

**EVOLUTIONARY ENGINEERING OF *CORIOLOPSIS POLYZONA*
FOR TEXTILE DYE DECOLORIZATION**

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**EVİRİM MÜHENDİSLİĞİ UYGULANAN *CORIOLOPSIS POLYZONA*
KÜFÜNÜN TEKSTİL BOYASI GİDERİM ÖZELLİKLERİ**

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ABBREVIATIONS

ABTS	: 2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid
AOP	: Advanced oxidation processes
CDW	: Cell dry weight
C.I.	: Color index
CNLC	: Carbon and nitrogen limited culture medium
COD	: Chemical oxygen demand
DMP	: 2,6-dimethoxyphenol
EMS	: Ethyl methane sulfonate
EPR	: Electronic paramagnetic resonance
ETAD	: Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry
GAC	: Granular activated carbon
1-HBT	: 1-hydrobenzotriazole
IME	: Inverse metabolic engineering
Lac	: Laccase
LiP	: Lignin peroxidase
LME	: Lignin-modifying enzymes
MnP	: Manganese peroxidase
NTG	: Nitroso-methyl guanidine
PAH	: Polycyclic aromatic hydrocarbons
PCB	: Polychlorinated biphenyls
PDA	: Potato dextrose agar
RBBR	: Remazol brilliant blue R
SSF	: Solid state fermentation
WT	: wild type
VA	: Veratryl alcohol

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LIST OF SYMBOLS

ϵ	: extinction coefficient
ΔA	: absorbance change at the given wavelength
A	: absorbance of residual the dye
A_0	: initial absorbance of the dye
d	: light path of the cuvette container cell
d_i	: dilution rate
V_t	: total reaction volume
V_E	: enzyme volume
W_1	: weight of filter paper after dried overnight at 80°C
W_2	: weight of the biomass filtered papers dried overnight at 80°C

EVOLUTIONARY ENGINEERING OF *CORILOPSIS POLYZONA* FOR TEXTILE DYE DECOLORIZATION

SUMMARY

Coriolopsis polyzona MUCL 38443 is a laccase-producing white-rot fungus with ability to decolorize textile dyes. Suitable evolutionary engineering strategies were design and apply to improve decolorization properties of *C. polyzona*. Initially, medium selection studies were performed to choose the most suitable culture medium for shake flask cultivations of *C. polyzona*, followed by evolutionary engineering studies. Chemical mutagenesis was applied to the wild-type strain by using ethylmethane sulfonate (EMS) to increase genetic diversity, and initial mutant populations were obtained. Afterwards, batch selection protocols were applied to these mutant populations during evolutionary engineering studies wherein important selection factor was determined to improve decolorization ability of *C. polyzona* as starvation stress, which was based on carbon and/or nitrogen limitation in culture medium. Biomass yields and decolorization properties were determined for mutant generations. The individual mutants of the final mutant populations were also analyzed by randomly selecting and separately plating single spores from the mutant cultures. The results of the mutant populations and individual mutants with respect to biomass yield and decolorization properties were compared and discussed.

To sum up, evolutionary engineering strategies were applied to enhance the decolorization ability of *C. polyzona* and mutant individuals from mutant population with best decolorization feature were obtained and compared with wild-type organism.

EVİRİM MÜHENDİSLİĞİ UYGULANAN *CORIOLOPSIS POLYZONA* KÜFÜNÜN TEKSTİL BOYASI GİDERİM ÖZELLİKLERİ

ÖZET

Lakkaz üreten beyaz çürütücül küfü *Coriolopsis polyzona* MUCL 38443 suşu aynı zamanda tekstil boyası giderebilme özelliğine de sahiptir. *C. polyzona*'nın boya giderim özelliklerini geliştirmek üzere evrim mühendisliği yöntemi seçilmiş ve uygulanmıştır. İlk başta *C. Polyzona* kültürlerini yetiştirmek için kullanılacak sıvı besi yeri seçimi çalışmaları gerçekleştirilmiştir. Seçilen sıvı besi yerinden sonra evrim mühendisliği uygulamalarına devam edilmiştir. Ana kültüre, genetik çeşitlilik elde etmek için, kimyasal bir mutajen olan etilmetilsülfonat eklenmiş ve mutasyona uğratılmış başlangıç popülasyonu elde edilmiştir. Daha sonra, kesikli seçim yöntemleri ile sıvı besi yerinde bulunan karbon ve/veya azot oranlarındaki değişimlere bağlı açlık stresi uygulanarak mutanat *C. polyzona* popülasyonlarının boya giderim özellikleri artırılmaya çalışılmıştır. Elde edilen mutant popülasyonların boya giderim özelliklerine ve kütle ağırlıklarına bakılmıştır. Elde edilen son mutant popülasyonundan gelişigüzel bireyler seçilerek ilk önce katı besi yerine ekilmiştir. Sonra mutant popülasyon ve elde edilen bireylerin karşılaştırmalı olarak boya giderim özelliklerine ve kütle ağırlıklarına bakılmıştır.

Özetlemek gerekilirse evrim mühendisliği kullanılarak *C. polyzona* küf suşunun boya giderim özellikleri artırılmaya çalışılmıştır. Elde edilen mutant popülasyonlardan ve onların bireylerinden en iyi boya giderim özelliğine sahip olanlar ana kültürle karşılaştırılmıştır.

1. INTRODUCTION

1.1. Wood structure

Earth's main renewable material wood is the largest commercial product provided from forests, which comprise 27% of the world's land area. World's wood consumption is approximately 3500 million m³ annually which improved over 65% since 1960 (Martinez *et al.*, 2005).

Wood is composed of three main polymeric materials, cellulose, hemicelluloses and lignin as shown in Figure 1.1 (Martinez *et. al.*, 2005).

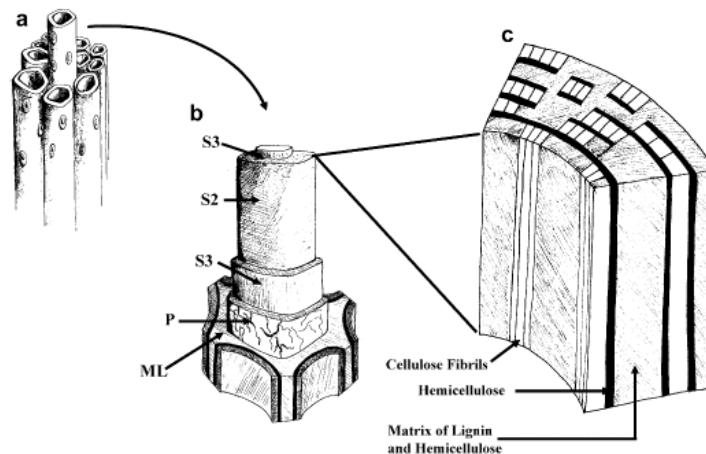


Figure1.1 Wood configuration: **a** shows the adjacent cells, **b** is the cell wall layers, *S1*, *S2* and *S3* are secondary cell wall layers, *ML* is the middle lamella and *P* is the primary wall, **c** shows lignin, cellulose and hemicelluloses distribution in the secondary cell wall (Perez *et al.*, 2002).

45-50% of wood is composed of cellulose, a linear polymer of cellobiose molecules formed from D-glucose subunits linked by β -1,4 glycosidic bonds, which make up long chains called elemental fibrils linked by hydrogen bonds and by van der Waals forces (Perez *et al.*, 2002, Martinez *et. al.*, 2005). Researches has been shown that major part of the cellulolytic microorganisms belong to eubacteria and fungi and some of the anaerobic protozoa and slime molds can be able to degraded cellulose and further investigated that under aerobic conditions water and carbon dioxide are

released and under anaerobic conditions carbon dioxide, methane and water are released from complete degradation of cellulose (Perez *et al.*, 2002).

Hemicelluloses, which compose the 25-30% of wood, is a highly complex carbohydrate polymer composed of polysaccharide that has a lower molecular weight than cellulose. Hemicelluloses are composed pentose and hexose residues as D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids which are bond by β -1,4- and occasionally by β -1,3-glycosidic bonds and are often acetylated, and generally form branched chains. In hardwood principal component of hemicelluloses is xylans together with variable percentages of galactose, arabinose, rhamnose and methylglucuronic acid units and acetyl groups, however in softwood is glucomannan (Perez *et al.*, 2002, Martinez *et al.*, 2005).

Lignin, which composes 20-35 % of wood, forms an amorphous matrix together with hemicelluloses that encloses the cellulose fibrils in cell walls, especially in the secondary cell walls, however its highest concentration is found in the middle lamella for acting as cement between wood fibers. This encirclement protects the hemicelluloses and cellulose from microbial depolymerization because lignin is highly resistant towards biological degradation as well as chemical degradation and besides the structural support also provides mechanical resistance and impermeability to wood (ten Have and Teunissen, 2001, Martinez *et al.*, 2005). Lignin, composed of phenylpropane units joined together by different types of linkages, is a non-water soluble and optically in active amorphous heteropolymer (Perez *et al.*, 2002). Lignin synthesis explained by Perez *et al.* as the generation of free radicals, which are released in the peroxidase-mediated dehydrogenation of three phenyl propionic alcohols, which are coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (*p*-hydroxyphenyl propanol) and sinapyl alcohol (syringyl propanol), furthermore the final result of this polymerization is a heterogeneous structure whose basic units are linked by C-C and aryl-ether linkages, with aryl-glycerol β -aryl ether being the predominant structure (Perez *et al.*, 2002).

Lignin can not be degraded as a sole source of carbon and energy, therefore degradation of lignin for gaining access to the holocellulose, cellulose with hemicelluloses, which is the actual carbon and energy source, is necessary for microbial attack. However, lignin's structural complexity as well as its high

molecular weight and insolubility make the degradation by microorganisms hardly difficult (Perez *et al.*, 2002).

Other than main three components of wood, there are non-structural components of wood include compounds extractable with organic solvents, called extractives, which can be either polar like phenols and tannins or non-polar like fats and sterols, water-soluble compounds like sugars and starch, as well as proteins and ashes which are together generally correspond to less than 5% of wood but can reach 20% in some softwoods (Martinez *et. al*, 2005).

1.2. Wood rotting fungi

Among the wood degrading microorganisms, fungi are the mainly known microorganisms capable of degrading the three polymers, cellulose, hemicelluloses and lignin, of wood structure (Perez *et al.*, 2002). The wood rotting fungi can be classified into three types, soft-rot fungi, brown-rot fungi and white-rot fungi, according to their mode of attacking the wood cell walls and components.

White-rot and brown-rot fungi includes basidiomycetes, soft-rot fungi includes ascomycetes and deuteromycetes. Brown-rot fungi mainly grow on softwood and seldom on hardwood, signify only 7% of wood-rotting basidiomycetes, which can degrade wood polysaccharides by a partial modification of lignin, resulting in a brown material consisting of oxidized lignin. However, white-rot basidiomycetes, which can grow on soft and hardwood, are characterized by their ability to degrade lignin, hemicelluloses, and cellulose, often results in a cellulose-enriched white material. Besides, white-rot and brown-rot basidiomycetes, some ascomycetes and their asexual states, called deuteromycetes, can degrade wood in contact with soil, which causes a decrease in the mechanical properties of wood, called as soft rot, a process that often involves bacteria. Moreover, soft-rot fungi can degrade wood under extreme environmental conditions like high or low water potential which can prohibit the activity of other fungi. Additionally, a limited number of ascomycetous fungi, called stain fungi, which causes a very limited degradation through wood extractives and water-soluble compounds by decaying wood through parenchymatic rays and resin channels causing discoloration of softwood tissues. Table 1.1 summarizes the characteristic properties of wood-rotting fungi types and stain fungi (Martinez *et. al*, 2005).

Table1.1 Types and characteristics of wood rotting fungi and stain fungi (Martinez *et. al*, 2005)

	White rot		Brown rot	Soft rot	Stain fungi
Decay aspects and consistency	Bleached appearance, lighter in color than sound wood, moist, soft, spongy, strength loss after advanced decay.		Brown, dry, crumbly, powdery, brittle consistency, breaks up like cubes, drastic loss of strength at initial stage of decay. Very uniform ontogeny of wood decay.	Soft consistency in wet environments. Brown and crumbly in dry environments. Generally uniform ontogeny of wood decay.	Discoloration sapwood areas (specks, spots and patches), blue (softwood), black (hardwood), red, or other colors. Discoloration due to colored hypha, or physiological response of tree against damage.
Host (wood-type)	Simultaneous rot	Selective delignification			
	Hardwood, rarely softwood	Hardwood and Softwood	Softwoods; seldom hardwoods. Forest ecosystems and wood in service.	Generally hardwoods (softwoods very slightly degraded). Forest ecosystems, waterlogged woods, historic archaeological wood, utility poles.	Both softwoods and hardwoods in forest ecosystems, and during transport and storage of timber.
Cell-wall constituents degraded	Cellulose, lignin and hemicellulose. Brittle fracture.	Initial attack selective for hemicelluloses and lignin, later cellulose also. Fibrous feature. Lignin degradation in middle lamella and secondary wall.	Cellulose, hemicelluloses. Lignin slightly modified. In some cases, extended degradation of hardwood (including middle lamella). Degradation at a great distance from hyphae (diffusion mechanism). Entire cell wall attacked rapidly with cracks and clefts.	Cellulose and hemicelluloses, lignin slightly altered. Cell wall attack in the proximity of hyphae starts from cell lumen. Logitudinal biconical cylindrical cavities in secondary wall (Type1). Secondary wall erosions from cell lumen (Type 2). Facultative soft-rot decay by some basidiomycetes.	Wood extractives and watersoluble compounds (sugars and starch). Colonization primarily affecting ray parenchyma and resin channels (produced through pits).
Anatomical features	Cell wall attacked progressively from lumen. Erosion furrows associated with hyphae.	Middle lamella dissolved by diffusion mechanism (not in contact with hyphae), radial cavities in cell wall.			
Casual Agents	Basidiomycetes (e.g. <i>T. versicolor</i> , <i>Irpex lacteus</i> , <i>P. chrysosporium</i> and <i>Heterobasidium annosum</i>) and some Ascomycetes (e.g. <i>Xylaria hypoxylon</i>).	Basidiomycetes (e.g. <i>Ganoderma australe</i> , <i>Phlebia tremellosa</i> , <i>C. subvermispora</i> , <i>Pleurotus</i> spp. and <i>Phellinus pini</i>).	Basidiomycetes exclusively (e.g. <i>C. puteana</i> , <i>Gloeophyllum trabeum</i> , <i>Laetiporus sulphureus</i> , <i>Piptoporus betulinus</i> , <i>Postia placenta</i> and <i>Serpula lacrimans</i>).	Ascomycetes (<i>Chaetomium globosum</i> , <i>Ustilina deusta</i>) and Deuteromycetes (<i>Alternaria alternata</i> , <i>Thielavia terrestris</i> , <i>Paecilomyces</i> spp.), and some bacteria. Some white (<i>Inonotus hispidus</i>) and brown-rot (<i>Rigidoporus crocatus</i>) basidiomycetes cause facultative soft-rot decay.	Ascomycetes (e.g. <i>Ophiostoma</i> and <i>Ceratocystis</i> spp.) and Deuteromycetes (e.g. <i>Aureobasidium pullulans</i> , <i>Phialophora</i> spp. and <i>Trichoderma</i> spp.)

1.3. White-rot fungi and their enzymes

Among the wood rotting fungi, white-rot fungi are the best degraders of lignin, which forms a matrix around the cellulose in wood cell walls to protect hemicelluloses and cellulose from microbial depolymerization. White rots get their name from the specific bleaching process that occurs during the fungal degradation of wood, causes cellulose enriched white material. White-rot fungi are the only known organisms that can completely degrade the lignin to carbon dioxide and water, and can further degrade cellulose and hemicelluloses (ten Have and Teunissen, 2001, Martinez *et al.*, 2005). Still, lignin cannot be degraded as a sole source of carbon and energy, white-rot fungi degrades lignin to gain access to the holocellulose, which is their actual carbon and energy source (ten Have and Teunissen, 2001). Therefore, white rots can also be considered as key regulators of the global C-cycle (Wesenberg *et al.*, 2003). Although, among fungi and other organisms, cellulose and hemicelluloses degradation is a primary metabolic process, however, lignin mineralization is obligatory which can be only carried out by the white rots. Most known white rots are basidiomycetes, even though a few ascomycete genera within the *Xylariaceae* are also capable of white rot break down (Pointing, 2001).

Degradation of wood by microorganisms is an enzymatic process. Cellulose and hemicelluloses transformation into monosaccharides is the simplest step, which can be accomplished by various cellulolytic and ligninolytic fungi as white-rots utilizing hydrolases to produce plenty of monosaccharides from polysaccharide components of wood. However, hydrolytic break down is inefficient and inhibits the degradation of cellulose and hemicelluloses in the presence of lignin matrix (Leonowicz *et al.*, 1999). Moreover, insolubility of wood substrates compels biodegradation occurrence exocellularly, either in association with the outer cell envelope layer or extracellularly. Hydrolytic break down is one type of extracellular enzymatic biodegradation. Other one is a unique oxidative extracellular ligninolytic system can able to degrade lignin. Thus, white rots are the most efficient lignin degraders due to their lignin-modifying enzymes (Perez *et al.*, 2002, Wesenberg *et al.*, 2003).

Three lignin-modifying enzymes (LME) of white-rot fungi are two glycosylated heme-containing peroxidases, lignin peroxidase (LiP, E.C. 1.11.1.14) and Mn

dependant peroxidase (MnP, E.C. 1.11.1.13) and a copper-containing phenoloxidase, laccase (Lac, E.C. 1.10.3.2) (Pointing, 2001). There are white rots capable of producing all of three extracellular enzymes, or two or only one of the enzymes. However, during lignin degradation various auxiliary enzymes, which can not degrade lignin by themselves, are combined with LMEs as glyoxal oxidase and superoxide dismutase for intracellular production of LiP and MnP co-substrate H_2O_2 , glucose oxidase, aryl alcohol oxidase and cellobiose dehydrogenase which are involved in feedback circuits and linking lignin break down with cellulose and hemicellulose degradation (Leonowicz *et al.*, 1999).

1.3.1. White rot peroxidases: Lignin peroxidase and Manganese peroxidase

Lignin peroxidase (LiP, 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol:hydrogen-peroxide oxidoreductase; E.C. 1.11.1.14) and manganese peroxidase (MnP, Mn(II):hydrogen-peroxide oxidoreductase; E.C. 1.11.1.13) were first discovered in mid 1980s. LiP discovered by Tien *et al.* in 1983 from white rot fungi *Phanerochaete chrysosporium* (Tien *et al.*, 1983). This discovery initiated the search for other oxidative enzymes secreted by white rot fungi and set off the discovery of MnP, which oxidizes Mn(II) to the Mn(III) (ten Have and Teunissen, 2001). White rot peroxidases oxidize their substrates by using H_2O_2 , therefore, as part of their ligninolytic system they can produce H_2O_2 generating oxidases as glucose oxidase, glyoxal oxidase and aryl alcohol oxidase. White rots, that could not secrete H_2O_2 generating enzymes oxidizes organic acids like oxalate and glyoxylate to provide their H_2O_2 need indirectly (ten Have and Teunissen, 2001, Martinez *et al.*, 2005).

LiP and MnP are both glycosylated proteins with a heme group in their active sites. Molecular weight of LiP ranges from 38 to 43 kDa. However, molecular weight of MnP is slightly higher than LiP ranges from 45 to 60 kDa even though MnPs are molecularly very similar to LiPs. LiPs are able to oxidize phenolic and non-phenolic compounds, amines, aromatic ethers and polycyclic aromatics with their ionization potential, which is a measure for the ease to abstract an electron from the highest occupied molecular orbital, of up to 9.0 eV. LiP catalyzes the oxidation reactions as the cleavage of the $Ca-C\beta$ and aryl Ca bond, aromatic ring opening and demethylation. Additionally, veratryl alcohol (VA), an excellent substrate of LiP and a secondary metabolite studied previously various times, enhances the oxidation of

poor substrates of LiP. MnPs use the conventional peroxidase catalytic cycle as LiP as however uses Mn(II) as substrate to oxidize it to Mn(III), which is stabilized by organic chelators, as oxalate and to a lesser extent malonate and glyoxylate (ten Have and Teunissen, 2001, Perez *et al.*, 2002). Mn(III) formed is a strong oxidant and relatively stable compound that can leave the active center to oxidize phenolic compounds, but not non-phenolic units of lignin, generates phenoxy-radicals, further undergo different reactions and causes the depolymerization (Perez *et al.*, 2002).

Oxidation reactions of these peroxidases cause the formation of a high redox potential oxo-ferryl intermediate during the reaction of the heme cofactor with H_2O_2 . Besides, Martinez *et al.*, point out the two aspects about their molecular structure which provide LiP and MnP their unique catalytic properties: (i) a heme environment, conferring high redox potential to the oxo-ferryl complex; and (ii) the existence of specific binding sites (and mechanisms) for oxidation of their substrates (Martinez *et al.*, 2005). The Figure 1.3 demonstrates the catalytic cycle of these white rot peroxidases, LiP and MnP.

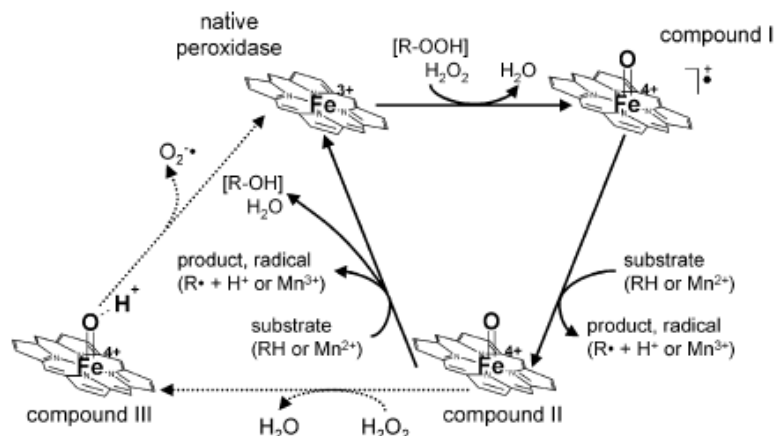


Figure 1.2 Catalytic cycles of white rot peroxidases, LiP and MnP

1.3.2. White rot phenoloxidase: Laccase

Laccase (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) is a multicopper blue oxidase, which is isolated from various fungi, plants and insects (Galhaup *et al.*, 2002, Martinez *et al.*, 2005). Laccase was first isolated from Japanese lacquer tree *Rhus vernicifera* by Yoshida in 1883 and has been studied since (Gianfreda *et al.*, 1999, Mayer and Staples, 2002). Laccase is one of the oldest enzymes that has been discovered and was the first ligninolytic enzyme that is investigated, even though its complete molecular structure was revealed in 2002 by the reported crystal structures

of laccases from basidiomycete *T. versicolor* and the ascomycete *Melanocarpus albomyces* (Mayer and Staples, 2002, Martinez *et al.*, 2005).

When first investigated in *Rhus vernicifera*, laccases were further discovered in entire family of *Anacardiaceae*, in which the lacquer tree is a member, in their resin ducts and in the secreted resins (Mayer and Staples, 2002). Mayer and Staples have mentioned in their review that “laccases can be roughly divided into two major groups which show clear differences, those from higher plants and those from fungi” (Mayer and Staples, 2002).

After its investigation in *Rhus vernicifera*, laccase is further discovered in sycamore (*Acer pseudoplatanus*) cells, apricots, mung bean hypocotyls cell walls, stems of *Zinnia elegans*, cell suspension cultures of rosemary, the sap of mango fruits and in the xylem from tobacco (Gianfreda *et al.*, 1999). Furthermore, eight laccases expressed in xylem tissues of *Pinus taeda*, laccase presence in leaves of *Aesculus parviflora* and in green shoots of tea and five laccases in xylem tissue of *Populus euramericana* have been detected (Mayer and Staples, 2002).

Even though, laccases are investigated in plants, insects and bacteria, the most important source of this enzyme is fungi. Among the fungi, wood-rotting fungi is the main producer of the laccases even it is isolated from various fungi including as *Aspergillus*, the thermophilic fungi *Myceliophora thermophila* and *Chaetomium thermophilum* (Perez *et al.*, 2002, Galhaup *et al.*, 2002) and ascomycetes, such as phytopathogenic ascomycetes *Gaeumannomyces graminis*, *Magnaporthe grisea* and *Ophiostoma novo-ulmi*, as well as from *Mauginella*, *Melanocarpus albomyces*, *Monocillium indicum*, *Neurospora crassa* and *Podospora anserina*, soil ascomycetes from the genera *Curvularia* and *Penicillium* and finally some freshwater ascomycetes (Baldrian, 2006). Lastly, although primary source of laccases are fungi, laccase property holder phenoloxidases were isolated from larval and adult cuticles of insects as *Drosophila melanogaster*, *Lucilia cuprina*, *Manduca sexta*, and *Sarcophaga bulata* and in bacteria *Azospirillum lipoferum* and in a heterocystous cyanobacterium *Anabaena azollae* (Gianfreda *et al.*, 1999).

In all these fungi, plants, bacteria and insects, laccases have various functions as participation in the radical-based mechanisms of lignin polymer formation and contribution to cell-wall reconstitution in regenerating protoplasts of higher plants

(Mayer and Staples, 2002, Baldrian, 2006), play a role in lignin degradation and or the removal of potentially toxic phenols arising because of degradation, morphogenesis, sporulation, or phytopathogenesis, fungal virulence (Galhaup *et al.*, 2002) and fungal plant-pathogen/host interaction, stress defenses (Baldrian, 2006) and synthesis of pigments in fungi (Martinez *et al.*, 2005), insect sclerotization, bacterial melanization and melanin-related virulence for humans as well (Li *et al.*, 1999). Although, laccases from various biological sources have different functions, all of them catalyzes polymerization and depolymerization processes (Riva, 2006) and related to the oxidation of aromatic substances which differs in resulted compounds and mechanisms as oxidation of monolignols to form polymeric lignins in plants, whereas degradation and depolymerization of lignins by white-rot fungi (Li *et al.*, 1999).

Laccases are larger than peroxidases with molecular weight around or higher than 60 kDa and are also glycosylated as peroxidases (ten Have and Teunissen, 2001). Laccases catalyzes the four electron oxidation by reduction of oxygen to water (ten Have and Teunissen, 2001, Baldrian, 2006). They are known as highly oxidizing that their ionization potential ranges from 450–480mV in *Myceliophthora thermophila* to 760–790mV in *Polyporus pinsitus* (Baldrian, 2006). Laccases mainly use phenolic compounds as hydrogen donors for reduction of O₂ to water, however, can catalyze single electron abstraction from various organic and inorganic substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, metal complexes such as ferrocene, ferrocyanide or iodide by simultaneously four electron reduction of O₂ to H₂O (Figure 1.4) (Galhaup *et al.*, 2002, Baldrian, 2006). Because its co-substrate oxygen usually there in the oxidation environment, therefore, any additional or synthetic low molecular weight cofactors as H₂O₂ for peroxidases are necessary (Baldrian, 2006).

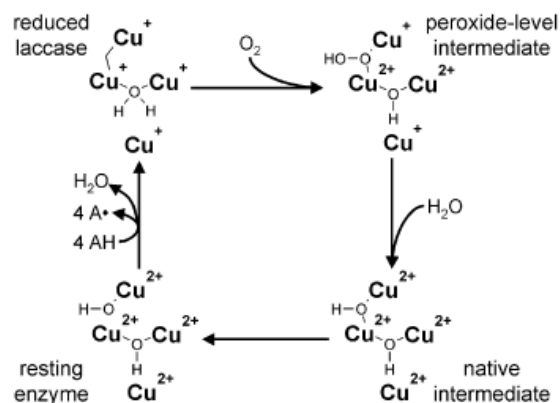


Figure 1.3 Catalytic cycle of laccase (Wesenberg *et al.*, 2003)

Laccase, molecularly as a dimeric or tetrameric glycoprotein, generally has four copper atoms per monomer distributed in three redox sites (Figure 1.4) (Couto and Herrera, 2006). Laccases, all blue multi-copper oxidases, contains at least one type-1 (T1) copper with at least additional three other copper ions as one type-2 (T2) and two type-3 (T3), arranged in trinuclear cluster (Baldrian, 2006), which are differ from each other in their characteristic electronic paramagnetic resonance (EPR) signals, coupled type-3 site activated at strong anion binding, type-1 and type-2 at strong electronic adsorption (Gianfreda *et al.*, 1999). Further, different copper sites can be identified by their spectroscopic characteristics as T1 copper shows strong absorption around 600 nm and also gives enzyme the typical blue color, however, T2 copper has weak absorption characteristic in the visible region and T3 coppers have an absorption maximum at 330 nm (ten Have and Teunissen, 2001). T1 copper, involved in the reaction with substrates, oxidizes the substrate and extracted electrons are transferred to T2/T3 site, which is a cluster in a triangular form and involved in the binding and reduction of oxygen as well as the storage of electrons transferred from the reduced substrates, where molecular oxygen is further reduced to water. It is purposed that electron transfer between copper sites are through from strongly conserved His-Cys-His tripeptide motif as shown in Figure 1.5 (ten Have and Teunissen, 2001, Baldrian, 2006).

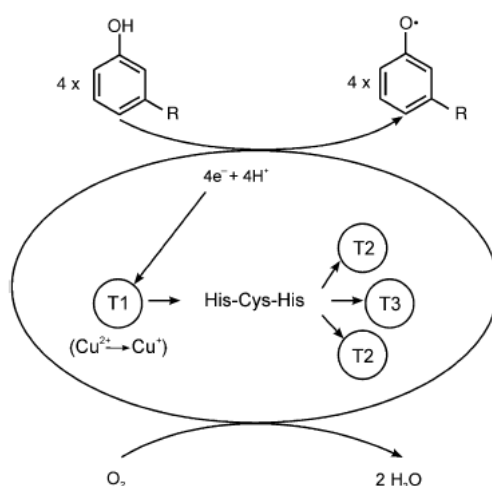


Figure 1.4 Four different copper sites of laccase and purposed oxidation mechanism (Baldrian, 2006)

Some laccases lack their T1 copper site, thus, couldn't be able to show characteristic absorption band around 600 nm, referred as “yellow laccases” (Baldrian, 2006). In addition, laccases with one atom of copper, two atoms of zinc and one atom of iron, referred as “white laccases”, have also been investigated in the white-rot fungus *Pleurotus ostreatus* (Arana-Cuenca *et al.*, 2004).

Usually, substrate molecules are oxidized by laccases due to direct interaction with the copper cluster. Nonetheless, the substrates cannot be oxidized directly by laccases, either because they are too large to penetrate into the active site or because they have a particularly high redox potentials compared to laccase's ionization potential. This can be overcome with the addition of ‘chemical mediators’, which are suitable compounds that act as intermediate substrates for the laccase, enables enzymes ability to oxidize non-phenolic structures (Riva, 2006, Couto and Herrera, 2006), thus, as well as capable of oxidizing mainly phenolic compounds laccases are able to oxidize non-phenolics. Some of these chemical mediators are 2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1-hydrobenzotriazole (1-HBT) and violuric acid, besides, there are various natural mediators that have been discovered as 4-hydroxybenzoic acid, 4-hydroxybenzyl alcohol, and 3-hydroxyanthranilate (ten Have and Teunissen, 2001).

Ligninolytic enzymes of white rots are exclusively extracellular, even this is a true situation for LiP and MnP, it is not the same with laccases, although nearly all laccases investigated so far are extracellular enzymes, the laccases of wood-rotting

fungi can be also found intracellularly. *Trametes versicolor*, grown on glucose, wheat straw and beech leaves, produces laccases both in extracellularly and intracellularly and also traces of intracellular laccase activity is found in *Agaricus bisporus*, even though more than 88% of the total laccase activity was in the culture supernatant, *Phanerochaete chrysosporium* and *Suillus granulatus* (Baldrian,2006).

White rots produce their ligninolytic enzymes during their secondary metabolism and because lignin oxidation doesn't provide net energy to the fungus, synthesis and secretion of these enzymes is often induced by limited nutrient levels, mostly in carbon or nitrogen limitation. Production of LiP and MnP is generally optimal at high oxygen tension but is repressed by agitation in liquid cultivation; yet laccase production is mostly enhanced by agitation. Usually, more than one isoforms of ligninolytic enzymes are expressed by different white rot species and under different culture conditions and encoded by gene families with complex regulation. Moreover, nutrient levels, mediator compounds and required metal ions, as Mn^{2+} for MnP, Cu^{2+} for laccase, affect transcription of respective genes (Wesenberg *et al.*, 2003).

With regard to their substrate range, ligninolytic enzymes, LiP, MnP and laccase, of white rots are highly non-specific; therefore, enzymes are capable of in vitro transformation or mineralization of a wide range of highly recalcitrant organopollutants with structural similarities to lignin, which are xenobiotics and white-rot fungi are the only organisms capable of their breakdown (Pointing, 2001). Thus, this attribution of white-rots makes them a strong candidate for research on their potential industrial applications.

1.4. Potential industrial applications of white-rot fungi

After the discovery of their lignin-modifying enzymes, potential applications of white rot fungi in manufacturing industries has turned out to be one of the significant research areas of industrial biotechnology and enzyme technologies.

Researches have been performed for possible usage of white rots in pulp and paper, food, textile, cosmetics industries and mainly in biological treatments of wastewaters from those manufacturing industries. Through the studies application of whole cells or purified ligninolytic enzymes from white rots are tested for their compatibility to the specific industrial processes.

White-rots attribution to breaking down wide variety of recalcitrant compounds, because of their substrate non-specific ligninolytic enzymes, like polycyclic aromatic hydrocarbons, chlorophenols, nitrotoluenes, dyes and polychlorinated biphenyls, besides phenols, provides them a noticeable application field on in situ bioremediation of contaminated soils (Perez *et al.*, 2002, Jounani *et al.*, 2006). Further application possibilities are the employment of white rots as biocatalysts in the production of fine chemicals and natural flavors such as vanillin and the biological treatment of several waste waters as bleach plant effluents or other waste waters containing lignin-like polymers, as is the cases of dyeing industry effluents and olive-oil-mill wastewaters (Perez *et al.*, 2002). For example, laccases can be used for various applications in industry as biocatalytic purposes in delignification of lignocellulosics and cross-linking of polysaccharides, for biological treatment of wastewater detoxification and textile dye transformations, usage in food technologies, as personal and medical care applications, further in biosensors and in analytical uses (Galhaup *et al.*, 2002).

1.4.1. Pulp and paper industry

Lignin elimination is necessary for production of kraft pulp, after the cooking with NaOH and Na₂S, from paper pulp by mechanical or chemical methods (Riva, 2006). Further, chlorine mediated chemical bleaching process is applied to remove color due to 10-15% residual lignin in pulp for high quality paper production (Li *et al.*, 1999). These chemical bleaching processes are very harsh to environment because of used chemicals produces chlorinated toxic organic compounds and low molecular toxic halogenated lignin degradation products as well and released into the aquatic environments from bleach plants. Thus, there is a growing pressure from environmentally concerned organizations to replace the conventional bleaching techniques with environmentally friendly ones (Li *et al.*, 1999, Pointing, 2001). Conventionally, chlorine, chlorine oxide and oxygen were further added to finalize the bleaching process, however due to environmental aspects usage of Cl₂ has been forbidden and the use of Cl₂O has been restricted. Consequently, new approaches for bleaching must be improved (Riva, 2006). Two different approaches can be stated as enzymatic bleaching process, bio-bleaching of pulp, or mineralization of toxic end-products by oxidative bioremediation treatments, in both approaches white rot fungi can be applied like laccase from white rot fungi is an ideal enzyme for bio-bleaching

in this matter and like ability of white rot fungus *Phanerochaete chrysosporium* in oxidative demethylation and dechlorination of bleach plant effluents, which has been studied previously (Li et al., 1999, Pointing, 2001).

1.4.2. Soil bioremediation

Soil bioremediation has taken the great attention these days because of releasing highly toxic, mutagenic and carcinogenic pollutants as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and BTEX compounds, which are benzene, toluene, ethylbenzene, and *o*-, *m*- and *p*-xylenes, into environment (Pointing, 2001, Levin *et al.*, 2003). PCBs are produced by chlorination of biphenyl and industrially used in dielectric fluids, flame retardants, heat-transfer fluids, hydraulic fluids, organic diluents, plasticizers and solvent extenders, therefore PCBs are persistence and everywhere in terrestrial environment (Pointing, 2001). Degradation of PAHs, major components of petroleum, is very little in case of higher PAHs with four and five rings, which are purely soluble in water and usually tightly bound to soil particles. BTEX compounds, components of gasoline and aviation fuels, and their nitro derivatives are widely used in industrial synthesis. Because of their carcinogenic neurotoxic effects, they are also one of primary organic pollutants (Levin *et al.*, 2003).

In situ bioremediation of contaminated soils can be applied in two different ways; one is changing the physicochemical nature of the soil by aeration or changing the nutrients, called bioremediation, and other is introduction of organopollutant degrading microorganisms to the contaminated soil, called bioaugmentation, in which physicochemical alterations can be further applied (Pointing, 2001).

Higher PAHs are persistent to bacterial degradation because of their molecular structures and even though both aerobic and anaerobic bacteria are able to degrade BTEX compounds with microbial consortium, no native strain of bacterium has been discovered to degrade all components of BTEX competently, where *o*-xylene is particularly resistant to bacterial degradation (Levin *et al.*, 2003). However, various researches have shown that white-rot fungi are capable of degrading these organopollutants, including in vivo removal of PCB, with 1-6 Cl substitutions, by *Coriolopsis polyzona*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, and *Trametes versicolor* (Pointing, 2001), oxidation of PAHs into more water-soluble

compounds for higher bioavailability resulted in increased rate of mineralization by bacteria than those of the parent compounds and simultaneous degradation of BTEX components by *Phanerochaete chrysosporium*. Thus, non-specific oxidases of white-rot fungi are able to break down PAHs, PCBs, chlorinated phenols, BTEX, dioxins, pesticides, explosives and dyes released into environment, to soil, from industrial effluents (Levin *et al.*, 2003).

1.4.3. Food industry

Enzymatic oxidation technologies developed as novel oxidation systems improved against conventional oxidation systems nowadays are one of the important application techniques in food industry. Enzymes purified from white rots have various applications in food industry. Especially laccases, which can be applied to certain processes that enhance or modify the color appearance of food or beverage, involves the elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity development in clear fruit juice, beer and wine. Besides, laccase from the white-rot fungus *Trametes hirsuta* increased the maximum resistance of dough and decreased the dough extensibility in both flour and gluten dough. Thus, there are various potential applications of laccase in different aspects of the food industry as bioremediation, beverage processing, ascorbic acid determination, sugar beet pectin gelation, baking and as a biosensor (Couto and Herrera, 2006). One of the significant bioremediation process applied in food industry is olive oil milling that produces more than 30 million tons of liquid effluent in the Mediterranean area annually. Effluent from olive oil milling is very detrimental to the environment owing to its high phenolic level, which structurally shows similarities with lignin, cause's toxicity and black color. Therefore, wastewaters from olive oil milling can be biologically treated with white rot fungi due to their feature of breaking down lignin and lignin model compounds efficiently (Jaouani *et al.*, 2006).

1.5. Industrial dyes and textile industry

Reported previous to that more than 10 000 dyes are manufactured and used above 700 000 metric tons worldwide annually (Fu and Viraraghavan, 2001). Due to world's economic growth since 2000, increase in production rate is estimated

between 3-4% which adds only approximately 30 000 metric tons per year to annual fabrication (Hunger, 2003).

Main manufacturer of dye industry was Germany in 1900s with other European countries, Switzerland, UK and France. Germany and other Western Europe countries hold the main production percentage in their hand even though USA and other lower cost countries such as India, China and Taiwan entered the dye market that's because European countries provides the supplementary of dye industry (Hunger, 2003) until 2000. In last decade, Asian countries become the largest manufacturer by holding 42% of the worldwide dye market (Wesenberg *et. al*, 2003). Textile, food, paper-making and cosmetic industries are the main customers of the dyes fabricated worldwide.

1.5.1. Dyes used in manufacturing industries and dye classification

Worlds first know synthetic dye, Mauveine, was produced by William Henry Perkin in 1856 (Wesenberg *et. al*, 2003). From since except one or two important dye, most of the dyes were discovered during 1800s (Hunger, 2003).

Classification as native and synthetic dyes is not sufficient because synthesis of natural substances now can be possible. Moreover, naming dyes as textile, leather, paper and food dyes can only gives an opinion on the characteristics of the dye. Therefore, dyes are classified mainly by two approaches: (1) due to their chemical properties, which is the most precise way or (2) due to their usage or method of application, which is preferred commonly by dye manufacturers as given in Table 1.1 (Hunger, 2003, Wesenberg *et. al*, 2003).

Table 1.2 Dye classifications due to usage (Hunger, 2003)

Class	Principal substrates	Method of application	Chemical type
Acid	Nylon, wool, silk, paper, inks and leather	Usually from neutral to acidic dyebaths	Azo(including premetallized), anthraquinone, triphenylmethane, azine, xanthene, nitro and nitroso
Azoic components and compositions	Cotton, rayon, cellulose acetate and polyester	Fiber impregnated with coupling component and treated with a solution of stabilized diazonium salt	Azo
Basic	Paper, polyacrylonitrile, modified nylon, polyester and inks	Applied from acidic dyebaths	Cyanine, hemicyanine, diazahemicyanine, diphenylmethane, triarylmethane, azo, azine, xanthene, acridine, oxazine and anthraquinone
Direct	Cotton, rayon, paper, leather and nylon	Applied from neutral or slightly alkaline baths containing additional electrolyte	Azo, phthalocyanine, stilbene and oxazine
Disperse	Polyester, polyamide, acetate, acrylic and plastics	Fine aqueous dispersions often applied by high temperature/pressure or lower temperature carrier methods; dye may be padded on cloth and baked on or thermofixed	Azo, anthraquinone, styryl, nitro and benzodifuranone
Fluorescent brighteners	Soaps and detergents, all fibers, oils, paints and plastics	From solution, dispersion or suspension in a mass	Stilbene, pyrazoles, coumarin and naphthalimides
Food, drug and cosmetic	Foods, drugs and cosmetics		Azo, anthraquinone, carotenoid and triarylmethane
Mordant	Wool, leather and anodized aluminum	Applied in conjunction with Cr salts	Azo and anthraquinone
Oxidation bases	Hair, fur and cotton	Aromatic amines and phenols oxidized on the substrate	Aniline black and indeterminate structures
Reactive	Cotton, wool, silk and nylon	Reactive site on dye reacts with functional group on fiber to bind dye covalently under influence of heat and pH (alkaline)	Azo, anthraquinone, phthalocyanine, formazan, oxazine and basic
Solvent	Plastic, gasoline, varnishes, lacquers, stains, inks, fats, oils and waxes	Dissolution in the substrate	Azo, triphenylmethane, anthraquinone and phthalocyanine
Sulfur	Cotton and rayon	Aromatic substrate vatted with sodium sulfide and reoxidized to insoluble sulfur-containing products on fiber	Indeterminate structures
Vat	Cotton, rayon and wool	Water-insoluble dyes solubilized by reducing with sodium hydrogen sulfite, then exhausted on fiber and reoxidized	Anthraquinone (including polycyclic quinones) and indigoids

Among both approaches, most systematic classification method is according to its chemical structure, given by Color Index (C.I.), which is shown in Table 1.2.

Table1.3 Dye classification due to chemical structure, Color Index (Wesenberg *et. al*, 2003)

Code	Chemical class	Code	Chemical class	Code	Chemical class
10,000	Nitroso	42,000	Triarylmethane	53,000	Sulfur
10,300	Nitro	45,000	Xanthene	55,000	Lactone
11,000	Monoazo	46,000	Acridine	56,000	Aminoketone
20,000	Disazo	47,000	Quinoline	57,000	Hydroxyketone
30,000	Trisazo	48,000	Methine	58,000	Anthraquinone
35,000	Polyazo	49,000	Thiazole	73,000	Indigoid
37,000	Azoic	49,400	Indamine/Indophenol	74,000	Phthalocyanine
40,000	Stilbene	50,000	Azine	75,000	Natural
40,800	Carotenoid	51,000	Oxazine	76,000	Oxidation Base
41,000	Diphenylmethane	52,000	Thiazine	77,000	Inorganic

Industrial dyes with different chromophores have different and stable chemical structures to provide requirements needed in various industrial dye applications. Major chromophores of commercially available dyes are azo, anthraquinone and indigo (Wong and Yu, 1999). Among industrially manufactured dyes azo dyes comprises the 50% of the world's production (Rodriguez, 1999) and with addition of anthraquinone dyes this amount increases to 90%. Due to their applications in industry, dyes can be classified as acid, azoic, basic, direct, disperse, reactive, mordant, sulfur, and vat dyes (Table 1.1) (Yang and McGarrah, 2005). Dyes can be further classified as direct, acid and reactive dyes as anionic, basic dyes as cationic and disperse dyes as nonionic dyes, in which chromophores of anionic and nonionic dyes mostly consist of azo or anthraquinone groups (Fu and Viraraghavan, 2001).

1.5.2. Textile industry and dyes used in textile industry

Textile industry is one of the main consumers of the dyes manufactured industrially. Textile manufacturing first begins with production of natural or synthetic textile fibers from raw materials. Fibers pass through four main processing stages: yarn production, fabric production, finishing and fabrication before they are further processed with bleaching, printing, dyeing, mechanical preshrinking and shaping (Yang and McGarrah, 2005). Among the manufacturing industries textile industry is one of the most complicated one due to usage of numerous toxic chemicals as complexing, sizing, wetting, softening, anti-felting and finishing agents, biocides, carriers, halogenated benzenes, surfactants, phenols, pesticides, dyes and many other additives, which are mainly used in wet processes called washing, scouring, bleaching, mercerizing, dyeing and finishing (Selcuk, 2005).

In textile industry mainly synthetic dyes derived from coal tar and petroleum-based intermediates are used in manufacturing. Furthermore, classifications of dyes are based on the fibers that dye can be applied. Chemical structure of dyes determines its affinity to particular textile fiber (Yang and McGarrah, 2005). Yang and McGarrah further explained particular dye affinity mechanisms by different dye classes as disperse, acid and reactive dyes.

Disperse dye because of their low solubility in water are applied as a distribution of finely grounded powders in the dye bath, because dyes with poor dissolution at low concentrations can transfer into synthetic textile fiber by its higher solubility in the substrate at aqueous dye bath. During application high temperatures and pressures are provided. Polyester and other synthetic fibers that couldn't dye with water-soluble dyes are dyed with disperse dyes (Yang and McGarrah, 2005).

Acid dyes and reactive dyes are water-soluble anionic dyes. Acid dyes, which contain one or more sulfonic or carboxylic acid groups in their molecular structure, are applied to nylon, wool, silk and some modified acrylic textiles in an acidic medium. Ionic bonds between sulfonic acid group of the dye and basic amino group of the fiber provides the dye-fiber affinity in acid dyes. Reactive dyes are used for dyeing of cellulosic fibers as cotton and rayon, but further can be used in silk, wool, nylon and leather dyeing. Reactive dyes have replaced the usage of direct, azoic and vat dyes. Reactive dyes form covalent bonds with textile fiber which enables dyes to become part of the fiber. Therefore, large amounts of salt are needed during application and significant amount of dye can remain unfixed to the fibers at the end of the process (Yang and McGarrah, 2005).

Every dye manufactured to be utilized in the textile industry are designed to resist fading against exposure to sweat, light, water, various chemicals including oxidizing agents, and microbial attack (Wesenberg et. al, 2003).

1.6. Textile industry wastewater treatment: Decolorization of textile industry effluents and degradation of textile dyes

In textile industry large volumes of water and diverse chemical compounds, varies from inorganic compounds to polymers and organic products, are consumed in wet processes (Robinson et al., 2001). Annually, huge amount of dyes are being used in

various industrial manufacturing processes. Besides, textile industry is one of these industries that use chemically different dyes and significant amount of these dyes release into aquatic environment through wastewaters and affects photosynthetic activity in aquatic life due to reduced light penetration through water surface. Because, dyes used are resistant to light, water and oxidizing agents, their degradation is very hard once dyes are released into aquatic system (Park *et al.*, 2006, Joo *et al.*, 2007). Hence, effluents of dyeing and textile industries have strong color, high pH, high temperature, high chemical oxygen demand (COD), and low biodegradability (Joo *et al.*, 2007). During manufacturing 10-15% of the dyes used are discharged into the water, because percent of dye that fixated on the fabrics depends on the fiber, depth of shade and application method, thus, due to dye 2-50% of dyes may enter the waste stream (Wong and Yu, 1999, Wesenberg *et al.*, 2003, Chatzisyneon *et al.*, 2006). Furthermore, due to textile produced and usage of various chemically different dyes even during single dyeing process causes a variation in dyeing effluent compositions and a release of extremely diverse wastewater effluent (O'Neill *et al.*, 1999).

Color is the first contaminant to be distinguished in effluents; therefore even small amounts of unfixed dyes (10-50 mg/L) in effluents are visible and affect the aesthetics, water transparency and gas solubility of aquatic systems (Wong and Yu, 1999, Robinson *et al.*, 2001). In textile industry, washing, scouring, peroxide bleaching, dyeing and finishing processes are the major wastewater sources, and consequently, production of highly toxic wastewater has to be treated efficiently under required standards to protect marine environment and diminish the environmental damage (Robinson *et al.*, 2001, Selcuk, 2005). For color removal or degradation, wastewater treatment can be applied in two possible locations; one is at the dyehouse, where color can be removed and treated water stream can be partially or fully re-used, second is before discharging at the sewage works (Pearce *et al.*, 2003). In concern of the environmental protection, Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry (ETAD) has established in 1974 to reduce the environmental damage, protect users and consumers and to collaborate fully with governments and public concerns over the toxicological impact of their products (Robinson *et al.*, 2001).

1.6.1. Conventional wastewater treatments

Effluents from textile industry have to be treated efficiently. Known conventional treatments present today are chemical and physical methods, as coagulation-flocculation, adsorption, advanced oxidation processes (AOPs), electrochemical degradation and membrane filtration (Park *et al.*, 2006, Bali and Karagozoglu, 2007). All of the conventional treatment systems used for the decolorization and degradation of the organic compounds in dye wastewater have different decolorization capabilities, capital costs and operation speeds and mostly preferred systems are coagulation and adsorption (Park *et al.*, 2006, Jo *et al.*, 2007).

1.6.1.1. Coagulation/Flocculation

Coagulation/flocculation method has been employed as wastewater treatment method using various different coagulants and flocculants that eliminates the colloidal materials and total suspended particles for color and turbidity removal (Aguilar *et al.*, 2005, Amuda and Alade, 2006). Coagulation/flocculation method mainly employed as pre-treatment process at industrial effluent treatments, because suspended solids and organic materials are eliminated by added coagulants and flocculants, which causes floc formation that further treated by floatation, adsorption, settling or filtration (Amuda and Amoo, 2007). Coagulants are used to form small flocs from dispersed colloids, where as flocculants are used for agglomeration of these small flocs into larger settable particles (Shuler and Kargi, 1992). Coagulants destabilizes the colloidal particles neutralizes the power that keeps them separated in suspension and allows them to aggregate, however, the aggregates formed are loosely bounded and could not settle rapidly. Thus, to enhance floc formation and bounds between the aggregates flocculants are added which do not cause significant surface charge change (Brostow *et al.*, 2007). Commonly used coagulants are usually simple electrolytes like acids-bases, salts and multivalent ions such as ferric salts as ferrous sulfate, ferric chloride, ferric chloro-sulfate and aluminum sulfate (alum) (Shuler and Kargi, 1992, Tatsi *et al.*, 2003, Alves *et al.*, 2005). Besides, flocculants can be anionic, cationic and nonionic either natural or synthetic polymeric compounds, high molecular weight and water soluble organic polyelectrolytes, as cationized natural polysaccharides, anionic or modified polyacrylamide, polystyrene sulfate, polyethylene imine (Shuler and Kargi, 1992, Aboulhassan *et al.*, 2006, Brostow *et*

al., 2007). Important parameters during coagulation/flocculation treatments are chemical type, optimum dosage and settling rate (Sabah and Erkan, 2006).

1.6.1.2. Advanced oxidation processes (AOPs)

Advanced oxidation systems are chemical treatments in which hydroxyl radicals ($\text{OH}\cdot$) are generated by different reactants, because these radicals have high oxidizing capacity and able to degrade toxic compounds to CO_2 and H_2O (Andreozzi *et al.*, 1999, Banerjee *et al.*, 2007). Moreover, free radical $\text{HO}_2\cdot$ and its conjugate $\text{O}_2^{\cdot-}$ are generated and included in degradation processes, except these radicals are much less reactive than hydroxyl radicals (Chiron *et al.*, 2000). Hydroxyl radical generation oxidizes various organic chemicals in dye wastewater and oxidation further causes an aromatic ring cleavage in the dye structure and produces oxygenated compounds and low molecular weight acids as well (Robinson *et al.*, 2001, Yang and McGarrah, 2005). Advanced oxidation processes can be applied in different combinations with different hydroxyl radical generating reactants like ozone with ultraviolet radiation (O_3/UV), ozone with hydrogen peroxide ($\text{O}_3/\text{H}_2\text{O}_2$), ozone, hydrogen peroxide with ultraviolet radiation ($\text{O}_3/\text{H}_2\text{O}_2/\text{UV}$), hydrogen peroxide with ultraviolet radiation ($\text{H}_2\text{O}_2/\text{UV}$), ozone (O_3), Fenton ($\text{Fe(II)}/\text{H}_2\text{O}_2$), Fenton-like ($\text{Fe(III)}/\text{H}_2\text{O}_2$), photo-Fenton ($\text{Fe(III)}/\text{H}_2\text{O}_2/\text{UV}$) and photocatalysis ($\text{TiO}_2/\text{UV}/\text{O}_2$) (Andreozzi *et al.*, 1999, Alnaizy and Akgerman, 2000).

Fenton and Fenton-like processes are most extensively studied advanced oxidation systems and observed to be efficient in textile effluent decolorization and detoxification. Moreover, Fenton reactions and ozonation are even now the most basic and practical advanced oxidation processes for the treatment of industrial effluents (Banerjee *et al.*, 2007).

Fenton reactions involves the direct addition of H_2O_2 and Fe(II) salts mixture into the wastewater stream, in which catalytic decomposition of H_2O_2 produces hydroxyl radicals that further decomposes the dyestuffs in textile effluents and the iron(III) ions generated during the oxidation endorse the removal of other pollutants by coagulation and sedimentation (Faouzi *et al.*, 2006). The degradation rate of Fenton and Fenton like reagents can be strongly accelerated by irradiation, applying UV-VIS light at wavelength values higher than 300 nm (Andreozzi *et al.*, 1999). Besides, Fenton processes usually are applied at acidic pH between 2 and 5 and generally are

the preferred process for heavily polluted and highly colored industrial wastewaters that are resistant to biological treatments or are toxic to live biomass (Robinson *et al.*, 2001, Alaton and Teksoy, 2007).

Presently, ozonation has become one of the widely used advanced oxidation processes, because ozone by itself is a very powerful oxidant and in certain conditions it can decompose and lead to the formation of hydroxyl radicals wherein the process efficiencies are strongly increased. Ozonation method comprise three main steps during wastewater treatments that the generation of ozone, the dissolution of ozone in the wastewater and the oxidation of organic matter (Faouzi *et al.*, 2006). Free radicals formed by ozone decomposition can be further react non-selectively with organic compounds present in the effluent (Chiron *et al.*, 2000). High color removal and significant amount of COD reduction can be achieved with ozone application and applied dosage is dependent on the total color and COD level to be removed (Yang and McGarrah, 2005, Robinson *et al.*, 2001). Ozonation can be preferred for degradation of double-bonded dye molecules and one major advantage of the processes is that ozone can be applied in its gaseous state and thus does not increase the volume of wastewater (Robinson *et al.*, 2001). Furthermore, combined oxidative systems with ozonation like ozone with ultraviolet radiation (O_3/UV), ozone with hydrogen peroxide (O_3/H_2O_2) and ozone, hydrogen peroxide with ultraviolet radiation ($O_3/H_2O_2/UV$), are applied to enhance degradation ability of the ozonation systems and developed to achieve better decolorization and detoxification percentages of textile effluents (Andreozzi *et al.*, 1999, Chiron *et al.*, 2000).

1.6.1.3. Electrochemical treatments

Electrochemical treatment techniques have been developed in mid 1990s. Techniques involve electrochemical oxidation, electrochemical coagulation and electroflotation (Robinson *et al.*, 2001, Chatzisyneon *et al.*, 2006). Several electrochemical processes involve the direct oxidation of pollutants at anode surfaces and others involve production of active species to react with target pollutants. It has been shown that electrochemical oxidation and coagulation are more effective and shows higher efficiency in textile effluent decolorization (Yang and McGarrah, 2005). Yang and McGarrah state that electrochemical oxidation depends on the type of electrolyte, electrolyte concentration, reaction temperature and current

density (Yang and McGarrah, 2005). Electrodes are made up of various diverse materials as cast iron, aluminum, graphite, Pt, TiO₂, PbO₂, several Ti-based alloys and, more recently, boron-doped diamond electrodes (Chatzisyneon *et al.*, 2006, Yang, 2007). A typical electrochemical treatment usually uses iron anode and dc supply is connected to the electrode sheets submerged into the effluent that is going to be treated. When electric current is given, iron anode is oxidized to Fe(II) which is further oxidized to Fe(III) (Yang, 2007). Electrochemical oxidation is capable of destroying the chromophore groups of dyes found in textile effluents at short treatment times and low energy consumption (Chatzisyneon *et al.*, 2006). Another important electrochemical treatment is electrocoagulation in which aluminum and iron sheets have been used as anodes to generate coagulants that adsorb and remove organic dyes that coagulants are electrochemically generated as destabilization agents, which cause charge neutralization for removal of xenobiotics from effluents. An aluminum coagulant removes colorant by simple adsorption without involving chemical reactions while an iron-based coagulant provides Fe(II) ions to further degrade the dyes. In iron employed electrochemical coagulation method a direct current passes through the iron anodes and oxidized Fe(II) and Fe(III) ions dissolve and combine with hydroxyl ions in water, which form metal hydroxyls that are partly soluble in water under distinct pH values and acquire a very high adsorption capacity. Therefore, coagulated particles attract and adsorb different ions and colloidal particles from the wastewater effluents (Khoufi *et al.*, 2007). Besides, the iron anode based electrochemical coagulation seems more effective for removal of reactive dyes while the aluminum based is superior for removal of disperse dyes (Yang and McGarrah, 2005).

1.6.1.4. Adsorption

For years, adsorption method have been employed to concentrate and eliminate organic materials in the industrial effluents, moreover this application gained more interest lately since their efficiency in the treatment and removal of recalcitrant materials, too stable to treat with other methods, from industrial effluents (Robinson *et al.*, 2001, Yang and McGarrah, 2005). In textile effluents, color removal occurs by two mechanisms, which are adsorption and ion exchange, and physical and chemical factors in the environment effects the treatment as dye and adsorbent interaction, adsorbents surface area, particle size, temperature, pH and contact time.

Adsorption happens with weak interactions as van der Waals forces whereas ion exchange utilizes much stronger interactions as ionic bonds (Shuler and Kargi, 1992). However, ion exchange is not commonly used method in dye effluent treatment because of its poor modification of wide range of dyes, even though it is efficient in the removal of soluble dyes, removal of both cation and anion dyes, there is no loss in adsorbent during regeneration and recovery of solvent after use, besides effluent exceeds over ion exchange resin until the all available exchange sites are saturated (Robinson *et al.*, 2001).

Major used adsorbent in wastewater treatments is powdered or granular activated carbon (GAC) that is effective in treatment of pulp and paper effluents and most commonly used color removal method from textile effluents (Amokrane *et al.*, 1997, Yang and McGarrah, 2005). Activated carbon is a crude form of graphite, which has a highly random or amorphous porous structure that has pore sizes range from visible cracks and crevices to crevices at molecular dimensions (Mohan and Pittman Jr., 2007). Effectiveness of the method depends on the carbon used to manufacture activated carbon like coconut shells, wood char, lignin, petroleum coke, bone-char, peat, sawdust, carbon black, rice hulls, sugar, peach pits, fish, fertilizer waste and waste rubber tire and properties of the effluent. Activated carbon is effective in removing cationic, mordant, and acid dyes and to a slightly lesser effective in removing dispersed, direct, vat and reactive dyes (Robinson *et al.*, 2001, Mohan and Pittman Jr., 2007). Other industrially used adsorbents are peat, wood chips, fly ash and coal mixtures, silica gel and other materials as natural clay, corn cobs and rice hulls. Peat by its cellular structure and with its much larger surface area is a good candidate for adsorbing transition metals and polar organic compounds from dye effluents. Peat can be used for steam rising when it is burned or as a substrate in solid state fermentation (SSF) for protein enrichment after its application is over. Wood chips are good adsorbents for acid dyes in textile effluents, which are also burned after their treatment to generate power. Fly ash and coal mixtures with higher fly ash concentration have higher surface area which increases the rate of adsorption. Silica gel is effective at removing basic dyes from dye effluents. Other materials such as natural clay, corn cobs and rice hulls are preferred due to their availability and cheapness; therefore they are economically attractive for dye removal and instead of

regenerated after dye adsorption, they are further used as substrates in SSF for protein enrichment like peat (Robinson *et al.*, 2001).

1.6.1.5. Membrane Filtration

Membrane filtration systems are used for clarification, concentration and mostly for separation continuously from effluents. Other than other conventional methods, membrane filtration systems are resistant to temperature, adverse chemical environment and microbial attack as well (Robinson *et al.*, 2001). Membrane filtration types are reverse osmosis, nanofiltration, ultrafiltration and microfiltration (Amokrane *et al.*, 1997, Allegre *et al.*, 2004). Membrane processes are feasible due to decrease in water consumption rate by water recycling; however, filtration is suitable when the effluent contains low concentration of dyes for water recycling in textile industry (Robinson *et al.*, 2001, Kim *et al.*, 2004). All of these membrane processes are pressure driven systems applied for color removal in dye effluents. Microfiltration and ultrafiltration are low pressure systems used to separate suspended solids, colloids and macromolecules from effluents. When both microfiltration and ultrafiltration processes are insufficient in color removal and couldn't provide the discharge requirements of the effluents, nanofiltration can be applied as an alternative due to its partly controlled membrane performance by its ion exchange capacity of the skin layer (Mutlu *et al.*, 2002).

1.6.2. Disadvantages of conventional wastewater treatments

Conventional wastewater treatment methods are still employed to treat industrial effluents; yet, these methods have some drawbacks that cause their applications unfeasible in certain cases for industrial effluent treatments like high cost, formation of hazardous by-products and intensive energy requirements (Kim *et al.*, 2004). Moreover, these methods realize the removal of pollutants from effluents by separation and they merely transfer them from one phase to another and leaving a difficulty of disposal as in coagulation/flocculation and adsorption processes where is a huge amount of sludge formed that is toxic and has the problem of its own disposal (Park *et al.*, 2006, Bali and Karagozoglu, 2007). Besides, another problem is the diversity of the industrial effluents with their large concentration of organic and inorganic materials in which conventional processes become unsatisfactory in

industrial wastewater treatments and in dye decolorization (Chatzisyneon *et al.*, 2006).

Besides all the given common disadvantages of the conventional treatment methods, each unique process has its own negative aspects also in decolorization of textile effluents. In coagulation, optimum coagulant concentration addition is necessary due to static charge of the dye effluent which further causes a difficulty in removal of sludge formed and a high disposal costs (Robinson *et al.*, 2001). Another disposable problem causing treatment system is adsorption on an activated carbon, in which also regeneration costs and cost of the material itself disrupts the activated carbon application in textile effluent decolorization methods; even though the dye removal rate of activated carbon can be developed with using massive doses, regeneration causes a performance reduction, 10-15 % adsorbent loss and efficiency of the method becomes unpredictable (Robinson *et al.*, 2001, Yang and McGarrah, 2005). Furthermore, other adsorbents have also disadvantages, for instance due to its hardness wood chips don't have the same adsorption quality as other adsorbents and longer contact time is necessary (Robinson *et al.*, 2001). In oxidative processes addition to toxic sludge formation, also methods like ozonation can cause toxic byproducts which can not be further degraded and stays in the effluent (Selcuk, 2005). Furthermore, during ozonation considerable doses of ozone is needed for decolorization of dyes and for additional degradation of auxiliary chemicals in dye effluents to be achieved as well and it has very short half-life (20 min), which causes a high cost when continuous ozone addition is necessary due to its short life, which can be further shortened when dyes are present (Robinson *et al.*, 2001, Yang and McGarrah, 2005). In membrane separations the concentrated residue causes a clogging on the membrane; therefore membrane replacement is necessary, which affects the cost and further causes disposal problems of the concentrated residues, besides dissolved solid materials couldn't reduced and water can not be re used (Robinson *et al.*, 2001).

1.6.3. Biological treatments: Bioremediation

Governments become more and more severe about decolorization of dyes in industrial effluents. However, textile wastewater streams are the most challenging ones to treat among the other industrial effluents (Couto and Herrera, 2006, Park *et*

al., 2006). Therefore, an effective, financially viable and environmentally friendly treatment processes have developed for decolorization of textile effluents (Park *et al.*, 2006). Most recent treatment technologies are physical and chemical treatments that have various disadvantages involve high amount of chemical usage, sludge formation which causes further disposal problems, high price infrastructure constraints and high operation costs (Toh *et al.*, 2003). Thus, biological treatments have developed into one of the significant area of research in recent years for treatment of industrial effluents, especially textile effluents.

Bioremediation is the usage of microorganisms to degrade pollutants in industrial effluents and can be employed by two methods, in situ treatments and bioreactors. Aim is to provide non-toxic and decolorized effluents with acceptable levels of discharge into environment and is to completely mineralize recalcitrant pollutants to CO₂ or transform them to less toxic materials (Pointing *et al.*, 2001). Besides, Pointing *et al.*, stated the major requirements for successful bioremediation in their recent review as microorganisms that provide the catabolic activity must stay alive, organisms must be able to transform the compound at reasonable rates and concentration must meet the levels of regulatory standards, organisms must not produce toxic products, concentrations or combinations of chemicals that inhibitory to the biodegrading organism must be avoided, or methods must exist to dilute concentrations or otherwise render innocuous the inhibitors, the target compound(s) must be available to the microorganisms, conditions during bioremediation must be made favorable to microbial growth or activity, e.g. sufficient supply of inorganic nutrients and O₂ or some other electron acceptor, optimum moisture content and temperature, a source of carbon and energy for growth if the pollutant is co-metabolized and the cost of the technology applied must be less or no more expensive than that of other treatment technologies that can degrade the compounds as well (Pointing *et al.*, 2001).

In textile effluents, treatment of brightly colored and water soluble reactive and acid dyes are the most challenging since these dyes can pass through the effluents unaffected during conventional treatments. Besides, dyes with anthraquinone chromophore are resistant to degradation because of their fused aromatic ring structures (Robinson *et al.*, 2001). Various microorganisms, that are anaerobic and aerobic species, have been researched and applied in bioremediation processes.

Nonetheless, textile dyes are produced to resist microbial degradation, thus, only little dye degradation can be accomplished in activated sludge process even though these are mixed and treated together with sewage (Yesilada *et al.*, 2001). Anaerobic microorganisms and their application in bioremediation have been researched for years. Dye degrading anaerobic bacteria have been employed in decolorization processes due to its simplicity and efficient color removal compared to other conventional methods. Anaerobic color removal can be achieved by biodegradation of dyes using azoreductase or by non-enzymatic azo reduction of dyes (Kapdan and Alparslan, 2005). The azo bonds of the dyes can be utilized as electron acceptors in electron transport pathway by anaerobic bacteria, however, azo bond cleavage produces low molecular weight amines, which are colorless, toxic, carcinogenic and cannot be degraded further by anaerobic bacteria. Besides, textile effluents can be further composed of salts, sulfur compounds, heavy metals and other toxic compounds that often inhibit the anaerobic microbial population (Yesilada *et al.*, 2001, Yang and McGarrah, 2005). Moreover, dye degrading aerobic bacteria strains are tried to be integrated into bioremediation systems but resulted in necessity of a specific strain for degradation of specific dye (Wong and Yu, 1999). Therefore, new organisms were searched and isolated with dye degradation feature and in recent years studies has demonstrated the fungi that are capable of dye degradation (Yesilada *et al.*, 2001). Studies, especially on white-rot fungi, revealed that their nonspecific and nonstereoselective lignin degrading mechanism by peroxidases, LiP, MnP, and phenoloxidases, laccase, is also able to degrade a wide variety of recalcitrant compounds in industrial effluents and dyes from textile industry as well (Aust, 1995). Hence, white-rots are preferred for decolorization studies due to their diversity in oxidizing more compounds than other microorganisms applied in bioremediation and with this feature; white rots gain advantage over anaerobic and aerobic bacteria. Bacteria catabolism uses pollutants as nutrient source, carbon and/or nitrogen, however, fungi oxidizes their substrate with no net energy gain so addition of carbon and nitrogen source is necessary for white rot fungi. White rots are aerobic organisms but bacteria can live and degrade certain xenobiotics under aerobic and anaerobic conditions. Moreover, white rot fungi have an ability to tolerate higher concentrations of toxic compounds than bacteria (Pointing *et al.*, 2001, Toh *et al.*, 2003). Most of the studies on degradation of dyes have been performed with white rot fungi *Phanerochaete chrysosporium* and *Trametes*

versicolor (Yesilada *et al.*, 2001). Ligninolytic enzymes, especially laccase, of white-rot fungi oxidizes the azo linkages of the dyes producing quinones and enzymatic oxidation further releases nitrogen from these linkages as molecular nitrogen that precludes the formation of toxic amines (Gianfreda *et al.*, 1999). Even though significant numbers of dye decolorization studies have performed with white rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor*, there are other various white rot species that isolated and searched for their dye degradation abilities like *Pycnoporus sanguineus*, which decolorizes azo and triphenylmethane dyes producing only laccase as ligninolytic enzyme. *Bjerkandera adusta* and *Pleurotus eryngii* can degrade dyes by producing manganese peroxidase. Purified laccase isoenzymes from *Trametes hispida* are used in decolorization of various synthetic dyes (Pointing *et al.*, 2001). Nilsson *et al.*, 2006 studied dye decolorization features of four different white rot fungi strains, *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus* and *Pleurotus sajor-caju*, and further studied real textile water decolorization ability of other white rot fungi, *Pleurotus flabellatus* (Nilsson *et al.*, 2006). After the studies on white rot fungi and their enzymes have become popular for dye decolorization, several researches with newly isolated white rots were performed. Furthermore, literature is full of various white rot fungi decolorization studies done with several different kinds of dyes as decolorization of anthraquinone and azo-based commercial dyes with *Trametes versicolor*, *Pleurotus ostreatus*, *Funilia trogii*, *Sclerotium rolfsii*, *Phanerochaete chrysosporium*, *Aspergillus niger*, *Neurospora crassa* and *Chrysonilia crassa* (Park *et al.*, 2006), decolorization of industrial disperse, reactive and acid complex dyes with *Dichomitus squalens*, *Daedalea flavida*, *Irpex flavus* and *Polyporus sanguineus* (Chander and Arora, 2007), decolorization of azo, anthraquinone and polymethine dyes with *Aspergillus foetidus*, *Aspergillus niger*, *Bjerkandera adusta*, *Geotrichum candidum*, *Lentinus edodes*, *Phanerochaete chrysosporium*, *Pleurotus cornucopiae*, *Pleurotus eryngii*, *Pleurotus ostreatus*, *Pleurotus saca*, *Schizophyllum commune* and *Trametes versicolor* (Mohocic *et al.*, 2006), decolorization of Remazol Brilliant Blue R (RBBR) and Orange G with *Dichomitus squalens*, *Ischnoderma resinsum* and *Pleurotus calyptratus* (Eichlerova *et al.*, 2005) and other fungi as *Hirschioporus larincinus*, *Inonotus hispidus* and *Phlebia tremellosa* (Robinson *et al.*, 2001). To enhance the decolorization ability of the white-rots different application methods have been studied such as immobilization of white rots on different solid phase

matrixes, enzymatic treatments of effluents by directly or immobilized ligninolytic enzymes purified from various white rot fungi strain, application of living or dead cells, alteration in the cultivation conditions including definition of various nutritional, physiological and environmental conditions, addition of mediators to broaden the application spectrum of the enzymes on more chemically different synthetic dyes and further studies on different bioreactor designs for wastewater treatments of textile effluents including stirred tanks, packed beds, airlifts, bubble columns and rotating disks (Moreira *et al.*, 2003, Wesenberg *et al.*, 2003).

1.7. Applications for strain improvement

In present day wide usage of microorganisms in industrial applications are well established. Microorganisms as whole cells or useful metabolites like enzymes that are isolated from microbial cells can be utilized as sources of interested products in food industry, especially in fermentation processes, pharmaceuticals and wastewater treatments as well as in other various industries. However, number one consideration of the industry is to accomplish optimum productivity in optimum time and conditions. Therefore, methods to enhance productivity of producer organisms have been searched and developed due to demand of the industry for large-scale productions. With the establishment of the recombinant DNA technology in field of genetics for strain improvement triggers the improvement of additional novel techniques. Metabolic engineering is one of these novel strategies evolved during these researches. However, obstacles confronted in metabolic engineering applications supported the discovery and development of another strain improvement strategy, inverse metabolic engineering. Moreover, recently, an inverse metabolic engineering strategy, evolutionary engineering has been further introduced and employed in strain development studies.

1.7.1. Metabolic engineering

With the increasing demand of the industry improvement of more productive strains with novel techniques have been introduced. Thus, different fields in science are combined, besides progress in field of molecular biology and genetics with the development of the recombinant DNA technology catalyzed the strain development

studies and new strategies that introduced with these recent innovations are metabolic engineering.

Bailey in 1991 defined metabolic engineering as “the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology” (Bailey, 1991). Thus, Stephanopoulos and Alper (2004) further simply have emphasized that ultimate purpose of the metabolic engineering is the improvement of cellular properties using modern genetic tools and have pointed out advances in molecular biology and genetic engineering empowers metabolic engineers with the increasing ability to create any desired cellular modification (Stephanopoulos and Alper, 2004). Most significant point of this approach is the specificity in the modification of the targeted biochemical reactions or in integration of newly introduced one, moreover detection of these targeted areas are necessary for further application of improved techniques to amplify, inhibit or delete, transfer or deregulate the corresponding genes or enzymes among these areas (Stephanopoulos, 1999). To verify the metabolic engineering approaches searches are reviewed under categories on approaches taken or on the aim. Among these groupings Nielsen (2001) categorized the metabolic engineering applications in most apparent way and reviewed them in seven categories as, recombinant protein production, engineering of microbial substrate utilization, gaining of new biosynthetic and catabolic properties, altering cellular physiology to improve microbial processes, reduction in by-product formation and yield and productivity improvement (Nielsen, 2001). These alterations on microorganism require the same specific steps to be covered, first, synthesis of improved strain followed by the examination of the strain performance in the desired conditions and further optimization of the strain (Nielsen, 2001, Petri and Schmidt-Dannert, 2004). Metabolic engineering has enabled yield and productivity improvement of industrial strains, production of novel recombinant proteins, engineering of microbial substrate utilization, gaining of new biosynthetic and catabolic properties, altering cellular physiology to improve microbial processes and decrease in by-product formation. However, there are some drawbacks of metabolic engineering applications, which are mentioned clearly, such as identification of the rate-limiting step in the specified metabolic pathway as well as the catalyses involved, all the possible steps of the pathway, reaction kinetics, concentrations of

the intermediates and metabolites must also be considered for a fully designed experimental applications (Bailey *et al.*, 1996). Thus, improvement of complex cellular organisms is very challenging because of the mentioned shortcomings (Sonderegger and Sauer, 2003). In the concept of metabolic engineering more complex networks are considered to be solved or construction of new networks over original ones are studied under the constructive metabolic engineering definition with rational and deductive approach using genetic engineering principles but desired constructive alterations on the metabolic activity can not show the prospective characteristic by the modified strain (Bailey *et al.*, 1996). Hence, there are some obstacles in the application of metabolic engineering approaches because of drawbacks mentioned above. As a solution another approach, inverse metabolic engineering strategy, is evolved to overcome these drawbacks (Bailey *et al.*, 1996).

1.7.2. Inverse metabolic engineering

Inverse metabolic engineering (IME) defined by Bailey *et al.* in 1996 as “the elucidation of a metabolic engineering strategy by: first, identifying, constructing, or calculating a desired phenotype; second, determining the genetic or the particular environmental factors conferring that phenotype; and third, endowing that phenotype on another strain or organism by directed genetic or environmental manipulation” (Bailey *et al.*, 1996). Hence, Sauer and Schlattner simply, over the definition of IME, explain the fact that “the key concept of this area is identification of the molecular basis of a desired phenotype and its subsequent transfer to an appropriate host organism” (Sauer and Schlattner, 2004). The method works through first, creation of the desired phenotype by evolutionary mechanisms operating in nature or in the laboratory conditions. Afterwards, desired phenotype is examined in genetic basis to provide information for further metabolic engineering applications. The genes identified can be integrated into more suitable host or natural host can be used for intended industrial application (Gill, 2003).

1.7.3. An inverse metabolic engineering approach: Evolutionary engineering

Continuous evolution processes which are based on the use of an appropriate selection procedure towards a desired phenotype in continuously growing cultures, is called evolutionary engineering. Spontaneous or induced mutagenesis results in the segregation of the initial monoclonal population and fitter variants periodically

dominate in the culture. Such evolutionary techniques, in contrast to recombinant DNA technology, have a higher likelihood of public acceptance as a more ‘natural’ procedure (Sauer, 2001, Çakar *et al.*, 2005). Inverse metabolic engineering is dependent on identification and construction of desired phenotype followed by genetic studies of that phenotype or environmental factors influencing that phenotype and lastly transferring of the genes of desired phenotype into more suitable strain for its application prospects or desired phenotype can directly used in industrial applications. Evolutionary engineering has been developed under these approaches in the face of integrating natural evolutionary features into laboratory scale applications. Thus, researchers have been able to apply adaptation and natural selection design cycle of microorganisms for fitting to the environment by controlling all aspects of the genetic diversification and functional selection of biological systems in the laboratory (Sauer, 2001, Koffas and Cardayre, 2005).

1.7.3.1. Selection algorithm: Evolutionary engineering strategies

Under the evolutionary engineering studies, rational experimental procedure can be employed through mutagenesis for increasing genetic diversity, selection after applied mutations and natural evolution of the organism followed by another selection process of the desired phenotype. Finally, the screening of the desired phenotype with improved desired characteristic and further analysis of the genes responsible for desired phenotype (Sauer, 2001, Çakar *et al.*, 2005).

Using their genetic diversification mechanism, organisms can be capable of natural adaptation. Besides, mutagenesis and recombination can be employed in this manner to provide genetic diversification among cultured population as well as for speeding up the evolutionary process. Hence, there are various mutation treatments like chemical mutagenesis or radiation treatment, spontaneous mutagenesis that uses mutator strains, tagged mutagenesis and in vivo recombination. Moreover, mutations for evolutionary engineering studies should be rationally designed to create an efficient evolutionary process (Sauer, 2001). In evolutionary engineering studies most frequently used mutagenesis treatments are chemical mutagenesis or radiation treatment. These mutations can be simply employed on almost any organism and resulted in base transitions, frame-shifts or there can be deletions of considerable lengths. Frequently used mutagens are ethyl methane sulfonate (EMS), nitroso-

methyl guanidine (NTG) and UV. Among these mutagens cultures usually are mutagenized randomly by EMS to increase genetic variability through acting on GC-rich regions, mostly on G-rich regions (Sonderreger and Sauer, 2003, Çakar *et al.*, 2005). Important factor is to arrange the mutation frequency and do not affect the survival rate as well as avoid multiple mutations away from desired ones. Another factor is to increase the number of desired mutants over unwanted colonies in the mutated medium; therefore, optimum dosage is very important for desired mutation effect because under the optimum dosage genetic diversity won't be accomplished or when overdosed the effect can be lethal (Sauer, 2001).

Selection follows the preliminary genetic diversification of the population. Most important factor in selection is to provide the conditions of process where the desired phenotype is going to be used. Selection conditions can be designed due the characteristics of the process conditions. Under selection methods natural evolution, solid media, batch and chemostat selections can be employed during evolutionary engineering studies (Sauer, 2001, Şeker, 2003).

Within the natural evolution studies thousands of species can be studied in the laboratory environment. The rules of evolution are simple: species are evolved using random variations followed by natural selection that selects the fittest species and finally these fittest species survive, reproduce and propagate their genetic material to future generations (Şeker, 2003). Hence, the genetic material of the organism consists of knowledge for surviving in the current environment and has a potential to develop novel functions to survive under alternative environmental conditions. This enables the activation of their adaptation mechanisms during evolution such as the expression of cryptic genes that are assumed to play a role in natural evolution, which are phenotypically silent DNA sequences that enhances the adaptive potential of the organism (Sauer, 2001). Furthermore, Sauer has mentioned that the evolutionary adaptation of species to changing environments occurs in all but the simplest cultivation systems. Therefore, evolutionary adaptation process in the laboratory conditions has examined for along period and is known as periodic selection, which means the periodic appearance and subsequent exponential take-over of the population by variants with a selection advantage over the currently present cells (Sauer, 2001). These mutations are completely neutral, gain-of-function reversions for such phenotypes, occur at a constant rate that equals the mutation rate

and thus these phenotypes should increase in a population in a constant size. If the mutation is not neutral, then the population size will change at a rate that is function of population structure, population strength and direction of the selection (Şeker, 2003, Sauer, 2001).

Solid media selection is frequently used because it enables the visual inspection of growth as a zone around the colony resulted from a diffusing product or a color change due to coupled reactions of the large numbers of mutants. For this technique to be successful differences between the fitness ability of the populations must be large and the advantageous populations are rare. Moreover, mainly significant advantage of the technique is to provide direct examination on the progress of evolutionary adaptation. However, drawback is that it is an inefficient technique for selection of complex phenotypes that require multiple mutations. Besides, after selection most of the desired phenotypes are used in bioreactor for production where the conditions of bioreactor are not included during selection. Therefore, there can be a danger in selecting non-reproducible phenotypes in liquid media from solid media (Sauer, 2001).

In liquid media, fitter variants in a particular environment evolve over time and eventually replace the parental population as a consequence of adaptation by selection. This characteristic of the organism is often studied in batch cultures. An important feature of selection in batch culture is dramatic changes in environmental conditions from feast to famine, so that the cells are subjected to alternating periods of growth and stasis upon serial transfer (Sauer, 2001, Şeker, 2003). Throughout the batch culture growth conditions alter by the time passes and microorganisms go through lag, log and stationary growth phases. Therefore, we can be able to investigate at any stage different evolutionary adaptations. However, continuous cultures, mostly chemostat, provide constant culture conditions. During continuous cultures selective factor is the growth rate for determining the survival because removal of cells from the growth medium is due to random overflow. Fitness in continuous culture is determined by the ability to compete with all other variants that are present at a given time under the applied conditions (Sauer, 2001). Therefore, the variation on growth parameters and other environmental conditions can be studied in a well-defined continuous culture (Şeker, 2003).

Before going through further improvements, desired phenotypes from evolved populations must be screened because, after the evolutionary process has been completed, a heterogeneous population can be evolved and most suitable variant must be indicated from these populations (Sauer, 2001). Therefore, an efficient screening and characterization assay should be employed (Şeker, 2003). Various methodologies can be employed as flow cytometry and cell sorting, which are the frequently preferred for single cell level analysis, as well as near infrared and Fourier transform infrared spectroscopy for examination of small scale microbial cultures and sensitive pyrolysis mass spectroscopy (Sauer, 2001). However, most of the methods cannot function at colony level so variants have to be characterized by culturing of individual colonies. If a desired phenotype is defined by growth under certain conditions, which is based on improved tolerance to certain unfavorable process conditions, then survival becomes a statistical process. Therefore, survival ability of the variants have to be monitored by the number of colony forming units or by measuring the most probable number of viable cells, based on the potential of various dilutions of the culture (Sauer, 2001, Şeker, 2003). Furthermore, data from these characterization stages might be used for metabolic flux analysis for to estimate the rates of intracellular reactions. Therefore, integration of metabolic flux analysis through genomic studies and with the aid of computational modeling of cellular metabolism might provide better screening methods and facilitates the identification of the physiological features of the desired phenotype (Sauer, 2001).

Most of the trails in evolutionary engineering processes are for to increase the productivity of the organism through alterations in original metabolic networks or to provide multiple resistances within the organism against multiple stress conditions. Evolutionary engineering of *Saccharomyces Cerevisiae* has been studied widely due to its wide usage in food industry for beer, wine, alcohol, baker's yeast and heterologous protein productions. During industrial production processes, *S. cerevisiae* is exposed to many different physiological stresses such as freezing and thawing, high temperature stress, high ethanol concentrations, and oxidative stress, thus, to develop multi-stress resistant *S. cerevisiae* strains, evolutionary engineering approach is used to obtain yeast mutant populations with significantly improved multi-stress resistance (Çakar *et al.*, 2005). Another evolutionary engineering study done by Sonderegger and Sauer was the evolutionary engineering of *S. cerevisiae* for

anaerobic growth on xylose due to commercial interest on xylose utilization as a carbon source for ethanol production (Sonderegger and Sauer, 2003).

1.8. Aim of the study

The aim of this study was to design and apply suitable evolutionary engineering strategies to improve decolorization properties of a white rot fungus, *Coriolopsis polyzona*. Chemical mutagenesis was applied to the wild-type strain by using ethylmethane sulfonate (EMS) to increase the genetic diversity of the starting population, and initial mutant populations were obtained. Batch selection protocols were applied to these populations by choosing starvation stress as the main selection strategy. Biomass yields and decolorization properties were determined for mutant generations. The individual mutants of the final mutant populations were also analyzed by randomly selecting and separately plating single spores from the mutant cultures. The results of the mutant populations and individual mutants with respect to biomass yield and decolorization properties were compared and discussed in this study.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Fungal strain

White rot fungus *Coriolopsis polyzona* MUCL 38443 was kindly provided by Sophie Vanhulle, Université Catholique de Louvain, Belgium.

2.1.2. Fungus culture media

2.1.2.1. Composition of solid medium

Potato Dextrose Agar (PDA) composition

Potato Infusion (dehydrated)	4	G
Dextrose	20	G
Agar	15	G

per liter of distilled water.

2.1.2.2. Composition of liquid medium

Glucose monohydrate	55	G
Bacteriological peptone	17	G
KH ₂ PO ₄	2.5	G
MgSO ₄ .7H ₂ O	1.027	G
CuSO ₄ .5H ₂ O	31.27	Mg
MnSO ₄ .H ₂ O	55.90	Mg
Thiamine – HCl	10	Mg

per liter of distilled water.

2.1.3. Chemicals

4-aminobenzoic acid was purchased from Fluka (Switzerland).

Ammonium heptamolybdate tetrahydrate was obtained from Riedel-de Haen (Germany).

Ammonium sulfate was purchased from Riedel-de Haen (Germany).

L-asparagine monohydrate was obtained from Merck (Germany).

2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was purchased from Sigma –Aldrich (Germany).

Bacteriological peptone was obtained from Acumedia (USA).

D-biotin was purchased from Fluka (Switzerland).

Boric acid was obtained from Merck (Germany).

Calcium chloride dihydrate was purchased from Merck (Germany).

Citric acid was obtained from Riedel-de Haen (Germany).

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

Copper sulfate pentahydrate was obtained from Merck (Germany).

Diammonium tartarate was purchased from Sigma-Aldrich (Germany).

2,6-dimethoxyphenol (DMP) was obtained from Fluka (Switzerland).

Disodium phosphate heptahydrate was purchased from Merck (Germany).

Disodium tartarate was obtained from Merck (Germany).

Ethyl methane sulfonate (EMS) was purchased from Sigma – Aldrich (Germany).

Folic acid was obtained from Fluka (Switzerland).

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

Glycerol was obtained from Carlo Erba (Italy).

Glycine was purchased from Merck (Germany).

Hydrogen peroxide was obtained from Merck (Germany).

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

Iron sulfate heptahydrate was obtained from Riedel-de Haen (Germany).

Magnesium chloride hexahydrate was purchased from Carlos Erba (Italy).

Magnesium sulfate heptahydrate was obtained from Riedel-de Haen (Germany).

Malt extract was purchased from Merck (Germany).

Maltose was obtained from Merck (Germany).

Manganese chloride tetrahydrate was purchased from Merck (Germany).

Manganese sulfate monohydrate was obtained from Merck (Germany).

Monosodium phosphate monohydrate was purchased from Merck (Germany).

Niacinamide was obtained from Fluka (Switzerland).

Nickel chloride hexahydrate was purchased from Lachema (Czech Republic).

D-pantothenic acid hemicalcium salt was from Fluka (Switzerland).

PDA was purchased from Acumedia (USA).

Potassium dihydrogen phosphate was obtained from Fluka (Switzerland).
 Di-potassium hydrogen phosphate was purchased from Merck (Germany).
 Riboflavin was obtained from Fluka (Switzerland).
 Rice used at spore formation was Reis (Turkey) or Sezon (Turkey) brand.
 Reactive Red 195 textile dye was kindly provided by Setaş Kimya AŞ. (Turkey).
 Remazol Black 5 was purchased from Dystar Textilefarben GmbH&Co. (Germany).
 Remazol Brilliant Blue was obtained from Dystar Textilefarben GmbH&Co. (Germany).
 Remazol Turquoise was purchased from Dystar Textilefarben GmbH&Co. (Germany).
 Sodium chloride was obtained from Riedel-de Haen (Germany).
 Tri-sodium citrate dihydrate was purchased from Riedel-de Haen (Germany).
 Sodium hydroxide was purchased from Riedel-de Haen (Germany).
 Sodium molybdate dihydrate was obtained from Merck (Germany).
 Sodium tartarate dihydrate was purchased from Panreac (Spain).
 Sodium thiosulfate was obtained from Merck (Germany).
 Soytone peptone was purchased from DIFCO (USA).
 Tartaric acid was obtained from Riedel-de Haen (Germany).
 Thiamine-HCl was purchased from Fluka (Switzerland).
 Tween 80 was obtained from Merck (Germany).
 Veratryl alcohol was purchased from Fluka (Switzerland).
 Yeast extract was obtained from Lab M (UK).
 Zinc chloride was purchased from Merck (Germany).
 Zinc sulfate heptahydrate was obtained from Riedel-de Haen (Germany).

2.1.4. Buffers and Solutions

ABTS in tartaric acid buffer	2.5 mM
2,6-dimethoxyphenol (DMP)	25 mM
Dye stock solutions (pH>12 in distilled water)	% 0.05 (w/v)
Glycerol	% 60 (v/v)
Glycine-HCl buffer (pH 3 – adjust with HCl)	50 mM
H ₂ O ₂	10 mM
Manganese sulfate	5.0 mM

Phosphate buffer (pH 7)	0.1 M
Sodium chloride	% 0.09 (w/v)
Sodium tartarate buffer (pH 5 - adjust with NaOH)	50 mM
Sodium thiosulfate	% 10 (w/v)
Tartaric acid buffer (pH 3 – adjust with NaOH)	0.1 M
Tween 80	% 0.1 (v/v)
Veratryl alcohol	500 µl

2.1.5. Laboratory Equipment

Autoclaves	Nüve OT 4060 Steam Sterilizer (Turkey) Tuttnauer 2549 ML (Switzerland) Tuttnauer 3870 ELVC (Switzerland)
Balances	Precisa XB620C (Germany) Precisa XB220A (Germany)
Blender	Waring (USA)
Centrifuge	Beckman Coulter Avanti J-30 I (USA)
Deep Freezers	- 20 °C Arçelik (Turkey) - 80 °C New Brunswick Scientific U410 Premium (England)
Incubator	Memmert UM400 (Germany) Nüve EN400 (Turkey)
Laminar flow	Faster BH-EN2003 (Italy)
Magnetic stirrers	Labworld (Germany) Velp Scientifica (Italy)
Microfuge	Beckman Coulter (USA)
Micropipettes	Eppendorf AG (Germany), 5000µl, 1000µl, 200µl, 100µl, 10µl
Orbital Shaker incubators	Shell lab 1575R-2E (USA) Thermo Electron Corporation (USA)
pH-meter	Mettler Toledo MP220 (Switzerland)
Refrigerator	+ 4 °C Arçelik (Turkey)
Rotor	Beckman JA-30.50 Ti – 30 000rpm (USA)

Ultrasonic Disintegrator	Soniprep 150 (UK)
UV-Visible Spectrophotometers	PerkinElmer Lambda 25 (USA) Shimadzu UV-Pharmaspec 1700 (Japan)
Vortex	Heidolph Reax top (Germany)

2.2. Methods

2.2.1. Cultivations on solid media

The white rot fungus *Coriolopsis Polyzona* was arrived from Université Catholique de Louvain, Belgium on a slant agar. *C. polyzona* was planted on PDA plates with wood chips on the plates and incubated at 28°C for 4-5 days. Fully-grown *C. polyzona* on wood chips, were transferred from PDA to screw top tubes to be stored at –80°C New Brunswick Scientific U410 Premium (England). *C. polyzona* stocks stored at –80°C were grown on PDA plates for 4-5 days at 28°C before spore formation, consistently.

2.2.2. Spore formation and harvesting

C. polyzona was grown on PDA plates for 5 days at 28°C. Agar pieces cut from the growing edge of the mycelium were used to inoculate the organism on rice at 28°C for 10 days. The spores were then harvested aseptically by extraction with 0.9% NaCl and filtered through six layers of cheesecloth. The spore suspensions were washed with 0.9 % NaCl until the color of the supernatants were removed.

2.2.2.1. Spore formation

A Buchner funnel covered with doubled layer cheesecloth was filled with rice. Rice was washed with distilled water for approximately 30 min. until it swelled and whitened. Rice was stirred during entire washing procedure. Approximately 140-150 grams rice was weighed and spread equally into bottom of Fernbach flasks, sealed and autoclaved for 15 min at Nüve OT 4060 Steam Sterilizer (Turkey). After sterilization, flasks were cooled to ambient temperature before inoculated with *C. polyzona* grown agar pieces removed from the growing edge of the mycelium and incubated at 28 °C for 8 days. Humidity was kept high in order to maintain rice moist for spore formation during incubation. Therefore, 5 ml sterile distilled water was added when condensation on the flasks were not inspected so as to maintain the

humidity. At the end of the incubation, the entire rice surface was covered with *C. polyzona* spores.

2.2.2.2. Spore harvesting

Initially, harvest equipment, 0.9% NaCl solution, Buchner funnels covered with six layers of cheesecloth, 500 ml empty flasks and glass pipettes, were prepared. Before harvesting procedure, entire prepared equipment were covered with foil and autoclaved for 15 min at Nüve OT 4060 Steam Sterilizer (Turkey). 0.9% NaCl solutions cooled to room temperature were added into rice flasks to harvest *C. polyzona* spores. The rice flasks were shaken first to break up the packed rice into smaller pieces and then putted into Thermo Electron Corporation (USA) orbital shaker incubator for 30 min. at 100-150 rpm. Subsequently, the spores were harvested by filtering through Buchner funnels covered with six layers of cheesecloth into sterilized 500 ml flasks and filled with 0.9% NaCl solution until the 500 ml line on the flasks. Flasks were placed at +4°C.

2.2.2.3. Spore washing and stocking

The spore suspensions were washed with 0.9 % NaCl daily until the color of the supernatants were removed. The spores were settled overnight to the bottom of the flasks. Supernatants were poured carefully, replaced with fresh 0.9 % NaCl solution and shaken gently to raise the settled spores. Once the supernatants were cleared, after removing the supernatant above the settled spores, spore suspensions were poured into sterilized 50 ml falcon tubes to store at +4°C. The spore suspensions were refreshed for shake flask cultivations every month.

2.2.3. Medium selection studies of *C. polyzona*

Medium selection studies of *C. polyzona* were performed with nine different media compositions. Compositions of all nine media used during suitable medium selection for *C. polyzona* were:

- Medium #1 20 g maltose, 1.84 g diammonium tartarate, 2.3 g disodium tartarate, 1.33 g KH_2PO_4 , 0.1 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.046 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.035 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.007 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.0 g yeast extract and vitamin solution (D-biotin 2 mg, D-pantothenic acid hemicalcium salt 0.2 mg, folic acid 0.2 mg, niacinamide 40 mg, thiamine-HCl 40 mg, *p*-aminobenzoic acid 20 mg and riboflavin 20 mg) per liter of distilled water.
- Medium #2 10 g glucose, 3.0 g L-asparagine monohydrate, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g KH_2PO_4 , 0.6 g K_2HPO_4 , 0.4 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.09 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.07 mg H_3BO_3 , 0.02 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1.0 mg FeCl_3 , 3.5 mg ZnCl_2 , 0.1 mg Thiamine - HCl and 5.0 μg Biotin per liter of distilled water.
- Medium #3 55 g glucose monohydrate, 17 g bacteriological peptone, 2.5 g K_2HPO_4 , 1.027 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 31.27 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 55.90 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and 10 mg Thiamine - HCl per liter of distilled water.
- Medium #4 15 g malt extract, 4.0 g yeast extract and 4.0 g glucose per liter of distilled water.
- Medium #5 20 g malt extract, 1.0 g peptone and 15 g glucose per liter of distilled water.
- Medium #6 10 g glucose, 10 g soytone peptone, 1 ml trace metals solution, 0.2 mM MnSO_4 per liter of distilled water and pH adjust with acetic acid at 5.0 [Trace metals solution: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 20mM, $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$ 40mM, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1mM, ZnCl_2 5mM, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 20mM, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 50mM, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 5mM, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1mM, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ 0.5mM (pH 2 - adjust with HCl)].
- Medium #7 10 g yeast extract, 0.25 g citric acid, 5.0 g $(\text{NH}_4)_2\text{SO}_4$, 5.0 g K_2HPO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.02 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ per liter of distilled water.
- Medium #8 200 g Boiled potato, 20 g dextrose (glucose) per liter of distilled water.
- Medium #9 15 g malt extract, 4.0 g yeast extract and 40 g glucose per liter of distilled water.

All the above nine media were examined for ligninolytic enzyme, laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP), activities. Enzyme activities were measured in order to select most suitable medium for shake flask cultivations of *C. polyzona*. In all media trials, *C. polyzona* spore stocks (10^8 spores/ml) were used to inoculate 100 ml medium in 500 ml shake flasks. The fungal cultures were grown in Thermo Electron Corporation (USA) Orbital shaker incubator at 150 rpm and 28°C in. The experiment was carried out triplicate.

2.2.4. Enzyme assays

Enzyme activities were measured from 300 µl sample aliquots withdrawn daily from the liquid cultures and centrifuged at 12500 rpm for 30 min at Beckman Coulter Microfuge (USA) before enzyme assays since the clear supernatants were analyzed for ligninolytic enzyme activity. Enzyme activities were monitored on PerkinElmer Lambda 25 UV-Vis Spectrophotometer (USA) using PerkinElmer UV KinLab version 2.85.00 (USA) kinetic assay. Spectrophotometer was warmed up approximately for 30 min before enzyme activity measurements.

2.2.4.1. Determination of laccase activity

Assay applied for determination of laccase activity was optimized by Prof. Dr. Tajalli Keshavarz's group, University of Westminster, due to standardized assay applied previously (Eggert *et al.*, 1996).

Tartaric acid buffer (0.1 M, pH adjusted to 3 using NaOH) and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) solutions (ABTS) (2.5 mM) in tartaric acid buffer were used in laccase activity determination assay and tartaric acid was further used as blank solution during measurements. 50 µl from centrifuged enzyme samples were added into quartz cuvette filled with 950 µl from buffer and 200 µl from ABTS, loaded into spectrophotometer and measured at 414 nm for 3 minutes of reaction time at 28 °C. Due to the oxidation of ABTS by laccase green color produced in the cuvette was detected at 414 nm.

The activity of laccase was measured from slope of the spectrogram formed during 3 minutes reading of sample absorbance using PerkinElmer UV KinLab version 2.85.00 (USA) kinetic assay. Laccase activity was calculated from the slope of the spectrogram between 120-180 seconds that was given directly by the kinetic assay.

When needed, enzyme samples were diluted during assays. Equation 2.1 was used to calculate the laccase activity in which ΔA was absorbance change at 414 nm, ε was extinction coefficient of ABTS at 414 nm ($36000 \text{ M}^{-1}.\text{cm}^{-1}$), d was light path of the cuvette container cell (cm), V_t was total reaction volume (1200 μl), V_E was enzyme volume (50 μl) and di was dilution rate.

$$\text{Laccase (U/l)} = \left(\frac{\Delta A/t}{\varepsilon.d} \right) \cdot (1 \times 10^6 \text{ } \mu\text{mol/mol}) \cdot (V_t/V_E).di \quad (2.1)$$

2.2.4.2. Determination of lignin peroxidase activity

LiP activity was measured by monitoring the oxidation of 500 μM veratryl alcohol in 50 mM glycine-HCl buffer at pH 3.0 adjust with HCl. Glycine-HCl buffer was used as blank in spectrophotometer measurements. During LiP activity determination assay, 500 μl veratryl alcohol and 390 μl glycine-HCl buffer were added initially into quartz cuvette with 100 μl centrifuged enzyme sample. Finally, 10 μl 10 mM H_2O_2 was added to total 1000 μl final volume in cuvette, loaded into spectrophotometer and measured at 310 nm for 2 minutes of reaction time at 28°C . Equation 2.1 was used to calculate LiP activity in which ΔA was absorbance change at 310 nm, ε was extinction coefficient of veratryl alcohol at 310 nm ($9.3 \text{ mM}^{-1}.\text{cm}^{-1}$), d was light path of the cuvette container cell (cm), V_t was total reaction volume (1000 μl), V_E was enzyme volume (100 μl) and di was dilution rate (Kirk and Tien, 1983).

2.2.4.3. Determination of manganese peroxidase activity

MnP activity was measured by oxidation of 25 mM 2,6-dimethoxyphenol (DMP) in 50mM sodium tartrate buffer at pH 5.0 adjust with NaOH. Sodium tartrate buffer was used as blank in spectrophotometer measurements. In MnP activity determination assay, 100 μl 2,6-dimethoxyphenol and 690 μl sodium tartrate buffer were added initially into quartz cuvette with 100 μl centrifuged enzyme sample. Lastly, 10 μl 10 mM H_2O_2 was added for initiation of enzyme reaction to total 1000 μl final volume in cuvette, loaded into spectrophotometer and measured at 469 nm and 30°C for 2 minutes of reaction time. Equation 2.1 was used to calculate MnP activity in which ΔA was absorbance change at 469 nm, ε was extinction coefficient of DMP at 469 nm ($49.6 \text{ mM}^{-1}.\text{cm}^{-1}$), d was light path of the cuvette container cell

(cm), V_t was total reaction volume (1000 μ l), V_E was enzyme volume (100 μ l) and di was dilution rate (Wariishi *et al.*, 1992).

2.2.5. Determination of cell dry weight

Membrane filters were dried at 80°C overnight in Memmert UM400 (Germany) incubator, cooled in a desiccator and weighed (W_1). Cultures filtered through pre-weighed membrane filters fitted in Buchner funnel placed on Nuche flask connected to vacuum. Filters were dried at 80°C overnight in incubator and cooled for 30 min in desiccator before second weighing (W_2). The cell dry weight (CDW) of the cells was determined by taking the difference between the two measurements multiplied by 10 to give biomass in g/l.

$$CDW = (W_2 - W_1) * 10 \quad (2.2)$$

2.2.6. Shake flask cultivations

The culture medium chosen for *C. polyzona* shake flask cultivations was LSK-27 medium composed of glucose monohydrate 55 g, bacteriological peptone 17 g, HK_2PO_4 2.5 g, $MgSO_4 \cdot 7H_2O$ 1.027 g, $CuSO_4 \cdot 5H_2O$ 31.27 mg, $MnSO_4 \cdot H_2O$ 55.90 mg and Thiamine – HCl 10 mg per liter of distilled water. Medium was prepared and heat sterilized for 15 min in an autoclave (Tuttnauer 2549 ML, Switzerland). 1 ml of Thiamine-HCl was added into each 100 ml medium after the sterilization by filter sterilization using 0.45 μ m filter. In every shake flask cultivation, 10^8 (spores/ml) spores concentration was inoculated into 100 ml liquid medium in 500 ml Erlenmeyer flasks. Flasks were incubated at 150 rpm and 28°C in orbital shaker incubator.

2.2.7. Sub-culturing

10^8 (spores/ml) *C. polyzona* spores concentration inoculated into 100 ml liquid medium in 500 ml flasks were incubated at 150 rpm and 28°C in orbital shaker incubator. At the 8th day of cultivation grown liquid cultures were taken for sub-culturing. *C. polyzona* cultures were blended using Waring (USA) blender and 1 ml of each blended culture was inoculated into 100 ml freshly prepared and sterilized culture medium. Sub-cultured *C. polyzona* cultures were incubated at 150 rpm and 28°C in orbital shaker incubator.

2.2.8. Selection algorithm

To improve the decolorization characteristics of *C. polyzona*, suitable evolutionary engineering strategies were applied due to designed selection algorithm (Figure 2.1).

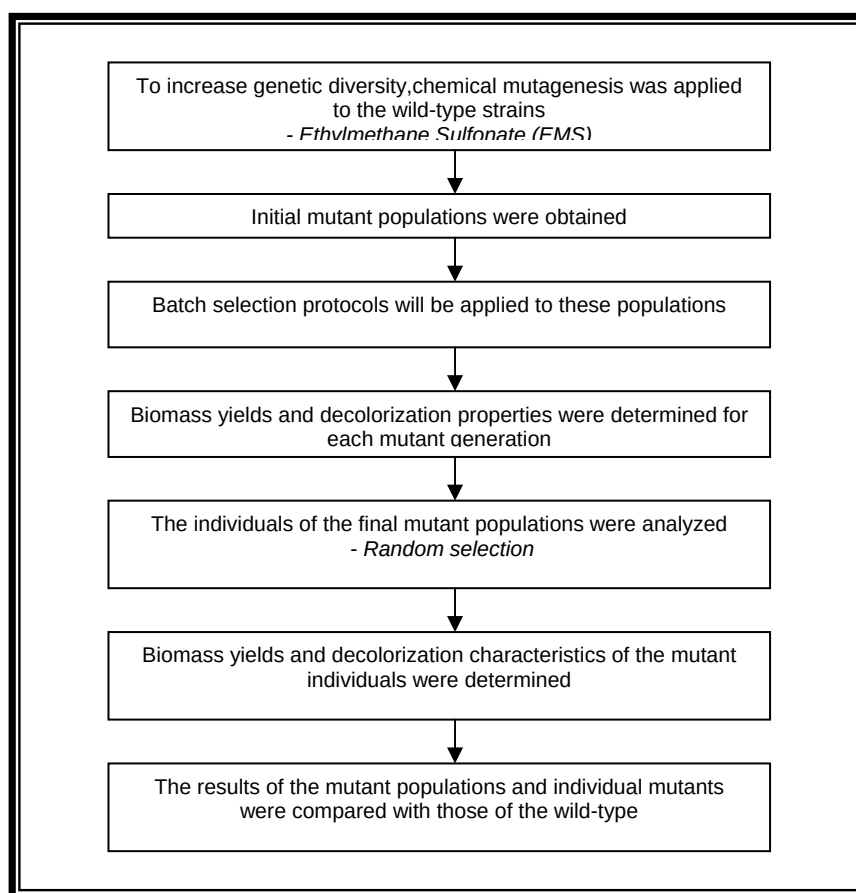


Figure 2.1 Selection algorithm designed for *C. polyzona*

2.2.9. EMS mutagenesis

EMS was applied to increase genetic diversity of the fungal population. Different EMS concentrations were applied for different time intervals given at Table 2.1.

Table 2.1 EMS amounts and applied intervals on *C. polyzona*

EMS amount (μl)	Time (min)		
300	5	10	15
150	15	30	-
100	15	-	-

EMS was applied to 15 ml spore suspension of *C. polyzona* according to procedure shown schematically at Figure 2.2.

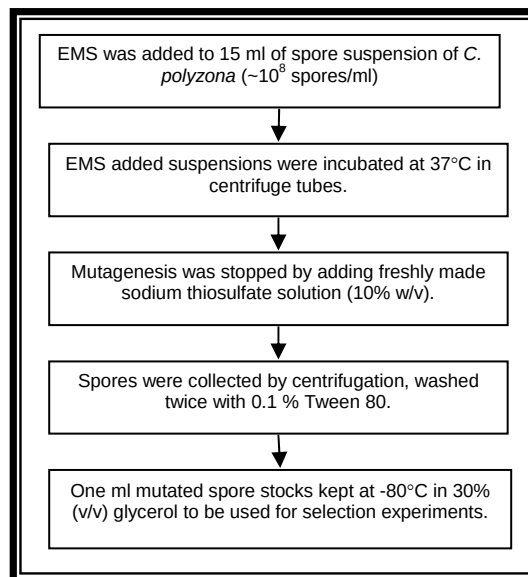


Figure 2.2 EMS mutagenesis method

2.2.10. Starvation stress

Various selective conditions can be applied during evolutionary engineering studies on the fungus to select for mutant generations with desirable characteristics. The selective condition chosen for applying to *C. polyzona* culture was starvation stress, which was based on carbon and nitrogen limitation in culture medium.

Firstly, a preliminary starvation study had been done with different concentrations of carbon and nitrogen in culture medium as shown in Table 2.2. Eleven different culture media inoculated with *C. polyzona* spore stocks (10⁸ spores/ml) into 100 ml media in 500 ml shake flasks were observed for 19 days of culturing at 150 rpm and 28°C.

From Culture #2 to #5 nitrogen limitation was applied by altering bacteriologic peptone concentrations. From Culture #6 to #11 carbon limitation was applied by altering glucose concentrations. Culture #1 was the actual medium used to inoculate *C. polyzona*. Laccase activities had been measured to compare effect of different media compositions and effect of starvation.

Table 2.2 Media compositions applied during preliminary starvation study

	Glucose (g/L)	Bactopeptone (g/L)	HK ₂ PO ₄ (g/L)	MgSO ₄ (g/L)	CuSO ₄ (mg/L)	MnSO ₄ (mg/L)	Thiamine–HCl (mg/L)
Culture #1	50	17	2.5	0.5	20	50	10
Culture #2	50	13	2.5	0.5	20	50	10
Culture #3	50	9	2.5	0.5	20	50	10
Culture #4	50	5	2.5	0.5	20	50	10
Culture #5	50	1	2.5	0.5	20	50	10
Culture #6	40	17	2.5	0.5	20	50	10
Culture #7	30	17	2.5	0.5	20	50	10
Culture #8	20	17	2.5	0.5	20	50	10
Culture #9	10	17	2.5	0.5	20	50	10
Culture #10	5	17	2.5	0.5	20	50	10
Culture #11	1	17	2.5	0.5	20	50	10

After this preliminary study a second trial was performed with different carbon and nitrogen concentrations in the media based on the results from the first trial to choose final starvation stress conditions for obtaining mutant generations. Again, laccase activities of each culture were measured every day for comparison. Culture conditions studied during second trial are given in Table 2.3.

Table 2.3 Media compositions studied during second starvation study

	Glucose (g/L)	Bactopeptone (g/L)	HK ₂ PO ₄ (g/L)	MgSO ₄ (g/L)	CuSO ₄ (mg/L)	MnSO ₄ (mg/L)	Thiamine–HCl (mg/L)
Control	50	17	2.5	0.5	20	50	10
Culture #1	50	9	2.5	0.5	20	50	10
Culture #2	40	9	2.5	0.5	20	50	10
Culture #3	40	17	2.5	0.5	20	50	10

Among these different media compositions studied during starvation experiments, the optimum one (40g Glucose/17 g bactopeptone per L of distilled water) with the highest laccase activity was chosen for studying in evolutionary engineering studies to obtain mutant generations.

2.2.11. Obtaining *C. polyzona* mutant generations

Under starvation stress condition *C. polyzona* generations were obtained from EMS mutagenized wild type organism by sub-culturing method. EMS mutagenized *C. polyzona* spore stocks (10⁸ spores/ml) were used to inoculate 100 ml starvation conditions applied medium in 500 ml shake flasks. The fungal cultures were grown in a rotary shaker (150 rpm) at 28°C. At the 8th day of culturing grown liquid cultures were taken to use in sub-culturing. Cultures were blended and 1 ml of each culture was inoculated to 100ml fresh medium each time. Every new sub-culturing referred to next generation obtained from the previous sub-culturing.

2.2.12. Selection of mutant individuals from stress applied mutant populations

Spore stocks of each mutant generation were obtained due to method explained in section 2.2.2. From this stress applied mutant populations mutant individuals were randomly selected by taking 10 μ l spore from spore number counted stock and diluted until obtaining 10spores/100 μ l with 90 μ l distilled water in 1,5ml microfuge tubes. From this diluted spore suspension, 10 μ l portions were inoculated on PDA plates and incubated at 28°C. From mycelium grown on PDA plates were cut to inoculate on rice for spore formation. Spores obtained from each harvest referred to mutant individuals obtained from each mutant population.

2.2.13. Decolorization studies

Decolorization effect of wild type *C. polyzona* were performed by using four different dyes, Remazol Brilliant Blue, Remazol Schwarz RL (Black 5), Reactive Red 195 and Remazol Turquoise. Decolorization effect of that mutant generations and mutant individuals were performed with Remazol Brilliant Blue and Black 5 dyes.

Stock solutions of each dye were prepared by adding to sterilized distilled water at pH>12 and concentration of 5g/L. Dyes were dissolved in the distilled water and constantly stirred at 80°C for overnight. One ml of dye was added to 100 ml liquid cultures from stock solutions to provide 50mg/L final concentration. Experiment set was planned as shown in Table 2.4.

Table 2.4 Decolorization experiment set

Name of the dye added	Definition	Number of replicate flasks
None (control)	Culture media+m/o	3
Remazol Schwarz RL (Black 5)	Abiotic control (culture media+dye)	3
	Culture media+dye+m/o	3
Remazol Brilliant Blue	Abiotic control (culture media+dye)	3
	Culture media+dye+m/o	3
Remazol Turquoise	Abiotic control (culture media+dye)	3
	Culture media+dye+m/o	3
Reactive Red 195	Abiotic control (culture media+dye)	3
	Culture media+dye+m/o	3

For every decolorization experiment with different *C. polyzona* cultures and dyes used, control flasks and abiotic controls of dyes were included in experiment sets and examined.

Every day, including day 0, one ml samples from each flask were withdrawn to measure absorbance values of dyes at 569nm for Black 5, 615nm for Remazol Brilliant Blue, 620nm for Remazol Turquoise and 521nm for Reactive Red 195. Wavelengths specific to dyes were determined from peaks formed by scanning dyes at spectrophotometer liquid medium used as blank. Before measuring the ABS values, samples were centrifuged for 30 min at 14000 rpm and samples withdrawn for laccase activity measurements were centrifuged at 12500 rpm for 30 min.

The decolorization effect was calculated according to Formula 2.3, in which A is the absorbance of residual dye and A_0 is the initial absorbance of the dye.

$$\text{Decolorization (\%)} = [(A_0 - A) \times 100] / A_0 \quad (2.3)$$

2.2.14 Cell Disruption

Wild type, mutant generation and selected mutant individual *C. polyzona* triplicate cultures were grown in RBBR added liquid medium for cell disruption at 5th and 7th day of cultivation to measure intracellular laccase activity during decolorization study. At 5th day of cultivation single flasks from triplicate cultures were selected, transferred to 50ml falcon tubes and centrifuged at 5000rpm for 30 min. Supernatants were poured and 10 ml of 0.1M phosphate buffer at pH 7.0 was added on to pellets. Cultures were vortexed and 1ml samples from buffered cultures were withdrawn for laccase activity determination. Cultures were stored at -20°C. The same procedure was applied for remained duplicate flasks at the end of decolorization experiment, day 7 of cultivation.

Cultures were disrupted with Soniprep 150 ultrasonic disintegrator (UK) for 2 min. at 10μm amplitude (Eriksen *et al.*, 1998, Cowley and Walters, 2002, Molloy, 2004) and 1ml samples were withdrawn for laccase activity measurements.

3. RESULTS

3.1. Medium selection for shake flask cultivations of *C. polyzona*

To choose the most suitable medium for *C. polyzona* liquid cultivations nine different media with different carbon, nitrogen sources and concentrations were tested. Media were included various other supplements such as trace metal and vitamin solutions as well. In nine different media, glucose, maltose, malt extract and potato were added as different glucose sources. Different nitrogen sources supplemented in media were yeast extract, bacteriological peptone and soytone peptone. Sample aliquots from every culture media were withdrawn daily for LiP, MnP and laccase activity analysis. No LiP activities were detected in any culture media. Negligible MnP activities were measured in media #1, #4 and #6 at days 3, 6 and 7, respectively. Significant laccase production by *C. polyzona* was examined in media #1, #3 and #6 (Table 3.1).

Table3.1 Laccase activity results from media #1, #3 and #6

Laccase activity (U/l)	Medium #1			Medium #3			Medium #6		
	1	2	3	1	2	3	1	2	3
0 th day	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1 st day	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2 nd day	13.93	2.27	3.40	0.00	0.00	0.00	4.80	5.40	5.67
3 rd day	299.20	88.53	77.40	0.00	0.00	0.00	25.60	60.33	10.87
4 th day	1670.68	475.67	387.67	4.20	3.40	3.80	130.00	174.93	83.60
5 th day	1516.01	2536.01	2420.68	16.00	12.80	8.87	310.00	178.00	400.67
6 th day	90.00	417.34	1214.67	16.27	13.13	19.60	89.33	318.67	453.34
7 th day	390.34	723.34	858.00	157.67	105.33	71.33	618.34	805.67	484.67
8 th day	225.33	496.00	296.00	3000.02	6496.70	1983.34	642.67	669.34	781.34
9 th day	521.00	710.34	454.34	11480.06	24213.45	9186.71	397.34	326.67	610.67
10 th day	609.00	799.34	392.00	46533.57	67867.01	34453.51	624.67	574.00	720.00
11 th day	760.34	1037.34	539.00	51680.26	136427.35	70373.69	565.34	547.34	731.00

In media #2, #4 and #5, the measured laccase activity varied between 3.00 and 12.67 U/l at cultivation days 3 to 6. In medium #8, laccase activity was varying between 5.33 and 56.00 U/l at cultivation days 2 to 6. In media #7 and #9, laccase production began later than other cultures, at the 4th day of the cultivation and activity was between 1.87 and 74.93 U/l at days 4 to 8 and 4 to 9, respectively. Laccase

production was examined in these media only at the stated cultivation days, before and after the days activity was 0 U/l.

In media #1, #3 and #6, laccase production was first detected at 2nd, 4th and 2nd day of the cultivation, respectively. Activities were measured for 11 days and in all three media laccase activity production was detected until the end of the cultivation set (Figure 3.1).

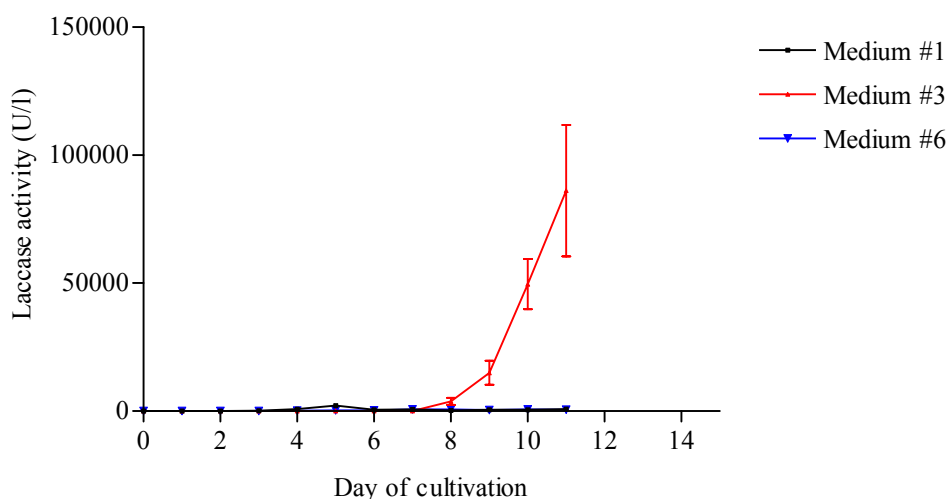


Figure 3.1 Laccase production by *C. polyzona* in medium #1 (■), medium #3 (▲) and medium #6 (▼)

From these tested nine media, medium #3 was chosen for shake flask cultivations of *C. polyzona*. In this medium no LiP and MnP activities were detected during 11 day cultivation period.

3.2. Sub-culturing study of *C. polyzona*

Before evolutionary engineering selection algorithm had been applied, an effective selection strategy had to be adopted. Spore formation and harvesting procedure of *C. polyzona* was a long period acquired approximately 20 days to inoculate, harvest and wash the spores ready to inoculate into liquid medium. Therefore, sub-culturing from liquid culture to fresh medium had been tried to overcome this problem. Cultivation period was 11 days and sub-culturing was performed at 8th day of the cultivation. From triplicate cultures two of them, culture #1 and #2, were sub-cultured and culture #3 was kept for further laccase activity measurements for comparison with sub-cultured duplicate. Laccase activities of the culture #1 and culture #2 at the 8th day of cultivation before sub-culturing were 13826.74 U/l and 15340.08 U/l, respectively. After the sub-culturing at the end of the cultivation period, 11th day, the

laccase activities were detected in sub-cultured cultures #1 and #2 as 183067.60 U/l and 180600.90 U/l, respectively and in culture #3 (not sub-cultured) as 136334.02 U/l (Figure 3.2).

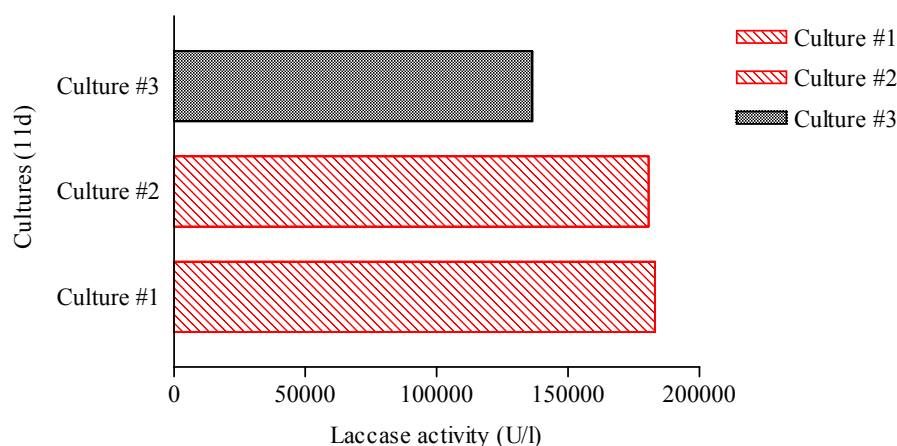


Figure 3.2 Laccase activities determined at the end of the sub-culturing cultivation period, day 11, of *C. polyzona*: Columns in red were laccase activities determined at the 11th day of the cultivation of sub-cultured *C. polyzona* cultures #1 and #2; column in black is the laccase activity at the 11th day of the culture #3 (not sub-cultured)

3.3. Starvation condition selection for to apply during evolutionary engineering of *C. polyzona*

Evolutionary engineering studies were performed with different stress conditions to obtain mutant generations. After a detailed literature search to choose an effective stress condition, starvation stress was decided to apply on *C. polyzona* strains to obtain mutant generations. A preliminary starvation study was done with different concentrations of carbon and nitrogen source of the culture medium to decide on the alteration range of the concentrations. The experiment was performed for 19 days in single flask and laccase activities were measured every day until the end of cultivation period to compare the effect of concentration changes in the control medium, from medium #2 to #5 nitrogen limitations was applied and from medium #6 to #11 carbon limitation was applied. Medium #1 was the original medium chosen for *C. polyzona* liquid cultivations. Table 3.2 demonstrates the results of laccase activities measured at enzyme analysis of these 11 cultures during 19 day of cultivation. In culture #11, no laccase activity was detected and in culture #10 negligible activities were measured between days 4 and 7, contains 5g and 1g glucose per liter of water, respectively.

Table 3.2 Laccase activity measurements from different carbon and nitrogen concentrations applied culture media of *C. polyzona*

Activity (U/l)	Control	Culture #2	Culture #3	Culture #4	Culture #5	Culture #6	Culture #7	Culture #8	Culture #9	Culture #10	Culture #11
1 st day	0	0	0	0	0	0	0	0	0	0	0
2 nd day	0	0	0	0	0	0	0	0	0	0	0
3 rd day	0	0	0	0	0	0	0	0	0	0	0
4 th day	0	0	0	13.60	73.67	0	0	0	0	1.87	0
5 th day	0	5.27	8.60	77.93	38.93	5.07	10.47	0	3.47	16.4	0
6 th day	492.67	2149.34	1347.01	436.67	22.67	43.33	38.00	5.73	155.53	6.87	0
7 th day	5938.36	7966.71	21623.44	836.00	14.00	26683.47	9976.72	95.53	2013.34	11.93	0
8 th day	833.34	12953.4	67033.67	93.53	33.00	59150.30	12740.06	2674.68	3020.02	0	0
9 th day	626.67	7233.37	73833.70	146.60	30.73	110567.20	20653.44	4471.69	2246.68	0	0
10 th day	2353.35	9573.38	65633.66	178.33	37.47	131867.30	16626.75	4851.69	1506.67	0	0
11 th day	3396.68	15920.08	78433.73	317.60	45.07	132334.00	16840.08	4013.35	2500.01	0	0
12 th day	5160.03	18080.09	58266.96	494.67	52.07	160534.10	24760.12	3813.35	2490.01	0	0
13 th day	5120.03	14186.74	20500.10	558.00	85.87	135600.70	23720.12	4390.02	2260.01	0	0
14 th day	4536.69	13513.40	14626.74	305.33	122.80	105600.50	23373.45	6158.36	2135.01	0	0
15 th day	12366.73	15926.75	4426.69	682.67	171.33	88900.44	23420.12	5546.69	2593.35	0	0
16 th day	1873.34	19520.10	12600.06	1128.01	247.33	80200.40	21446.77	5040.03	3486.68	0	0
17 th day	1423.34	13940.07	11606.72	1096.01	190.33	83433.75	23253.45	4466.69	2740.01	0	0
18 th day	2150.01	11493.39	11580.06	1140.67	153.33	81300.41	22053.44	4376.69	2521.68	0	0
19 th day	2636.68	10466.72	15493.41	1013.34	125.67	58700.29	20960.10	4605.02	2626.68	0	0

From cultures with nitrogen limitation (colored in red in Table 3.2), culture #3 (50g glucose/9g bactopectone) showed the highest laccase activity at 11th day of the cultivation as 78433.73 U/l and in carbon limited cultures (colored in blue in Table 3.2), culture #6 (40g glucose/17g bactopectone) showed the highest laccase activity as 160534.10 U/l at day 12 (Figure 3.3).

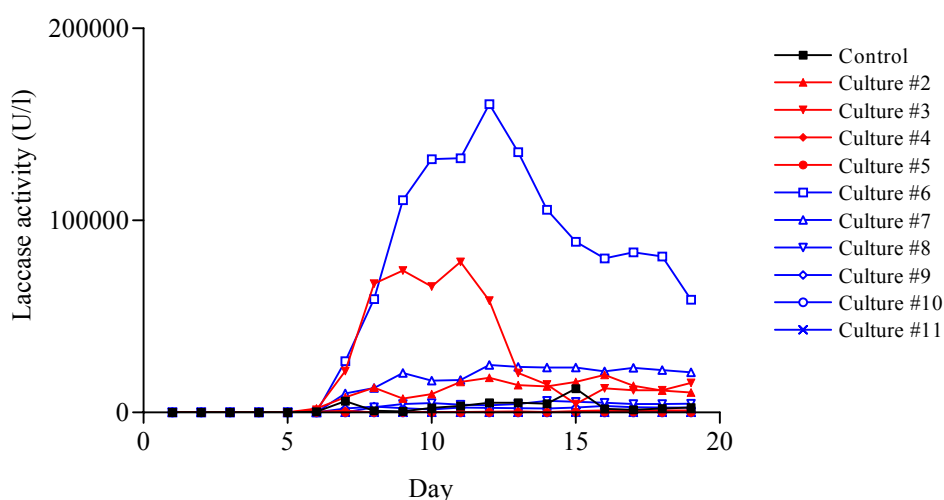


Figure 3.3 Laccase activity results of different carbon and nitrogen concentration inclusive cultures of *C. polyzona*: Carbon-limited cultures, culture #2 (▲), culture #3 (▼), culture #4 (◆), culture #5 (●); Nitrogen-limited cultures, culture #6 (□), culture #7 (△), culture #8 (▽), culture #9 (◇), culture #10 (○), culture #11 (×); control (■)

Cell dry weight results (CDW) of the *C. polyzona* cultures also measured to examine the effect of carbon and nitrogen concentration alterations in the culture medium (Table 3.3).

Table 3.3 CDW results of the *C. polyzona* cultures measured at the end of the 19 day cultivation period

CDW (g/l)	
Culture #1	5.20
Culture #2	6.93
Culture #3	4.82
Culture #4	6.47
Culture #5	4.70
Culture #6	4.72
Culture #7	2.72
Culture #8	2.17
Culture #9	2.00
Culture #10	1.27
Culture #11	0.84

CDW results of the limited cultures were slightly lower than that of the control culture, except culture #2 which had higher CDW values than that of the control culture. In cultures #10 and #11, there was negligible and no laccase activity detected, respectively and biomass results were also very low when compared to control culture (Figure 3.4).

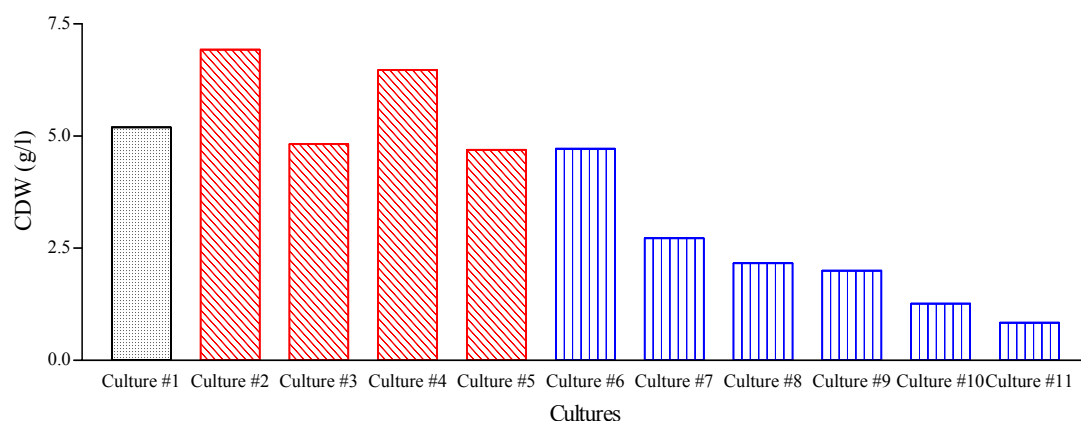


Figure 3.4 CDW results of the cultures measured at the end of the cultivation period, day 19

Following the first trial to find concentration alteration range, another starvation study was performed. The experiment was designed by choosing cultures with highest laccase activity from carbon limited and nitrogen limited cultures, culture #3 and culture #6, respectively. Besides these two cultures from the first study, another culture medium was added with carbon and nitrogen limitation, CNLC, 40g glucose/9g bactopectone per liter of distilled water. Laccase activities were measured to compare the effect of concentration changes in the culture medium (Table 3.4).

Table 3.4 Laccase activities measured during second starvation experiment includes nitrogen-limited culture #3, carbon-limited culture #6 and carbon and nitrogen limited CNLC of *C. polyzona*

Laccase activity (U/l)	Control 1	Control 2	Control 3	Culture #3 1	Culture #3 2	Culture #3 3
0 th day	0	0	0	0	0	0
1 st day	0	0	0	0	0	0
2 nd day	0	0	0	0	0	0
3 rd day	0	0	0	0	0	0
4 th day	0	0	0	9.00	12.60	13.20
5 th day	11.93	145.53	13.93	1057.01	413.34	488.00
6 th day	768.67	5238.36	2038.68	7750.04	5770.03	6056.70
7 th day	3142.02	7306.70	6155.03	12860.06	12940.06	15493.41
8 th day	7126.70	33400.17	6633.37	6240.03	12306.73	15746.75
9 th day	10106.72	36653.52	5646.69	2480.01	4253.35	7706.71
	CNLC 1	CNLC 2	CNLC 3	Culture #6 1	Culture #6 2	Culture #6 3
0 th day	0	0	0	0	0	0
1 st day	0	0	0	0	0	0
2 nd day	0	0	0	0	0	0
3 rd day	0	0	0	0	0	0
4 th day	14.67	13.80	12.87	21.27	45.93	17.87
5 th day	1460.01	1170.01	1492.67	3812.69	9091.71	945.34
6 th day	11896.73	9180.05	10273.38	11556.72	9083.38	7316.70
7 th day	12320.06	9386.71	11626.72	33586.83	10673.39	23093.45
8 th day	8666.71	4240.02	6333.37	51186.92	5013.36	46746.90
9 th day	1773.34	2320.01	3520.02	64320.32	3213.35	78160.39

Laccase activities in culture #6 were doubled when compared to the control cultures of *C. polyzona*. Nitrogen-limited culture #3 laccase activities were higher than control culture between days 5 and 8. However, activities in culture #3 decreased after 8th day of the cultivation. The same laccase activity trend was also examined in the CNLC medium and the measured activities were lower than both the carbon limited culture #6 and the nitrogen limited culture #3 (Figure 3.5).

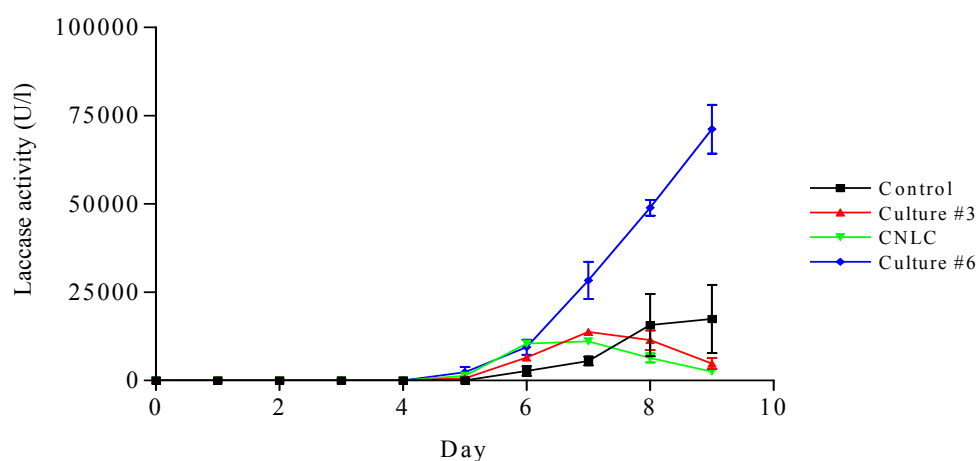


Figure 3.5 Laccase activities of the starvation cultures of *C. polyzona*; control (■), culture #3 (▲), CNLC (▼) and culture #6 (◆)

Besides the laccase activity comparison of the cultures, also CDWs were measured at the end of the cultivation period to compare biomass formation (Table 3.5).

Table 3.5 CDW results from second starvation study at the end of the cultivation period of *C. polyzona*

CDW(g/l)	
Control 1	9.77
Control 2	9.80
Control 3	10.16
Culture #3 1	7.21
Culture #3 2	7.00
Culture #3 3	7.92
CNLC 1	5.96
CNLC 2	6.34
CNLC 3	7.60
Culture #6 1	8.33
Culture #6 2	5.67
Culture #6 3	8.29

Even though the biomass values of the control cultures were higher than those of the other cultures (Figure 3.6), measured laccase activities were better successively in the cultures#6, control, #3 and CNLC.

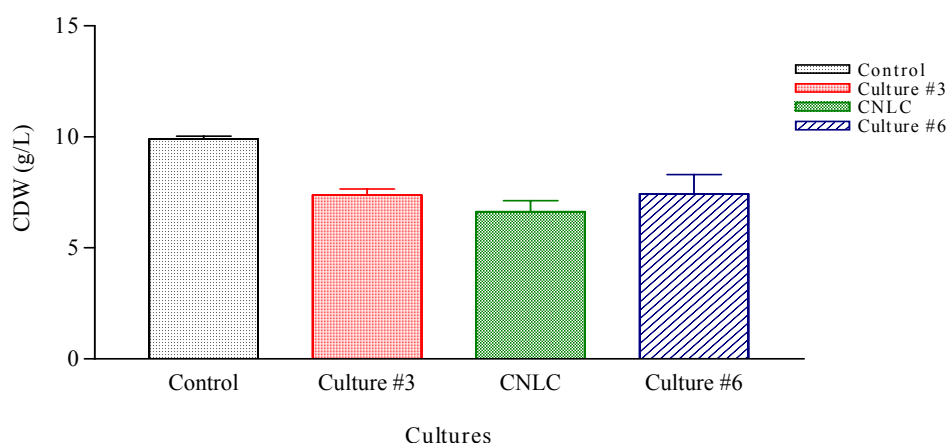


Figure 3.6 Measured CDWs of the starvation cultures from second study of *C. polyzona*

From both starvation studies, highest laccase activities were detected in the carbon limited culture #6 with 40g glucose/17g bactopeptone per distilled water. *C. polyzona* CNLC cultures with both carbon and nitrogen limitation showed average of 13764.51 U/l at the day 7 which is only the half of the activity measured from control cultures. Therefore, medium concentrations of culture #6 were adopted as starvation stress conditions in evolutionary engineering of *C. polyzona* cultures.

3.4. Application of designed selection algorithm to evolutionary engineering of *C. polyzona*

3.4.1. EMS mutagenesis applied on wild type *C. polyzona* to obtain mutant population

Prior to designed selection algorithm was applied for evolutionary engineering studies; first step was to provide mutant populations from EMS mutagenized wild type *C. polyzona* cultures. The aim was to increase genetic diversity of the wild type strain with no loss in wild type characteristics. First EMS trial was unsuccessful due to high concentration and extensive application time, 300µl for 1 hour, of the EMS. The dosage was lethal that no survivors were observed either on PDA plates or in liquid medium. Therefore, a second EMS mutagenesis experiment was designed with different concentrations at different time intervals, 300µl for 5, 10 and 15 minutes, 150µl for 30 and 15 minutes. EMS mutagenized cultures were inoculated on PDA plates and into liquid medium to examine the mutant populations' viability compared to wild type. No growth was examined in the liquid cultures. However, 150µl/30'

and 300 μ l/15' applied cultures were grown on PDA plates. From these cultures agar pieces were cut from the growing edge of the mycelium and inoculated on the rice for spore formation. Spores were inoculated into duplicate liquid medium with wild type control culture to examine laccase activity at the end of the 11 day cultivation period.

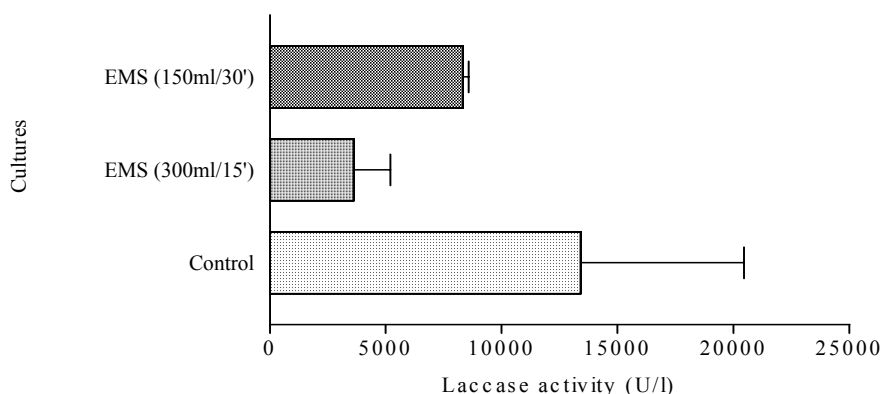


Figure 3.7 Laccase activities measured at day 11, the end of the cultivation period

Measured activity of the wild type culture was 13413.40 U/l. Activities of the 150 μ l/30' and 300 μ l/15' EMS applied cultures were 8321.71 U/l and 3624.19 U/l, respectively. Activities of the cultures were low compared to wild type *C. polyzona* culture. Final laccase activity of 150 μ l/30' culture was 62% and 300 μ l/15' was the 27% of the wild type activity. Therefore, final concentration of EMS to apply wild type was decided as 100 μ l for 15'. Inoculated mutagenized population both on PDA plate and in liquid cultivation medium were viable and the examined laccase activity was the 89% of the wild type and measured CDWs were higher than the wild type culture, 10.25 g/l and 8.18 g/l, respectively.

3.4.2. Obtaining mutant generations

Selection strategy for evolutionary engineering study was constructed as the cultivation of primary EMS mutagenized *C. polyzona* mutant population at starvation conditions for 8 day cultivation period. At the end of the cultivation period, mutant populations were sub-cultured into fresh starvation conditions applied culture medium to obtain mutant generations and on PDA plates for spore formation of each generation. Laccase activities were measured during these 8 day intervals (Table 3.6).

Table 3.6 Laccase activities of the *C. polyzona* mutant generations obtained from EMS mutagenized wild type organism

Laccase activity (U/l)	Control (wild type)	EMS	1 st generation	2 nd generation	3 rd generation	4 th generation	5 th generation
0 th day	0	0	0	0	0	0	0
1 st day	0	0	0	0	0	0	0
2 nd day	5.62	0	0	0	1.80	11.60	11.20
3 rd day	2.58	0	0	17.47	3.00	8.13	11.53
4 th day	132.50	0	0	33.00	24.47	58.73	31.67
5 th day	425.40	214.93	6385.03	91.13	52.80	136.80	93.13
6 th day	4430.58	1404.01	11563.39	72.60	76.47	242.00	123.60
7 th day	14397.85	2838.35	18353.43	84.13	888.67	7056.70	154.13
8 th day	15093.41	4136.69	25413.46	99.73	5300.03	11420.06	742.67
Laccase activity (U/l)	6 th generation	7 th generation	8 th generation	9 th generation	10 th generation	11 th generation	
0 th day	0	0	0	0	0	0	
1 st day	0	0	0	0	0	0	
2 nd day	3.47	32.07	77.00	46.93	0	2.40	
3 rd day	5.13	17.00	52.20	15.20	1.40	2.27	
4 th day	39.20	125.47	213.87	8.53	0.93	60.73	
5 th day	82.13	1761.34	587.34	6.00	2.07	301.60	
6 th day	105.93	3115.35	1344.67	82.33	6.93	762.00	
7 th day	203.33	6173.36	2643.35	2070.01	18.27	474.00	
8 th day	7676.71	11750.06	7283.37	5790.03	26.53	1166.67	

8 day cultivation period under starvation conditions were enough to force mutant populations to adapted stress condition due to rapid increase in laccase activity between 4 and 8 days of cultivation. There was a rapid increase at the first generation after the first sub-culture; however, following the 1st generation there was a sudden decrease in the laccase activity at the second generation. At the 4th generation laccase activity was 76% of the wild type culture. Until the 7th generation again there was a decrease in laccase activity, yet at 7th generation laccase activity was 78% of the wild type activity. Following the 8th generation laccase activity was decreased again and final mutant population was ended at the 11th generation (Figure 3.8).

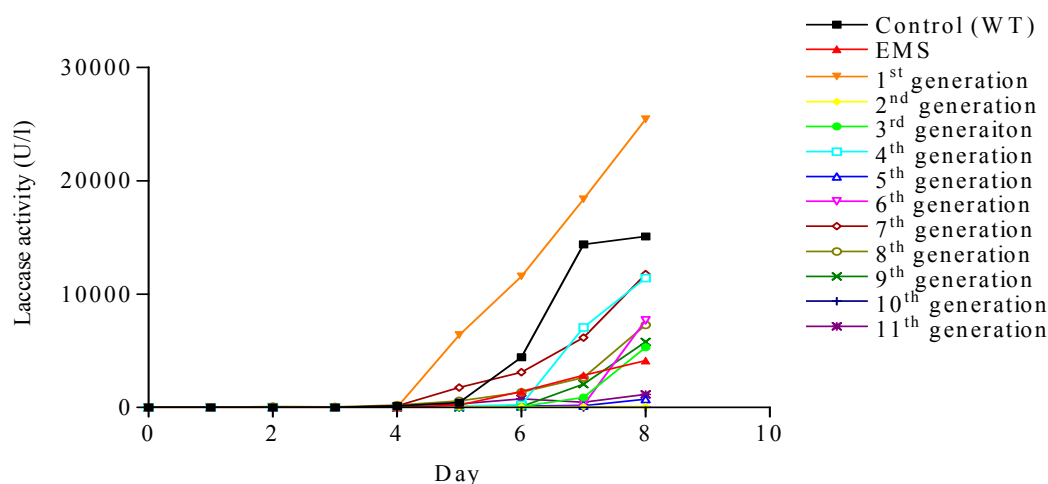


Figure 3.8 Laccase activities measured during obtaining mutant generations of *C. polyzona* from EMS mutagenized primary mutant population; control (■), EMS (▲), 1st generation (▼), 2nd generation (◆), 3rd generation (●), 4th generation (□), 5th generation (Δ), 6th generation (▽), 7th generation (◇), 8th generation (○), 9th generation (×), 10th generation (+), 11th generation (*)

Even so, the highest laccase activity was examined at the 7th generation as 11750.06 U/l that is 78% of the wild type activity, 70% of the generations obtained showed higher laccase activities than EMS mutagenized primary mutant population.

Biomass concentration of each generation was also determined to compare with that of the wild type and EMS mutagenized cultures (Table 3.7).

Table 3.7 CDWs of the generations obtained from EMS mutagenized *C. polyzona* wild type culture

CDW (g/l)			
Control (WT)	7.11	6 th generation	8.222
EMS	8.18	7 th generation	6.083
1 st generation	9.739	8 th generation	6.441
2 nd generation	10.25	9 th generation	6.915
3 rd generation	8.903	10 th generation	9.156
4 th generation	7.731	11 th generation	8.793
5 th generation	9.541		

Until the 7th generation biomass production was higher than the wild type *C. polyzona* culture. At next there generations, 7th, 8th and 9th generations biomass production was slightly lower than the wild type culture and increased again at the 10th and 11th generations (Figure 3.9).

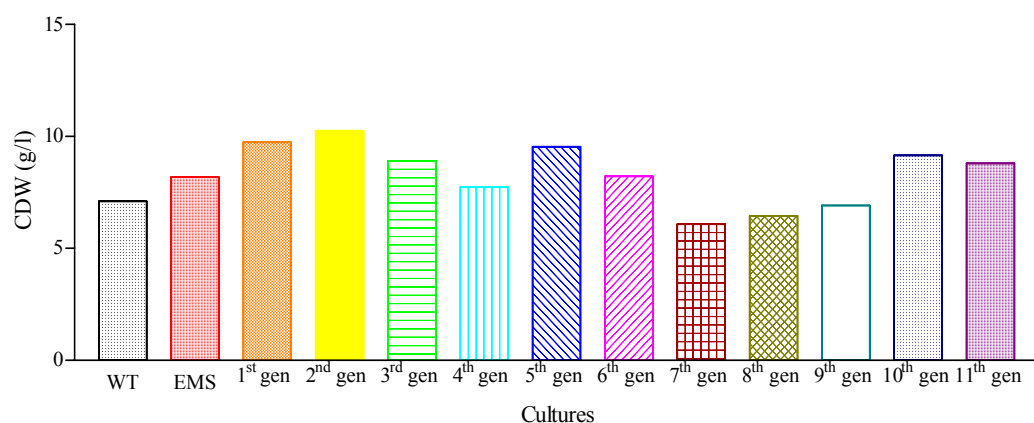


Figure 3.9 Measured biomass concentrations of *C. polyzona* mutant populations and wild type culture

3.4.3. Selection of mutant individuals from final mutant populations

From obtained mutant populations, 7th generation with highest laccase activity among the generations and 8th generation was chosen for obtaining mutant individuals. Randomly selected individuals from 7th and 8th generations were inoculated on PDA plates. Only individuals from 7th mutant generation and 9 from 10 randomly selected individuals were grown on plates. No individuals from 8th generation were grown on plates. Nine mutant generations were inoculated into liquid medium and laccase activities were measured for 12 day cultivation (Table 3.8).

Table 3.8 Measured laccase activities of the mutant individuals obtained from 7th mutant generation of *C. polyzona*

Laccase activity (U/L)	CP701	CP702	CP703	CP704	CP705	CP706	CP707	CP708	CP709
0 th day	0	0	0	0	0	0	0	0	0
1 st day	0	0	0	0	0	0	0	0	0
2 nd day	0	0	0	0	0	0	0	0	0
3 rd day	0	0	0	0	0	0	0	0	0
4 th day	0	0	0	1.20	10.34	0	3.74	0	85.98
5 th day	0	0	0	8.20	6.74	2.40	7.94	0	43.22
6 th day	0	0	5.00	8.00	11.21	11.87	21.88	0	18.88
7 th day	0	3.80	16.21	4.54	14.61	211.64	26.41	0	6.00
8 th day	0	3.20	14.54	5.27	16.34	879.77	10.67	0	2.93
9 th day	0	3.07	15.61	9.54	26.75	1511.42	7.80	0	2.53
10 th day	0	4.00	16.34	5.27	44.76	2103.72	4.34	0	2.13
11 th day	0	5.54	33.08	3.40	163.55	4854.09	7.54	0	3.27
12 th day	0	10.94	69.57	2.67	392.20	8460.90	154.01	0	179.36

From nine individuals, in cultures of CP701 and CP702, the measured laccase activity was zero during 12 days cultivation period. The highest laccase activity measured among the individual mutants was from individual CP706 as 8460.90 U/l,

which was the 75% of the wild type activity and higher than the laccase activity measured from the 7th generation mutant population (Figure 3.10).

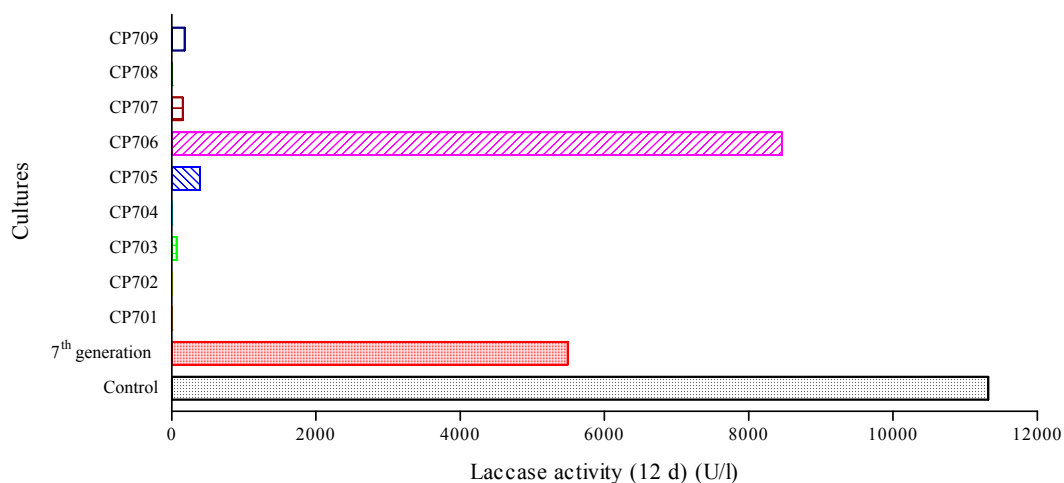


Figure 3.10 Measured laccase activities at the end of the cultivation period, day 12, of the mutant individuals compared to wild type and 7th mutant population

Biomass production during 12 day cultivation period was also measured (Table 3.9) and compared with the wild type culture and 7th mutant population as well (Figure 3.11).

Table 3.9 Measured CDWs of the mutant individuals with wild type and 7th mutant generation at the end of the cultivation period

CDW (g/l)	
Control	6.22
7 th generation	8.58
CP701	5.18
CP702	10.47
CP703	5.75
CP704	5.46
CP705	5.98
CP706	10.04
CP707	9.73
CP708	1.39
CP709	9.02

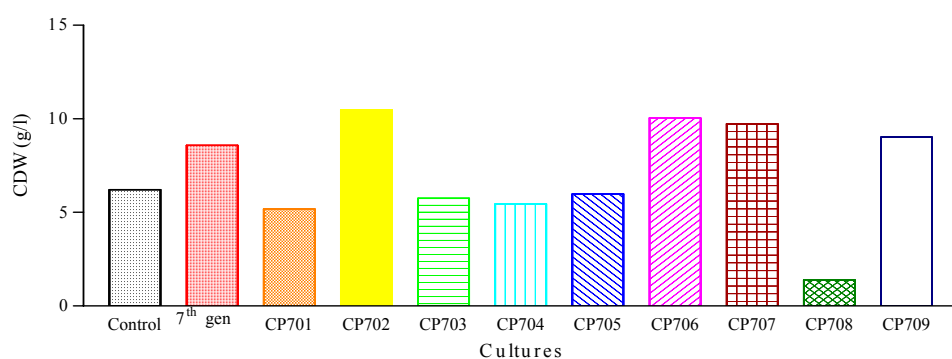


Figure 3.11 Measured biomass data of the mutant individuals with the wild type and 7th mutant generation of *C. polyzona* at the end of the cultivation period

Biomass production of mutant individuals CP706 and CP707 were higher than those of the wild type and 7th mutant population of *C. polyzona*.

3.5. Decolorization studies of *C. polyzona*

Two different reactive dyes, reactive black 5 and RBBR, were used to examine decolorization abilities of the wild type, mutant 7th generation and selected individuals, CP705, CP706, CP707 and CP709, of *C. polyzona*. Beside these two dyes, wild type *C. polyzona* was further examined in decolorization of the additional reactive dyes, remazol turquoise blue G133 and reactive red 195 (Table 3.10).

Table 3.10 Decolorization characteristics of the *C. polyzona* wild type, mutant 7th generation and selected mutant individuals, CP705, CP706, CP707 and CP709

		Reactive Black 5	RBBR	Remazol turquoise blue G133	Reactive red 195
WT	Decolorization (%)	81.65±6.22	92.33±6.77	86.32±3.75	76.62±2.47
	Laccase activity (U/l)	19988.99±3411.48	7361.70±1101.08	26726.80±1291.66	16550.92±5035.79
	CDW (g/l)	7.51±0.12	7.57±1.26	7.04±0.90	7.23±1.00
7 th generation	Decolorization (%)	75.90±7.19	84.81±2.13		
	Laccase activity (U/l)	139.60±33.75			
	CDW (g/l)	4.90±2.21			
CP705	Decolorization (%)	76.85±0.18	84.51±14.22		
	Laccase activity (U/l)	3.70±1.27			
	CDW (g/l)	5.98±1.39			
CP706	Decolorization (%)	81.20±0.18	91.32±1.23		
	Laccase activity (U/l)	39.37±10.32			
	CDW (g/l)	11.02±2.57			
CP707	Decolorization (%)	82.48±1.99	94.94±1.23		
	Laccase activity (U/l)	8.13±5.47			
	CDW (g/l)	7.97±2.27			
CP709	Decolorization (%)	77.49±4.34	58.97±13.61		
	Laccase activity (U/l)	11.47±14.15			
	CDW (g/l)	6.72±2.75			

Wild type *C. polyzona* cultures were able to decolorize 81.65% of the Reactive black 5, 92.33% of the RBBR, 86.32% of the remazol turquoise blue G133 and 76.62% of the Reactive red 195.

Reactive black 5 and RBBR was chosen to compare decolorization properties of 7th mutant generation (Figure 3.13) and selected individuals, CP705, CP706, CP707 and CP709 (Figure 3.14) with wild type *C. polyzona* cultures (Figure 3.12).

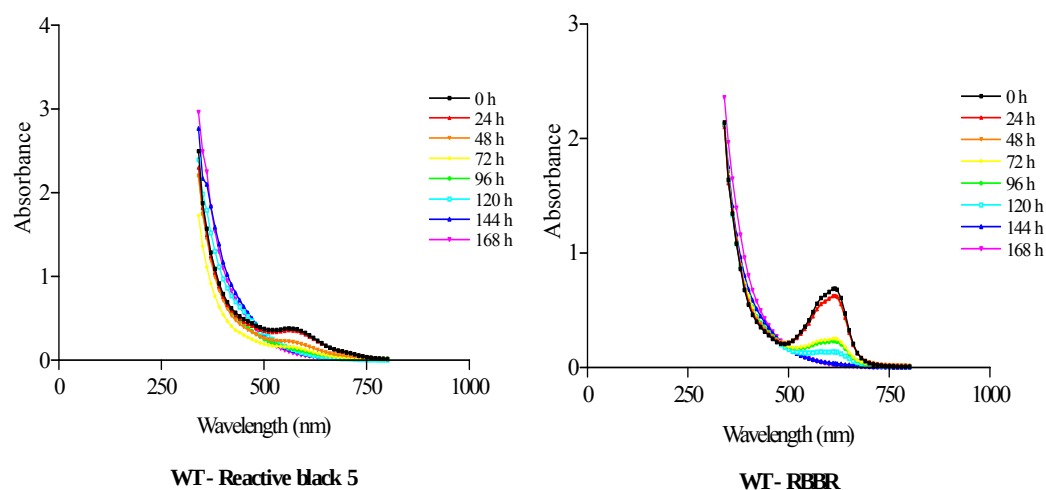


Figure 3.12 Visible absorbance spectra of wild type *C. polyzona* decolorization of reactive black 5 (on the left) and RBBR (on the right)

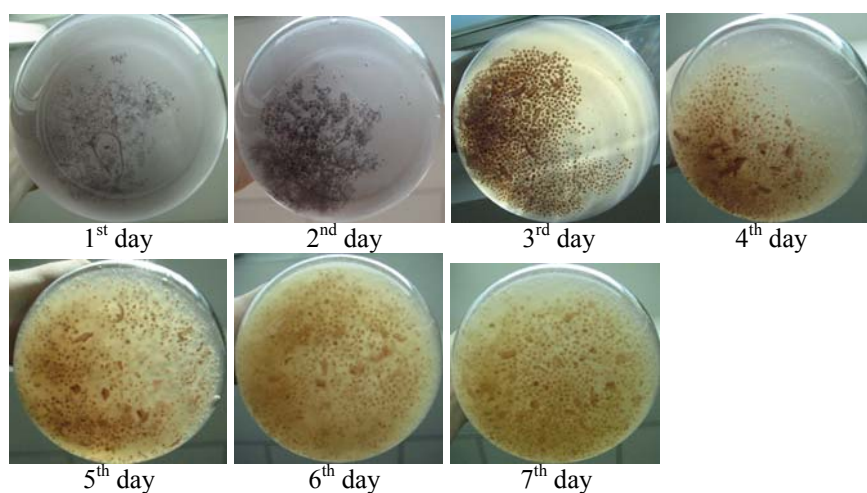


Figure 3.13 Images of black 5 added wild type *C. polyzona* liquid cultures taken during 7 day decolorization study

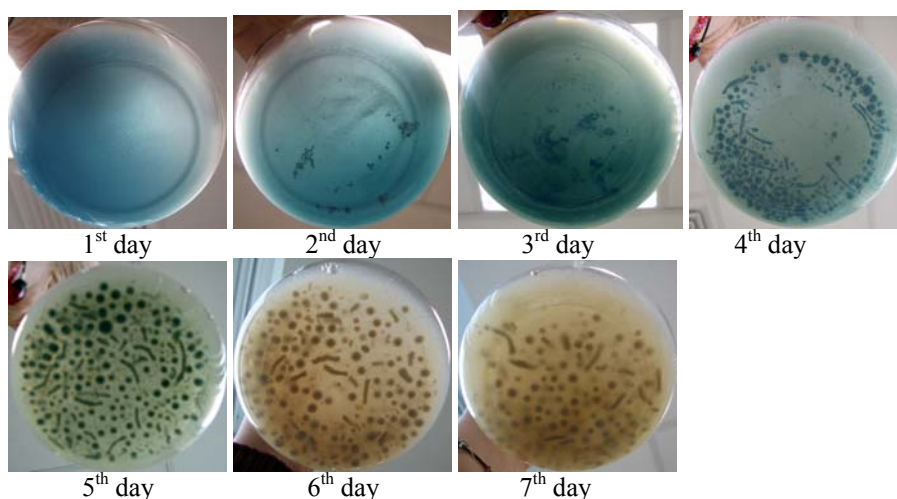


Figure 3.14 Images of RBBR added wild type *C. polyzona* liquid cultures taken during 7 day decolorization study

Mutant 7th generation was able to decolorize 75.90% of the reactive black 5 and 84.81% of the RBBR. When compared to wild type culture, effectiveness of the decolorization by population was 93% and 92% of the wild type for reactive black 5 and RBBR, respectively, based on the wild type decolorization percentages.

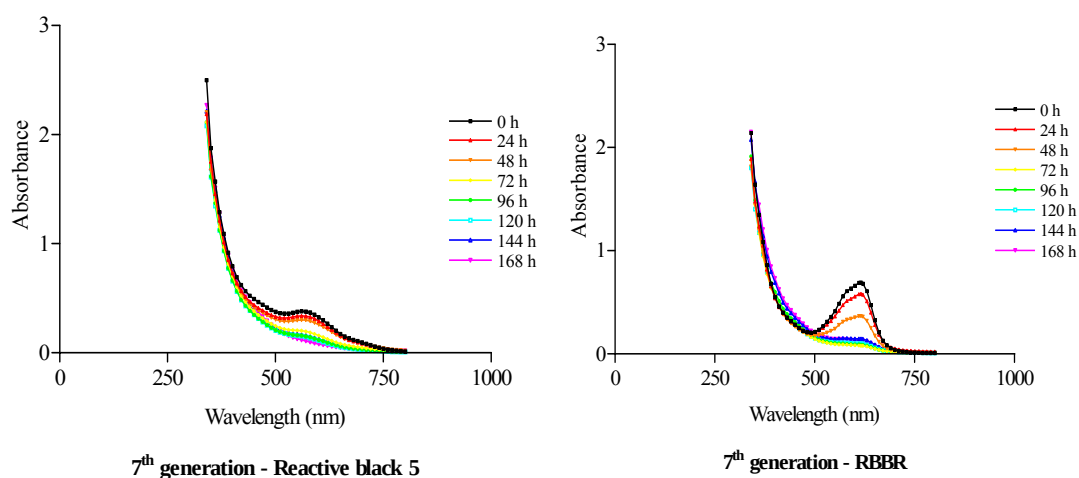


Figure 3.15 Visible absorbance spectra of 7th mutant *C. polyzona* generation decolorization of reactive black 5 (on the left) and RBBR (on the right)

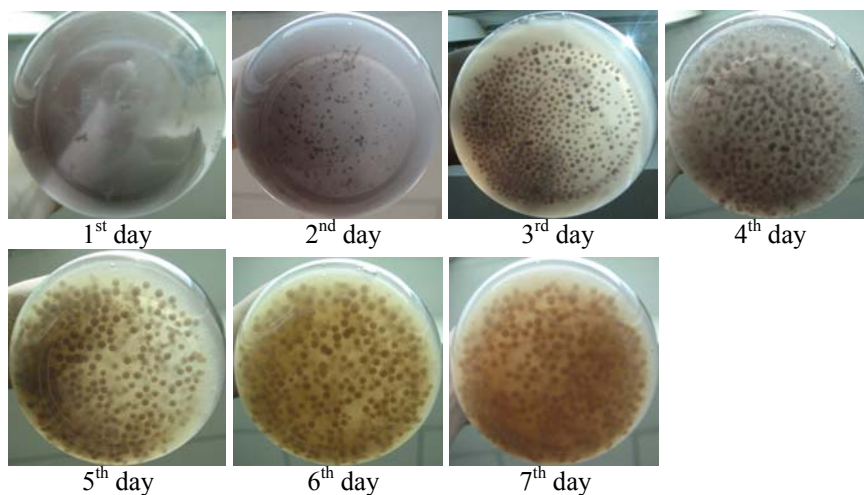


Figure 3.16 Images of black 5 added 7th mutant generation *C. polyzona* liquid cultures taken during 7 day decolorization study

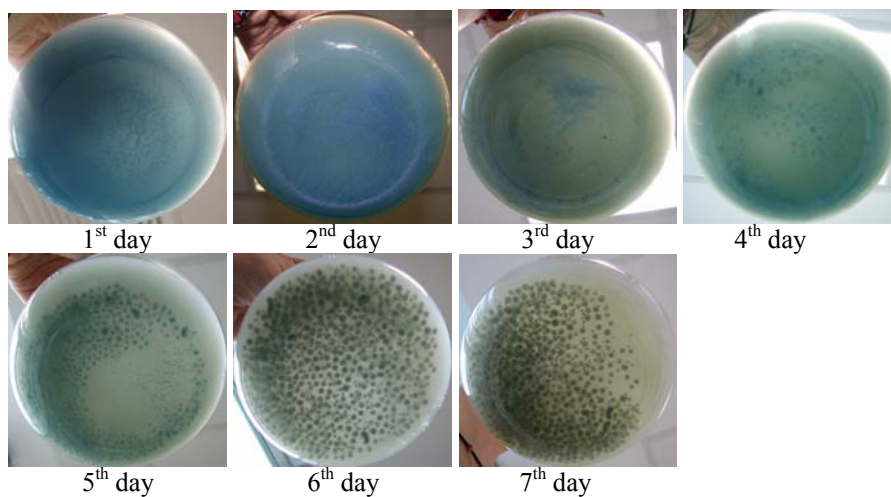


Figure 3.17 Images of RBBR added 7th mutant generation *C. polyzona* liquid cultures taken during 7 day decolorization study

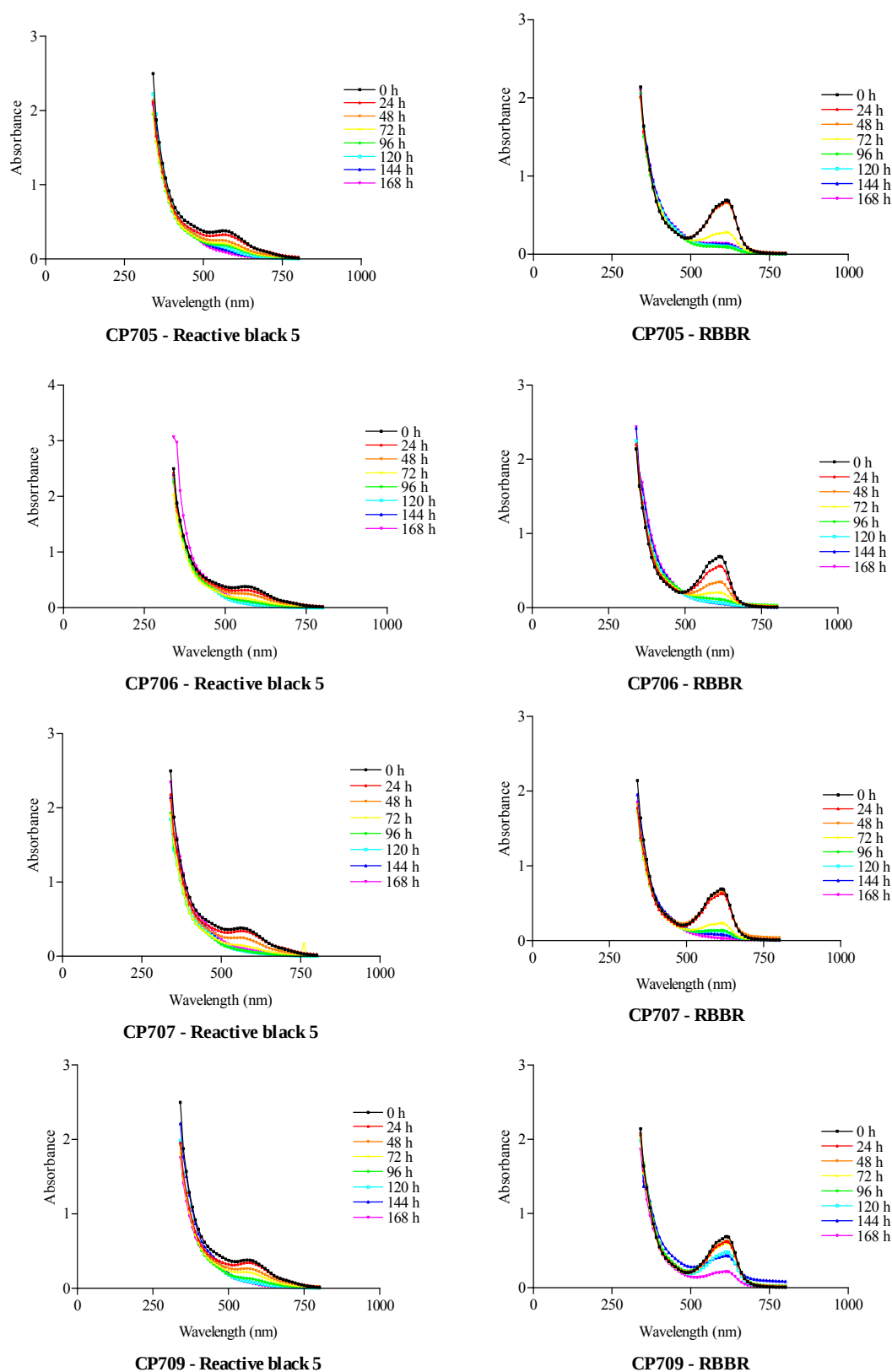


Figure 3.18 Visible absorbance spectra of the *C. polyzona* mutant individuals, CP705, CP706, CP707 and CP709, decolorization of reactive black 5 (on the left) and RBBR (on the right)

From mutant individuals CP705, CP706, CP707 and CP709 were selected for examining their decolorization properties compared with the wild type and also with the mutant population. Decolorization by the wild type of the reactive black 5 and RBBR were 81.65% and 92.33%, respectively, where CP705 decolorize 76.85% of black 5 and 84.51% of RBBR, CP706 decolorize 81.20% of black 5 and 91.32% of RBBR, CP707 decolorize 82.48% of black 5 and 94.94% of RBBR and CP709 decolorize 77.49% of black 5 and 58.97% of RBBR. Among these selected individuals CP706 and CP707 were effective in decolorization and CP707 was even slightly effective than the wild type decolorization properties.

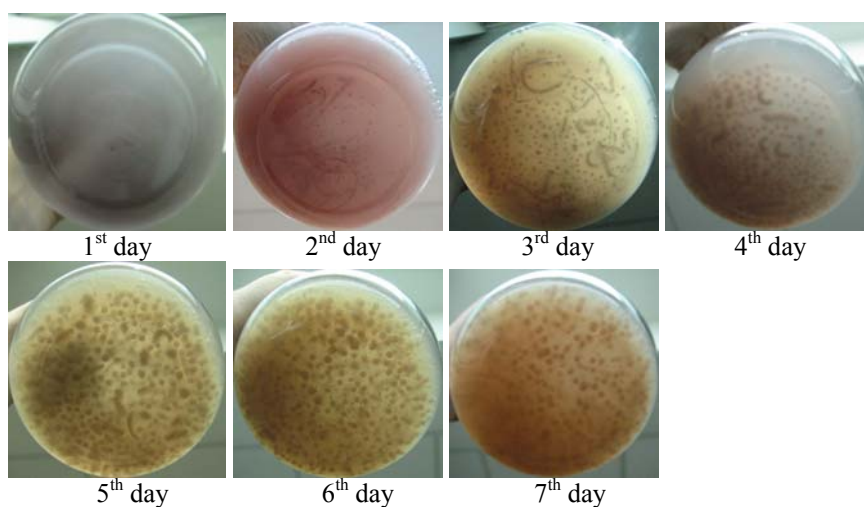


Figure 3.19 Images of black 5 added *C. polyzona* mutant individual CP705 liquid cultures taken during 7 day decolorization study

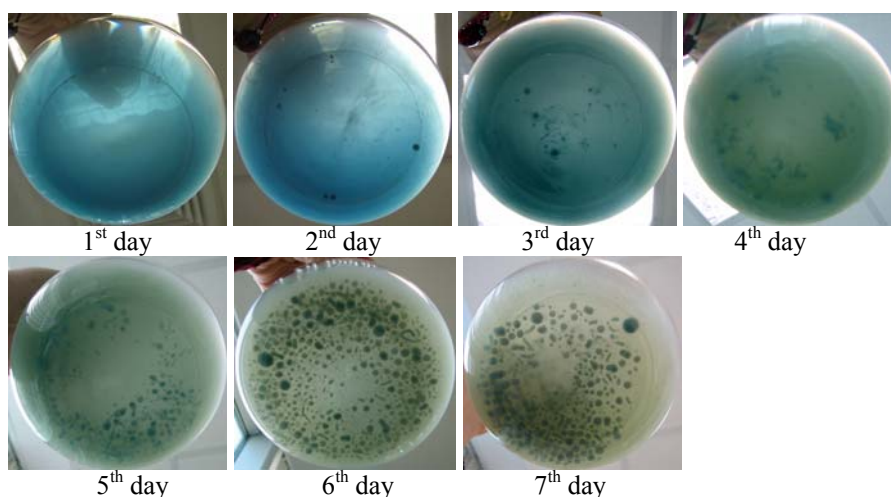


Figure 3.20 Images of RBBR added *C. polyzona* mutant individual CP705 liquid cultures taken during 7 day decolorization study

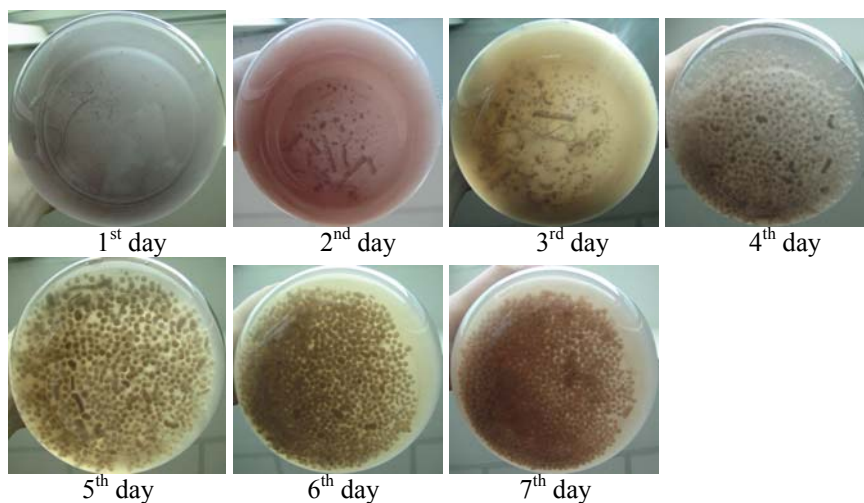


Figure 3.21 Images of black 5 added *C. polyzona* mutant individual CP706 liquid cultures taken during 7 day decolorization study

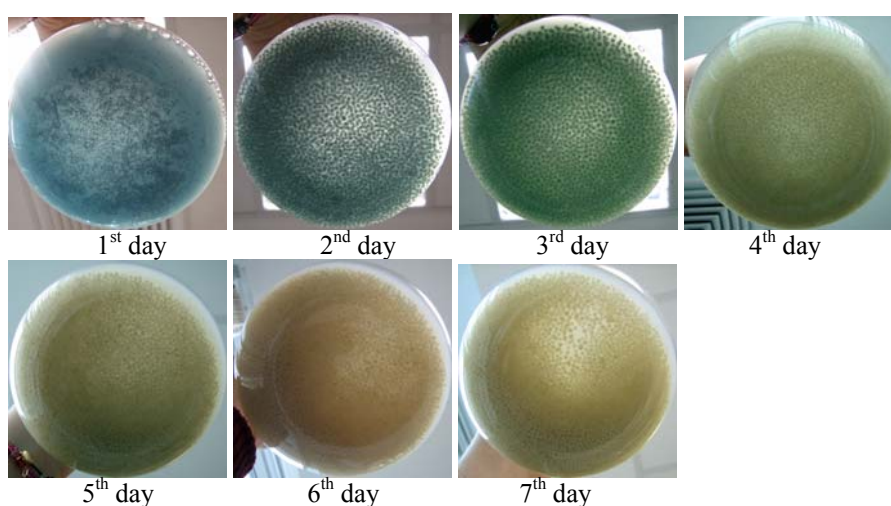


Figure 3.22 Images of RBBR added *C. polyzona* mutant individual CP706 liquid cultures taken during 7 day decolorization study

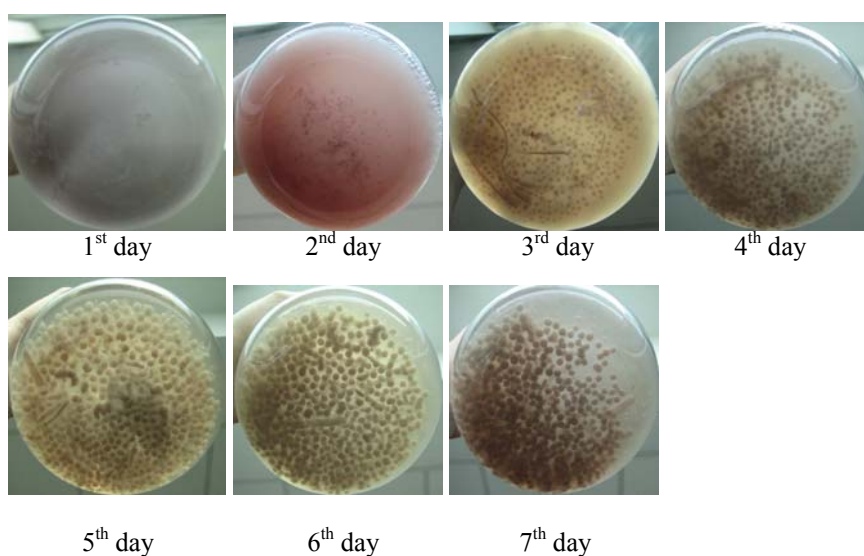


Figure 3.23 Images of black 5 added *C. polyzona* mutant individual CP707 liquid cultures taken during 7 day decolorization study

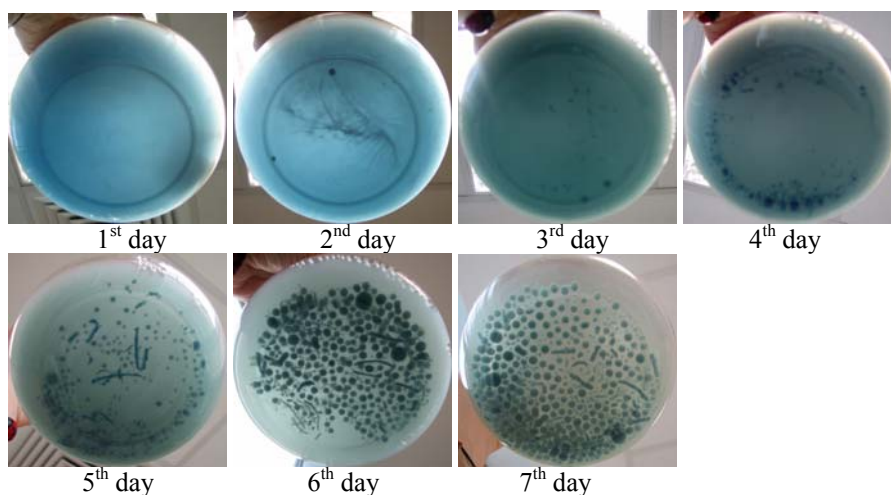


Figure 3.24 Images of RBBR added *C. polyzona* mutant individual CP707 liquid cultures taken during 7 day decolorization study

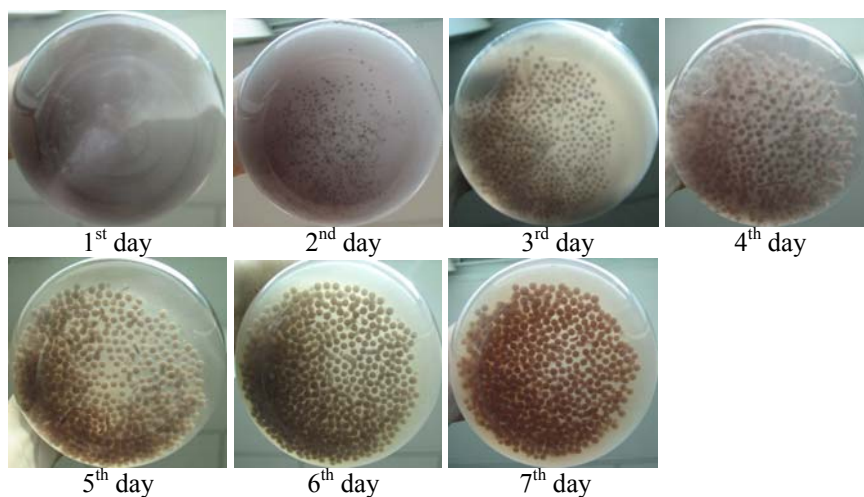


Figure 3.25 Images of black 5 added *C. polyzona* mutant individual CP709 liquid cultures taken during 7 day decolorization study

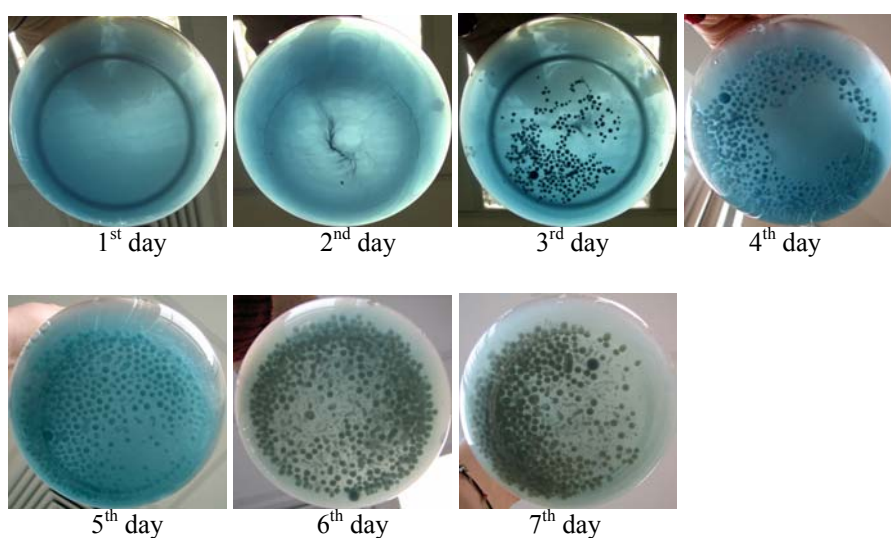


Figure 3.26 Images of RBBR added *C. polyzona* mutant individual CP709 liquid cultures taken during 7 day decolorization study

3.6 Intracellular laccase determination

During decolorization studies selected mutant 7th generation and mutant individuals CP705, CP706, CP707 and CP709 showed lower extracellular laccase activity than wild type *C. polyzona* cultures. However, mutant cultures decolorization efficiency was comparable and even slightly higher than wild type culture. Based on the images taken during decolorization experiments, biosorption of the dye on fungal pellets may be possible. To clarify this, intracellular laccase activity was measured in RBBR added *C. polyzona* cultures (Table 3.11) due to the possibility that instead of secreting the enzyme extracellularly, laccase could be kept inside the pellets for dye decolorization.

Table 3.11 Intracellular laccase activities of RBBR added *C. polyzona* cultures

RBBR			
Laccase activity (U/l)		Buffer	Sonication
WT (day 5)	3,27	3,40	12,00
WT (day 7)	233,33	173,27	628,67
7 th generation (day 5)	7,53	3,53	27,07
7 th generation (day 7)	11,67	55,80	112,73
CP705 (day 5)	1,33	1,27	16,87
CP705 (day 7)	2,53	0,93	22,73
CP706 (day 5)	4,07	0,00	24,13
CP706 (day 7)	4,87	2,80	35,40
CP707 (day 5)	1,80	6,73	0,80
CP707 (day 7)	1,60	1,60	26,20
CP709 (day 5)	1,40	0,00	15,33
CP709 (day 7)	1,07	0,00	25,47

For intracellular laccase determination, fungal pellets were disrupted at two different times during 7 day cultivation periods. Yellow highlighted areas in Table 3.11 refer to cell disruption at day 5 of cultivation which was the day after the first laccase activity was measured during *C. polyzona* cultivations. Not highlighted areas show the laccase activities measured at the end of the cultivation, day 7. First column shows the activities from the culture supernatants before they are transferred into phosphate buffer, second column shows the activities in buffer solution and third column shows the intracellular laccase activity after sonication.

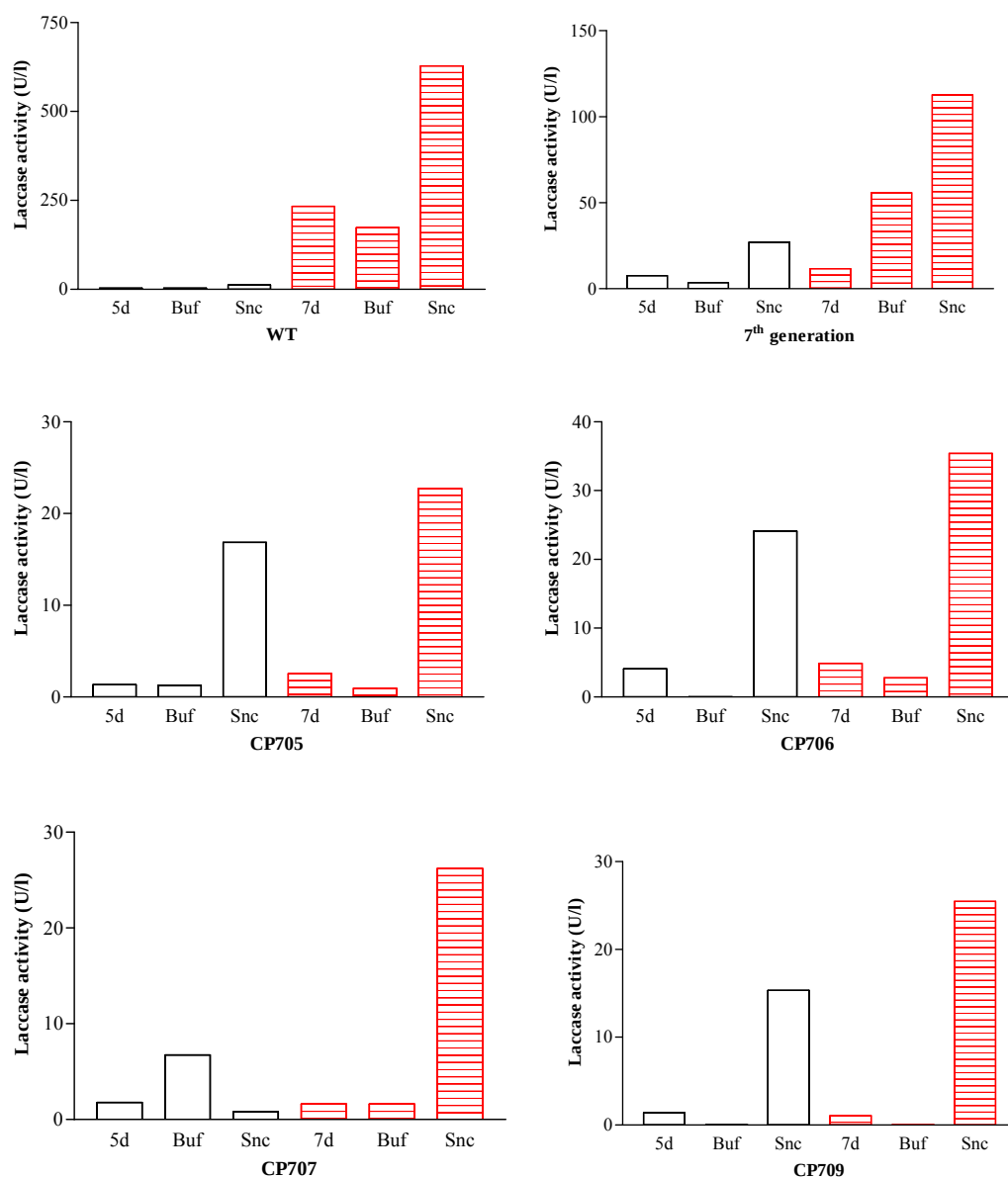


Figure 3.27 Intracellular laccase activities of the RBBR added *C. polyzona* liquid cultures

Additional to intracellular laccase activity measurements, pH of the cultures during 7 day cultivation period were measured as well (Table 3.12).

Table 3.12 pH of the RBBR added *C. polyzona* cultures during decolorization study

RBBR							
pH	Control	WT	7 th generation	CP705	CP706	CP707	CP709
1 st day	5	6	6-5	6	6-5	6-5	6-5
2 nd day	5	6-5	6	5	5	6-5	6
3 rd day	4	4	6	5	5	5	5
4 th day	4	4	5	5	5	5	5
5 th day	4	4	5	5	5	5	5
6 th day	4	4	5	5	5	5	5
7 th day	4	4	4	4	4	4	4

4. DISCUSSION AND CONCLUSION

In this study, decolorization characteristics of the white-rot fungus *C. polyzona* was attempted to be improved through design and application of suitable evolutionary engineering strategies. For this purpose, preliminary studies were performed to choose the most suitable cultivation medium for *C. polyzona* followed by additional studies performed to design a selection algorithm for evolutionary engineering of *C. polyzona*. Subsequent to the application of evolutionary engineering selection algorithm, all the obtained mutant populations and mutant individuals from selected populations were compared with the wild type according to their decolorization abilities by using two different dyes, an azo-based reactive dye Remazol Black RL and an anthraquinone-based reactive dye RBBR.

Nine different media were selected and examined due to the ligninolytic enzymes production level of *C. polyzona* in these media. Media with different carbon, nitrogen sources and concentrations were tested. Besides, media included various other supplements such as trace metal and vitamin solutions. No LiP activities were detected in any culture media. Negligible MnP activities were measured in media #1, #4 and #6 at days 3, 6 and 7, respectively. Significant laccase production by *C. polyzona* was examined in media #1, #3 and #6. Among these three, medium #3 was chosen for *C. polyzona* shake flask cultivations and in this medium no LiP or MnP activity was detected. In specific researches, about decolorization of olive oil mill wastewaters with potential fungi (Jaouani *et al.*, 2003) and with *C. polyzona* (Jaouani *et al.*, 2006), it was stated that *C. polyzona* was a producer of all three ligninolytic enzymes. However, enzyme production level by white-rots could be different in various different conditions. In Mn^{2+} supplemented media, LiP activity was completely repressed (Jaouani *et al.*, 2006), in which such LiP repression could also be explained in our medium conditions since it contained 55.90 mg/l $MnSO_4$. Beside the media supplements, also agitation could affect the enzyme production profile of the white rots as in agitated cultures MnP and LiP production was repressed, however laccase production was enhanced (Pointing, 2001, Wesenberg *et al.*, 2003).

Evolutionary engineering approach comprehended the laboratory evolution of organisms under selective stress conditions. Various applicable selective conditions were searched through the literature to choose a selective stress condition for obtaining mutant *C. polyzona* generations. During decolorization processes temperature, pH, dye concentration, agitation, the amount of pellet and cultivation medium were important factors influencing the decolorization percentage. Different white-rot strains grow under different optimum temperatures between 27°C and 30°C (Knapp *et al.*, 2001). 28°C was chosen as the optimum temperature for *C. polyzona* cultivations. White rots usually prefer growth under acidic pH and moreover production of organic acids from carbohydrate utilization during growth causes the pH change in cultivation medium (Knapp *et. al*, 2001). Therefore, pH could not be considered as a selective stress condition because pH might not be stabilized due to pH changes occurred during batch *C. polyzona* cultivations, unless a bioreactor was used. Limited nutrient levels can affect the synthesis and secretion of the ligninolytic enzymes (Wesenberg *et al.*, 2003). Influence of limited carbon and nitrogen sources in growth media were examined by various research groups. High laccase production was observed at high nitrogen concentrations; however, change in laccase activity level also depends on the carbon source included in the medium. In a study with *Phanerochaete chrysosporium* grown in high nitrogen containing cellulose medium, relatively more laccase activity was measured than those grown in low nitrogen containing cellulose medium. When carbon source changed with glucose, no difference was observed at high or low nitrogen containing glucose media (Gianfreda *et al.*, 1999). Moreover, it had to be stated that some white rot strains respond differently to the nitrogen limitation, some strains show higher enzyme production in N-limited cultures whereas some strains can show higher enzyme production in high nitrogen concentrations (ten Have and Teunissen, 2001). Following the literature search, starvation stress was decided to apply on *C. polyzona* strains to obtain mutant generations by changing the nitrogen and carbon concentrations of the cultivation medium. To choose applicable starvation stress concentrations two studies were performed. In first study, from cultures with nitrogen limitation, culture #3 (50g glucose/9g bactopectone) showed the highest laccase activity at 11th day of the cultivation as 78433.73 U/l and from carbon limited cultures, culture #6 (40g glucose/17g bactopectone) showed the highest laccase activity as 160534.10 U/l at 12th day. Following the preliminary study, the second one was designed by choosing

cultures with highest laccase activity from carbon-limited and nitrogen-limited cultures and addition of another culture medium with carbon and nitrogen-limitation, CNLC (40g glucose/9g bactopectone). From both starvation studies, highest laccase activities were detected at the carbon-limited culture #6 (40g glucose/17g bactopectone) rather than nitrogen-limited cultures. Hence, medium concentrations of culture #6 with carbon limitation were adopted for selective stress conditions during evolutionary engineering of *C. polyzona*.

Before designed selection algorithm was applied for evolutionary engineering studies, the first step was EMS mutagenesis on wild type *C. polyzona* cultures. The aim was to increase genetic diversity of the starting population of selection with no loss in wild type characteristics. EMS was used and it can be simply employed on almost any organism (Sauer, 2001). Therefore, 100µl EMS was applied for 15 minutes on *C. polyzona* wild type strain in order to obtain primary mutant cultures.

From EMS mutagenized primary mutant culture, *C. polyzona* mutant populations were obtained at starvation conditions with 8 day cultivation period and at the end of the cultivations, cultures were sub-cultured into fresh starvation conditions applied medium to obtain further mutant generations. Eleven mutant *C. polyzona* generations were obtained. There was a rapid increase in laccase activity at the first generation after the first sub-culture; however, effect of mutations and stability might not be in the desired level, thus we continued to obtaining mutant generations instead of using the first generation. Besides, following the 1st generation there was a sudden decrease in the laccase activity of the 2nd generation, and during the 4th generation, laccase activity increased to 76% of the wild type culture. Afterwards, another decrease was observed in laccase activities of the generations until the 7th generation, which showed 78% of the wild type laccase activity. However, following the 8th generation laccase activity was decreased again and final mutant population was ended at the 11th generation.

The 7th generation was the highest laccase activity producer among all mutant generations. From the 7th and 8th generations, mutant individuals were randomly selected. Only individuals from 7th mutant generation and 9 from 10 randomly selected individuals were grown on PDA plates. No individuals from the 8th generation were able to grow on PDA plates. Probably, accumulation of mutations through generations was high in the 8th generation and therefore, no randomly

selected individuals were able to grow and show any wild type property. From nine individuals, laccase activities of CP701 and CP702 were zero during the 12 day cultivation period. The highest laccase activity measured among the individual mutants was from individual CP706 as 8460.90 U/l, which was the 75% of the wild type activity, also higher than the laccase activity of 7th generation.

Two different reactive dyes, reactive black 5 and RBBR, were used to examine decolorization abilities of the wild type, mutant 7th generation and selected individuals, CP705, CP706, CP707 and CP709, of *C. polyzona*. Wild type *C. polyzona* cultures were able to decolorize 81.65% of the reactive black 5, 92.33% of the RBBR, 86.32% of the remazol turquoise blue G133 and 76.62% of the reactive red 195. Mutant 7th generation was able to decolorize 75.90% of the reactive black 5 and 84.81% of the RBBR. When compared to wild type culture, effectiveness of the decolorization by 7th population was 93% and 92% of the wild type for reactive black 5 and RBBR, respectively, based on the wild type decolorization percentages. Mutant individuals, CP705 decolorize 76.85% of black 5 and 84.51% of RBBR, CP706 decolorize 81.20% of black 5 and 91.32% of RBBR, CP707 decolorize 82.48% of black 5 and 94.94% of RBBR and CP709 decolorize 77.49% of black 5 and 58.97% of RBBR. Among these selected individuals CP706 and CP707 were effective in decolorization and CP707 was even slightly effective than the wild type when decolorization percentages were compared. Besides, the removal of the reactive black 5 by wild type, 7th generation and by selected individuals, CP705, CP706, CP707 and CP709, was 62%, 47%, 53%, 71%, 78% and 57%, respectively, before laccase activity was detected at the 4th day of the cultivation and the removal of RBBR by wild type, 7th generation and by selected individuals, CP705, CP706, CP707 and CP709, was 68%, 84%, 84%, 71%, 70% and 8%, respectively, before laccase activity was detected at the 4th day of the cultivation. In literature, different RBBR and Reactive black 5 decolorization percentages were accomplished at diverse laccase and MnP activity levels. RBBR decolorization by *T. versicolor* CNPR8107 and ATCC20869 was 96% and 98%, respectively with 3 U/l laccase and 65 U/l MnP activities measured from CNPR8107 and 10 U/l laccase and 50 U/l MnP activities measured from ATCC20869 (Toh *et al.*, 2003). *P. ostreatus* decolorized >90% of RBBR with 1200 U/l laccase activity (Palmieri *et al.*, 2005). *T. rubrum* LSK-27 decolorized 83% of reactive black 5 with 98 U/l laccase activity and 86% of

RBBR with 125 U/l laccase activity (Yesiladali *et al.*, 2006). *I. lacteus* decolorized 95% of RBBR by producing 1.0 U/l laccase, 6.4 U/l LiP and 0.6 U/l MnP (Kasinath *et al.*, 2003). *D. squalens* decolorized 84% of RBBR by producing 153600 U/l laccase, however in the same paper it was stated that *I. resinoum* was able to decolorize 97% of RBBR by producing 3100 U/l (Eichlerova *et al.*, 2005). In another paper, RBBR decolorization by *D. squalens* determined as 96-98% with 114 U/l laccase activity (Revankar and Lele, 2007). Low extracellular laccase activities were detected as 11.67 U/l, 2.53 U/l, 4.87 U/l, 1.60 U/l and 1.07 U/l from RBBR added cultures of 7th generation and mutant individuals CP705, CP706, CP707 and CP709, respectively. However, decolorization abilities were similar to wild type; therefore due to suggested biosorption mechanism intracellular laccase activities were examined. After sonication, laccase activities were measured as 112.73 U/l, 22.73 U/l, 35.40 U/l, 26.20 U/l and 25.47 U/l from RBBR added cultures of 7th generation, CP705, CP706, CP707 and CP709, respectively, at the end of the cultivation period. No LiP and MnP activities were detected during RBBR decolorization experiment.

Researches on various isolated white rots were become more favorable in recent years due to their potential in dye decolorization processes. These studies were also joined with strain improvement trials to obtain more suitable organisms for intended industrial applications. In scope of these researches, suitable evolutionary engineering strategies were designed and applied to improve decolorization ability of *C. polyzona*. Obtained mutant generations and selected individuals might further be tested in a suitable bioreactor to determine the feasibility of the use of *C. polyzona* in industrial bioremediation applications. Moreover, to enhance decolorization ability of *C. polyzona*, immobilization on a suitable matrix could also be tried and could be further tested in bioreactor environment. Comparison of dead and living cell decolorization abilities and addition of mediators to broaden the decolorization spectrum of *C. polyzona* could be examined. Finally in long term, alterations, in genetic bases, on desired *C. polyzona* phenotypes obtained by evolutionary engineering can be determined and afterwards further application of rational metabolic engineering can be realized to integrate identified characteristics into a more suitable host for industrial applications or direct utilization of natural host.

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