid residues near the substrate (the upper number for *MaLac* residue, lower number for *MmLac* residue). Note: 3DM, 2,6-DMP.

4.13. Industrial Applications of *MmLac*

4.13.1. Synthetic Dye Decolorization

The highest decolorization value was determined after 120 hours of incubation conducted with synthetic dyes used in different industrial applications. All samples prepared decolorization was carried out in the presence of ABTS as a mediator and values for Rhodamine 6 G, Remazol Brilliant Yellow, Crystal violet, Amido Black 10 B were calculated at $43.0 \pm 0.9\%$, $58.4 \pm 0.4\%$, $33.3 \pm 3.0\%$, $62.5 \pm 0.5\%$ at 120 hours with negative control reactions not having the enzyme. The decolorization rate was determined as Amido Black 10B > Remazol Brilliant Yellow > Rhodamine 6 G > Crystal violet at the same concentration (Figure 4.27).

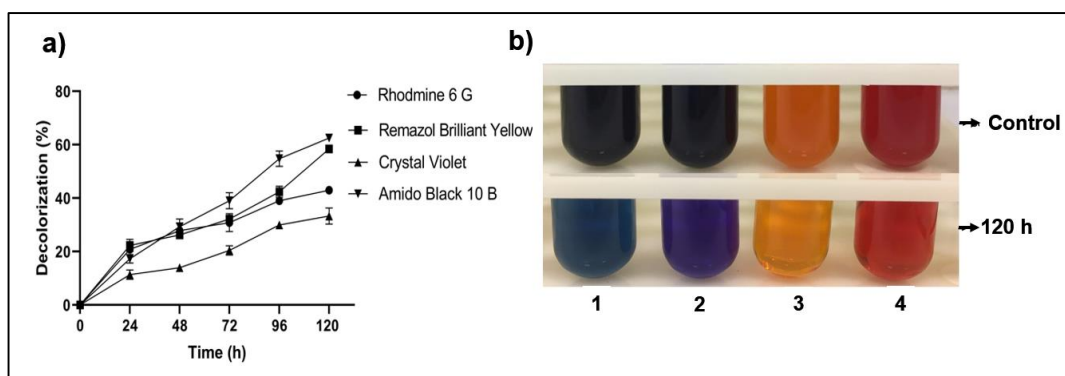


Figure 4.27: Decolorization effect of *MmLac*. (a) Decolorization (%) of synthetic dyes by the laccase; (b) decolorization for 4 synthetic dyes with *MmLac*:1: Amido Black, 2: Crystal Violet, 3: Brilliant Yellow, 4: Rhodamine 6 G.

4.13.2. Textile Bleaching

Twelve experimental treatments were carried out in the textile laboratory; detailed experimental parameters are given in Figure 4.28a for 55 °C and 80 °C. The bleaching effect of H_2O_2 alone on the sample is calculated as Berger unit presented in Figure 4.28b. According to the results, the whiteness after bleaching of raw fabrics treated with H_2O_2 ranged from 30 to 38 Berger degrees. The bleaching capacity of *MmLac* was tested at the optimum pH for enzyme catalysis (5.0), at 80 °C.

The WI value was calculated at ~29 for the laccase-only pre-treatment (treatment 2). This shows that the laccase pre-treatment improved the WI of the cotton fabric compared to the control (treatment 1). WI constants were calculated as approximately 42, 45 and 45 for treatment 5, 6 and 7, respectively (Figure 4.28c). Comparably, the highest WI were observed in treatments 9 to 12. The highest WI constant (~60) was obtained with treatment 11 which used 6 g/L enzyme, 3 g/L H_2O_2 and 5 g/L NaOH (Figure 4.28a).

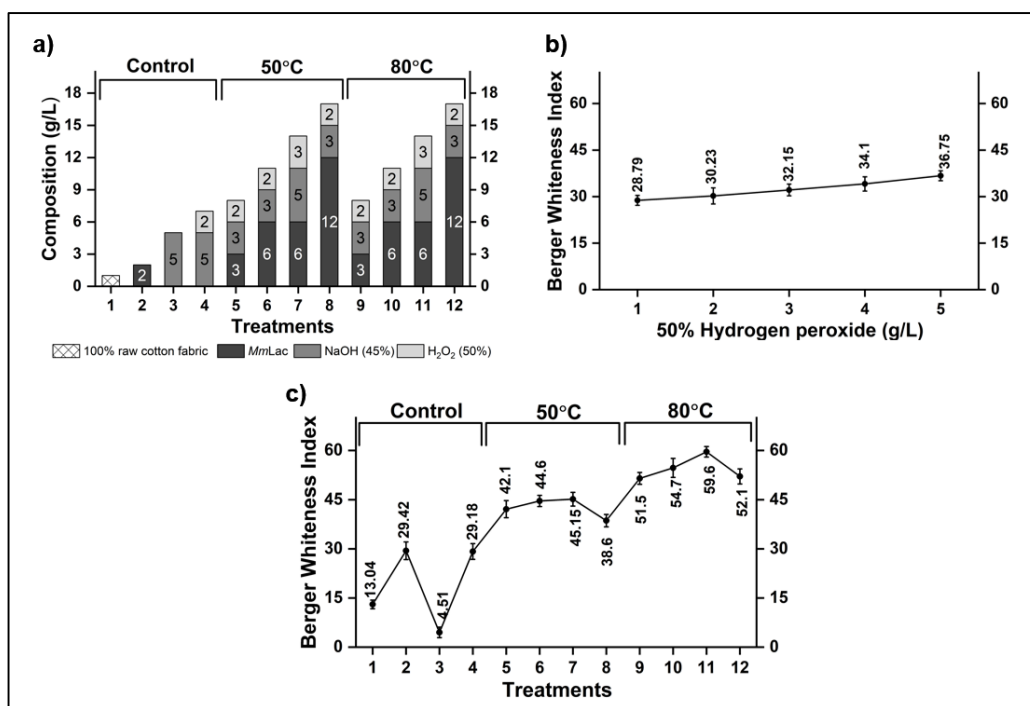


Figure 4.28: Components and results used in fabric bleaching. (a) Each composition has a wetting agent, 0.2 g/L stabilizer (blend of anionic phosphonates) and 0.05 mM ABTS; (b) bleaching effect of H₂O₂; (c) Berger Whiteness Index (WI) of recipes used in experiments.

5. DISCUSSION

Asco-laccases have the potential for versatile industrial applications that have been studied structurally and biochemically in many organisms [130, 131]. Cloning, structural and biochemical characterization of a new laccase enzyme from *M. mycetomatis* were performed in the heterologous expression host, the methylotrophic yeast *P. pastoris*. Production of ligninolytic enzymes like laccases at an industrial scale with the use of *P. pastoris* as the heterologous expression system is difficult. Low laccase yield in natural and genetically modified hosts constitute as a bottleneck for industrial-scale applications [132]. However, the screening results and a high amount of active recombinant protein demonstrate the efficiency of the *mmlac2* construct in the expression of the laccase gene in *P. pastoris* [133]. Extracellular secretion of original mature laccase was achieved with a robust contribution of extending peptide (ETEAEF) placed upstream of the His-tag followed by our gene in the N-terminal, all of which was added to the end of the α -mating factor signal sequence. The extending peptide expressed was reported as an enhancer for efficient secretion without deteriorating the enzyme function [134, 135, 136]. In contrast, the polyhistidine tag is located in C-terminal in the literature [134, 137, 138]. *Pichia* expression system for the constructed plasmid is a good candidate for heterologous expression of laccases.

Furthermore, 30 °C was widely reported as the optimum temperature for protein expression in *P. pastoris* [139]. However, 18 °C is an optimum temperature for better expression of *mmlac2* construct in *P. pastoris*. This might be due to the enhanced protein folding pathway, as well as increased cell viability at lower temperatures [140].

CuSO₄, temperature, pH, and storage temperature were tested to detect optimum conditions for *MmLac* activity. Copper ion is the most critical parameter for the expression and activity of laccases [141]. The activity increased ~15-fold over the control cultures an accustomed case for laccases due to their dependency of Cu²⁺ for activity (Figure 4.18) [142]. Both forming green color around of the colonies in Cu²⁺ including medium plates and various activity assays in the presence of Cu²⁺ (Figure 4.14) support that the laccase is strict copper dependence.

MmLac showed the best activity under acidic conditions like almost reported laccases [143, 123, 144] since *MmLac* has His⁴⁹⁰ in catalytic site (Figure 4.26). Generally, laccases have basic histidine amino acids in this position in their active site that is crucial for the oxidation of substrates [112]. Under acidic conditions, His⁴⁹⁰ is positive that playing the main role with the other active site amino acids for interacting substrates. Moreover, *MmLac* shows activity over a wide range of pH (2.0–8.0) that is a crucial property for enzymes as reported by various laccase applications performed in different pHs.

MmLac is a mesophilic enzyme due to having maximum activity at 55–60 °C (Fig. 4.20b). However, the enzyme conserves nearly half of its activity at 75 °C and noticeably active even after 80 °C that makes it partially thermostable. This thermostability makes the enzyme desirable for some biotechnological applications operated under 80 °C temperature like the decolorization of textile dyes [145].

Although laccases are known as catalysts do not require cofactors metal ions, as well as halides such as NaCl, are known to interact with the catalytic region of laccase enzymes, thereby altering enzyme stability and activity [146]. In most cases, some metals such as Cu²⁺ and Mn²⁺ can increase the enzyme's catalytic activity, while others such as Ag²⁺, Hg²⁺ can inhibit [147]. Therefore, we examined *MmLac* activity in the presence of different metals. While Zn²⁺, Mg²⁺, and Cu²⁺ caused an increase in *MmLac* activity, Hg²⁺, Ag²⁺, Fe²⁺, and all metals at a concentration of 100 mM resulted in an opposite outcome. This inhibition may have occurred due to the competition caused by these metals [148]. This creates a potential for industrial applications in heavy metal-containing conditions under certain predicaments. The effect of NaCl was also investigated due to chloride's inhibition of enzymatic activity by affecting electron transfer in the T2/T3 copper regions of laccase enzymes (Figure 4.22) [149, 150]. The high concentration of NaCl (1 M) completely inhibited the laccase. Laccases have no tolerance for salts due to their highly competitive potential [150, 151].

Organic solvents are widely used with substrates for industrial applications during enzyme catalysis. While DMSO was seen as an optimum buffer for maximum activity, acetonitrile, isopropanol, and acetone inhibited the enzyme by destroying the structure of the enzyme. Different organic solvents have been reported to inactivate laccase activity by affecting protein folding or enhance it by maintaining

their structure by causing microenvironmental changes around the active site [152, 153].

The effects of different storage temperatures on *MmLac* enzyme activity were assayed (Figure 4.23). -20°C is slightly better than -80°C but both of them can be evaluated as the most effective storage temperatures for the laccase even after 8 weeks. Having 60% activity after 8 weeks makes *MmLac* enzyme novel due to its long-term stability.

Experimental and computational studies enlightened the structural properties of the laccase. MALDI-TOF MS results confirm that *MmLac* is a 67 kDa monomer (Figure 4.25a), which is approximately 5 kDa larger than the molecular mass calculated from the amino acid sequence. The large mass inconsistency showed the presence of posttranslational modifications (PTMs) on *MmLac*, and the observed broad peak and poor mass resolution in the MALDI-TOF spectrum were indicative of heterogeneous glycoforms. Thus, the intact protein was deglycosylated by EndoH directly on the MALDI target to confirm the existence of N-linked glycosylation. After 60-min digestion, a new peak appeared at about 5.5 kDa less than the intact protein peak, indicating that the recombinant *MmLac* was modified by N-linked glycans having a hybrid structure (Figure 4.25b).

Despite *MmLac* and *MaLac* having partially low sequence similarity, the overall shape of the substrate-binding pocket and active site residues of the two enzymes have conserved (Figure 4.26) [112]. Figure 4.26a highlights the predicted substrate-binding residues, which is predominantly His in laccases from *Melanocarpus albomyces*, *Trametes pubescens*, *Aspergillus niger*, *Thielavia arenaria*, and *Botrytis aclada* as well as in the *Madurella mycetomatis* (PDBID: 3FU8, 2HZH, 5LM8, 3PPS, 3SQR respectively) (Figure 4.5). Substrate-binding pocket residues of *MmLac* is formed by residues (Gly¹⁸⁵, Pro¹⁸⁶, Asp²³⁰, Leu³⁵⁵, Phe³⁶⁶, Trp³⁶⁸, Phe⁴¹⁸, Leu⁴²⁰, Trp⁴⁸⁹, and His⁴⁹⁰) which is well conserved among *Ascomycete*-type laccases [112]. The fresh oxygen molecules required for catalytic cycle enter through near the trinuclear site by the allowance of the cavity formed by the last four residues (Asp, Ser, Gly, and Leu) of the C-terminus of *MmLac* [154].

The hydroxyl group of C1 of phenolic substrate 2,6-DMP was bound in the active site through H-bond with the methyl group of Leu⁴²⁰ and His⁴⁹⁰ (CD1–3.1 Å and NE2–2.8 Å) in the *MmLac* structure (Figure 4.26). Since the surface exposed

His⁴⁹⁰ has easy access to the 2,6-DMP's surface and been suggested to be a primary electron acceptor, this residue relevantly has a role in electron transfer mechanism [155].

In literature, laccase mutagenesis studies have shown that the Glu²³⁵ in *MaLac* is crucial in the oxidation of phenolics by forming a hydrogen bond to the OH group of phenolic substrates [112]. Since the *MaLac* Glu²³⁵Asp and Glu²³⁵Thr mutants preferred more the 2,6-DMP and the ABTS as substrates the Glu²³⁵ to Asn²³⁰ substitution (which shortens the side chain as -CH₃) in *MmLac* may lead to higher catalytic turnover. Even though Asp¹⁹⁰ to Glu¹⁸⁵, Ala¹⁹¹ to Pro¹⁸⁶ and Glu²³⁵ to Asn²³⁰ substitutions showed a decrease in substrate affinity to the phenolic substrates, binding affinity to the non-phenolic substrates (such as ABTS) was better in *MmLac*. These positions may good mutagenesis candidates to enlighten the role of them in forming a substrate-binding pocket.

The catalytic efficiency of *MmLac* is 67, 2, which is 1.6-fold better than *MaLac* for ABST, SGZ, and 2,6-DMP substrates (Table 4.5). Although the comprehensive pattern of the binding pocket of *MmLac* is similar to *MaLac*, it is slightly more spacious because of the Ala¹⁹¹ to Pro¹⁸⁶ and Glu²³⁵ to Asn²³⁰ substitutions [112] which may lead to differences in kinetic rates (Figure 4.26b). Since the enzyme was more efficient in non-phenolic substrate oxidation, *MmLac* may be an interesting template to use to engineer the enzyme to increase its phenolic substrate oxidation activity.

Table 4.5: Kinetic parameters of *MmLac* and the selected laccases from various organism.

Organism	Origin	ABTS			SGZ			2,6-DMP			Ref.
		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)	
<i>M. mycetomatis</i>	Asco-fungus	181.3	848.7	4.7	239.3	525.1	2.2	273.0	399.3	1.5	This study
<i>Myriocoonium sp.</i>	Asco-fungus	104.9	2.96	28.2	4.2	0.29	67.3	67.8	3.18	46.9	[156]
<i>M. verrucaria NF-05</i>	Asco-fungus	85.9	267.1	3.1	n.r.*	n.r.	n.r.	n.r.	n.r.	n.r.	[157]
<i>F. oxysporum</i>	Asco-fungus	721.8	7.48	0.01	n.r.	n.r.	n.r.	739	12.34	0.02	[158]
<i>S. macrospora k-hell</i>	Asco-fungus	58.45	1.17	0.02	21.1	0.45	0.02	75	0.3	0.004	[159]
<i>P. variabile</i>	Asco-fungus	203	56	0.27	n.r.	n.r.	n.r.	1176	17.6	0.01	[160]
<i>A. oryzae</i>	Asco-fungus	110	63.3	0.5	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	[161]
<i>T. harzianum</i>	Asco-fungus	180	5.2	0.02	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	[162]
<i>Chaetomiaceae</i>	Asco-fungus	652	26	0.04	9	4	0.44	12	34	2.83	[163]
<i>X. polymorpha</i>	Asco-fungus	20	550	27.5	3.5	199	56.85	97.5	247	2.53	[164]
<i>M. roreri</i>	Basidio-fungus	24.1	316	13.1	12.5	21	1.7	358	74	0.207	[143]
<i>T. trogii</i>	Basidio-fungus	7.3	260	35.5	6.7	35.9	5.3	302	50.3	0.2	[165]
<i>Cerrena sp.</i>	Basidio-fungus	93.4	2468	26.4	n.r.	n.r.	n.r.	200	703.7	3.52	[166]
<i>T. versicolor</i>	Basidio-fungus	169	125	0.7	n.r.	n.r.	n.r.	249	49	0.1	[167]
<i>P. sanguineus</i>	Basidio-fungus	77	68	0.9	91	51.9	0.6	203	6.9	0.03	[168]
<i>G. fornicatum</i>	Basidio-fungus	103.9	65.6	0.63	n.r.	n.r.	n.r.	276.7	38.5	0.14	[169]
<i>M. perniciosus FA553</i>	Basidio-fungus	170	21.2	0.12	21	0.45	0.02	108	0.3	0.003	[135]
<i>P. ostreatus HP-1</i>	Basidio-fungus	46.51	244.32	5.25	n.r.	n.r.	n.r.	400	208.33	0.52	[170]
<i>C. unicolor BBP6</i>	Basidio-fungus	49.1	3078.9	62.7	n.r.	n.r.	n.r.	3430.8	11.3	0.0033	[171]
<i>Pycnoporus sp. SYBC-L10</i>	Basidio-fungus	19.1	1156.2	60.53	n.r.	n.r.	n.r.	71.2	214.52	3.012	[172]
<i>L. gongylophorus</i>	Basidio-fungus	131.9	110.7	0.84	5.1	11.7	2.3	n.r.	n.r.	n.r.	[173]

*n.r., not reported

Continuation of Table 4.2.

<i>P. brevispora</i> BAFC 633	Basidio-fungus	445	194	0.435	n.r. *	n.r.	n.r.	117	3118	26.64	[174]
<i>C. perennis</i> SKU0322	Basidio-fungus	119.6	2217	18.5	n.r.	n.r.	n.r.	72.8	1012	13.9	[175]
<i>F. fraxinea</i>	Basidio-fungus	270	208	0.77	n.r.	n.r.	n.r.	426	130.2	0.24	[176]
<i>P. tigrinus</i> CBS 577.79	Basidio-fungus	31	185	5.9	n.r.	n.r.	n.r.	119	366	3	[177]
<i>Pleurotus sajor-caju</i>	Basidio-fungus	56	52	0.92	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	[178]
<i>C. bulleri</i>	Basidio-fungus	37	54	1.4	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	[179]
<i>A. faecalis</i>	Bacteria	500	1243.6	2.5	n.r.	n.r.	n.r.	480	1375	2.9	[180]
<i>A. elongatus</i>	Bacteria	39.2	2150	54.8	16.1	918.8	57.1	8.2	1009.4	123.1	[181]
<i>T. thermophilus</i>	Bacteria	36	0.4	0.011	27	1.001	0.037	152.1	0.13	0.001	[182]
<i>Cohnella</i> sp. A01	Bacteria	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	686	20.3	0.029	[183]
<i>B. licheniformis</i>	Bacteria	44	5.6	1.27	4.1	3.3	0.8	57.5	1.4	0.024	[184]
<i>B. subtilis</i>	Bacteria	124	322	2.59	18	80	4.4	216	29	0.134	[185]
<i>Agrobacterium</i> sp. S5-1	Bacteria	230.8	32.8	0.142	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	[186]
<i>Achromobacter</i> sp. HWN16	Bacteria	0.625	6940	11000	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	[187]
<i>Citrobacter</i> sp. LLJ16	Bacteria	0.484	5370	11000	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	[188]
<i>Geobacillus</i> sp. JS12	Bacteria	3990	0.3	0.00007	n.r.	n.r.	n.r.	4230	45.17	0.01	[188]
<i>K. pneumoniae</i>	Bacteria	5330	1.02	0.0001	n.r.	n.r.	n.r.	1240	2.93	0.002	[189]
<i>S. meliloti</i>	Bacteria	88	3.46	0.039	19	11.2	0.589	342	48.7	0.142	[190]
<i>S. viridochromogenes</i>	Bacteria	300	8	0.026	n.r.	n.r.	n.r.	450	1.9	0.004	[191]
<i>C. hirsutus</i>	Plant	56.7	260	4.5	n.r.	n.r.	n.r.	53	126	2.3	[192]
<i>M. sexta</i>	Insect	460	1.66	0.003	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	[193]

*n.r., not reported

The capability of laccases to use chromophore compounds as substrate leads to their use in industrial decolorization processes [14, 194, 195] *MmLac*'s observed decolorization performance was determined as; Amido Black 10B>Remazol Brilliant Yellow>Rhodamine 6 G>Crystal violet at the same concentration for all times. The structure of the laccase enzyme, utilized mediators and the experimental procedures affect the enzymatic decolorization efficiency of dyes [196].

Laccases fulfil these issues with their capacity to degrade the environmentally hazardous dyes that are currently used in this industry. The capability of laccases to use chromophore compounds as a substrate has led to their use in industrial decolourisation processes [14, 195]. The decolourisation performance of *MmLac* was between 33% and 62%. Although the decolourisation rates of this enzyme are similar to those in the literature [144, 197, 198], the laccase-mediated dye degradation can be enhanced by optimisation of experimental variables like enzyme and dye concentrations, incubation time, temperature and pH [196].

Cotton bleaching is used to decolourise natural pigments [199]. Due to health and environmental concerns about conventional bleaching methods, laccases are becoming acknowledged as promising enzymes to overcome these issues in the textile industry [200].

The optimal conditions to treat cotton fabrics with the enzyme prior to bleaching, to obtain greater whiteness, without damaging fabrics was investigated. Surprisingly, the whiteness of the enzyme-only treated fabric (treatment 2) was higher than that of the control fabric by 23 WI units.

The significant effect of the laccase on bleaching was seen by following treatments 5 and 6. The enzyme amount doubled, while the amount of H_2O_2 and NaOH remains constant, therefore, the increase in the WI can be directly related to the increase in enzyme concentration. This correlation demonstrates the bleaching effect of the laccase [201].

The effects of H_2O_2 alone are known to be negligible in the presence of the enzyme [201], however, the WI is higher in treatment 7 than treatments 5 and 6 due to an increase of H_2O_2 and NaOH. H_2O_2 is an oxidizing agent and provides bleaching by breaking down to oxygen and water. NaOH contributes to the bleaching process via the formation of hydroxyl ions and improves whiteness by supporting the bleaching mechanism of H_2O_2 [201]. However, the WI decreased with increased

laccase (12 g/L) in treatment 8. In high concentrations, the enzyme may form aggregates, making it less active in the bleaching process [202]. The accumulation of reaction products may also inhibit enzyme activity [203]. Therefore, developing a method to overcome possible aggregation, without damaging textile fibers, may enable the use of these enzymes in more of the bleaching process.

The higher WI, calculated in treatments 9 to 12 (Figure 4.28c) can be explained by the activation of H₂O₂ at temperatures > 80 °C. The highest WI (~60) is obtained by treatment 11 (Figure 4.28c). In textile processes, a WI of between 50 and 55 is obtained by the traditional alkali scouring process, in which a higher amount of H₂O₂ and NaOH is used at high temperatures (~98 °C) [204]. Higher temperatures are also used in enzymatic bleaching processes; a study by [205] found that whiteness of 25 to 30 is obtained by pectinase scouring applications, and greater whiteness could be achieved at high temperatures ≥90 °C [201, 206–208].

A high amount of H₂O₂ is beneficial to reduce fiber damage caused by the high temperatures and alkalinity of the bleaching process. In the present study, a lower amount of H₂O₂ and caustic, combined with the use of laccase enzyme, provided high WI values up to 60. This is an unusual finding since normally a high amount of H₂O₂ is necessary to reach this level of fabric whiteness [201]. These results show that the application of laccase facilitates high WI values, with fewer chemicals even at low temperatures of ~80 °C. Previous enzymatic scouring applications could only achieve these values at temperatures ≥90 °C. To our knowledge, *MmLac* has achieved the highest level of whiteness (~60) with the least amount of chemicals. Due to the Berger constants being obtained under different conditions, a direct comparison of whiteness could not be made in the present study. Most of the high WI values obtained in other studies are from expensive, non-environmentally friendly methods, such as using more chemicals, or combining of sequential enzymatic techniques enhanced through the use of mechanical systems like ultrasound technology [202, 208–213]. Therefore, our results indicate that the enzymatic bleaching process could deliver significant savings in energy, chemicals, cost and time, compared to traditional alkali and combined mechanic processes.

The achromatizing efficiency of the laccase was investigated to reveal enzyme performance for textile biotechnology application. The calculated scores showed that the new bleaching system, with laccase enzyme application, has a powerful

decolourising ability under mild process conditions. With the new bleaching process, fabrics achieved satisfactory whiteness in the range of 39 to 60, at much lower temperatures (between 55 °C and 80 °C, at pH 5.0), compared to temperatures of 98 °C applied in traditional processes. Remarkably, high WI values were obtained at 80 °C due to the activation of H₂O₂, and support results from alkali scouring methods. Additionally, lower amounts of NaOH and H₂O₂ were used, compared to the traditional processes. Using fewer chemicals results in a significant reduction of the undesirable emissions created in the process, which is an environmentally significant issue. Although the enzyme has moderate kinetic rates, it showed prominent performance and stability in bleaching tests, both alone and with H₂O₂, under alkali conditions and high temperatures. Therefore, future work should focus on extensive structural analysis, with particular emphasis on surface residues.

To reveal enzyme performance for the textile biotechnology application, the achromatizing efficiency of the laccase was investigated in the research. The calculated scores showed that the new bleaching system with a laccase enzyme application has a powerful decolorizing ability under mild process conditions. With the new bleaching process, the fabrics with a satisfactory whiteness degree range of 38.6 – 59.6 were obtained with lower temperatures of 55 and 80 °C in pH: 5.0 compared to 98 °C applied in traditional processes. Especially higher whiteness indexes were obtained at 80 °C due to the activation of H₂O₂ and support of alkali scouring. Additionally, a low amount of caustic and H₂O₂ was used compared to the traditional processes. Usage of less chemical provides a reduction of the undesirable odors created in the process which is an environmentally significant issue. Although the enzyme has moderate kinetic rates, it showed unexpected performance and stability in bleaching tests even in the presence of H₂O₂, alkali condition, and under high temperatures. Therefore, an extensive structural analysis focusing on surface residues could be planned as future work.

In conclusion, the increase in demand for cost-effective stable laccase for harsh conditions of applications and high performance in applications makes attractive fungal laccases to study. In this study, cloning of the designed plasmid construct having a laccase gene from *M. mycetomatis* and overexpressing was achieved in *P. pastoris*. Long term storage stability, having activity in a wide range pH even in the presence H₂O₂ and thermostability demonstrate the laccase robustness in extreme

application conditions from the industrial perspective. Furthermore, calculated industrial scores make the enzyme a good candidate in textile applications despite the kinetic rates. Hence, a study measuring the enzyme performance in environmental and food technology applications may contribute to its application in various areas. Lastly, the reported study introduces new challenges to produce the new recombinant laccase having the feature of various industrial applications on a large scale by fermentation technology.

6. CONCLUSION

Cloning of the designed plasmid construct with a laccase gene from *M. mycetomatis* was achieved in *P. pastoris*. The recombinant laccase enzyme has been actively produced in high amounts. The enzyme was characterised after purification and has been tested under industrial conditions for bleaching. The long term storage stability, thermostability and activity at a wide pH range, even in the presence of H₂O₂ (for industrial applications), demonstrate the robustness of this laccase under extreme application conditions. Furthermore, the whiteness index of conventional fabric is lower, compared to the enzyme-bleached sample. As concentrations of the laccase increase, the whiteness index also increases, and, significantly, is higher than the high-cost, non-ecofriendly combined processes. This study introduces new challenges to produce the novel recombinant laccase, which has a wide range of industrial applications, on a large scale by fermentation technology. Therefore, scaled-up studies of enzyme production, tests on the bleaching effect, and tests of the enzyme's performance in environmental and food technology applications, may contribute to its widespread use for a range of applications.

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BIOGRAPHY

Ahmet was born in Tokat, Turkey, on May 15, 1985. He received his BSc Degree in Biology from Ankara University in 2008. Later, He joined the MSc program in Molecular Biology at Ankara University under the supervision of Prof. Dr. Özlem YILDIRIM and was graduated from this program in 2010. Furthermore, He joined the MSc. program in Molecular Biology and Genetics at Gebze Institute of Technology under the supervision of Assistant Prof. F. İnci ÖZDEMİR and was graduated from this program in 2014. He has been a part of the PhD program of Molecular Biology and Genetics at Gebze Technical University since 2016 under the supervision of Associated Prof. Dr. Barış BİNAY. He also has been working as a forensic science expert at Istanbul Police Department since 2015.

APPENDICES

Appendix A: Publications related with the thesis

Tülek A., Karataş E., Yılmazcan Ö., Aydın D., Binay B., (2020) “Optimisation of the Production and Bleaching Process for a New Laccase from *Madurella mycetomatis*, Expressed in *Pichia pastoris*: from Secretion to Yielding Prominent”, Molecular Biotechnology, 281, doi: 10.1007/s12033-020-00281-9.

Tülek A., Karataş E., Binay B., “Cloning of a novel laccase gene from *Madurella mycetomatis* and expression in *Pichia pastoris*”, 2nd International Eurasia Biochemical Approaches & Technologies (EBAT) Congress, O-76, Antalya, Turkey, 26-29 October 2019.

Tülek A., Karataş E., Yücebilgiç G., Yıldırım D., Binay B., “Immobilization of *Madurella mycetomatis* laccase in metal-organic framework composite with mesoporous silica shells”, 2nd Enzyme and Bioprocess Days (EBDAYS) Congress, O-13, Kocaeli, Turkey, 01-03 September 2020.

Tülek A., Ünlü A., Karataş E., Binay B., “Expression and Biochemical Characterization of *Madurella mycetomatis* Laccase Enzyme in *Pichia pastoris*”, 2nd Enzyme and Bioprocess Days (EBDAYS) Congress, O-18, Kocaeli, Turkey, 01-03 September 2020.

Tülek A., “A New Laccase from *Madurella mycetomatis* expressed in *Pichia pastoris*: From discovery to yielding biotechnological performance in the textile industry”, CA18229_Yeast4bio COST Action 2nd WG Meeting 11th-12th November 2020, Working Groups Meeting.

Appendix B: Plasmid Maps of Recombinant *mmlac* Gene Constructs

The images were created with SnapGene software.

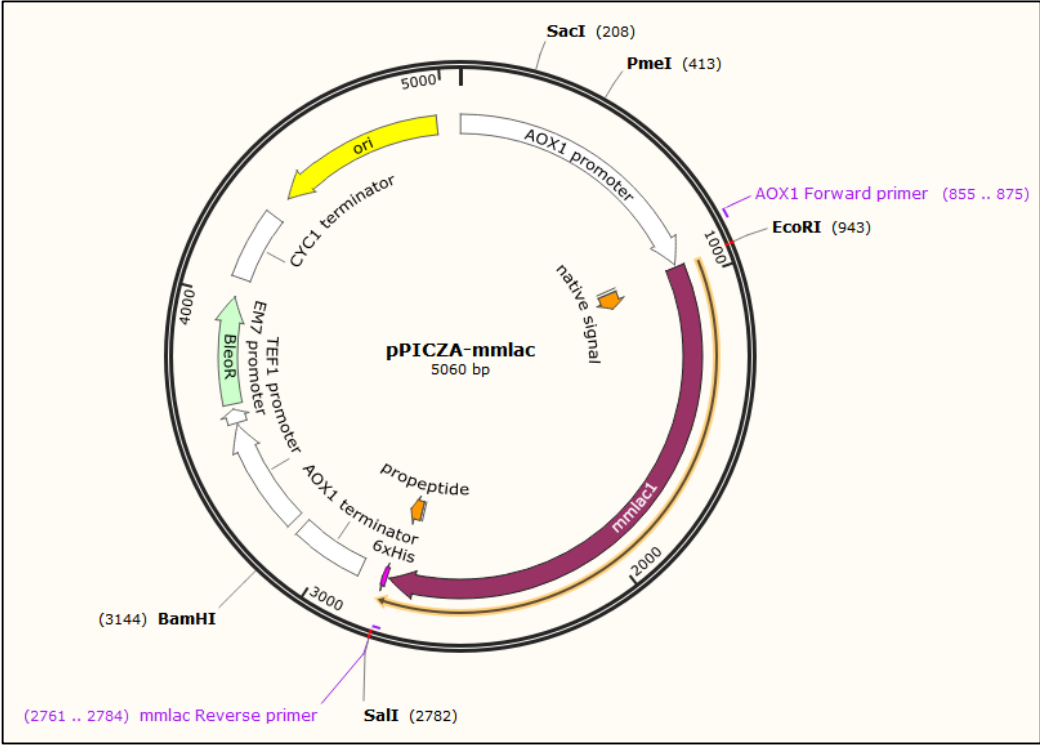


Figure B1.1: Recombinant plasmid map of pPICZA-mmlac.

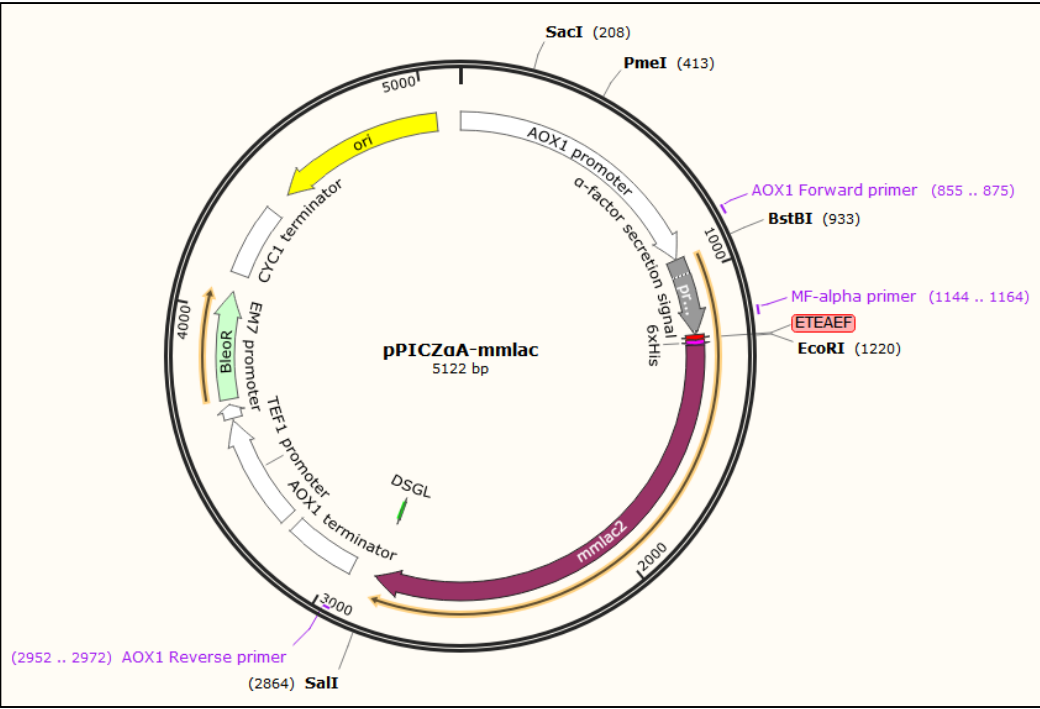


Figure B1.2: Recombinant plasmid map of pPICZαA-mmlac.