DOKUZ EYLUL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE INVESTIGATION OF LIGNINOLYTIC ENZYME ACTIVITY VARIATIONS DEPEND ON GROWTH CONDITIONS

by

Merve AKPINAR

July, 2011

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THE INVESTIGATION OF LIGNINOLYTIC ENZYME ACTIVITY VARIATIONS DEPEND ON GROWTH CONDITIONS

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Merve AKPINAR

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We have read the thesis entitled **"THE 1NVESTIGATION OF LIGNINOLYTIC ENZYME ACTIVITY VARIATIONS DEPEND ON GROWTH CONDITIONS"** completed by **MERVE AKPINAR** under supervision of **ASSOC. PROF.DR. RAZİYE ÖZTÜRK ÜREK** and we certify that in our opinion it is fully adcquate, in scope and in quality, as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. Raziye ÖZTÜRK ÜREK

Supervisor

Prof. Dr. Leman TARHAN

(Jury Mcmber)

Prof. Dr. Ayşegül PALA

(Jury Mcmber)

Prof.Dr. Mustafa SABUNCU
Director
Graduate School of Natural and Applied Sciences

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Merve AKPINAR

THE INVESTIGATION OF LIGNINOLYTIC ENZYME ACTIVITY VARIATIONS DEPEND ON GROWTH CONDITIONS

ABSTRACT

Lignin degradation by fungi and their specific enzymes has been focus of a large number of biotechnological studies. The most efficient lignin degraders are fungi of the white rot group. Of these, *Pleurotus eryngii* is commercially important edible mushroom species. This fungus can be cultivated in synthetic medium. Therefore, there has been an increasing trend to ligninolytic enzymes in recent years.

In this study; the aim is to investigate optimal growth conditions for maximum ligninolytic enzyme activities, which are laccase (Lac), manganese peroxidase (MnP), lignin peroxidase (LiP) and aryl alcohol oxidase (AAO) enzymes from *P. eryngii* in submerged fermentation (SF). In addition, reducing sugar, nitrogen and protein levels, biomass and pH levels were also researched. For this purpose, the effects of basal medium and flask volume, different liquid medium, inoculation type and temperature, cultivation mode, nitrate, manganese and copper concentrations were studied for SF conditions. In SF, cultivation was performed with plug inoculation and stationary incubation at degree of 28.

According to the results, the highest Lac and MnP activities were detected as 973.333 and 756 U/L, while LiP and AAO were obtained as 42.527 and 271.828 U/L, respectively. The reducing sugar and nitrogen levels in SF were sharply decreased on the fifth day. Finally, decolorization of methyl orange, reactive red 2 and reactive black 5 was performed with the ligninolytic enzyme obtained by SF. These results showed that the investigated ligninolytic enzymes can be used decolorization of azo dyes.

Keywords: *Pleurotus eryngii*, submerged fermentation, laccase, manganese peroxidase, lignin peroxidase, aryl alcohol oxidase

LİGNİNOLİTİK ENZİM AKTİVİTE DEĞİŞİMLERİNİN BÜYÜME KOŞULLARINA BAĞIMLI İNCELENMESİ

ÖZ

Funguslar ve spesifik enzimleri tarafından lignin degradasyonu pek çok biyoteknolojik çalışmanın odak noktasını oluşturmaktadır. En etkili lignin degrade edicileri, beyaz çürükçül grubu funguslardır. Bunlardan *Pleurotus eryngii* ticari açıdan önemli yenilebilir mantar türlerindendir. Bu fungus sentetik ortamlarda ya da bitki rezidülerini içeren çok çeşitli substratlar üzerinde kültive edilebilir. Bundan dolayı, son yıllarda ligninolitik enzimlere artan bir ilgi bulunmaktadır.

Bu çalışmada amaç, derin kültür fermentasyonunda (DKF) *P. eryngii* den lignin peroksidaz (LiP), mangan peroksidaz (MnP), aril alkol oksidaz (AAO) ve lakkaz (Lak) ligninolitik enzimlerinin maksimum aktiviteleri için optimum büyüme koşullarının araştırılmasıdır. Buna ilaveten, indirgen şeker, azot ve protein seviyeleri, biyokütle ve pH seviyeleri de incelenmiştir. Bu amaçla, bazal ortam ve erlenmayer hacimlerinin, farklı sıvı ortamların, aşılama tipi ve sıcaklığın, kültivasyon modunun, nitratın, mangan ve bakır iyonları derişimleri SF koşulları için çalışılmıştır. DK de kültivasyon, agar kesitiyle aşılama ve durağan inkübasyonda 28 derecede gerçekleştirildi.

Sonuçlara göre, *P. eryngii* tarafından en yüksek Lak ve MnP aktiviteleri sırasıyla 973.333 ve 756 U/L iken, LiP ve AAO aktiviteleri 42.527 ve 271.828 U/L dir. DK de indirgen şeker ve azot seviyeleri inkubasyonun beşinci gününde süratle azalmıştır. Son olarak, Metil Oranj, Reaktif Kırmızı 2 ve Reaktif Siyah 5 boyalarının dekolorizasyonları DKF den elde edilen ligninolitik enzimlerle gerçekleştirilmiştir. Bu sonuçlar incelenen ligninolitik enzimlerin azo boyaların dekolorizasyonunda kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Pleurotus eryngii*, derin kültür fermentasyonu, lakkaz, mangan peroksidaz, lignin peroksidaz, aril alkol oksidaz

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CHAPTER ONE

INTRODUCTION

1.1 Lignin

Lignin is one of the three major polymeric components found in the cell walls of higher order plants. Along with the other two major components, cellulose and hemicellulose, lignin forms a highly efficient composite system that is synthesized entirely from carbon, oxygen, hydrogen, and energy from the sun. Lignin's role in this composite is to act as a matrix material that binds the plant polysaccharide microfibrils and fibers, thereby imparting the strength and rigidity to the plant stem necessary for vertical growth (Feldman, 2002). Lignin also performs other biological functions; including helping protect plants from biological attack and assisting in water transport by sealing plant cell walls against water leaks. Though the total lignin content varies widely from plant to plant, it is estimated that a total of 30% of the organic carbon in plant biomass worldwide is contained in lignin (Boudet, 2000).

Industrially, lignin is of great interest due to the existence of large scale manufacturing processes dependent on retrieving the polysaccharide component of plants. Among these processes is wood pulping, one of the largest industries in the world. The main objective of wood pulping is to separate individual fibers from wood, and to do so lignin must first be removed. Huge amounts of lignin are extracted by wood pulping and other industries annually, though the bulk of it is either burned to recover energy or is otherwise considered waste. Only about 1 to 2% of this lignin is used to make other products (Lora, & Glasser, 2002).

Lignin is the second most abundant biopolymer on earth after the plant polysaccharide cellulose (Sarkanen, & Ludwig, 1971), and it is abundant plant biopolymers accounting for approximately 30% of the organic carbon in the biosphere. Because of the essential role of lignin for plant life and its relevance for a

number of agro-industrial processes, the biosynthetic pathway leading to the monolignols, their polymerization and the final structure of the lignin polymer have been intensively studied over many decades (Ralph, Brunow, & Boerjan, 2007).

Lignin is a phenolic heteropolymer associated with cellulose and hemicellulose in the secondary cell walls of vascular elements, fibres and sclereids in vascular plants. Lignin, especially which in trees, accounts for an enormous reservoir of organic carbon in the biosphere. This polymer is synthesized by the generation of free radicals, which are released in the peroxidase-mediated dehydrogenation of three phenyl propionic alcohols: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (p-hydroxyphenyl propanol), and sinapyl alcohol (syringyl propanol). This heterogeneous structure is linked by C–C and aryl-ether linkages, with aryl-glycerol β-aryl ether being the predominant structures (Croteau, Kutchan, & Lewis, 2000). Lignin monomers are differentially targeted to discrete regions (lignin initiation sites) of various lignifying cell walls; for example, p-coumaryl alcohol is mainly targeted to the middle lamella and coniferyl alcohol to the secondary wall of the xylem elements (Figure 1.1) (Fukushima, & Terashima, 1991).

Lignin deposition occurs after the completion of cell growth and when the three layers of secondary cell walls, the outer (S1), middle (S2) and inner (S3), are assembled during thickening of the secondary cell wall. Lignin deposition begins at the cell corners in the primary cell wall and in the middle lamella when S1 formation has initiated Figure 1.1. After the deposition of polysaccharides, lignification then proceeds in two distinct stages through the S2 and S3 layers (Boerjan, Ralph, & Baucher, 2003).

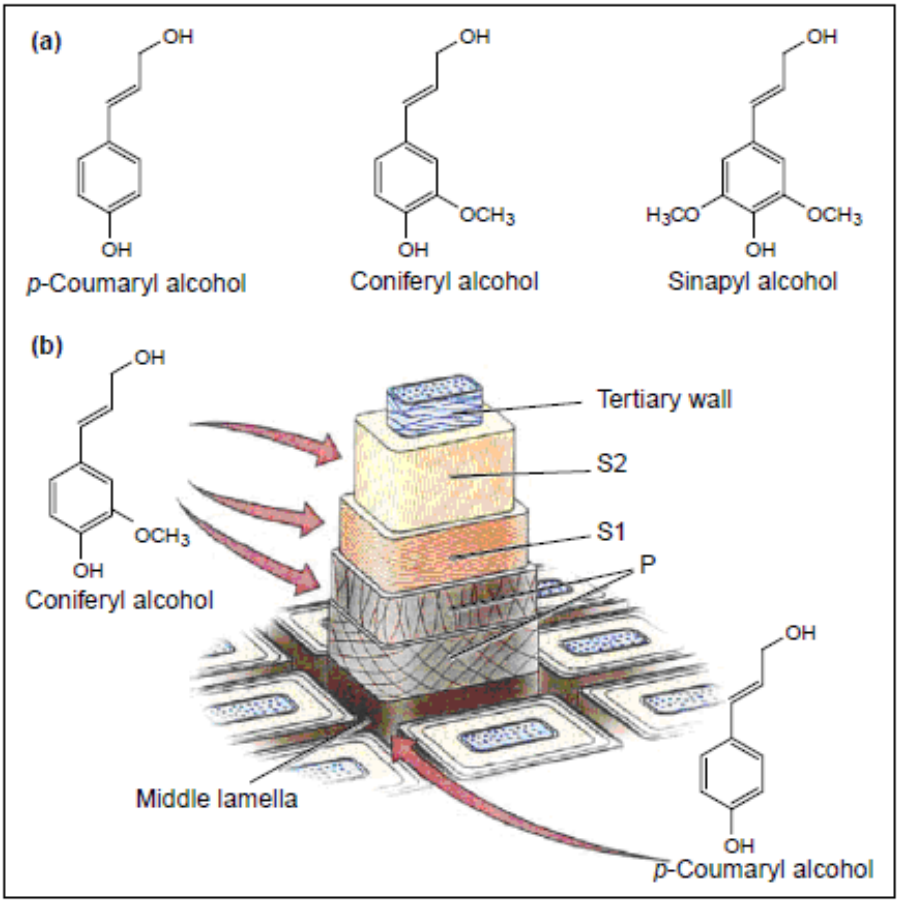


Figure 1.1 Monolignols and differential cell-wall targeting.(a) chemical structures of monolignols and (b) telescopic representation of a conifer tracheid (Croteau, Kutchan, & Lewis, 2000).

Lignin is one of the main structural elements of wood, and in angiosperms it is mainly composed of guaiacyl (G) and syringyl (S) monomers. Considerable scientific interest has focused on the formation of S lignin in woody angiosperms due to the association between the wood S lignin content and the delignification properties i.e. in chemical pulping processes. In gymnosperms lignin is composed mainly of G units, whereas in angiosperms S units are also involved. The third phenylpropanoid unit, p-hydroxyphenyl (H), is mostly involved in grass (Gramineae) lignins (Boerjan, Ralph & Baucher, 2003), but it is also a minor element both in gymnosperm and angiosperm lignin (Cabane, et al., 2004) (Figure 1.2).

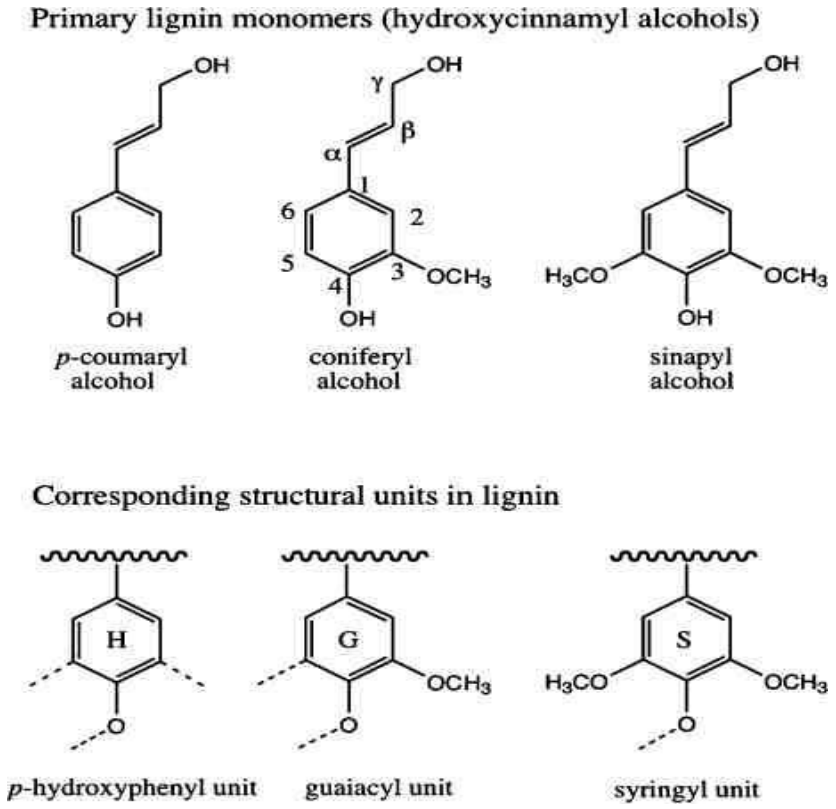


Figure 1.2 Primary lignin monomers and corresponding lignin unit.

Lignin composition in terms of the H: G: S ratio varies between different vascular plant groups. Woody gymnosperms (softwoods) have the highest lignin content, and their lignin is made up mostly of G units. By contrast, lignin of woody angiosperms (hardwoods) consists of S and G units, and that from non-woody angiosperms contains also H units (Martínez, et al., 2005).

The ability to synthesize lignin has created the necessary conditions for the terrestrial lifestyle of plants. The main function of lignin is to provide structural integrity of the cell walls, which is crucial for woody plants with a high need for structural support and stem rigidity. As a hydrophobic molecule, lignin waterproofs the cell walls and is hence elementary for the transport of water and solutes in the xylem (the vascular system) (Boerjan, Ralph, & Baucher, 2003). The insolubility and complex structure of lignin polymers lead to high resistance against microbial degradation, and they thus play an important role in plant defence (He, & Wolyn,

2005). From the human point of view, however, lignin has a negative impact on wood pulping because the need to extract lignin from cellulose fibres both decreases the yield of cellulose and is an economically and environmentally costly process (Baucher, Halpin, Petit-Conil, & Boerjan, 2003).

Lignin quantity and quality vary naturally (Campbell, & Sederoff, 1996). Depending on the species of woody plant the lignin content may vary from 15 to 36% of the dry weight of wood (Higuchi, 2006). Angiosperm wood is typically composed of vessels, fibres and ray parenchyma cells, and the secondary cell walls of vessels have a higher lignin concentration compared to the fibres and ray parenchyma cells (Boerjan, Ralph, & Baucher, 2003). The vessel wall lignins are also enriched with G units, whereas in the fibre and ray cell wall lignin S units predominate in Betula (Saka, & Goring, 1988) and many other hardwood species (Musha, & Goring, 1975). In general, during secondary cell wall assembly the G units are deposited prior to the S units (Terashima, Fukushima, Tsuchiya, & Takabe, 1986; Bhalerao, Nilsson, & Sandberg, 2003). The S/G ratio may also change during the development of the plant, and the S unit content of lignin especially has been found to increase with age (Grand, Boudet, & Ranjeva, 1982). The increased proportion of S monomers is also indicative of tension wood, i.e. the reaction wood specific to angiosperm species that is formed as a result of the mechanical stress of stem bending (Sarkanen, & Hergert, 1971).

Biological role of lignin is to strengthen the plant cell walls by adhesion of the layers of cellulose microfibrils, and thereby also enhance water transport and resistance to pathogens (Boerjan, Ralph, & Baucher, 2003; Higuchi, 1997).

1.2 Lignin Biodegradation and Ligninolytic System

Lignocellulose degradation is a central step for carbon recycling in land ecosystems (Martínez, et al., 2005). Wood-rotting basidiomycetes are classified as

white-rot and brown-rot fungi based mainly on macroscopic aspects (Schwarze, Engels, & Mattheck, 2000).

Lignin is chemically difficult to degrade because the free radical coupling mechanism responsible for its biosynthesis from phenolic cinnamyl alcohols results in a polymer interconnected through diverse carbon–carbon and ether bonds that are not hydrolyzable under biological conditions (Boerjan, Ralph, & Baucher, 2003). The predominant structures, making up about half the total, are β-O-4-linked ethers, followed by phenylcoumarans, resinols, and various minor subunits. It has clearly been shown that lignin is racemic, and consequently evens a simple β-O-4-linked dimer, with two asymmetric carbons, exists as four stereo isomers. Since the number of isomers increases geometrically with the number of subunits, the three-dimensional surface presented by lignin is complex and non-repeating (Ralph, et al., 1998).

There were several structural models of lignin (Brunow, 2001). Due to its complicated structure and nonhydrolyzable bonds, lignin is more difficult to breakdown than cellulose or hemicellulose. The molecular weight (MW) of lignin is high, about 1000 kDa or more, which prevents its uptake inside the microbial cell (Erikson, Blanchette, & Ander, 1990). Thus, the biological degradation of macromolecular lignin must occur through the activity of extracellular enzymes (Kuhad, Singh, & Eriksson, 1997).

The degradation of lignin by filamentous fungi is a major route for the recycling of photosynthetically fixed carbon, and the oxidative mechanisms employed have potential biotechnological applications. The lignin peroxidases (LiPs), manganese peroxidases (MnPs), and closely related enzymes of white rot basidiomycetes are likely contributors to fungal ligninolysis. Many of them cleave lignin model compounds to give products consistent with those found in residual white-rotted lignin, and at least some depolymerize synthetic lignins. However, none has yet been shown to delignify intact lignocellulose *in vitro*. The likely reason is that the

peroxidases need to act in concert with small oxidants that can penetrate lignified tissues. Recent progress in the dissolution and NMR spectroscopy of plant cell walls may allow new inferences about the nature of the oxidants involved. Furthermore, increasing knowledge about the genomes of ligninolytic fungi may help us decide whether any of the peroxidases has an essential role (Hammel, & Cullen, 2008).

1.2.1 Lignin Degrading Fungi

Fungal wood decay may lead to white or brown rot, or so called soft rot. The white rot basidiomycetous fungi are the only organisms able to depolymerize and even mineralize (to CO_2 and H_2O) all the components of wood, primarily cellulose, hemicellulose and lignin (Kertsen, & Cullen, 2007) with a remarkable ability to decompose the coloured, aromatic, heterogenous and persistent lignin phenylpropanoid units naturally synthesized by plants in their cell walls (Higuchi, 2006).

The organisms commonly known as fungi are a tremendously diverse group found in virtually all habitats. Because of their diverse nature, the fungi are a difficult group to define. Perhaps the best way to describe these fascinating creatures and introduce them to you is to consider the characteristics that most of them share (Kirk, Connors, & Zeikus, 1976).

Concepts of wood decay continue to be largely based on a limited range of fungi, primarily in the families *Polyporaceae*, *Hymenochaetaceae* and *Corticiaceae* (Aphyllophorales) (Worrall, Anagnost, & Zabel, 1997). Other basidiomycetes can also cause degradation of wood, but comprehensive studies on decay by such fungi are rare (Seifert, 1983).

The only organisms reported to degrade lignin efficiently are the white-rot fungi that under natural conditions mostly colonize dead or living wood (Erikson, Blanchette, & Ander, 1990). Wood-degrading fungi are divided into 3 groups based

on the type of rot they cause in wood: white-rot, brown-rot and soft-rot fungi. White-rot fungi attack the lignin component of wood and leave the cellulose and hemicellulose less affected. White-rot and brown-rot fungi both belong to the basidiomycetes, whereas soft-rot fungi are ascomycetes, and their activity is usually related to high or low moisture content of wood (Blanchette, 1995). Selective lignin degraders are especially interesting from the standpoint of biotechnological applications, since they remove lignin and leave the valuable cellulose intact. Lignin degradation by these fungi is thought to occur during secondary metabolism and typically under nitrogen starvation. However, a wide variety of lignin degradation efficiency and selectivity abilities, enzyme patterns and substrates enhancing lignin degradation are reported from these fungi (Hatakka, 2001; Hofrichter, 2002).

Soft-rot fungi can degrade wood under extreme environmental conditions (high or low water potential) that prohibit the activity of other fungi (Martínez, et al., 2005).

Brown-rot fungi, which grow mainly on softwoods, represent only 7% of wood-rotting basidiomycetes. This group of basidiomycetes can degrade wood polysaccharides after only a partial modification of lignin, resulting in a brown material consisting of oxidized lignin, which represents a potential source of aromatic compounds for the stable organic matter fraction in forest soils (Martínez, et al., 2005).

1.2.1.1 White Rot Fungi

In the beginning of the white rot decay, the cellulose macromolecules are mainly left intact, which also gives the white appearance for the decayed wood (Lundell, Mäkelä, & Hildén, 2010).

White rot fungi is a rather heterogeneous group of organisms. They have in common the capacity to degrade lignin as well as the other wood components and the ability to produce extracellular enzymes which also oxidize phenolic compounds

related to lignin. This is the reason why phenolic compounds have been utilized for the identification of white rot fungi. Their ability to metabolize large amounts of lignin in wood is unique among microorganisms. The relative amounts of lignin and polysaccharides degraded and utilized by these fungi vary and so does the order of preferential attack. Some species preferentially remove lignin from wood leaving pockets of white, degraded cells that consist entirely of cellulose, while others degrade lignin and cellulose simultaneously. They often cause a bleaching of normal wood coloration. The normal method of wood degradation by white-rot fungi is for the celluloses and the lignin to be attacked simultaneously. However, there are examples of a specific degradation of the middle lamella lignin (Kaarik, 1974). It has been demonstrated that a totally specific attack on the lignin by white-rot fungi cannot be undertaken. This is most likely so because so much energy is required to degrade lignin that an additional, more easily accessible energy source is also necessary. A progressive erosion of the cell wall occurs when components are degraded simultaneously or a diffuse attack of lignin may occur by species that preferentially remove lignin (Ander, & Eriksson, 1976).

White rot fungi are the most efficient ligninolytic organisms (Stalpers, 1976; Seifert, 1983). This capability to degrade lignin is due to their extracellular non-specific and non-stereoselective enzyme system composed by laccases, lignin peroxidases and manganese peroxidases, which function together with H_2O_2-producing oxidases and secondary metabolites (Kirk, & Farrell, 1987).

White rot fungi are basidiomycetes that are capable of degrading a lignocellulose substrate. There are other fungi capable of doing this, but they do not produce the same enzymes that are portentous for the research into pollution removal. They are called white rot because the degradation process results in a bleaching of the wood substrate. Fungi are robust organisms that have a high tolerance to toxic environments, making them ideal to use for bioremedial purposes. They can also withstand high temperatures and a wide range of pH, further enhancing their hardy capabilities. *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus ostreatus* are

examples of the white rot fungi (Kirk, & Farrell, 1987; Erikson, Blanchette, & Ander, 1990; Hatakka, 1994).

1.2.1.2 Pleurotus eryngii

The genus *Pleurotus* (Jacq.: Fr.) Kumm. (Pleurotaceae, higher Basidiomycetes) is a cosmopolitan group of mushrooms with high nutritional value, therapeutic properties and various environmental and biotechnological applications (Cohen, Persky, & Hadar, 2002). *Pleurotus* spp are saprophytic fungi that are cultivated on lignin and cellulose-containing substrates, such as cotton stalks, wheat/rice straw and sawdust. Utilization of these materials is clearly dependent on *Pleurotus*' ability to secrete a range of enzymes, including peroxidases, laccases, cellulases, hemicellulases and xylanases. One of the advantages of the *Pleurotus* group is their ability to grow on lignocellulosic substrates without the need for a composting or casing layer. Since *Pleurotus* spp can decompose lignocellulose efficiently without chemical or biological pretreatment, a large variety of lignocellulosic wastes can be utilized and recycled. Some examples of the agricultural wastes studied as substrates for *Pleurotus* spp are coffee pulp, flax shive, corn cob, sugarcane bagasse, citronella bagasse and rice hulls (Wood, & Smith, 1987).

Lignin biodegradation is an oxidative process, involving enzymes such as lignin peroxidase (LiP), MnP, VP and laccase (Kirk, & Farrell, 1987; Erikson, Blanchette & Ander, 1990; Hatakka, 1994; Tuor, Winterhalter, & Fiechter, 1995; Camarero, Sarkar, Ruiz-Due˜nas, Martínez, & Martínez, 1999). Different fungal species can either modify or completely degrade all the major components of wood. The ligninolytic system of *Pleurotus* spp has been extensively studied in recent years. Three ligninolytic enzyme families have been characterized: MnP, versatile peroxidase (VP) and laccase (Hatakka, 1994; Tuor, Winterhalter, & Fiechter, 1995; Camarero, et al., 1999). These enzymes can be used for various biotechnological and environmental applications. *Pleurotus* spp. and their enzymes may serve as an

efficient alternative for the bioremediation of resistant pollutants, as compared with non-ligninolytic micro-organisms (Cohen, Persky, & Hadar, 2002).

1.2.2 Ligninolytic Enzymes

Lignin degrading enzymes (LDEs) belong to two classes viz the heme-containing peroxidases and the copper-containing laccases. The peroxidases comprise of MnP, LiP and versatile peroxidase (VP). A series of redox reactions initiated by the LDEs degrade lignin or structures analogous to lignin subunits such as aromatic compounds .The LDEs oxidize the aromatic compounds until the aromatic ring structure is cleaved, which is followed by further degradation with other enzyme (Figure 1.3).

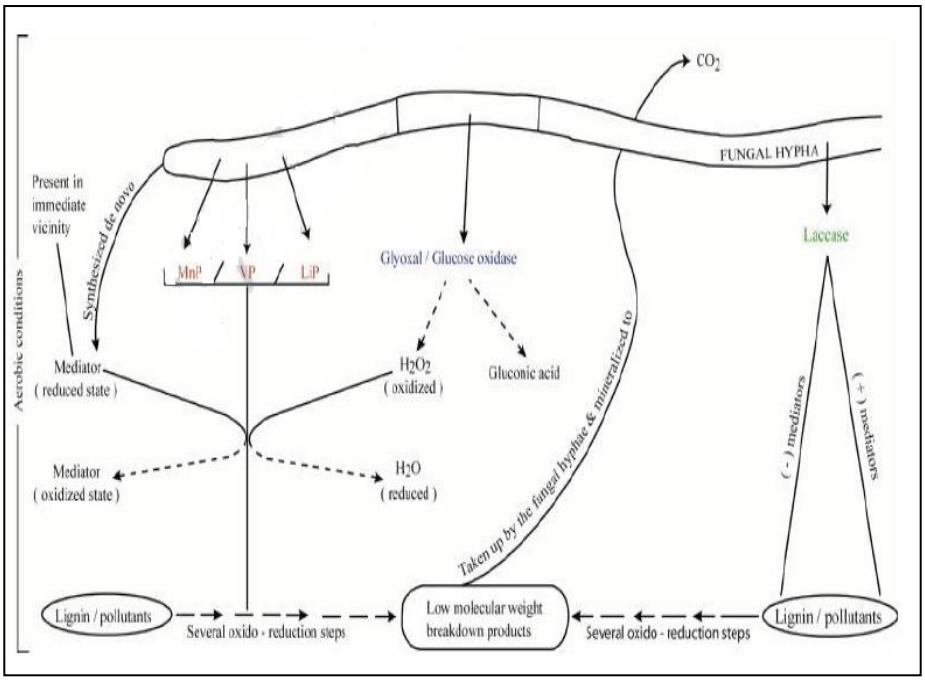


Figure 1.3 Shematic diagram showing the role an activity of various extracellular lignin-degrading enzymes produced by fungi (Raghukumar, 2008).

Lignin breakdown is thought to occur by concomitant action of ligninolytic enzymes. The main extracellular enzymes participating in lignin degradation are heme-containing ligninase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC

1.11.1.13) and Cu-containing laccase (Lac, benzenediol: oxygen oxidoreductase, EC 1.10.3.2) (Hatakka, 2001). These types of peroxidase were isolated from the white-rot fungi, *P. ostreatus* (Cohen, Persky, & Hadar, 2002), *Bjerkandera* sp. BOS55 (Palma, Martínez, Lema, & Martínez, 2000). *Lentinula edodes* Mn-dependent peroxidase also oxidizes veratryl alcohol (D'Annibale, Crestini, Mattia, & Sermanni, 1996), while MnP from *Panus tigrinus* is able to degrade nonphenolic lignin model compounds (Maltseva, Niku-Paavola, Leontievsky, Myasoedova, & Golovleva, 1991).

The lignin-degrading enzymes known so far are extracellular and nonspecific, participating in different oxidative reactions where the aromatic structure of lignin and bonds between the basic units are broken (Erikson, Blanchette & Ander, 1990; Orth & Tien, 1995; Kuhad, Singh, & Eriksson, 1997). The resulting small-molecular-weight compounds can then be transported inside the cell for further breakdown by fungi and also bacteria. Cell-free mineralization of synthetic ^{14}C-labelled and natural lignin by one of the ligninolytic peroxidases MnP, was first reported by Hofrichter (2002). This may point to the extracellular mineralization (outside the fungal cell) of lignin.

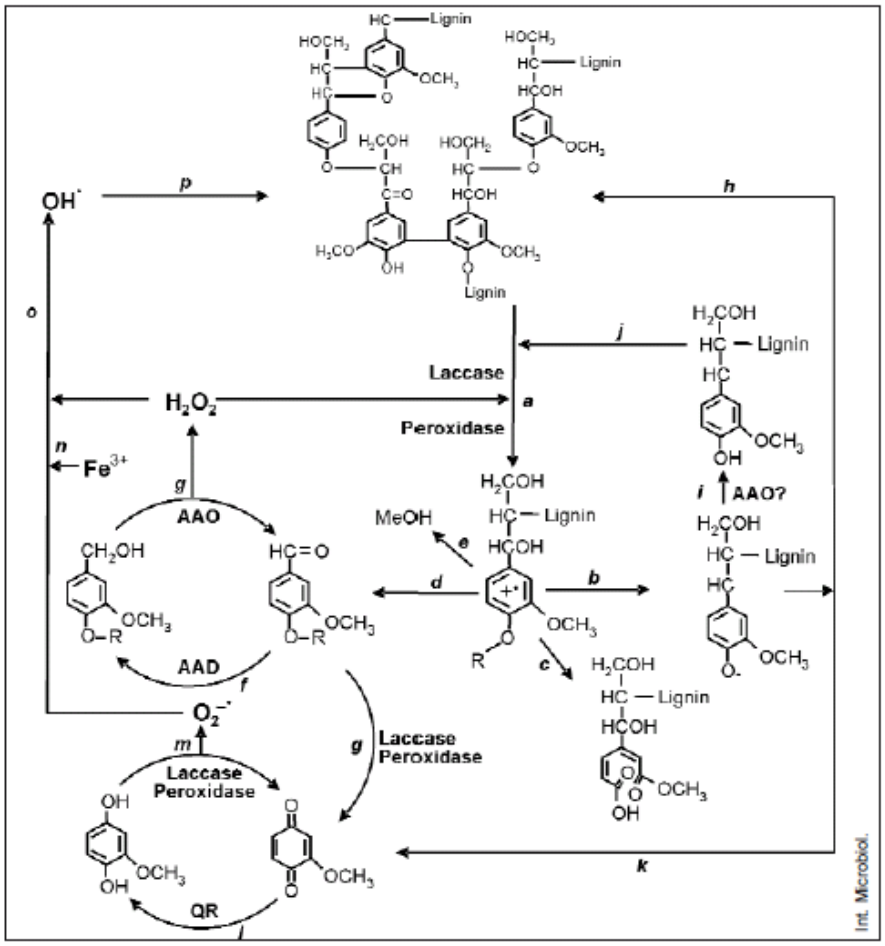


Figure 1.4 Scheme for lignin biodegradation including enzymatic reactions and oxygen activation (Gutiérrez, & Martínez 1996).

As shown in Figure 1.4, laccases or ligninolytic peroxidases (LiP, MnP, and VP) produced by white-rot fungi oxidize the lignin polymer, thereby generating aromatic radicals (a) (Erikson, Blanchette, & Ander, 1990). These evolve in different non-enzymatic reactions, including C4-ether breakdown (b), aromatic ring cleavage (c), Cα-C_β breakdown (d), and demethoxylation (e) (Gutiérrez, & Martínez, 1996). The aromatic aldehydes released from Cα-C_β breakdown of lignin, or synthesized de novo by fungi (f, g) (Gutiérrez, Caramelo, Prieto, Martínez, & Martínez, 1994) are the substrate for H_2O_2 generation by aryl alcohol oxidase (AAO) in cyclic redox reactions involving also aryl alcohol dehydrogenase (Guillén, Martínez, Martínez, & Evans, 1994). Phenoxy radicals from C4-ether breakdown (b) can repolymerize on

the lignin polymer (h) if they are not first reduced by oxidases to phenolic compounds (i), as reported for AAO (Marzullo, Cannio, Giardina, Santini, & Sannia, 1995). The phenolic compounds formed can be again reoxidized by laccases or peroxidases (j). Phenoxy radicals can also be subjected to Cα-C_β breakdown (k), yielding p-quinones. Quinones from g and/or k contribute to oxygen activation in redox cycling reactions involving qinone reductase, laccases, and peroxidases (l, m) (Guillén, Martínez, Muñoz, & Martínez, 1997). This results in reduction of the ferric iron present in wood (n), either by superoxide cation radical or directly by the semiquinone radicals, and its reoxidation with concomitant reduction of H_2O_2 to hydroxyl free radical ($OH^{\cdot}$) (o) (Guillén, Gómez-Toribio, Martínez, & Martínez, 2000). The latter is a very strong oxidizer that can initiate the attack on lignin (p) in the initial stages of wood decay, when the small size of pores in the still-intact cell wall prevents the penetration of ligninolytic enzymes (Evans, Dutton, Guillén, & Veness, 1994). Then, lignin degradation proceeds by oxidative attack of the enzymes described above. In the final steps, simple products from lignin degradation enter the fungal hyphae and are incorporated into intracellular catabolic routes (Martínez, et al., 2005).

1.2.2.1 Lignin peroxidases

Lignin peroxidise (LiP) is a monomeric heme-glycoprotein peroxidase secreted by wood-degrading fungi. LiPs were first found in the lignin-degrading fungus *P.chrysosporium*, it is part of the extracellular enzyme system of this white-rot fungus (Venkatadri, & Irvine, 1993). They catalyze the oxidation of nonphenolic aromatic compounds. The resulting cation radicals are further decomposed chemically (Conesa, Punt, & van den Hondel, 2002; Martínez, 2002). LiP was shown to mineralize a variety of recalcitrant aromatic compounds and to oxidize a number of polycyclic aromatic and phenolic compounds (Aitken, Massey, Chen, & Heck, 1994). The role of LiP's in lignin depolymerization has also been confirmed (Cornwell, Tinland-Butez, Tardone, Cabasso, & Hammel, 1990). LiP plays a central role in the biodegradation of the plant cell wall constituent lignin. The molecular

mass of LiPs from different white rot fungi strains varies from 37 to 50 kDa (Hirai, Sugiura, Kawai, & Nishida, 2005), pH and temperature activity profiles of LiPs from different sources vary significantly with optimum activities shown between pH 2–5 and 35–55˚C, respectively (Yang, Yuan, & Chen, 2004; Asgher, Asad, Bhatti, & Legge, 2007).

LiP catalyzed veratryl alcohol oxidation; veratryl alcohol has played a pivotal role in the study of the lignin biodegradation process and the optimum pH of the enzyme is rather low (pH = 2.5). The fact that LiP will oxidize (non-phenolic) compounds with a relatively high redox potential has been interpreted by many authors as the result of an unusually high redox potential of the oxidized enzyme intermediates, lignin peroxidase Compound I (LiP-I) or Compound II (LiP-II).

The general mechanism of LiP catalyzed reaction consists of two steps:

(1) A $2e^-$ oxidation of the native ferric enzyme [Fe(III)] to yield compound I intermediate that exists as a ferry iron porphyrin radical cation [$Fe(IV)=O^+$, LiP-I], with the peroxide substrate (H_2O_2) cleaved at the O – O bond (Figure 1.5).

$$\text{LiP [Fe(III)]} + H_2O_2 \rightarrow \text{LiP I [Fe (IV) = O}^+\text{]} + H_2O$$

(2) A two consecutive $1e^-$ reduction of LiP-I by electron donor substrates to the native enzyme. The first $1e^-$ reduction of LiP-I by a reducing substrate, such as VA, yields compound II [Fe (IV) = O, LiP-II] and a VA radical cation (VA^+). A second $1e^-$ reduction returns the enzyme to the ferric oxidation state, completing the catalytic cycle. LiP-I can also return to the native (resting) enzyme by a direct $2e^-$ reduction in some cases.

$$\text{LiP-I} + \text{AH} \rightarrow \text{LiP-II [Fe (IV) = O]} + A'^+$$

$$\text{LiP-II} + \text{AH} \rightarrow \text{LiP} + A^+$$

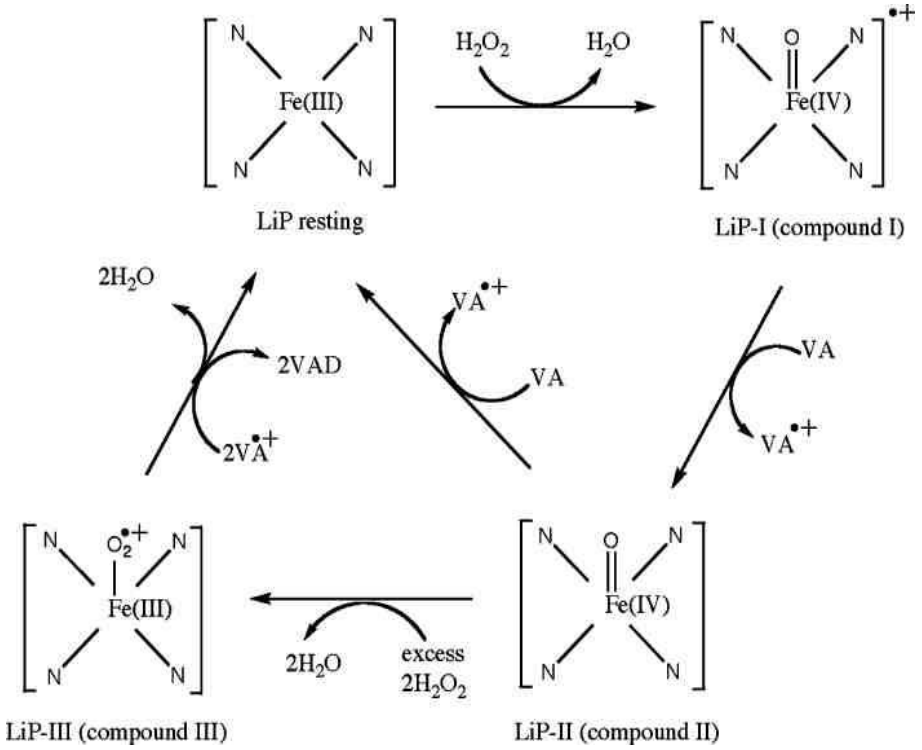


Figure 1.5 Catalytic cycle of lignin peroxidase.

Fungal LiPs are globular and mostly helical glycoproteins of about 40 kDa (including carbohydrate), with 343-344 amino acids (pI 4.15), depending on the isozyme (Piontek, Smith, & Blodig, 2001). The tree-dimension structure of LiP is presented in Figure1.6.

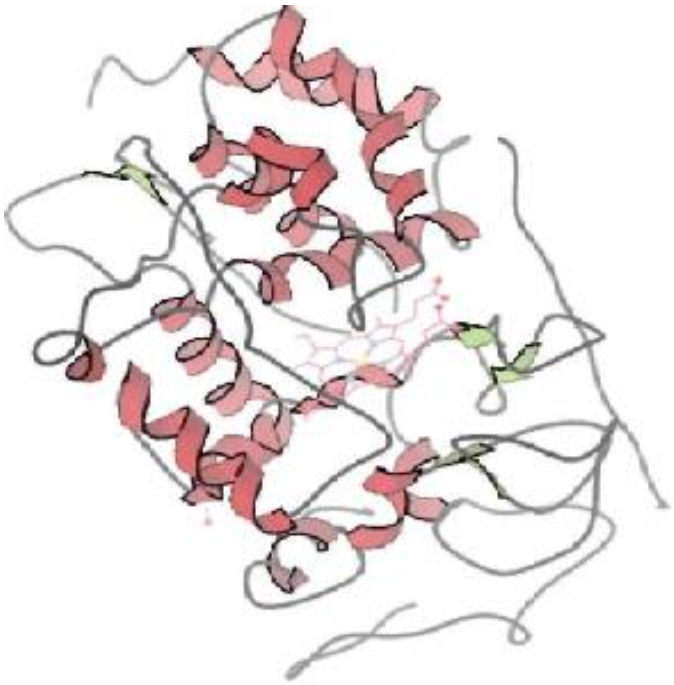


Figure 1.6 Three-dimensional structure of *P. chrysosporium* lignin peroxidase.

The stability of LiP, which influences greatly the economic and technical feasibility of the enzyme's application in waste treatment, was investigated (Aitken, & Irvine, 1989). Cornwell et al. (1990) reported an immobilization of LiP on porous ceramic supports did not adversely affect its stability and showed a good potential for degradation of environmentally-persistent aromatics. Venkatadri, & Irvine (1993) developed a silicon membrane reactor that could be used for hazardous waste treatment and LiP production.

1.2.2.2 Manganese peroxidase

The manganese peroxidises (MnPs) are produced by lignin degrading fungi. MnPs are extracellular glycoproteins with an iron protoporphyrin IX (heme) prosthetic group (Asgher, Kausara, Bhattia, Shah, & Ali, 2008). MnPs oxidize phenolic compounds to phenoxy radicals by oxidation of Mn (II) to Mn (III) with H_2O_2 as an oxidant. Mn (III) is chelated by organic acids (e.g. oxalate or malate in

nature). Chelated Mn (III) oxidizes phenolic lignin compounds to phenoxy radicals that degrade spontaneously (Hofrichter, 2002). MnP oxidizes a wide range of compounds from lignin to polycyclic aromatic hydrocarbons (Steffen, 2003). The MW of extracellular fungal MnPs varies from 40 to 50 kDa, and the isoelectric point (pI) is usually acidic (pI 3-4), but neutral MnPs have also been found. (Figure 1.7) Optimum pH of 4–7 and optimum temperature of 40–60ºC of MnPs are found (Ozturk Urek, & Pazarlioglu, 2007; Baborova´, Moder, Baldrian, Cajthamlova´, & Cajthaml, 2006).

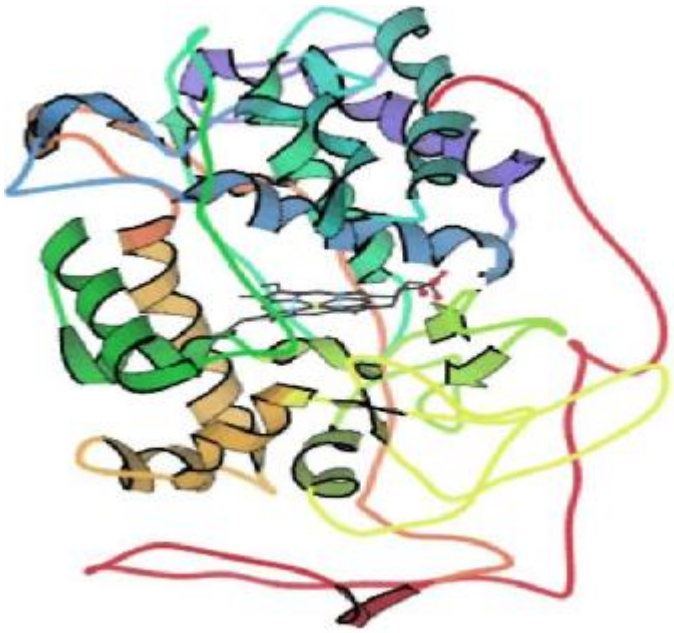


Figure 1.7 Three-dimensional structure of *P. chrysosporium* manganese peroxidase.

The MnP, produced by *P. chrysosporium* has also been observed to catalyze the oxidation of several monoaromatic phenols, and aromatic dyes, but these reactions depend on the presence of both divalent manganese and certain types of buffers (Aitken, & Irvine, 1989). The catalytic mechanism was presented in the Figure 1.8. In fact, MnP catalyzes the oxidation of Mn (II) to Mn (III) in the presence of Mn (III)-stabilizing ligands. The resulting Mn (III) complexes can then carry out the oxidation of organic substrates (Aitken, et al., 1994). MnPs are also strongly

oxidizing and undergo a classical peroxidase cycle but do not oxidize nonphenolic lignin-related structures directly because they lack the invariant tryptophan residue required for electron transfer to aromatic substrates. Instead, they have a manganese-binding site that consists of several acidic amino acid residues plus one of the heme propionate groups. Accordingly, one-electron transfer to Compound I of MnP occurs from bound Mn^{2+} and Compound II of MnP is formed (Sundaramoorthy, Kishi, Gold, & Poulos, 1994; Wariishi, Valli, & Gold, 1992). Also, the other product, Mn^{3+}, is released from the active site if various bidentate chelators are available to stabilize it against disproportionation to Mn^{2+} and insoluble Mn^{4+}. The physiological chelator is thought to be oxalate, an extracellular metabolite of many white rot fungi (Kuan, Johnson, & Tien, 1993).

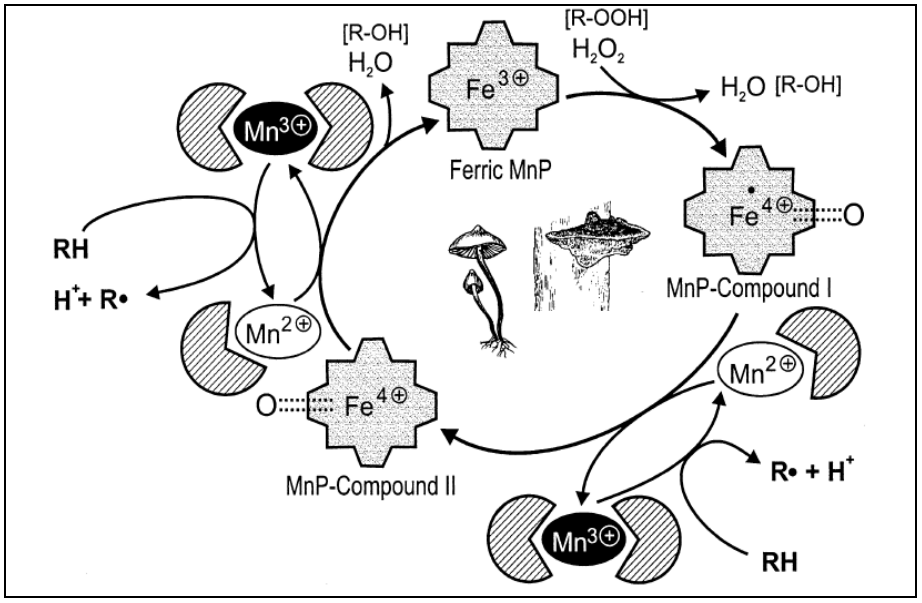


Figure 1.8 The catalytic cycle of manganese peroxidase (MnP).

This reaction is evidently to transfer the oxidizing power of MnP to a small agent – Mn^{3+} – that can diffuse into the lignified cell wall and attack it from within. This is an attractive feature of MnP action, as the low permeability of intact lignocellulose is directly addressed. However, the stability conferred by anionic chelators such as

oxalate increases the electron density on the Mn^{3+}, thus making it a considerably weaker oxidant. As a result, the Mn^{3+}-organic acid chelates produced by MnPs are unable to oxidize the predominating nonphenolic structures of lignin by electron transfer. Mn^{3+} chelates can attack the infrequent phenolic structures in lignin, but these units probably occur largely as end groups on the polymer (Boerjan, Ralph, & Baucher, 2003) and their oxidation does not result in extensive ligninolysis (Hammel, et al., 1993).

1.2.2.3 Laccase

Laccases (Lacs) have been known for many years in plants, fungi, and insects, where they play a variety of roles, including synthesis of pigments, fruit-body morphogenesis, and detoxification (Mayer, & Staples, 2002). Fungal phenoloxidases have a low redox potential that allows direct oxidation only of phenolic lignin units, which often comprise less than 10% of the total polymer (Martínez, et al., 2005).

Fungal laccases are mainly extracellular glycoproteins with carbohydrate content of 10–30%, typical molecular mass of 60–80 kDa, and acidic pI values of 3–6 (Hildén, Hakala, & Lundell, 2009), optimum temperature of Lacs is 40 to 65˚C (Asgher, et al., 2008).

The Lacs are multicopper phenol oxidases that catalyze the oxidation of a variety of phenolic and lower-redox potential compounds (Baldrian, 2006; Hildén, Hakala, & Lundell, 2009) with the concomitant reduction of molecular oxygen to water.

One catalytic cycle involves several enzyme intermediates, and overall 4 e^- are donated by the phenolic substrate compounds (Phe-OH). The reactive phenoxy radicals undergo further chemical reactions leading to oxidized quinones or coupled oligomeric products (Leonowicz, et al., 2001; Solomon, Chen, Metz, Lee, & Palmer, 2001). Unlike the peroxidases, it does not contain heme as the cofactor but copper; neither does it need H_2O_2 as the co-substrate but rather molecular oxygen (Baldrian,

2006). In the presence of a mediator such as 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonate) (ABTS) or 1- hydroxybenzotriazole, Lacs are capable of oxidation of nonphenolic compounds (Eggert, Temp, Dean, & Eriksson, 1996).

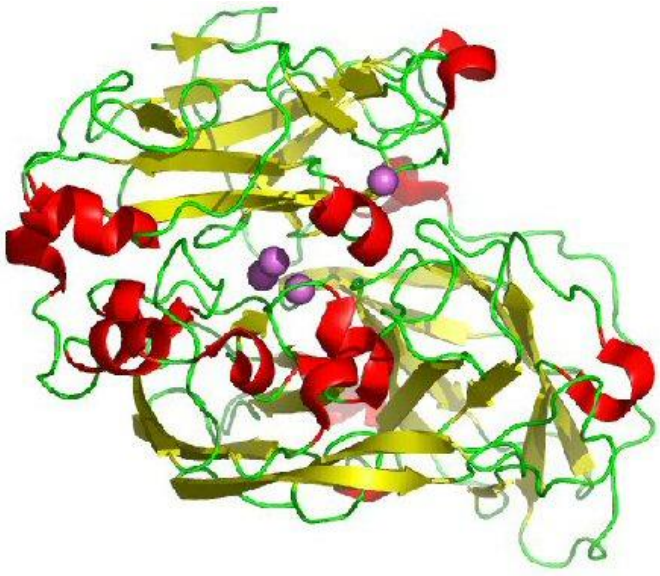


Figure 1.9 3D structure of *Trametes versicolor* laccase.

The Lac holoenzyme can either exist as a monomer, dimer or even as a tetramer. Each monomer contains 4 atoms of copper, which are distirubuted into three redox sites are designated as type1 (T1), type 2 (T2) and type 3 (T3) copper. Fungal "blue" laccases contain four copper atoms located in two metal centers (T1, T2/T3) (Figure1.9) (Lyashenko, et al., 2006; Ferraroni, et al., 2007; Morozova, Shumakovich, Gorbacheva, Shleev, & Yaropolov, 2007). T1 copper acts as electron acceptor from substituted phenols or amines (the typical laccase substrates); and T2 copper, which transfers the electrons to the final acceptor, dioxygen, which is reduced to water. The two T3 coppers act as intermediates in the electron transfer pathway that also includes one cysteine and two histidine protein residues. The molecular environment of Lac T1 copper seems to regulate the redox potential of the enzyme (Piontek, Antorini, & Choinowski, 2002).

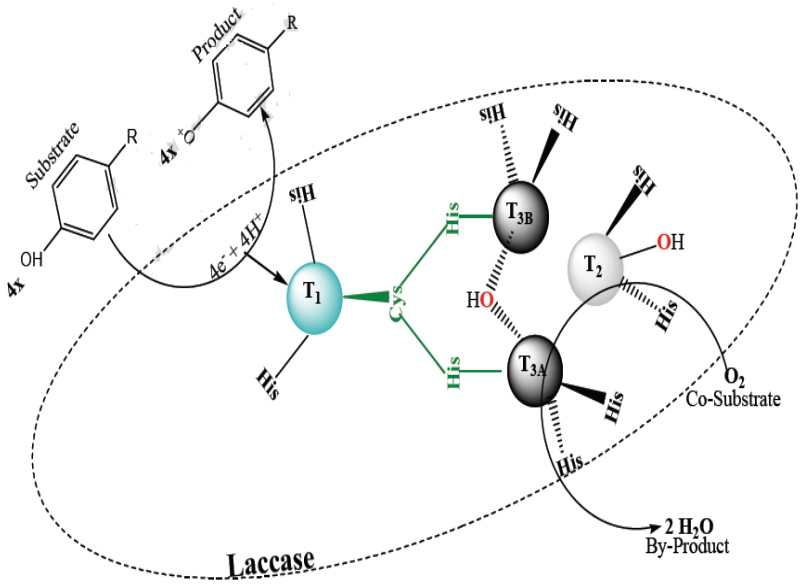


Figure 1.10 Mechanism of laccase catalysis (Baldrian, 2006).

Lac catalysis is believed to comprise of three major steps (Gianfreda, Xu, & Bollag, 1999) (Figure 1.10):

1. Reduction of mononuclear copper center: The reducing substrate (usually phenolic compounds) loses an electron to laccase. This electron reduces the T1 copper (at the mononuclear copper center), which is positioned just below the susbstrate-binding site (Piontek, Antorini, & Choinowski, 2002). The oxidized substrate now becomes a radical, which can either donate the second electron to the T1 copper end become a quinone or directly take part in any non enzymatic reactions leading to either polymerization or depolymerization. The reduced T1 copper oxidizes itself by transferring the electron to the trinuclear copper cluster. In this way, there are four such mono-electronic reductions of the T1 copper which occur sequentially (Gianfreda, Xu, & Bollag, 1999).

2. Internal electron transfer from the mononuclear copper to the trinuclear copper center: The O_2 molecule first binds to the T2 and any one of the T3 copper atoms. This then undergoes asymmetric activation leading to formation of four O-H bonds during the generation of two molecules of water. The oxygen-binding pocket appears to restrict the access of oxidizing substrate, which is molecular oxygen (Gianfreda, Xu, & Bollag, 1999) as opposed to its low affinity for the reducing substrate.

3. Reduction of molecular oxygen at the trinuclear copper center takes place at the trinuclear cluster with the concomitant release of water (Claus, 2004).

Lacs have several biotechnological applications including delignification of lignocellulosics, biopulping and biobleaching, textile dye transformation, removal of phenolics from must and wine and detoxification of phenolic waste effluent (Alberts, Gelderblom, Botha, & Vanzyl, 2009).

1.2.2.4 Aryl Alcohol Oxidase

Aryl alcohol oxidase (AAO) constitutes a source for H_2O_2 required in lignin biodegradation. AAO (EC 1.1.3.7) catalyzes the oxidation of aryl alcohols to the corresponding aldehydes with concomitant reduction of O_2 to H_2O_2. The enzyme has wide substrate specificity, oxidizing primary, polyunsaturated alcohols. The reaction of AAO (Figure 1.11) is initiated by the oxidative dehydrogenation of the substrate (reductive half-reaction), and is completed by flavin reoxidation by molecular oxygen with the production of hydrogen peroxide (oxidative half-reaction).

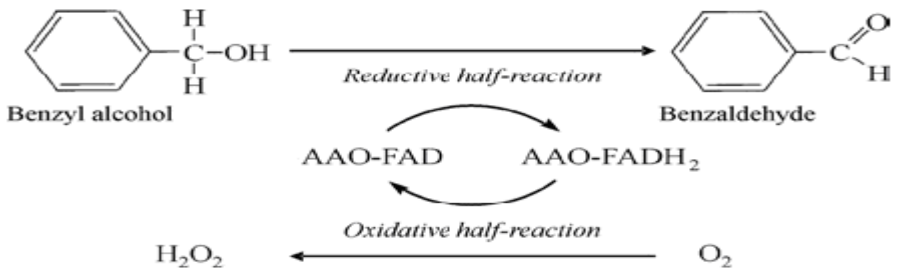


Figure 1.11 Redox reaction catalysed by AAO.

AAO, a monomeric glycosylated protein of 73 kDa (pI 3.9), is one of the many flavoenzymes that contain a non-covalently bound FAD cofactor (Hefti, Vervoort, & van Berkel, 2003) (Figure 1.12).

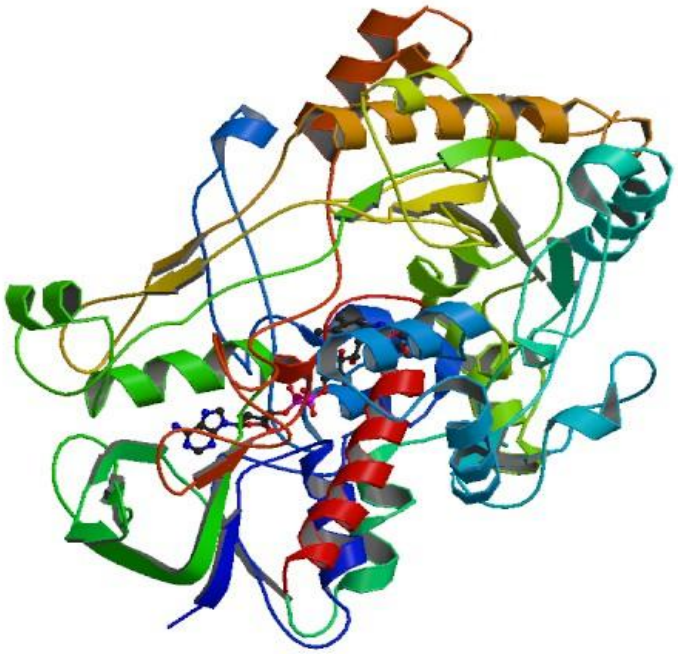


Figure 1.12 3D structure of AAO.

1.3 Submerged Fermentation Processes

Submerged fermentation (SF) is the cultivation of microorganisms in liquid nutrient broth. It usually consists of water, sources of energy, carbon, nitrogen, mineral elements and possibly vitamins and oxygen. As the microorganisms break down the nutrients, they release the desired enzymes into solution. Most industrial enzymes are secreted by microorganisms into the fermentation medium in order to break down the carbon and nitrogen sources. Besides nutrients, aeration is also important for the growth of biomass and production of enzymes. Parameters like temperature, pH, oxygen consumption and carbon dioxide formation can be measured and controlled to optimize the fermentation process for the purpose of

enzyme production. Firstly, in harvesting enzymes from the fermentation medium one must remove insoluble products, e.g. microbial cells. This is normally done by centrifugation. As most industrial enzymes are extracellular, they remain in the fermented broth after the biomass has been removed. In SF, the measure of process parameters is easy, whereas high costs due to the expensive media are the disadvantages (Deindoerfer, Mateles, & Humphrey, 1962).

1.4 Biotechnological Applications of Ligninolytic Enzymes

1.4.1 Decoloration of Synthetic Dyes and Waste Water

Importance of dyes to human civilization both ancient and contemporary is well documented. Modern developments have been a switch from natural to synthetic dyes (Paszczynski, Pasti, Coszczynskii, Crawford, & Crawford, 1991). Synthetic dyes are used extensively for textile dying, paper printing, and colour photography and as additives in petroleum products (Knapp, Newby, & Reece, 1995). Approximately 10000 different dyes and pigments are used industrially and over $7x10^5$ tons of these dyes are produced annually worldwide (Spadarao, Gold, & Renganathan, 1992).

Dyestuff and textile industries are the major producers and users of the dye respectively. The discharges of highly colored effluents from these industries are not only aesthetically unpleasing but also impede light penetrations and upset the biological aqua system. Moreover it is dangerous to surface as well as underground waters.

Removal of color from these effluents is one of the major important problems. The treatment of such waters by physical and chemical methods such as adsorption, coagulation, flocculation, oxidation, filtration and electro chemical methods have become difficult and are quite expensive (Kapdan, Kargi, McMullan, & Marchart, 2000). Biological treatment of the effluent may become an economically and

environmentally attractive alternative to the present physico-chemical methods of treatment.

Currently, textile effluents are treated by physico-chemical methods that are often quite expensive. Various physical/ chemical methods, such as adsorption, chemical precipitation, photolysis, chemical oxidation and reduction, electrochemical treatment, have been used for the removal of dyes from wastewater (Saratale, Saratale, Chang, & Govindwar, 2011).

In addition these methods do not generally degrade the pollutant, thereby causing an accumulation of the dye as sludge creating a disposal problem. Currently available technologies have been reviewed (Robinson, Chandran, & Nigam, 2001), and therefore special attention is given to biological processes because they are cost effective and environmentally friendly. Removing dyes in aerobic conditions is mainly achieved by adsorbing the dyes on bacteria, rather than oxidation. Some anaerobic textile wastewater treatment methods have been developed at a laboratory scale and have shown to remove color efficiently (Kapdan, Kargi, McMullan, & Marchart, 2000) but the anaerobic treatment of some azo dyes may result in the formation of toxic aromatic amines. Furthermore, there may be a risk of reverse colorization when anaerobic degradation products are exposed to oxygen (Knapp, Newby, & Reece, 1995).

Many white rot fungi e.g. *P. chrysosporium, Ganoderma sp WR.1, Trametes trogii, Irpex lacteus, Dichomitus squalens, Pycnoporus strains etc*. have been intensively studied in connection with their ligninolytic enzyme production and their decolorization abilities of dyes (Kasikara Pazarlioglu, Ozturk Urek, & Ergun, 2005; Revankar, & Lele, 2007; Susla, Novotny, & Srobodora, 2007). Moreover, In biological treatment processes, various physicochemical operational parameters, such as the level of agitation, oxygen, temperature, pH, dye structure, dye concentration, supplementation of different carbon and nitrogen sources, electron donor and redox

mediator, directly influence the decolorization performance of dyes (Saratale, et al., 2011).

1.4.2 Other Applications of Ligninolytic Enzymes

1.4.2.1 Biopulping

Biopulping is the treatment of lignocellulosic materials with natural wood degrading fungi prior to thermomechanical pulping. Biological pulping has the potential to improve the quality of pulp, properties of paper and to reduce energy costs and environmental impact relative to traditional pulping operations. It has been suggested that energy savings alone could make the process economically viable. Other benefits include improved burst strength and tear indices of the product and reduced pitch deposition during the production process. The technology has focused on the white rot fungi, which have complex extracellular ligninolytic enzyme systems that can selectively remove or alter lignin and allow cellulose fibers to be obtained (Wesenberg, Kyriakides, & Agathos, 2003).

1.4.2.2 Biobleaching

New environmentally benign elemental chlorine free and totally chlorine free bleaching technologies are necessary for minimizing the hemicellulose content in dissolving pulp, adjusting the brightness at a high level and improving. Simultaneously, the quality of the effluents in terms of toxicity and adsorbable organic halogen biological methods of pulp prebleaching using xylanases provide the possibility of selectively removing up to 20% of xylane from pulp and saving up to 25% of chlorine containing bleaching chemicals. Alternatively, pulp can be bleached with white-rot fungi and their ligninolytic enzymes, enabling chemical savings to be achieved and chlorine free bleaching process to be established (Scott, Akhtar, & Kirk, 2000).

1.4.2.3 Biodegradation of Toxic Compounds

Ligninolytic enzymes from *P. chrysosporium* have the capacity to oxidize a wide range of organic toxic compounds converting them to non-toxic metabolites or to CO_2 and H_2O (Bumpus, & Aust, 1987). White rot fungi like *P.chrysosporium* have also been reported to treat vinasses a highly hazardous environmental pollutant organopollutants such as 2,4,6-trinitrotoluene polychlorinated biphenyls, organochlorines, PAHs and wood preservatives were shown to be degraded by white-rot fungi (Pointing, 2001). Many reports demonstrate that the complex ligninolytic machinery of basidiomycetous fungi is involved in many of these degradation processes (Leonowicz, et al., 2001).

1.4.2.4 Improvement of Digestibility of Lignocellulosic Animal Feed

The bioconversion of plant residues into feed and food as well as the realization of large-scale technology belong to the most important aims of ecological and economical housekeeping of mankind. The digestibility of lignocellulose used as animal feed to rumen microorganisms is limited by its lignin content. Therefore low-cost techniques for selective removal of lignin from the lignocellulose matrix are needed (Hossain, Das, Ibrahim, & Anantharaman, 2004).

CHAPTER TWO

MATERIAL AND METHODS

This chapter describes the experimental procedures used in this study and the materials used in all procedures.

2.1 Material

Within the scope of this thesis, *Pleurotus eryngii* was utilized for obtaining ligninolytic enzyme activity.

2.1.1 Microorganism

The white-rot fungus, *Pleurotus eryngii* (DC.) Gillet (MCC58) was used in this study and taken from Agroma Mushroom Cultivation (Denizli, Turkey) as a gift.

2.2 Methods

In this thesis, the determination methods were carried out as spectrophotometric except the pH and dry biomass levels.

2.2.1 Microorganism's Maintenance

It has been maintained on agar medium at 4˚C, and transferred every month to fresh medium.

For this purpose, five different agar medium was tried and their contents are as follows:

- The malt extract-peptone-agar (MPA) medium contained 30.0 g/L malt extract, 3.0 g/L peptone and 15.0 g/L agar and pH was adjusted 5.6.
- The yeast extract-glucose-peptone-agar (YGPA) medium contained 5 g/L yeast extract, 10 g/L glucose, 5 g/L peptone and 15.0 g/L agar, and pH was adjusted 6.0.
- The malt extract-yeast extract-peptone-agar (MYPA) medium contained 20.0 g/L malt extract, 2 g/L yeast extract, 1 g/L peptone 20.0 g/L agar and pH was adjusted 5.5.
- The medium malt extract-agar (MEA) contained 20.0 g/L malt extract, 20.0 g/L agar and pH was adjusted 6.0.
- The modified yeast extract-potato dextrose agar (YPDA) medium contained 50 g/L yeast extract, 24 g/L potato-dextrose agar and pH was adjusted 5.6.

To prepare the medium, the mixture of the contents was stirred until agar was completely dissolved. Then, the medium was sterilized at 121˚C in an autoclave for 20 minutes. After the medium was cooled, the medium was transferred on plate. When the medium solidified, it was inoculated and incubated at 25˚C.

Figure 2.1 The growth of *P.eryngii* was completely covered on MPA.

The growing of *P.eryngii* was started after day four and covered the surface of the solid medium completely after day twelve (Figure 2.1).

2.2.2 Preparation of Inoculation

When the inoculation was carried out with spore suspension, the spores were harvested from plates under aseptic conditions. Optical density (OD) of spore suspension at 500 nm was adjusted 0.5. The spore suspension (1 mL or 5 mL) was transferred to growth medium as 10 mL or 50 mL of total volume, respectively.

When the inoculation was carried out with plug, three 1-cm disks of fungal spore, excised from agar plates, were transferred to 100 mL erlenmeyer flasks containing 10 mL of growth medium.

When the inoculation was carried out with mycelium, five 0.8-cm disks of the fungal spore, excised from agar plates, or 10 mL of spore suspension (OD_{500}=0.5) were transferred to 250 mL Erlenmeyer flasks containing 100 mL or 90 mL of growth medium, respectively to form mycelium. The mycelium growth medium composed of: 10 g/L glucose, 2 g/L NH_4NO_3, 0.8 g/L KH_2PO_4, 0.75 g/L Na_2HPO_4 x $7H_2O$, 0.5 g/L $MgSO_4.7H_2O$, 2 g/L yeast extract. The initial pH was adjusted to 6.0 prior to sterilization. After 7 days of cultivation, mycelial pellets were harvested and homogenized using a laboratory homogenizer at 10000 rpm and 4ºC. The homogenized mycelium (5mL) was transferred to 250 mL Erlenmeyer flasks containing 45 mL of only growth medium (2) (Stajić et.al., 2006).

2.2.3 Growth Media and Submerged Fermentation Process

The liquid media used in submerged fermentation to obtain ligninolytic enzyme activity from *Pleurotus eryngii* was showed in Table 2.1.

Table 2.1 The growth medium for *P.eryngii*

Medium 1	**Medium 2**	**Medium 3 (for 1000 mL)**
20 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 2 g/L yeast extract, 0.5 g/L $MgSO_4$, 0.56 g/L $CaCl_2$, 0.012 g/L $FeSO_4$, 0.0845 g/L $MnSO_4.H_20$ pH 5.5	10 g/L glucose, 2 g/L yeast extract, 0,8 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 0,75 g/L $Na_2HPO_4x7H_2O$, 2 g/L NH_4NO_3, 0.002 g/L $ZnSO_4.7H_20$, 0.005 g/L $FeSO_4.7H_2O$, 0.06 g/L $CaCl_2.2H_2O$, 0.02 g/L $CuSO_4.7H_2O$, 0.05 g/L $MnSO_4.7H_2O$ pH 5.6	100 mL basal III medium, 100 mL %10 glucose 100 mL of 20 mM acetate buffer 10 mL of tiamin-HCl (100mg/mL) 12.5 mL of 0.086 M Na-tartarate 12.5 mL of 0.086 M $(NH_4)_2SO_4$ 100 mL of spore suspension (OD_{500}=0.5) 60 mL trace element solution 100 mL of inducer solution (174 μM Mn^{2+}) 405 mL sterilized distilled water pH 6.0
		Basal III medium: 20 g/L KH_2PO_4 1 g/L $CaCl_2$ 5 g/L $MgSO_4$ 100mL/L trace element solution

The trace element solutions for medium 3 composed of 3 g/L $MgSO_4$, 0.5 g/L $MnSO_4$, 1.0 g/L NaCl, 0.1 g/L $FeSO_4.7H_2O$, 0.1 g/L $CoCl_2$, 0.1 g/L $ZnSO_4.7H_2O$, 0.1 g/L $CuSO_4$, 10mg/L $AIK(SO_4)_2.12H_2O$, 10 mg/L H_3BO_3, 10mg/L $Na_2MoO_4.2H_2O$, 1.5 g/L nitrilotriacetate (Tien, & Kirk, 1988).

The 10 mL and 50 mL of liquid medium were transferred into 100 and 250 mL flask, respectively. These flasks were sterilized at 121˚C for 20 minutes, cooled to room temperature, and then inoculated.

The effects of volumes of liquid medium and flasks, different liquid media (medium 1, medium 2 or medium 3), inoculation type (spore suspension, plug or mycelium), cultivation mode (shaking at 180 rpm or stationary) and temperature (22 or 28˚C), Mn^{2+} (180, 250, 500, 750 and 1000 μM) and Cu^{2+} (0.07, 0.14, 0.5, 1.0, 2.0, 3.0 mM) concentrations and also NH_4NO_3 (2 or 4 g/L) on the ligninolytic enzyme activities from *P. eryngii* were investigated in the submerged fermentation process.

2.2.4 Crude Enzyme Preparation

In order to optimize culture conditions for the ligninolytic enzyme activities secreted by *P. eryngii*, different methods of crude enzyme preparation were used according to inoculation type.

To obtain crude enzyme extract in SF when inoculating spore suspension or mycelium, the culture was centrifuged at 12000 rpm for 10 min at 4˚C after separating the biomass. The homogeneous supernatant obtained was used in enzyme activity assays, determination of protein, reducing sugar and nitrogen levels.

When the inoculation was performed with plug in SF, the culture was stirred at 180 rpm for an hour on ice bath to extract the samples. Then, biomass was separated by centrifugation at 5000 rpm and 4˚C for 10 minutes after the culture extracted. Then, the supernatant was collected and was centrifuged at 12 000 rpm at and 4˚C for 10 minutes again. This supernatant was called the crude enzyme extract.

2.2.5 Analysis of Samples

Samples taken from the submerged cultures were analyzed for reducing sugar and nitrogen levels in the medium, as well as dry biomass, ligninolytic enzyme activities and protein production. And also, changes in pH were measured.

2.2.5.1 Determination of Dry Biomass

A simple procedure was used for biomass measurement. After the content of flasks was centrifuged at 5000 rpm and 4˚C for 10 minutes, the supernatant was collected and the pellet was separated. The biomass was dried in an oven at 105˚C for two hours until reaching to a constant weight. Thereafter, the sample was cooled to room temperature and scaled. The differences in tare and sample were equal to dry biomass.

2.2.5.2 Determination of Reducing Sugar Concentration

After the crude enzyme extract was obtained, the supernatant was used for analysis of reducing sugar content by the dinitrosalicyclic acid (DNS) method (Miller, 1959). The solution used for DNS method was prepared by first making solutions A and B as follows:

Solution A: 1g of dinitrosalicyclic acid was dissolved in 20 ml of 2N NaOH solution.

Solution B: 30 g of sodium-potassium tartarate was dissolved in 50 ml of distilled water.

Solution B was added to A while stirring and the total medium was covered to 100 ml with distilled water. The final solution (DNS) was kept in the dark. The first step of the analysis method was to mix 500 µL of supernatant with 500 µL of DNS

solution. The mixture was then boiled for ten minutes and cooled for one minute in an ice bath. 5 ml of distilled water was added and mixed. The absorbance was measured at 546 nm against a reference sample. The reference sample was prepared by using 500 µL of distilled water instead of the sample supernatant and the same analysis procedure was followed. Standard curves were prepared in the range of 0-165.1 µg of glucose per milliliter of liquid, and the same procedure was performed. The calibration curve was given in Figure 2.2.

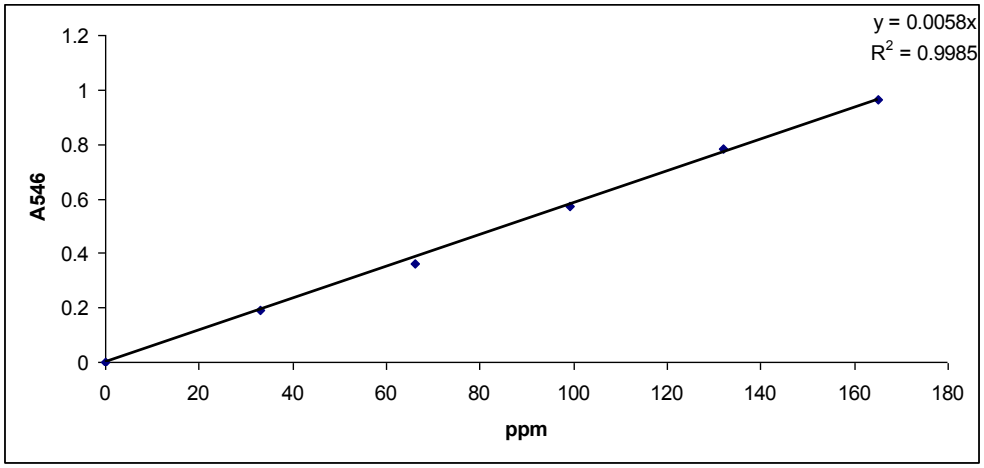


Figure 2.2 Calibration curve of reducing sugar level

2.2.5.3 Determination of Nitrogen Concentrations

When the crude enzyme extract was obtained, the supernatant was used for analysis of nitrogen content by phenol-hypochlorite method (Weatherburn, 1967). The reagent solutions used for phenol-hypochlorite method were prepared as follows:

Phenol reagent solution was prepared by mixing equal volume from solution A and B.

5 g of phenol was dissolved in 50 ml of distilled water (solution A), and 25 mg of sodium-nitroprusside was dissolved in 50 ml of distilled water (solution B).

Alkaline hypochlorite solution was prepared by mixing equal volume from solution A and B.

Solution A: 5 g of sodium hydroxide was dissolved in 100 ml of distilled water.

Solution B was commercial hypochlorite solution containing 26 g/L of NaOCl.

The first step of the analysis method was to incubate 2.0 mL of supernatant at room temperature for fifteen minutes. Later, 500 µL of phenol solution and 500 µL of alkaline hypochlorite solution were respectively added to incubated sample. The last solution was mixed with vortex and incubated for five minutes in 60^0C-water bath. The absorbance was measured at 630 nm against a blank sample. The blank sample was prepared by using 2.0 mL of distilled water instead of the sample supernatant and the same analysis procedure was followed. Standard curves were prepared in the range of 0-500 µg of ammonium sulfate ($(NH_4)_2SO_4$) per mL of liquid, and the same procedure was performed. The calibration curve was given in Figure 2.3.

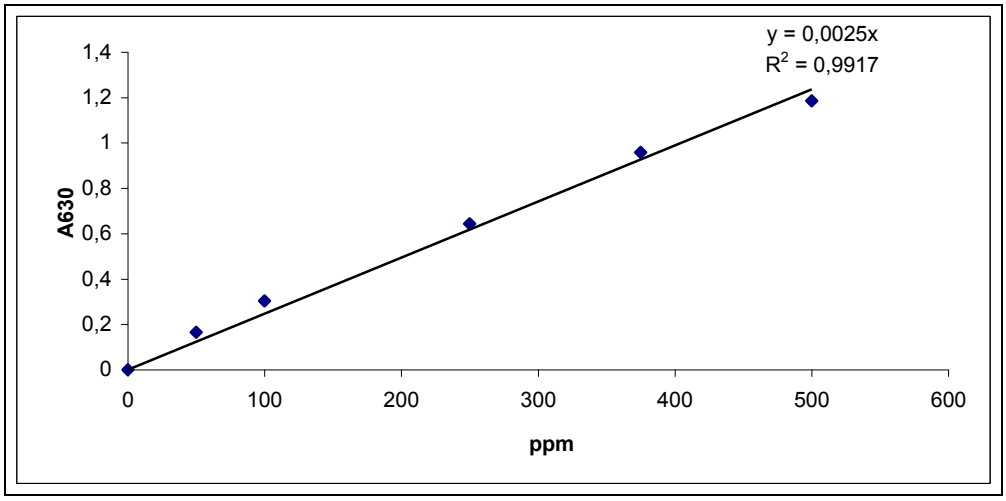


Figure 2.3 Calibration curve of nitrogen level

2.2.5.4 Determination of pH Levels

The pH of the supernatant was measured using a pH-meter.

2.2.5.5 Determination of Protein Concentration

After the crude enzyme extract was obtained, the supernatant was used for analysis of protein production by the Bradford method (Bradford, 1976). The solution used for Bradford method was prepared as follows:

Dye stock reagents: 100 mg of Coomassie Brillant Blue G-250 is dissolved in 50 mL of 95% ethanol. The solution is added to 100 mL of 85% H_3PO_4, and diluted to 1000 mL of total volume with water. The solution is stable indefinitely in a dark bottle at 4°C. Protein concentrations of sample were determined by comparing the absorbance of the protein-dye binding complex at 595 nanometers (nm). 100 µL of sample was added to 900µL dye reagents and then mixed. This solution was waited for two minutes at room temperature. Right after, the absorbance was measured at 595 nm against a reference sample. The reference sample was prepared by using 100 µL of distilled water instead of the sample supernatant and the same analysis procedure was followed. Calibration curves were plotted in the range of 0-200 µg of bovine serum albumine (BSA) per millilitre of liquid, and the same procedure was performed. The calibration curve was depicted in Figure 2.4.

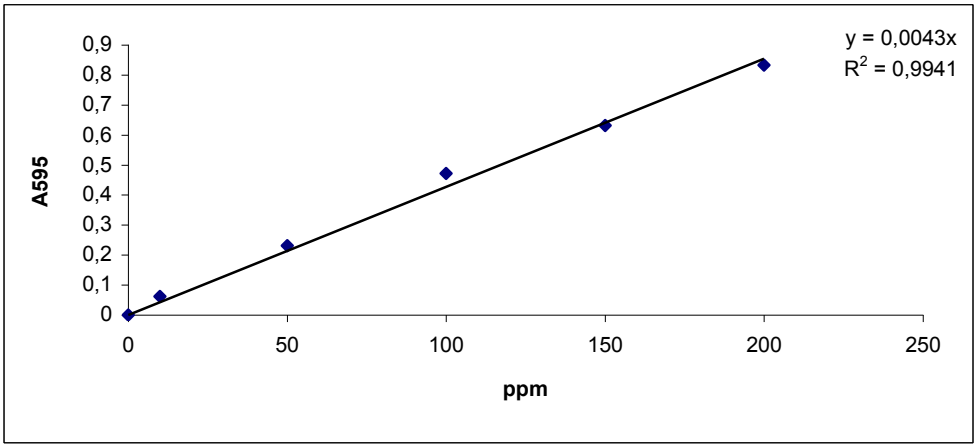


Figure 2.4 Calibration curve of protein

2.2.5.6 Determination of Ligninolytic Enzyme Activities

The enzyme assay conditions were indicated as follows:

Laccase (Lac) activity was assayed by measuring the oxidation of ABTS (ε_{max} = 36000 cm^{-1} M^{-1}). The reaction mixture (total volume 1ml) contained 700 µL of 100 mM sodium acetate buffer at pH 4.5, 100 µL of 2.0 mM 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS-$(NH_4)_2$) in distilled water and 200 µL of sample or its dilution. The reaction mixture was well mixed in a quartz cuvette and the reaction was monitored by measuring the absorption at 420 nm and 25^{o}C in a UV-spectrophotometer. The reaction mixture showed green color and increased in intensity at the initial stage of the reaction. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 mmol of ABTS per min. The activities were expressed in unit per liter (Johannes C., & Majcherczyk A., 2000).

Manganese peroxidase (MnP) activity was measured by 2, 6-dimethoxyphenol (2, 6-DMP; ε_{max}=27500 M^{-1} cm^{-1}) oxidation (Kuwahara, Glenn, Morgan, & Gold, 1984). The reaction mixture contained 600 µL of 250 mM Na-tartrate buffer at pH 4.5, 50 µL of 20 mM 2,6-DMP, 50 µL of 20 mM $MnSO_4.H_2O$, 100 µL of 4 mM

H_2O_2 and 200 µL of enzyme solution. The reaction was started by adding H_2O_2 and monitored by the absorbance at 469 nm and 25˚C. One unit (U) of enzyme activity is defined as the amount of MnP that oxidizes 1 µmol of 2, 6-DMP per minute. The activities were expressed in unit per liter.

Lignin peroxidase (LiP) activity (modified) was determined by the oxidation of veratryl alcohol to veratraldehyde. The reaction mixture contained 650 µL of 125 mM sodium tartarate buffer at pH 2.5, 100 µl of 20 mM veratryl alcohol, 50 µl of 4 mM hydrogen peroxide solution and 200 µl of the sample or its dilution. The reaction was started by adding hydrogen peroxide and the increase in absorbance at 310 nm and at 30˚C was monitored (ε_{max} = 9300 cm^{-1} M^{-1}). One unit of enzyme activity was 1 µ mole of veratraldehyde produced per minute per ml of the sample. The activities were expressed in unit per liter (Tien, & Kirk, 1988).

Aryl alcohol oxidase (AAO) activity was assayed spectrophotometrically using veratryl alcohol as a substrate. The reaction mixture of the standard assay contained 100 µL of 10 mM veratryl alcohol, 700 µL of 100 mM potassium phosphate buffer at pH 6.0, and 200 µL of enzyme solution in a total volume of 1.0 ml. Oxidation of the substrate at 30°C was monitored based on an absorbance increase at 310 nm resulting from the formation of veratraldehyde (ε_{max}=9300 cm^{-1} M^{-1}). One unit of the enzyme activity was defined as the amount of enzyme required to produce 1 µmol of veratraldehyde per minute under the assay conditions. (Okamoto, & Yanase, 2002). The activities were expressed in unit per liter.

2.2.6 Decolorization

Three different dyes belonging to azo class, methyl orange, reactive red 2 and reactive black 5 were analyzed. Decolorization was determined by monitoring the decrease in absorbancy at the maximum absorbance of each dye (methyl orange 505 nm, reactive red 2 538 nm, reactive black 5 597 nm). A control test in which the

enzyme was replaced by distilled water was conducted in parallel. The stock dye solution concentration was 240 mg/L.

The reaction was carried out at 25˚C and the reaction mixture contained 718.75 µL of distilled water, 31.25 µL of stock dye solution and 750 µL of enzyme solution (containing Lac activity 774.167 U/L and MnP activity 721.091 U/L) (for 10 mg/L of dye concentration).

CHAPTER THREE

RESULTS

3.1 Optimization of Spore Production

In this study, five different agar media, namely MPA, YGPA, MYPA, MEA, YPDA, were tried for the *Pleurotus eryngii* spore production. The best medium was MPA. This fungus covered the surface of the solid medium completely after day twelve.

3.2 Submerged Fermentation Process

Although many factors have to be considered to design a suitable medium for a specific fermentation process, certain basic requirements must be met. These requirements include the elemental constituents for cell biomass and metabolite production as well as an adequate supply of energy for biosynthesis and cell maintenance.

In this study, *P.eryngii* was grown in three different basal liquid media so as to optimize submerged fermentation conditions for maximum ligninolytic enzyme activities. The effects of inoculation type, liquid medium and flasks volumes, different liquid media, temperature, NH_4NO_3 concentration, Mn^{2+} and Cu^{2+} concentrations, shaking or stationary cultivation mode have been examined.

3.2.1 The Effect of Liquid Medium and Flask Volumes

In this study, 100 mL and 250 mL of flasks were involved 10 mL and 50 mL of first basal medium including 250 μM Mn^{2+} and inoculated with spore suspension. The cultivation was carried out at 180 rpm for including 50 mL of basal medium and on stationary condition including 10 mL of basal medium at 28˚C.

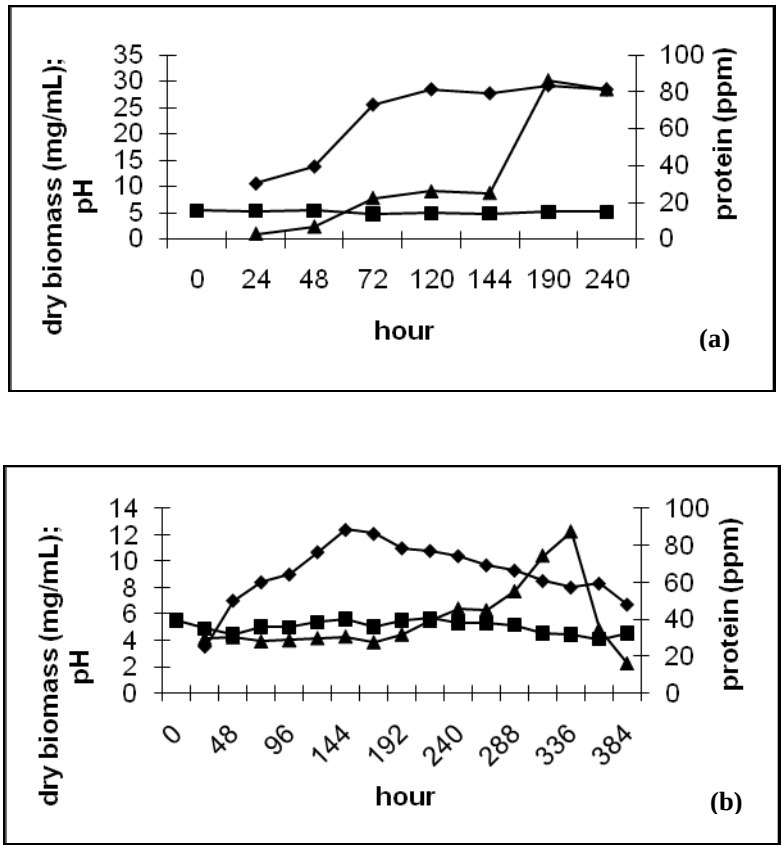


Figure 3.1 The effect of liquid medium and flask volume on dry biomass, pH and protein levels. (a) 50 mL of basal medium in 250 mL of flask; (b) 10 mL of basal medium in 100 mL flask (□■□; pH, □♦□; dry biomass, □▲□; protein).

As shown in Figure 3.1a, dry biomass constantly increased up to 72th hour (h), and it was reached to stationary phase. Also, it was determined non-significant changes in pH values. Initially, the protein level was slowly increased and the highest level was detected as 86.364 ppm at 190th h. As shown in Figure 3.1b, dry biomass constantly increased up to 144th h, then it was not much changed because reached to stationary phase. The pH value was almost constant. Initially, the protein level was slowly increased and it was reached to a maximum at 336th h as 87.571 ppm.

3.2.2 The Effect of Liquid Media

Firstly, these cultivations were performed at 180 rpm and 28˚C. 50 mL of basal medium 1 and medium 2 containing 250 and 180 μM Mn^{2+}, respectively, was prepared. There was no Cu^{2+} in both media. Cu^{2+} is a prosthetic group for laccase enzyme while Mn^{2+} for MnP. Spore suspension was transferred to each flask for inoculating.

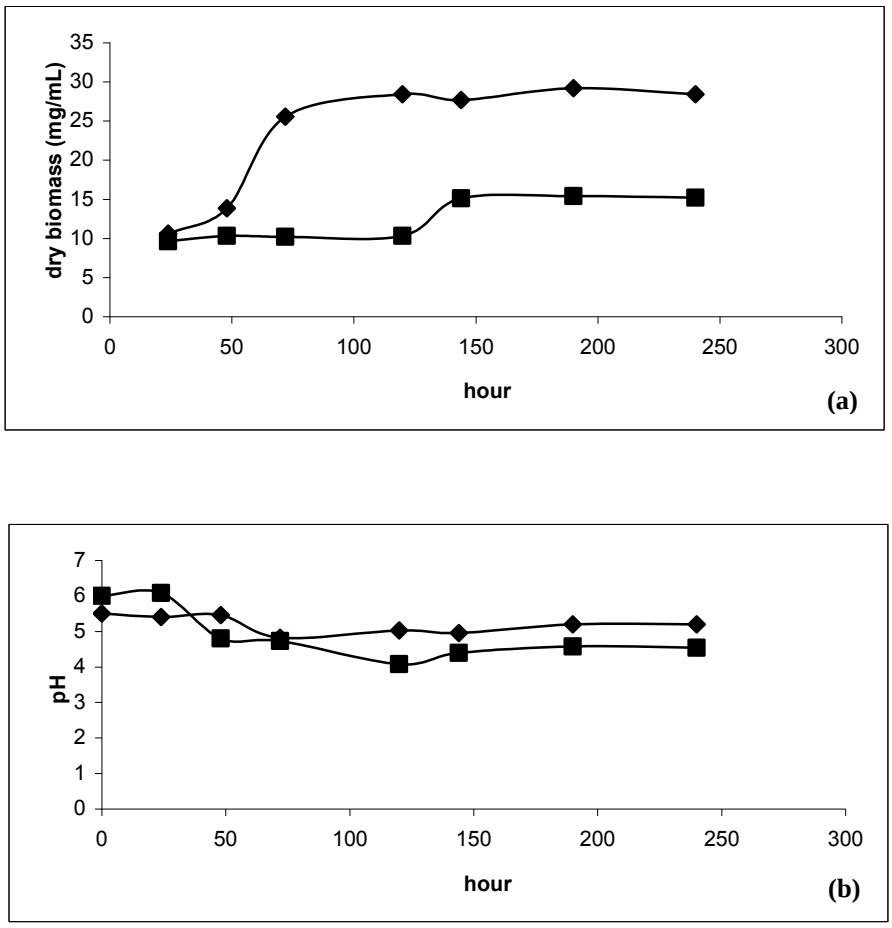


Figure 3.2 The effect of liquid medium on dry biomass (a), pH levels (b) and protein production (c); (□♦□; medium 1, □■□; medium 2).

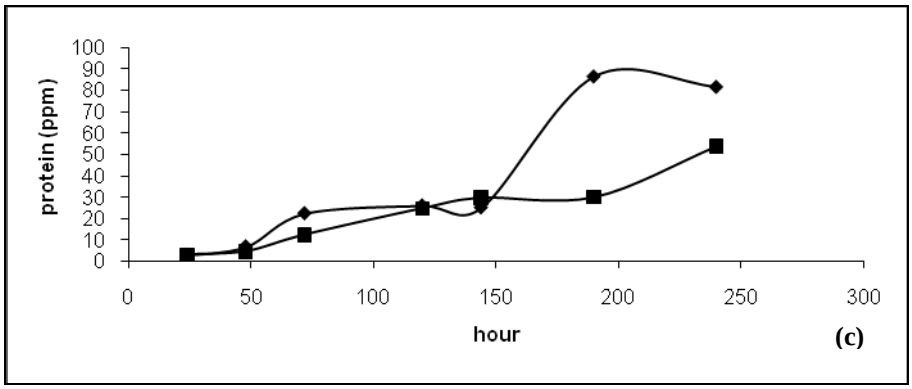


Figure 3.2 The effect of liquid medium on dry biomass (a), pH levels (b) and protein production (c); (□♦□; medium 1, □■□; medium 2) (continue).

The dry biomass in medium 1 was nearly two-fold higher than that of medium 2 ($p< 0.05$). In this study, *P. eryngii* was reached to stationary phase at 72th and 144th h on medium 1 and medium 2, respectively (Figure 3.2a). As shown in Figure 3.2b, the pH levels were very similar ($p>0.05$). As illustrated in figure 3.2c, protein production by *P. eryngii* in medium 1 was higher when compared to medium 2. The reason for this may be due to high dry biomass in medium 1.

Then, on stationary conditions, the cultivation temperature was selected to be 25˚C and the other conditions, basal medium contents and volumes were same as above (Figure 3.3).

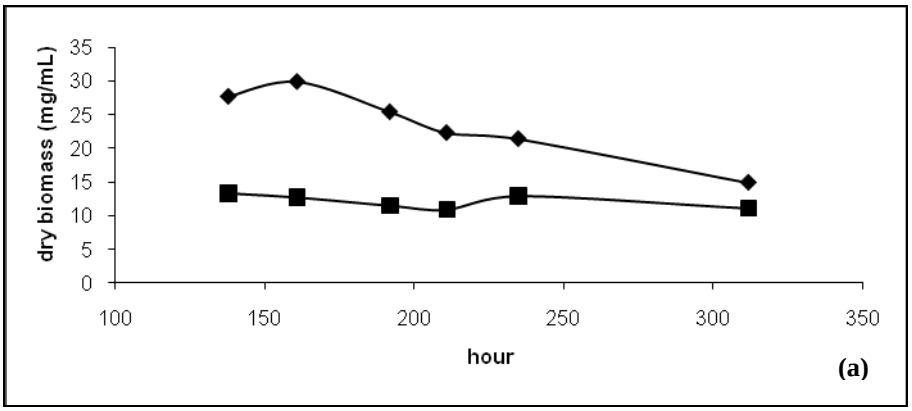


Figure 3.3 The effect of liquid medium on dry biomass (a) and differences in pH levels (b); (□♦□; medium 1, □■□; medium 2).

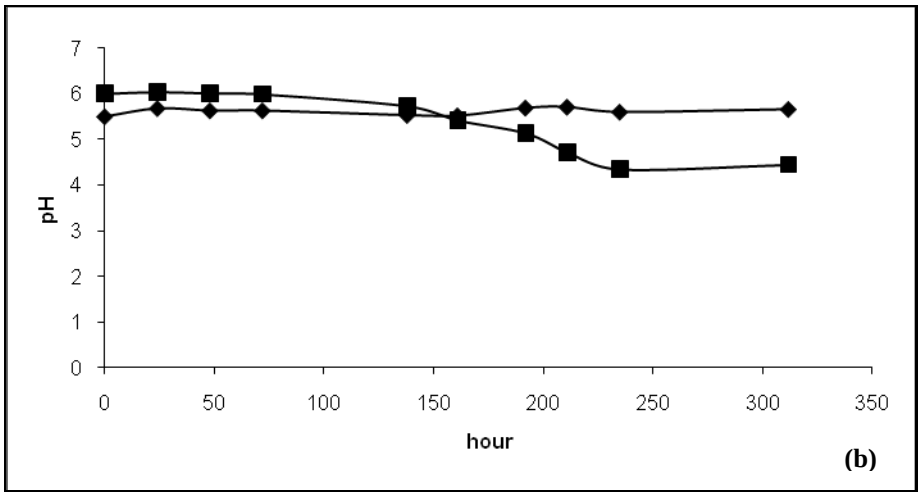


Figure 3.3 The effect of liquid medium on dry biomass (a) and differences in pH levels (b); (□♦□; medium 1, □■□; medium 2) (continue).

The dry biomass in medium 1 was higher than that of medium 2 (Figure 3.3a). The pH level showed similar trends in both media up to 161th hour, and then it was stable in medium 1, but in medium 2 decreased (Figure 3.3b). It might be said that the dry biomass in medium 2 was not affected by pH changes.

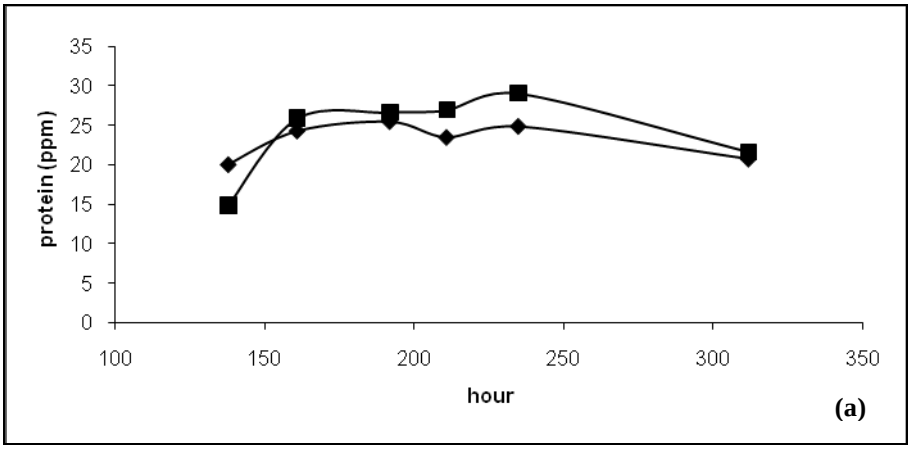


Figure 3.4 The effect of liquid medium on protein production (a) and MnP activity (b); (□♦□; medium 1, □■□; medium 2).

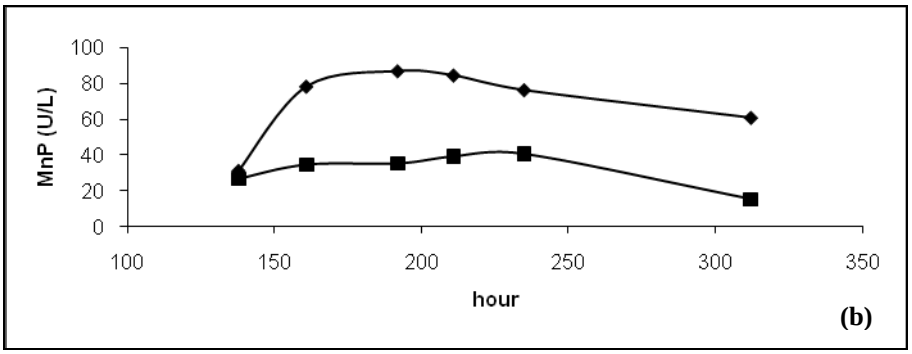


Figure 3.4 The effect of liquid medium on protein production (a) and MnP activity (b); (□♦□; medium 1, □■□; medium 2) (continue).

In both media, the protein levels were very similar (Figure 3.4a). The MnP activity in medium 1 was higher than in medium 2 and the highest activity was detected as 86.836 U/L at 192th h in medium 1 (Figure 3.4b).

Also, the other medium, namely Tien and Kirk medium, was used for this purpose. In this study, 10 mL of liquid medium including 174 μM of Mn^{2+} as an inducer was used for obtaining maximum ligninolytic enzyme activities. The cultivation was performed at 28°C and 180 rpm. Beside inoculation was performed with spore suspension.

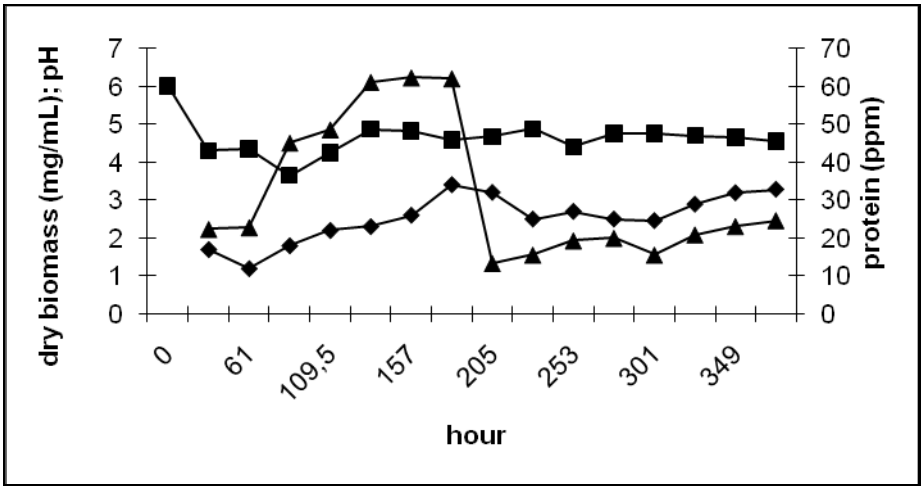


Figure 3.5. The effect of liquid medium on dry biomass (羊♦羊), pH levels (羊■羊), protein production (□▲□).

As shown in Figure 3.5, there was not much growth in this medium and were changes in the pH level. The protein production increased up to 133th h, then suddenly decreased and then remained stable.

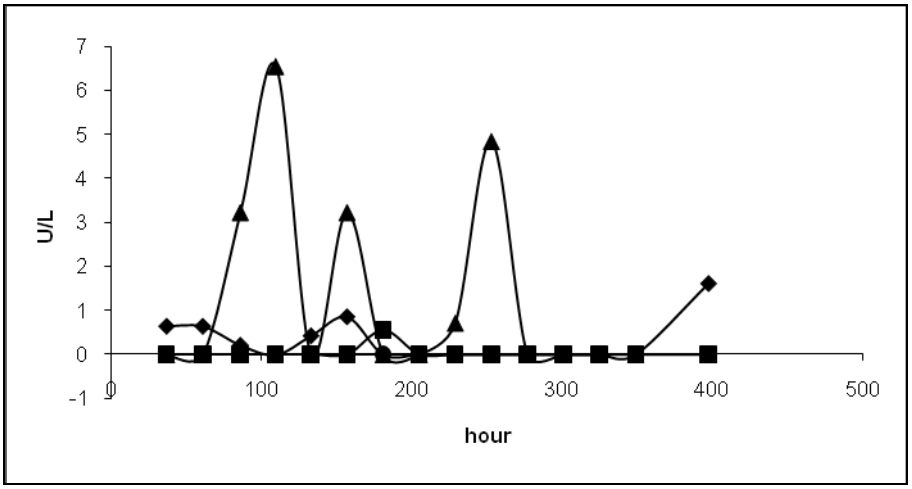


Figure 3.6. The effect of liquid medium on LiP (□♦□), MnP (□■□), Lac (□●□) and AAO (□▲□) activities.

As shown in Figure 3.6, the maximum enzyme activity in this medium was observed in AAO and detected as 6.542 U/L. Lac activity was not determined. Also, LiP and MnP activities were little detectable.

When the calcium nitrate was used instead of calcium chloride as same amounts of Ca^{2+} in medium 1, there were insignificantly changes in dry biomass, pH levels, and protein and ligninolytic enzyme activities.

3.2.3 The Effect of Inoculation Type

Ligninolytic enzyme activities from white rot fungi also depend on the inoculation type. In accordance with this purpose, submerged fermentation process was performed three inoculation type: spore suspension, plug and mycelium.

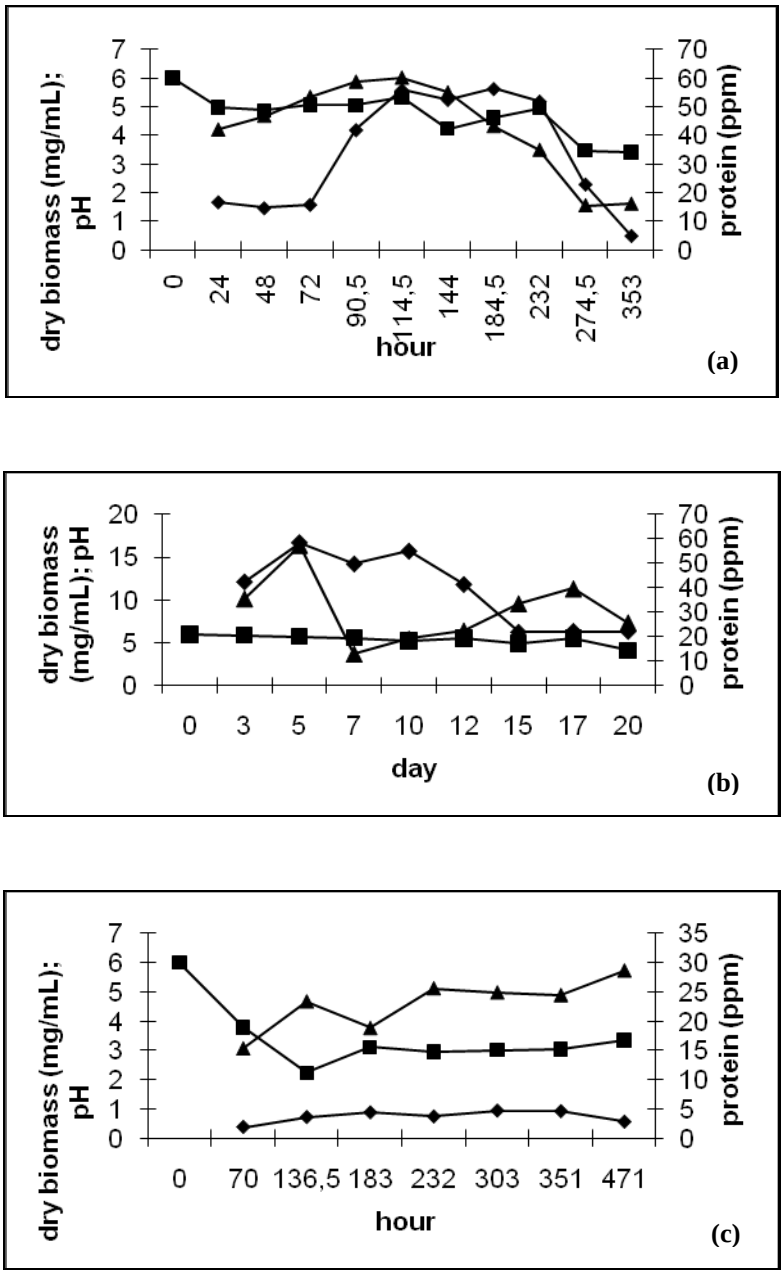


Figure 3.7 The effects of inoculation type on dry biomass (□♦□), pH levels (□■□) and protein production (□▲□). (a) Spore suspension, (b) plug, (c) mycelium formed by spore suspension, (d) mycelium formed by plug.

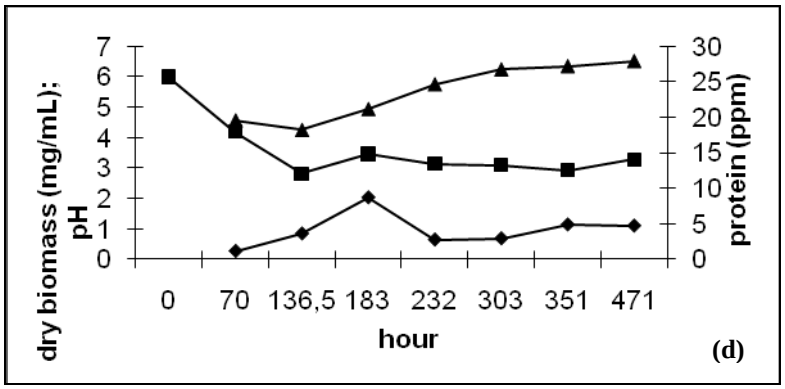


Figure 3.7 The effects of inoculation type on dry biomass (□♦□), pH levels (□■□) and protein production (□▲□). (a) Spore suspension, (b) plug, (c) mycelium formed by spore suspension, (d) mycelium formed by plug (continue).

As seen from Figure 3.7, the increase in dry biomass was higher when inoculation was carried out with plug. The pH levels showed differences in inoculation performing spore suspension and mycelium but not plug. Compared to all the inoculation types, the highest protein production was obtained with the plug as 57.5 ppm on 5th day (Figure 3.7b).

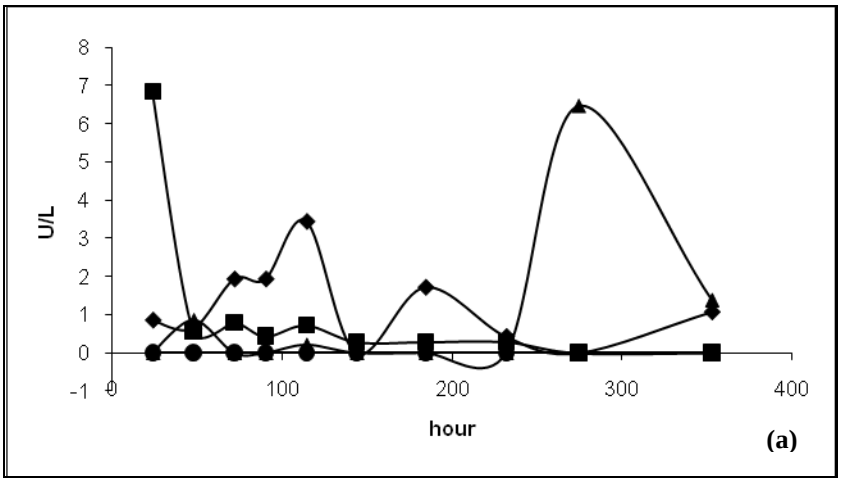
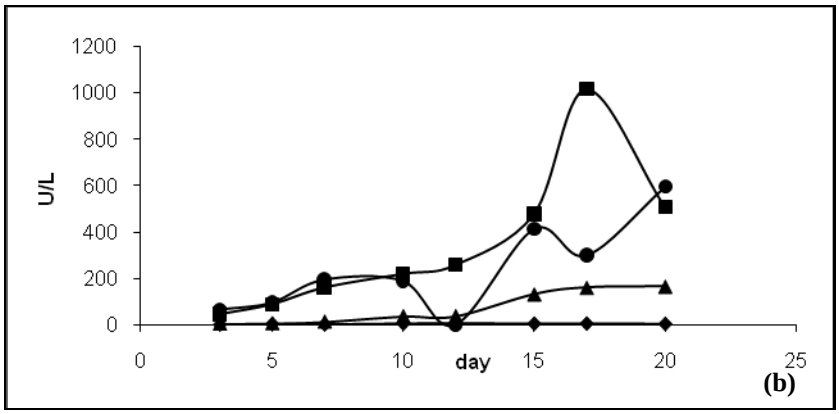
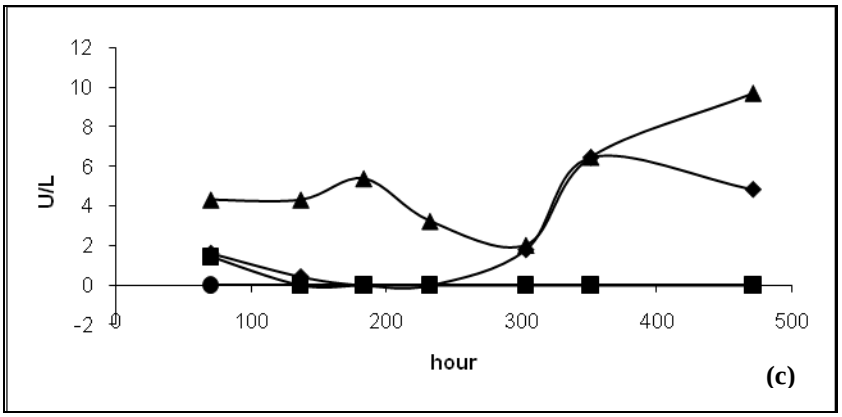


Figure 3.8 The effect of inoculation on Lac (□●□), MnP (□■□), LiP (□♦□) and AAO (□▲□) activities. (a) spore suspension, (b) plug, (c) mycelium formed by spore suspension, (d) mycelium formed by plug.





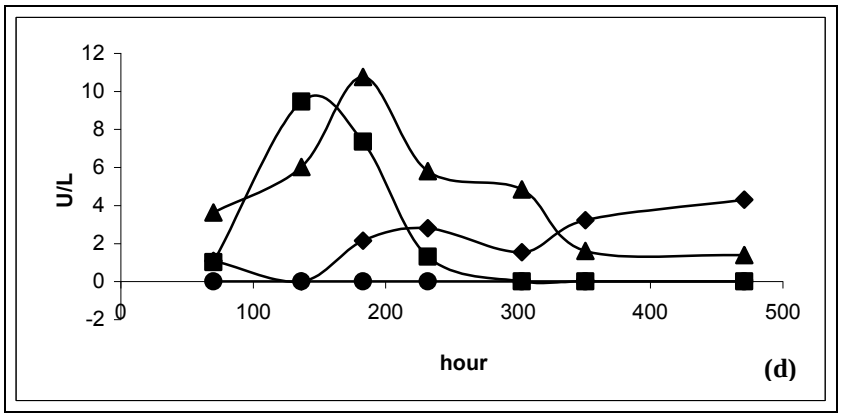


Figure 3.8 The effect of inoculation on Lac (□●□), MnP (□■□), LiP (□♦□) and AAO (□▲□) activities. (a) spore suspension, (b) plug, (c) mycelium formed by spore suspension, (d) mycelium formed by plug (continue).

As depicted in Figure 3.8, the highest Lac activity was detected as 595.833 U/L on 20th day when the inoculation was carried out with plug, and it was not

determined with other inoculation types. The maximum MnP activity was determined as 1016,727 U/L on 17th day by carrying out plug inoculation, while it was determined as 6.836 U/L on first day by performing with spore suspension inoculation. Similar to these results, when inoculation was performed with mycelium, the highest MnP activity was detected as 9.454 U/L at 136.5th h in mycelium formed by the plug. There was not detected any significant differences in LiP activity in all inoculation types, whereas AAO activity was determined as 165.161 U/L on 20th day when the inoculation was carried out with plug.

According to the results, the best inoculation type for ligninolytic enzyme activity by *P. eryngii* was determined as plug.

3.2.4 Cultivation Mode and Temperature

The other optimization condition for ligninolytic enzyme production by *P. eryngii* is whether cultivation is carried out shaking or not. Firstly, the Tien and Kirk medium, described earlier, was used and the cultivation by inoculating with spore suspension was performed both shaking (180 rpm) and stationary condition at 28˚C (Figure 3.9).

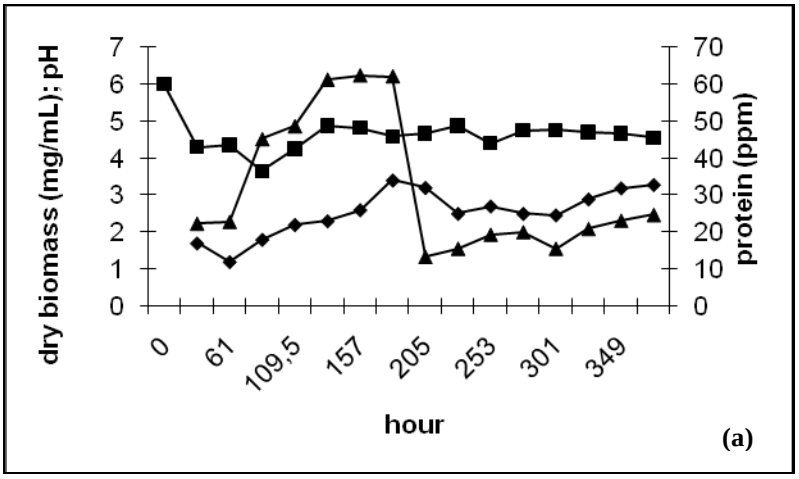


Figure 3.9 The effect of shaking on dry biomass (□♦□), pH levels (□■□) and protein production (□▲□). (a) shaking and (b) stationary conditions.

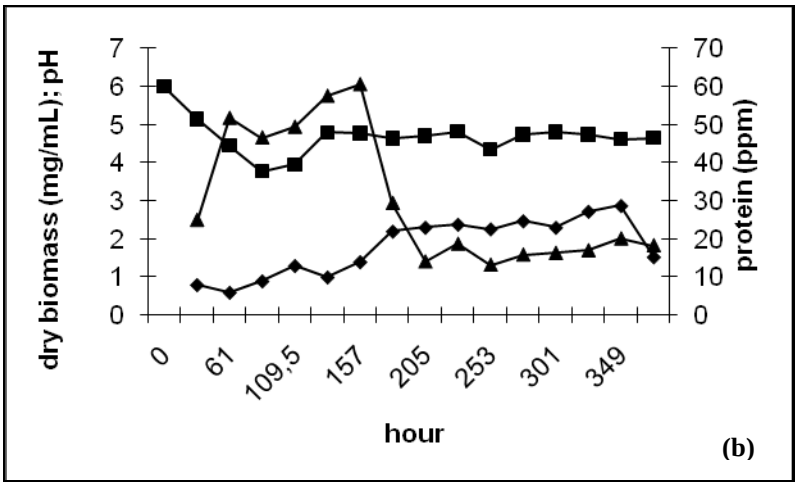


Figure 3.9 The effect of shaking on dry biomass (□♦□), pH levels (□■□) and protein production (□▲□). (a) shaking and (b) stationary conditions (continue).

The variations in dry biomass, pH and protein levels were showed same trends in both shaking and stationary fermentation conditions. The highest protein production was obtained on time of 181 and 157 h, respectively and then suddenly decreased.

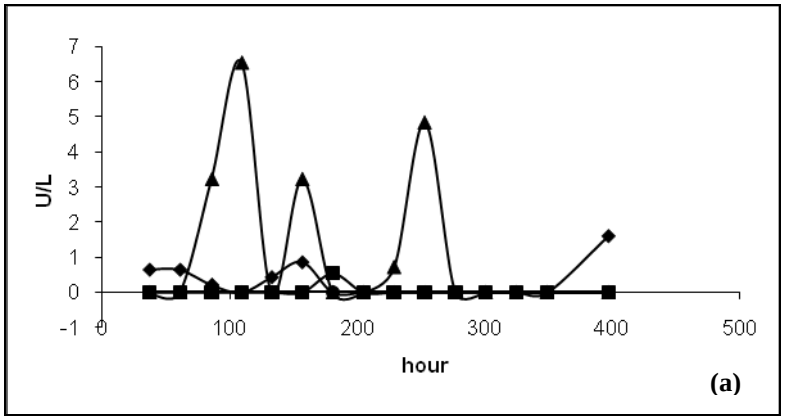


Figure 3.10 The effect of shaking on LiP (□♦□), MnP (□■□), Lac (□●□) and AAO (□▲□) activities. (a) shaking and (b) stationary cultivation conditions.

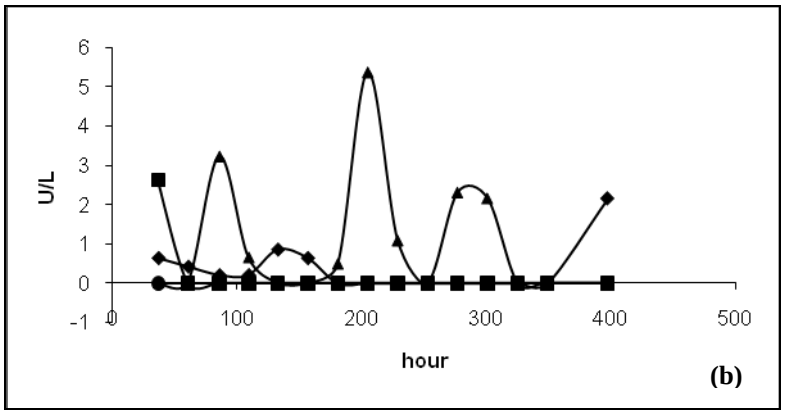
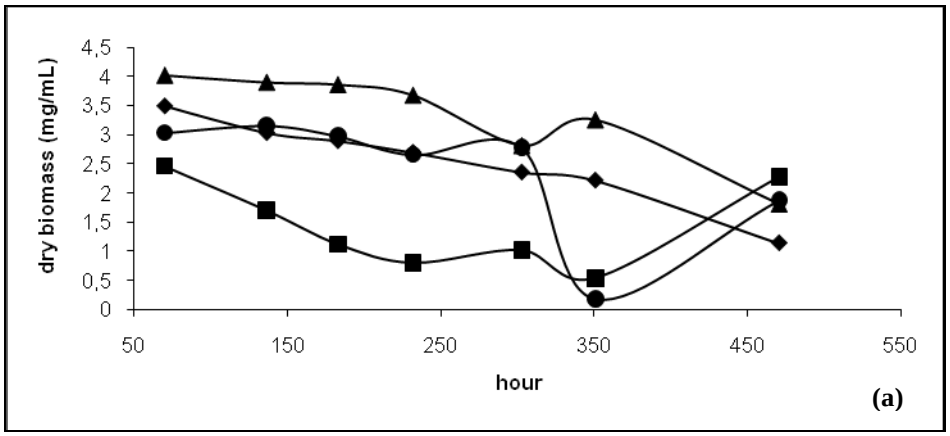


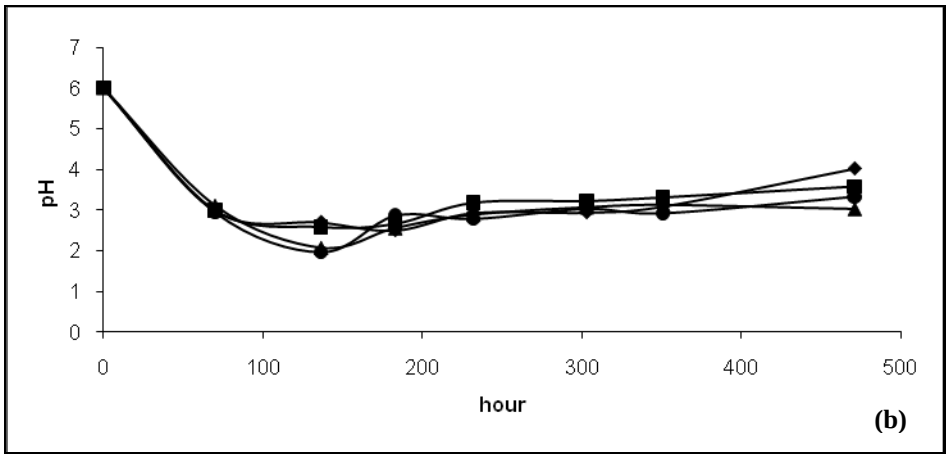
Figure 3.10 The effect of shaking on LiP (□♦□), MnP (□■□), Lac (□●□) and AAO (□▲□) activities. (a) shaking and (b) stationary cultivation conditions (continue).

As can be seen from Figure 3.10, no Lac activity was determined in this growth medium on both shaking and stationary conditions. Maximum AAO activity in this medium was detected as 6.542 U/L at 109.5th h when cultivation was carried out with shaking condition. There were not any significant differences on both shaking and stationary cultivation conditions in LiP and MnP activities.

Secondly, after the mycelium formed by both spore suspension and plug was inoculated to the growth medium, the incubation was carried out eight different conditions. These were presented as follows:

1.The mycelium formed with spore suspension, shaking condition (180 rpm) and at 28˚C.
2.The mycelium formed with plug, shaking condition (180 rpm) and at 28˚C.
3.The mycelium formed with spore suspension, shaking condition (180 rpm) and at 22˚C.
4.The mycelium formed with plug, shaking condition (180 rpm) and at 22˚C.
5.The mycelium formed with spore suspension, stationary condition and at 28˚C.
6.The mycelium formed with plug, stationary condition and at 28˚C.
7.The mycelium formed with spore suspension, stationary condition and at 22˚C.
8.The mycelium formed with plug, stationary condition and at 22˚C.





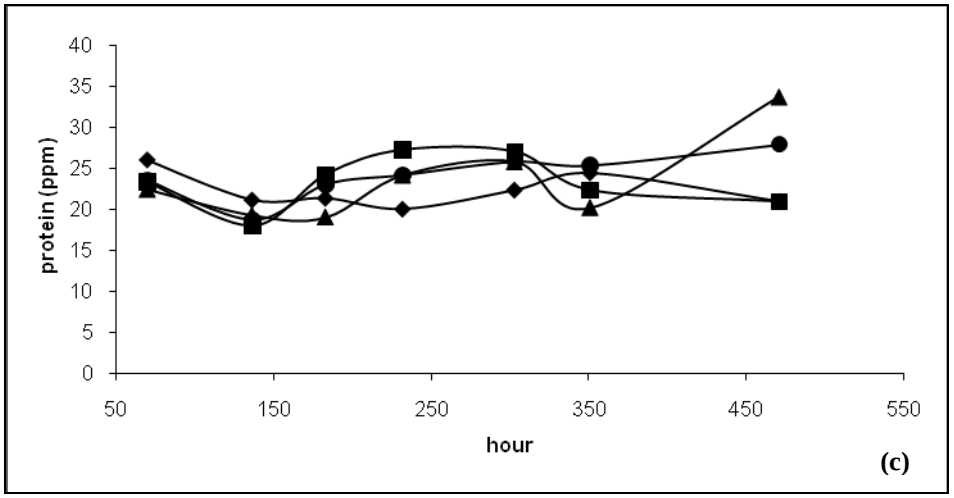


Figure 3.11 The effect of cultivation type and temperature on dry biomass (a), pH levels (b) and protein production (c); 1 (□♦□), 2 (□■□), 3 (□▲□), 4 (□●□).

As shown in Figure 3.11, the highest dry biomass was obtained when the cultivation was performed at 180 rpm, 22˚C and inoculation was carried out by mycelium formed by spore suspension. In all conditions, pH levels were showed same trends. The protein production in all conditions was very similar and maximum production, similarly dry biomass was obtained as 33.721 ppm at 471th h when the cultivation was performed at 180 rpm, 22˚C and inoculation was carried out by mycelium formed by spore suspension.

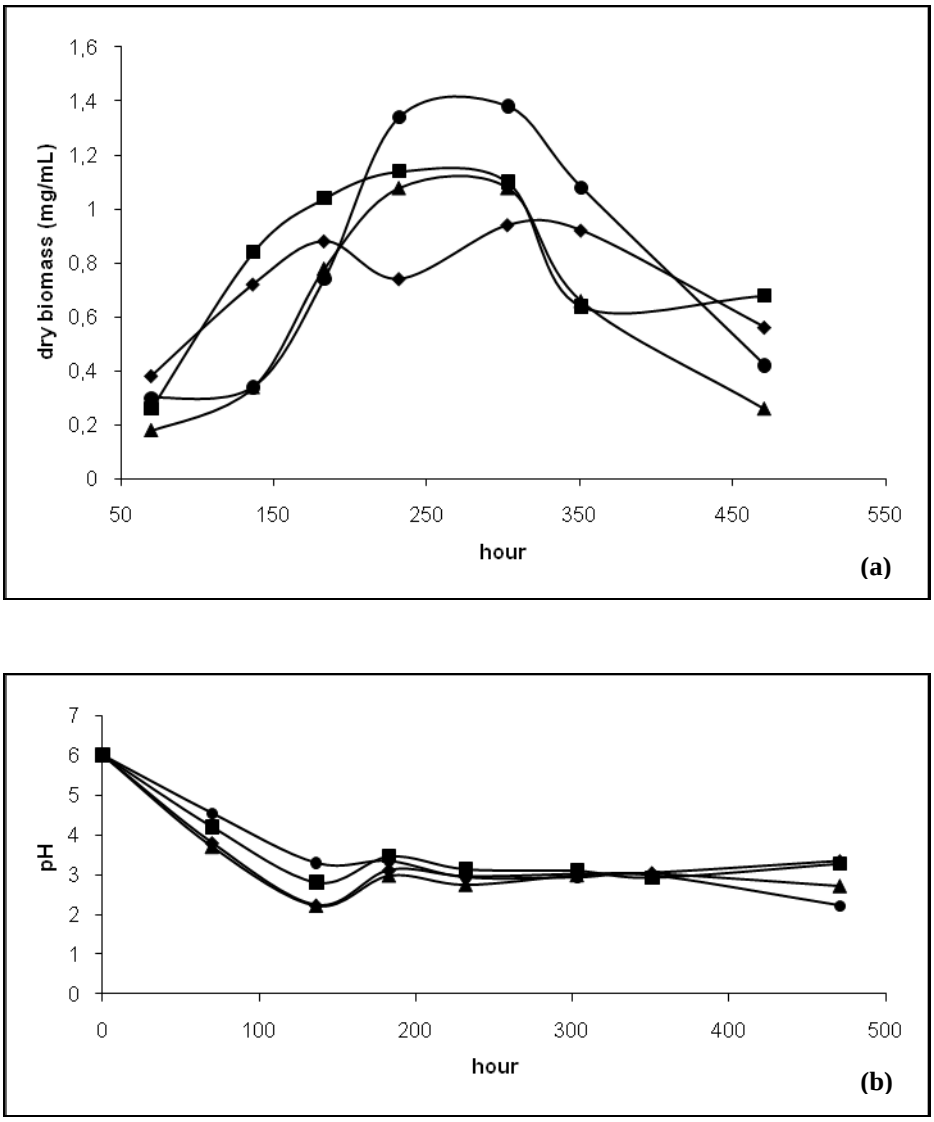


Figure 3.12 The effect of cultivation type and temperature on dry biomass (a), pH levels (b) and protein production (c); 5 (□♦□), 6 (□■□), 7 (□▲□), 8 (□●□).

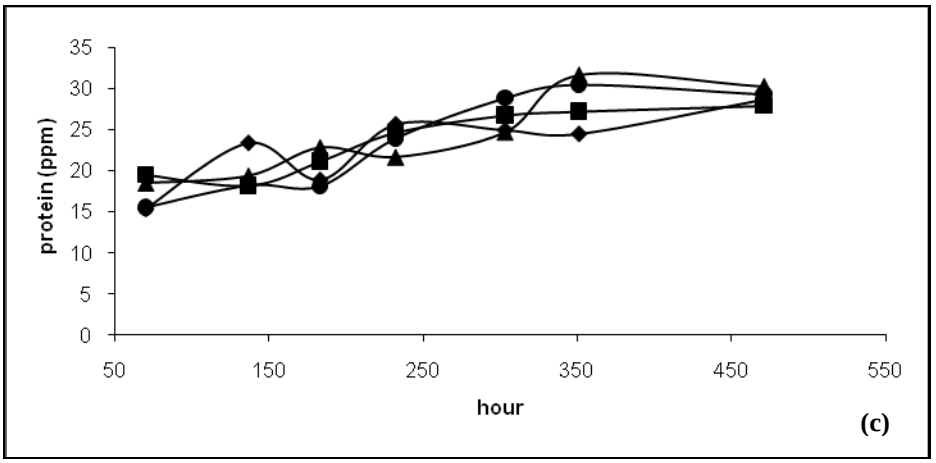


Figure 3.12 The effect of cultivation type and temperature on dry biomass (a), pH levels (b) and protein production (c); 5 (□♦□), 6 (□■□), 7 (□▲□), 8 (□●□) (continue).

As is depicted in Figure 3.12, the highest dry biomass was obtained when the cultivation on stationary condition was performed at 22˚C and inoculation was carried out by mycelium formed by plug. In all conditions, pH levels were showed same trends in incubation period. The protein production in all tested conditions was very similar and maximum production was obtained as 31.628 ppm at 351th h when the cultivation on stationary condition was performed at 22˚C and inoculation was carried out by mycelium formed by spore suspension.

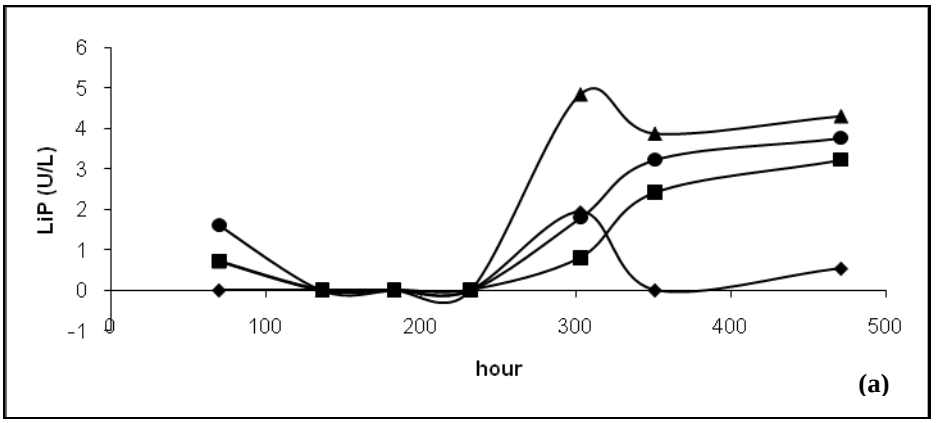
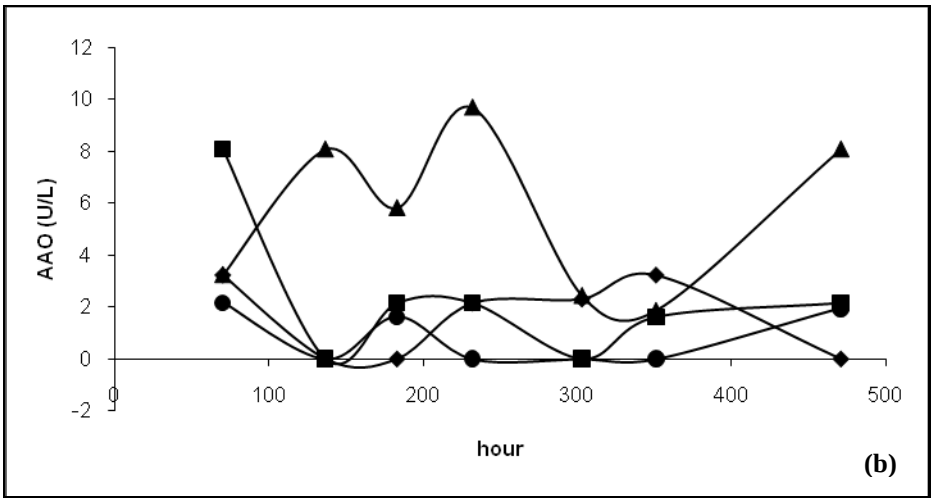


Figure 3.13 The effect of cultivation type and temperature on LiP (a), AAO (b) and MnP (c) activities; 1 (□♦□), 2 (□■□), 3 (□▲□), 4 (□●□).



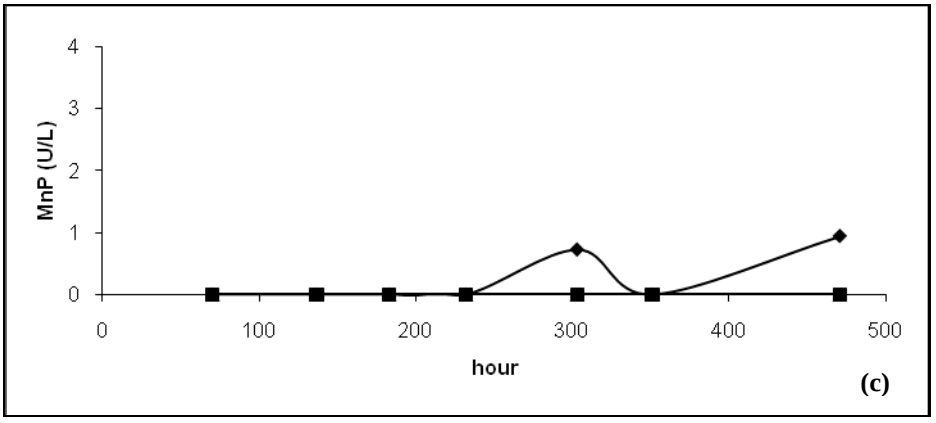
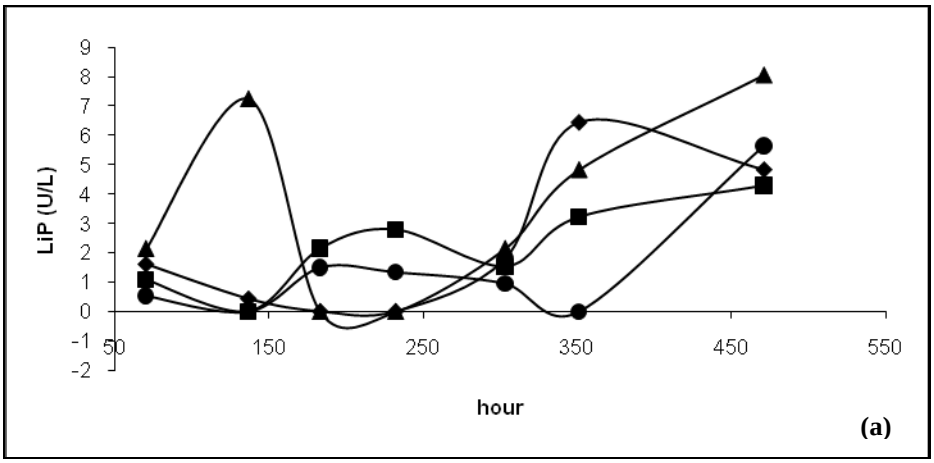
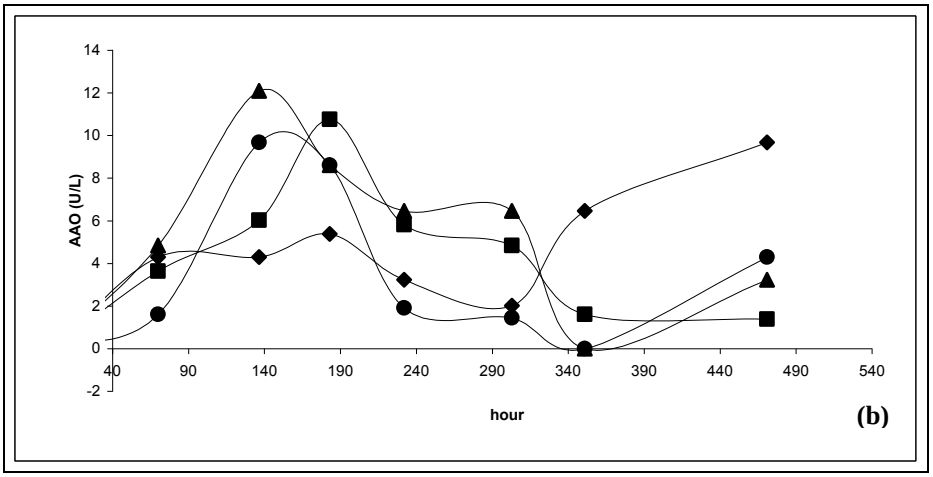


Figure 3.13 The effect of cultivation type and temperature on LiP (a), AAO (b) and MnP (c) activities; 1 (□♦□), 2 (□■□), 3 (□▲□), 4 (□●□) (continue).

The highest LiP activity was detected as 4.839 U/L at 303th h when the cultivation was performed at 180 rpm and 22˚C, and inoculation was carried out by mycelium formed by spore suspension (Figure 3.13a). The maximum AAO activity was confirmed as 9.677 U/L at 232th h when the cultivation was carried out when the cultivation was performed at 180 rpm and 22˚C, and inoculation was carried out by mycelium formed by spore suspension (Figure 3.13b). MnP activity was very low when the cultivation was performed at 180 rpm and 28˚C and inoculation by mycelium formed by spore suspension (Figure 3.13c). No Lac activity was obtained on all conditions. According to results, the obtained maximum LiP and AAO activity conditions are consistent with dry biomass and protein productions.





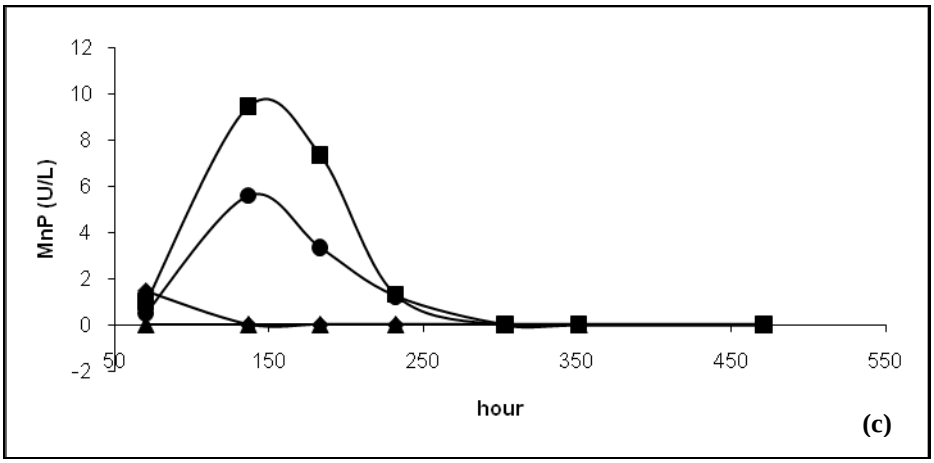


Figure 3.14. The effect of cultivation type and temperature on LiP (a), AAO (b) and MnP (c) activities; 5 (□♦□), 6 (□■□), 7 (□▲□), 8 (□●□).

As seen from Figure 3.14, the highest LiP activity was detected as 8.065 U/L at 471th when the cultivation on stationary condition was performed at 22˚C and inoculation by mycelium formed by spore suspension. The maximum AAO activity was confirmed as 12.097 U/L at 136.5th h when the cultivation was performed on stationary condition and 22˚C and inoculation by mycelium formed by spore suspension. The maximum MnP activity was obtained as 9.454 U/L when the cultivation on stationary condition was performed at 28˚C and inoculation by mycelium formed by plug. No Lac activity was obtained on all tested conditions. According to results about eight different conditions, stationary cultivation condition is suitable for ligninolytic enzyme activity from *P.eryngii*.

3.2.5 The Effect of Mn^{2+} Concentration

The ligninolytic enzyme activity variations by white rot fungi were affected especially growth medium components and their concentrations. In this study, 10 mL of liquid medium (1) was used for this purpose. This medium contained 250 and supplemented 500 µM Mn^{2+}. The inoculation was carried out by spore suspension and the stationary cultivation was performed at 28˚C.

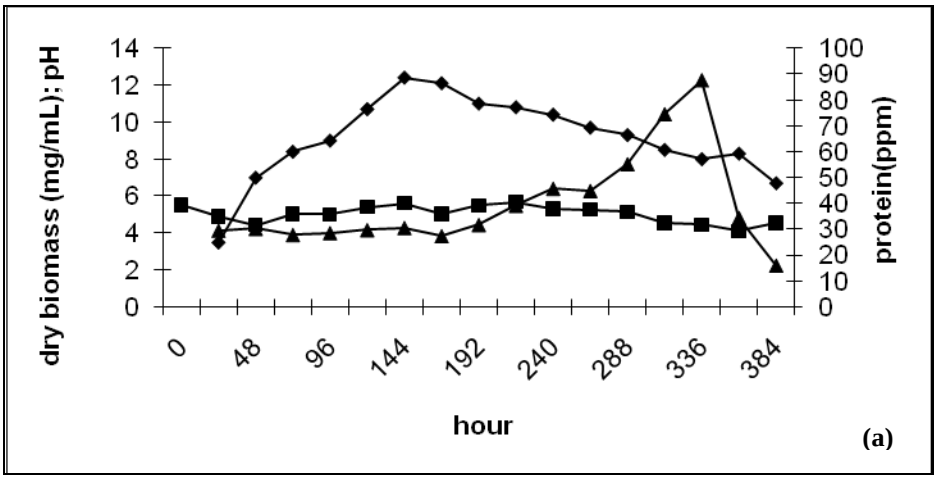


Figure 3.15 The effect of manganese concentration on dry biomass (□♦□), pH levels (□■□) and protein production (□▲□) on growth medium (1) in the presence of 250 (a) and 500 (b) µM Mn^{2+}.

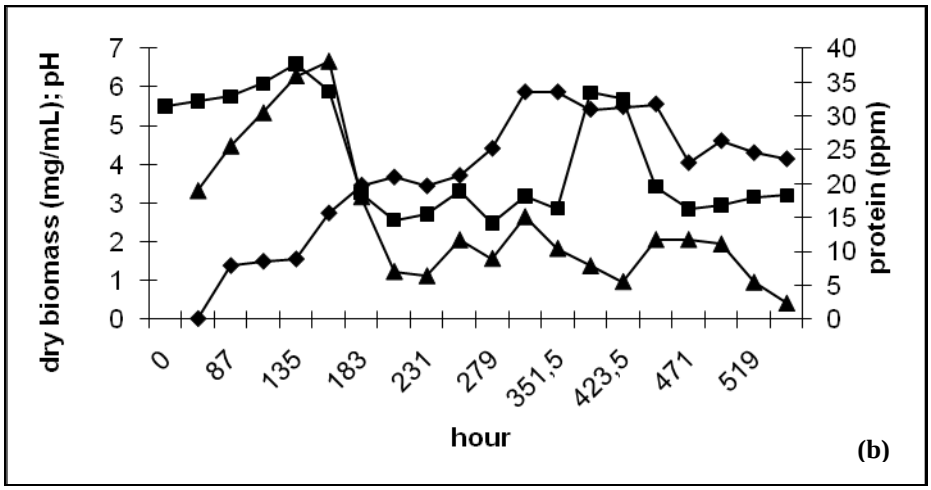
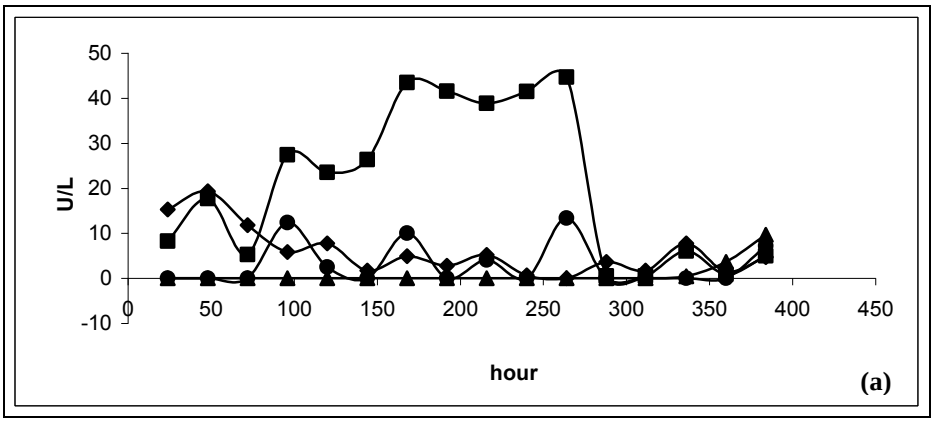


Figure 3.15 The effect of manganese concentration on dry biomass (□♦□), pH levels (□■□) and protein production (□▲□) on growth medium (1) in the presence of 250 (a) and 500 (b) µM Mn^{2+} (continue).

As shown in Figure 3.15, dry biomass in the presence of 250 µM Mn^{2+} constantly increased up to 145.5th h, then it was not much changed. The dry biomass in 500 µM Mn^{2+} gradually increased up to 327th hour and it reached to stationary phase at that time. The pH value in the presence of 250 µM Mn^{2+} was almost constant, but in 500 µM Mn^{2+} changed. Initially, the protein level in 250 µM Mn^{2+} was slowly increased and it was reached to maximum at 145.5th h as 31.321 ppm and the protein level in 500 µM Mn^{2+} was reached to maximum at 159th h as 38.043 ppm, then suddenly decreased and it was not much changed.



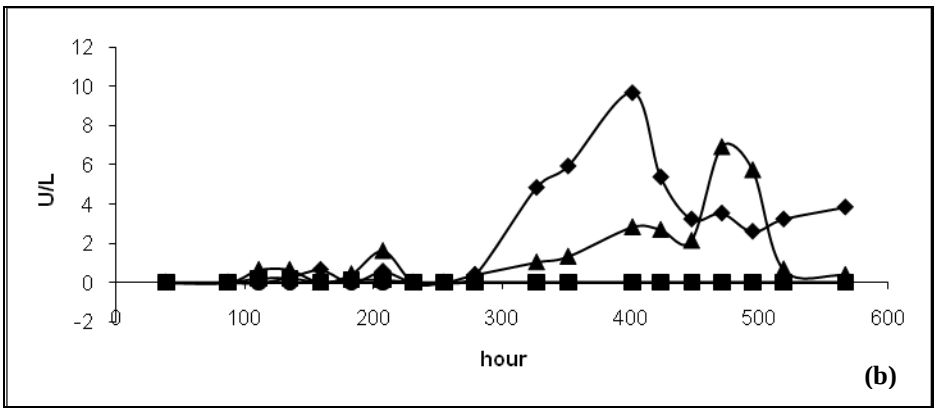


Figure 3.16 The effect of manganese concentration on LiP (□♦□), MnP (□■□), Lac (□●□) and AAO (□▲□) activities on growth medium (2) in the presence of 250 (a) and 500 (b) μM Mn^{2+}.

As can be seen from Figure 3.16, there was no Lac activity in the presence of 500 μM Mn^{2+}, however in 250 μM Mn^{2+} Lac activity showed two peaks as 12.333 and 13.389 U/L at 96 and 264th h, respectively. The maximum LiP activity was two-fold higher in the presence of 250 μM Mn^{2+} in comparison with 500 μM Mn^{2+} ($p<0.05$). The highest MnP activity was observed in 250 μM Mn^{2+} as 44.675U/L at 264th hour, whereas AAO activity demonstrated maximum as 9.677 U/L at 384th hour in 250 μM Mn^{2+}.

To optimize this parameter for ligninolytic enzyme activity, the experiences were performed using 10 mL of culture medium (2) containing 4g/L of ammonium nitrate, 1 mM copper sulphate and in the presence or absence of different manganese

concentrations. Inoculation was carried out with plug. Each flask on stationary cultivation condition was incubated at 28˚C.

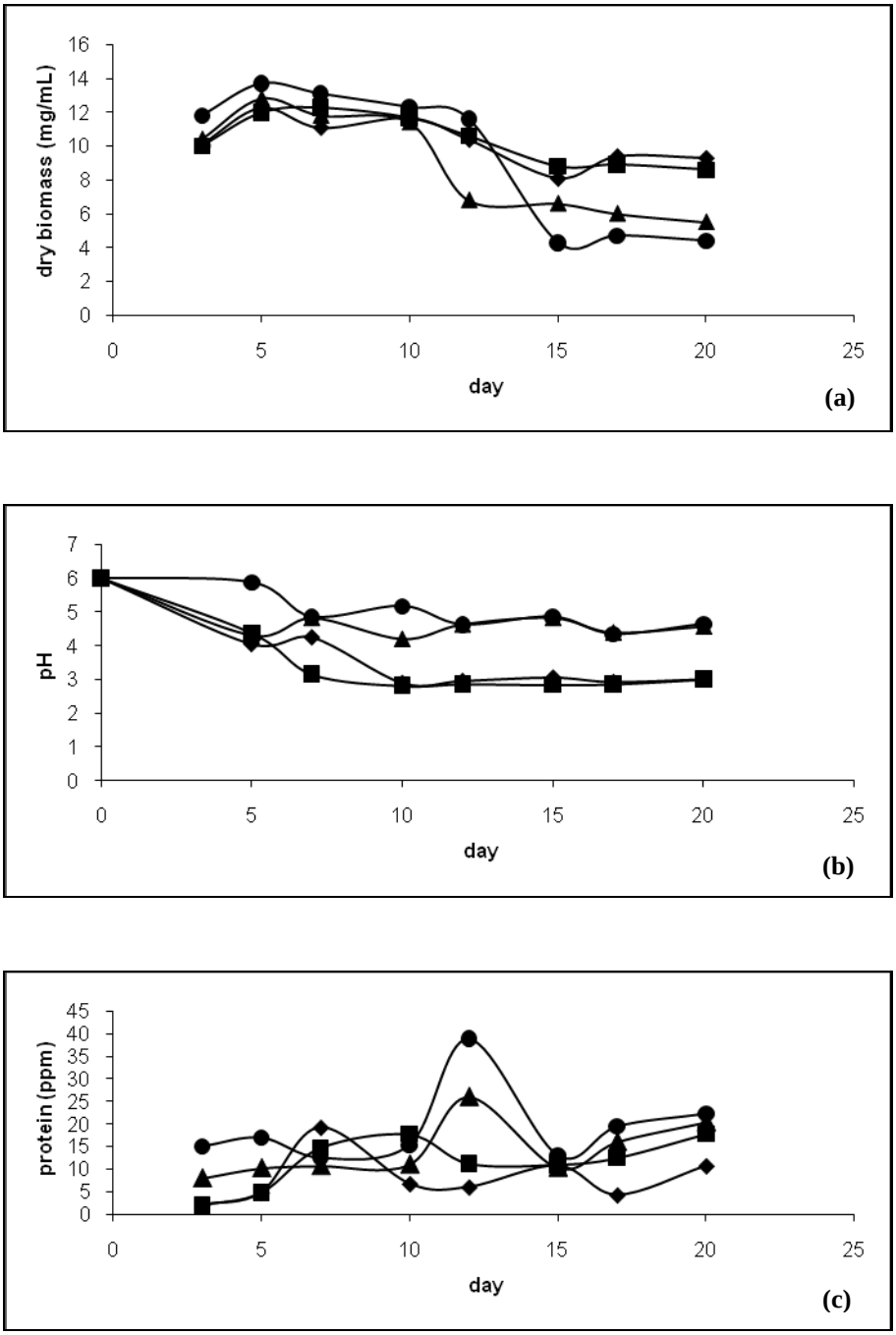


Figure 3.17 The effect of manganese concentrations on dry biomass (a), pH levels (b) and protein production (c) in the presence of 180 (□♦□), 500 (□■□), 750 (□▲□) and 1000 (□●□) µM Mn^{2+} concentration on growth medium (2).

P. eryngii reached to its stationary phase on 5th day in all tested growth condition. The pH values in all condition were gradually decreased. The maximum protein production was observed in 1000 following 750 µM Mn^{2+} on 12th day.

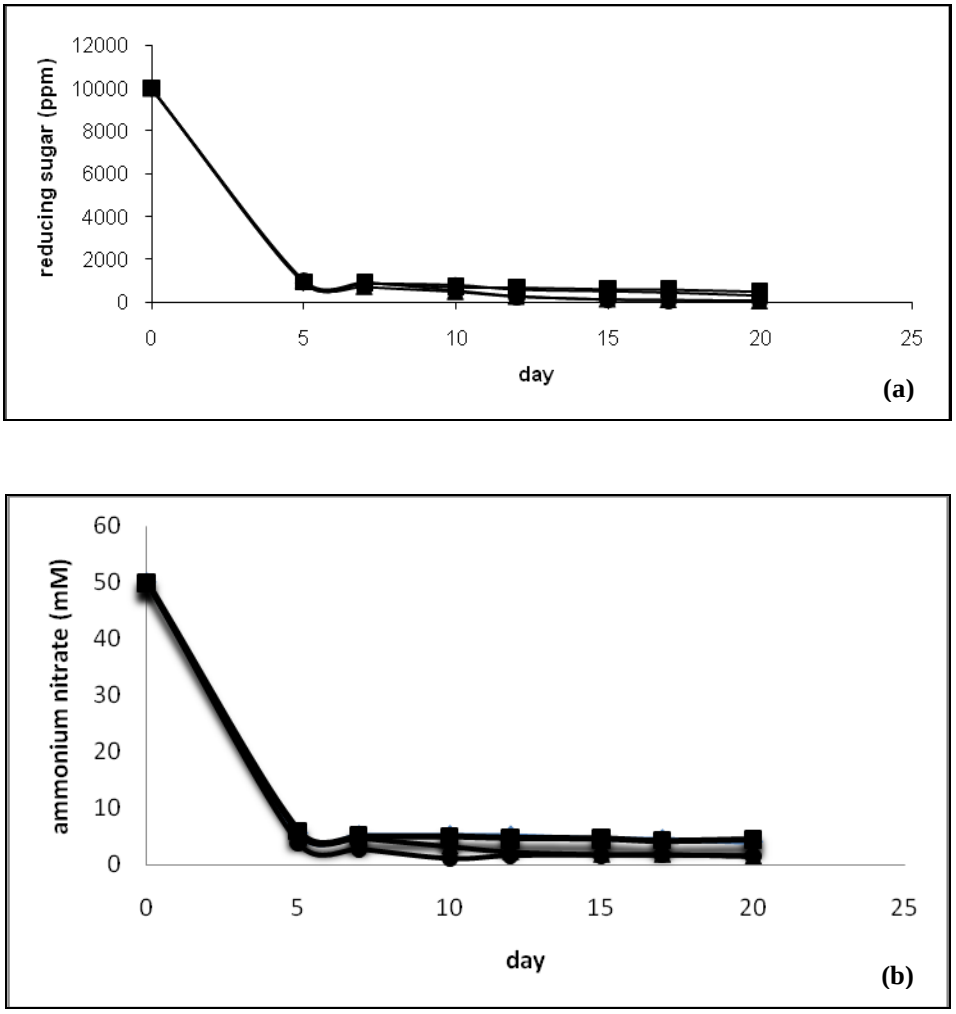
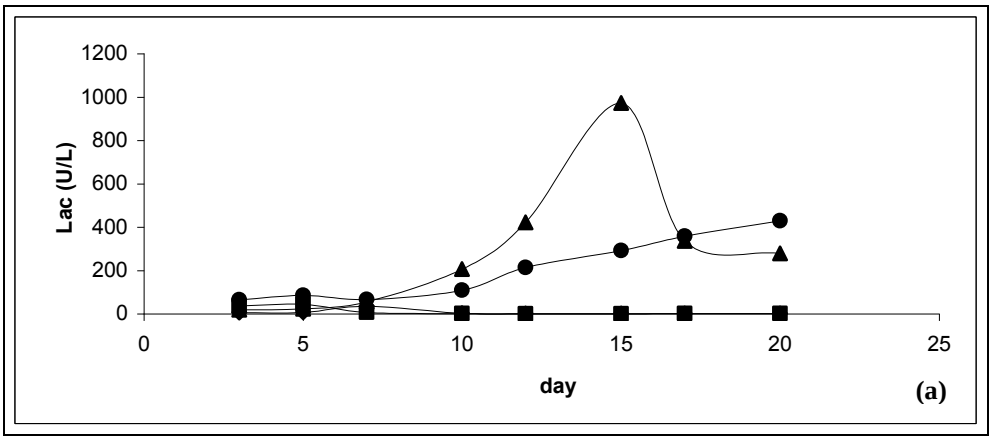


Figure 3.18 The effect of manganese concentrations on reducing sugar amounts (a) and nitrogen levels (b) in the presence of 180 (□♦□), 500 (□■□), 750 (□▲□) and 1000 (□●□) µM Mn^{2+} concentration on growth medium (2).

The reducing sugar and nitrogen amounts in all growth medium was rapidly decreased up to 5th day, but not depleted fully by *P.eryngii.*



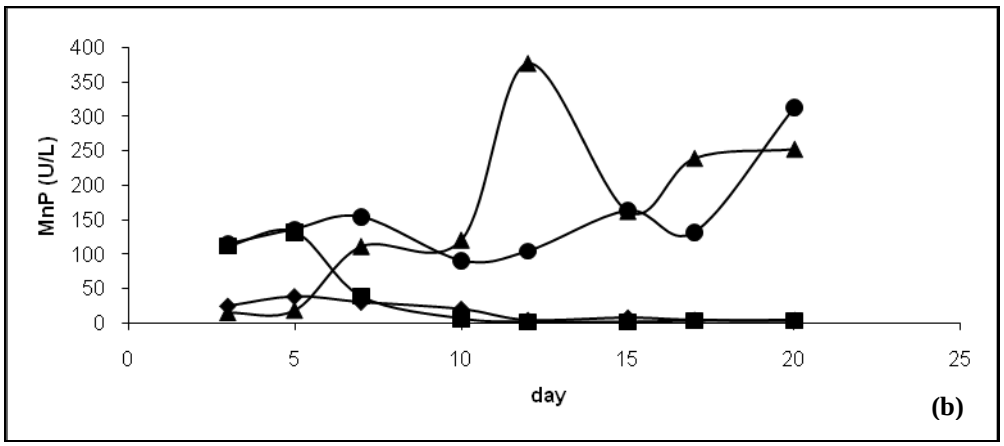


Figure 3.19 The effect of manganese concentrations on laccase (a) and MnP (b) activities in the presence of 180 (□♦□), 500 (□■□), 750 (□▲□) and 1000 (□●□) μM Mn^{2+} concentration on growth medium (2).

The maximum Lac and MnP activities were determined on 15th day as 3.623 and 3.233 U/L, respectively, on control growth medium, which did not contain Mn^{2+} and Cu^{2+}. The highest Lac activity was detected as 973.333 U/L on 15th day in the presence of 750 μM Mn^{2+}. The highest MnP activity was detected as 376.519 U/L on 12th day in the presence of 750 μM Mn^{2+} (Figure 3.19).

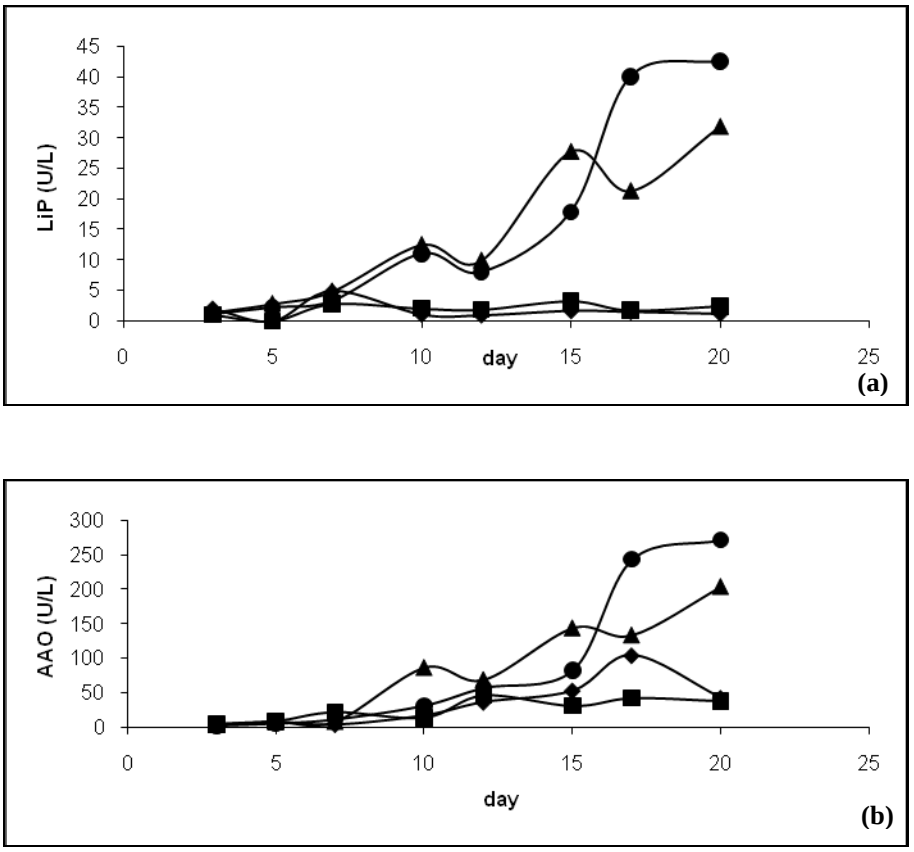


Figure 3.20 The effect of manganese concentrations on LiP (a) and AAO (b) activities in the presence of 180 (□♦□), 500 (□■□), 750 (□▲□) and 1000 (□●□) μM Mn^{2+} concentration on growth medium (2).

The maximum LiP and AAO activities were determined as 42.527 and 271.828 U/L, respectively, on 20th day in the presence of 1000 μM Mn^{2+} (Figure 3.20). Besides, under control condition not including Mn^{2+} and Cu^{2+} growth medium, the maximum LiP and AAO activities were detected as 5.018 and 8.602 U/L on 15th and 10th day, respectively.

3.2.6 The Effect of Cu^{2+} Concentration

In this study, 10 mL of liquid medium (1) was used for optimizing copper concentration to obtain maximum ligninolytic enzyme activities. This medium contained 500 μM Mn^{2+} and Cu^{2+} or not. The inoculation was carried out by spore suspension and the stationary cultivation was performed at 28˚C.

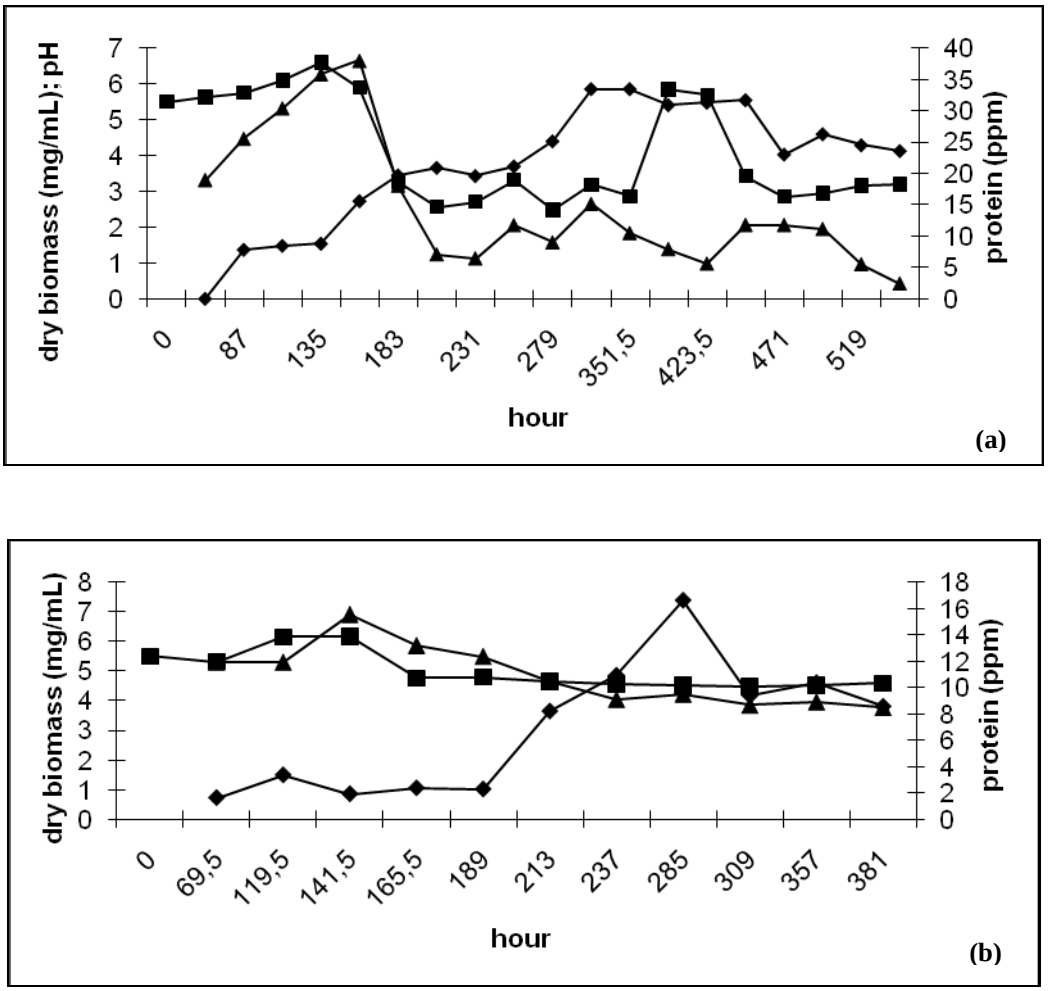


Figure 3.21 The effect of copper concentration on dry biomass (□♦□), pH levels (□■□) and protein production (□▲□) on growth medium (1) in the absence of copper sulphate (a) and presence of 500 µM (b) Cu^{2+}.

In absence of Cu^{2+}, the dry biomass gradually increased up to 327th h and pH value slowly increased till 135th h and then rapidly decreased and again increased. pH level in presence of 500 µM Cu^{2+} decreased from 5.5 to 4.59 depending incubation time. Protein production in absence of Cu^{2+} was reached to maximum at 159th h as 38.043 ppm, then suddenly decreased and thereafter not much changed. Also, the maximum protein production in growing medium including 500 µM Cu^{2+} was obtained as 15.532 ppm at 141.5th h (Figure 3.21). According to results, increasing Cu^{2+} concentrations may have a negative effect on protein production.

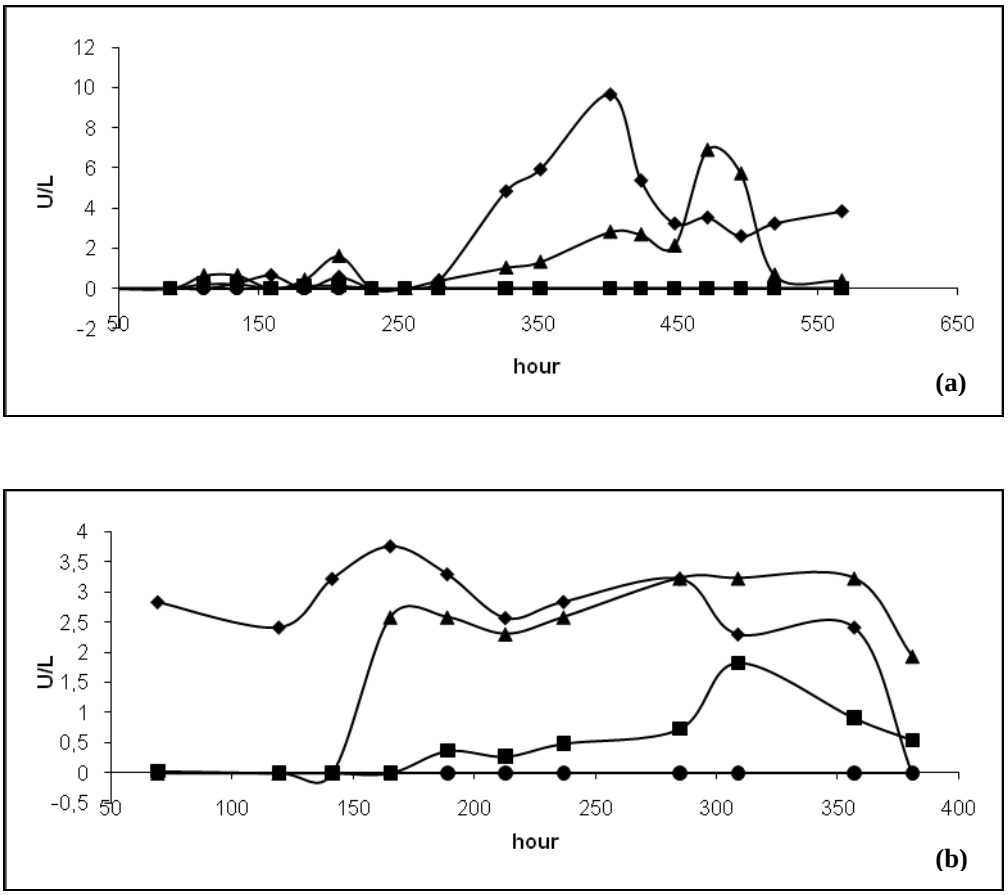


Figure 3.22 The effect of copper concentration on LiP (□♦□), MnP (□■□), Lac (□●□) and AAO (□▲□) activities on growth medium (1) in the absence of copper sulphate (a) and presence of 500 μM (b) Cu^{2+}.

MnP activity was determined in very low values compared to the LiP and AAO enzyme activities. The highest LiP and AAO activities were detected as 9.677 and 6.912 U/L at 401.5th and 471th h, respectively, in absence of Cu^{2+}. No Lac activity was observed in growth medium (1) including both culture conditions. That's way; this growth medium is not suitable for laccase enzyme activity.

Also, the other culture medium (2) including 0.70 or 3 mM copper sulphate and 180 μM Mn^{2+} was used for maximum ligninolytic enzyme activity. The cultivation conditions and inoculation type were the same as medium (1).

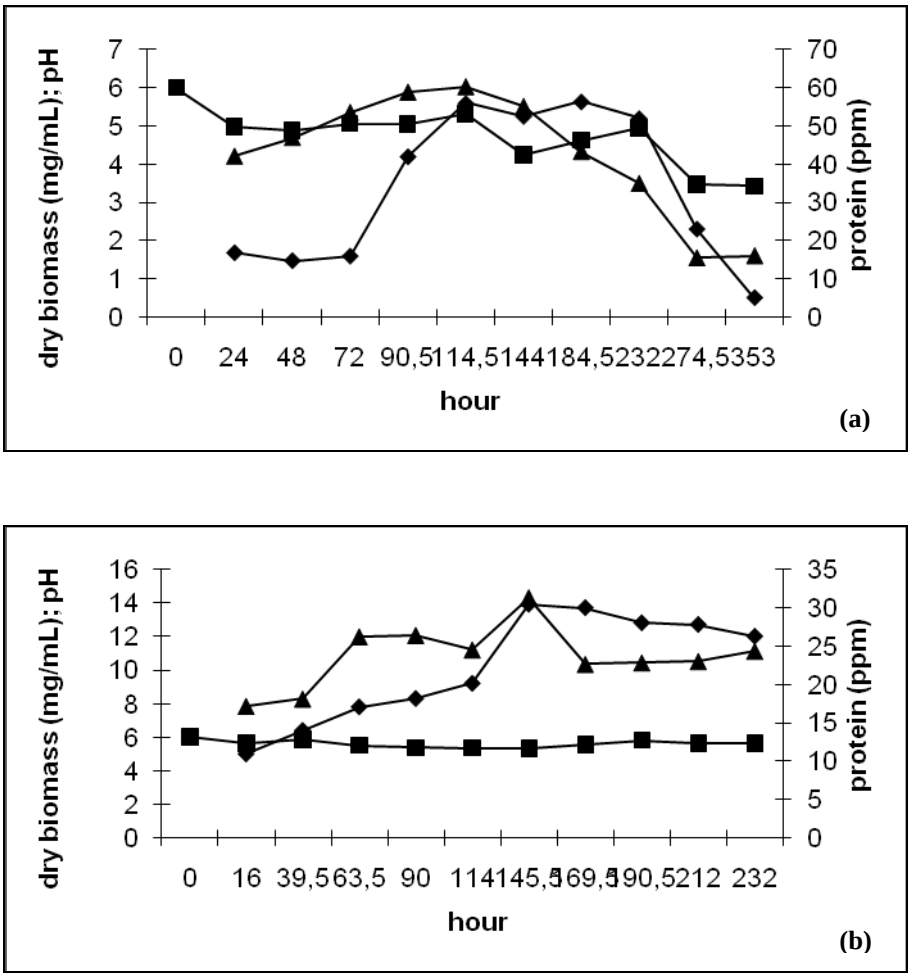


Figure 3.23 The effect of copper concentration on dry biomass (□♦□), pH levels (□■□) and protein production (□▲□) on growth medium (2); (a) 0.70 and (b) 3 mM copper sulphate.

As shown in Figure 3.23, the cultivation was reached to stationary phase at 114.5th hour on growing medium including 70 μM Cu^{2+}, however it was reached to it at 145.5th when the cultivation was carried out in presence of 3.0 mM Cu^{2+}. There were not any changes in pH levels in 3mM Cu^{2+}. But it was gradually decreased in 70 μM Cu^{2+}. Compared to the all cultivation conditions, the maximum protein production was obtained as 60.143 ppm at 114.5th h in 70 μM Cu^{2+}.

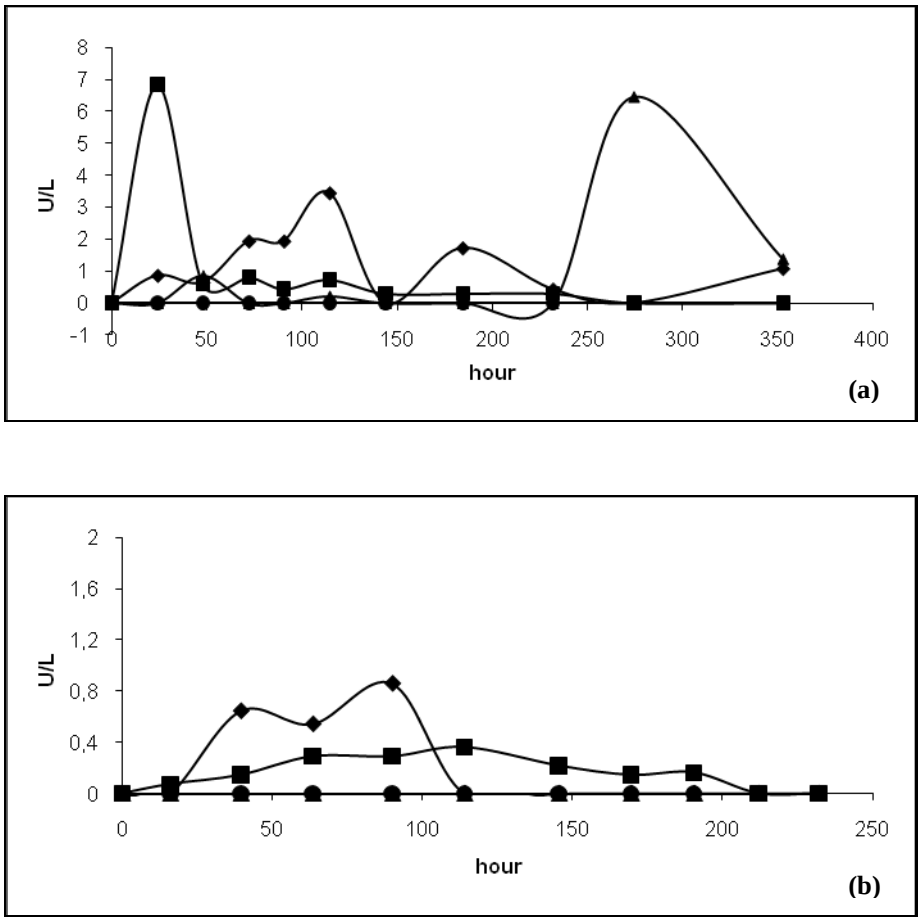


Figure 3.24 The effect of copper concentration on LiP (□♦□), MnP (□■□), Lac (□●□) and AAO (□▲□) activities on growth medium (2); (a) 0.70 and (b) 3 mM copper sulphate.

No Lac and AAO activities including 3 mM Cu^{2+} was observed. While there was no Lac activity was obtained, the maximum AAO activity was detected as 6.452 U/L at 274.5th h on growth medium including 70 μM Cu^{2+}. The highest LiP and MnP activities were determined as 3.441 and 6.836 U/L at 114.5th and 24th h in 70 μM Cu^{2+}. And also, the low LiP and MnP activities were determined in 3 mM Cu^{2+}.

To optimize this parameter for maximum ligninolytic enzyme activity, the another experiences were performed using 10 mL of culture medium (2) containing 4g/L of ammonium nitrate, 500 μM manganese sulphate and in the presence or

absence of different copper concentrations. Inoculation was carried out with plug. Each flask on stationary culture condition was incubated at 28˚C.

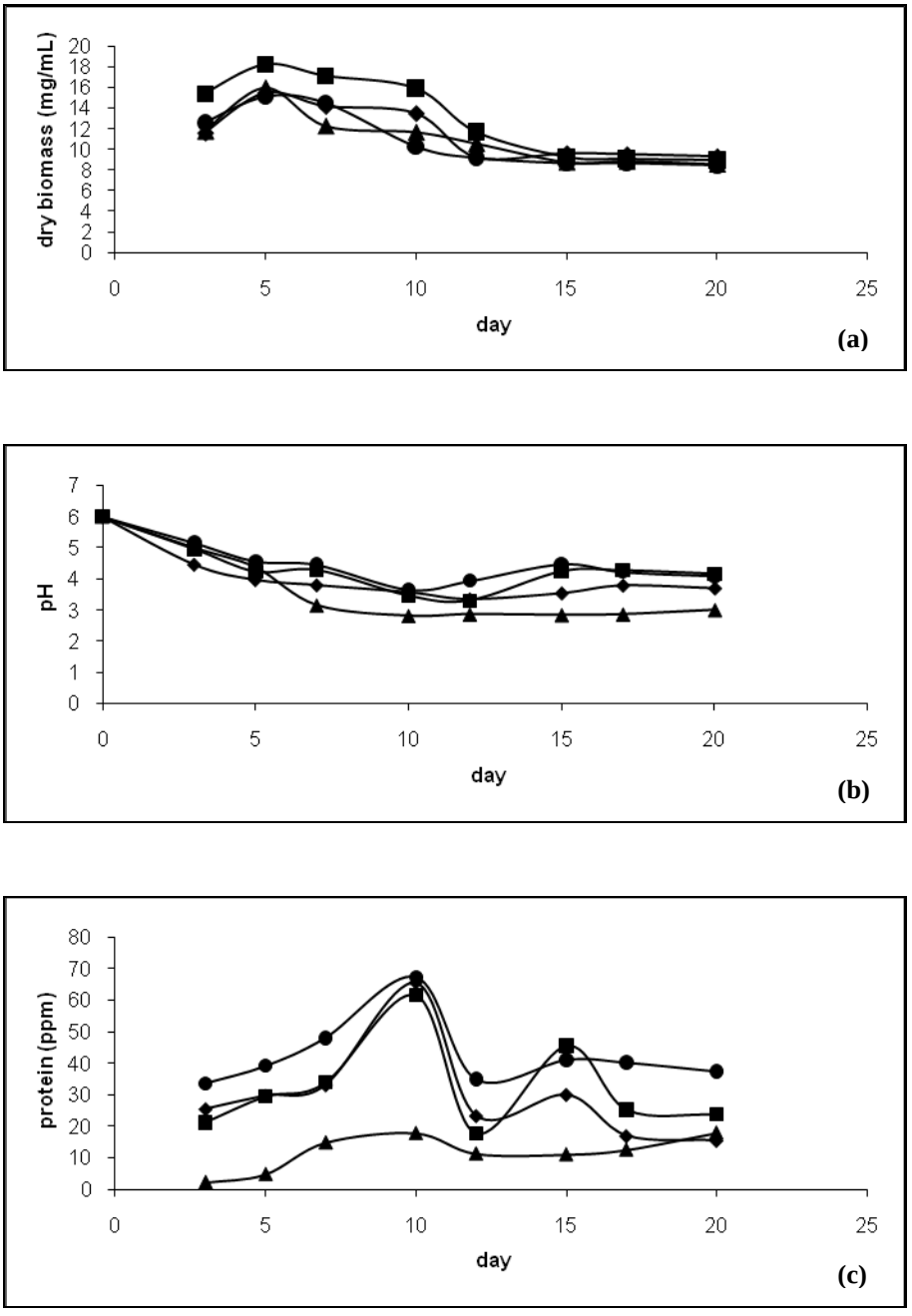


Figure 3.25 The effect of copper concentrations on dry biomass (a), pH levels (b) and protein production (c) in the presence of 0.14 (□♦□), 0.5 (□■□), 1.0 (□▲□) and 2.0 (□●□) mM Cu^{2+} concentration on growth medium (2).

P. eryngii cultivation reached to stationary phase on 5th day in all growth conditions. The pH values in all conditions were indicated similar trend. The maximum protein production was observed in 2 mM Cu^{2+} as 67.222 ppm on 12th day (Figure 3.25).

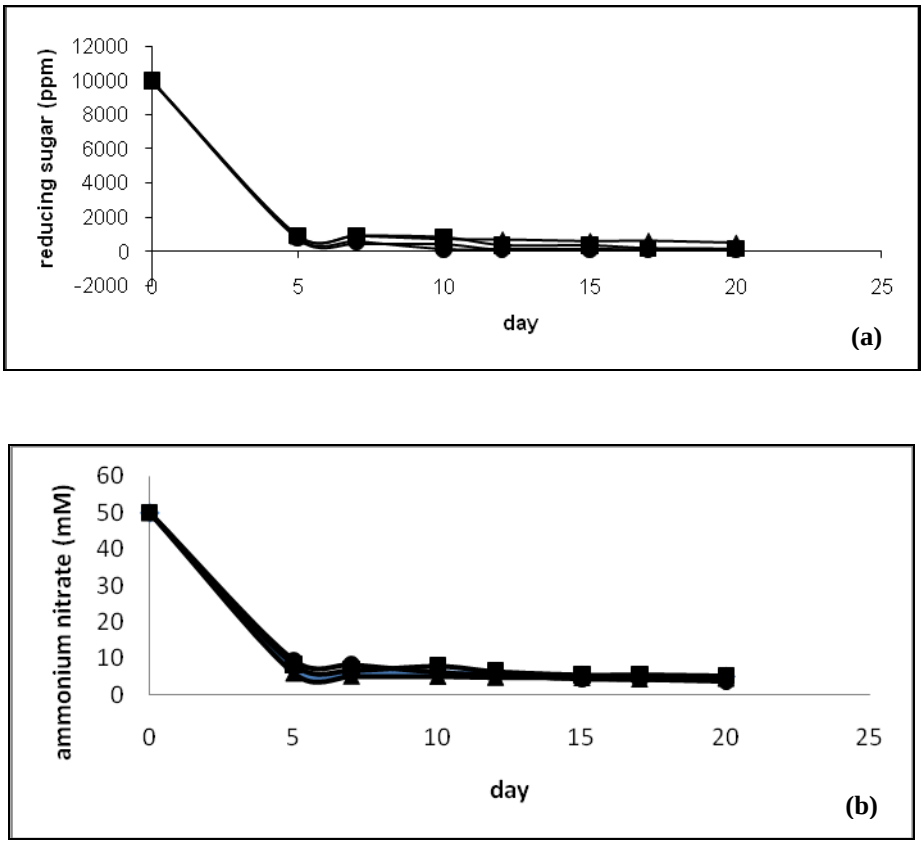


Figure 3.26 The effect of copper concentrations on reducing sugar amounts (a) and nitrogen level (b) in the presence of 0.14 (□♦□), 0.5 (□■□), 1.0 (□▲□) and 2.0 (□●□) mM Cu^{2+} concentration on growth medium (2).

The glucose and nitrogen consumption in all growth conditions was increased up to 5th day, and then slowly increased but it was not completely depleted (Figure 3.26).

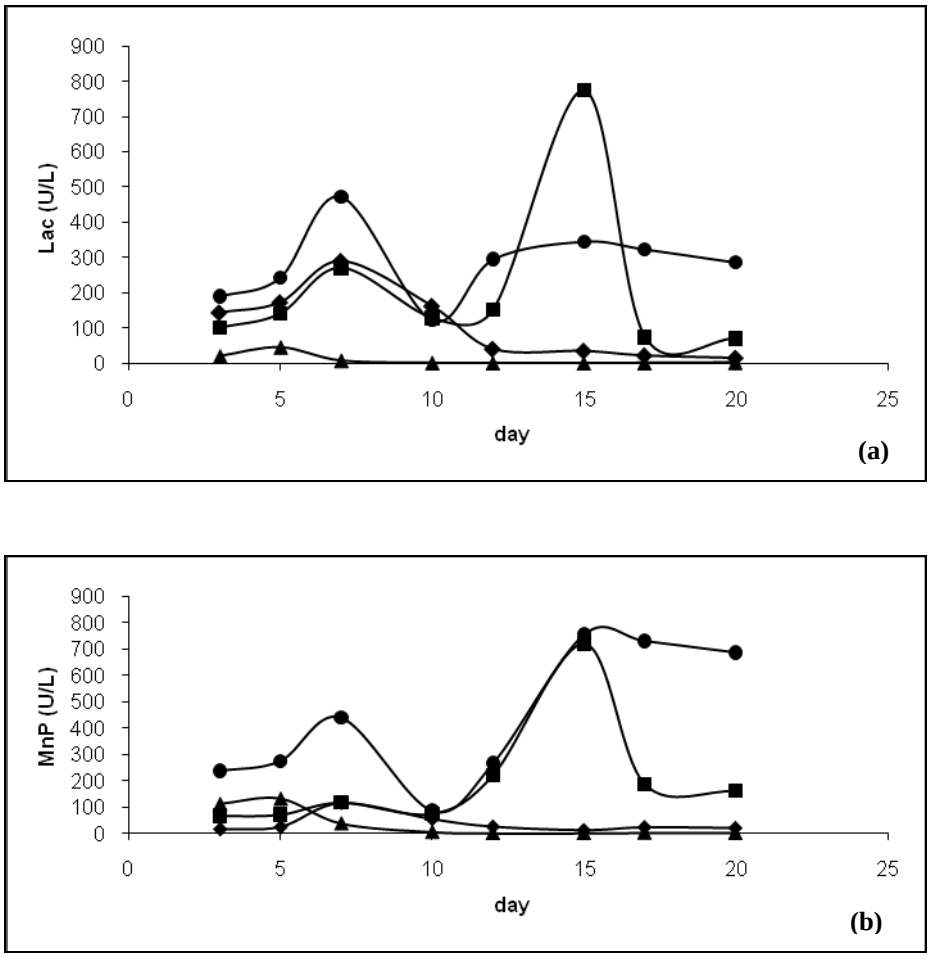


Figure 3.27 The effect of copper concentrations on Lac (a) and MnP (b) activities in the presence of 0.14 (□♦□), 0.5 (□■□), 1.0 (□▲□) and 2.0 (□●□) mM Cu^{2+} concentration on growth medium (2).

The highest Lac and MnP activities were determined on 15th day as 3.623 and 3.233 U/L, respectively, on control growth medium, which did not contain Mn^{2+} and Cu^{2+}. The maximum Lac activity was determined as 774.167 U/L on 15th day in the presence of 0.5 mM Cu^{2+}. The MnP showed maximum activity on 15th day as 756 U/L in 2 mM Cu^{2+}. The MnP activity in the presence of 0.5 mM Cu^{2+} was very close to the activity of 2 mM Cu^{2+} and it was detected as 721.091 U/L on 15th day (Figure 3.27).

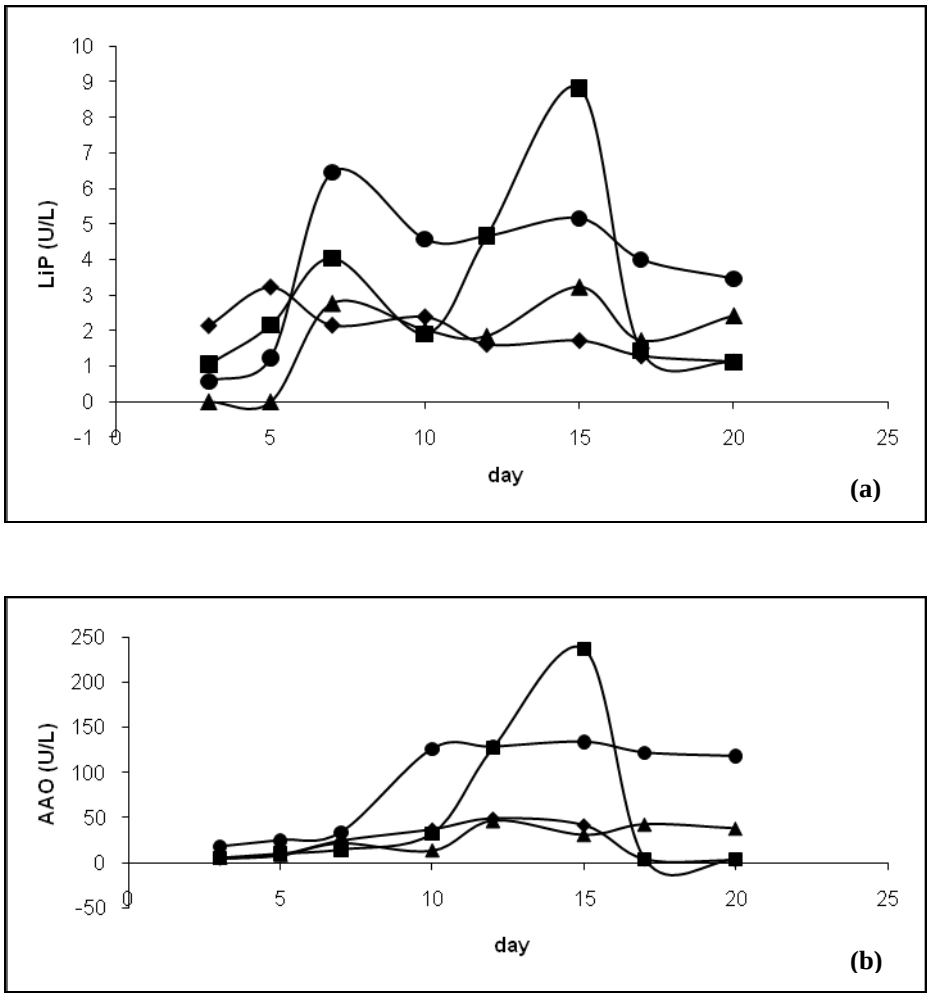


Figure 3.28 The effect of copper concentrations on LiP (a) and AAO (b) activities in the presence of 0.14 (□♦□), 0.5 (□■□), 1.0 (□▲□) and 2.0 (□●□) mM Cu^{2+} concentration on growth medium (2).

Similarly Lac activity, the maximum LiP and AAO activities were determined on 15^{th} day as 8.817 and 236.989 U/L in the presence of 0.5 mM Cu^{2+}, respectively (Figure 3.28). Besides, under control condition not including Mn^{2+} and Cu^{2+} growth medium, the highest LiP and AAO activities were detected as 5.018 and 8.602 U/L on 15^{th} and 10^{th} day, respectively.

3.3 Decolorization

Synthetic dyes such as methyl orange, reactive red 2 and reactive black 5 were treated with ligninolytic enzymes. The highest decolourisation was performed methyl orange as % 9.92 after 10 minutes of treatment.

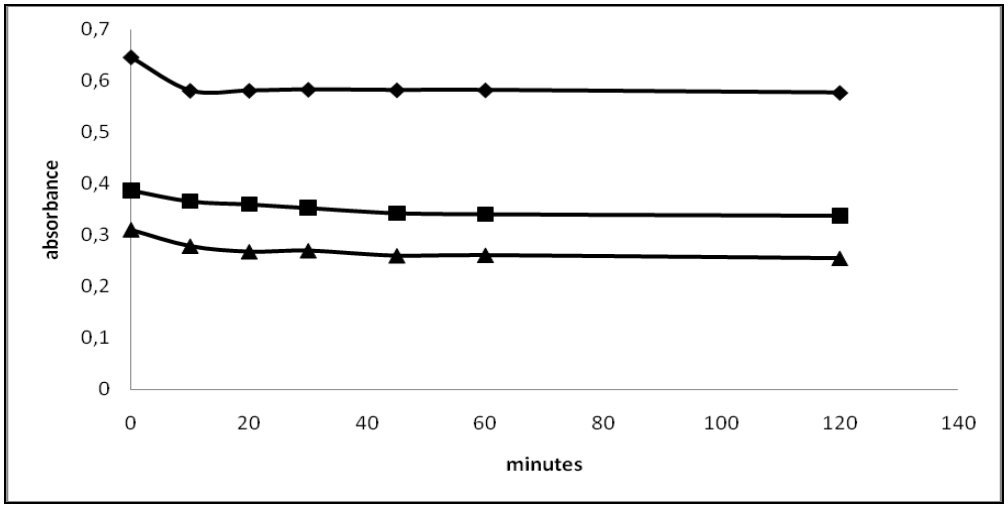


Figure 3.29 The decolorization of some azo dyes; MO, (□♦□), 50 mg/L (□●□), RR, (□■□), RB, (□▲□).

CHAPTER FOUR

DISCUSSION

The present study evaluated ligninolytic enzyme activity variations from *P.eryngii* in SF conditions. *P.eryngii* has been extensively studied as edible mushroom and a little is known on the ligninolytic systems of this fungus.

On SF cultivation, the effects of different inoculation types, synthetic growth media and their volumes as well as Mn^{2+} and Cu^{2+} concentrations, temperature and cultivation mode were investigated so as to obtain maximum ligninolytic enzyme activity. 750 μM Mn^{2+} and 0.5 mM Cu^{2+} concentration were determined as optimum under stationary condition at 28ºC in 100 mL flasks in the presence of 10 mL of growth medium (2, initial pH 6.0) after inoculation 1 cm disks of plug for attaining maximum ligninolytic enzyme activity from *P.erngii*. According to results, the maximal ligninolytic enzyme activities from *P.eryngii* in SF were more suitable under stationary than shaking conditions. Similar to these results, the Mu˜noz, et al. (1997) found that when the cultivation was performed in shake-flask, there were no detectable LiP or MnP activities by *Pleurotus* spp. Moreover, it has been suggested that ligninolytic enzymes production is influenced by environmental factors such as temperature, pH, inducers, culture conditions, and medium composition (Giardina, et al., 1999; Téllez-Téllez, Fernandez, Montiel-Gonzalez, Sanchez, & Díaz-Godinez, 2008) as the mentioned above.

When the SF cultivation was carried out without Mn^{2+} and Cu^{2+} concentrations as a control condition, the highest Lac and MnP activities were detected as 3.623 and 3.233 U/L, respectively, on the 15^{th} day, while LiP and AAO activities were obtained as 5.018 and 8.602 U/L on the 15^{th} and 10^{th} day, respectively. Under the optimal SF conditions, Lac activity demonstrated the 268.65-fold increases in the presence of 750 μM Mn^{2+} and 1.0 mM Cu^{2+}, and the 213.68-fold increases in the presence of 500 μM Mn^{2+} and 0.5 mM Cu^{2+} when compared to control's values. Similar results were attained by Lorenzo, et al. (2002), who found that grape stalk cultures presented

activities around 600-800 U/L, whereas grape seed and barley straw cultures exhibited values of 300-500 U/L from the 20th to the 30th day under SF conditions by *T. versicolor*. The MnP activity showed the 116.46-fold increment in 750 μM Mn^{2+} and 1.0 mM Cu^{2+}, and the 233.84-fold increment in 500 μM Mn^{2+} and 2.0 mM Cu^{2+} in comparison with the control's values. In addition, the maximum LiP activity was the 8.47-fold and AAO activity was the 31.6-fold higher than that of control's values in the presence of 1000 μM Mn^{2+} and 1.0 mM Cu^{2+}.

The pH on all fermentation process is an important factor to control of ligninolytic enzyme activities. According to the obtained results, the pH levels generally remained between 4.5 and 5.5 during the cultivation time and these results supported by Cabaleiro, et al. (2002). The optimal pH was found in the range of 4.0-5.0 to attain maximum Lac activity. Bettin, et al. (2011) found that the Lac production was decreased at a pH above 5 and the optimal pH for Lac production indicated three different peaks at 2.4, 3.2 and 4.4. Similarly, using a different strain of *P. sajor-caju*, Murugesan, et al. (1995) showed that the optimal pH for Lac production was 5.0.

The reducing sugar levels were sharply decreased up to 5th day on SF cultivation, then nearly stable, so that the cultivation was reached to carbon-limited medium (critical reducing sugar concentration), thus significant ligninolytic enzyme secretion by *P.eryngii* started. This result is supported by Galhaup, et al. (2002) who produced laccase by *T. pubescens*. As well, the lignin degradation and ligninolytic enzymes production are understood to be secondary metabolic processes triggered by the depletion of nutrients such as carbon or nitrogen sources from a medium. When the reducing sugar was reached to critical concentration on 5th day (Bettin, et al., 2011), the maximum biomass production was obtained at the stationary phase. Similar to these results, Galhaup, & Haltrich (2001) investigated that Lac production by *T. pubescens*, they obtained that the time of glucose depletion also coincided with the maximum amount of biomass formed.

On SF cultivation, the nitrogen consumption by *P.eryngii* was increased up to 5th day, then approximately stable similar to glucose concentration. In the literature,

there is contradictory evidence on the effects of nitrogen sources (nature and concentration) on ligninolytic enzyme activity. While high nitrogen media gave the highest laccase activity in *L.edodes, Rigidoporus lignosus and T.pubescens,* the nitrogen limited conditions enhanced enzyme production in *Pycnoporus cinnabarinus, P. sanguineus, and Phlebia radiata* (Mester & Field, 1997; Liliana, Feng, & Jean-Marc, 1999; Galhaup, Wagner, Hinterstoisser, & Haltrich, (2002). Some reports suggested that nitrogen regulation is strain-dependent and affected by the available carbon source (Buswell, & Odier, 1987). Thus, it is unclear whether this regulatory system is effective in all white-rot fungi including *Pleurotus* spp. (Kamitsuji, Honda, Watanabe, & Kuwahara, 2004).

The copper concentration is another factor affecting the ligninolytic enzyme activity, especially Lac. In this study, it was not observed much difference in fungal growth by changing the copper concentration of the medium. This result was supported by Palmieri et al. (2000), who found that the fungal growth were the same in the presence and in the absence of copper.

In SF conditions, the optimal Cu^{2+} concentration for Lac activity was 0.5 mM, and on the other conditions it was decreased. On the contrary, the optimal Cu^{2+} concentration for Lac formation by *T. pubescens* was found to be 1.5–2.0 mM (Galhaup, & Haltrich, 2001). The decreases in Lac activity might be provided that free copper ions, as well as the production of a toxic compound, could result in oxidative stress at an advanced stage of fungal growth (Fernandez-Larrea, & Stahl, 1996).

The highest Lac activity determined in 750 μM Mn^{2+} on SF conditions. There was a relationship between the Lac production and Mn^{2+} concentration. It was reported that Lac and Mn^{2+} have been shown to be much more important for operation of the enzymatic lignin-degrading complex of wood-degrading fungi than it was thought before (Schlosser, & Höfer, 2002). Mn^{2+} with dicarboxylic acids (oxalic, malonic or tartaric) as chelating agents are Lac substrates, oxidizable by dioxygen to Mn^{3+}. The

trivalent manganese ions can then react with dicarboxylic acids to yield hydrogen peroxide (Morozova, et al., 2007).

Another important effect on ligninolytic enzyme activity was manganese concentration and it is especially responsible for MnP activity (Steffen, et al., 2002; Ozturk Urek, & Pazarlioglu, 2005). In this study, when the cultivation was carried out without manganese and copper concentration, there was insignificantly ligninolytic enzyme activity from *P.eryngii*. Similar to these results, Kamitsuji, et al. (2004) showed that MnP activity was not detected in growth medium without $MnSO_4$. Thus, manganese in the culture is speculated to affect the production of MnP. In *P. ostreatus*, the addition of manganese promoted the production of MnP, although a depressive effect of manganese has also been found (Martínez, et al., 1996).

One very interesting issue is the role of the concentration of enzymes and their substrates in the action of MnP. It was demonstrated that high production of the enzyme itself cannot be clearly correlated with substantial degradation of target molecules and the availability of Mn and H_2O_2 is thus perhaps the most important factor (Eichlerová, et al., 2000). In addition, hydrogen peroxide is a substrate of other important enzymes of the lignin degrading complex of basidiomycetes: manganese peroxidase and lignin peroxidase (Morozova, et al., 2007). The production of H_2O_2 is supported the ligninolytic enzyme activities, especially AAO by *P.eryngii*.

The interesting result was that the LiP activity was obtained on SF conditions by *P.eryngii*. Because several authors demonstrated that LiP activity was not detectable by *Pleurotus spp.* (Fukushima, & Kirk, 1995). In this study, the highest LiP activity was obtained as 42.527 U/L in the presence of 1000 μM Mn^{2+} and 1.0 mM Cu^{2+}. It was reported that LiP was readily inactivated at low pH (below 4.0), but in this study the pH levels generally were determined in the range of 4.5-5.5. Thus, there may be related with LiP activity and pH levels. Moreover, Aitken, & Irvine (1989) was indicated that the enzyme's stability was improved by increasing the pH, increasing

enzyme concentration or incubating the enzyme in the presence of its substrate veratryl alcohol.

This is a short period to achieve azo dye decolorization. According to results, the ligninolyic enzyme activity secreted by *P.eryngii* is able to play a role in the decolorization of these azo dyes. The contributions of LiP, MnP and laccase to the decolourization of dyes may be different for each fungus (Stolz, 2001; McMullan, et al., 2001). Kasikara Pazarlioglu, Ozturk Urek, & Ergun (2005) showed that the degradation of the dye is efficiently with MnP activities. In addition, it was shown that certain MnP (e.g. from *Pycnoporus cinnabarinus*) are also able to decolourize complex industrially relevant azo dyes, such as Reactive Black 5 or Direct Blue 1 (Schliephake, et. al., 2000). These results showed that the investigated ligninolytic enzymes can be used decolorization of some azo dyes.

REFERENCES

Aitken, M. D., & Irvine, R. L. (1989). Stability testing of ligninase and Mn-peroxidase from *Phanerochaete chrysosporium. Biotechnology and Bioengineering, 34,* 1251-1260.

Aitken, M. D., Massey, I. J., Chen, T. P., & Heck, P. E. (1994). Characterization of reaction products from the enzyme catalyzed oxidation of phenolic pollutants. *Water Research, 28,* 1879-1889.

Alberts, J.F., Gelderblom, W.C.A., Botha, A., & Vanzyl, W.H. (2009). Degradation of aflatoxin B1 by fungal laccase enzymes. *International Journal of Food Microbiology, 135*, 47–52.

Ander, P., & Eriksson, K. E. (1976). The importance of phenol oxidase activity in lignin degradation by the white-rot fungus *Sporotrichum pulverulentum*. *Archices of Microbiology, 109,* 1-9.

Asgher, M., Asad, M. J., Bhatti, H.N., & Legge, R. L. (2007) Hyperactivation and thermostabilization of *Phanerochaete chrysosporium* lignin peroxidase by immobilization in xerogels. *World Journal of Microbiology and Biotechnology, 23*, 525–531.

Asgher, M., Kausara, S., Bhattia, H.N., Shah, S.A.H., & Ali, M. (2008). Optimization of medium for decolourization of solar golden yellow R direct textile dye by *Schizophyllum commune* IBL-06. *International Biodeterioration & Biodegradation, 61*, 189–93.

Baborova´, P., Moder, M., Baldrian, P., Cajthamlova, K., & Cajthaml, T. (2006). Purification of a new manganese peroxidase of the white-rot fungus *Irpex lacteus* and degradation of polycyclic aromatic hydrocarbons by the enzyme. *Research in Microbiology, 157*, 248–253.

Baldrian, P. (2006). Fungal laccases – occurence and properties. *FEMS Microbiology Reviews, 30*, 215–242.

Baucher, M., Halpin, C., Petit-Conil, M., & Boerjan, W. (2003). Lignin: genetic engineering and impact on pulping. *Critical Reviews in Biochemistry and Molecular Biology, 38*, 305–350.

Bettin, F., da Rosa, L.O., Montanari, Q., Calloni, R., Gaio, T.A., Malvessi, E., et al. (2011). Growth kinetics, production, and characterization of extracellular laccases from *Pleurotus sajor-caju* PS-2001. *Process Biochemistry, 46,* 758–764.

Bhalerao, R., Nilsson, O., & Sandberg, G. (2003). Out of the woods: forest biotechnology enters the genomic era. *Current Opinion in Biotechnology, 14,* 206–213.

Blanchette, R. A. (1995). Biodeterioration of archeological wood. *CAB Biodeterioration Abstracts, 9,* 113-127.

Boerjan, W., Ralph, J., & Baucher, M. (2003). Lignin biosynthesis. *Annual Review of Plant Biology, 54,* 519–546.

Boudet, A. M. (2000). Lignins and lignification: selected issues. *Plant Physiology Biochemistry, 38,* 1–16.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical *Biochemistry, 72*, 248-254.

Brunow, G. (2001). Methods to reveal the structure of lignin. In M. Hofrichter, & A. Steinbüchel, (Ed.). *Lignin, Humic Substances and Coal* (Vol.1) (89-116). Weinheim: Wiley-VHC.

Bumpus, J. A., & Aust, S. D. (1987). Biodegradation of environmental pollutants by *Phanerochaete chrysosporium*: involvement of the lignin degrading system. *BioEsays, 6,* 166-170.

Buswell, J.A., & Odier, E. (1987). Lignin biodegradation. *CRC Critical Reviews in Biotechnology, 6,* 1-60.

Cabaleiro, D.R., Rodrı´guez-Couto, S., Sanroma´ n, A., & Longo, M.A. (2002). Comparison between the protease production ability of ligninolytic fungi. *Process Biochemistry, 37,* 1017–1023.

Cabane´, M., Pireaux, J. C., Leger, E., Weber, E., Dizengremel, P., Pollet, B., et al. (2004). Condensed lignins are synthesized in poplar leaves exposed to ozone. *Plant Physiology, 134,* 586–594.

Camarero, S., Sarkar, S., Ruiz-Due˜nas, F. J., Martínez, M. J., Martínez, A.T. (1999). Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. *Journal of Biological Chemistry, 274,* 10324–10330.

Campbell, M. M., & Sederoff, R. R. (1996). Variation in lignin content and composition-mechanisms of control and implications for the genetic improvement of plants. *Plant Physiology, 110,* 3-13.

Claus, H. (2004). Laccases: structure, reactions, distribution. *Micron, 35,* 93–96.

Cohen, R., Persky, L., & Hadar, Y. (2002). Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*. *Applied Microbiology and Biotechnology, 58,* 582-594.

Conesa, A., Punt, P. J., & van den Hondel, C. A. (2002). Fungal peroxidases: molecular aspects and applications. *Journal of Biotechnology, 93*, 143-158.

Cornwell, K. L., Tinland-Butez, M. F., Tardone, P. J., Cabasso, I., & Hammel, K. E. (1990). Lignin degradation and lignin peroxidase production in cultures of *Phanerochaete chrysosporium* immobilized on porous ceramic supports. *Enzyme Microbiology and Technology, 12*, 916–920.

Croteau, R., Kutchan, T. M., & Lewis, N. G. (2000). Natural products (secondary metabolites. In B. Buchanan, R. Jones, & W. Gruissem, (Ed.). *Biochemistry and Molecular Biology of Plants* (1250-1318). Rockville: American Society of Plant Physiologists.

Deindoerfer, F. H., Mateles, R. İ, & Humphrey, A. E. (1962). Fermentation process review. *Applied Microbiology*, 273-303.

D'Annibale, A., Crestini, C., Mattia, E. D. & Sermanni, G. G. (1996). Veratryl alcohol oxidation by manganese-dependent peroxidase from *Lentinus edodes*. *Journal of Biotechnology, 48*, 231-239.

Eggert, C., Temp, U., Dean, J. F. D., & Eriksson, K. E. L. (1996). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Letters, 391*, 144–148.

Eichlerová, L., Homolka, F., Nerud, F., Zadrazil, P., Baldrian, & Gabriel, J. (2000). Screening of *Pleurotus ostreatus* isolates for their ligninolytic properties during cultivation on natural substrates, *Biodegradation, 11*, 279–287.

Erikson, K. E. L., Blanchette, R. A., & Ander, P. (1990). Microbial and enzymatic degradation of wood and wood components. In *Wood decomposition* (41-62). New York: Springerverlag.

Evans, C. S., Dutton, M. V., Guillén, F., & Veness, R. G. (1994). Enzymes and small molecular mass agents involved with lignocellulose degradation. *FEMS Microbiology Reviews, 13*, 235-240.

Feldman, D. (2002). Lignin and its polyblends—a review. In T. Q. Hu, (Ed.) *Chemical modification, properties, and usage of lignin* (81–100). New York: Kluwer Academic/Plenum Publishers.

Ferna´ndez-Larrea, J., & Stahl, U. (1996). Isolation and characterization of a laccase gene from *Podospora anserina. Molecular Genetics and Genomics, 252*, 539–551.

Ferraroni, M., Myasoedova, N., Schmatchenko, V., Leontievsky, A. A., Golovleva L. A., Scozzafava, A., et al. (2007). Crystal structure of a blue laccase from *Lentinus tigrinus*: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. *BMC Structural Biology, 7*, 60–72.

Fukushima, K., & Terashima, N. (1991). Heterogeneity in formation of lignin. Part XV: Formation and structure of lignin in compression wood of *Pinus thunbergii* studied by microautoradiography. *Wood Science and Technology, 25*, 371-381.

Fukushima, Y., & Kirk, T. (1995). Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system. *Applied and Environmental Microbiology, 61*, 872-876.

Galhaup, C., & Haltrich, D. (2001). Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Applied Microbiology and Biotechnology, 56*, 225–232.

Galhaup, C., Wagner, H., Hinterstoisser, B., & Haltrich, D. (2002). Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. *Enzyme and Microbial Technology, 30*, 529–536.

Gianfreda, L., Xu, F., Bollag, J. M. (1999). Laccases: a useful group of oxidoreductive enzymes. *Bioremediation Journal, 3*, 1-25.

Giardina, P., Palmieri, G., Scaloni, A., Fontanella, B., Faraco, V., Cennamo, G., et al. (1999). Protein and gene structure of a blue laccase from *Pleurotus ostreatus. Biochemical Journal, 34*, 655-663.

Grand, C., Boudet, A. M., & Ranjeva, R. (1982). Natural variations and controlled changes in lignification process. *Holzforschung, 36*, 217-223.

Guillén, F., Martínez, A. T., Martínez, M. J., & Evans C. S. (1994). Hydrogen peroxide- producing system of *Pleurotus eryngii* involving the extracellular enzyme aryl-alcohol oxidase. *Applied Microbiology and Biotechnology, 41*, 465-470.

Guillén, F., Martínez, M. J., Muñoz, C., & Martínez, A.T. (1997). Quinone redox cycling in the ligninolytic fungus *Pleurotus eryngii* leading to extracellular production of superoxide anion radical. *Archives of Biochemistry and Biophysics, 339*, 190-199.

Guillén, F., Gómez-Toribio, V., Martínez, M. J., & Martínez, A. T. (2000). Production of hydroxyl radical by the synergistic action of fungal laccase and aryl alcohol oxidase. *Archives of Biochemistry and Biophysics, 383,* 142-147.

Gutiérrez, A., Caramelo, L., Prieto, A., Martínez, M. J., & Martínez, A. T. (1994). Anisaldehyde production and aryl-alcohol oxidase and dehydrogenase activities in ligninolytic fungi of the genus *Pleurotus*. *Applied and Environmental Microbiology, 60*, 1783-1788.

Gutiérrez, A., & Martínez, A. T. (1996). Biodegradación de la lignina: Una perspectiva actual. *Revista Iberoamericana de Micología, 13*, 18-23.

Hammel, K. E., Jensen, Jr., K. A., Mozuch, M. D., Landucci, L. L., Tien, M., & Pease, E. A. (1993). Ligninolysis by a purified lignin peroxidase. *Journal of Biological Chemistry, 268,* 12274–12281.

Hammel, K. E., & Cullen, D. (2008). Role of fungal peroxidases in biological ligninolysis. *Current Openion on Biotechnology, 19,* 166–172.

Hattaka, A. (1994). Lignin-modifying enzymes from selected white rot fungi: production and role in lignin degradation. *FEMS Microbiology Reviews, 13,* 125–35.

Hatakka, A. (2001). Biodegradation of lignin. In M. Hofrichter, & A. Steinbüchel, (Ed.). *Biopolymers* (Vol. 1) (129-180). Weinheim: Wiley-VCH.

He, C. Y., & Wolyn, D. J. (2005). Potential role for salicylic acid in induced resistance of asparagus roots to *Fusarium oxysporum f.sp.asparagi*. *Plant Pathology, 54,* 227–232.

Hefti, M. H., Vervoort, J., & van Berkel, W. J. H. (2003). Deflavination and reconstitution of flavoproteins. *European Journal of Biochemistry, 270,* 4227-4242.

Hildén, K., Hakala, T. K., & Lundell, T., (2009). Thermotolerant and thermostable laccases. *Biotechnology Letters, 31,* 1117–1128.

Higuchi, T. (1997). *Biochemistry and Molecular Biology of Wood*. London: Springer Verlag.

Higuchi, T. (2006). Look back over the studies of lignin biochemistry. *Journal of Wood Science, 52,* 2-8.

Hirai, H., Sugiura, M., Kawai, S., & Nishida, T. (2005). Characteristics of novel lignin peroxidases produced by white-rot fungus *Phanerochaete sordida* YK-624. *FEMS Microbiology Letters, 246*, 19–24.

Hofrichter, M. (2002). Review: Lignin conversion by manganese peroxidase (MnP). *Enzyme and Microbial Technology, 30,* 454-466.

Hossain, S. M., Das, M., Ibrahim, S. H., & Anantharaman, N. (2004). Studies on biopulping of jute stick using *Phanerochate chrysosporium*. *IPPTA Journals, 16(2)*, 47-56.

Johannes, C., & Majcherczyk, A. (2000). Laccase activity tests and laccase inhibitors. *Journal of Biotechnology, 78,* 193–199.

Kaarik, A. (1974). Decomposition of wood. In C. H. Dickinson, G.J.F. Pugh, (Ed.). *Biology of plant litter decomposition* (129-174). London: Academic Press.

Kamitsuji, H., Honda, Y., Watanabe, T., & Kuwahara, M. (2004). Production and induction of manganese peroxidase isozymes in a white-rot fungus *P. ostreatus*. *Applied Microbiology and Biotechnology, 65(3),* 287-294.

Kapdan, I., Kargi, F., McMullan, G., & Marchart, R. (2000). Comparison of white-rot fungi cultures for decolorization of textile dye stuffs. *Bioprocess Engineering, 22,* 347.

Kasikara Pazarlioglu, N., Ozturk Urek, R., Ergun, F. (2005). Biodecolourization of direct blue 15 by immobilized *Phanerochaete chrysosporium*. *Process Biochemistry, 40*, 1923–1929.

Kersten, P., & Cullen, D. (2007). Extracellular oxidative systems of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Fungal Genetics and Biology, 44*, 77–87.

Kirk, T. K., Connors, W. J., & Zeikus, G. (1976). Requirements for a growth substrate during lignin decomposition by two wood-rotting fungi. *Applied and Environmental Microbiology, 32*, 192–194.

Kirk, T. K., & Farrell, R.L. (1987). Enzymatic 'combustion': the microbial degradation of lignin. *Annual Review of Microbiology, 41*, 465–505.

Knapp, J. S., Newby, P. S., & Reece, L. P. (1995). Decolourization of dyes by wood-rotting basidiomycetes fungi. *Enzyme Microbiology and Technology, 17*, 664–668.

Kuan, I.C., Johnson, K. A., & Tien, M. (1993). Kinetic analysis of manganese peroxidase. *Journal of Biological Chemistry, 268*, 20064–20070.

Kuhad, R.C., Singh, A. & Eriksson, K. E. L. (1997). Microorganisms and enzymes involved in the degradation of plant fiber cell walls. In *Advances in biochemical engineering biotechnology* (Vol. 57) (46-125). Germany: Springer-Verlag.

Kuwahara, M., Glenn, J. K.. Morgan, M. A., & Gold, M. H. (1984). Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. *FEBS Letters, 169*, 247–250.

Leonowicz, A., Cho, N.S., Luterek, J., Wilkolazka, A., Matuszewska, A., Hofrichter, M., et al. (2001). Fungal laccase: properties and activity on lignin. *Journal of Basic Microbiology, 41*, 185–227.

Liliana, G., Feng, X., & Jean-Marc, B. (1999). Laccases: A Useful Group of oxidoreductive Enzymes. *Bioremediation Journal, 3*, 1-26.

Lora, J. H., & Glasser, W.G. (2002). Recent industrial applications of lignin: a sustainable alternative to nonrenewable materials. *Journal of Polymers and the Environment, 10*, 39–48.

Lorenzo, M., Moldes, D., Rodr´ıguez Couto, S., & Sanroman, A. (2002). Improvement in laccase production by employing different lignocellulosic wastes in submerged cultures of *Trametes versicolor*, *Bioresource Technology, 82*, 109–113.

Lundell, T. K., Mäkelä, M. R., & Hildén, K. (2010). Lignin-modifying enzymes in filamentous basidiomycetes –ecological, functional and phylogenetic review. *Journal of Basic Microbiology, 50,* 5–20.

Lyashenko, A.V., Bento, I., Zaitsev, V. N., Zhukhlistova, N.E., Zhukova, Y.N., Gabdoulkhakov, A.G., et al. (2006). X-ray structural studies of the fungal laccase from *Cerrena maxima*. *Journal of Biological Inorgic Chemistry, 11*, 963–973.

Maltseva, O. V., Niku-Paavola, M. L., Leontievsky, A. A., Myasoedova, N. M. & Golovleva, L. A. (1991). Ligninolytic enzymes of the white rot fungus *Panus tigrinus*. *Biotechnology and Applied Biochemistry, 13*, 291-302.

Martinez, M., Ruiz-Duenas, F. J., Guillen, F., & Martinez, A. T. (1996). Purification and catalytic properties of two manganese peroxidase isoenzymes from *Pleurotus eryngii. European Journal of Biochemistry, 237*, 424–432.

Martínez, A.T. (2002). Molecular biology and structure-function of lignin-degrading heme peroxidases. *Enzyme and Microbial Technology, 30*, 425-444.

Martinez, A. T., Speranza, M., Ruiz-Duenas, F. J., Ferreira, P., Camarero, S., Guillen, F., et al. (2005). Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology*, *8*, 195–204.

Marzullo, L., Cannio, R., Giardina, P., Santini, M. T., & Sannia, G. (1995). Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and

prevents polymerization of laccase-oxidized substrates. The *Journal of Biological Chemistry, 270*, 3823-3827.

Mayer, A.M. & Staples, R.C. (2002). Laccase: new functions for an old enzyme. *Phytochemistry, 60*, 551-565.

McMullan, G., Meehan, C., Conneely, A., Kirby, N., Rbinson, T., Nigam, P., et al. (2001). Microbial decolourization and degradation of textile dyes. *Applied Microbiology and Biotechnology, 56*, 81–7.

Mester, T., & Field, J. A. (1997). Optimization of manganese peroxidase production by the white rot fungus *Bjerkandera* sp. strain BOS55. *FEMS Microbiology Letters, 155*, 161-168.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*, 426-428.

Morozova, O. V., Shumakovich, G. P., Shleev, S. V., & Yaropolov. Y.I. (2007). Laccase–mediator systems and their applications: A review. *Applied Biochemistry and Microbiology, 43(5)*, 523–535.

Morozova, O. V., Shumakovich, G. P., Gorbacheva, M. A., Shleev, S.V., & Yaropolov, A. I. (2007). "Blue" laccases. *Biochemistry, 72*, 1136–1412.

Murugesan, A. G., Vijayalakshmi, G. S., Sukumaran, N., & Mariappan, C. (1995). Utilization of water hyacinth for oyster mushroom cultivation. *Bioresource Technology, 51*, 97–98.

Musha, Y., & Goring, D. A. I. (1975). Distribution of syringyl and guaiacyl moieties in hardwoods as indicated by ultraviolet microscopy. *Wood Science and Technology, 9*, 45-58.

Mu˜noz, C., Guillén, F., Martínez, A. T., & Martínez, M. J. (1997). Induction and characterization of laccase in the ligninolytic fungus *Pleurotus eryngii*. *Current Microbiology, 34*, 1–5.

Orth, A. B., & Tien, M. (1995). Biotechnology of lignin degradation, In U. Kück, (Ed.). *The Mycota* (Vol. 2) (287-302). Germany: Springer-Verlag.

Okamoto, K., & Yanase, H. (2002). Aryl alcohol oxidases from the white-rot basidiomycete *Pleurotus ostreatus. Mycoscience 43*, 391–395.

Ozturk Urek, R., & Kasikara Pazarlioglu, N. (2005). Production and stimulation of manganese peroxidase by immobilized *Phanerochaete chrysosporium. Process Biochemistry, 40,* 83-87.

Ozturk Urek, R., & Kasikara Pazarlioglu, N. (2007). Production of manganese peroxidase by *Phanerochaete chrysosporium. Brazilian archives of biology and technology, 50(6),* 913-920.

Palma, C., Martínez, A. T., Lema, J. M., & Martínez, M. J. (2000). Different fungal manganese-oxidizing peroxidases: a comparison between *Bjerkandera* sp. and *Phanerochaete chrysosporium*. *Journal of Biotechnology, 77*, 235-245.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B., & Sannia, G. (2000). Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus. Applied and Environmental Microbiology, 66(3),* 920-924.

Paszczynski, A., Pasti, M.B., Coszczynskii, S., Crawford, D.L., & Crawford, R.L. (1991). New approach to improve degradation of recalcitrant azo dyes by *Streptomyces* spp. and *Phanerochaete chrysosporium*. *Enzyme and Microbial Technology, 13*, 378-384.

Piontek, K., Smith, A. T., & Blodig, W. (2001). Lignin peroxidase structure and function. *Biochemical Society Transactions, 29,* 111–116.

Piontek, K., Antorini, M., & Choinowski, T. (2002). Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. *Journal of Biological Chemistry, 277,* 37663–37669.

Pointing, S.B. (2001). Feasibility of bioremediation by white-rot fungi. *Applied Microbiology and Biotechnology, 57,* 20–33.

Raghukumar, C. (2008). Marine fungal biotechnology: an ecological perspective. *Fungal Diversity, 31*, 19-35.

Ralph, J., Hatfield, R. D., Piquemal, J., Yahiaoui, N., Pean, M., Lapierre, C. et.al. (1998). NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase. *Proceedings of the National Academy of Sciences, 95*, 12803-12808.

Ralph, J., Brunow G., & Boerjan W. (2007). Lignins. In: F. Rose, & K. Osborne, (Ed.). *Encyclopedia of life sciences* (1-10). Chichester: Wiley & Sons.

Revankar, M. S., Lele, S. S. (2007). Synthetic dye decolorization by white rot fungus, *Ganoderma* sp. WR-1. *Bioresource Technology, 98*, 775-780.

Robinson, T., Chandran, B., & Nigam, P. (2001). Studies on the production of enzymes by white rot fungi for the decolourization of textile dyes. *Enzyme and Microbial Technology, 29,* 575-579.

Saka, S., & Goring, D.A. I. (1988). Distribution of lignin in white birch wood as determined by bromination with TEM-EDXA. *Holzforschung, 42(3),* 149 - 153.

Saratale, R. G., Saratale, G. D., Chang, J.S., & Govindwar, S. P. (2011). Bacterial decolorization and degradation of azo dyes: a review, *Journal of the Taiwan Institute of Chemical Engineers, 42*,138–157.

Sarkanen, K. V., Hergert, H. L. (1971). Classification and distribution. In: K. V. Sarkanen, & C. H. Ludwig, (Ed.). Lignins: occurrence, formation, structure and reactions (43-94). New York: Wiley & Sons.

Sarkanen, K.V., & Ludwig, C.H. (1971). Definition and nomenclature. In *Lignins, occurrence, formation, structure and reactions* (1–18). New York: Wiley-Interscience.

Schliephake, K., Mainwaring, D. E., Lonergan, G. T., Jones, I. K., & Baker, W. L. (2000). Transformation and degradation of disazo dye Chikago sky blue by a purified laccase from *Pycnoporus cinnabarinus*. *Enzyme and Microbial Technology, 27*, 100–7.

Schlosser, D., & Höfer, C. (2002). Laccase-catalyzed oxidation of Mn^{2+} in presence of natural Mn^{3+} chelators as a novel source of H_2O_2 and its impact on manganese peroxidase. *Applied and Environmental Microbiology, 68*, 3514-3521.

Schwarze, F. W.M. R., Engels, J., & Mattheck, C. (2000). *Fungal strategies of wood decay in trees*. New York: Springer.

Scott, G. M., Akhtar, M. & Kirk, T. K. (2000). An update on biopulping commercialization. In *Proceedings of the 2000 Pulping Conference*. Atlanta: Tappi Press.

Solomon, E. I., Chen, P., Metz, M., & Lee, S. K., & Palmer, A. E. (2001). Oxygen binding, activation, and reduction to water by copper proteins. *Andgewandte Chemie, 40*, 4570–4590.

Spadarao, J. T., Gold, M., & Renganathan, V. (1992). Degradation of azo dye by the lignin-degrading fungus *Phanerochaete chrysosporium. Applied and Environmental Microbiology, 58*, 23-97.

Stalpers, J. A. (1976). Identification of wood-inhabiting fungi in pure culture. *Studies in Mycology, 16,* 1-248.

Steffen, K.T., Hofrichter, M., & Hatakka, A. (2002). Purification and characterization of manganese peroxidises from the litter-decomposing basidiomycetes *Agrocybe praecox* and *Stropharia coronilla*. *Enzyme and Microbial Technology, 30,* 550-555.

Steffen, K. (2003). Degradation of recalcitrant biopolymers and polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi, Ph. D. Thesis. Helsinki: *Department of Applied Chemistry and Microbiology*.

Seifert, K. A. (1983). Decay of wood by the *Dacrymycetales*. *Mycologia, 75,* 1011-1018.

Stolz, A. (2001). Basic and applied aspects in the microbial degradation of azo dyes. *Applied Microbiology and Biotechnology, 56,* 69–80.

Sundaramoorthy, M., Kishi, K., Gold, M. H., & Poulos, T. L. (1994). The crystal structure of manganese peroxidase from *Phanerochaete chrysosporium* at 2.06-Δ resolution. *Journal of Biological Chemistry, 269*, 32759–32767.

Susla, M., Novotny, C., & Srobodora, K. (2007). The implication of *Dichomitus squalens* laccase isoenzymes in dye decolourization by immobilized fungal cultures. *Bioresource Technology, 98*, 2109–2115.

Stajić, M., Persky, L., Friesem, D., Hadar, Y., Wasser, S.P., Nevo, E., et al. (2006). Effect of different carbon and nitrogen sources on laccase and peroxidases

production by selected *Pleurotus* species. *Enzyme and Microbial Technology, 38(1),* 65-73.

Téllez-Téllez, M., Fernandez, F. J., Montiel-Gonzalez, A. M., Sanchez, C., & Díaz-Godinez, G. (2008). Growth and laccase production by *Pleurotus ostreatus* in submerged and solid-state fermentation. *Applied Microbiology and Biotechnology, 81,* 675-679.

Terashima, N., Fukushima, K., Tsuchiya, S., & Takabe, K. (1986). Heterogeneity in formation of lignin. VII. An autoradiographic study on the formation of guaiacyl and syringyl lignin in poplar. *Journal of Wood Chemistry and Technology, 6,* 495-504.

Tien, M., & Kirk T. K. (1988). Lignin peroxidase of *Phanerochaete chrysosporium. Methods in Enzymology, 161,* 238-249.

Tuor, U., Winterhalter, K., & Fiechter, A. (1995). Enzymes of white-rot fungi involved in lignin degradation and ecological determinants for wood decay. *Journal of Biotechnology, 41,* 1–17.

Venkatadri, R., & Irvine, R. L. (1993). Cultivation of *Phanerochaete chrysosporium* and production of lignin peroxidase in novel biofilm reactor system: hollow fibre reactor and silicone membrane reactor. *Water Research, 27,* 591–596.

Wariishi, H., Valli, K., & Gold, M. H. (1992). Manganese (II) oxidation by manganese peroxidase from the Basidiomycete *Phanerochaete chyrsosporium*. *Journal of Biological Chemistry, 267,* 236888-23695.

Weatherburn, M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical Chemistry, 39 (8),* 971-974.

Wesenberg, D., Kyriakides, I., & Agathos, W. (2003). White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology Advances, 22*, 161-187.

Worrall, J. J., Anagnost, S. E. & Zabel, R. A. (1997). Comparison of wood decay among diverse lignicolous fungi. *Mycologia, 89*, 199-219.

Wood, D. A., & Smith., J. F. (1987). The cultivation of mushroom. In J. R. Norris, & G.L. Pettipher, (Ed.). *Essays in agricultural and food microbiology* (310-343). Chichester: John Wiley & Sons.

Yang, J. S., Yuan, H. L., & Chen, W. X. (2004). Studies on extracellular enzymes of lignin degrading fungus *Penicillium* sp. P6. *China Environmental Science, 24*, 24–27.