DOKUZ EYLUL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

PRODUCTION OF SOME LIGNINOLYTIC AND HYDROLYTIC ENZYMES WITH SOLID STATE FERMENTATION

by

Merve AKPINAR

July, 2017

İZMİR

PRODUCTION OF SOME LIGNINOLYTIC AND HYDROLYTIC ENZYMES WITH SOLID STATE FERMENTATION

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Merve AKPINAR

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Ph.D. THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"PRODUCTION OF SOME LIGNINOLYTIC AND HYDROLYTIC ENZYMES WITH SOLID STATE FERMENTATION"** completed by **MERVE AKPINAR** under supervision of **PROF. DR. RAZİYE ÖZTÜRK ÜREK** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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PRODUCTION OF SOME LIGNINOLYTIC AND HYDROLYTIC ENZYMES WITH SOLID STATE FERMENTATION

ABSTRACT

In this thesis, the productions of ligninolytic and hydrolytic enzymes, laccase, manganese peroxidase, lignin peroxidase, carboxymethyl cellulase, xylanase, exoglucanase, β-glucosidase, from *Pleurotus eryngii* were optimized using peach and cherry wastes under solid state fermentation conditions. Firstly, the chemical compositions of these wastes from fruit juice industry were determined in terms of cellulose, lignin, reducing sugar, total carbohydrate and protein, nitrogen and some metals. According to chemical analysis, these wastes could be named as lignocellulosic. *P. eryngii* was incubated on both peach and cherry agroindustrial wastes by solid state fermentation for thirty days without-with pretreatment conditions. The lignocellulosic substrates were pretreated with dilute acid and alkaline solutions, hot water before incubation. The used pretreatment methods had generally negative effects on these enzymes productions. Also, the effects of different compounds; copper, iron, Tween 80, ammonium nitrate and manganese, and their variable concentrations on ligninolytic and hydrolytic enzymes productions were investigated in detail.

In the second part of the thesis, the purifications of laccases were carried out by ammonium sulfate precipitation, anion exchange and gel filtration chromatographies, respectively due to their biotechnological and industrial importance. The laccase fractions with high yield and high purification factor were subjected to electrophoretic analysis. Also, the purified laccases were partially characterized. The effects of selected chemical agents such as ethylenediaminetetraacetic acid, phenylmethane sulfonyl fluoride, iodoacetamide, β-mercaptoethanol and sodium azide on the purified laccase activities were also investigated.

In the third part of the thesis, the decolorization of some azo dyes was carried out by crude extracts and purified laccases from both cultures, and the effects of the dyes

on laccase enzymes stabilities were investigated in addition to optimization of productions, purification and characterization studies. Finally, Fourier transform infrared spectroscopy analyzes were performed to determine whether there were the changes of methyl orange dye.

Keywords: Decolorization, hydrolytic enzymes, ligninolytic enzymes, *Pleurotus eryngii*, purification, solid state fermentation.

BAZI LİGNİNOLİTİK VE HİDROLİTİK ENZİMLERİN KATI HAL FERMENTASYONUYLA ÜRETİMİ

ÖZ

Bu tez kapsamında, *Pleurotus eryngii*'nin ligninolitik ve hidrolitik enzimlerinden lakkaz, mangan peroksidaz, lignin peroksidaz, karboksimetil sellülaz, ksilanaz, ekzoglukanaz, β-glukozidazın üretimleri şeftali ve vişne atıkları kullanılarak katı hal fermentasyonu koşullarında optimize edilmiştir. İlk olarak, meyve suyu endüstrisinden elde edilen bu atıkların sellüloz, lignin, indirgen şeker, total karbohidrat ve protein, azot ve bazı metaller açısından kimyasal bileşimleri belirlenmiştir. Kimyasal analizlere göre, bu atıklar lignosellülozik olarak adlandırılabilmektedir. *P. eryngii* hem şeftali hem de vişne atıkları üzerinde otuz gün boyunca katı hal fermentasyonu ile ön muamelenin olduğu ve-veya olmadığı koşullarda inkübe edilmiştir. Lignosellülozik substratlar inkubasyon öncesinde seyreltik asit ve baz çözeltileri, sıcak su ile ön muamele edilmiştir. Uygulanan ön işlem yöntemleri genellikle bu enzimlerin üretimlerini olumsuz etkilemiştir. Ayrıca, ligninolitik ve hidrolitik enzimlerin üretimleri üzerine farklı bileşiklerin; bakır, demir, Tween 80, amonyum nitrat ve manganın ve bunların değişen derişimlerinin etkileri detaylı olarak araştırılmıştır.

Tezin ikinci kısmında, biyoteknolojik ve endüstriyel önemleri sebebiyle lakkazların saflaştırılmaları sırasıyla amonyum sülfat çöktürmesi, anyon değişim ve jel filtrasyon kromatografileri ile gerçekleştirilmiştir. Yüksek verim ve yüksek saflaştırma faktörü olan lakkaz fraksiyonları elektroforetik analizlere tabi tutulmuştur. Ayrıca, saflatırılan lakkazlar kısmi olarak karakterize edilmiştir. Saflaştırılan lakkaz aktiviteleri üzerine etilendiamin tetraasetik asit, fenilmetan sülfonil florid, iyodoasetamid, β-merkaptoetanol gibi seçilen kimyasal ajanların etkileri de incelenmiştir.

Tezin üçüncü kısmında, üretim optimizasyonları, saflaştırma ve karakterizasyon çalışmalarına ek olarak bazı azo boyaların dekolorizasyonları her iki kültürden elde edilen ham ekstrakt ve saflaştırılan lakkazlarla gerçekleştirilmiştir ve lakkaz enzimlerinin stabiliteleri üzerine boyaların etkisi de araştırılmıştır. Son olarak, Fourier

Dönüşümlü Infrared Spektrofotometre analizleri metil oranj boyasında değişimlerin olup olmadığını belirlemek için yapılmıştır.

Anahtar kelimeler: Dekolorizasyon, hidrolitik enzimler, ligninolitik enzimler, *Pleurotus eryngii*, saflaştırma, katı hal fermentasyonu.

CONTENTS

Page

LIST OF FIGURES

Page

Page

Page

Page

LIST OF TABLES

Page

CHAPTER ONE
INTRODUCTION

Nowadays, major problems are the rising generation of waste with its consequent environmental problems and the search for new sources of energy due to the increasing demand on energy sources and the concern about the remaining amount of fossil resources. One significant feedstock that could be utilized as a renewable energy source is lignocellulose which has recently attracted important attention (Mansour et al., 2016).

Lignocellulosic materials are the most promising feedstock as natural and renewable resource essential to the functioning of modern industrial societies. A large amount of such materials as waste byproducts are being generated through agricultural practices mainly from various agro-industries. Sadly, much of the lignocellulosic biomass is often disposed of by burning, which is not restricted to developing countries alone (Anwar, Gulfraz & Irshad, 2014).

Lignocellulose wastes are accumulated every year in large quantities, causing environmental problems (Table 1.1). Such wastes contain a variety of materials such as poplar trees, sawdust, waste paper, sugarcane bagasse, brewer's spent grains, husks, switchgrass, leaves, stalks, stems, shells and peels from cereals like rice, wheat, corn, barley, among others (Mussatto & Teixeira, 2010).

Table 1.1 Lignocellulosic residues generated from different agricultural sources (Sánchez, 2009)

Lignocellulosic Residue	**10^6 ton/year**	**Lignocellulosic Residue**	**10^6 ton/year**
Sugar cane bagasse	317–380	Bean straw	4.9–5.9
Maize straw	159–191	Rye straw	4.3–5 2
Rice shell	157–188	Coffee straw	1.6–1.9
Wheat straw	154–185	Almond straw	0.4–0.49
Barley straw	35–42	Hazelnut husk	0.2–0.24
Yuca straw	40–48	Cotton fiber	17–20

Recently lignocellulosic biomasses have gained rising research interests and special significance owing to their renewable nature (Anwar et al., 2014). They could be utilized for the production of a number of value added products, such as enzymes, organic acids, ethanol, food additives and others owing to their chemical composition based on sugars and other compounds (Mussatto & Teixeira, 2010). On the other hand, the large amounts of lignocellulosic biomass can potentially be converted into different high value products containing value added chemicals, biofuels, and cheap energy sources for microbial fermentation and enzyme production (Anwar et al., 2014). Therefore, besides the environmental problems caused by their accumulation in the nature, the non-use of these materials constitutes a loss of potentially valuable sources (Mussatto & Teixeira, 2010).

1.1 Composition of Lignocellulosic Residues

Lignocellulose is a generic word used to describe key structural components of nonwoody and woody plants and represents plant biomass, the most promising, naturally abundant, renewable organic resource. The building blocks of lignocellulose are the noncarbohydrate polymer lignin and the carbohydrate polymers cellulose, hemicellulose, and pectin, with protein, ash, and extractive components as minor constituents. All of them have a significant chemical effect on the tertiary structure of lignocellulose (Toushik, K. Lee, J. Lee & Kim, 2017; Malgas, Thoresen, Dyk & Pletschke, 2017).

Plant cell walls are classified into two types, i.e. primary and secondary cell walls, which differ both in their chemical composition and in the physiological roles they play. Plant cell walls are laid down around dividing and elongating cells and comprise largely of polysaccharides (hemicelluloses, cellulose, and pectin) and approximately 10% protein in addition to phenolic esters linked to wall polysaccharides. The structure of plant cell walls is thin and flexible, thus making it ideal to encase growing and expanding plant cells. Specific cells synthesize secondary cell walls that are made up of across-linked matrix of cellulose, hemicelluloses, and lignin, and are laid down on the interior of the plant cell walls. In addition to providing mechanical support,

secondary cell walls fulfil critical biological processes, such as water and nutrient transport, silique shattering, plant organ movement and defense against pathogens (Guerriero, Hausman, Strauss, Ertan & Siddiqui, 2016).

The main component of lignocellulosic plant materials is cellulose, followed by hemicellulose and lignin. Cellulose and hemicellulose are macromolecules constructed from different sugars; whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors (Chen, 2014). When evaluated in more detail, cellulose is a major structural component of plant cell walls, which is responsible for mechanical strength, while hemicellulose macromolecules are often repeated polymers of pentoses and hexoses. Lignin contains three aromatic alcohols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) produced through a biosynthetic process and forms a protective seal around the other two components i.e., hemicelluloses and cellulose (Figure 1.1) (Anwar et al., 2014).

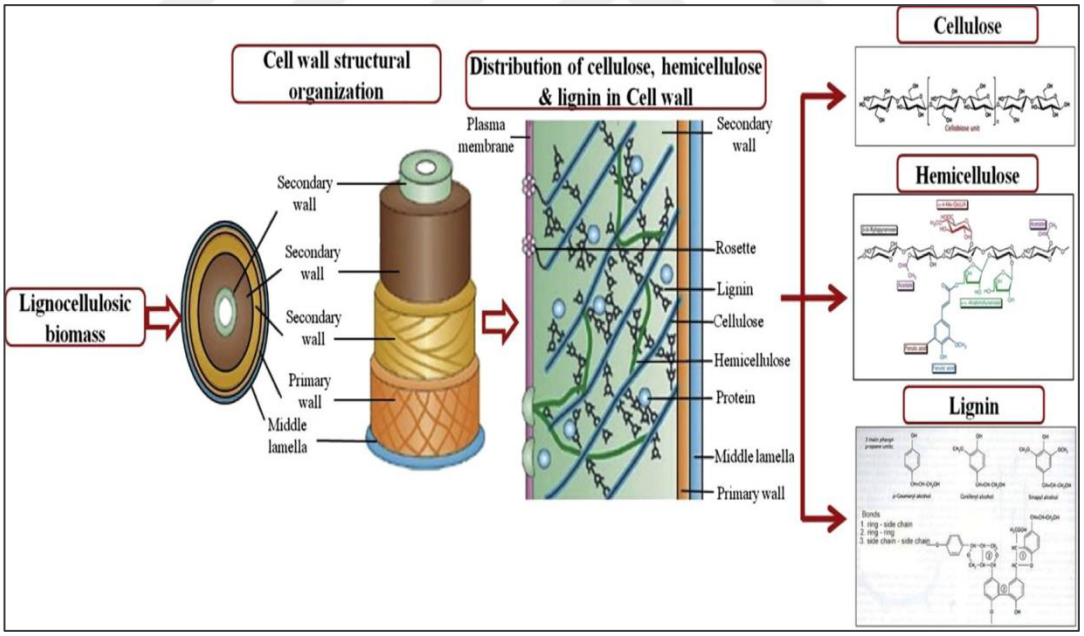


Figure 1.1 Diagrammatic illustration of the framework of lignocellulose; cellulose, hemicellulose and lignin (Anwar et al., 2014)

Cellulose is a high molecular weight linear homopolymer that is consist of *D*-glucose subunits linked by β-1,4 glycosidic bonds forming the dimer cellobiose (Chen, 2014). The chemical formula of cellulose is $(C_6H_{10}O_5)_n$, where "n" indicates the

number of glucose groups, which is called the degree of polymerization (Toushik et al., 2017). Plant biomass include 40-50% of cellulose molecules which are held together by intermolecular hydrogen bonds and in native state. However, they have a strong tendency to form intermolecular and intramolecular hydrogen bonds and this tendency increases the rigidity of cellulose and make highly insoluble and highly resistant to most organic solvents. Cellulose molecules are naturally exists as bundles which aggregated together in the form of microfibrils order i.e., crystalline and amorphous regions (Anwar et al., 2014). Also, cellulose chains include approximately 36 glucan chains and each chain contains an average of 500 to 15000 glucose molecules with an approximate molecular weight of 10 to 150 kDa (Toushik et al., 2017).

Cellulose basically forms a skeleton which is surrounded by hemicellulose and lignin (Figure 1.2) (Mussatto & Teixeira, 2010). Cellulose and hemicellulose, two major carbohydrates, are covalently cross-linked with noncarbohydrate lignin, which imparts strength to the cell wall of plant materials, and removal of lignin can accelerate the hydrolysis rates of them (Toushik et al., 2017).

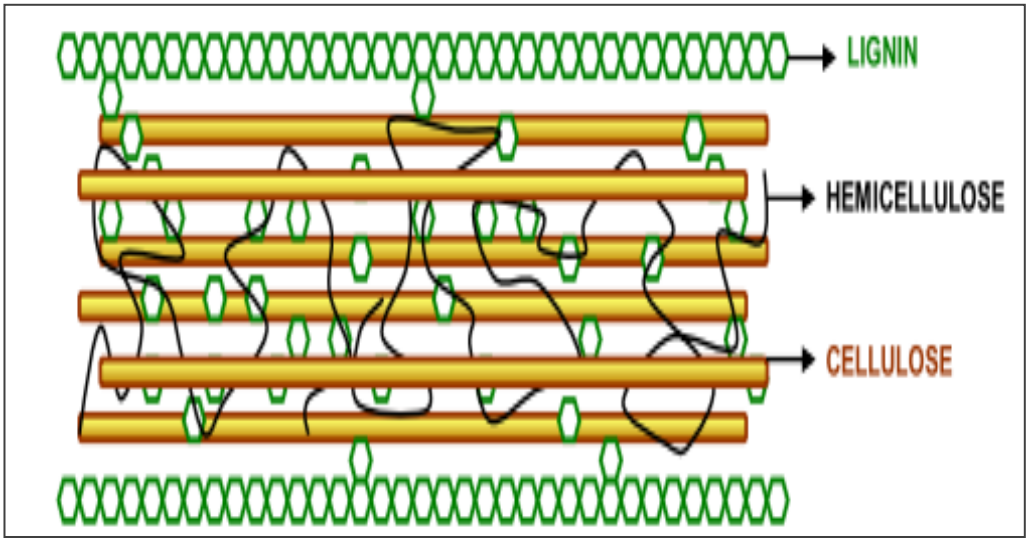


Figure 1.2 Representation of lignocellulose structure showing cellulose, hemicellulose and lignin fractions (Mussatto & Teixeira, 2010)

Hemicellulose is one of the most important polysaccharide which mainly comprise glucomannan, glucuronoxylan and trace amounts of other polysaccharides. Grasses

and straws include arabinan, galactan and xylan, whereas mannan is a component of hardwood and softwood hemicellulose. They are catalogued with sugar as a backbone, i.e., mannans, xylans, and glucans, with mannans and xylans being the most common. Arabinans, galactans, and arabinogalactans are included in the hemicellulose group; however, they do not share the equatorial β-1,4 linked backbone structure. In hardwoods, glucuronoxylan (O-acetyl-4-O-methyl-glucurono-β-D-xylan) is the predominant component. Xylospyranose is the backbone of the polymer and connected with β-1,4 linkages. Lignocellulosic biomass contains 25-35% of hemicellulose (Anwar et al., 2014). Hemicelluloses are tightly bound to other main polysaccharide, celluloses, via non-covalent interactions of hydrogen bonds, and bound to lignin through covalent bonds. As a copolymer, hemicelluloses are composed of different quantities of various saccharide molecules, which make it distinct from cellulose. Hemicellulose comprises approximately 15-19% in dry weight of cell wall polysaccharides, and the hemicellulose backbone contains approximately 500 to 30000 sugar units per chain with an approximate molecular weight of 30 kDa (Toushik et al., 2017).

Lignin is the second most abundant biopolymer on Earth after plant polysaccharide cellulose (Lundell, Mäkelä & Hildén, 2010). Also, it is generally the most complex and smallest fraction, representing about 10-25% of the biomass by weight. It has a heterogeneous polymer, long-chain composed largely of phenyl-propane units most commonly linked by ether bonds. Lignin acts like a glue by filling the gap between and around the hemicellulose and cellulose complexion with the polymers. Lignin is comprised of complex and large polymer of phenyl-propane, methoxy groups and noncarbohydrate poly phenolic substance, which bind cell walls component together. Phenylpropanes (3 carbons attached with 6 carbon atom rings) are main block of lignin. These phenylpropanes denoted as 0, I, II methoxyl groups attached to rings give special structure I, II and III. These groups depend on the plant source which they are obtained. Structure I exist in plants (grasses) and structure II found in the wood (conifers) while structure III present in deciduous wood (Anwar et al., 2014).

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 (Lundell et al., 2010).

The amounts of carbohydrate polymers and lignin vary from one plant species to another. Besides, the ratios between various constituents in a single plant may also vary with stage of growth, age, and other conditions. Average values of the main components in some lignocellulose wastes are shown in Table 1.2.

Table 1.2 Composition of some lignocellulosic materials (Bilal, Asgher, Iqbal, Hu & Zhang, 2017a; Toushik et al., 2017)

Lignocellulosic waste	**Cellulose (wt %)**	**Hemicellulose (wt %)**	**Lignin (wt %)**
Wheat bran	10.5–14.8	35.5–39.2	8.3–12.5
Banana waste	13.2	14.8	14
Nut shells	25–30	25–30	30–40
Peach pomace	28.7-30.0	18.6-20.0	5.35-6.0
Coffee pulp	33.7–36.9	44.2–47.5	15.6–19.1
Wheat straw	29–35	26–32	16–21
Barley straw	36–43	24–33	6.3–9.8
Sugarcane bagasse	42	25	20
Apple pomace	40.0-43.6	19.0-24.4	15.0-20.4
Rice husk	28.7–35.6	11.96–29.3	15.4–20
Corn stover	38	26	19
Corn straw	42.6	21.3	8.2
Rice straw	32.1	24	18
Cherry pomace	16-20	10.7	69.4
Oat straw	31–35	20–26	10–15
Newspaper	40–55	25–40	18–30
Grape pomace	6-17.75	18.0-31.0	59.0-64.0
Olive tree biomass	25.2	15.8	19.1

The most critical step in the efficient utilization of these lignocellulosic feedstocks is delignification, which expedites the separation of the main biomass components such as hemicellulose, cellulose, and lignin. Nevertheless, the bioconversion is hindered by the structural complexity of substrate making them a challenge to be used.

Therefore, bioconversion of lignocellulosic materials to useful products including pulp and paper, composite, animal feed, fine chemical, biofuels and enzymes requires multi-step processes including pretreatment, enzymatic digestibility and fermentation (Bilal et al., 2017a).

Pretreatment process generally depolymerizes lignin in the lignocellulosic materials, so the resulted biomass becomes more amenable to consequent cellulolytic enzymes attack for improved saccharification. It is worth mentioning that following suitable delignification; the cellulose conversion to glucose becomes easier owing to greater biodigestibility and approachability of the cellulase enzymes to cellulose. A large variety of pretreatment procedures have been experimented in the past decades and are generally distinguished as mechanical (e.g., milling, grinding,), physicochemical (e.g., steam, autohydrolysis, liquid hot water, supercritical fluids), chemical (e.g., acid, alkali, oxidizing agents, organic solvents), and biological (e.g., fungi) processes and/or combinations of these methods. Chemical pretreatment is deliberated as the current leading delignification approach; however, scaling up of the treatment proves to be impractical and uneconomical, since chemical routes rely on the appliance of expensive equipment and toxic/corrosive chemicals (Bilal et al., 2017a). Enzymatic pretreatment approach appears to be encouraging in this scenario to disrupt plant cell wall (lignin) and modify lignocellulosic structures with appreciable benefits like no chemical treatment, important biomass conversion, optimum product recovery, least by-product generation, ecofriendly conditions, low energy input (Bilal et al., 2017a).

1.2 Biodegradation of Lignocellulosic Residues

Degradation of the polysaccharides into their respective monomeric sugars within the lignocellulosic biomass is achieved biologically through the synergistic attack of glycoside hydrolases (GH), lignin modifying enzymes and other accessory enzymes. This enzyme synergism facilitates an enhanced hydrolytic activity, where the resulting activity is greater than the theoretical sum of the individual enzyme activities. The

released monomer sugars can subsequently be fermented into value added products by microorganisms (Malgas et al., 2017).

Organisms such as bacteria and fungi have evolved several mechanisms for the lignocellulose degradation. The common mechanism for the degradation of it is the free enzyme system, which mainly exists in fungi and aerobic bacteria (Malgas et al., 2017). Among the fungi that can infect and colonize wood, brown, white and soft rot fungi have been extensively studied mainly due to their ability to degrade wood cell walls, which can cause extensive damage and failure of wooden products in service (A.P. Singh & T. Singh, 2014).

The fungal degradation of lignocellulose takes place exocellularly, either in association with the outer cell envelope layer or extracellularly, owing to the insolubility of cellulose, lignin and hemicellulose. Fungi have two types of extracellular enzymatic systems for carrying out this bioprocess: the unique oxidative and extracellular ligninolytic system, which degrades lignin and opens phenyl rings; and a hydrolytic system, which generates hydrolases that are responsible for the degradation of polysaccharide (Sánchez, 2009).

In nature, brown rot fungi are most common in coniferous forests, but are also present in other environments and habitats, such as soils. Brown rotted wood has a brownish appearance. Upon drying, the degraded wood develops characteristic cubical cracks and turns into a fine powder when crushed, because of significant strength losses due to extensive depolymerisation of cellulose. Lignin can also be modified during cell wall degradation but much less readily than cellulose, and often remains as a residue in degraded cell wall regions. Cell wall degradation is diffuse, and although cell walls become distinctly porous, degraded wood tissues retain their original form if not subjected to a load or pressure, giving a false impression of being intact and sound (A.P. Singh & T. Singh, 2014).

White rot fungi (WRF) are common in nature. They are particularly abundant in forest ecosystems; rotting stumps as forest residues with a ‘bleached’ appearance are

a common site in hardwood forests, as hardwoods are more susceptible to white rot attack than softwoods. WRF employ both non-enzymatic and enzymatic mechanisms to degrade wood cell walls, and can simultaneously degrade all wood cell wall components (simultaneous degraders) or preferentially remove lignin from cell walls. Micromorphological patterns of degradation change depending on wood species and cell type, environmental conditions, cell wall erosion being the most common form of degradation (A.P. Singh & T. Singh, 2014).

Soft rot fungi are widely present in nature and can tolerate a wide range of temperature and moisture conditions. Based on cell wall degradation patterns produced soft rot fungi are distinguished as cavity producers (type 1) and as those causing cell wall erosion (type 2). Type 1 fungi, which have been more extensively studied, can efficiently degrade cellulose and hemicellulose and to a limited extent also lignin (A.P. Singh & T. Singh, 2014).

Most enzyme synergy studies have described synergistic application of GH enzymes and other ligninolytic and hydrolytic enzymes to combat lignocellulose recalcitrance and improve biomass saccharification. Efficient and complete substrate degradation requires the co-operation of two or more enzymes. This interaction is commonly referred to as enzyme synergy, whereby the combined activities of more than one enzyme significantly enhance the efficiency of substrate hydrolysis (Malgas et al., 2017). On the other hand, substrate complexity and multiplicity of enzymes are the main obstacles of lignocellulose degradation.

1.2.1 Lignin Biodegradation and Ligninolytic System

Lignin is chemically difficult to degrade since 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 which 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 resinols, phenylcoumarans and various minor subunits. It has clearly been

illustrated that lignin is racemic, and consequently evens a simple β-O-4-linked dimer, with two asymmetric carbons, exists as four stereo isomers. Because the number of isomers geometrically increases with the number of subunits, the three-dimensional surface presented by lignin is non-repeating and complex (Ralph et. al., 1998).

There were several structural models of lignin. Lignin is more difficult to breakdown than cellulose or hemicellulose because of its nonhydrolyzable bonds and complicated structure. The molecular weight (MW) of lignin is high, about 100 kDa or more, which prevents its uptake inside the microbial cell (Eriksson, Blanchhette & Ander, 1990). Therefore, the lignin biodegradation must occur through the activity of extracellular enzymes (Kuhad, Singh & Eriksson 1997).

The lignin biodegradation by filamentous fungi is a major route for the recycling of photosynthetically fixed carbon, and the oxidative mechanisms employed have potential biotechnological applications (Hammel & Cullen, 2008). WRF are a physiological group of basidiomycetes that possess a remarkable capability to degrade lignin and lignin-like substances by giving a bleached white appearance to the wood. The lignin peroxidases (LiPs), manganese peroxidases (MnPs), laccases (Lacs) and closely related enzymes of white rot basidiomycetes are likely contributors to fungal ligninolysis. Different WRF strains secrete individual and multiple ligninolytic enzymes complexes in various proportions under suitable fermentation environment. Therefore, WRF have been divided into four groups based on their ligninolytic enzymes composition and secretion in various categories that are LiP, MnP and Lac producing, MnP and Lac producing, LiP and MnP producing, LiP and Lac producing (Bilal et al., 2017b).

1.2.1.1 Lignin Peroxidases

Lignin peroxidase (LiP), a glycoprotein of 38–46 kDa (and pI of 3.2–4.0) containing 1 mol of iron protoporphyrin IX per 1 mol of protein, catalyzes the H_2O_2-dependent oxidative depolymerization of lignin. LiP was shown to mineralize a variety of recalcitrant aromatic compounds and to oxidize a number of phenolic and

polycyclic aromatic compounds. LiP demontrates a very high redox potential (E_0' ~ 1.2 V at pH 3.0) when compared to Lacs (~ 0.8 V at pH 5.5) and MnP (~ 0.8 V at pH 4.5): this property enables LiP to catalyze the oxidation of nonphenolic aromatic compounds, even in the absence of a mediator (Pollegioni, Tonin & Rosini, 2015). pH and temperature activity profiles of LiPs from different sources change significantly with optimum activities shown between pH 2–5 and 35–55˚C, respectively (Asgher, Asad, Bhatti & Legge, 2007).

LiP catalyzes veratryl alcohol (VA) oxidation; veratryl alcohol has played a pivotal role of the lignin biodegradation process. 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 (Pollegioni et al., 2015; Wong, 2009):

(1) A $2e^-$ oxidation of the native ferric enzyme [Fe(III)] to yield compound I intermediate that occurs 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 1.3).

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

(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 (Pollegioni et al., 2015; Wong, 2009).

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

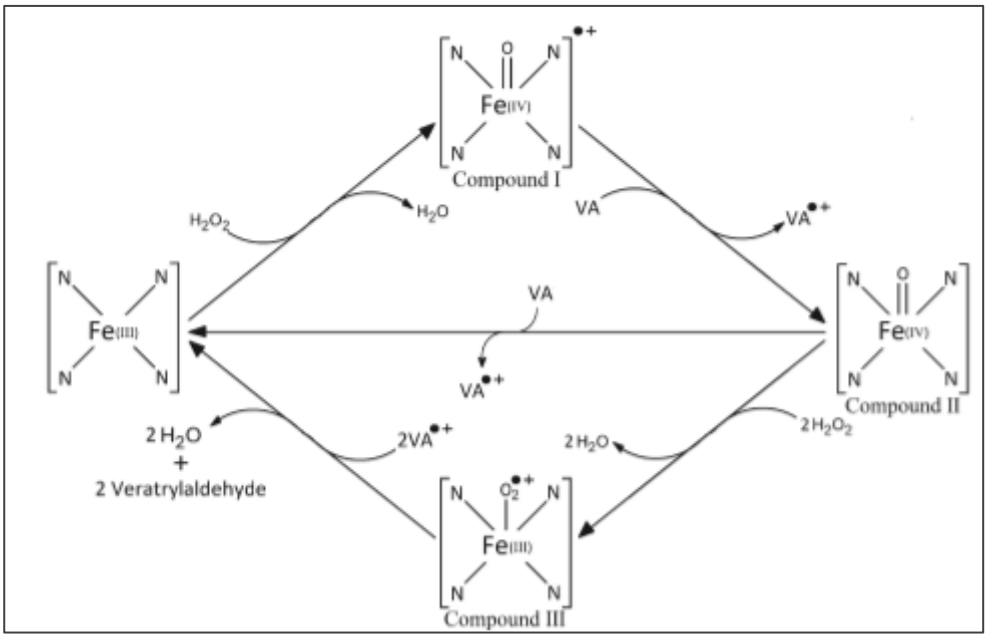


Figure 1.3 Catalytic cycle of lignin peroxidase (Pollegioni et al., 2015)

1.2.1.2 Manganese Peroxidases

Manganese peroxidases (MnP) are heme-containing glycoprotein which requires H_2O_2 as an oxidant. MnP acts on both phenolic and non-phenolic lignin units. It oxidizes Mn^{2+} to Mn^{3+} with H_2O_2 which oxidizes phenol rings to phenoxy radicals causing decomposition of compounds (Moilanen, Winquist, Mattila, Hatakka & Eerikäinen, 2015). The MW of extracellular fungal MnPs varies from 40 to 50 kDa, and the isoelectric point (pI) is usually acidic (pI 3-4). Optimum pH of 4–7 and optimum temperature of 40–60 ºC of MnPs are found (Ozturk Urek & Kasikara Pazarlioglu, 2007).

The catalytic mechanism of MnP was given in the Figure 1.4. In fact, MnP catalyzes the oxidation of Mn^{2+} to Mn^{3+} in the presence of Mn^{3+}-stabilizing ligands. The resulting Mn^{3+} complexes can then carry out the oxidation of organic substrates (Aitken, Massey, Chen & Heck, 1994). MnPs are also strongly oxidizing and undergo a classical peroxidase cycle but do not oxidize nonphenolic lignin-related structures directly since they lack the invariant tryptophan residue required for electron transfer to aromatic substrates. Hence, one-electron transfer to Compound I of MnP happens from bound Mn^{2+} and Compound II of MnP is formed. 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 WRF (Tripathi & Dixit, 2016).

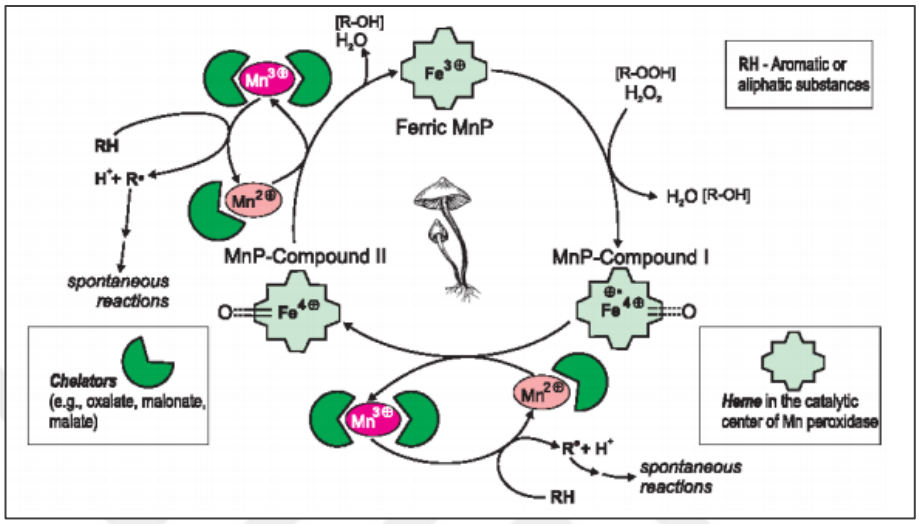


Figure 1.4 Catalytic cycle of manganese peroxidase (Tripathi & Dixit, 2016)

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 et. al, 2003).

1.2.1.3 Laccases

Fungal laccases (Lacs) are mainly extracellular glycoproteins with carbohydrate concentration of 10–30%, typical MW of 60–80 kDa, and acidic pI values of 3–6 (Hildén, Hakala & Lundell, 2009), optimum temperature of Lacs are 40 to 65˚C (Baldrian, 2006).

Lacs are biologically significant enzymes that belong to the oxidase group and also useful as green enzymes for cleaner industrial application to reduce the environmental pollution. These enzymes that include blue copper proteins or copper oxidases can oxidize a variety of compounds like carbohydrates, non-aromatic and aromatic compounds, e.g. phenolic and non-phenolic compounds, by degrading them into smaller components that help to reduce molecular oxygen to water (Senthivelan, Kanagaraj & Panda, 2016).

The Lac holoenzyme can either occur 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” Lacs contain four copper atoms located in two metal centers (T1, T2/T3). 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 (Cys) and two histidine (His) protein residues. The molecular environment of Lac T1 copper seems to regulate the redox potential of the enzyme (Ferraroni et al., 2007).

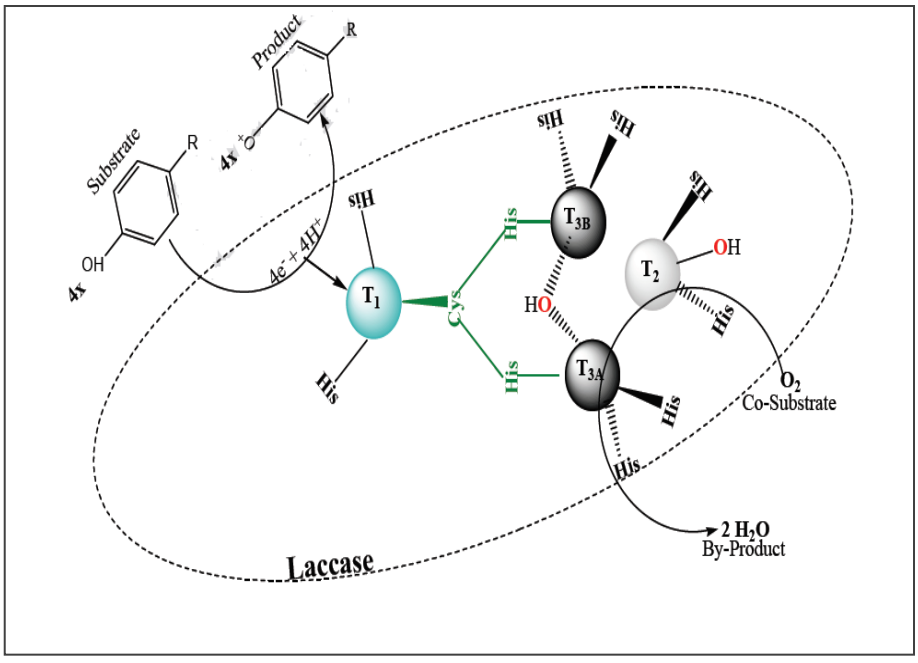


Figure 1.5 Catalytic cycle of laccase (Gianfreda, Xu & Bollag, 1999)

Lac catalysis is believed to comprise of three major steps (Gianfreda, Xu & Bollag, 1999) (Figure 1.5):

1. Reduction of mononuclear copper center: The reducing substrate (usually phenolic compounds) loses an electron to Lac. This electron reduces the T1 copper (at the mononuclear copper center), which is positioned just below the susbstrate-binding site. 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.

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 causing 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 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.

Other enzymes are associated with mainly lignin degrading enzymes in lignin breakdown, but are unable to degrade lignin alone. Glyoxal oxidase and superoxide dismutase produce the H_2O_2 required by LiP and MnP. Other enzymes are involved in feedback mechanism and serve to link lignocellulose biodegradation pathways. These consist of glucose oxidase, aryl alcohol oxidase, and cellobiose dehydrogenase (Pointing, 2001).

1.2.2 Cellulose Biodegradation and Cellulolytic Enzymes

Cellulolytic microorganisms can establish synergistic relationships with non-cellulolytic species in cellulosic wastes; the interaction cause complete degradation of cellulose. Cellulases responsible for the hydrolysis of cellulose, are composed of a complex mixture of enzyme proteins with different specificities to hydrolyze the β-1,4-glycosidic linkages bonds (Toushik et al., 2017). Cellulases can be divided into three major enzyme activity classes. These are endoglucanases or endo-1-4-β-glucanase, cellobiohydrolase and β-glucosidase. Endoglucanases, often called carboxymethylcellulases (because of the artificial substrate used for their detection), are thought to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre which opens-up sites for subsequent attack by the cellobiohydrolases. Cellobiohydrolase, often called exoglucanase can hydrolyze highly crystalline cellulose. Cellobiohydrolase remove monomers and dimers from the end of the glucan chain. β-glucosidase hydrolyzes glucose dimers and in some cases cellulose-oligosaccharides to glucose (Figure 1.6) (Vaithanomsat et al., 2011).

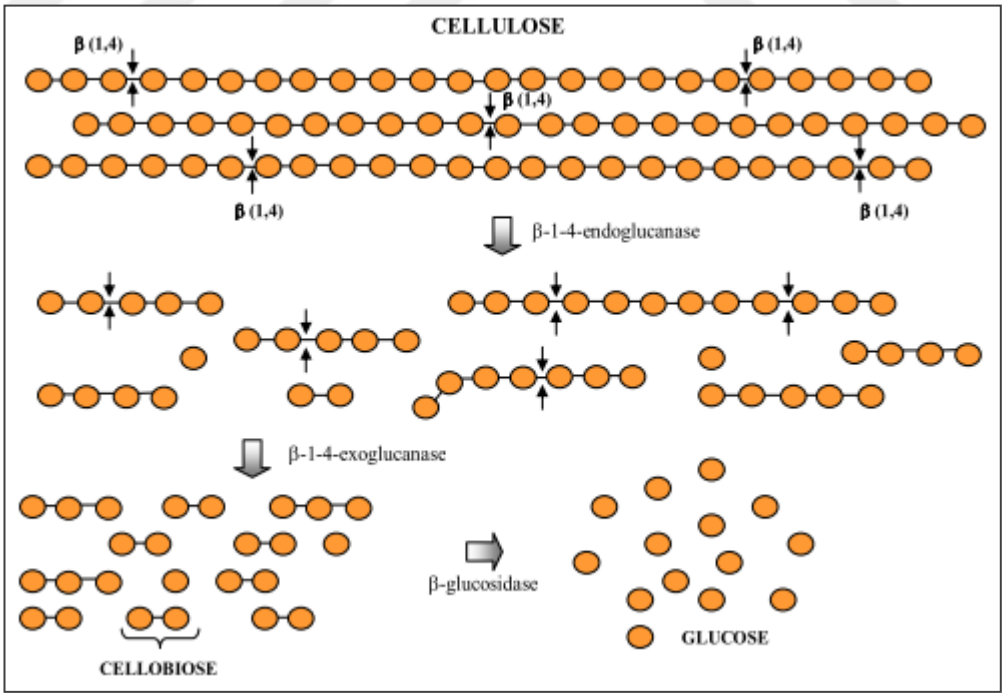


Figure 1.6 Schematic representation of the cellulase enzymes over the cellulose structure (Mussatto & Teixeira, 2010)

Fungal cellulases have frequently been reported to act synergistically in the cellulose degradation. A commonly held model for synergism between endoglucanases and exoglucanases suggests that hydrolysis is initiated by endoglucanases that cleave random β-1,4-linkages within the cellulose chain. This activity is followed by exoglucanase action that releases cellobiose from the non reducing ends of cellulose chain. The fact that the active centers of these enzymes are structurally different causes the substrate specificity (Cullen & Kersten, 1992).

1.2.3 Hemicellulose Biodegradation and Hemicellulolytic Enzymes

Hydrolysis of hemicellulose by the hemicellulases is crucial not only for the degradation of the cell wall structure but also for enhancing the hydrolysis of tightly linked cellulose by the cellulase enzyme. Formation of valuable polysaccharides from hemicellulose is initiated after the breakdown of hemicellulose by two hemicellulase enzymes, endoxylanases and exoxylanases, because xylan is the most abundant hemicellulose in the plant cell walls. Hemicellulases are classified based on their catalytic activity into glycoside hydrolases (GHs), carbohydrate esterases (CEs), and polysaccharide lyases (PLs). In addition to xylanase, hemicellulases include mannanase, glucuronidase, arabinase, β-glucanase and acetyl xylan esterase (Toushik et al., 2017).

Hemicelluloses are biodegraded to monomeric sugars and acetic acid. Xylan is the main carbohydrate found in hemicellulose. Its complete degradation requires the cooperative action of a variety of hydrolytic enzymes. Hemicellulases are frequently classified according to their action on distinct substrates, endo-1,4-β-xylanase generates oligosaccharides from the cleavage of xylan, 1,4-β-xylosidase produces xylose from oligosaccharides. Also, hemicellulose degradation needs accessory enzymes such as xylan esterases, ferulic and p-coumaric esterases, α-4-O-methyl glucuronosidases and α-1-arabinofuranosidases, acting synergistically to efficiently hydrolyze wood xylans and mannans. In the case of O-acetyl-4-O-methylglucuronxylan, which is one of the most common hemicelluloses, four different enzymes are required for degradation: endo-1-4-β-xylanase (endoxylanase), acetyl

esterase, α-glucuronidase and β-xylosidase. The degradation of O-acetylgalactoglucomannan starts with rupture of the polymer by endomannases. Acetylglucomannan esterases remove acetyl groups, and α-galactosidases eliminate galactose residues. Finally, β-mannosidase and β-glycosidase break down the endomannase-generated oligomeric β-1,4 bonds (Pérez, Munoz-Dorado, de la Rubia & Martinez, 2002).

1.3 White Rot Fungi

Many of fungi are capable of hydrolyzing polysaccharide components of wood. However; when these components occur in a complex with lignin, they are resistant to hydrolytic breakdown. 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 et al., 2010).

WRF 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 WRF (Akpinar, 2011). Therefore, organisms performing lignocellulose degradation are of great importance and they have been identified as some fungi (Table 1.3).

Table 1.3 Enzymes produced by some fungi in several agricultural residues (Sánchez, 2009)

Fungus	Type of rot fungus	Substrate	Enzymes
Phanerochaete chrysosporium	White	Grape seeds, barley bran and wood shavings	LiP, MnP
Trametes versicolor	White	Wood shaving, carozo maize and compost of gardening wheat straw	Lac
Pleurotus ostreatus	White	Bagasse of cane maize straw	Cellulases, xylanases, Lac, MnP
Aspergillus niger	Brown	Sugarcane bagasse	Cellulases, Xylanases
Bjerkandera adusta	White	Shavings of wood, carozo maize, compost of gardening wheat straw	MnP, LiP
Fusarium sp.	Brown	Maize plants, young plant leaves	Xylanases, cellulases
Pycnoporus cinnabarinus	White	Softwood pulp	Lac, LiP, MnP
Fomitopsis palustris	Brown	Microcrystalline cellulose	Cellulases
Strobilurus ohshimae	White	Sugi Word	LiP, MnP

1.3.1 Pleurotus eryngii

Pleurotus spp. represent the third largest group of cultivated edible mushrooms in the world, grown on a variety of plant residues; and they have been found to be nutritionally important. Since *Pleurotus* spp. can use many substrates, they may be cultivated on a large number of substrates, according to local availability in different regions of the world. *Pleurotus* spp. have also been studied for their potential in upgrading lignocellulosic agricultural wastes for animal feed. The advantage of utilizing *Pleurotus* spp. for this particular application is their preferential degradation of lignin: the lignin is selectively degraded and the cellulose is exposed and can be employed by ruminants (Akyüz, Kirbag, Karatepe, Güvenç & Zengin, 2011).

Pleurotus spp. are among the easiest mushrooms to cultivate. They grow on wood, usually on dead, standing trees or on fallen logs in nature. Various substrates that include lignin and cellulose can be used for *Pleurotus* cultivation, such as wood chips, rice straw, corn, cotton stalks, waste hulls, wheat and other agricultural wastes, some of which can be recycled and upgraded for use as animal feed or in the preparation of

other products. The main advantage of using *Pleurotus* spp. to upgrade lignocellulosic waste is their selective degradation of lignin and hemicelluloses. Utilization of these materials is clearly dependent on *Pleurotus*' ability to secrete a range of enzymes, including peroxidases, Lacs, 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 flax shive, coffee pulp, corn cob, citronella bagasse, sugarcane bagasse and rice hulls. The substrates used in each region depend upon the locally available agricultural wastes (Cohen, Persky & Hadar, 2002).

The ligninolytic system of *Pleurotus* spp. has been studied in recent years. Two ligninolytic enzyme families have been characterized: MnP and Lac. These enzymes can be used for various environmental and biotechnological applications. *Pleurotus* spp. and their enzymes may serve as an efficient alternative for the bioremediation of resistant pollutants, as compared with non-ligninolytic microorganisms. *P. ostreatus* presents promise in its ability to degrade and mineralize toxic chemicals, such as atrazine, organophosphorus, polycyclic aromatic hydrocarbons and wastewaters (Cohen et al., 2002).

1.4 Solid State Fermentation

Solid state fermentation (SSF) is defined as fermentation with almost nofree water, but with substrate moist enough to support growth of microorganisms. Hence, SSF is the process which best mimics the natural habitat of most filamentous fungi (Hansen, M. Lübeck, Frisvad, P.S. Lübeck & Andersen, 2015). In SSF, solid material is non-soluble that acts both as source of nutrients and physical support. Solid material could be a naturally occurring solid substrate such as agricultural crops, agro-industrial residues or inert support (Barrios-González, Baños, Covarrubias & Garay-Arroyo, 2008). Both types of solid are insoluble in water but can absorb water into their matrix,

thereby providing the required moisture. Selection of the C-source or substrate (C+N-source) is a major aspect of SSF, and for production of enzymes it is necessary to screen a range of C-sources to find the optimal one corresponding to the chosen ascomycete and desired enzyme(s) (Hansen et al., 2015).

The moisture necessary for microbial growth exists in an absorbed state or complexed within the solid material. The lower moisture level at which SSF can perform is approximately 12% since below this level all biological activities cease. The upper limit is a function of absorbency and hence moisture content, which varies depending upon the nature of the substrate. In addition, there are several factors affecting SSF process such as initial moisture, pH and pretreatment of the substrate, particle size, temperature of incubation, age and size of the inoculum, relative humidity, agitation and aeration, supplementation of nutrients such as N, P and trace elements, supplementation of C-source and inducers (Rahardjo, Tramper & Rinzema, 2006).

One of the most positive points of SSF is the possibility of using low-cost agro-industrial by-products as substrates in order to produce high-added value products, such as polymers, chemical compounds and mainly enzymes, which can be applied in various industries, for example in foods or textile or even wastewater treatment (Bück, Casciatori, Thoméo & Tsotsas, 2015).

The morphology of fungi growing in solid state is of a mycelial form, where both aerial and substrate penetrating hyphae are produced. This characteristic phenotypical growth pattern is common for all types of SSF (Hansen et al., 2015). Growth of a fungus on a solid material involves modification of the substrate by secreted enzymes and this subsequently allows the growing (penetrating) hyphae to enter the substrate. The microbial biomass inside the substrate material and on the substrate surface secretes metabolites and enzymes and consumes the liberated nutrients (Barrios-González, 2012).

There are several advantages in employing many SSF processes over the conventional submerged fermentation (SmF) ones, like higher yields enzymes. The most advantages of SSF are a consequence of the different physiology shown by fungi and other microorganisms on a solid material, in relation to the one presented in SmF. Several relatively apparent differences were originally presented for enzyme production in SSF in relation to the same produced in SmF (Table 1.4). These include higher productivities and less prone to problems with substrate inhibition. In addition, there are many cases where enzymes produced in SSF differ from the ones produced in SSF: enzymes with different characteristics like higher optimal temperature or pH stability, different kinetic parameters or even enzymes that were not secreted to the medium in SmF were secreted in SSF (Barrios-González, 2012). Energy expenditure is lower for SSF compared to SmF since there is less water requirements, no mechanical mixing and the downstream processing also requires less energy (Hansen et al., 2015).

Table 1.4. Special physiology shown by fungi in SSF that contrasts with the one shown in SmF (Barrios-González, 2012)

1	Enzymes are generally produced in much higher concentrations in SSF.
2	Some enzymes from SSF show different characteristics (molecular weight, kinetic parameters, optimal conditions) in relation to the ones obtained in SmF.
3	Some enzymes that are intracellular in SmF are extracellular in SSF.
4	Strains that are good enzyme producers in SmF are not so good in SSF and vice versa.
5	Higher secondary metabolite production, often in shorter times.
6	Some secondary metabolites are only produced in SSF, like coniosetin, pyrrocidienes A, B and acremonidins A–E.
7	Concentrated medium increases secondary metabolite production in SSF.
8	Energy expenditure is lower for SSF since there is less water requirements.

There has been growing interest in SSF because the amounts of enzymes (proteins in general) secreted by filamentous fungi in this system are large and very frequently exceed those secreted in SmF. For example, in SSF on wheat bran *Aspergillus oryzae* produced 500-fold-higher yield of heterologous protein (chymosin) than in SmF (Barrios-González, 2012).

Also, Table 1.5 is demonstrated that several industrial enzymes and secondary metabolities are generated in SSF (Sánchez, 2009).

Table 1.5 Variety of some substrates, products and microorganisms involved in SSF (Sánchez, 2009)

Substrates	**Product**	**Microorganism**
Almond mea	Lipase	*Rhizopus oligosporus*
Apple pomace, corncob, barley husk	Dye degradation	*WRF*
Banana waste	Ligninolytic enzymes	*Pleurotus sp.*
Cacao jelly	Endo-polygalacturonase	*Peacilomyces clavisporus*
Coconut cake	Lipases	*Candida rugosa*
Corn stover	Cellulolytic enzymes	*Fusarium oxysporum*
Eucalyptus kraft pulp	Xylanase	*Streptomyces sp.*
Pineapple, mixed fruit, maosmi waste	Citric acid	*Aspergillus niger*

1.5 Applications of Ligninolytic and Hydrolytic Enzymes

Ligninolytic and hydrolytic enzymes actively degrade lignocellulosic substrates, and are used in various applications in several food industries, such as fruit and vegetable juice, vegetable oil processing, winemaking, brewing, and baking industries (Toushik et al., 2017).

Enzymes have always played a substantial role in the production of variety of commercial products from many industries such as food processing, animal feed, textile and detergent. These enzymes not only make the process environmentally benign but also play an important role in improving the productivity and eventually the cost of product formation. Since last two decades, among various industrial enzymes, celluloses, xylanases and Lacs (some of lignocellulolytic enzymes) are gaining enormous attention for their potential applications in bioconversion of lignocellulosic materials into value added products (Deswal, Sharma, Gupta & Kuhad, 2012). Some applications by lignocelluloytic enzymes are demonstrated in Table 1.6.

Table 1.6 Ligninolytic and hydrolytic enzymes used in various industries and their applications (Kirk, Borchert & Fuglsang, 2002)

Industry	Enzyme class	Application
Detergent (laundry and dish wash)	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
Starch and fuel	Xylanase	Viscosity reduction (fuel and starch)
Baking	Xylanase and ligninolytic enzymes	Dough conditioning, dough strengthening
Animal feed	Hemicellulases, cellulases and ligninolytic enzymes	Digestibilty
Textile	Cellulases and ligninolytic enzymes	Denim finishing, cotton softening, bleaching, excess dye removal
Pulp and paper	Hemicellulases, cellulases and ligninolytic enzymes	Bleach boosting, de-inking, drainage improvement, fiber modification
Personal care	Ligninolytic enzymes	Bleaching, antimicrobial

1.6 The Importance and Aim of This Thesis

According to literature, lignocellulose is the most common organic polymer, representing approximately 1.2 x 10^9 tons of the total annual biomass production through photosynthesis, and it is considered to be inexhaustible source of raw material for different products (Sánchez, 2009). It is the most abundant and renewable biopolymer on earth and the dominating waste from agriculture. A promising strategy for efficient utilization of this renewable resource is the microbial hydrolysis of lignocellulosic waste and fermentation of the resultant reducing sugar for economically production of desired metabolites and enzymes, especially ligninolytic and hydrolytic enzymes.

The aim is to investigate the ligninolytic and hydrolytic enzyme productions by *P. eryngii* with SSF using peach and cherry wastes from fruit juice industry though SSF. To the best of our knowledge, these wastes are the first substrate material for the ligninolytic and hydrolytic enzyme productions under SSF conditions by any WRF. Therefore, the biotechological potential of *P.eryngii* was revealed with using these

wastes as a substrate by it. Firstly, the chemical contents of these wastes were determined, and then the SSF culture conditions were optimized to produce higher ligninolytic and hydrolytic enzymes by *P.eryngii*. Secondly, the Lacs enzymes from peach and cherry cultures were purified and partially characterized. Moreover, approximately 10 000 different dyes and pigments, mainly for use in the dye and printing industries, are produced annually worldwide. They are of vital importance due to their ability of enviromental contaminants. When the high amounts and activities of enzymes were produced, decolorization of some dyes such as methyl orange (MO), reactive black 5 (RB), tartrazine (TT) and reactive red 2 (RR) were performed. Lastly, fourier transform infrared spectroscopy (FTIR) analyzes were performed to determine whether there were the chemical changes of MO dye.

CHAPTER TWO
MATERIAL AND METHODS

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

2.1 Material

All chemicals used were of analytical grades or higher where appropriate.

2.1.1 Microorganism

The white-rot fungus, *Pleurotus eryngii* (DC.) Gillet (MCC58) was used and obtained from Agroma Mushroom Cultivation Turkey, Denizli.

2.2 Methods

2.2.1 Microorganisms' Maintenance

The white rot fungus, *P. eryngii* (DC.) Gillet (MCC58) was maintained on malt extract peptone agar (MPA) medium at 4˚C, and transferred every month to fresh medium. The MPA medium consisted of 30.0 g/L malt extract, 3.0 g/L peptone and 15.0 g/L agar and pH was adjusted 5.6 (Akpinar & Ozturk Urek, 2014). To prepare the medium, the mixture of these components was stirred until agar was completely dissolved. Then, the medium was sterilized at 121˚C in an autoclave for 20 minutes. After cooling, the medium was transferred on plate. When the medium solidified, it was inoculated and incubated at 25˚C for 12 days.

Figure 2.1 *P.eryngii* covered the MPA surface after 12 day (Akpinar, 2011)

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

2.2.2 Lignocellulosic Substrates and Their Chemical Compositions

Peach and cherry agro-industrial wastes from the Dimes fruit juice factory (Izmir, Turkey) were collected and used as the solid substrate for the SSF. According to given knowledge from Dimes fruit juice factory, the peach and cherry fruits cultivated in the Aegean region of Turkey were harvested at optimum technological maturity. Their wastes came for two or three pressing to make fruit juice.

To investigate the wastes' chemical content, 1 g of solid wastes were mixed with 12 mL of potassium phosphate buffer at pH 6.0, 10 mM (0.174 g of K_2HPO_4 and 0.136 g of KH_2PO_4 of dissolving 100 mL distilled water) and homogenized at 10 000 rpm and 4ºC for five minutes with 30 seconds intervals. The homogenized samples were centrifugated at 12 000 rpm and 4˚C, for 15 min. Then, the supernatants were collected. The nitrogen, total protein, carbohydrate, reducing sugars contents of them were colorimetrically determined by the phenol-hypochlorite method (Weatherburn, 1967), Bradford dye-binding assay (Bradford, 1976), phenol-sulfuric acid method (Saha & Brewer, 1994), and the dinitrosalicyclic acid (DNS) method (Miller, 1959), respectively. In addition, the lignin and cellulose contents of the wastes were determined independently of the samples prepared just before. The lignin levels were

spectrophotometrically analyzed by the thioglycolic acid method (Bruce & West, 1989), whereas the cellulose levels after acid hydrolysis in these wastes were determined gravimetrically (Anonim, 2013). Experiments were done triplicate and samples were analyzed in triplicate.

2.2.2.1 Determination of Nitrogen Concentrations

When the samples were centrifugated, the supernatants of them were used for analyses of nitrogen contents by phenol-hypochlorite method (Weatherburn, 1967). The reagent solutions used in this procedure were prepared as follows:

Phenol reagent solution was prepared by mixing equal volume from solution A and B. Solution A; 5 g of phenol was dissolved in 50 mL of distilled water. Solution B; 25 mg of sodium-nitroprusside was dissolved in 50 mL of distilled water.

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 60ºC-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 curve was 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 2.2.

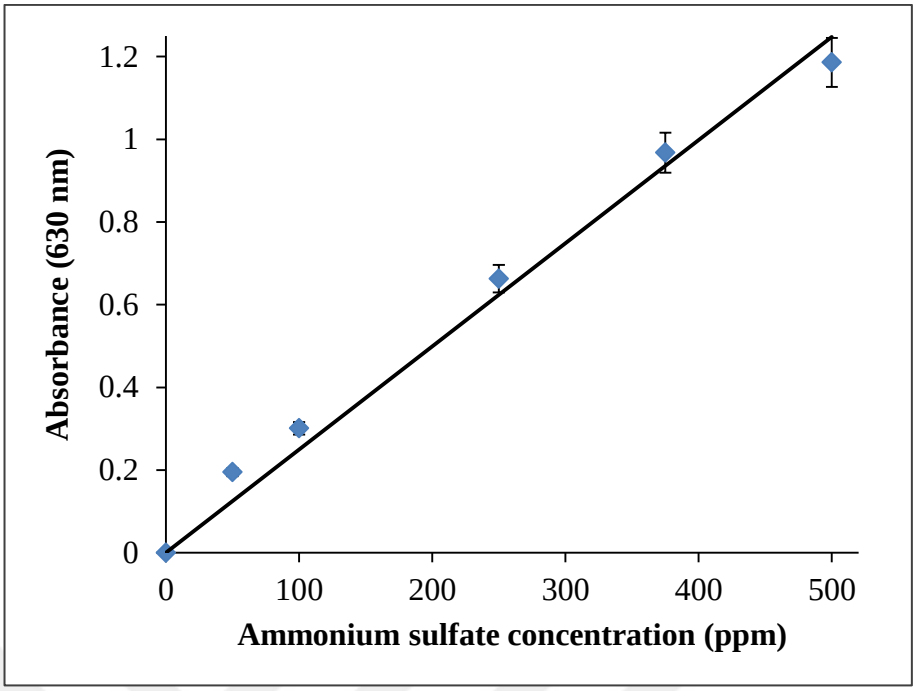


Figure 2.2 Calibration curves of nitrogen levels (y=0.0025x; R^2=0.99)

2.2.2.2 Determination of Protein Concentration

After the samples were homogeneously obtained from centrifugation, the supernatants were used for analysis of protein levels 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 was dissolved in 50 mL of 95% (v/v) ethanol. The solution is added to 100 mL of 85% v/v H_3PO_4, and diluted to 1000 mL of total volume with water. Ethanol and H_3PO_4 solutions were added directly from their stok solutions. The solution is stable indefinitely in a dark bottle at 4°C for a month. Protein concentrations of samples were determined by comparing the absorbance of the protein-dye binding complex at 595 nm. 100 µL of sample was added to 900 µL 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 blank sample. The blank sample was prepared by using 100 µL of distilled water instead of the sample supernatant and the same analysis procedure was followed. Calibration curve was plotted in the range of 0-200 µg of bovine serum albumine

(BSA) per mL of liquid, and the same procedure was performed. The calibration curve was depicted in Figure 2.3.

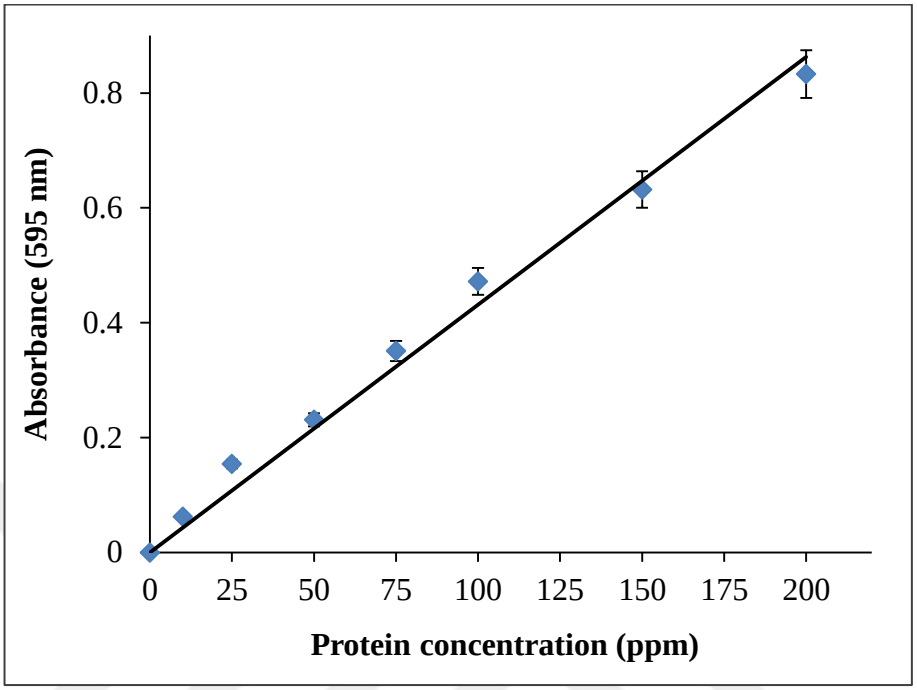


Figure 2.3 The calibration curves of protein levels (y=0.0043x; R^2=0.99)

2.2.2.3 Determination of Carbohydrate Concentration

After homogenizing and collecting sample, the homogeneous sample was used for analysis of carbohydrate concentration by the phenol-sulfuric acid method (Saha & Brewer, 1994). The solution used for phenol-sulfuric acid method was prepared as follows:

Phenol solution was contained 5 g of phenol dissolved in 100 mL of distilled water. The first step of the analysis method was to mix 1.0 mL of supernatant or its dilution with 1.0 mL of 5% (w/v) phenol solution and 5.0 mL of concentrated sulfuric acid. After waiting for twenty minutes at room temperature, the absorbance was measured at 470 nm against a blank sample. The blank sample was prepared by using 1.0 mL of distilled water instead of the sample supernatant and the same analysis procedure was followed. Standard curve was prepared in the range of 0-100 μg of glucose per mL of

liquid, and the same procedure was performed. The calibration curve was given in Figure 2.4.

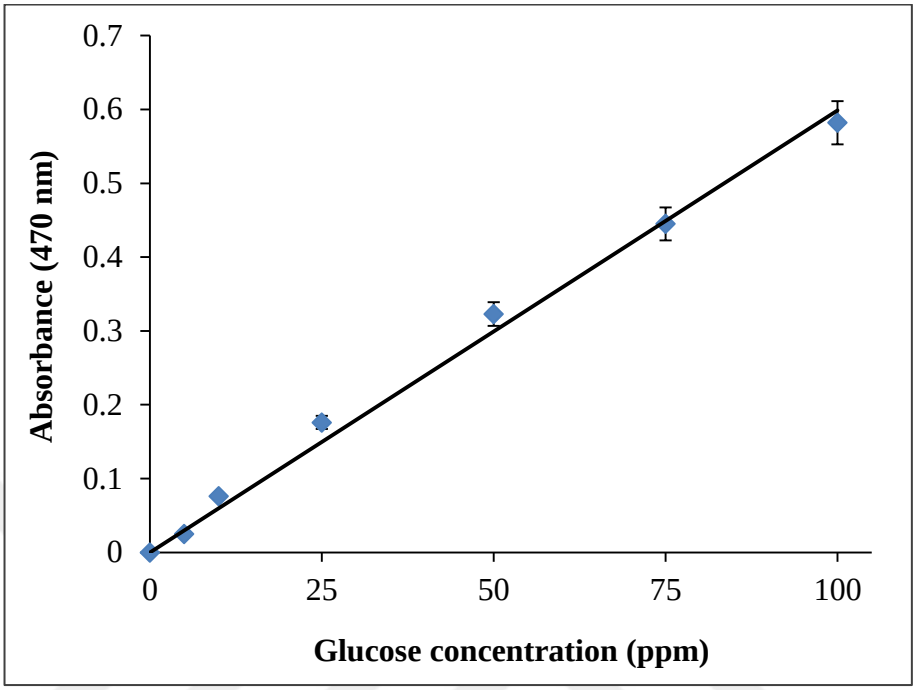


Figure 2.4 The calibration curves of carbohydrate levels (y=0.006x; R^2=0.99)

2.2.2.4 Determination of Reducing Sugar Concentration

The reducing sugar contents of the wastes were analyzed by the dinitrosalicyclic acid (DNS) method (Miller, 1959). The solutions used for DNS method were 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. NaOH solution (2.0 N); 1.6 g of NaOH was dissolved in 20 mL distilled water. Solution B; 30 g of sodium-potassium tartarate was dissolved in 50 mL of distilled water.

Solution B was added to solution 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 blank sample. The blank sample was prepared by using 500 µL of distilled water instead of the sample supernatant and the same analysis procedure was followed. Standard curve was prepared in the range of 0-165.1 µg of glucose per mL of liquid, and the same procedure was performed. The calibration curve was given in Figure 2.5.

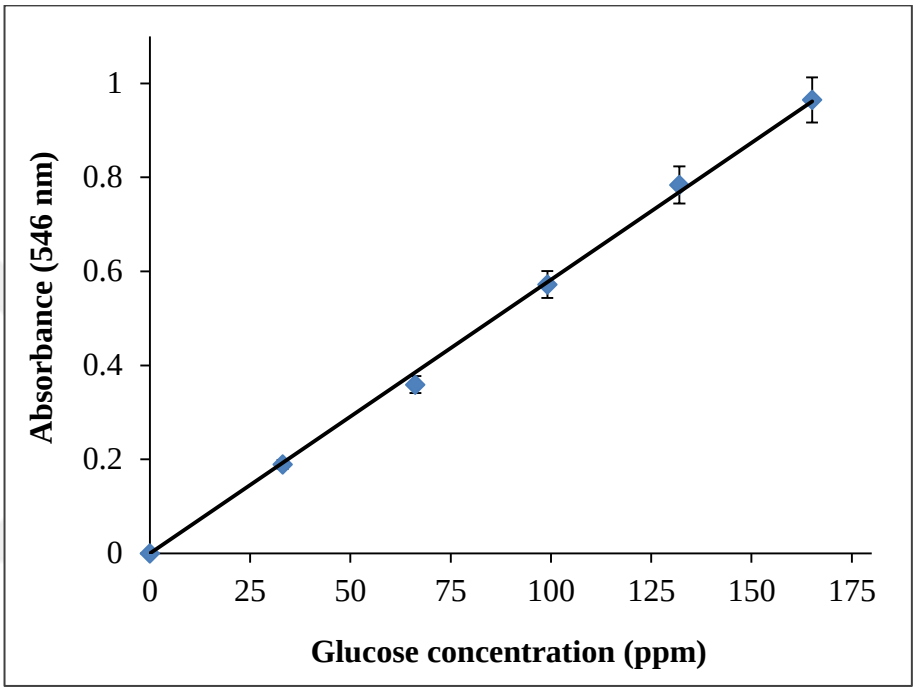


Figure 2.5 Calibration curves of reducing sugar levels (y=0.0058x; R^2=0.99)

2.2.2.5 Lignin Determination

The wastes materials were cut into small pieces, dried at 65˚C, and were ground to a fine powder (<0.08 mm) in a blender. This analysis was performed at two stage:

Firstly, isolation of structural biomass for lignin analysis was used by modifying (Bruce & West, 1989). The samples were dried in an oven at 105˚C until they reached to a constant weight. 200 mg of dried sample was suspended in 20 mL of washing buffer (100 mM K_2HPO_4/KH_2PO_4 at pH 7.8, 0.5%, v/v, Triton X-100), slowly stirred for 30 minutes at room temperature, and centrifuged at 5000 rpm for fifteen minutes. The pellet was first resuspended in washing buffer, and then washed as described

above and centrifuged at 5000 rpm for fifteen minutes again. The resulting pellet consisted mainly of cell walls, i.e., structural biomass (SBM). The SBM pellet was dried at 80˚C for twelve hours, weighed, and used for lignin analysis by the thioglycolate method (Bruce & West, 1989).

Secondly, lignin analysis with thioglycolic acid (TGA) method was used by modifying. Aliquots of 1-2 mg of SBM pellet (3 replicates per individual sample) were weighed into Eppendorf tubes and mixed with 1.5 mL of 2 N HCl and 0.3 mL of TGA. Samples were incubated at 95˚C for four hours and repeatedly mixed. Samples were rapidly cooled on ice and centrifuged at 10 000 rpm for twelve min. The supernatant was discarded. Pellets were washed 3-times with distilled water (1 mL). Thereafter, pellets were incubated with 1 mL of 0.5 N NaOH for 18 hours on a shaker at 150 rpm and 25˚C. The suspension was centrifuged at 10 000 rpm for twelve min. The supernatant carefully transferred into a 2-mL eppendorf tube. The pellet was resuspended in 0.5 mL of 0.5 N NaOH, vigorously mixed, and centrifuged. The resulting supernatant was combined with the first alkaline supernatant and mixed with 0.3 mL of concentrated HCl. Samples were incubated for four hours at 4˚C to precipitate the lignothioglycolate derivates. The samples were centrifuged, the supernatant discarded, and the pellet solubilized in 1 mL of 0.5 N NaOH. Absorbance of the resulting solution was measured at 280 nm. Calibration curve was generated by subjecting increasing amounts of 0-1.5 mg of commercial lignin (alkaline spruce lignin, Aldrich) to the same procedure and it was illustrated in Figure 2.6.

Washing buffer preparation: 4.355 g of K_2HPO_4 ve 3.402 g of KH_2PO_4 were dissolved in 250 mL distilled water and pH was adjusted 7.8. After that, 1250 µL of Triton X-100 was added this solution.

HCl solution (2.0 N) preparation: 6.36 mL of HCl was dissolved in distilled water and total volume was completed with distilled water to 100 mL.

NaOH solution (0.5 N) preparation: 2 g of NaOH was dissolved in 100 mL distilled water.

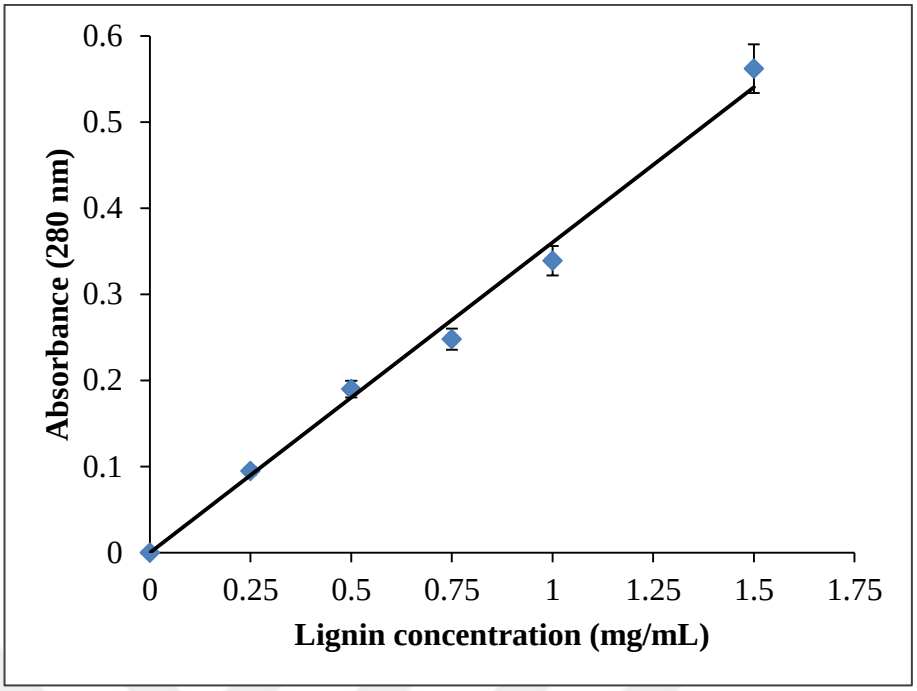


Figure 2.6 The calibration curve of lignin levels (y=0.3604x; R^2=0.99)

2.2.2.6 Cellulose Determination

1 g of sample (either peach or cherry wastes) was mixed with covering solution and then the system connect to the back cooler. After boiling for 30 minutes, the mixture was cooled to room temperature. The cooled solution was filtered from the filter paper been taring (M_1). Filter paper and vessel were washed with %70 (v/v) acetic acid solution once. After completely filtered, the sample was washed with hot distilled water. The filter process was continued until the filtrate did not change litmus paper colour. And then, the samples were washed with acetone three times after finishing wash process with distilled water. After the acetone was completely filtered, the samples were washed with ether once a more. The filter paper was dried at 105˚C and cooled to room temperature. Finally it was weighted (M_2) (Anonim, 2013). The cellulose content was calculated bu the formula 2.1.

Covering solution contained 70 mL of 70% acetic acid, 5 mL of nitric acid and 2 g of trichloroacetic acid.

Acetic acid solution preparation (%70 v/v); 700 mL acetic acid was dissolved in distilled water, and total volume should be 1000 mL.

Calculation:
M_1=The weight of filter paper; M_2=The weight of filter paper and sample; m=The weight of sample.

$$\% \, cellulose = \left[\frac{\mathrm{M2} - \mathrm{M1}}{\mathrm{m}}\right] x \, 100 \qquad (2.1)$$

2.2.2.7 The Determination of Metal Contents of These Wastes

The determination of the Cu, Fe and Mn metal contents of the wastes to be used as substrates in the SSF processes was carried out at the ARGEFAR laboratories, Ege University by their experts using inductively coupled plasma mass spectrometry (ICP-MS).

2.2.3 Solid State Fermentation

The medium for SSF was prepared by 5 g of solid waste (peach or cherry waste) obtained from Dimes Fruit Juice Factory (Izmir, Turkey) with basal medium volume (10 mL) in a 100 mL flask. The basal medium components were shown in Table 2.1. The initial pH of the synthetic medium was adjusted to 6.0 prior to sterilization. The flask was sterilized for 20 minutes at 121˚C, cooled to room temperature, and then inoculated with mycelium plug (three 1.0 cm of disks). Solid state culture was placed in a stationary incubator at 28˚C during the cultivation (Akpinar & Ozturk Urek, 2012). Incubation period was initially designated as twenty days, then this time was extended to 30 days after the obtaining results.

Table 2.1 Basal medium components in per liter (Stajić et al., 2006)

Component	Concentration	Component	Concentration
Yeast extract	2.0 g	$ZnSO_4.7H_20$	0.002 g
KH_2PO_4	0.8 g	$FeSO_4.7H_2O$	0.005 g
$MgSO_4.7H_2O$	0.5 g	$CaCl_2.2H_2O$	0.06 g
$Na_2HPO_4x7H_2O$	0.75 g	$CuSO_4.7H_2O$	0.02 g
NH_4NO_3	2.0 g	$MnSO_4.7H_2O$	0.05 g

2.2.3.1 Harvesting of Sample and Crude Enzyme Preparation

The *P. eryngii* was incubated under SSF conditions using peach/cherry waste as a substrate during incubation period. Samples from flasks were harvested after 3, 5, 7, 10, 12, 15, 17, 20, 26 and 30 days of incubation. The cultures obtained in SSF were mixed with 25 mL of 50 mM sodium acetate buffer (pH 5.0) two times and stirred at 180 rpm for an hour on ice bath to extract the samples. When the inoculation was performed with plug in SSF, solid waste and biomass were separated by centrifugation at 5000 rpm and 4˚C for 10 minutes after the culture extracted. Then, the supernatant was collected and centrifuged at 12 000 rpm at and 4˚C for 10 minutes again. This supernatant was called the crude enzyme extract (Akpinar & Ozturk Urek, 2012; 2014).

Sodium acetate buffer preparation (50 mM, pH 5.0); 4.102 g of sodium acetate was dissolved in distilled water, and then acetic acid solution containing 2.85 mL acetic acid was added to this solution. After pH was adjusted 5.0, the total volume of this buffer was completed to 1000 mL with distilled water.

When the crude enzyme extract was obtained, the supernatants were used for measurements of the extracellular ligninolytic and hydrolytic enzymes activities, and the analysis of protein, reducing sugar and nitrogen amounts in all fermentation conditions. Experiments were done triplicate and samples were analyzed in triplicate.

2.2.3.2 Evaluation of Fermentation Conditions on Enzymes Activities

Process parameters were varied to study their effects on SSF for ligninolytic and hydrolytic enzymes activities by *P.eryngii.* For ligninolytic and hydrolytic enzymes productions with higher activities, pretreatment of wastes was carried out, while basal medium shown in Table 2.1 was optimized in terms of some compositions such as copper, iron, Tween 80, ammonium nitrate, manganese concentrations. Fermentation process was carried out so that one parameter was changed at a time. The graphs showing the activity changes (in chapter three) were drawn by considering the parameter being examined. Since the solubility of waste components in the fermentation medium might be very low, the effect of these components on the basal medium content during the optimization could be negligible. Also, these enzymes productions by *P. eryngii* were carried out under control conditions when using untreated each waste (5 g) and non-optimized basal medium (10 mL), shown in Table 2.1.

2.2.3.2.1 The Pretreatment of Lignocellulosic Wastes Used as Substrates in Solid State Fermentation. The both peach and cherry wastes, lignocellulosic biomass, were pretreated with hot water, dilute acid and base solutions (Bilal et al., 2017a). The hot water treatment was named as wet oxidation. These pretreatment methods were expressed as follows:

The wet oxidation (WO) treatment: The biomass at a solid loading of 10% (w/w) was mixed with water and pretreated at 100˚C with 60 min. After pretreatment, the biomass was filtered.

Dilute acid treatment: The biomass at a solid loading of 10% (w/w) was mixed with dilute sulfuric acid (concentration of 1.0% (w/v)) and pretreated at 100˚C with 60 min. After pretreatment, the biomass was washed until the pH adjusted to 5.6 and then filtered.

Dilute base treatment: The biomass at a solid loading of 10% (w/w) was mixed with dilute sodium hydroxide (concentration of 1.0% (w/v)) and pretreated at 100˚C with 60 min. After pretreatment, the biomass was washed until the pH adjusted to 5.6 and then filtered.

Sulfuric acid solution (1.0% w/v): 10 g of sulfuric acid (5.435 mL) was dissolved in distilled water and the total volume of this solution was completed to 1000 mL with distilled water.

Sodium hydroxide solution (1.0% w/v): 10 g of sodium hydroxide was dissolved in 1000 mL distilled water.

2.2.3.2.2 Copper Supplementations. The influence of heavy metals on ligninolytic and hydrolytic enzymes activities of *P. eryngii* was determined. For this aim, various copper concentrations (0; 35; 70; 100; 500; 1000; 2000; 3000 and 5000 µM) were incorporated into the basal medium using peach or cherry wastes. Each copper amounts were added at the time of inoculum transfer. The non-optimized basal medium contained 70 µM copper.

2.2.3.2.3 Iron Supplementations. The influence of heavy metals on ligninolytic and hydrolytic enzymes activities by *P. eryngii* was determined. In order to study, various iron concentrations (0; 10; 18; 25; 40; 100; 500; 1000; 2000 and 5000 µM) were incorporated into the basal medium. Each iron amounts were added at the time of inoculum transfer. The non-optimized basal medium contained 18 µM iron.

2.2.3.2.4 Tween 80 Supplementations. The influence of surfactant on ligninolytic and hydrolytic enzymes activities by *P. eryngii* was determined. In order to study, various Tween 80 concentrations (0.025; 0.05; 0.1 and 0.2% (v/v)) were incorporated into the fermentation medium. Each Tween 80 amounts were added at the time of inoculum transfer. The non-optimized basal medium did not contain Tween 80.

2.2.3.2.5 Ammonium Nitrate Supplementation. The influence of nitrogen sources' concentrations on ligninolytic and hydrolytic enzymes activities by *P. eryngii* was determined. In order to study, various ammonium nitrate concentrations (1.0, 2.0 and 4.0 g/L) were incorporated into the basal medium. Each ammonium nitrate amounts were added at the time of inoculum transfer. The non-optimized basal medium contained 2.0 g/L ammonium nitrate.

2.2.3.2.6 Manganese Supplementation. The influence of heavy metals on ligninolytic and hydrolytic enzymes activities by *P. eryngii* was determined. In order to study, various manganese concentrations (180, 250, 500, 750 and 1000 μM) were incorporated into the basal medium. Each manganese amounts were added at the time of inoculum transfer. The non-optimized basal medium contained 180 μM manganese.

2.2.3.3 Analysis of Fungal Extracts for Ligninolytic and Hydrolytic Enzymes Activities

2.2.3.3.1. Laccase (Lac) Activity. This assay measured the oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS-$(NH_4)_2$) (ε_{max} = 36000 M^{-1} cm^{-1}). The reaction mixture (total volume 1 mL) was contained 700 μL of 100 mM sodium acetate buffer at pH 4.5, 100 μL of 5.0 mM 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°C in a UV-spectrophotometer. The reaction mixture showed green color and increased in intensity at the initial stage of the reaction. One unit (U) of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min. The activities were expressed in unit per L (Johannes & Majcherczyk, 2000).

Sodium acetate buffer preparation (100 mM, pH 4.5); 2.050 g of sodium acetate was dissolved in distilled water. 1.50 g of acetic acid (1.423 mL) was dissolved in distilled water. Both of solutions were mixed. After the pH of this solution was

adjusted to 4.5, the total volume of this buffer was completed to 250 mL with distilled water.

ABTS solution (5 mM); 0.0274 g of ABTS was dissolved in 10 mL distilled water.

2.2.3.3.2 Manganese Peroxidase (MnP) Activity. It was measured by 2,6-dimethoxyphenol (2,6-DMP; ε_{max} =27 500 M^{-1} cm^{-1}) oxidation (Kuwahara, Glenn, Morgan & Gold, 1984). The reaction mixture was contained 600 µL of 250 mM Na-citrate buffer at pH 4.5, 50 µL of 20 mM 2,6-DMP, 50 µL of 30 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 L.

Sodium citrate buffer preparation (250 mM, pH 4.5); 18.381 g of sodium citrate dihydrate was dissolved in distilled water. 12.008 g of citric acid was dissolved in distilled water. Both of solutions were mixed. After the pH of this solution was adjusted to 4.5, the total volume of this buffer was completed to 250 mL with distilled water.

2,6-DMP solution (20 mM); 0.031 g of 2,6-DMP was dissolved in 10 mL distilled water.

Manganese sulfate solution (30 mM); 0.051 g of $MnSO_4.H_2O$ was dissolved in 10 mL distilled water.

H_2O_2 solution (4 mM); 0.0014 g of H_2O_2 (1.225 µL) was dissolved in distilled water and total volume should be 10 mL. This solution was also used in LiP assay and kept at 4 ℃ in dark.

2.2.3.3.3 Lignin Peroxidase (LiP) Activity. It was determined by the oxidation of VA to veratraldehyde. The reaction mixture was contained 650 µL of 125 mM sodium

tartarate buffer at pH 2.5, 100 µL of 20 mM VA, 50 µL of 4 mM H_2O_2 solution and 200 µl of the sample or its dilution. The reaction was started by adding H_2O_2 and the increase in absorbance at 310 nm and 30˚C was monitored (ε_{max} = 9300 M^{-1} cm^{-1}). One unit (U) of enzyme activity is 1 µmol of veratraldehyde produced per minute per mL of the sample. The activities were expressed in unit per L (Tien & Kirk, 1988).

Sodium tartarate buffer preparation (125 mM, pH 2.5); 7.19 g of sodium tartarate dihydrate was dissolved in distilled water. 4.69 g of tartaric acid was dissolved in distilled water. Both of solutions were mixed. After the pH of this solution was adjusted to 2.5, the total volume of this buffer was completed to 250 mL with distilled water.

Veratryl alcohol solution (20 mM); 0.0336 g of VA (29.08 µL) was dissolved in distilled water and total volume should be 10 mL. It was kept in dark.

2.2.3.3.4 Carboxymethyl Cellulase (CMCase; Endo-1,4-β-glucanase) Activity. It was measured with the azo-dyed substrate, carboxymethyl cellulose. A 1 mL reaction mixture was contained 0.5 mL of approximately diluted supernatant and 0.5 mL of 2%, w/v, carboxymethyl cellulose in pH 4.8 sodium citrate-citric acid buffer (50 mM, pH 4.8). The mixture was incubated at 50 °C for 30 minutes, and the reducing sugar was assayed by the dinitrosalicylic acid procedure (Miller, 1959), with glucose as the standard. One unit (U) of CMCase activity is defined as the amount of enzyme that released 1 µmol of reducing sugar (expressed as glucose equivalent) per minute at 50 °C and pH 4.8 (Kumaran, Sastry & Vikineswary, 1997). The calibration curve was given in Figure 2.7.

Sodium citrate buffer preparation (50 mM, pH 4.8); 3.676 g of sodium citrate dihydrate was dissolved in distilled water. 2.402 g of citric acid was dissolved in distilled water. Both of solutions were mixed. After the pH of this solution was adjusted to 4.8, the total volume of this buffer was completed to 250 mL with distilled water. It was also used in xylanase assay.

Carboxymethyl cellulose solution (2%, w/v); 200 mg of carboxymethyl cellulose was dissolved in 10 mL of sodium citrate buffer (50 mM, pH 4.8).

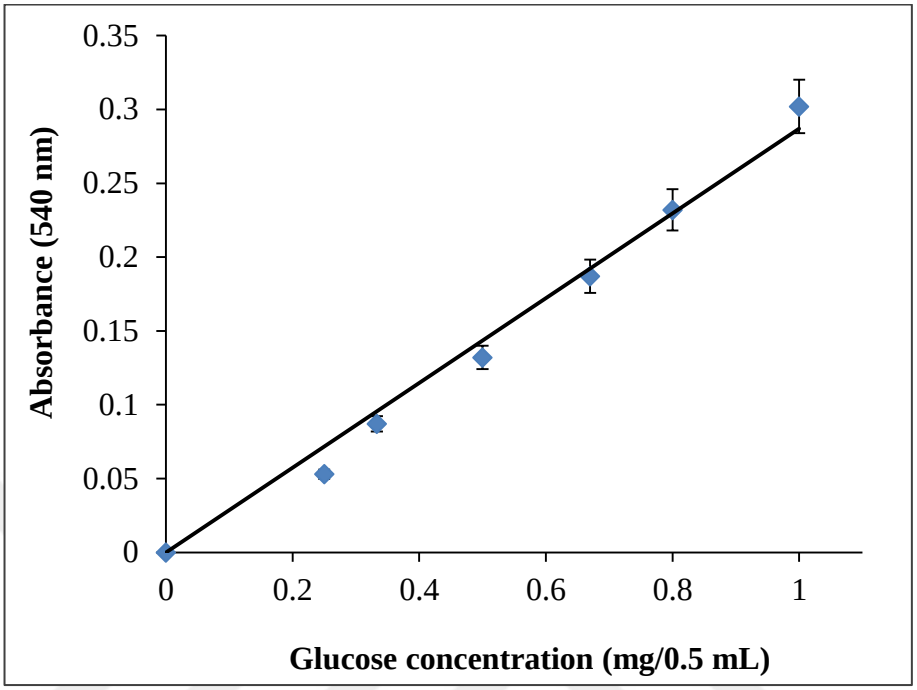


Figure 2.7 Calibration curve of CMCase enzyme using glucose as a standard (y=0.287x; R^2=0.99)

2.2.3.3.5 Xylanase Activity. It was estimated according to modified carboxymethyl cellulase assays (Kumaran et al., 1997). A 1.0 mL reaction mixture was contained 0.5 mL of approximately diluted supernatant and 0.5 mL of 2% oat spelts xylan in pH 4.8 sodium citrate-citric acid buffer (50 mM, pH 4.8). The mixture was incubated at 50 °C for 30 minutes, and the reducing sugars were assayed by the DNS procedure (Miller, 1959), with xylose as the standard. One unit (U) of xylanase activity is defined as the amount of enzyme that released 1 µmol of reducing sugar (expressed as xylose equivalent) per minute at 50 °C and pH 4.8 (Kumaran et al., 1997). The calibration curve was given in Figure 2.8.

Oat spelts xylan solution (2%, w/v); 200 mg of oat spelts xylan was dissolved in 10 mL of sodium citrate buffer (50 mM, pH 4.8).

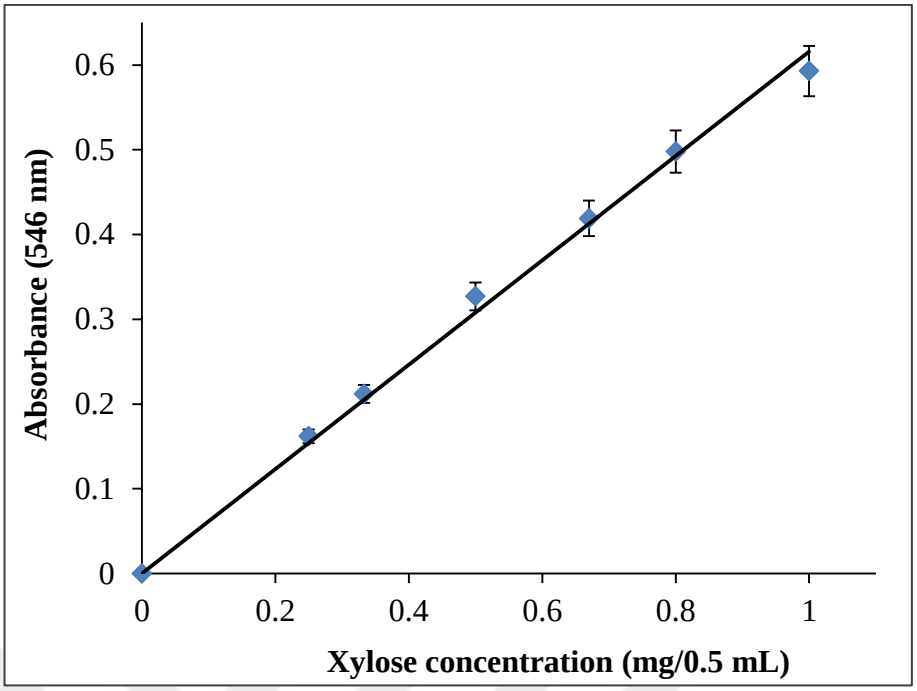


Figure 2.8 Calibration curve of xylanase enzyme using xylose as a standard (y=0.6157x; R^2=0.99)

2.2.3.3.6 Exo-1,4-β-glucanase Activity. It was assayed in microplates using *p*-nitrophenyl-β-D-cellobioside (PNPC). The reaction mixture was contained 0.16 mL of 1.2 mM PNPC in 50 mM sodium acetate buffer (pH 5.0) and 0.04 mL sample. Reaction mixtures were incubated at 40 ˚C for 20–60 minutes. The reaction was stopped by adding 0.1 mL of 0.5 M sodium carbonate, and absorbance was read at 400 nm. Enzyme activity was calculated using the molar extinction coefficient of p-nitrophenol (11 600 M^{-1} cm^{-1}). One unit (U) of enzyme activity is defined as the amount of enzyme releasing 1 µmol of p-nitrophenol per min (Baldrian, Valaskova, Merhautova & Gabriel, 2005).

Sodium acetate buffer (50 mM; pH 5.0) was previously prepared in Section 2.2.3.1.

PNPC solution (1.2 mM); 5.56 mg of PNPC was dissolved in 10 mL of sodium acetate buffer (50 mM, pH 5.0).

Sodium carbonate solution (0.5 M); g of sodium carbonate was dissolved in 50 mL distilled buffer. Both sodium acetate buffer and sodium carbonate solutions were also used in BGLA assay.

2.2.3.3.7 1,4-β-glucosidase Activity (BGLA). It was assayed using *p*-nitrophenyl- β-D-glucoside as a substrate. The reaction mixture was contained 0.16 mL of 1.2 mM *p*-nitrophenyl- β-D-glucoside in 50 mM sodium acetate buffer (pH 5.0) and 0.04 mL sample. The reaction mixture was incubated at 40 °C for 20-60 minutes. The reaction was stopped by adding 0.1 mL of 0.5 M sodium carbonate, and the absorbance was read at 400 nm. Enzyme activity was calculated using the molar extinction coefficient of p-nitrophenol (11 600 M^{-1} cm^{-1}). One unit (U) of enzyme activity is defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per minute (Baldrian et al., 2005).

p-nitrophenyl- β-D- glucopyranoside solution (1.2 mM); 3.62 mg of *p*-nitrophenyl-β-D- glucopyranoside was dissolved in 10 mL of sodium acetate buffer (50 mM, pH 5.0).

2.2.3.4 Determination of Protein, Reducing Sugar and Nitrogen Concentrations

The total protein, reducing sugar and nitrogen concentrations of supernatants were respectively determined as described previously depending on incubation period.

2.2.4 The Purifications of Laccase Enzymes from SSF Using Peach and Cherry Wastes

After the optimization of fermentation medium, it was decided to purify the Lac enzymes due to their biotechnological importance and wide utilization among all enzymes.

2.2.4.1 Ammonium Sulfate Precipitation

Ammonium sulfate fractionation has been commonly used to partially purify laccases from the crude filtrate of their sources for many years. Ammonium sulfate is the most common salt used in enzyme purification because it combines many useful features, such as salting out effectiveness, high solubility and low price. In principle, the fractionation method depends on the ability of high concentration of ammonium

sulfate to bind available water molecules and thus prevent the solvation of proteins (Taqi, 2012). Before the partial purification of Lac by ammonium sulfate, the crude enzyme extract was concentrated by ultrafiltration using membrane with a cut off 10 kDa. The concentrated crude enzyme extracts were partially purified by ammonium sulfate precipitation at 4℃.

Solid ammonium sulfate was added with continuous stirring to the enzyme suspension at 35, 45, 55, 65, 85% (w/v) of saturation, and the resulting suspension was allowed to settle for 30 min. The precipitate was obtained by centrifugation (12 000 rpm, 15 min) at 4℃, lower phase and upper phase were separated. The precipitate was dissolved in a minimal amount of sodium acetate buffer (20 mM, pH 4.5). The Lacs activities and protein amounts were determined in both phases.

Sodium acetate buffer solution (250 mM, pH 4.5); 5.1275 g of sodium acetate was dissolved in distilled water, and then acetic acid solution containing 3.56 mL acetic acid was added to this solution. After pH was adjusted 4.5, the total volume of this buffer was completed to 250 mL with distilled water. For preparing 20 mM sodium acetate buffer, this solution was diluted at ratio 1:12.5.

2.2.4.2 DEAE-Sepharose Ion Exchange Chromatography

After ammonium sulfate precipication methods, the partially purified enzyme was loaded to anion exchange column. Column dimensions were 15 x 1.5 cm and flow rate was 0.5 mL/min. 50 mM sodium acetate buffer (pH 4.5) was used as an equilibration buffer. A 2 mL samples of the enzymes were loaded. A gradient elution system consisted of sodium acetate buffer solutions (50-250 mM, pH 4.5) and NaCl salt solutions (1-2 M). A 5 mL of gradient solutions were added to column by increasing their concentrations. The eluted enzymatic fractions (1.0 mL/eppendorf) were collected and analyzed in terms of Lac activity and protein levels at 280 nm. The fractions with Lac activity were also analyzed with Bradford method instead of other direct UV method for the correction of protein amounts more sensitively.

For preparing 50 mM sodium acetate buffer; the solution, previously prepared in Section 2.2.4.1, was diluted at ratio 1:5.

2.2.4.3 Sephadex G-100 Gel Filtration Chromatography

The sample from anion exchange column was concentrated by ultrafiltration (10 kDa) and then applied to the Sephadex G-100 gel filtration column. Column dimensions were 75 x 1.5 cm and flow rate was 0.2 mL/minute. 20 mM sodium acetate buffer (pH 4.5) was used as equilibration and elution buffer. A 2 mL enzyme sample was loaded to column and 2.5 mL fractions were collected. The eluted fractions (2.5 mL/tube) were monitored at 280 nm for protein levels and at 420 nm for Lac activity. 20 mM sodium acetate buffer (pH 4.5) was previously prepared in Section 2.2.4.1.

Blue dextran and standard protein mixture instead of sample were applied to the gel filtration column under same conditions in order to determine the molecular weight of purified Lacs. Same analyzes were carriout out by gel filtration marker kit (MWGF70, Sigma, molecular weights of standard marker kit; 6500- 66 000 Da). The all samples were dissolved in equilibration buffer containing 5% glycerol.

2.2.4.4 Electrophoretic Analysis

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed, using a Mini-Protean Tetra Cell (Bio-Rad Laboratories; Philadelphia, N.J.), according to the method of Laemmli using a vertical slab gel apparatus (Laemmli, 1970). The samples from gel filtration were first concentrated by ultrafiltration (10 kDa) and then applied on the surface of a 4% stacking gel polymerized on top of a 12% separating gel. The molecular weight (Mr) estimates were determined through protein markers obtained from Sigma-Aldrich (M3913) including bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsinogen inhibitor (20 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa). Molecular weight markers and purified Lacs were dissolved with sample buffer

and denaturated by incubation in a boiling water-bath for 4 min. A current of 120 V was applied for approximately 2 h. The proteins were stained with Comassie Brilliant Blue R250 overnight prior to destaining. Molecular weights of purified Lacs were determined. Table 2.2 showed the components of stacking and seperating gels. Acrylamide/Bis solution and tetramethylethylenediamine (TEMED) were directly used.

Table 2.2 The components of stacking and separating gels

	Stacking Gel	**Seperating Gel**
Acrylamide/Bis	1.3 mL	4.0 mL
Sterile distilled water	6.1 mL	3.4 mL
1.5 M Tris-HCl, pH 8.8	-	2.5 mL
0.5 M Tris-HCl, pH 6.8	2.5 mL	-
%10 SDS	0.1 mL	0.1 mL
%10 ammonium persulfate (fresh)	50 µL	50 µL
TEMED	10 µL	5 µL
Total Monomer	10 mL	10 mL

SDS solution (10%, w/v); 1 g of SDS was dissolved in 9 ml water with gentle stirring and brought to 10 mL with deionized water.

Tris-HCl buffer preparation (1.5 M, pH 8.8); 27.23 g of Tris base was dissolved in 80 mL deionized water. The pH was adjusted at 8.8 by using 6 N HCl. Right after, the total volume was completed to 150 mL with deionized water.

Tris-HCl buffer preparation (0.5 M, pH 6.8); 6.0 g of Tris base was dissolved in 60 mL deionized water. The pH was adjusted at 6.8 by using 6 N HCl. Right after, the total volume was completed to 100 mL with deionized water.

Ammonium persulfate solution (APS; 10%, w/v); 100 mg of APS was dissolved in 1.0 mL deionized water.

Sample buffer contained 3.55 mL deionized water, 1.25 mL Tris-HCl buffer (0.5 M, pH 6.8), 2.5 mL glycerol, 2.0 mL SDS (10%, w/v), 0.2 mL bromophenol blue

(0.5%, w/v). 50 µL of β-mercaptoethanol was addet to sample buffer before use. When the samples were contained higher amounts of protein, they could diluted with sample buffer.

10x running buffer (pH 8.3); 30.3 g of Tris base, 144.0 g of glycine and 10.0 g of SDS was dissolved and brought total volume up to 1000 mL with deionized water. The pH was not adjusted with acid or base. 10x stock solution was diluted at ratio 1:10 before use.

2.2.5 The Effects of Chemical Agents on Purified Laccases Activities

Due to the variable biotechnological applications of Lacs, it is crucial to know the effect of different agents on its enzymatic activity as well as stability. Therefore, the effect of different chemical agents, namely EDTA (ethylenediaminetetraacetic acid), PMSF (phenylmethanesulfonyl fluoride), iodoacetamide, β-mercaptoethanol, N-ethyl-5-phenylisoazolium-3'-sulfonate (WRK), 1-bromo-2,5-pyrolidinedione (NBS), on purified Lacs activities were investigated. Firstly, the enzymatic assays were performed in the reaction mixtures as described above, with EDTA, PMSF and β-mercaptoethanol solutions at final concentrations of 1.0 mM, iodoacetamide solution at 0.5 mM. After the enzyme solutions were treated these chemicals for 10 min, the absorbances were measured. The purified protein samples were treated with various amounts of Woodward's reagent K and NBS solutions in the ratio 1:1 at 37 ℃ for 30 min. Namely, 200 µL WRK and NBS solutions (0.1 M) were added to protein samples (200 µL). After incubation, WRK or NBS treated protein samples were analyzed in terms of activity changes of Lacs.

In addition, the spectral properties of the purified Lacs were investigated by recording the absorbance of the enzyme solution at wavelengths between 200 and 800 nm with a UV–*Vis* spectrophotometer.

2.2.6 Kinetic Characterization of P. eryngii Laccases from Different SSF cultures

2.2.6.1 K_m and V_{max}

Purified *P. eryngii* Lacs activities were measured with different substrate concentrations (1.0-5.0 mM ABTS) under standard assay conditions. Lineweaver-Burk graphics were drawn and K_m – V_{max} values were calculated by using Lineweaver-Burk diagram.

2.2.6.2 Determination of Inhibition Effect of Sodium Azide

For the determination of the inhibition effects of sodium azide, the purified Lacs activities were measured with variable substrat (1.0-5.0 mM ABTS) and sodium azide concentrations (0.001, 0.005, 0.01 mM). Then, Lineweaver-Burk graphics were drawn and inhibiton type was decided by using Lineweaver-Burk diagram.

2.2.7 In Vitro Decolorization

Dye decolorization studies were analyzed with four different dyes (methyl orange (MO), tartrazine (TT), reactive red 2 (RR) and reactive black 5 (RB)) which are belong to azo class. 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 crude enzyme solution (for 50 mg/L of dye concentration). In addition, the reaction was carried out at 25˚C and the reaction mixture contained 687.5 µL of distilled water, 62.5 µL of stock dye solution and 750 µL of enzyme solution (for 100 mg/L of dye concentration). The concentration of stock dye solution was 2.4 mg/mL (Akpinar & Ozturk Urek, 2012).

Decolorization was spectrofotometrically determined by monitoring the decrease in absorbance at the maximum absorbance of each dye (MO at 505 nm, TT at 425 nm, RR at 538 nm and RB at 597 nm). In parallel, control samples were maintained with heat inactivated enzyme. Also, the dye decolorization performances of purified Lacs

were detected for MO under the same conditions and concentrations. The decolorization degree was calculated using the following formula (2.2):

$$D = \frac{100\ (Aini - Aobs)}{Aini} \qquad (2.2)$$

where D is the decolorization degree (in percent), A_{ini} the initial absorbance, and A_{obs} the observed absorbance.

In addition, the effect of each dye on Lac and MnP enzymes activities from peach and cherry cultures, dye-treated enzyme samples, were monitored during 3 hours at 10 min intervals at 25°C in the dark and in the absence of mediators. The enzymes activities were measured as described before during the time. In this study, the dye solutions for MO, TT, RR and RB was mixed with crude enzyme solutions in the ratio 1:1. The each dye concentration in the reaction medium was 50 ppm and total volume of treated samples were 10 mL.

Fourier Transform Infrared Spectroscopy (FTIR) analysis of dye treated crude enzyme samples was carried out using Perkin Elmer 783 Spectrophotometer. The FTIR analysis was done in the mid IR region of 600–4000 cm^{-1} with 16 scan speed. For this analysis, the reactions between the each of dyes and the enzymes solutions must be stopped and sodium azide (NaN_3) was chosen as an inhibitiory of these reactions. Firstly, the effects of NaN_3 concentrations (0.5, 1.0, 2.0, 2.5, 5.0 mM) on Lacs and MnPs activities from SSF cultures were investigated and the optimal concentration was determined. Secondly, the reactions were stopped by sodium azide at the relevant time, which was specified in the decolorization performance after the enzyme solutions were treated with the different dyes (in the ration 1:1) for each time period the dye under the conditions just stated. Each of dye concentration in the reaction medium was 50 ppm. After that, approximately 10 mL of each sample was taken to examine changes in dye structures and FTIR assays were directly performed with attenuated total reflection (ATR) probe.

Stock sodium azide solutions (10 mM); 0.0065 g of sodium azide was dissolved in 10 mL distilled water.

2.2.8 Statistical Analysis

All statistical analyses were performed with the program SPSS 15.0 for Windows. The all values were the mean of three separate experiments.

CHAPTER THREE
RESULTS AND DISCUSSION

Selection of appropriate organisms for SSF is one of the important factors via useful agricultural solid substrates for producing ligninolytic and hydrolytic enzymes. *Pleurotus* sp. are new model organisms for extracellular ligninolytic enzyme productions via different fermentative methods and main ligninolytic enzyme families have been reported as the enzymatic complex from *Pleurotus* species; MnP and Lac but lack LiP (Díaz-Godínez, Téllez-Téllez, Sánchez & Díaz, 2017). The fungus *P. eryngii* has been extensively investigated for Lac and MnP production, but there were little studies regarding its hydrolytic enzyme systems (Asgher, Khan & Bilal, 2016). The isolation of *P. eryngii* from the decayed woods is easily carried out (Hadibarata, Yusoff, Aris & Kristanti, 2012). When compared to other ligninolytic fungi, *P. eryngii* has great advantages in terms of their nutritional values, numerous medicinal features, potent ligninolytic and other enzyme system, the ability of degrading recalcitrant and aromatic compounds and the their roles of many biotechnological applications such as food production (edible mushroom), biotransformation of raw plant materials to feed, biopulping and biobleaching of paper pulp, as well as bioremediation of soil and industrial waters. It is another preference that the cultivation of this organism is easy (Stajić, Vukojević & Duletić-Laušević, 2009). Also, *P. eryngii* should confer advantages over other fungi for its capability to grow on nonfermented lignocellulosic wastes with its reproduction abilities (Akpinar & Ozturk Urek, 2014). In this thesis, *P. eryngii* was preferred to reveal its lignocelluloytic abilities. Different agroindustrial wastes were screened for lignocelluloytic enzyme production under SSF (Mussatto & Teixeira, 2010), however peach and cherry wastes have not been reported in production of various industrially important enzymes including lignocelluloytic enzymes by fungi as per the published literature so far. Namely, these wastes were firstly investigated for the production of ligninolytic and hydrolytic enzymes by *P.eryngii*, and also the use of them for SSF as substrates with or without any pretreatment conditions.

3.1 The Chemical Contents of Agricultural Wastes

The chemical compositions of untreated peach and cherry wastes are demonstrated in Table 3.1. According to the obtained results, all tested concentrations for cherry waste except lignin and protein concentrations were higher than that of peach waste. The lignin concentrations in both wastes were higher when compared to other lignocellulosic wastes; while the cellulose concentrations in peach and cherry wastes were lower (Mussatto & Teixeira, 2010). The lignocellulosic wastes were generally analyzed in terms of cellulose, hemicellulose and lignin, sometimes pectin and ash. In this thesis, the metal contents of lignocellulosic wastes were firstly investigated. The metal analysis of both wastes were determined by ICP-MS method in the laboratory of ARGEFAR, Ege University. According to their results, cherry wastes contained higher copper, iron and manganese in comparison to peach wastes'. Also, the iron amounts in both wastes were higher than copper and manganese amounts.

Table 3.1 Chemical composition of peach and cherry wastes

Chemical Contents (g/g %)		**Peach**	**Cherry**
Nitrogen		4.32±0.3	17.1±1.1
Protein		0.08±0.005	0.05±0.002
Cellulose		4.63±0.3	14.04±1.0
Lignin		40.56±3.1	38.8±2.6
Reducing Sugar		0.6±0.04	1.59±0.008
Carbohydrate		18.59±1.1	19±1.1
Metal Contents (mg/kg %)	**Cu**	1.734	5.917
	Fe	13.14	119.4
	Mn	1.183	4.454

The pretreatment methods could be very important for microbial growth and enzyme productions. Because, the lignocellulosic complex is made up of cellulose and lignin bound by hemicelullose chains. Lignin hinders in microbial attack, so pretreatment may be required to make surface area accessible for microbial utilization to facilitate the release of sugars from a lignocellulosic biomass prior to fermentation (Singh & Bishnoi, 2012). Pretreatment technologies are usually classified into physical, chemical, physicochemical and biological. Recently, some pretreatment

methods have been tried with ionic liquids, but they were still expensive and unpractical (Hendriks & Zeeman, 2009). The other chemical treatments such as organic solvents, steam-explosion, ammonia fiber explosion, lime were used for structural changes of lignocellulosic substrates. In this thesis, lignocellulosic substrates were pretreated with three methods, dilute acid and base solutions, hot water. These pretreatment methods commonly used for improving digestibility of lignocellulosic biomass due to economic, eco-friendly and basic methods. Also, these methods have been found to be effective on variety of lignocellulosic feedstocks. Especially, dilute acid (H_2SO_4) and base (NaOH) treatment were effective in fractionation of lignocellulosic materials from biomass and required neutralization step and high energy input (Hendriks & Zeeman, 2009). The both peach and cherry wastes were pretreated with dilute acid and base solutions, hot water. The hot water treatment was named as neutral pretreatment or wet oxidation. Then, the changes of main components of these wastes after all pretreatments were determined.

Akpinar & Ozturk Urek (2012) found that the component analysis of grape waste revealed that it contains about 40% total carbohydrate, 19.28% cellulose, 50.26% lignin, and 0.21% protein. In addition; they researched extracellular ligninolytic enzymes production by *P. eryngii* on agroindustrial wastes using pomegranate and apricot wastes. In that study, the apricot contents were obtained as 52.48% lignin and 4.47% cellulose, while the pomegranate wastes contained as 45.84% lignin and 8.67% cellulose (Akpinar & Ozturk Urek, 2014). These results were similar to the findings in this dissertation. The lignin contents of some lignocellulosic biomasses such as oil palm shells, coconut husk are higher than that of cellulose. The lignin and cellulose contents in oil palm shells are 50.7 and 20.8% of dry weight, respectively (Gaurav, Sivasankari, Kiran, Ninawe & Selvin, 2017).

Table 3.2 The changes of main components of peach wastes after all pretreatments

	Protein (g/g %)	**Cellulose (g/g %)**	**Lignin (g/g %)**	**Total Carbohydrate (g/g %)**
Hot water	0.08±0.006	4.72±0.4	38.45±3.3	23.46±1.8
Dilute acid	0.072±0.006	4.44±0.3	34.07±2.6	28.46±1.9
Dilute base	0.074±0.005	4.12±0.3	31.23±2.2	36.06±2.8

Peach wastes pretreated with hot water contained as 4.72±0.4 and 38.45±3.3% (w/w) cellulose and lignin, respectively (Table 3.2). The increase in cellulose content was not much higher in comparison with untreated waste's value. However, the cellulose concentrations in wastes treated by dilute acid and base were reduced. There were insignificant changes lignin concentrations between the hot water treated and untreated wastes, while the reductions of lignin concentrations were very high in other treated biomass ($p>0.05$). In addition, total carbohydrate concentrations of all pretreated wastes were higher than that of untreated biomass. When these wastes treated with base solution, the total carbohydrate concentrations were reached to maxima (36.06±2.8% (w/w)) among the others. Also, there were insignificant differences in protein concentrations at all treated and untreated conditions ($p>0.05$).

Table 3.3 The changes of main components of cherry wastes after all pretreatments

	Protein (g/g %)	**Cellulose (g/g %)**	**Lignin (g/g %)**	**Total Carbohydrate (g/g %)**
Hot water	0.05±0.004	14.46±1.1	37.24±3.4	24.12±1.7
Dilute acid	0.044±0.003	13.34±0.9	33.59±2.7	29.74±1.8
Dilute base	0.047±0.002	12.56±0.9	30.65±2.4	36.86±2.7

When the lignocellulosic biomasses were treated different physicochemical techniques, many changes in chemical composition of cherry wastes were observed. There were insignificant differences in protein concentrations under all treated and untreated conditions. Cherry wastes pretreated with hot water contained as 14.46±1.1 and 37.24±3.4% (w/w) cellulose and lignin, respectively (Table 3.3). Namely, there were insignificant changes in cellulose and lignin concentrations between the hot water treated and untreated wastes ($p>0.05$). The cellulose and lignin concentrations in these wastes treated with dilute acid and base solutions were reduced, especially the reduction of lignin concentration (base treated biomass) was very high. On the other hand, total carbohydrate concentrations of all pretreated wastes were higher than that of untreated biomass. When the wastes treated with base solution, the total carbohydrate concentrations were reached to maxima (36.86±2.7% (w/w)) among the others. The main advantage of acid and base pretreatments is the reduction of lignin contents, but some inhibitory materials could be generated during the processes (S.

Sun, S. Sun, Caoa & R. Sun, 2016). The increments in total carbohydrate concentrations of peach and cherry wastes may be due to solubilization of lignin in all liquids during pretreatment. In other words, there was a synergism between lignin and total carbohydrate concentrations in both pretreated wastes. Moreover, large amounts of total carbohydrates make it a potential and renewable raw material for enzyme and bioethanol production. Singh & Bishnoi (2012) treated lignocellulosic wastes, wheat straw, which was commonly used for producing ligninolytic and hydrolytic enzymes with alkali (NaOH) concentrations (2.75% (w/v)) and composition of wheat straw in lignin amounts were decreased after pretreatment. This finding showed similar trends with our results. After process, the cellulose concentration in it was also increased.

After pretreatment, the lignocellulosic biomasses were separated by filtration, and then they were ready for the utilization as substrates in SSF. The obtained filtrates following biomasses washing in all pretreatments conditions were not chemically analyzed and were threw away, but they could primarily composed of soluble and non-soluble cellulose, lignin and their derivatives such as hexoses, pentoses, phenolic and non-phenolic compounds, which stimulate/suppress the ligninolytic and hydrolytic enzyme activities, proteins, carbohydrates, etc.

The peach and cherry wastes had high lignin content, which envisages the application of effective delignification methods for biotechnological process. Thus, the production of ligninolytic and hydrolytic enzymes must be optimized for high efficiency degradation of especially lignin and other polysaccharides.

3.2 The Optimization of Ligninolytic and Hydrolytic Enzyme Activities of Peach and Cherry Cultures by *P.eryngii*

3.2.1 Effect of Different Pretreatment Methods on Ligninolytic and Hydrolytic Enzymes Activities

3.2.1.1 Effect of Different Pretreatment Methods on Ligninolytic Enzymes Activities

The maximum Lac activity in control conditions of peach cultures were attained, and also the activities of Lac were decreased slightly under all pretreatment conditions similar to obtained results in other cultures using as substrates, treated or untreated cherry wastes. Besides, the highest Lac activity in peach cultures was determined as 2193.06±50.4 U/L (17^{th} day) (Figure 3.1 (a)). When the lignocellulosic biomass was treated, the Lac activity decreased about 9.03-fold under other conditions. As depicted in Figure 3.1 (a) and (b), the maximal Lac activity was obtained under control condition followed by base, hot water and acid pretreatments of peach cultures in similar to MnP activities in all conditions. The maximum MnP activity was obtained under control condition as 732.73±19.8 U/L. Concurrently, the maximum activity in this culture was detected on 17^{th} day as similar to activity of Lac. Also, these enzyme activities were reduced much in peach waste treated by dilute acid. In this study, insignificant levels of LiP activities were detected in all untreated and pretreated of both cultures with similar trends ($p>0.05$). The highest value was detected as 94.09±4.1 U/L under control conditions, and following 43.01±2.6 U/L on the 7^{th} day of incubation with pretreated peach cultures with dilute base (Figure 3.1 (c)). The decline in LiP activities was observed after pretreatment of peach cultures. The interesting result was that the LiP activity was obtained on SSF conditions by *P. eryngii*, because *Pleurotus* sp. belong to a subclass of lignin degrading microorganisms that produce Lacs and MnPs but no LiPs (Palmieri et al., 1997).

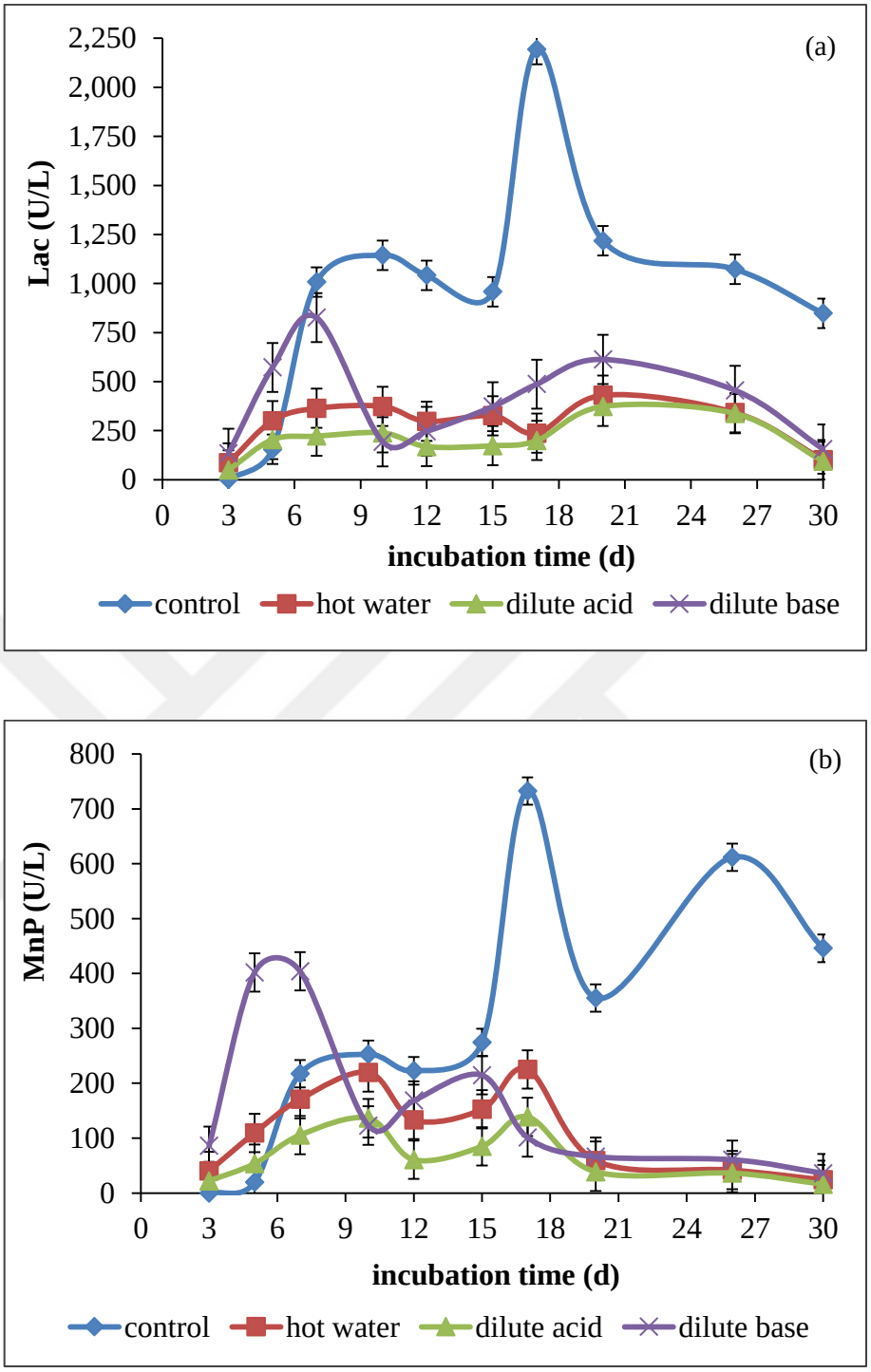


Figure 3.1 Variations of ligninolytic enzymes activities (Lac (a), MnP (b) and LiP (c)) on SSF cultures using peach wastes with and without pretreatment. The values are the mean ±SD for experiments of three separate experiments

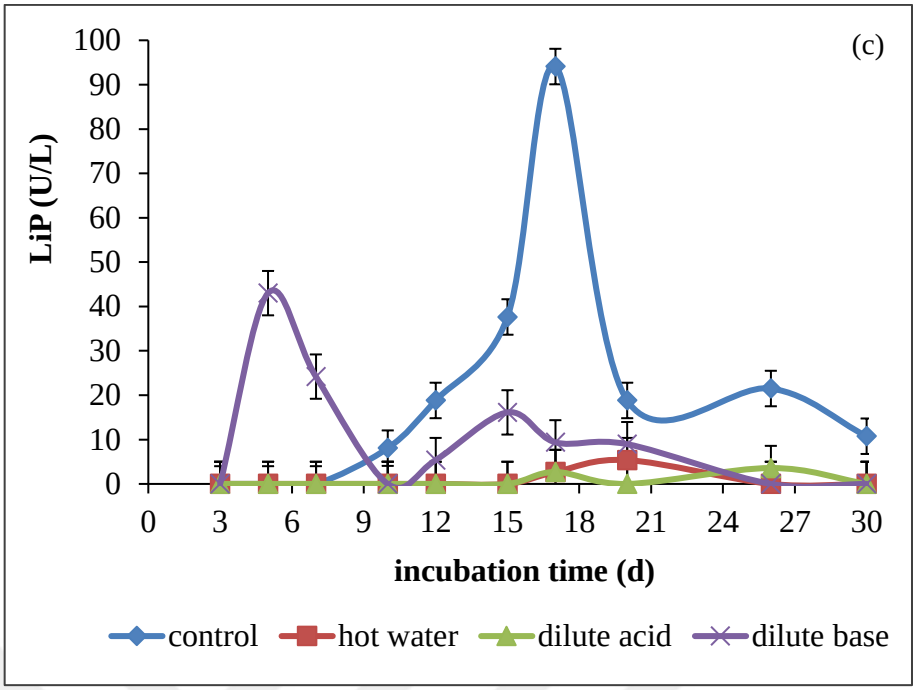


Figure 3.1 Variations of ligninolytic enzymes activities (Lac (a), MnP (b) and LiP (c)) on SSF cultures using peach wastes with and without pretreatment. The values are the mean ±SD for experiments of three separate experiments (continued)

Lignocellulosic materials are recalcitrant to fungal hydrolysis owing to their composite structure (Limayem & Ricke, 2012). Effective pretreatments should improve the ligninolytic and hydrolytic enzyme production, but selection of these methods is great importance for economic SSF process. According to the literature, there is no study about lignocelluloytic enzyme production by *P.eryngii* using peach and cherry wastes with and/or without any pretreatment conditions. The maximum Lac activity in control condition was attained, and also the activities were decreased slightly under all pretreatment conditions. Besides, the maximum Lac activities in cherry cultures were determined as 1297.22±34.6 U/L (10^{th} day) (Figure 3.2 (a)). There were insignificant differences among Lac activities in control conditions of cherry cultures on 7^{th}, 10^{th} and 12^{th} day of incubation ($p>0.05$). Also, the Lac activities in hot water and dilute acid were very near to close to each other between the 7^{th} and 17^{th} incubation days. It was shown that the maximum Lac activity by *P. ostreatus* was attained as 746.1 U/L on the 10 day of incubation using grapevine sawdust as a substrate (Stajić, Vukojević, Knežević & Milovanović, 2013). When compared to other research, the four WRF screened for Lac production during 46 days and only

Euc-1 produced this enzyme (the highest activity of 100 U/L; at the end of the incubation period) using wheat straw (Dias et al., 2010). Similar to Lac activities, the MnP activities were also affected negatively after the pretreatment of cherry waste. The highest MnP activity was obtained under control condition followed by base, hot water and acid pretreatments of cherry cultures. Concurrently, the highest activity in this culture was detected on 26th day as 430.91±8.6 U/L and there was a small extra peak under control conditions (Figure 3.2 (b)). Also, the MnP activities were approximately same in acid and neutral pretreatments of cherry cultures, while their levels were lower in activity of base pretreatment. Stajić et al. (2013) showed that the maximum MnP activity by *P. ostreatus* was attained as 10.3 U/L (10th day) using grapevine sawdust as a substrate and the highest activity obtained was 41.84-fold higher than that of their results, while Isikhuemhen et al. (2012) researched the ligninolytic and hydrolytic enzymes activities from corn stalks under SSF by white rot fungus, *Lentinus squarrosulus* Mnt., during 30 days of incubation and they determined the highest MnP activity as 13 U/L on the 6th day.

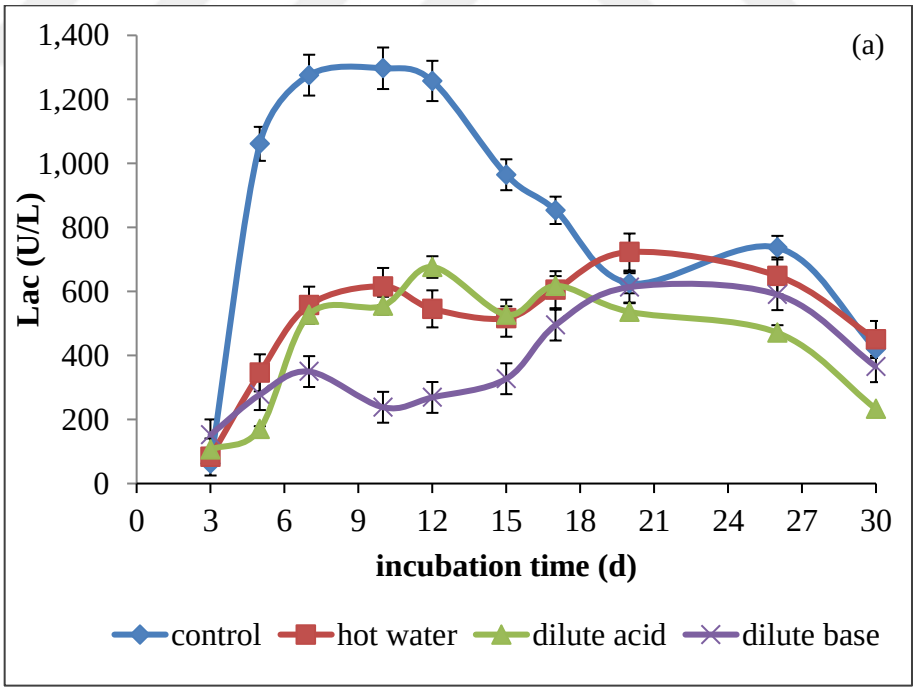
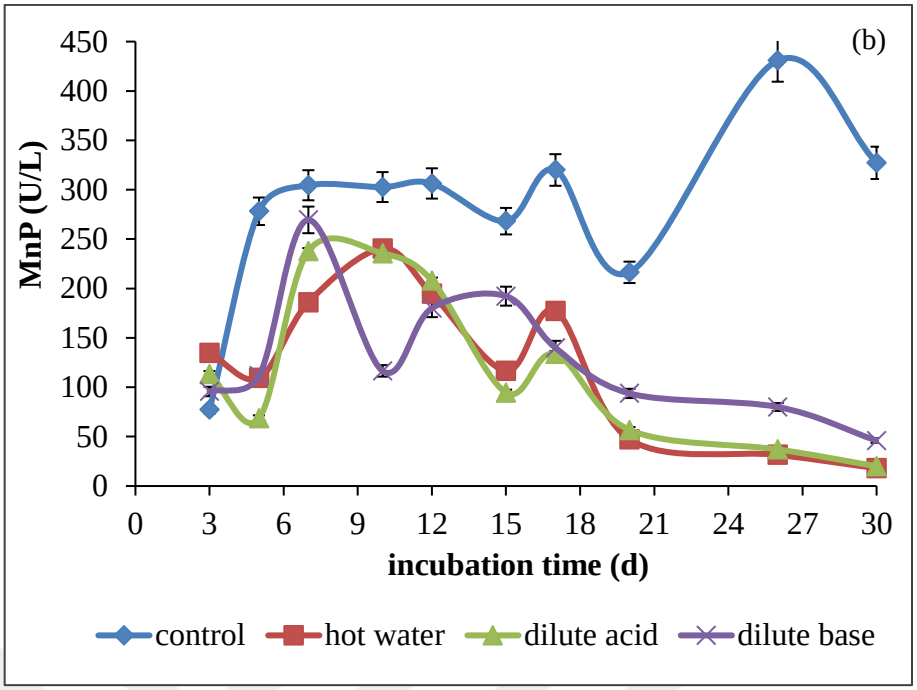


Figure 3.2 Variations of ligninolytic enzymes activities (Lac (a), MnP (b) and LiP (c)) on SSF cultures using cherry wastes with and without pretreatment. The values are the mean ±SD for experiments of three separate experiments



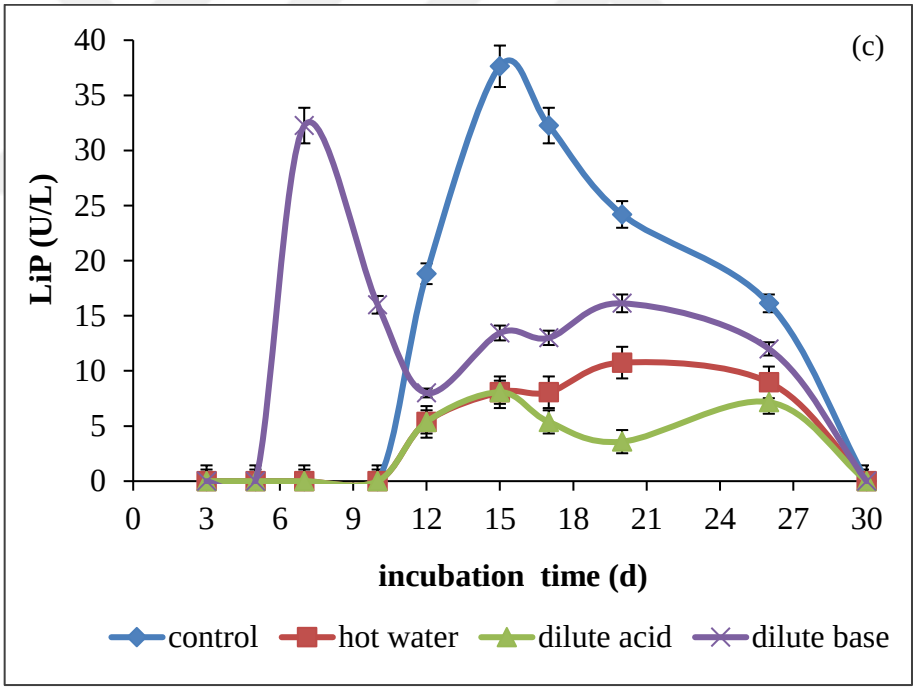


Figure 3.2 Variations of ligninolytic enzymes activities (Lac (a), MnP (b) and LiP (c)) on SSF cultures using cherry wastes with and without pretreatment. The values are the mean ±SD for experiments of three separate experiments (continued)

Generally, there were little LiP activities in *Pleurotus* sp. (Díaz-Godínez et al., 2017). In this study, insignificant levels of LiP activities were detected in all untreated

and pretreated of cherry cultures with similar trends ($p>0.05$). The highest value was detected as 37.63±1.1 U/L under control conditions as shown in Figure 3.2 (c). The decline in LiP activities was observed after pretreatment of cherry cultures. In addition, *I. lacteus* and Euc-1 strains showed their maximum LiP activities as 60 and 80 U/L after 23 and 35 days of incubation during SSF, these results were higher than our highest LiP activity value (Dias et al., 2010). According to literature, most WRF secrete at least two ligninolytic enzymes, whereas *P.eryngii* secretes three of them during SSF conditions in this study.

3.2.1.2 Effect of Different Pretreatment Methods on Hydrolytic Enzymes Activities

The activities of CMCase and xylanase showed similar trends under control and hot water-treatment condition of peach cultures. The both hydrolytic enzyme activities under control conditions of peach cultures were increased up to 5th day of incubation, and then decreased. The highest levels of these enzymes were determined as 3.64±0.02 U/mL and 3.08±0.02 U/mL, respectively (Figures 3.3 (a) and (b)). The pretreatment methods on these enzymes productions showed negative effect. Moreover, Saritha, Arora & Nain (2012) illustrated that *Trametes hirsuta* used paddy straw as a carbon source and produced the highest CMCase activity 0.71 U/mL on the 15th day of incubation and this value was 5.13-fold lower than that of our result in obtained peach culture.

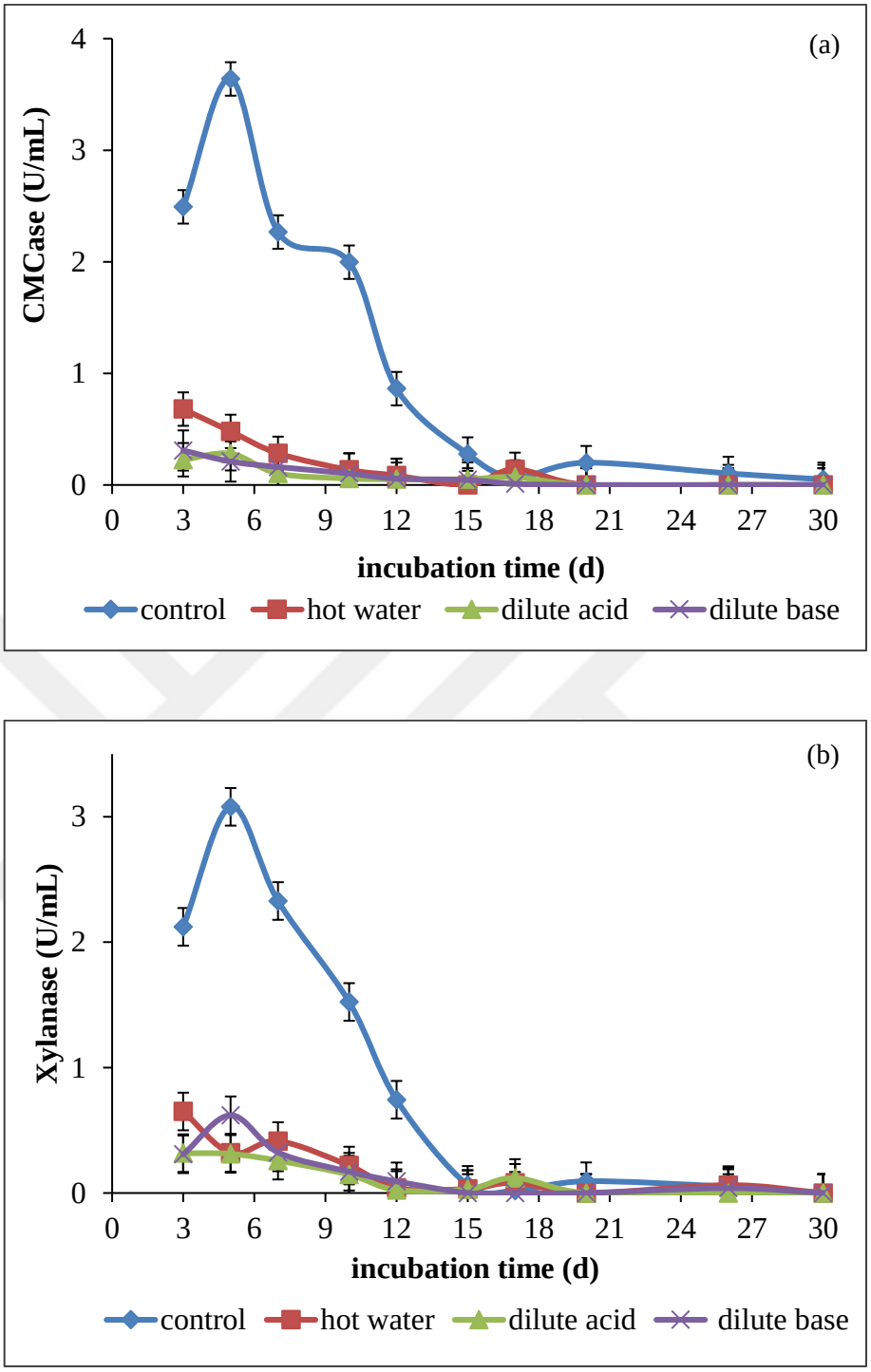
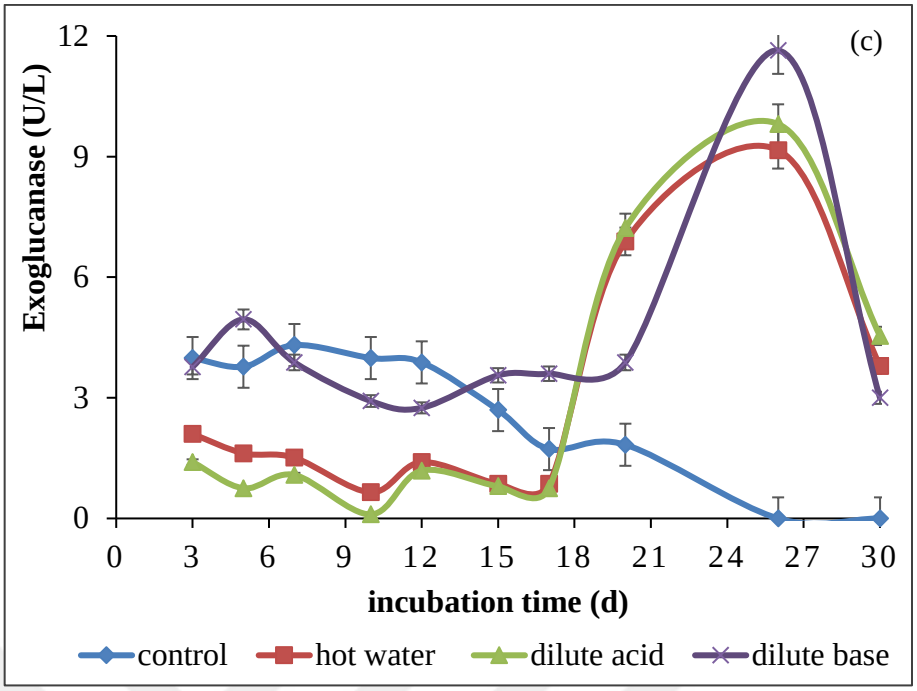


Figure 3.3 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes with and without pretreatment. The values are the mean ±SD for experiments of three separate experiments



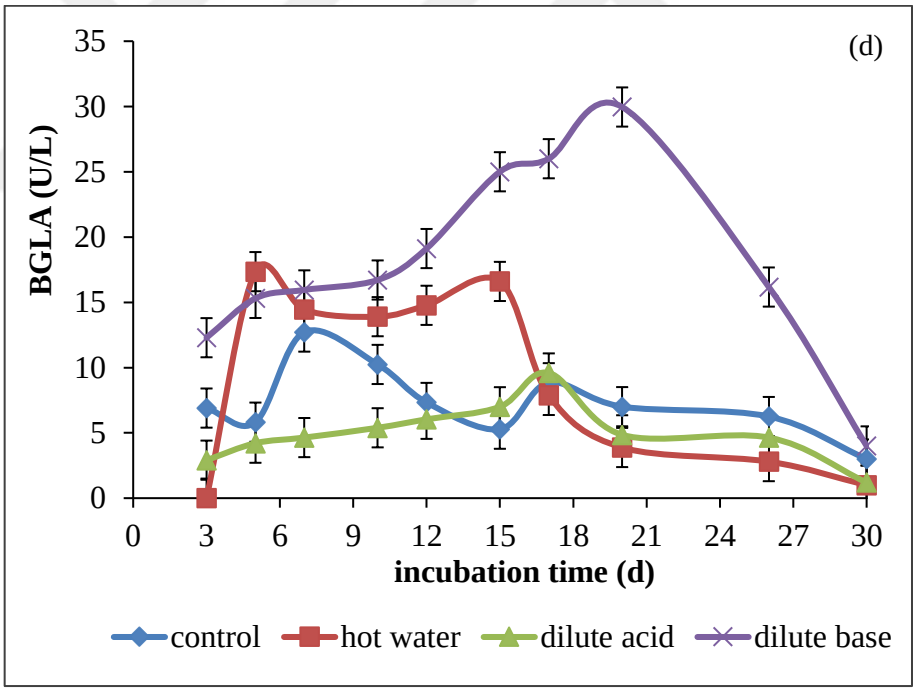


Figure 3.3 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes with and without pretreatment. The values are the mean ±SD for experiments of three separate experiments (continued)

Also, the very low exoglucanase activities were detected in all conditions of peach cultures. The highest exoglucanase activity was detected as 11.64±0.2 U/L (26th day)

in peach cultures using substrate pretreated with dilute base (Figure 3.3 (c)), while its maximum activity under control conditions was 4.31±0.2 U/L. There were a few studies about exoglucanase and BGLA production by *Pleurotus* spp. (Téllez-Téllez, Díaz, Sánchez & Díaz-Godínez, 2013). The base and hot water pretreatment methods demonstrated positive effect on BGLA enzyme production on peach cultures. The BGLA activities were increased up to 20th day of incubation, then decreased on peach cultures using base treated substrates. In peach cultures, the maximum BGLA activity was attained in base-treated cultures followed hot water, control and acid conditions. As can be seen in Figure 3.3 (d), the maximum activity was found as 29.96±0.9 U/L, which was 2.35-fold higher than that of control ($p<0.05$). Similar to these activity values in the thesis, BGLA activities of *Pleurotus* spp. were extremely low (Naraian, Singh, Verma & Garg, 2010). According to literature, fungi generally have not produced all of these hydrolytic enzymes. Thus, the consortium cultures have stood out in fungal hydrolytic system and *Pleurotus* spp. secrete dominantly CMCase of them in peach cultures of SSF (Chi, Hatakka & Maijala, 2007).

The maximum values of CMCase and xylanase enzymes were detected as 1.30±0.01 and 1.16±0.01 U/mL on the 3rd day of incubation under control condition of cherry cultures (Figures 3.4 (a) and (b)). The decrement in both enzymes was observed after all treatments in cherry cultures. That is, the CMCases were produced simultaneously with xylanases by the *P.eryngii* during the cultivation period. Dias et al. (2010) have noted that during the SSF period the very low CMCase activities were detected in both *Irpex lacteus* and Euc-1 strains compared to our maximum CMCase activities of cherry cultures by *P.eryngii*. In that study, *I. lacteus* and Euc-1 showed a peak of xylanase activity (0.08 U/mL) after 10 days of incubation. On the other hand, Naraian et al. (2010) subtracted ligninolytic and hydrolytic enzyme profiles of *Pleurotus* spp.; *Pleurotus florida*, *Pleurotus sajor-caju* and *P.eryngii*. The CMCase and xylanase activities separately ranged from 198 to 317 U/L and from 178 to 269 U/L. Moreover, Saritha et al. (2012) illustrated that *T. hirsuta* used paddy straw as a carbon source and produced the highest CMCase activity 0.71 U/mL on the 15th day of incubation, and this enzyme activity was reached to maximum in this study earlier.

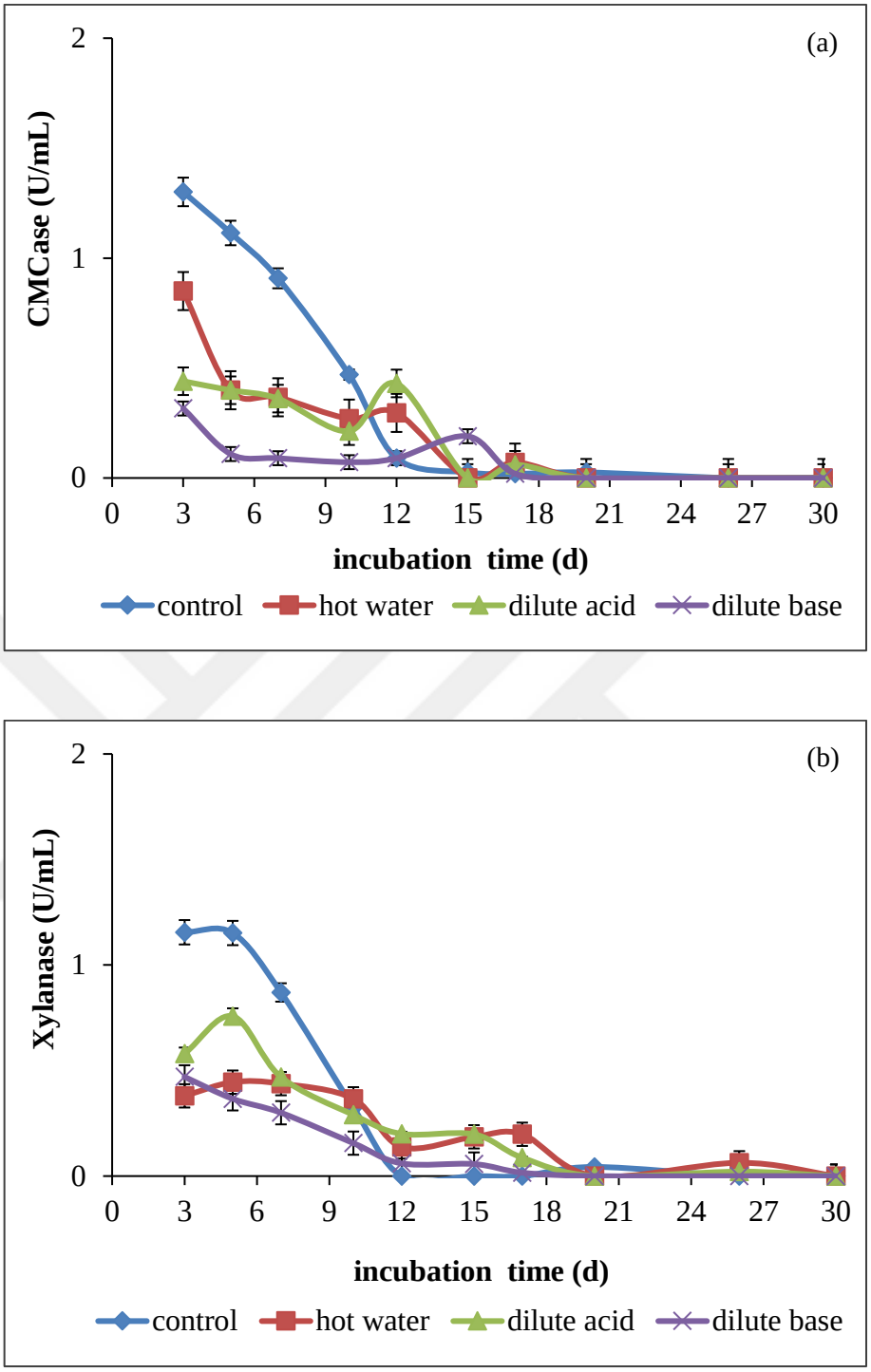
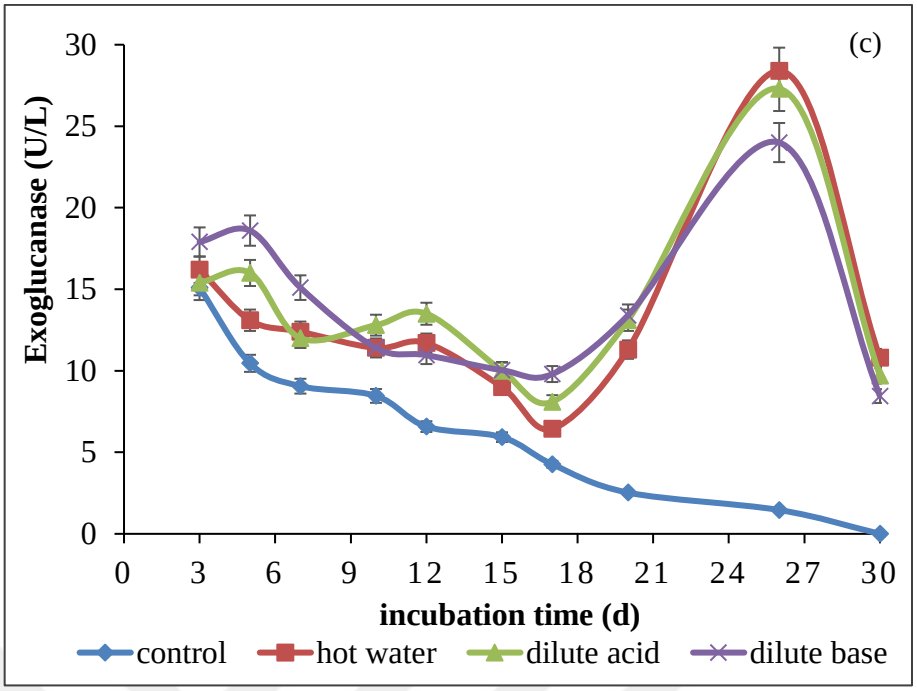


Figure 3.4 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes with and without pretreatment. The values are the mean ±SD for experiments of three separate experiments



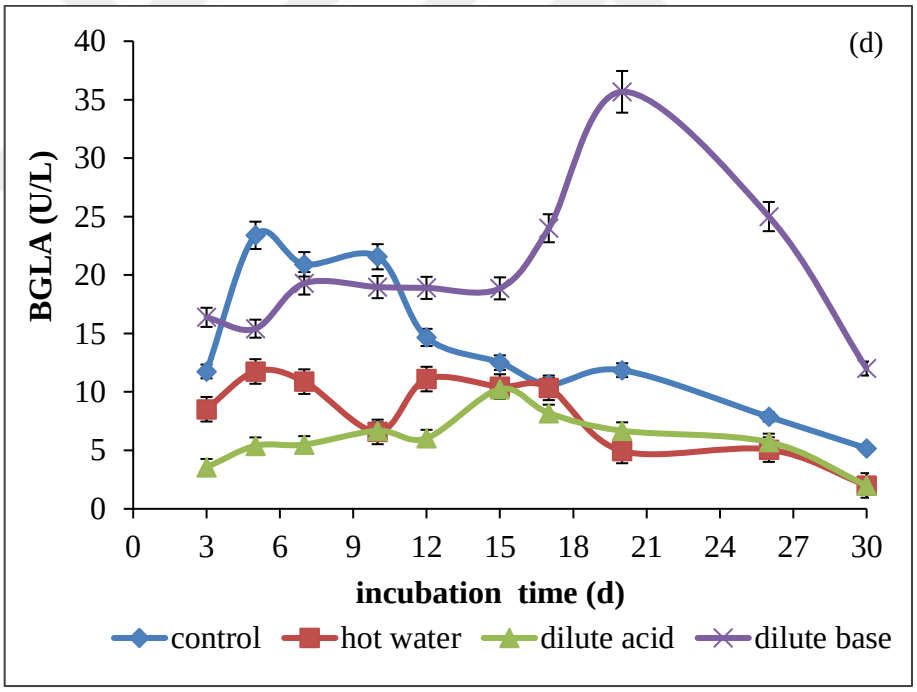


Figure 3.4 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes with and without pretreatment. The values are the mean ±SD for experiments of three separate experiments (continued)

The very low exoglucanase activities were detected in all conditions of cherry cultures. The exoglucanase activities in *P.eryngii* enzyme supernatant were changed

up to 28.4±0.5 U/L for cherry wastes with and without pretreatment conditions during 30 days of cultivation (Figure 3.4 (c)). The highest exoglucanase activity was detected as 28.4±0.5 U/L (26^{th} day) in hot water pretreatment of cherry cultures. The BGLA activities were increased up to 20^{th} day of incubation, then decreased on cherry cultures (Figure 3.4 (d)). In cherry cultures, the maximum BGLA activity was attained in base-treated cultures followed control, hot water and dilute acid conditions. The highest BGLA activity was obtained as 35.67±1.1 U/L in base-treated of cherry cultures, which was 1.52-fold higher than that of control ($p<0.05$). In addition, when the cherry wastes were pretreated with dilute acid solution and hot water, this enzyme activity decreased approximately 50% ($p<0.05$) (Figure 3.4 (c)). There were a few studies about exoglucanase and BGLA production by *Pleurotus* spp. (Téllez-Téllez et al., 2013).

Generally, the physicochemical pretreatment methods are able to remove lignin from the lignocellulosic biomass, thereby increasing cellulose content and making it more susceptible to enzymatic hydrolysis (Janu et al., 2011). This theory is only compatible with the results obtained after hot water treatment of peach and cherry wastes. The lignin and cellulose contents of these wastes decreased after dilute and base pretreatments due to their dissolution properties, and thus the total carbohydrate concentrations were increased. All pretreated and untreated lignocellulosic wastes were further used as substrates for ligninolytic and hydrolytic enzyme productions by *P. eryngii* via SSF. According to results, these pretreatment methods usually were unsufficient for enzyme productions using both wastes. The ligninolytic enzymes from both cultures were negatively affected, the maximal enzyme productions were obtained using untreated lignocellulosic wastes. Among the hydrolytic enzymes, CMCase and xylanase activities were significantly reduced when compared to controls' values. The exoglucanase and BGLA activities were very low in all conditions. The decreases in ligninolytic, CMC and xylanase activities could be depend on the formation of furfural and hydroxymethylfurfural after pretreatments in addition to microbial growth. These are main inhibitor for fermentation processes and mainly generated as a result of dehydration of pentose and hexose sugars from cellulose and hemicellulose (Ye et al., 2016). This can be related to the increase in

total carbohydrate concentration after pretreatments. There was no any comparison with ligninolytic and hydrolytic enzymes activities using pretreatmented lignocellulosic wastes in here. Because, studies in the literature examined the changes in the lignocellulosic structure of the pretreatment and its effect on the final product. There is very little study of the synergy between the enzyme production after the treatment and the final product. In these studies, the pretreatment process generally induced enzyme production in contrast to results in the thesis. Therefore, the further studies were carried out untreated peach and cherry wastes for ligninolytic and hydrolytic enzymes production by *P. eryngii*. On the other hand, the CMCase and xylanase enzymes were secreted at the initial days of incubation, then ligninolytics secretion started. Thus, the degradation of lignocellulose structure firstly were performed by CMCase and xylanase in contrast to literature (Ashger et al., 2016).

3.2.2 Effect of Different Copper Concentrations on Ligninolytic and Hydrolytic Enzymes Activities

3.2.2.1 Effect of Different Copper Concentrations on Ligninolytic Enzymes Activities

One of the important factors affecting biodegradation processes is the presence of metals in fungal environment. Heavy metals can be toxic for WRF and affect their growth and the activity of extracellular enzymes. In comparison with heavy metals, little is known what the function of essential metals, such as copper, iron and manganese in lignocellulose degradation (Baldrian, 2003). Cu^{2+} is an important nutritional factor for several extracellular and intracellular enzyme synthesis and activities by microorganisms. Also, copper atoms served as cofactors in the catalytic core of Lac; thus, a minimum concentration (mM range) of Cu^{2+} was necessary for production of the active enzyme (Bertrand, Martínez-Morales & Trejo-Hernandez, 2013; Patel, A. Gupte & S. Gupte, 2009). In this thesis, the effects of Cu^{2+} concentration on ligninolytic and hydrolytic enzyme activities were investigated in two stages. Firstly, the basal medium was enriched by supplementation of Cu^{2+} up to 2.0 mM. After the enzymes activity measurements, new concentrations of Cu^{2+} on these

enzyme activities were also researched. Thus, seven Cu^{2+} concentrations were designated for SSF during the stationary cultivation and time-dependent.

Figure 3.5 (a) illustrates the role of increasing Cu^{2+} concentrations in Lac production by *P. eryngii*. The Lac production increased as the concentration of Cu^{2+} increased up to 70 µM, then decreased. The addition of Cu^{2+} to the culture medium above 70 µM resulted in maximum Lac activities inferior to the obtaining activities in the presence of 70 µM. On the other hand, the incubation time obtaining maximum Lac activities was observed in the 15^{th}, 17^{th}, 20^{th} day of incubation in the presence of 35, 70, 100 µM Cu^{2+}, respectively, while the Lac activities reached to maximum at the same day of incubation (10^{th} day) with supplemented 500 and 1000 µM Cu^{2+}. The highest Lac activity was determined as 2193.06±50.4 U/L in the presence of 70 µM Cu^{2+}, whereas the maximum Lac activity was measured as 903.47±24.8 U/L on the 12^{th} day in the absence of Cu^{2+} and it was lowest when compared to those maxima in other supplemented conditions. The Lac secretion by *P. eryngii* was later when Cu^{2+} was added to basal medium up to 100 µM. According to our results, conditions promoting Lac expression by *P. eryngii* appeared to be different to those reported by many other fungi. Especially, higher Lac activity was indicated with increasing Cu^{2+} concentrations in the medium to other reports (Patel et al. 2009; Piscitelli et al., 2011), whereas our findings in this research demonstrated that the rising Cu^{2+} concentrations did not positively affect Lac activity. Cu^{2+} concentrations above 70 µM could showed the toxic effect on Lac productions, and thus reduce the activity. Xin & Geng (2011) found that the supplementation of Cu^{2+} (1 mM) to the defined basal medium induced Lac production by *T. versicolor* under SSF conditions. Different studies have shown that Lac activity is regulated by Cu^{2+} through gene expression induction/translational or post-translational regulation (Fonseca, Shimizu, Zapata & Villalba 2010). On the other hand, our results were supported by Elsayed et al. (2012). They reported that among the tested Cu^{2+} concentrations (50, 100, 250, 500, 750, 1000, 1250, and 1500 µM), 100 µM supported the maximum Lac production by *P. ostreatus* ARC280 compared to the original medium containing 50 µM Cu^{2+}, and significant decrease in Lac activity was observed when the concentration of Cu^{2+} was increased beyond 100 µM.

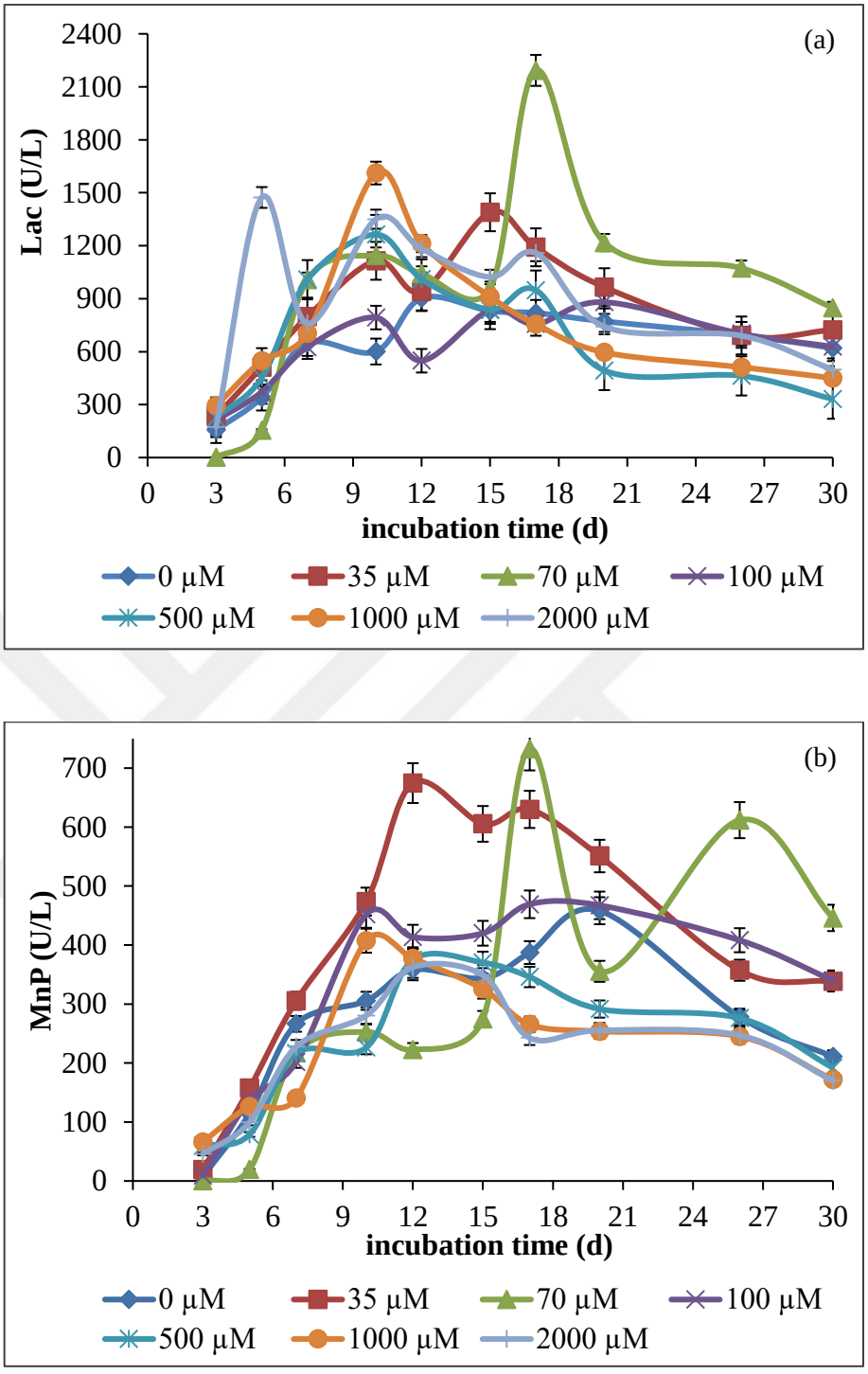


Figure 3.5 The influence of copper concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments

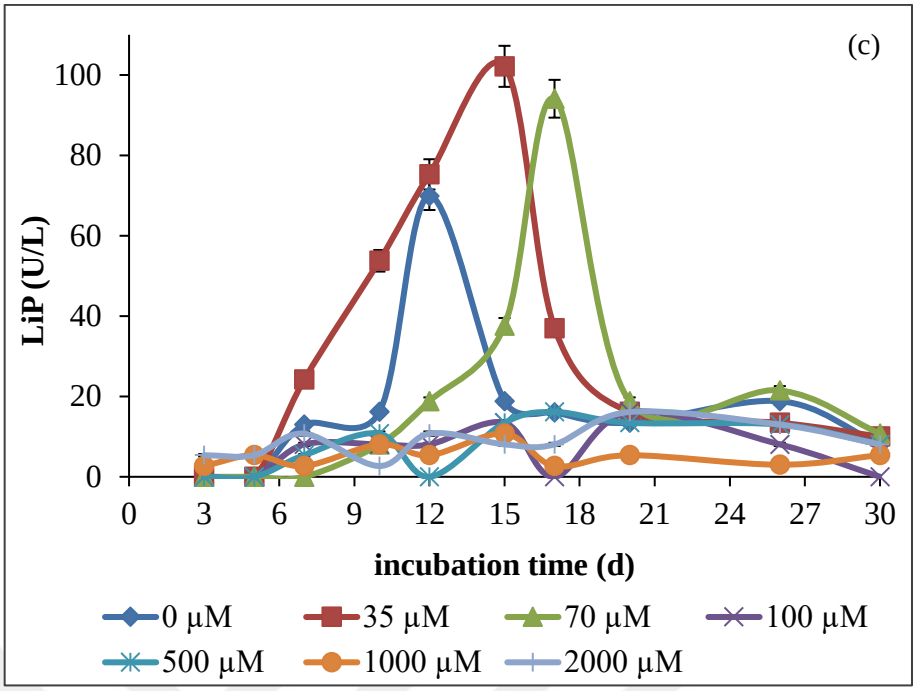


Figure 3.5 The influence of copper concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

Similar to Lac activities, different Cu^{2+} concentrations above 70 µM when added to basal solution suppressed the MnP production of *P. eryngii*. 70 µM copper supported MnP production with high activity (732.23±19.8 U/L, 17th day) as can be seen in Figure 3.5 (b). Also, its maximum activity in the absence of Cu^{2+} was determined as 458.18±3.8 U/L. In higher concentrations, the production of MnP was earlier when compared to the attaining it in the medium containing 70 µM Cu^{2+}. The maximal MnP activity in 2000 µM was recorded as 362.42±2.6 U/L, and this value was 49% lower than that of it (732.23±19.8 U/L). Otherwise, low LiP activities in Cu^{2+} concentrations above 70 µM were obtained. This enzyme activity reached to its maxima in the presence of 35 µM as 102.15±1.8 U/L on the 15th day (Figure 3.5 (c)), and there was unimportant difference between this value and the obtained activity in the presence of 70 µM Cu^{2+}. In the absence of Cu^{2+}, the maximal LiP activity was determined as 69.89±0.5 U/L on the 12th day. The increments in Cu^{2+} concentrations up to 70 µM affected LiP activities positively, while the supplementations of Cu^{2+} changed the LiP activities negatively in other conditions.

Some culture conditions such as presence/absence of micronutrients are believed to have a profound effect on ligninolytic and hydrolytic enzyme activities production by fungi. In the present study, different Cu^{2+} concentrations were added to basal media of cherry cultures at the beginning of incubation. In the absence of Cu^{2+}, the maximal Lac, MnP and LiP activities were observed as 1209.72±25.2, 428.12±7.8 and 34.95±0.08 U/L, respectively, on the day of 10 (Figure 3.6). There were unsignificant differences in Lac, MnP and LiP activities in the presence of 0.07 mM Cu^{2+} when compared to the activities in the absence of copper ($p>0.05$), but the secretions of MnP and LiP by *P. eryngii* were late (Figure 3.6). The Lac activities in 0.07 mM Cu^{2+} were nearly same on the 7^{th}, 10^{th}, 12^{th} days. Also, the highest Lac activity was detected as 3403±76.4 U/L on the 15^{th} day with the 1.0 mM copper. The increments of Cu^{2+} concentrations up to 1.0 mM affected Lac activities positively, while Lac activities were decreased in higher concentrations than 1.0 mM. In all Cu^{2+} concentrations, there were peaks in these enzyme activities on the 10 day of incubation. On the other hand, the maximal MnP activities increased in the increments of Cu^{2+} up to 2.0 mM, these activities decreased in other concentrations as can be seen in Figure 3.6 (b). Moreover, the MnP activities in 1.0 and 2.0 mM Cu^{2+} were approximately same, but this activity in 1.0 mM was obtained earlier. The highest MnP activity was recorded as 821.36±10.8 U/L on the 5^{th} day (in 1.0 mM Cu^{2+}) and this value was 1.91-fold higher than that of it in 0.07 mM Cu^{2+}. There were insignificant alterations among LiP activities in the supplementation of Cu^{2+} up to 0.5 mM. LiP activities in all conditions ranged from 0 to 91.39±0.08 U/L during incubation, and all of them were too low (Figure 3.6 (c)). The highest LiP activity was attained in 5.0 mM Cu^{2+} and 2.43-fold higher than the value in 0.07 mM Cu^{2+}. According to results, Cu^{2+} concentration of 1.0 mM is optimal for ligninolytic enzyme production via SSF process of cherry cultures. The effect of Cu^{2+} on laccase activity was studied in several reports for many of fungi. The availability of Cu^{2+} in the medium might allow the synthesis of the enzyme, but Cu^{2+} present in high concentration was toxic to microbial cells (El-Batal, ElKenawy, Yassin & Amin, 2015). Cu^{2+} has also an effect at the transcriptional level. This metal regulates mRNA levels of laccase-encoding genes in different fungi, for example *T. versicolor*, *Trametes pubescens* and *P. ostreatus*. To date, it is not known with certainty how copper activates the transcription of laccase genes. The laccase activity in fungal

cultures, however, does not necessarily correspond to the level of gene expression (Vrsanska et al., 2016). Also, the reason for the induction of ligninolytic enzymes by Cu^{2+} additions can be explained by the reduction of extracellular activity proteases which might degrade Lac, MnP and LiP.

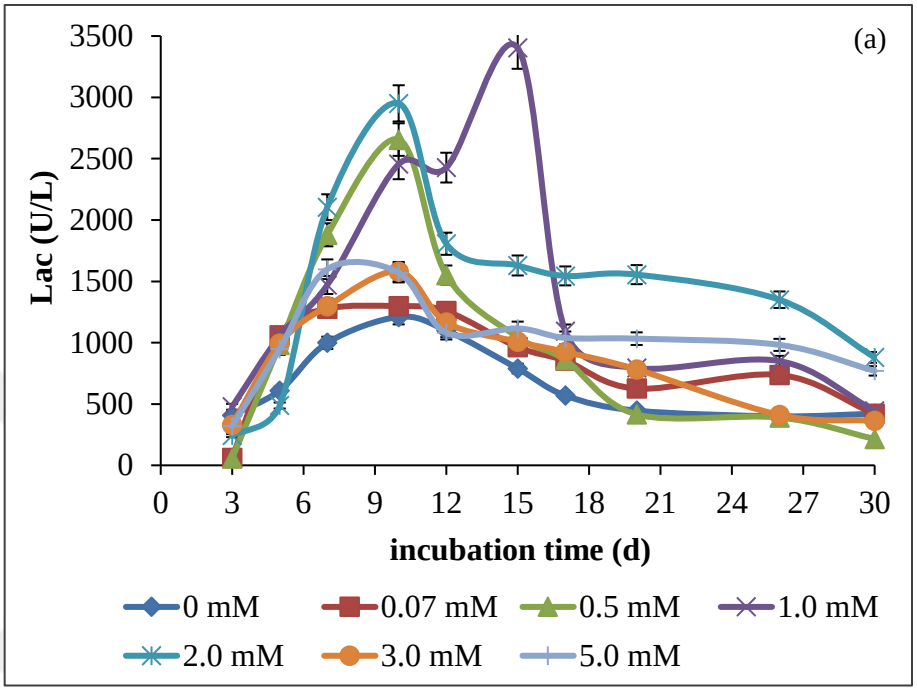
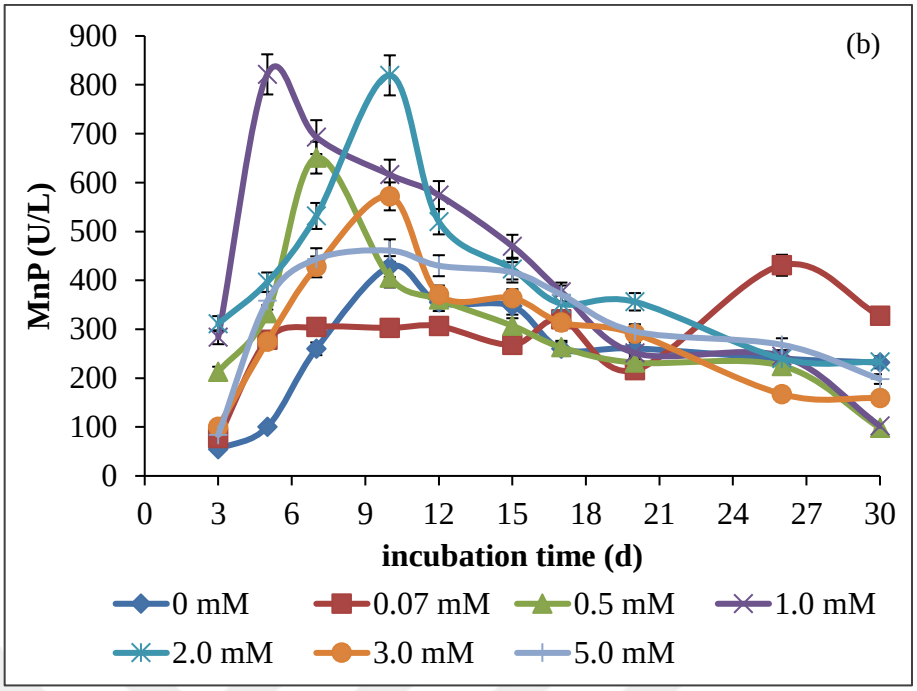


Figure 3.6 The influence of copper concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments



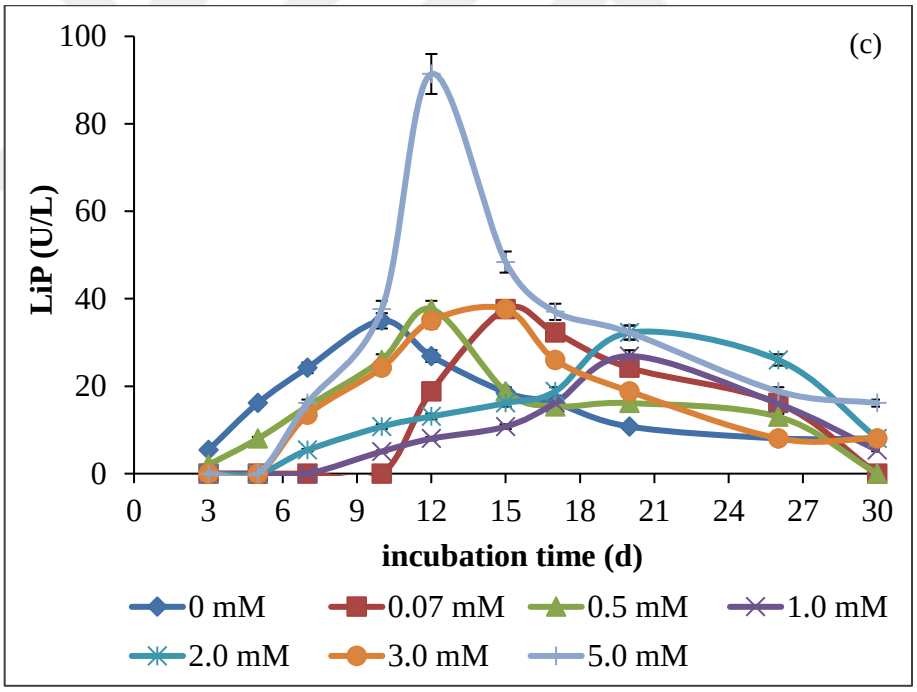


Figure 3.6 The influence of copper concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

3.2.2.2 Effect of Different Copper Concentrations on Hydrolytic Enzymes Activities

The extracellular hydrolytic enzyme activities of *P. eryngii* were tested under varying Cu^{2+} concentrations. Both CMCase and xylanase activities in all conditions except including 2000 μM Cu^{2+} were reached to their maxima on the 5 day of incubation, and their maximum activities in 2000 μM were recorded on 3rd day (Figures 3.7 (a) and (b)). Xylanases were induced in peach cultures with Cu^{2+} concentrations above 70 μM, reaching the highest activity as 4.63±0.02 U/mL in the presence of 2000 μM Cu^{2+}. This activity value was 1.50-fold higher than that of the value in the presence of 70 μM Cu^{2+} (3.08±0.02 U/L) ($p<0.05$). The CMCase and xylanase activities in Cu^{2+} concentrations below 70 μM were lower in comparison with their activities in the presence of 70 μM Cu^{2+}. Also, the highest CMCase activity was detected as 3.64±0.02 U/mL in the presence of 70 μM Cu^{2+} (Figure 3.7 (a)). The maximal reductions of both enzyme activities were observed in the presence of 35 μM Cu^{2+}. Interestingly, their activities in the absence of Cu^{2+} were higher than the already mentioned values. Also, the exoglucanase and BGLA activities were very low in all conditions. The highest exoglucanase and BGLA activities were recorded as 4.85±0.09 and 33.41±1.1 U/L in Cu^{2+} of 500 and 2000 μM (Figures 3.7 (c) and (d)).

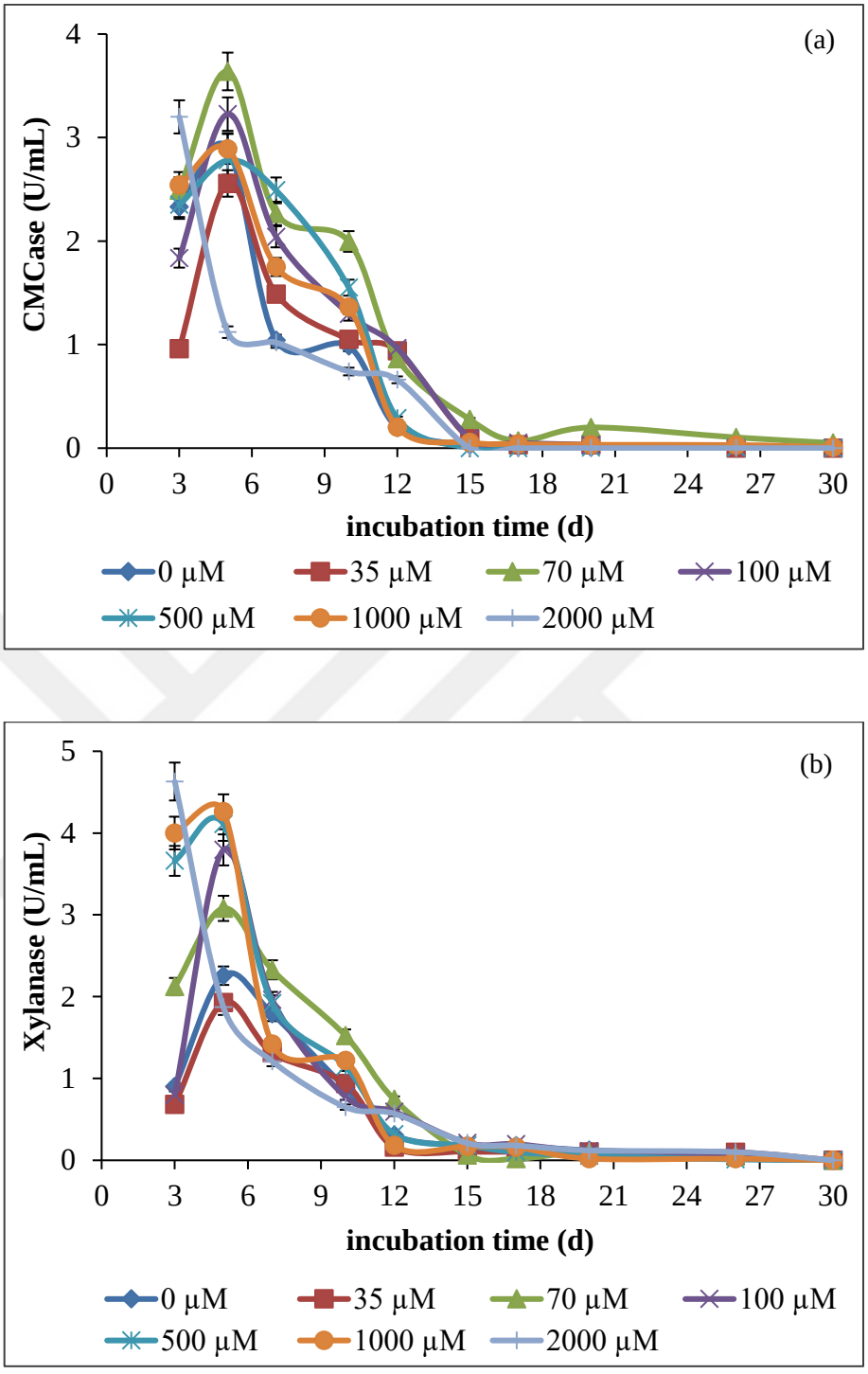
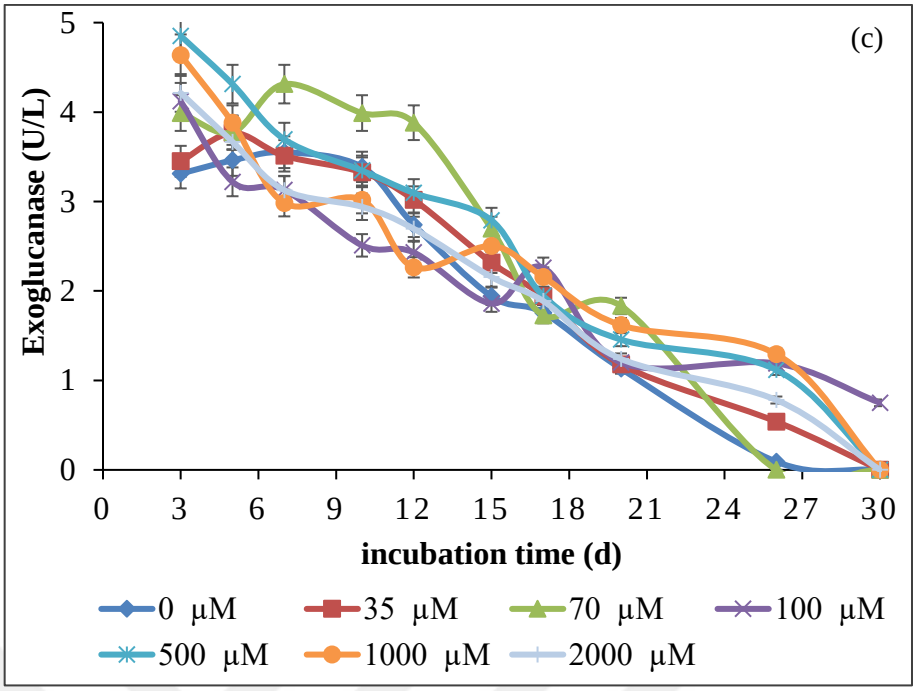


Figure 3.7 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on copper concentrations. The values are the mean ±SD for experiments of three separate experiments



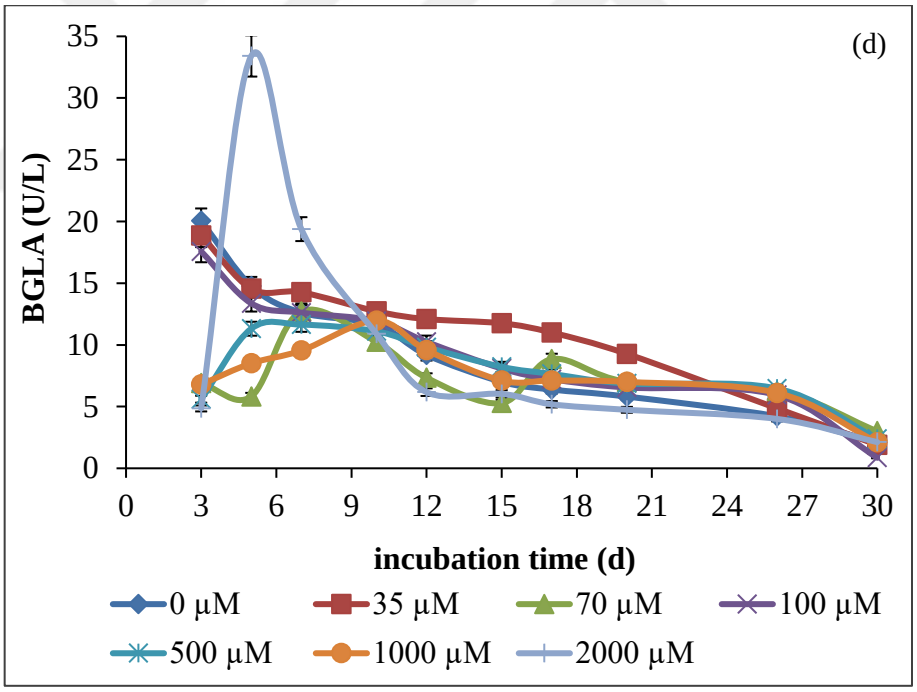


Figure 3.7 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on copper concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

An experiment was designed to investigate time-dependently the effects of different Cu^{2+} concentrations on the *P. eryngii* hydrolytic enzymes productions during SSF process in cherry cultures. After 5 days of incubation, the CMCase and xylanase

activities were as high as 4.29±0.02 and 6.67±0.03 U/mL with 2.0 mM Cu^{2+}, which were 3.30 and 5.75-fold higher than the values in the presence of 0.07 mM Cu^{2+} as shown in Figures 3.8 (a) and (b). Investigating the effects of various individual Cu^{2+} concentrations revealed that Cu^{2+} of 2.0 mM was the better than the amount for hydrolytic enzyme productions of *P. eryngii* in the presence of 0.07 mM Cu^{2+}. BGLA activities were significantly increased when the culture medium was amended with 0.5, 1.0 and 2.0 mM Cu^{2+}. The hydrolytic enzyme activities increased by supplementation of Cu^{2+} except 3.0 and 5.0 mM. The exoglucanase activity was only induced in the presence of 1.0 mM Cu^{2+} in comparison with the values in the presence of 0.07 mM Cu^{2+} (Figure 3.8 (c)). Also, the highest BGLA activity was determined as 51.4±1.2 U/L (on the 12th day) in the presence of 1.0 mM Cu^{2+} and it was 2.20-fold higher than that of the obtaining value in the presence of 0.07 mM Cu^{2+} as shown in Figure 3.8 (d). Also, both CMCase and xylanase activities reached to maxima on the 5th day.

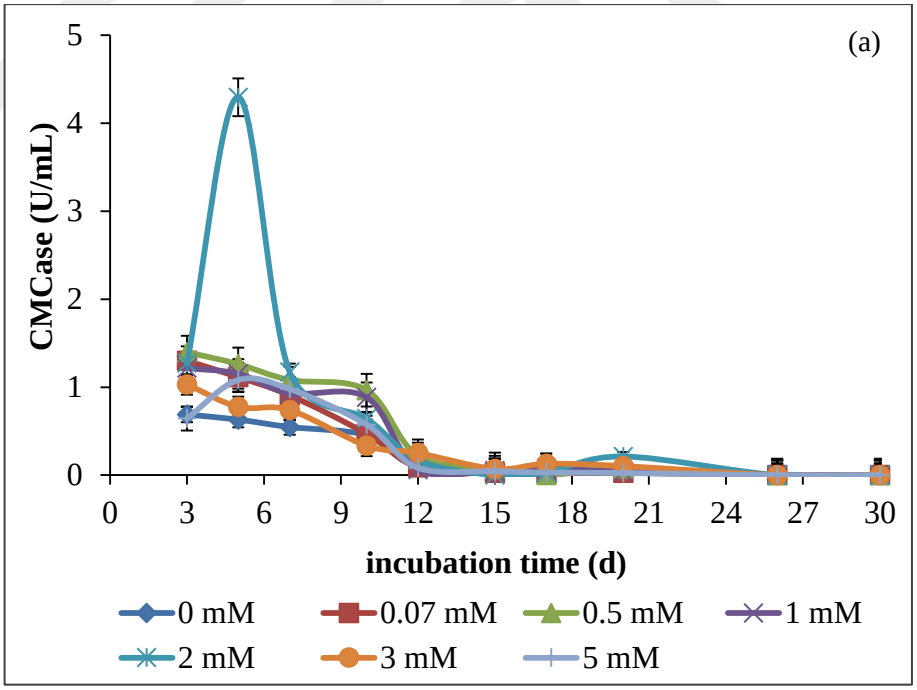
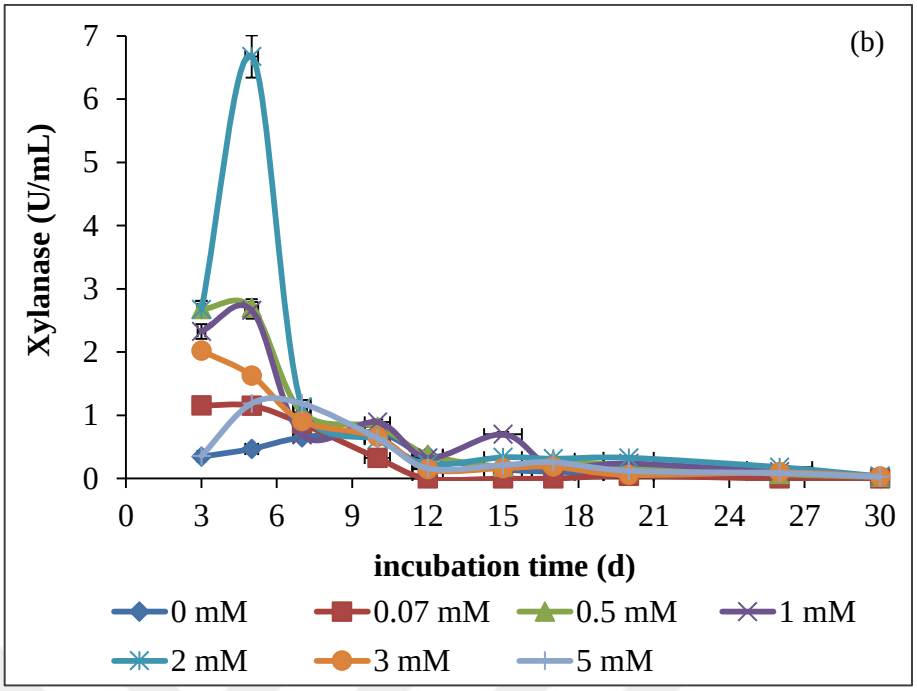


Figure 3.8 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on copper concentrations. The values are the mean ±SD for experiments of three separate experiments



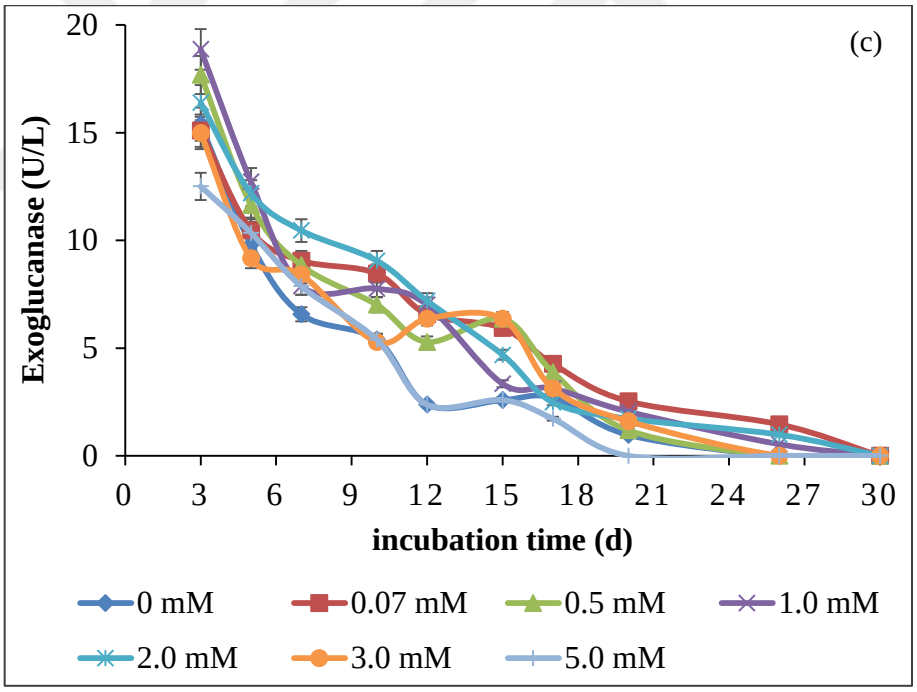


Figure 3.8 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on copper concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

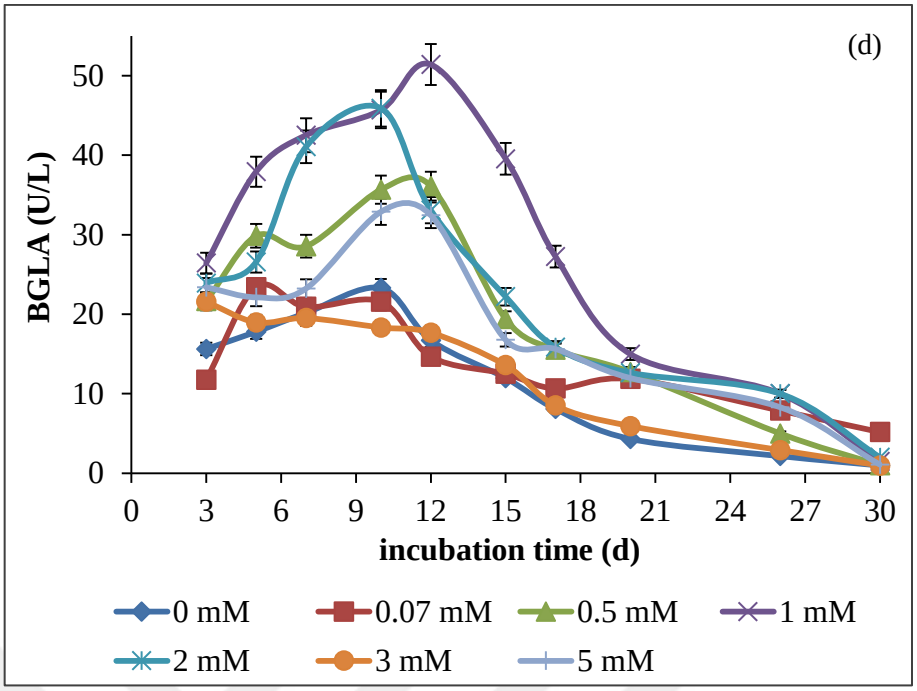


Figure 3.8 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on copper concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

The fungal lignocellulolytic enzyme cocktail including ligninolytic and hydrolytic ones is of vital importance for lignocellulose degradation and its biotechnology, and thus the optimization of ligninolytic and hydrolytic enzyme productions at high yield must be necessary. Copper is a well-known Lac inducer, and this had been confirmed by many previous reports (Wang, Liu, Ning, Liao & Jia, 2017). Therefore, an attempt was made in this study to investigate the effect of copper sulfate on ligninolytic and hydrolytic enzyme production by *P. eryngii*. In peach cultures, only xylanase, exoglucanase and BGLA activities were positively affected when the culture media were amended by increasing Cu^{2+} concentrations above 70 µM. In otherwords, the main enzymes of *P.eryngii* playing a role in lignocellulose degradation except xylanase were not stimulated by Cu^{2+} supplementation. In contrast to activities from peach cultures, the all enzymes from cherry cultures of *P. eryngii* were induced by the enrichments of Cu^{2+}. Especially, the Cu^{2+} effect on the MnP enzyme was remarkable. Since, the production of this enzyme took place in a very short time in the presence of 1.0 mM Cu^{2+}, while MnP production under control conditions occurred on the 26^{th} day of incubation. The Lac enzymes of peach and cherry cultures were chosen as reference

enzyme for determining optimal Cu^{2+} concentration and another optimization parameters due to their biotechnological importance. The optimal value was detected as 70 μM for peach cultures, whereas it was 1.0 mM for cherry cultures. The optimal Cu^{2+} concentrations for both cultures were usually appropriate for most of enzymes especially ligninolytics. In contrast to literature, there is no stimulatory effects of Cu^{2+} on Lac and MnP in peach cultures. This may be due to the adsorption of these enzymes on the lignocellulosic waste surface when the Cu^{2+} concentrations were increased. This is a limiting factor for lignocellulose depolymerization. Also, it becomes difficult to detect extracellular enzyme activities when the enzyme adsorption occurs (Taherzadeh & Karimi, 2008).

3.2.3 Effect of Different Iron Concentrations on Ligninolytic and Hydrolytic Enzymes Activities

3.2.3.1 Effect of Different Iron Concentrations on Ligninolytic Enzymes Activities

The many nutritional factors such as Fe^{2+} were responsible for major ligninolytic and hydrolytic enzyme production with high activities, so these enzyme activities by *P. eryngii* were evaluated under SSF conditions. Firstly, the fungal cultures of *P. eryngii* growing in SSF were supplemented with $FeSO_4$ to final concentrations of 0, 10, 18, 25, 100, 500 and 1000 μM in basal medium before inoculation. The non-optimized basal medium contained 18 μM Fe^{2+} and there were no changes in other nutrient concentrations. According to results, these enzyme activities were suppressed in the presence of 1000 μM Fe^{2+} and they were not induced in Fe^{2+} concentrations below 18 μM. Then, it was decided that the effect of Fe^{2+} concentration (40 μM) on these enzyme activities was subsequently researched in terms of enzyme release. Namely, the ligninolytic and hydrolytic enzyme activities of *P. eryngii* at ranging Fe^{2+} concentrations of 18-500 μM were interpreted.

The addition of this metal ion to the basal medium up to including 18 μM Fe^{2+} enhanced the production of Lac by *P.eryngii* of peach cultures. Figure 3.9 (a) shows that Lac secretion was negligible (< 2.0 U/L) in the early day of incubation on cultures

containing 18 μM Fe^{2+}. The Lac activity in this cultures showed two peaks, Fe^{2+} at 18 μM resulted in a maximum Lac activity of 2193.06±50.4 U/L on the day of 17. The optimal Fe^{2+} concentration for Lac synthesis by *P.eryngii* under SSF cultures using peach waste was found to be 18 μM in the presence of 70 μM Cu^{2+}. The Fe^{2+} concentrations above 18 μM negatively affected Lac activity. The increments in Fe^{2+} concentration decreased Lac enzyme activities. The highest reduction was observed as 37% in comparison with the value, 2193.06±50.4 U/L (Figure 3.9 (a)). Many researches showed that the Lac production by the Fe^{2+} was regulated at transcriptional level (Zhu & Williamson, 2003). However, the supplementation of Fe^{2+} was found to be less effective to induce the Lac activity according to our results. In other words, 18 μM of Fe^{2+} concentration was enough for Lac production and synthesis by *P. eryngii* under SSF conditions at the transcriptional level. It can be originated from that ions such as iron may interrupt the electron transport system of Lac and substrate conversion (Kim & Nicell, 2006). Similar observations were recorded for MnP and LiP activities of *P.eryngii* under SSF condition with supplemented Fe^{2+} concentrations of peach cultures. Also, the strain produced maximum MnP and LiP activities of 732.73±19.8 and 94.09±4.1 U/L after 17^{th} day of incubation, respectively, (Figures 3.9 (b) and (c)). The maximal MnP activities were recorded in the presence of 18 μM Fe^{2+} followed by 25, 40, 100 and 500 μM. When the Fe^{2+} was added to basal medium up to 25 μM, the MnP secretion was carried out earlier. In addition, the second maximal LiP activity was obtained as 40.32±2.6 U/L in the presence of 40 μM. The increments didn't showed positive effects on ligninolytic enzyme activities of peach cultures, and optimal concentration of Fe^{2+} was 18 μM.

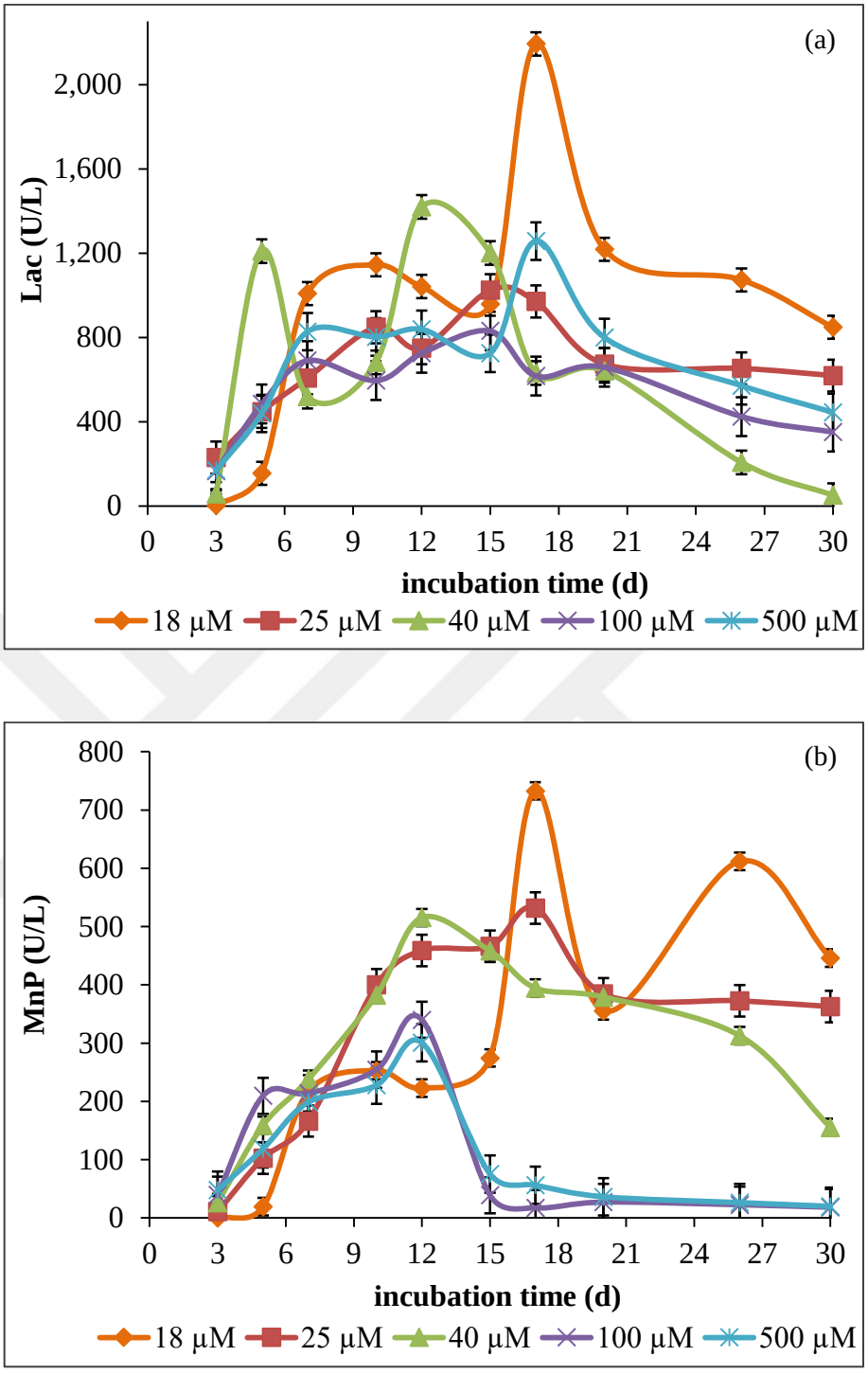


Figure 3.9 The influence of iron concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments

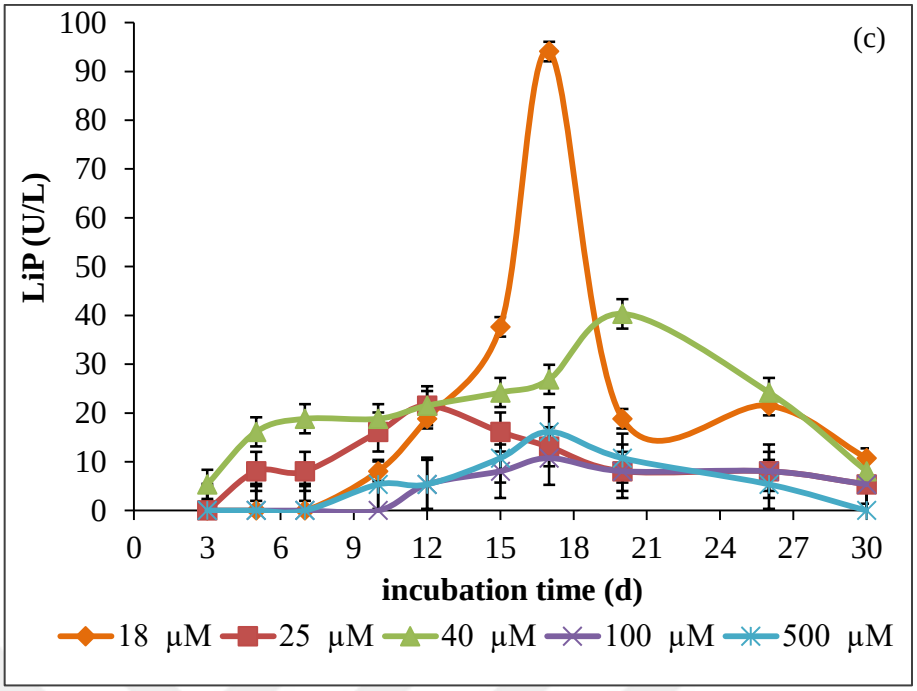
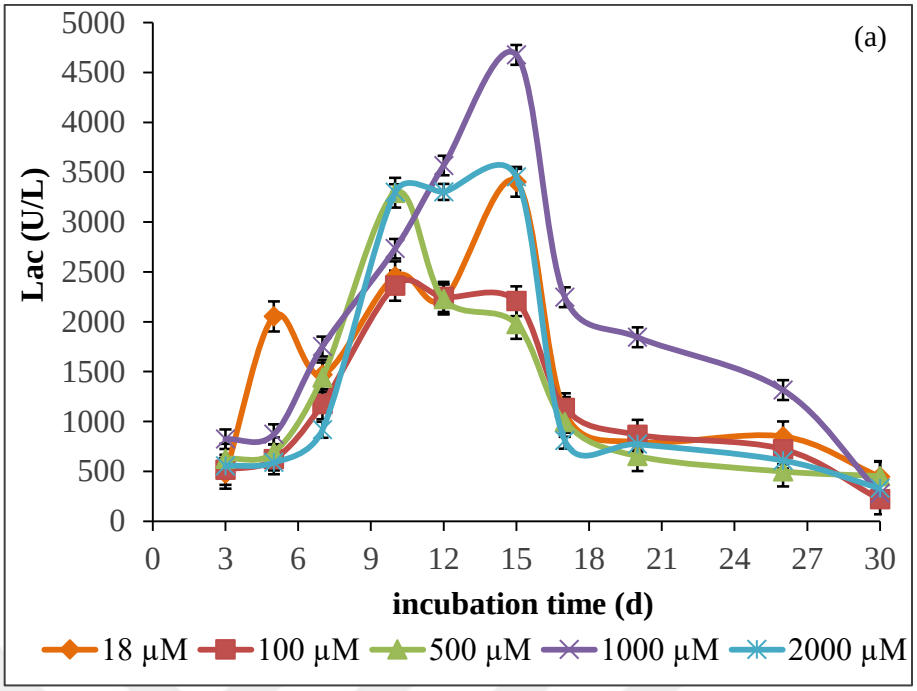


Figure 3.9 The influence of iron concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

The production of major lignocellulosic degrading enzymes indicating ligninolytic, cellulolytic and hemicellulolytic activities have significantly affected by the supplements such as Fe^{2+}. Thus, these enzyme activities by *P. eryngii* were evaluated under SSF conditions in varying Fe^{2+} concentrations during 30 days. Firstly, the Fe^{2+} was added to growing fungal cultures in various concentrations from 0 to 5000 µM. The basal medium contained 18 µM Fe^{2+} and there were no changes in other nutrient concentrations. According to results, the addition of Fe^{2+} in medium with every concentration exhibited strong effects on ligninolytic and hydrolytic enzyme activities by *P. erngii* using cherry waste as a substrate. In absence of Fe^{2+}, these enzyme activities were affected negatively. Also, their activities except CMCase and xylanase increased with the rise in Fe^{2+} concentrations and their values were higher than those obtained in the culture including 18 µM Fe^{2+}. The variations in Fe^{2+} concentrations up to 1000 µM showed positive effects on CMCase and xylanase activities, while the activities were lower than those obtained in the culture including 18 µM Fe^{2+} when the concentrations above 1000 µM were added to culture medium. Therefore, the effects

of Fe^{2+} supplementation to the basal medium before inoculation, containing 1000 μM Cu^{2+}, on ligninolytic and hydrolytic activities of *P. eryngii* were further investigated.

As depicted in Figure 3.10 (a), the increment in Fe^{2+} concentrations up to 1000 μM positively affected Lac activities by *P.eryngii*. The highest Lac activity was determined as 4677.26±75 U/L in the presence of 1000 μM Fe^{2+} on the 15^{th} day of incubation. Similar to Lac activities, the MnP activities were increased depending on some Fe^{2+} concentrations. The highest value was attained as 2064.68±35 U/L on the 5^{th} of incubation in 1000 μM Fe^{2+}, and it was 2.51-fold higher than that of it in the presence of 18 μM Fe^{2+} (Figure 3.10 (b)) ($p<0.05$). In addition, the highest LiP activities were detected as 69.89±2.5 U/L in both 500 and 1000 μM Fe^{2+} in Figure 3.10 (c). The highest Lac activity was also 1.37-fold higher than that of the value in the basal medium containing 18 μM Fe^{2+} ($p<0.05$). According to these results, the optimal Fe^{2+} concentration was found as 1000 μM for ligninolytic enzyme production by *P. eryngii* of cherry SSF cultures. The Lac activities reached to their maxima between the 10^{th} and 15^{th} days of incubation in all tested conditions, but the secretions of MnP by *P. eryngii* were earlier when compared to Lac activities. LiP secretions with higher activities were observed towards the last days of incubation.



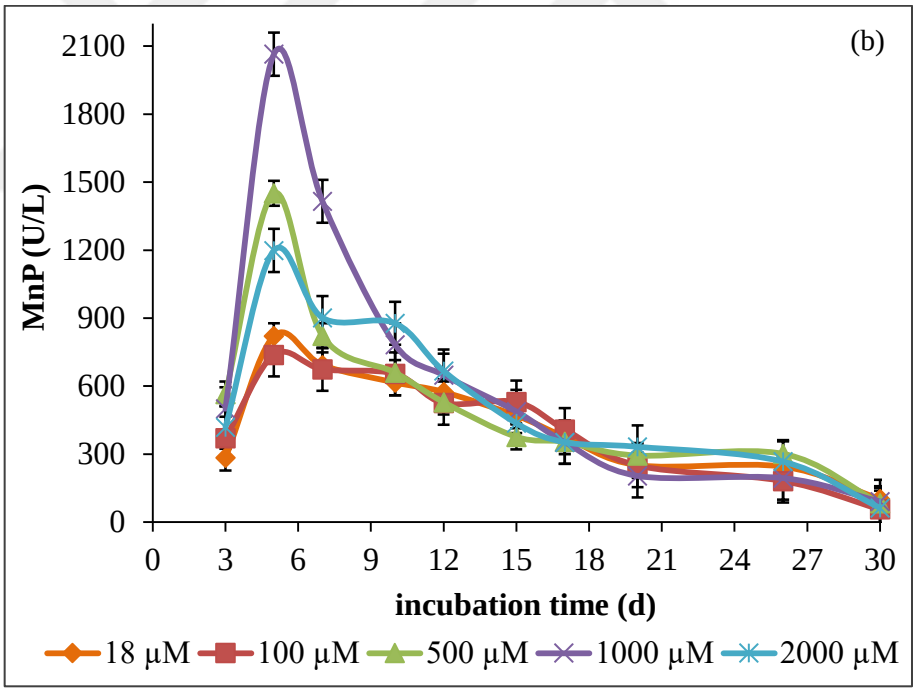


Figure 3.10 The influence of iron concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments

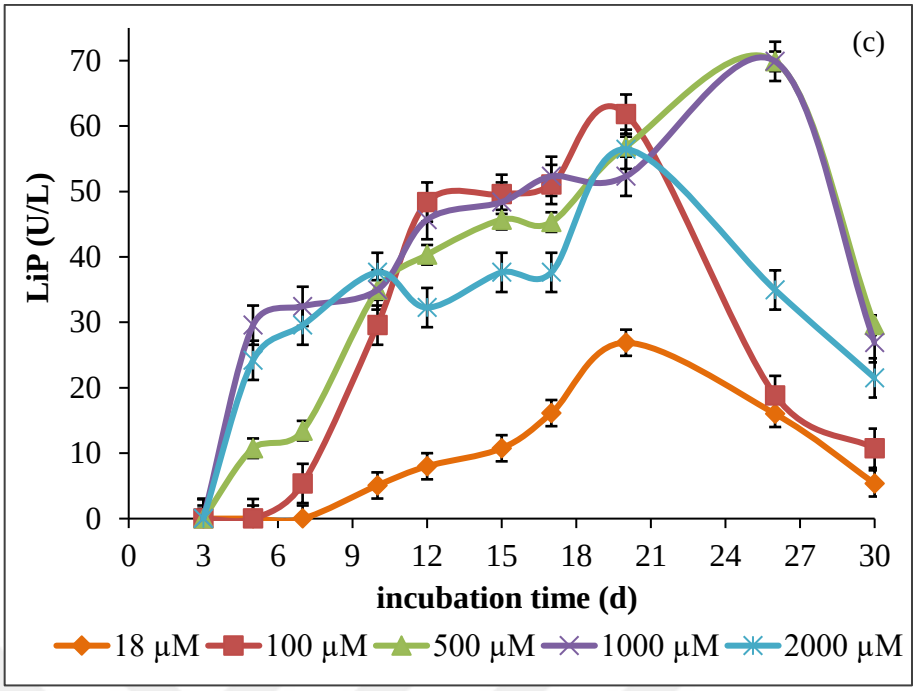


Figure 3.10 The influence of iron concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

3.2.3.2 Effect of Different Iron Concentrations on Hydrolytic Enzymes Activities

Figure 3.11 illustrated the role of Fe^{2+} concentrations on hydrolytic enzyme activities of *P.eryngii* of peach cultures time-dependent. Results of experiments implied that enhancement of Fe^{2+} concentrations played significant roles in enhancing the hydrolytic enzyme productions. Among the tested concentrations of Fe^{2+}, supplementation of 40 µM expressed the CMCase activity of about 4.85± 0.02 U/mL on the 3rd day, but the activity in the presence of 18 µM Fe^{2+} was obtained as 3.64±0.02 U/mL (Figure 3.11 (a)). The CMCase activities including 18 µM and 25 µM Fe^{2+} in basal medium showed similar trends during incubation, and they had similar trends in other conditions, too. The increase in CMCase activity was at least 3.6%, while this enzyme activity was reduced in 25 µM Fe^{2+}. Similar to CMCase activities, xylanase activities in 18 µM and 25 µM Fe^{2+} similar trends during incubation. On the other hand, the addition of Fe^{2+} to culture media was increased the xylanase activities. The maximum activity of xylanase was recorded as 3.79±0.02 U/mL in 500 µM Fe^{2+} and 1.23-fold higher than the value in the medium containing 18 µM Fe^{2+} (Figure 3.11

(b)). The maximum exoglucanase activity was detected as 5.25±0.02 U/L in the medium containing 500 µM Fe^{2+}, while the maximum BGLA activity was attained as 95.95±2.6 U/L in 25 µM Fe^{2+} (Figures 3.11 (c) and (d)). In other Fe^{2+} conditions, the BGLA activities were very close to each other. These experimental results emphasized that iron was the suitable inducer for hydrolytic enzyme productions in *P.eryngii*.

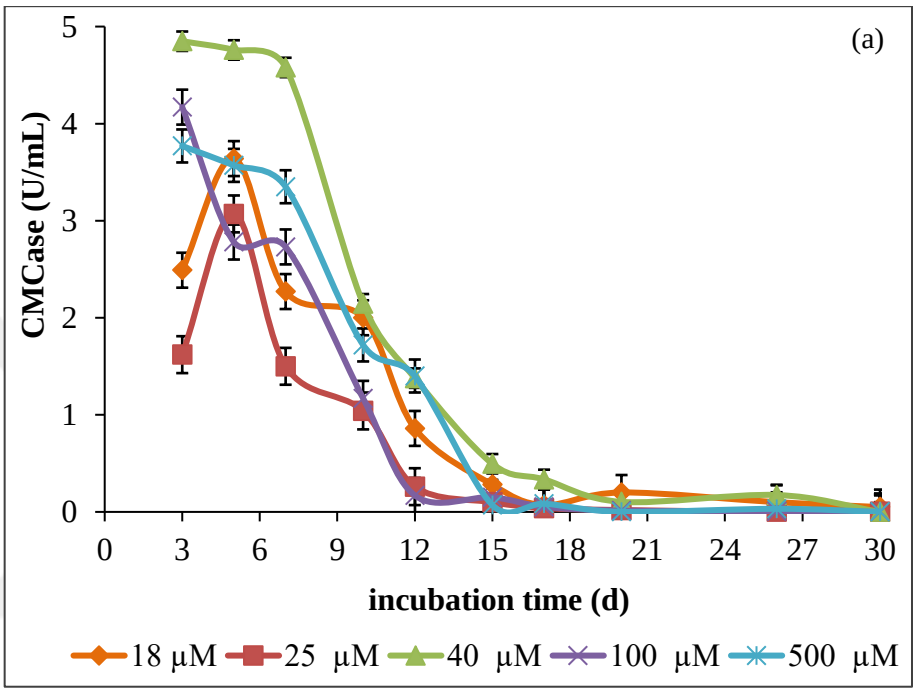
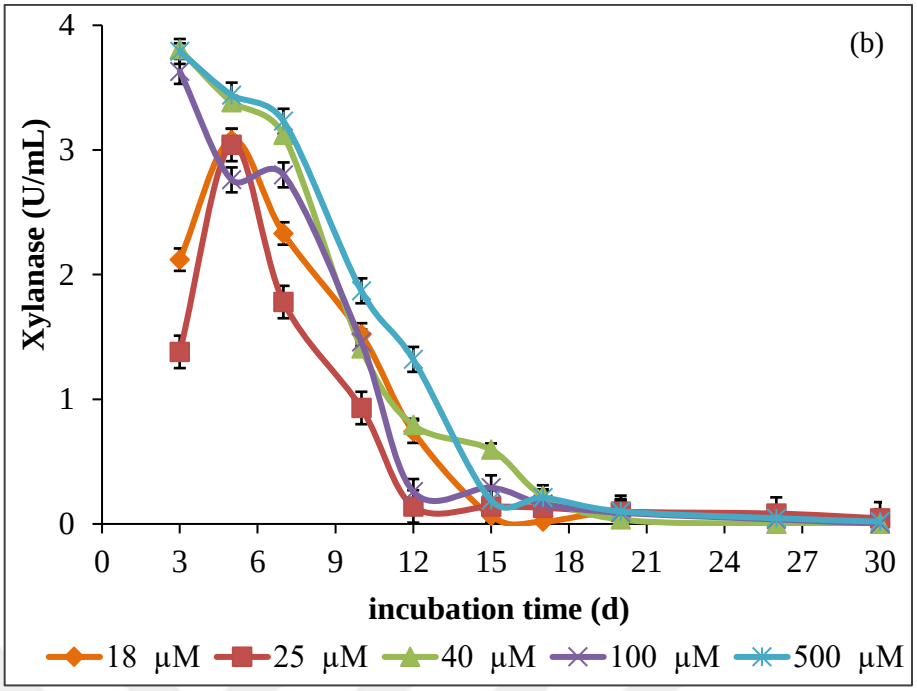


Figure 3.11 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on iron concentrations. The values are the mean ±SD for experiments of three separate experiments



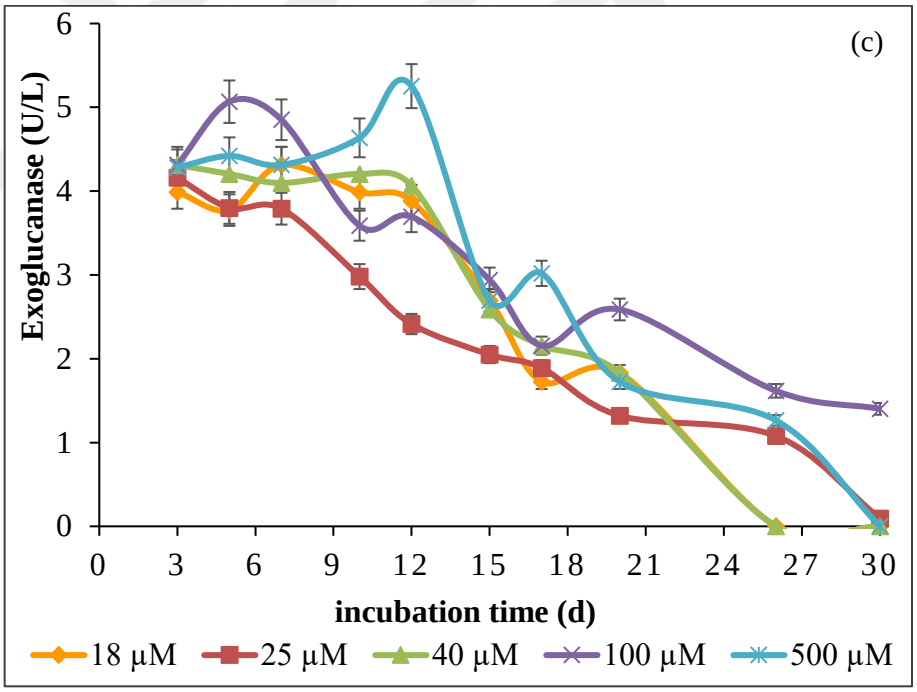


Figure 3.11 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on iron concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

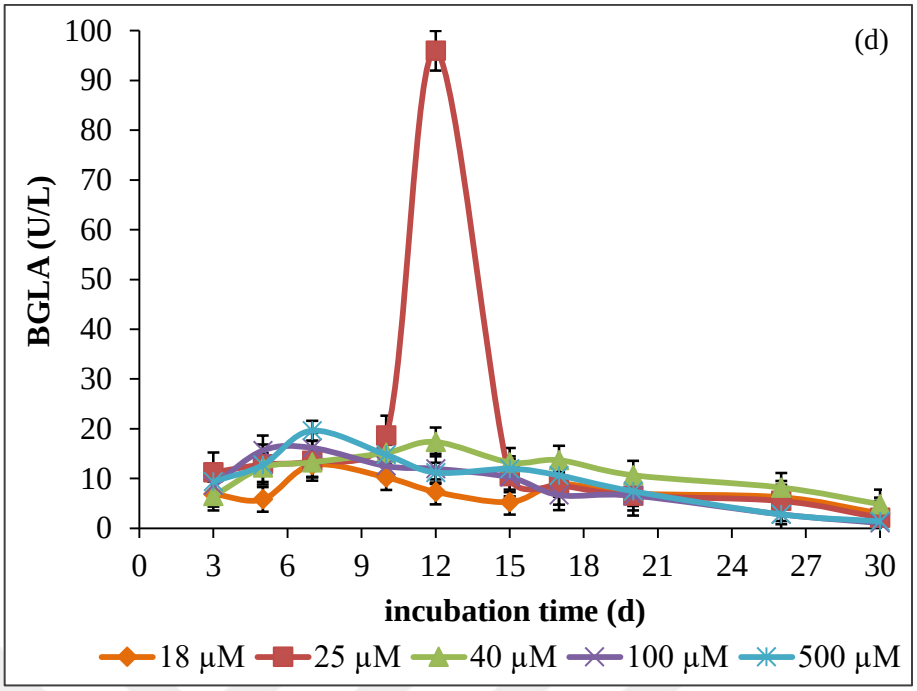


Figure 3.11 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on iron concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

As depicted in Figures 3.12 (a) and (b), when the cherry culture media were supplemented with Fe^{2+}, CMCase and xylanase activities increased nearly 39 and 4% in comparison to their activities determining 18 µM Fe^{2+}, respectively. The maximum CMCase and xylanase activities were obtained as 1.68±0.01and 2.76±0.01 U/mL, respectively, in the presence of 100 µM Fe^{2+} on cherry cultures and day of 5^{th}. Otherwise, the highest BGLA activity was attained as 66.95±3.7 U/L in 1000 µM Fe^{2+} (5^{th} day) as shown in Figure 3.12 (c). There were insignificant differences between this value and the other maximum BGLA activity in 500 µM on the day of 26 ($p>0.05$). The maximal xylanase activities were attained on the 5^{th} day of incubation with all Fe^{2+} conditions. CMCase and xylanase activities were detected at the beginning of incubation. These hydrolytic enzymes activities were induced when the Fe^{2+} concentration was increased in the medium. For xylanase activity, only Fe^{2+} concentration of 500 µM had a negative effect. On the other hand, CMCase secretions with maximal activities were delayed from 3^{rd} day to 5^{th} day depending on the increment in Fe^{2+} concentrations. The highest exoglucanase activity was also 2.04-fold higher than that of the medium containing 18 µM Fe^{2+} ($p<0.05$). Also, the

exoglucanase activity reached to maximum in the presence of 18 µM Fe^{2+} on the 3rd day of incubation, and the activities under other conditions were lower than this value (Figure 3.12 (c)). Also, the BGLA activities reached to maxima in the presence of 500 and 1000 µM and there were insignificant differences between these values ($p>0.05$) (Figure 3.12 (d)).

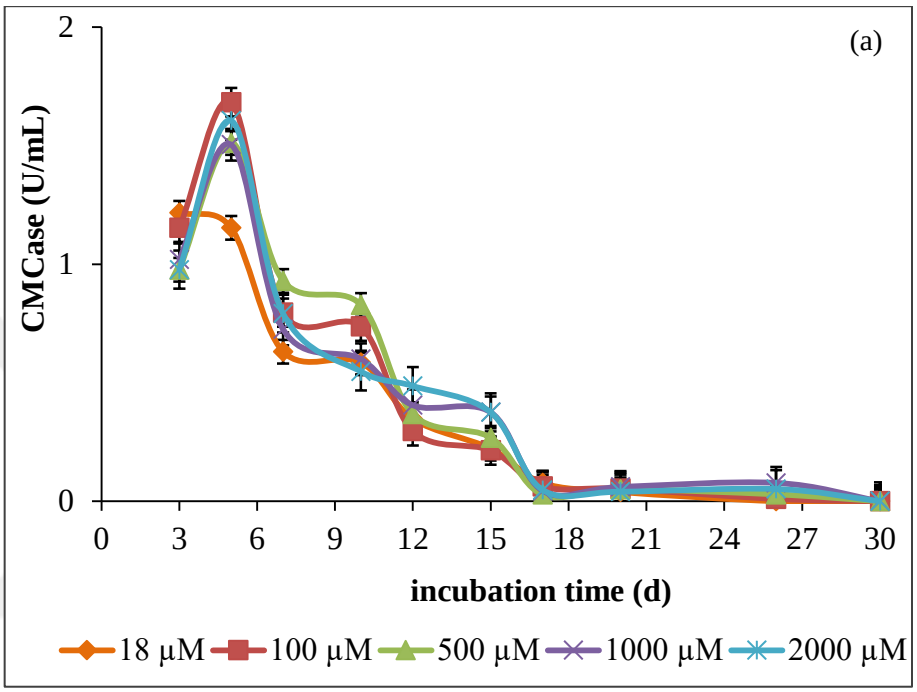
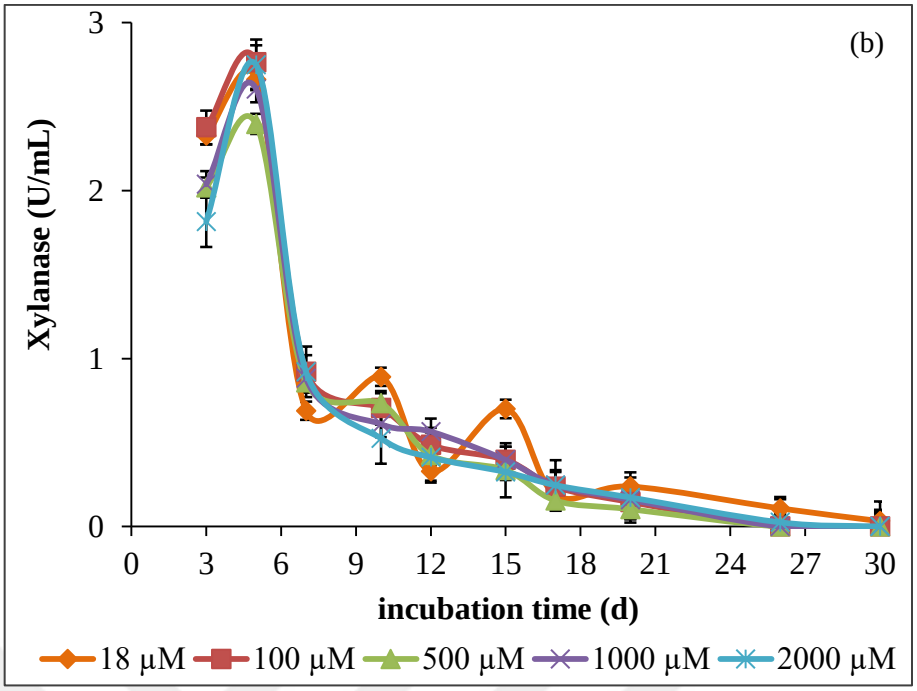


Figure 3.12 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on iron concentrations. The values are the mean ±SD for experiments of three separate experiments



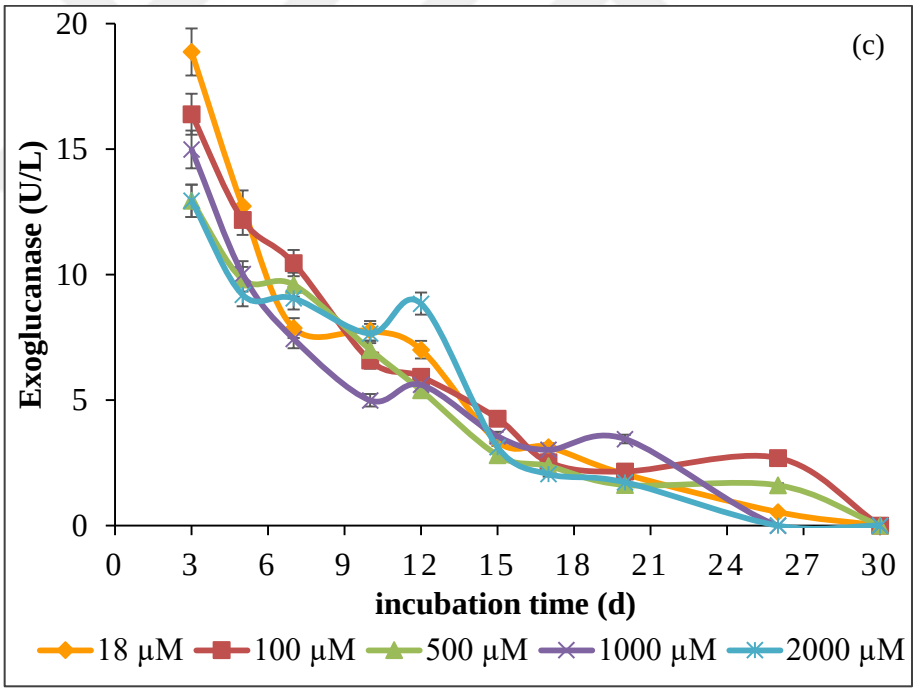


Figure 3.12 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on iron concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

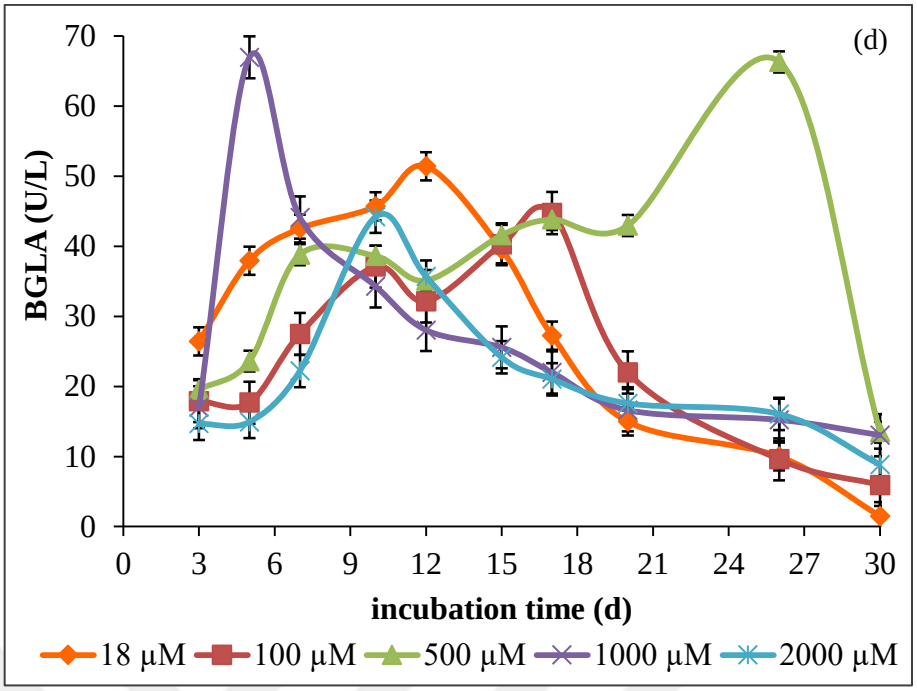


Figure 3.12 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on iron concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

One of the most effective approaches to increase the yield of ligninolytic and hydrolytic enzymes is the supplementation of the nutrient medium with an appropriate inducer. For enhanced the productions of these enzymes from *P.eryngii*, the effect of different concentrations of Fe^{2+} was studied under SSF conditions using peach and cherry wastes as substrates. Although cherry wastes were richer in Fe^{2+} compared to peach wastes, the effects of Fe^{2+} on the productions of ligninolytic and hydrolytic enzymes were researched at a wider range of concentrations after preliminary results. The ligninolytic enzyme activities from peach wastes were negatively affected by increasing Fe^{2+} concentrations, and their maximum activities were obtained on the 17 day of incubation. In both cultures, the effects of Fe^{2+} concentrations on exoglucanase and BGLA activities among hydrolytic enzymes were at considerable levels since their activities were very low. Also, the levels of all ligninolytic enzyme activities from cherry cultures were found to reach maximum peaks in the presence of 1000 µM Fe^{2+} at different days of fermentation. The Lac and LiP activities reached to maximum on the days of 15 and 26, respectively. Also, the activity in 1000 µM was approximately two fold higher when the LiP activities were compared to the obtaining values in the

presence of 18 and 1000 μM Fe^{2+} on the 20 day of incubation ($p<0.05$). In contrast to peach cultures, the maximum MnP activities were detected at the initial days of incubation. The CMCase activities from both cultures were generally induced by the supplementation of Fe^{2+} above 18 μM, while the additions of Fe^{2+} above 25 μM to the peach culture medium affected positively the xylanase activities and their activities from cherry cultures was only suppressed in the 500 μM. Generally, the dominant enzymes in lignocellulose, CMCase and xylanase, were secreted earlier, and then the ligninolytic ones were produced by *P. eryngii* under tested conditions. The high activities of CMCase and xylanase during the beginning of incubation could probably be due to the fact that the ligninolytic enzymes were repressed by them. This also suggests that cellulose and hemicellulose are utilized in the beginning by the growing of the fungus. The optimal value of Fe^{2+} concentration was detected as 18 μM for peach cultures, whereas it was 1.0 mM for cherry cultures. The optimal Fe^{2+} concentrations for both cultures were usually appropriate for most of enzymes especially ligninolytics. There was no observation on the induction of ligninolytic enzymes from peach cultures by adding Fe^{2+} in the thesis. Similar to the optimization results of Cu^{2+} supplementation to peach cultures, the adsorption of these enzymes on the lignocellulosic waste surface may have occurred when the Fe^{2+} concentrations were increased. In briefly, the obtained results illustrate that the higher amounts of Fe^{2+} have a significant positive influence on the ability of *P.eryngii* to produce lignocellulose degrading enzymes under SSF conditions using cherry waste, but on peach cultures vice versa.

3.2.4 Effect of Different Tween 80 Concentrations on Ligninolytic and Hydrolytic Enzymes Activities

3.2.4.1 Effect of Different Tween 80 Concentrations on Ligninolytic Enzymes Activities

The present study focused on the obtaining of high ligninolytic and hydrolytic enzyme activity levels by optimizing concentrations of additive compound, Tween 80, affecting secretion of enzymes under SSF conditions of *P. eryngii*. First, the effects of

Tween 80 (0.05% v/v) on these enzymes were evaluated under control conditions namely using non-optimized basal medium. Since the ligninolytic and hydrolytic enzymes activities except CMCase and xylanase were stimulated by this (data not shown), various concentrations were incorporated in the fermentation medium of both cultures at the time of inoculums transfer as can be seen in Figures 3.13-3.16 in order to study the effect of Tween 80 on their activities.

The Tween 80, non-ionic detergent, influenced the Lac activities in all culture conditions supplemented with different concentrations (0.025, 0.05, 0.1, 0.2%, v/v). The maximal Lac activities in all Tween 80-supplemented conditions were attained on the 20 days of incubation, but it was on 17^{th} day in the absence of Tween 80. As shown in Figure 3.13 (a), the maximum Lac activity was observed as 24 987.57±2145 U/L in the presence of 0.025%, v/v, Tween 80, and this value was 11.4-fold higher than that obtained in the absence of Tween 80 ($p<0.01$). Thus, these results clearly show the positive effect of Tween 80 as an inducer of Lac activity. Surfactants such as Tween 80 that modify the fungal membrane are known to be promoting Lac secretion by many fungal strains (Dekker, Barbosa, Giese, Godoy & Covizzi, 2010). Elsayed et al. (2012) studied the effect of different concentration of Tween 80 (0.1-0.75%; v/v) on Lac activity, and they found that the enzyme activity reached to its maximum value at a concentration of 0.1% (v/v Tween-80) and increased by about 44% than control (without Tween-80). The increase in Lac activity in the presence of Tween 80 might be due to increased permeability of the cell membrane's lipid bilayer, facilitating more rapid secretion of enzymes out of the cell. On the other hand, this surfactant could protect the enzyme structure and its activity from environmental factors by forming micelles around the enzyme. Moreover, the solubility of compounds in lignocellulosic structure may be increased, and thus better surface area may be provided for the utilization of the fungus in the presence of Tween 80. Similar to Lac activities, the joint effect of Tween 80 on both MnP and LiP was observed. The induction of Lacs in culture enriched by 0.025%, v/v, Tween 80 was quite high in similar to MnP and LiP activities. The enrichment of culture medium in all Tween 80 concentrations demonstrated the induction of Lac activity. The lowest Lac activity was recorded in the absence of Tween 80. On the other hand, the addition of Tween 80 stimulated MnP

and LiP activities in all tested conditions. The highest MnP and LiP activities of peach culture were attained as 4134.35±45 and 1613.04±18 U/L on the 20th day of incubation in the 0.025%, v/v, Tween 80 supplementation, and these values were approximately 5.65- and 17.16-fold higher than those in the absence of Tween 80 (Figures 3.13 (b) and (c)) ($p<0.01$).

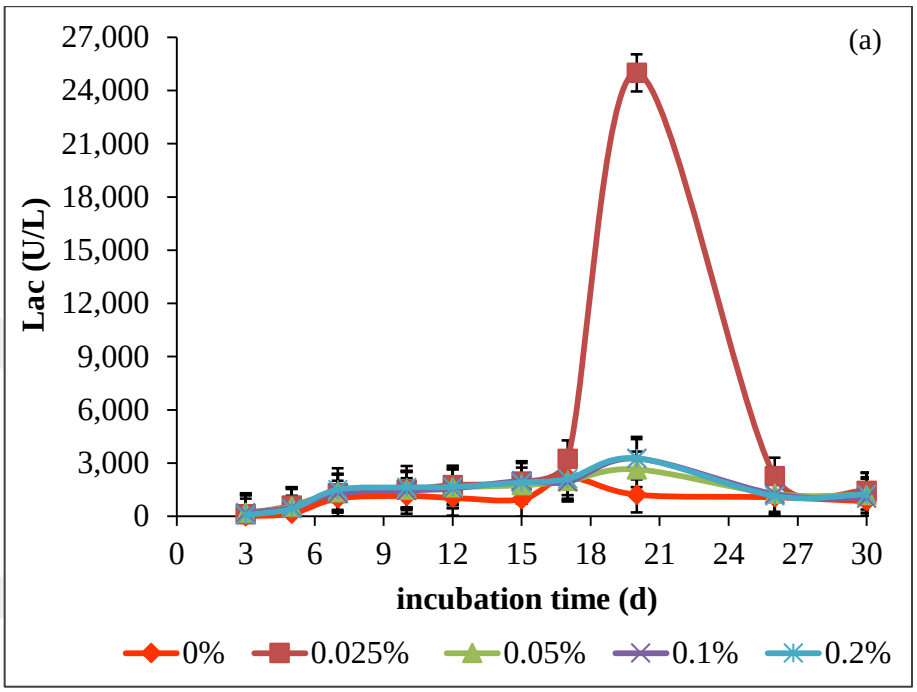
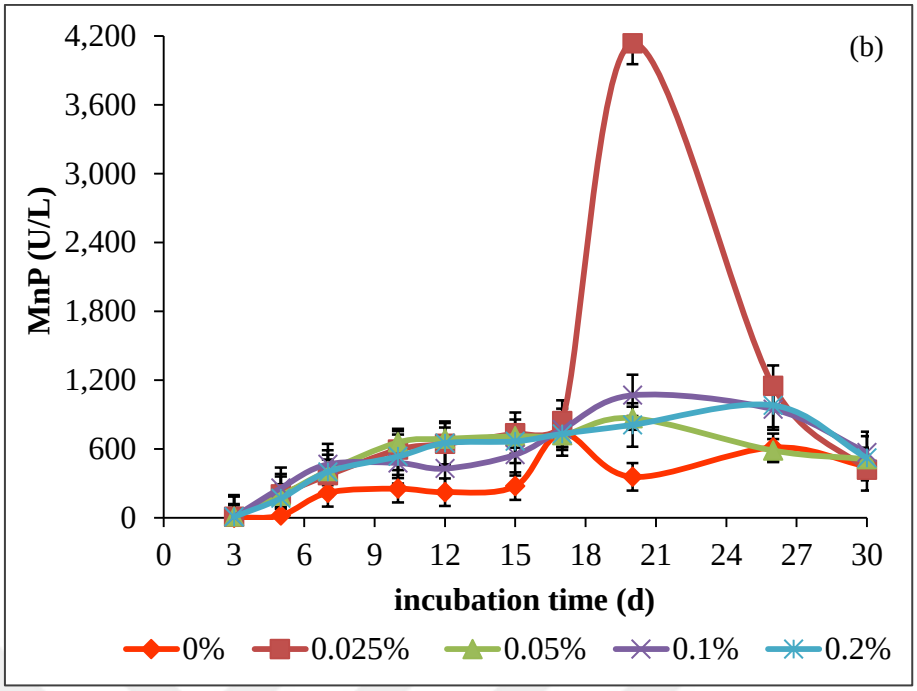


Figure 3.13 The influence of Tween 80, nonionic detergent, concentrations (%, v/v) on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments



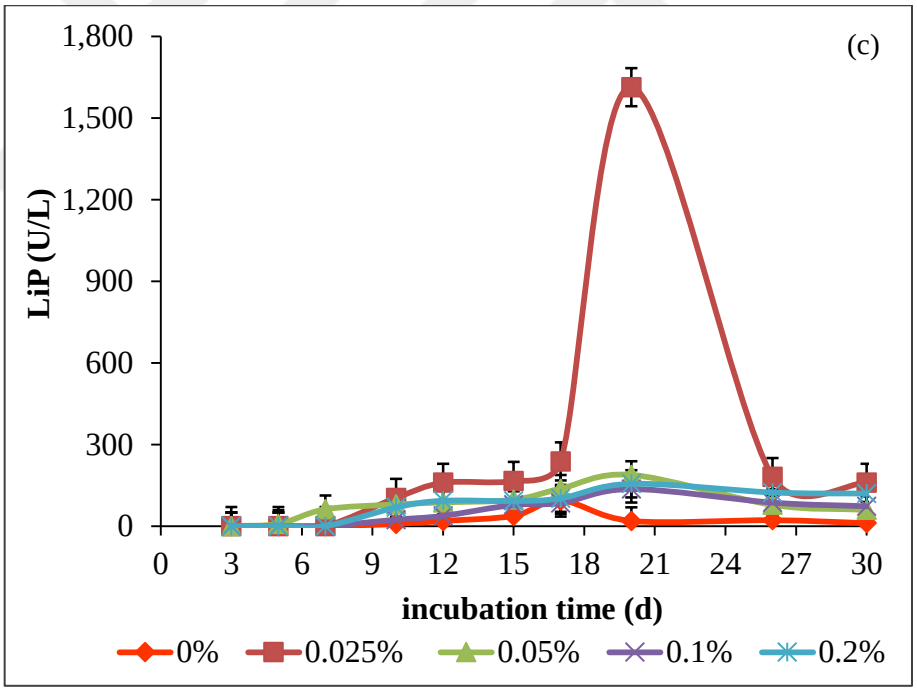


Figure 3.13 The influence of Tween 80, nonionic detergent, concentrations (%, v/v) on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

The Tween 80 when supplemented to basal medium induced especially MnP, xylanase and BGLA activities of cherry cultures by *P. eryngii*. According to these

results, the supplementation of Tween 80 (0.05% v/v) with basal medium including 18 μM Fe^{2+} and 70 μM Cu^{2+} had an additive effect on their activities. This compound is very important for the most of extracellular enzyme productions due to the impact on the stability and production of these ligninolytic and hydrolytic enzymes. Thus, whether the different concentration of Tween 80 could affect activities of ligninolytic and hydrolytic enzymes during SSF of cherry waste by *P. eryngii* under optimized conditions until now was the further question that provided the goal for this section.

The effect of Tween 80 supplementations (0.025, 0.05, 0.1, 0.2%, v/v) on the extracellular ligninolytic enzyme activities of *P.eryngii* was tested in cherry cultures shown in Figure 3.14. There were insignificant differences in Lac activities between 4677.26±75 U/L (in the absence of Tween 80) and 4606.9±72 U/L (0.2%, v/v, Tween 80 supplemented medium) ($p>0.05$), but the Lac activities very lowered under the other conditions. The decreases in maximum Lac activities were nearly attained 12.7% in both supplemented 0.025 and 0.05%, v/v, Tween 80 conditions (Figure 3.14 (a)). When the culture medium was enriched by 0.1%, v/v, Tween 80, the maximum Lac activity was lowered approximately 9.7%. On the other hand, no obvious effects on MnP activities were observed after the addition of Tween 80 in concentrations 0.025 and 0.2%, v/v. The MnP activity was 1878.25±14 U/L on the 7^{th} day of cultivation in medium supplemented with 0.05%, v/v, Tween 80. The highest MnP activity was measured as 2263.25±45 U/L on the 10^{th} day of incubation in the presence of 0.1%, v/v, Tween 80 and it was 9.6-fold higher than the activity obtained in the absence of this surfactant (Figure 3.14 (b)). In addition, the LiP activities in all investigated Tween 80 concentrations decreased when compared to the detected value in the absence of it (69.89±2.5 U/L) (Figure 3.14 (c)). When all these results were summarized, the addition of Tween 80 to the cherry culture under SSF did not show a significant effect on inducing ligninolytic enzymes activities. El-Batal et al. (2015) reported that the addition of the surfactant Tween 80 resulted in higher yields of ligninolytic enzymes in certain fungi, but these enzymes activities of *P. eryngii* by cherry cultures were insignificantly induced in this thesis ($p>0.05$).

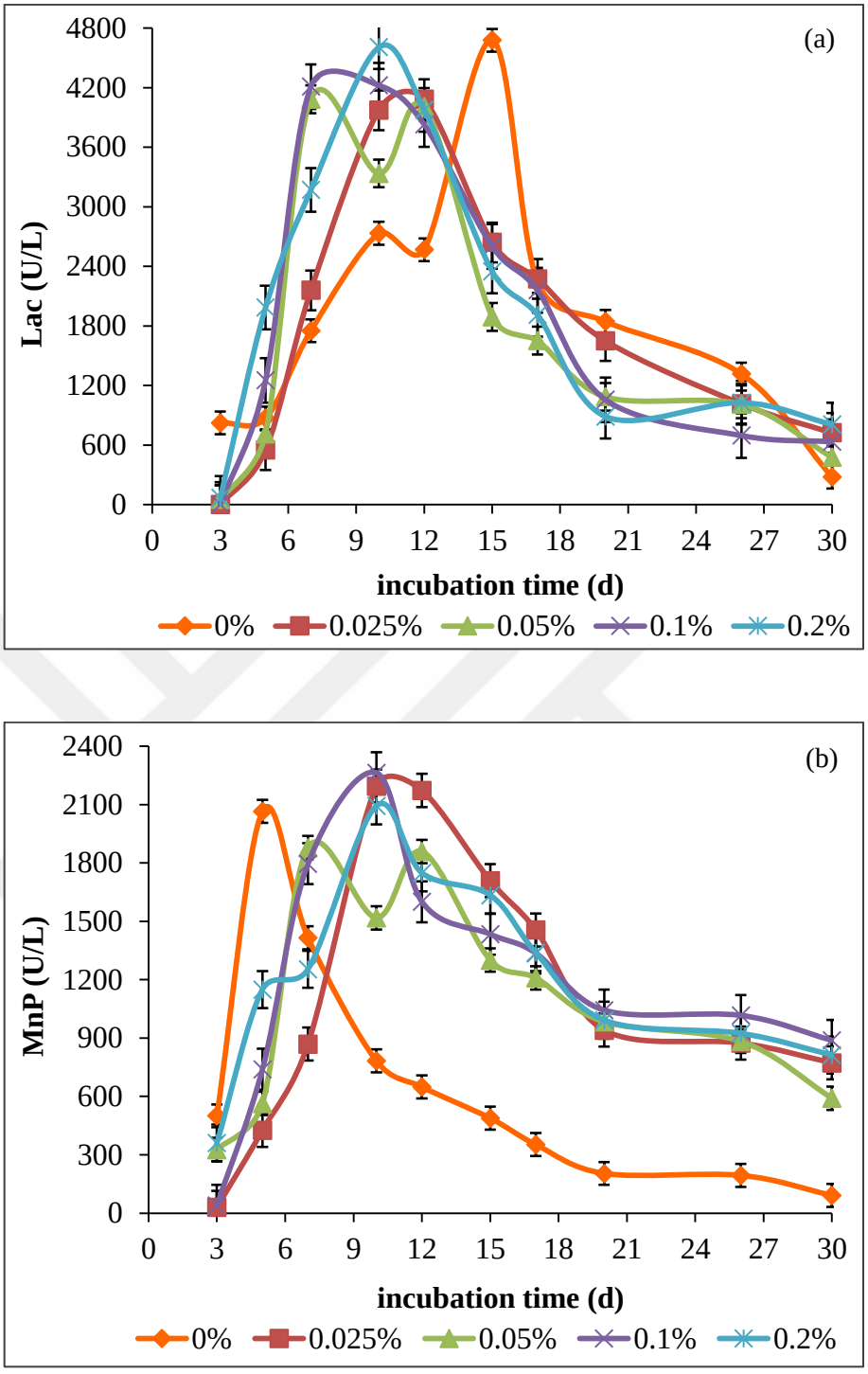


Figure 3.14 The influence of Tween 80, nonionic detergent, concentrations (%, v/v) on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments

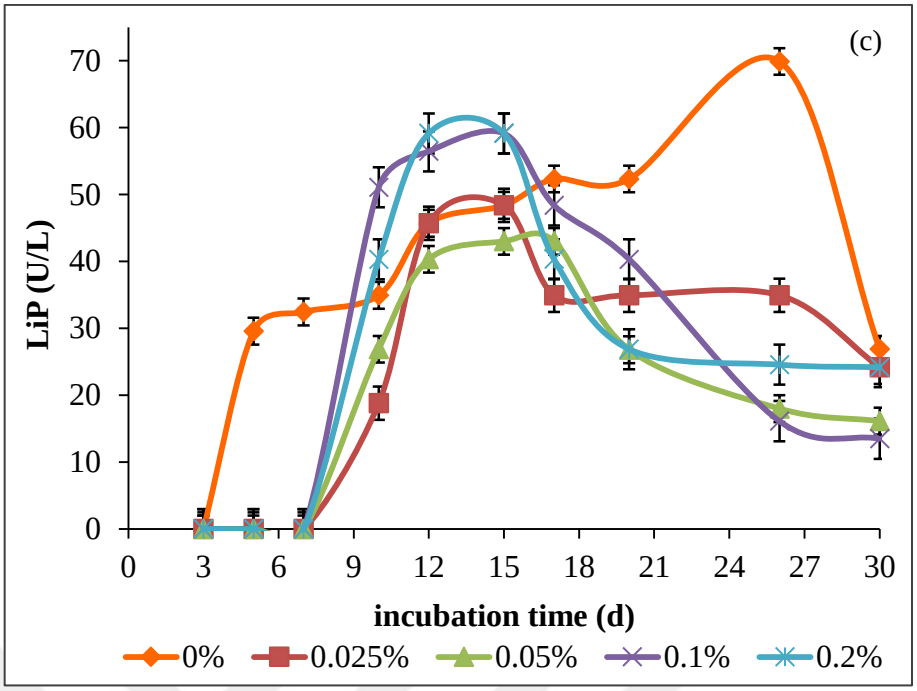


Figure 3.14 The influence of Tween 80, nonionic detergent, concentrations (%, v/v) on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

3.2.4.2 Effect of Different Tween 80 Concentrations on Hydrolytic Enzymes Activities

Figure 3.15 illustrated the role of Tween 80 on hydrolytic enzyme activities of peach cultures. The CMCase and xylanase activities were negatively affected by Tween 80 in all concentrations, whereas BGLA activities were positively. In all concentrations of Tween 80, the CMCase and xylanase activities were inhibited. The all exoglucase activities were increased by the Tween 80 supplementation as can be seen in Figure 3.15 (c). The highest BGLA activity (25.12±1.1 U/L) was found in the peach culture supplemented with 0.2% Tween 80, and also there were insignificantly differences when compared to culture supplemented with 0.01%, v/v, (23.39±1.1 U/L) and 0.025%, v/v, Tween 80 (24.26±1.5 U/L) as shown in Figure 3.15 (d) ($p>0.05$). Thus, these experimental results explained that Tween 80 was the suitable inducer for ligninolytic enzyme activities in *P.eryngii* of peach cultures under SSF, while it was unsuitable for hydrolytic enzyme activities.

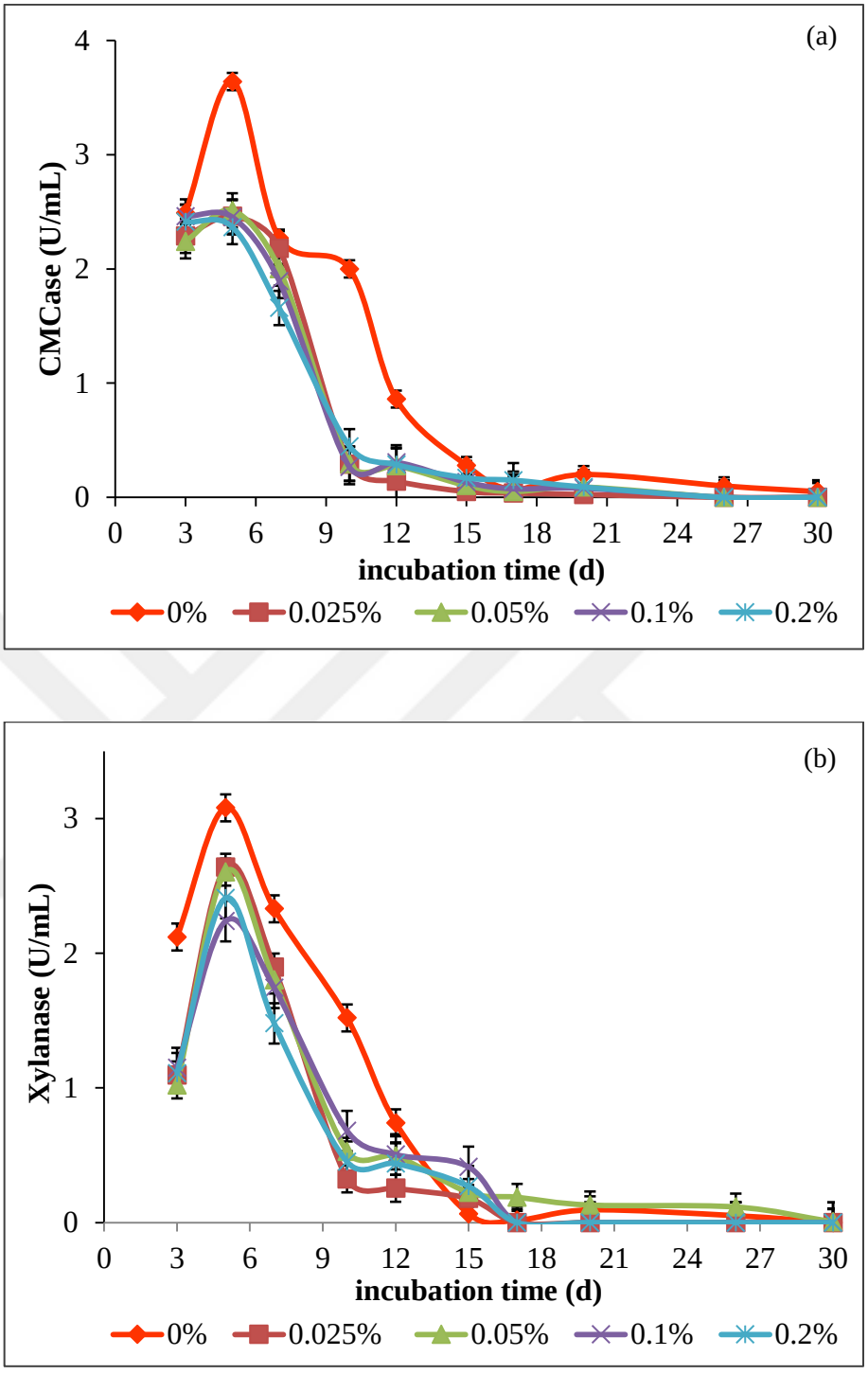
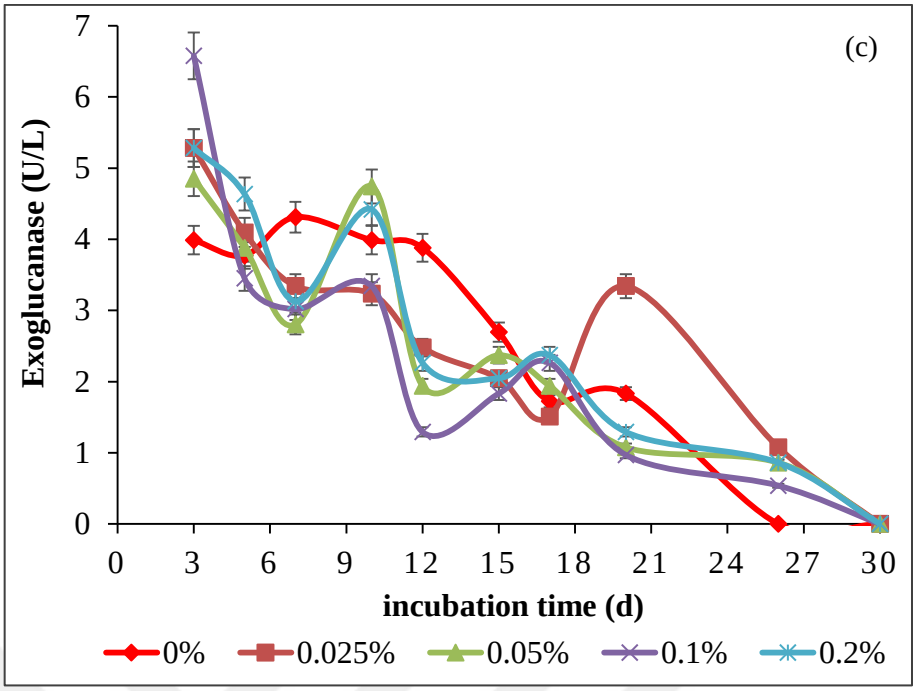


Figure 3.15 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on Tween 80 concentrations (%, v/v). The values are the mean ±SD for experiments of three separate experiments



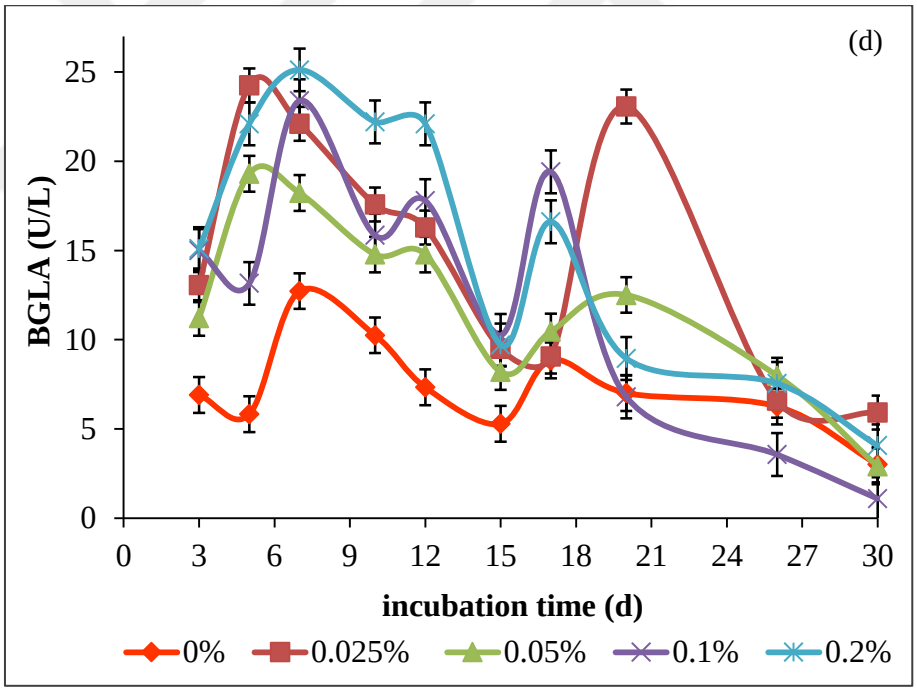


Figure 3.15 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on Tween 80 concentrations (%, v/v). The values are the mean ±SD for experiments of three separate experiments (continued)

Four different concentrations of Tween 80 were tested for their effects on hydrolytic activities of *P.eryngii* in cherry cultures as seen in Figure 3.16. In all experiments, the

Tween 80 induced xylanase activities, but not CMCase activities. There were insignificantly differences among the CMCase activities in all conditions except the medium containing 0.2%, v/v, Tween 80, its activity was very lowered in that culture ($p>0.05$) (Figure 3.16 (a)). Thus, the CMCase activities were suppressed when the medium was enriched with this compound. Also, the maximal xylanase and BGLA activities were respectively determined as 3.10±0.01 U/mL and 76.32±2.5 U/L in the presence of 0.05 and 0.025%, v/v, as can be seen in Figures 3.16 (b) and (d). The xylanase activities in made rich media with Tween 80 were determined earlier. BGLA activity in the presence of Tween 80 was induced, while it was decreased in all other condition in comparison to the activity obtained in the absence of Tween 80. The highest xylanase activity was 18.85-fold higher than that of it in medium not including Tween 80 ($p<0.01$). The all exoglucanase activities were also increased when the production media were enriched by Tween 80 (Figure 3.16 (c)).

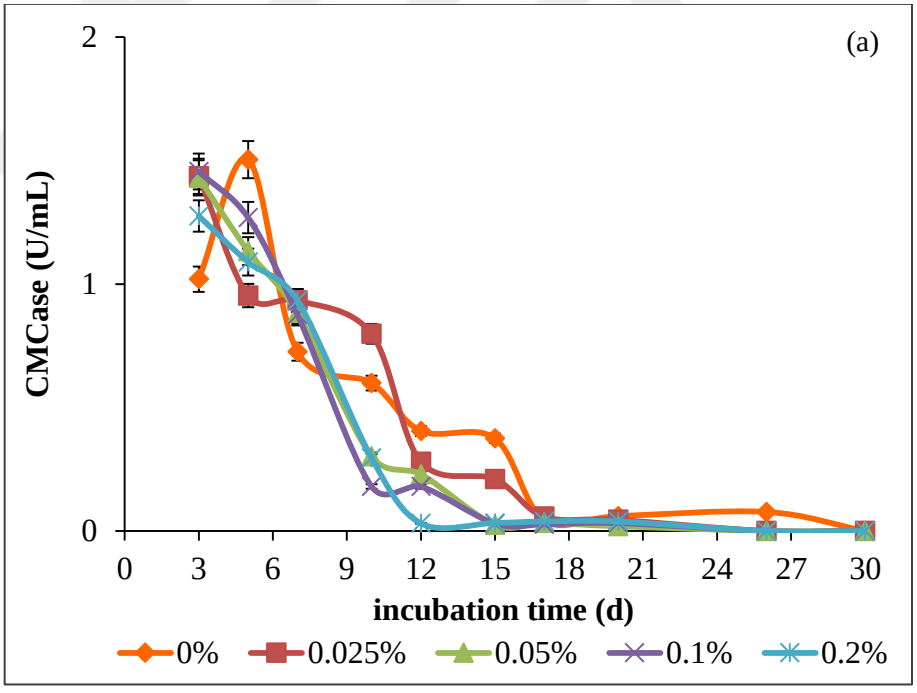
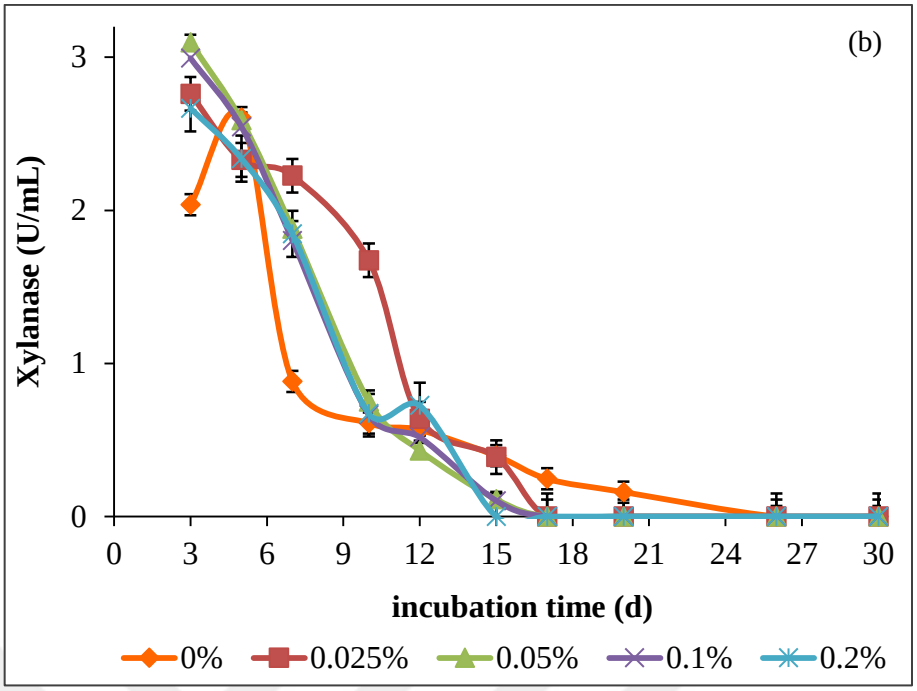


Figure 3.16 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on Tween 80 concentrations (%, v/v). The values are the mean ±SD for experiments of three separate experiments



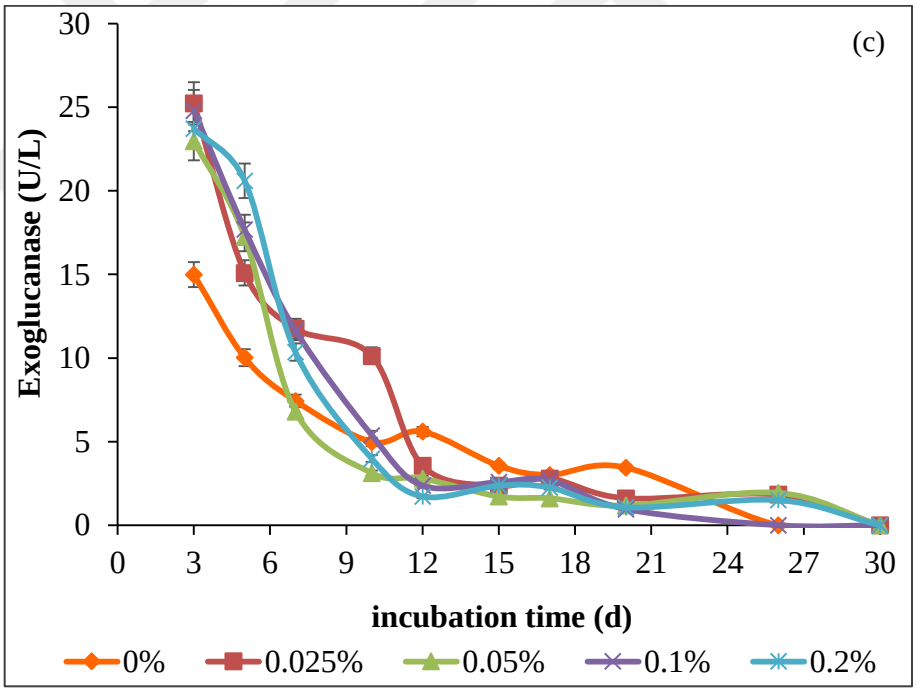


Figure 3.16 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on Tween 80 concentrations (%, v/v). The values are the mean ±SD for experiments of three separate experiments (continued)

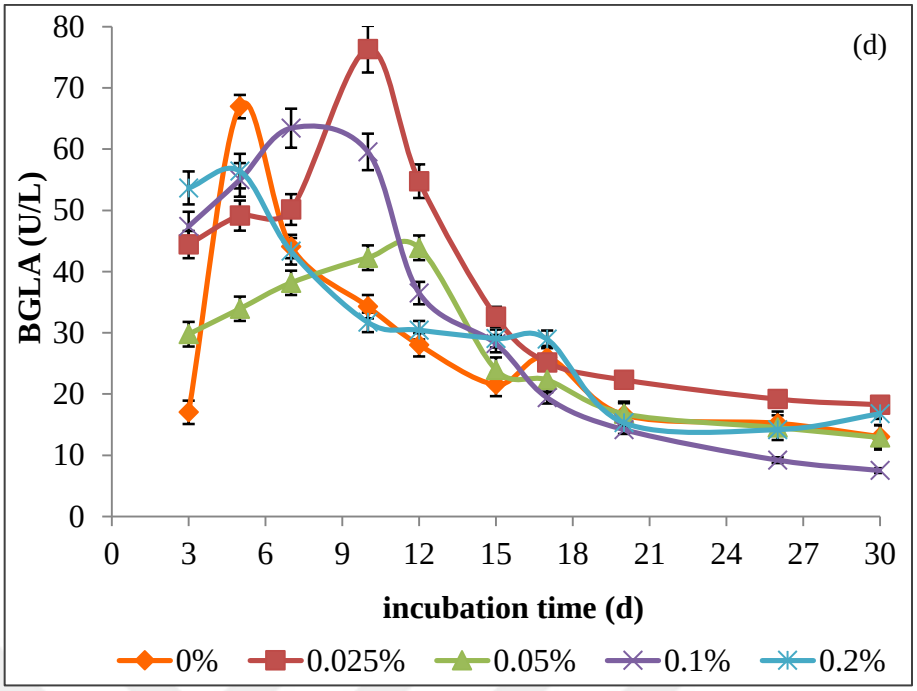


Figure 3.16 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on Tween 80 concentrations (%, v/v). The values are the mean ±SD for experiments of three separate experiments (continued)

Lignocellulose is a potential substrate for biotechnological applications, especially biofuel production, but high lignocellulose conversion requires high enzyme loading, which makes the process less economically feasible. Thus, methods to increase enzyme effectiveness are important for reduction of enzyme consumption. Non-ionic surfactant was found to be most effective, however the mechanism is not known for the increase of lignocellulose hydrolysis by surfactant addition (Eriksson, Börjesson & Tjerneld, 2002). Thus, the effects of different Tween 80 concentrations on the productions of ligninolytic and hydrolytic enzymes playing significant roles in lignocellulose degradation were researched during the incubation time. According to obtained results, the best induction of ligninolytic enzymes of *P. eryngii* on peach cultures was performed by adding Tween 80 at the concentration of 0.025%, v/v. The activities of CMCase and xylanase enzymes were negatively affected by this compound, but other hydrolytic ones were positive. Similar results in terms of hydrolytic enzymes except BGLA activities from cherry cultures were determined. The BGLA activities increased only in Tween 80 of 0.025%, v/v. Among ligninolytic enzymes, the only MnP activities showed increaments in their activities with

increasing Tween 80 concentrations, but others were negatively affected by these. In this thesis, the Tween 80 was chosen for the additive compound to fermentation medium, because it was a non-ionic surfactants. The stimulatory effects of the detergent may be protection from enzyme denaturation, decreasing adsorption of ligninolytic and hydrolytic enzymes to lignocellulose. Also, the activity increments in the presence of Tween 80 may be due to the increasing enzyme stabilities by forming micellar like structures between the surfactant and any enzyme. On the other hand, this surfactant may change the nature of peach and cherry wastes. In contrast, no significant effect of Tween 80 addition on ligninolytic and hydrolytic enzymes from cherry cultures were detected, and these results were supported by Eriksson et al. (2002). As a result, the peach cultures were supplemented by the Tween 80 at a concentration of 0.025%, v/v, while there was no necessary to enrich cherry cultures with this compound for further studies.

3.2.5 Effect of Different Ammonium Nitrate Concentrations on Ligninolytic and Hydrolytic Enzymes Activities

3.2.5.1 Effect of Different Ammonium Nitrate Concentrations on Ligninolytic Enzymes Activities

In fungi, the nitrogen catabolite repression, homologous to the economic theory of microbial metabolism for carbon sets that, simple sources of nitrogen (e.g. ammonium) are preferred and consumed before. For some enzymes linked to secondary metabolism such as ligninolytic and hydrolytic enzymes, it is well known that this mechanism drives their extracellular productions; thus, some studies pointed that these enzymes are regulated by the nitrogen catabolite repression system and besides, their productions, it responds differentially to diverse nitrogen sources and/or concentration in the culture medium (Hernández et al., 2015).

As it can be observed in Figure 3.17, the joint effect of ammonium nitrate concentrations (1.0, 2.0, 4.0 g/L) on Lac activities by *P.eyngii* was studied under peach cultures containing 70 μM Cu^{2+}, 18 μM Fe^{2+} and 0.025%, v/v, Tween 80. This effect

was very significant and the maximum Lac activity was obtained by culture supplemented with 4.0 g/L ammonium nitrate, recorded as 28124.98±2345 U/L on the 20 day of incubation (Figure 3.17 (a)). This maximum value was 12.82-fold higher than that obtained in the peach culture including 2.0 g/L ammonium nitrate ($p<0.01$). So, this effect was considered in the subsequent experiments. There were many studies proving the stimulatory effect of both the nature and concentration of nitrogen sources on Lac activity (Patel et al., 2009). In secondary metabolic pathways such as Lac production, nutritional requirements have been differed depending on culture conditions and fungal strains. Some of them produced the high activity of Lac enzyme under nitrogen-limited conditions or vice versa (Mikiashvili, Wasser, Nevo & Elisashvili, 2006). The reason of obtaining a higher Lac activity might be better regulated the synthesis the enzyme in the presence of higher concentrations of ammonium nitrate when compared to control condition. According to results in this study, all ligninolytic enzymes reached to their maxima on the day of 20. Also, both Lac and MnP activities were induced by increase in ammonium nitrate concentrations in spite of LiP activities. As depicted in Figures 3.17 (b) and (c), the highest MnP activity was detected as 4697.38±102 U/L in the presence of 4.0 g/L ammonium nitrate, whereas the highest activity of LiP was 2333.18±45 U/L in 1.0 g/L ammonium nitrate. The highest activities of both MnP and LiP were approximately 1.14 and 1.45-fold higher than those in the peach culture including 2.0 g/L ammonium nitrate, respectively, ($p<0.05$). According to these results, the optimal ammonium nitrate concentration was found as 4.0 g/L for ligninolytic enzyme production by *P.eryngii* of peach SSF cultures. So, this effect was considered in the subsequent experiments. This result supported by El-Batal et al. (2015). According to their reports, Lac production by *P. ostreatus* was in excess with the higher concentration of nitrogen source, while some fungi such as *Lentinula edodes* and *P. chrysosporium* provide examples of Lac production in nitrogen sufficient media.

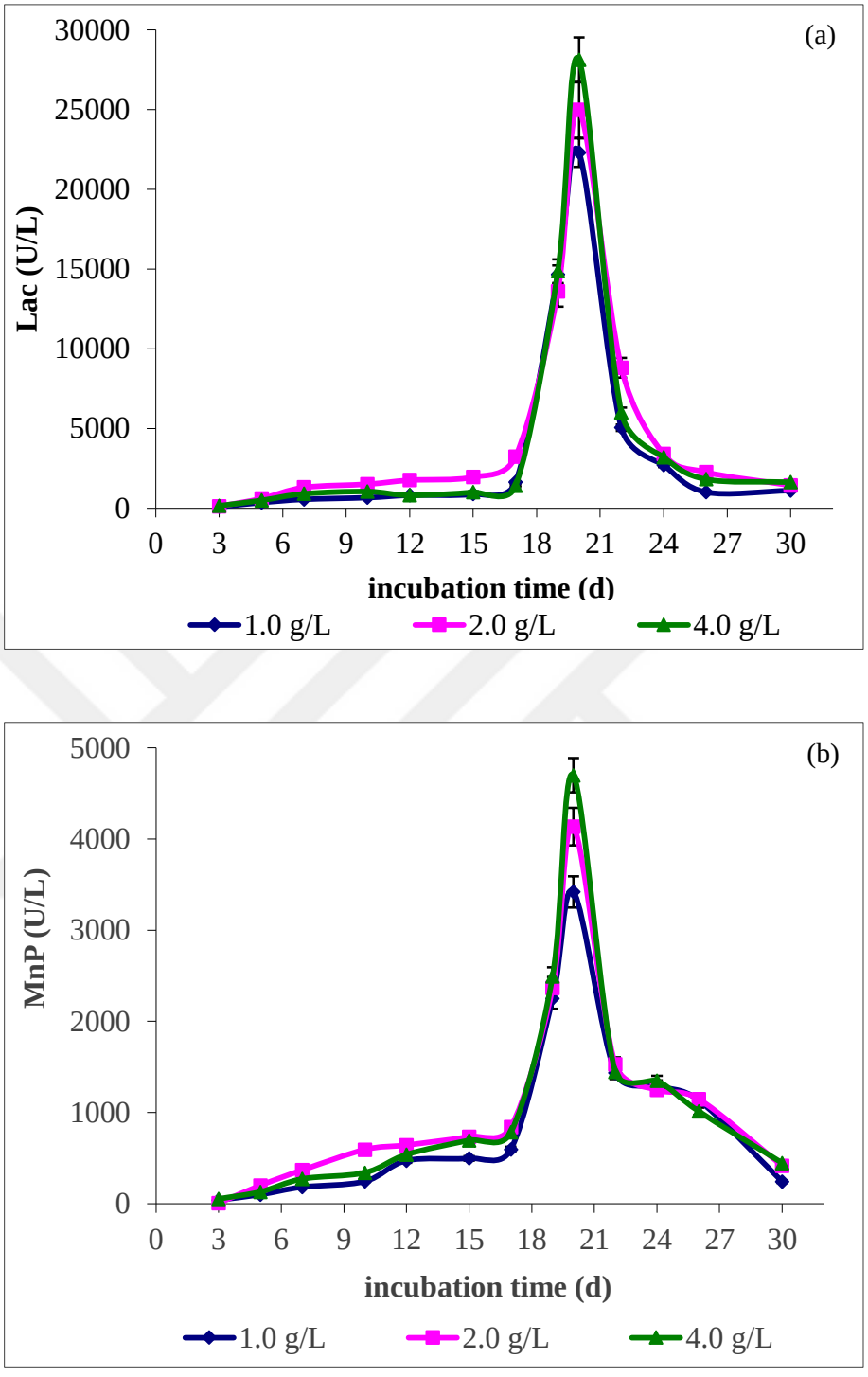


Figure 3.17 The influence of ammonium nitrate concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments

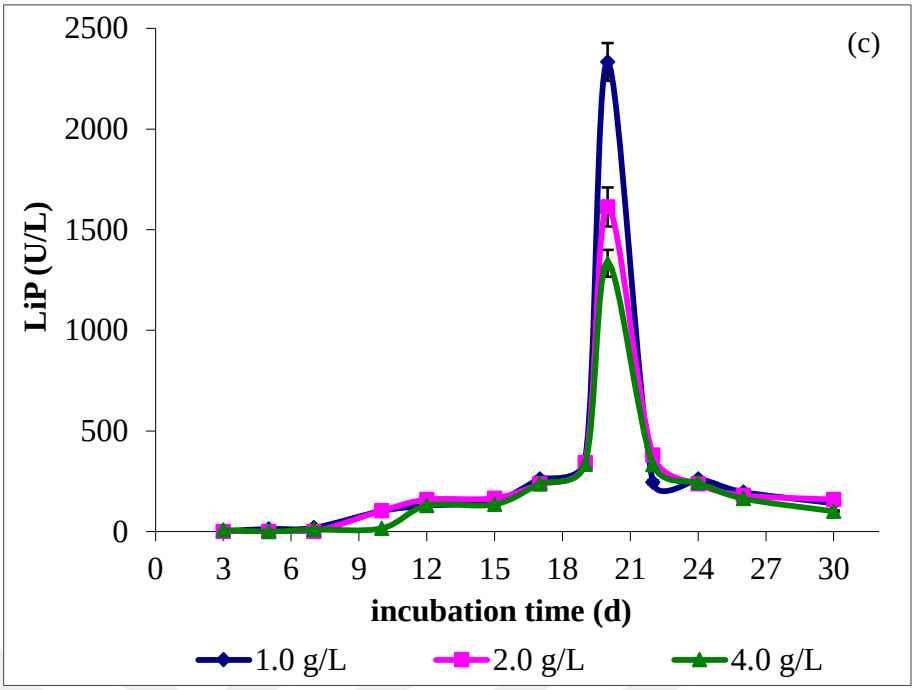


Figure 3.17 The influence of ammonium nitrate concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

As depicted in Figure 3.18 (a), the increment in ammonium nitrate concentrations up to 4.0 g/L, positively affected Lac activities by *P.eryngii* via cherry culture. The highest Lac activity was determined as 4976.63±75 U/L in the presence of 4.0 g/L ammonium nitrate on the 15th day of incubation. The maximum Lac activities were recorded on the 15th day of incubation in all investigated ammonium nitrate concentrations, and all of them were very close to each other. Similar to Lac activities, the MnP activities increased depending on ammonium nitrate concentrations. The highest value was attained as 2064.68±35 U/L on the 5th of incubation in 2.0 g/L ammonium nitrate, and it was 2.67-fold higher than that of obtained activities in 4.0 g/L ammonium nitrate shown in Figure 3.18 (b) ($p<0.05$). In addition, the highest LiP activities were detected as 69.89±2.5 U/L in the presence of 2.0 g/L ammonium nitrate as seen in Figure 3.18 (c). Also, in the cultures supplemented with 4.0 g/L ammonium nitrate, an inhibition of LiP activities was observed. According to these results, the optimal ammonium nitrate concentration was found as 2.0 g/L for ligninolytic enzyme production by *P.eryngii* of cherry SSF cultures.

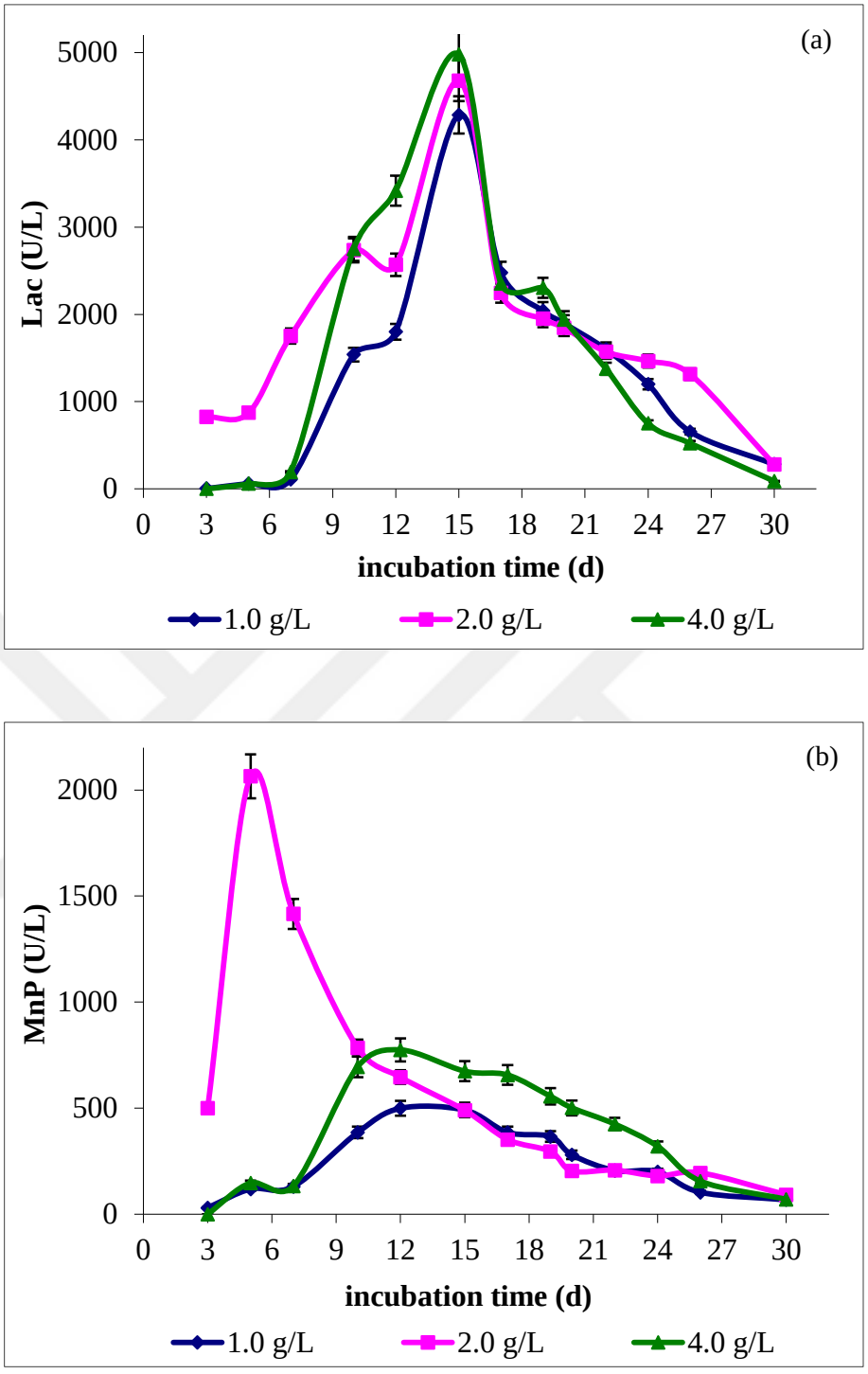


Figure 3.18 The influence of ammonium nitrate concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments

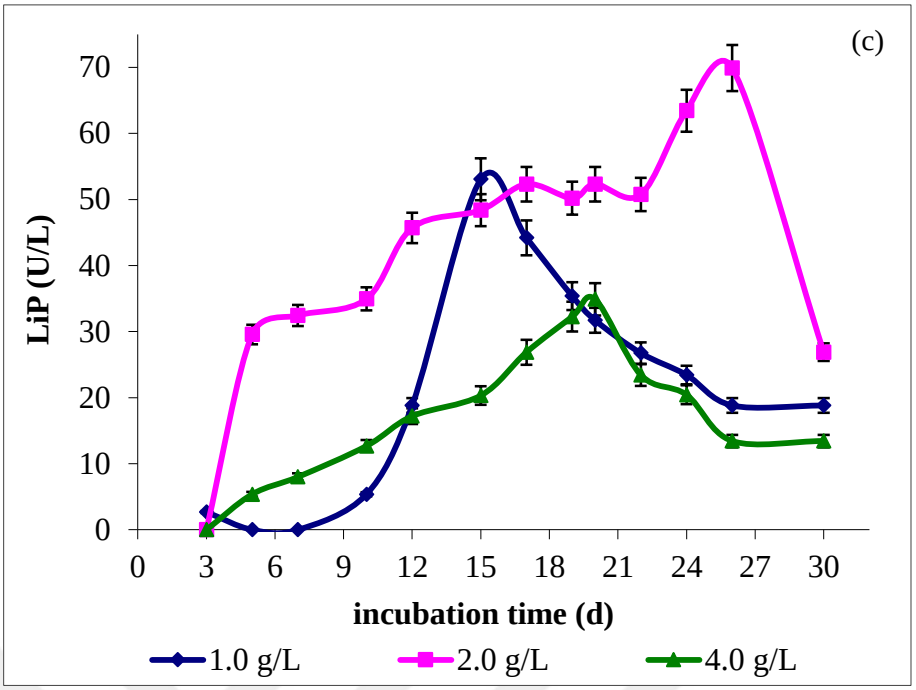


Figure 3.18 The influence of ammonium nitrate concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

3.2.5.2 Effect of Different Ammonium Nitrate Concentrations on Hydrolytic Enzymes Activities

As depicted in Figure 3.19, the ammonium nitrate concentrations have been analyzed in order to find the optimal ones for hydrolytic enzyme productions as well as ligninolytic enzymes by *P.eryngii* of peach cultures under SSF conditions. For this, experiments at different ammonium nitrate concentrations (1.0; 2.0; 4.0 g/L) with 70 µM copper, 18 µM iron, 0.025%, v/v, Tween 80 were carried out. The cultures respectively showed the highest CMCase and xylanase activities of about 11.7±0.09 and 3.28±0.02 U/mL when the medium was supplemented with 4.0 and 1.0 g/L ammonium nitrate (Figures 3.19 (a) and (b)), and there were insignificant differences in xylanase activities between *P. eryngii* cultures including 1.0 and 4.0 g/L ammonium nitrate ($p>0.05$). In addition, the highest activity of exoglucanase were determined as 5.28±0.02 U/L in 2.0 g/L concentration of ammonium nitrate, while the highest BGLA activity was obtained as 28.14±1.5 U/L in 4.0 g/L concentration of ammonium nitrate and nearly higher that attained in the cultures with 1.0 g/L ammonium nitrate (Figures

3.19 (c) and (d)). According to results, the maximal activities of CMCase and xylanase were observed at the beginning of incubation, but they in conditions including 2.0 g/L ammonium nitrate were determined on the 5th day.

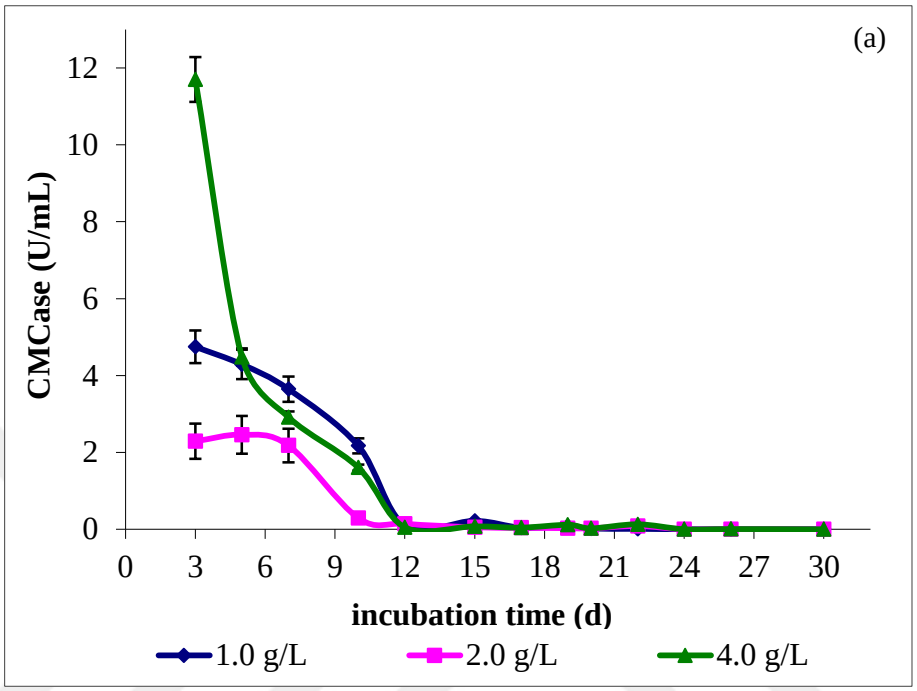
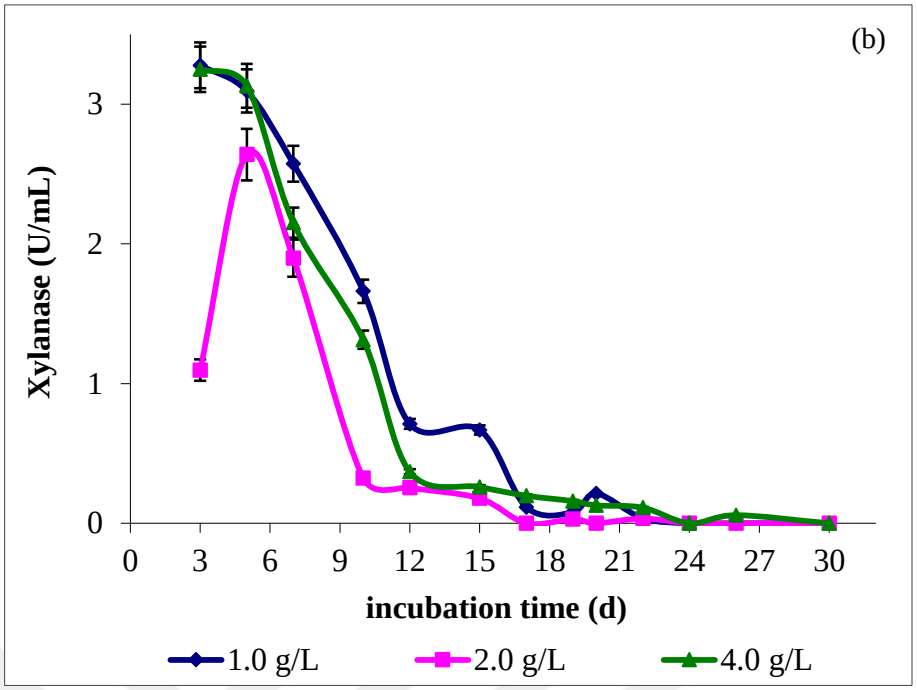


Figure 3.19 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on ammonium nitrate concentrations. The values are the mean ±SD for experiments of three separate experiments



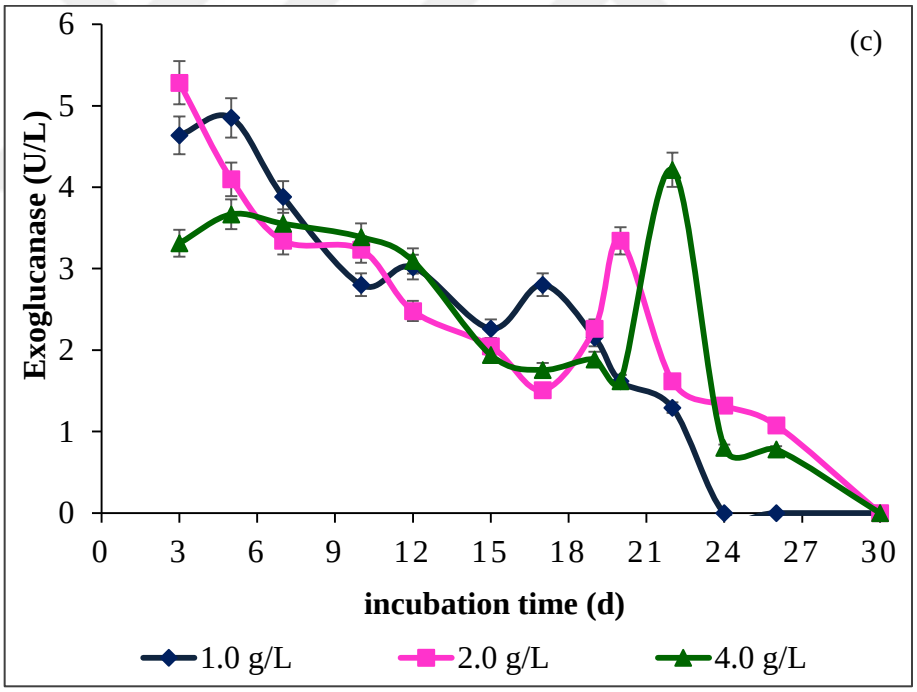


Figure 3.19 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on ammonium nitrate concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

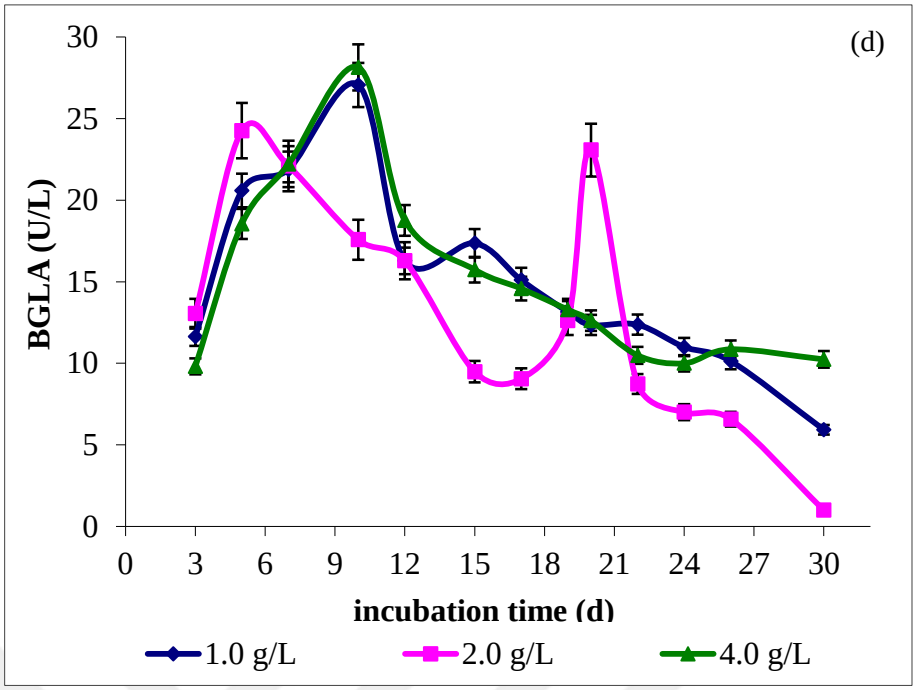


Figure 3.19 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on ammonium nitrate concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

As depicted in Figures 3.20 (a), when the cherry culture media were supplemented with ammonium nitrate concentrations, the maximum CMCase and xylanase activities were obtained as 3.44±0.02 and 2.60±0.01 U/mL in the presence of 1.0 g/L and 2.0 g/L ammonium nitrate of cherry cultures on the day of 3rd and 5th, respectively, (Figures 3.20 (a) and (b)). Very low BGLA and exoglucanase activities were determined in all cultures, supplemented with different ammonium nitrate concentrations (Figures 3.20 (c) and (d)). Otherwise, the highest BGLA activity was attained as 71.59±3.2 U/L in 4.0 g/L ammonium nitrate (17th day) as shown in Figure 3.20 (d). There were insignificant differences between maximal CMCase activities in supplemented with 1.0 and 4.0 g/L ammonium nitrate cultures ($p>0.05$), but very low maximal CMCase activity was detected when compared to others in aforementioned conditions similar to comparison of maximal xylanase activities.

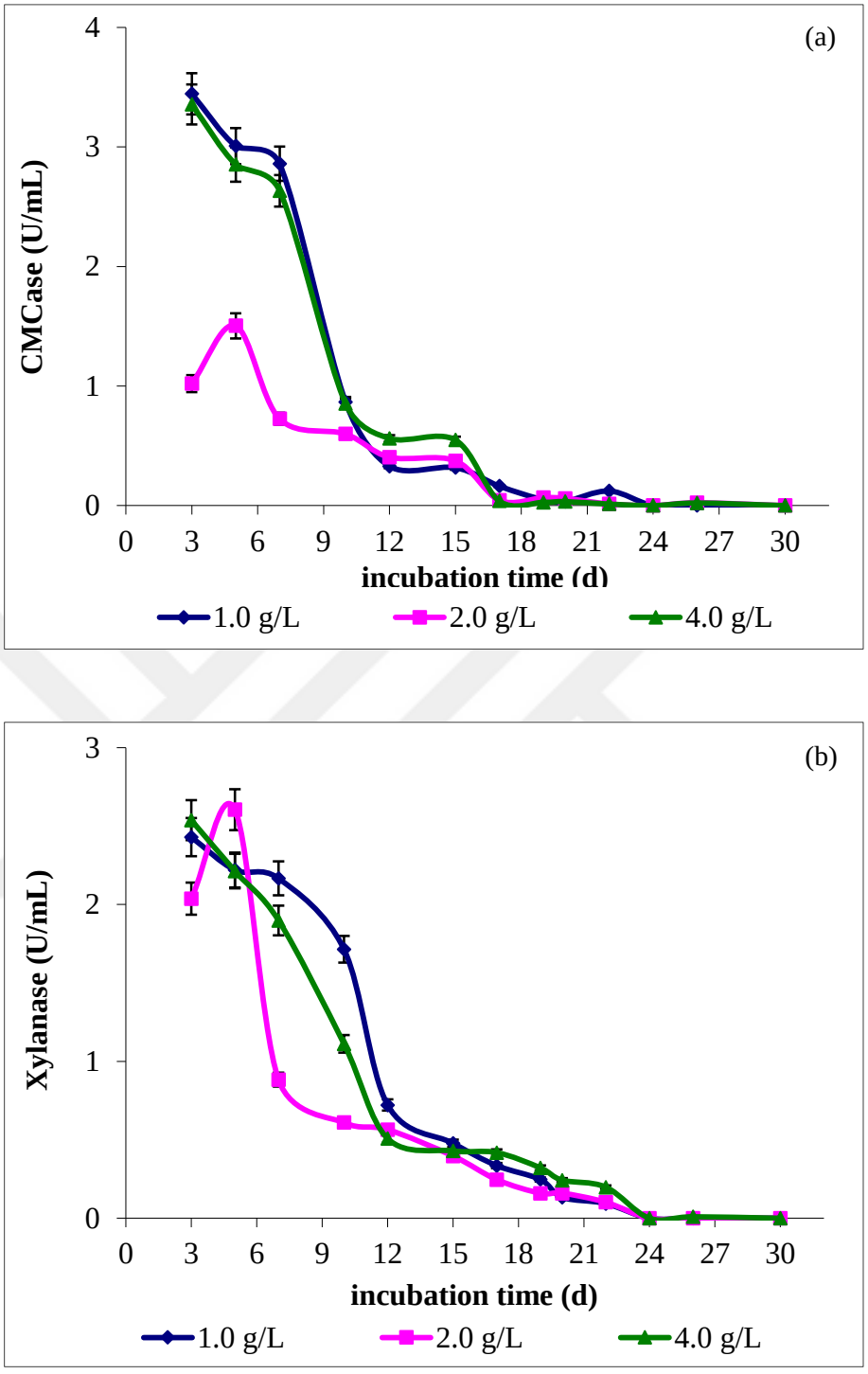
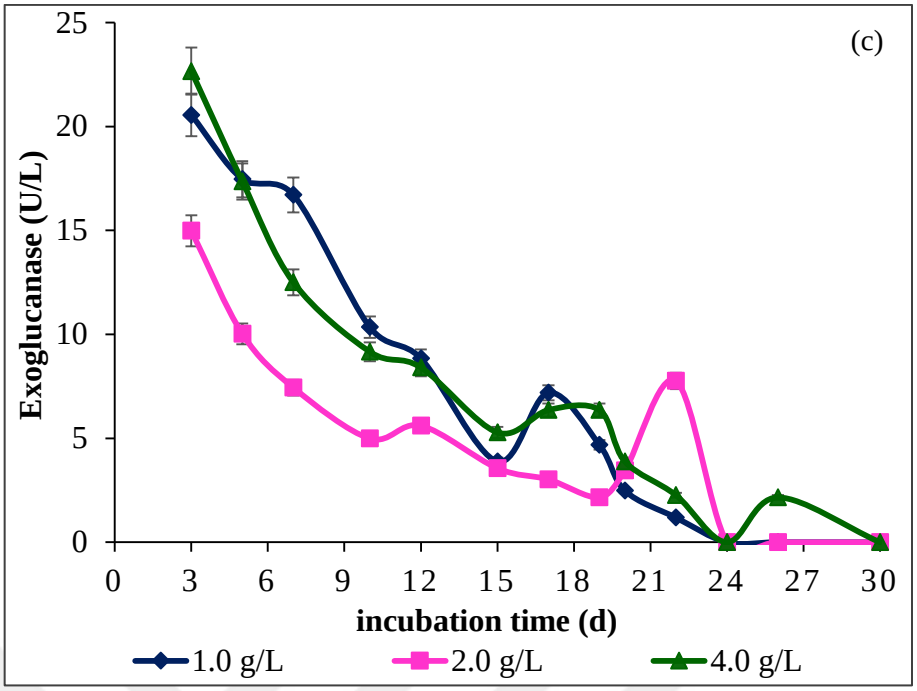


Figure 3.20 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on ammonium nitrate concentrations. The values are the mean ±SD for experiments of three separate experiments



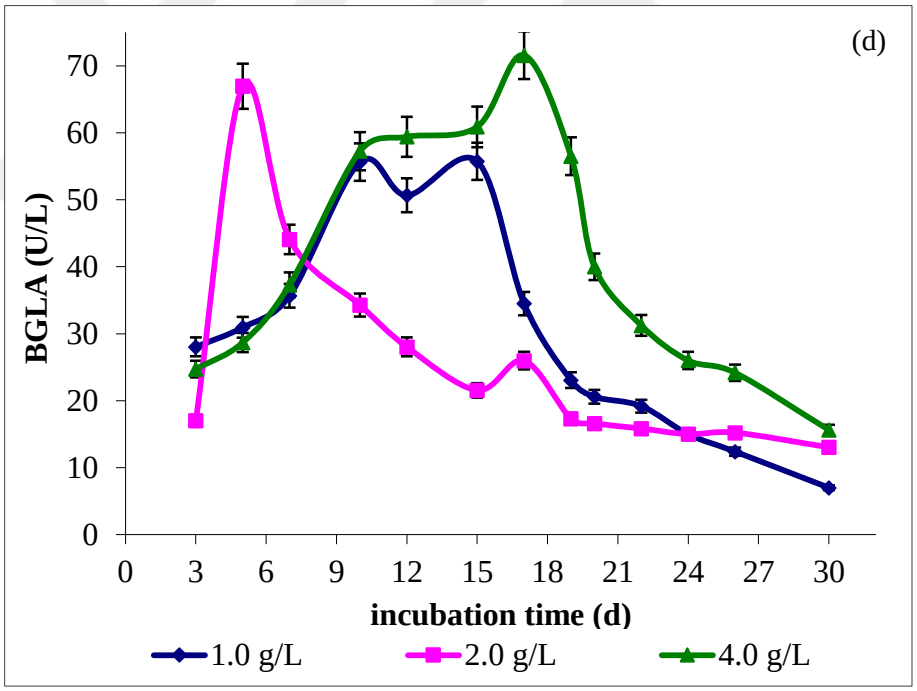


Figure 3.20 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on ammonium nitrate concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

There are a few studies that have been initiated by different authors to know the effect of nitrogen sources and/or amounts on ligninolytic enzymes production of some WRF (Prasher & Chauhan, 2015). The aim of the present study is to elucidate the

influence of nitrogen amounts (ammonium nitrate) on ligninolytic and hydrolytic enzymes productions of a white rot fungus, *P. eryngii* under SSF conditions. The obtained results demonstrate that in SSF of peach waste the supplementation of nitrogen source above 2.0 g/L significantly affect the most of enzymes yield except LiP and exoglucanase. Thus, high amounts of nitrogen stimulated the ligninolytic and hydrolytic enzymes productions of *P. eryngii*. On the other hand, the maximal ligninolytic activities of cherry cultures were determined in the presence of 2.0 g/L ammonium nitrate. The hydrolytic ones except xylanase were reached to their maxima when the culture medium was supplemented by 4.0 g/L ammonium nitrate. Also, the xylanase activities in 2.0 and 4.0 g/L ammonium nitrate were very close to each other. Since decolorizations of some dyes will be carried out at a later stage of thesis, ligninolytic enzyme activities are taken into account when determining the optimum concentration of ammonium nitrate. The optimal concentration was found as 4.0 g/L for peach cultures, whereas it was 2.0 g/L for cherry cultures.

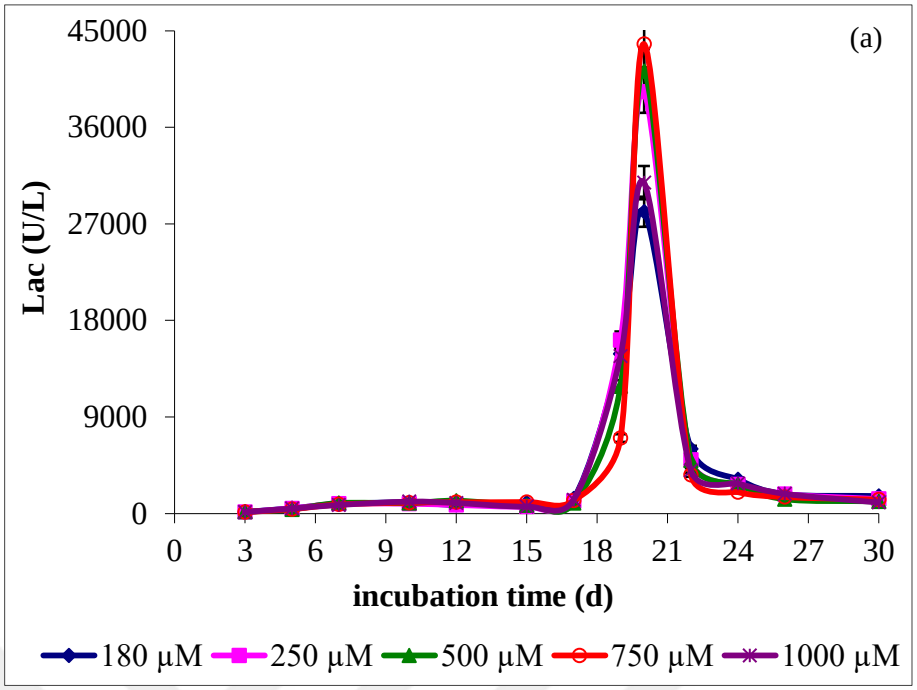
3.2.6 Effect of Different Manganese Concentrations on Ligninolytic and Hydrolytic Enzymes Activities

3.2.6.1 Effect of Different Manganese Concentrations on Ligninolytic Enzymes Activities

Manganese has often been discussed as being inducers, enhancers or mediators of some extracellular enzymes, e.g. ligninolytic and hydrolytic ones. It is known that supplementing growth medium of fungi with Mn^{2+} leads to significantly enhance these enzymes activities.

As depicted in Figure 3.21 (a), the Lac activity by *P.eryngii* ranged from 136.76±9.2 to 43 761.33±3845 U/L for all researched Mn^{2+} concentrations (180, 250, 500, 750, 1000 μM in basal medium) under SSF conditions of peach cultures. The highest activity value of this enzyme was detected as 43 761.33±3845 U/L on the 20^{th} day of incubation. This value was 19.95-fold greater than that of the value in non-optimized culture medium ($p<0.01$). Lac production was therefore enhanced under such

condition. According to Figure 3.21 (a), the Lac productions by *P. eryngii* were gradually increased up to 750 μM Mn^{2+} concentrations then decreased. Contrary to the Cu^{2+} and Fe^{2+}, the Mn^{2+} showed stimulatory effect on Lac activity in *P. eryngii* under SSF. According to literature, Mn^{2+} was also effective inducer of Lac in many WRF (Piscitelli et al., 2011). Positive impact of some Mn^{2+} concentrations on Lac activity, Piscitelli et al. (2011) may explained by induction of gene transcription. The increments in Mn^{2+} concentration increased Lac enzyme activities. Similar observations were recorded for MnP and LiP activities of *P.eryngii* under SSF condition with supplemented Mn^{2+} concentrations of peach cultures. Also, the strain produced maximum MnP and LiP activities of 5036.82±48 and 1791.61±15.5 U/L in the presence of 750 and 500 μM Mn^{2+}, respectively (Figures 3.21 (b) and (c)). Actually, the all ligninolytic enzymes reached to their maximum activities on day of 20 in all tested Mn^{2+} concentrations. Differences of Lac activities in both 250 and 750 Mn^{2+} conditions was nearly 10%, and the activity in 500 μM was observed as 41 232.67±2845 U/L. On the other hand, there were insignificant differences among MnP activities in all conditions ($p>0.05$), and also the highest value of this enzyme activity was 1.07-fold higher than obtaining from the culture with 180 μM Mn^{2+} (4768.04 U/L). In addition, the highest activity of LiP was 1.34-fold higher than the activity from the just mentioned culture.



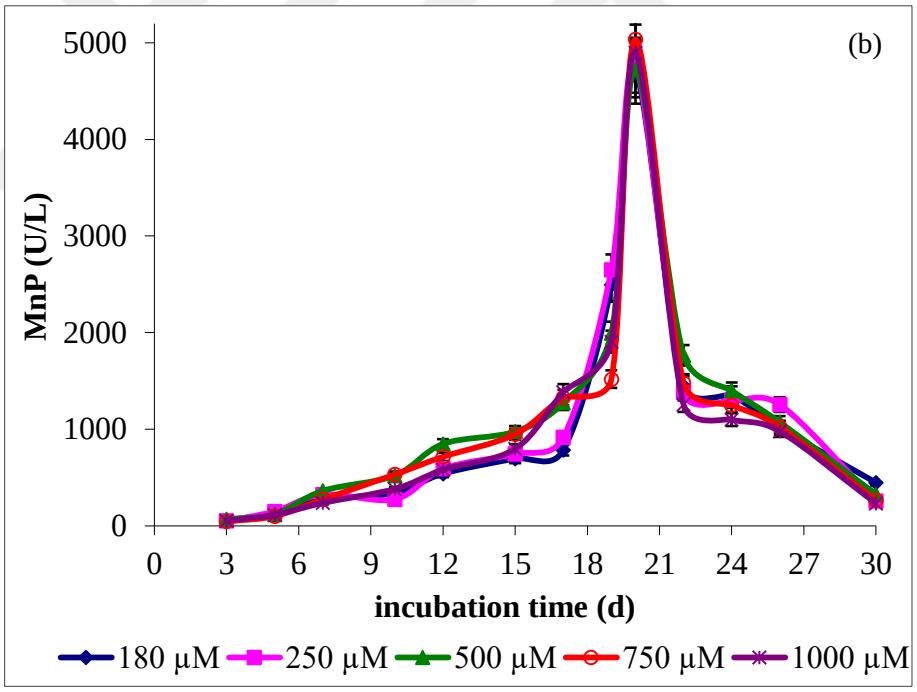


Figure 3.21 The influence of manganese concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments

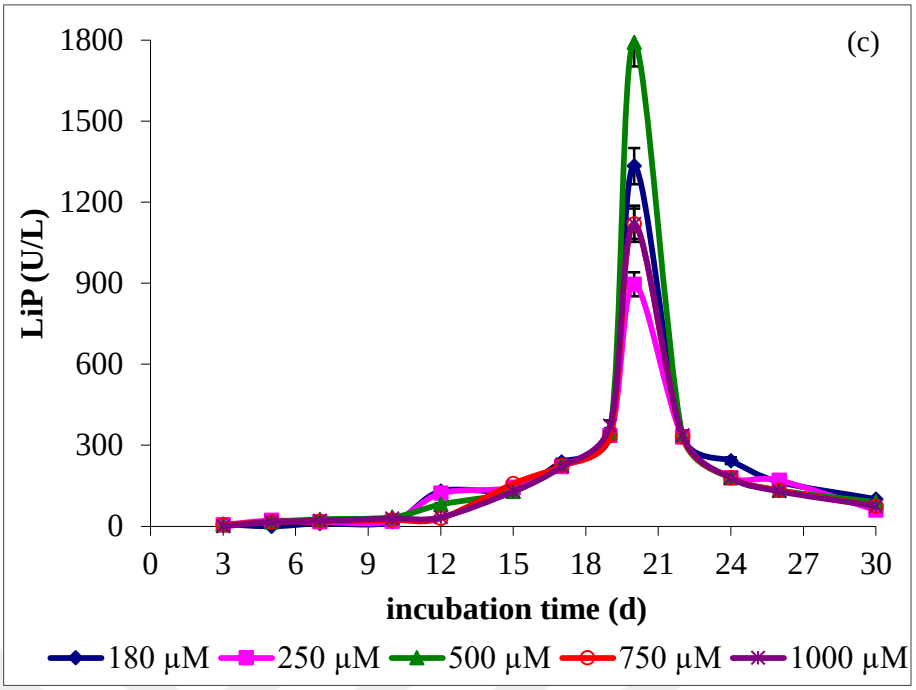


Figure 3.21 The influence of manganese concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using peach wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

Recently, the capability of white-rot fungus, *P. eryngii*, to produce of Lac with high activity on SSF systems of cherry cultures in other investigated parameters was described. Herein, the aim of this study was to research the effect of Mn^{2+} on the production of ligninolytic and hydrolytic enzymes by *P. eryngii* cultivated in cherry cultures. The effect of Mn^{2+} supplementations (180, 250, 500, 750, 1000 µM) on the extracellular ligninolytic enzyme activities of *P.eryngii* was tested shown in Figure 3.22. There were insignificant differences in Lac activities obtained from cultures 180 µM (4677.26±75 U/L) and 250 µM Mn^{2+} supplemented medium (4658.63±72 U/L) ($p>0.05$), but the Lac activities very lowered under 500 and 1000 µM manganese supplemented conditions as can be seen in Figure 3.22 (a). The Lac and MnP activities generally showed similar trends in all conditions. On the other hand, the MnP activities were very affected by different Mn^{2+} concentrations, and the maximum MnP activity was found as 2064.68±35 U/L on the 5^{th} day of incubation in the presence of 180 µM Mn^{2+} (Figure 3.22 (b)). The MnP activity in 250 µM Mn^{2+} reached to its maxima on the 5^{th} day as 1297.45±30 U/L and this value was 1.59-fold lower than the highest MnP activity ($p>0.05$). The maximal MnP activities in all conditions were

demonstrated on the same day of incubation. In addition, the LiP activities in all investigated Mn^{2+} concentrations except 500 µM Mn^{2+} increased when compared to the activity obtaining from the culture with 180 µM Mn^{2+} (69.89±2.5 U/L) (Figure 3.22 (c)).

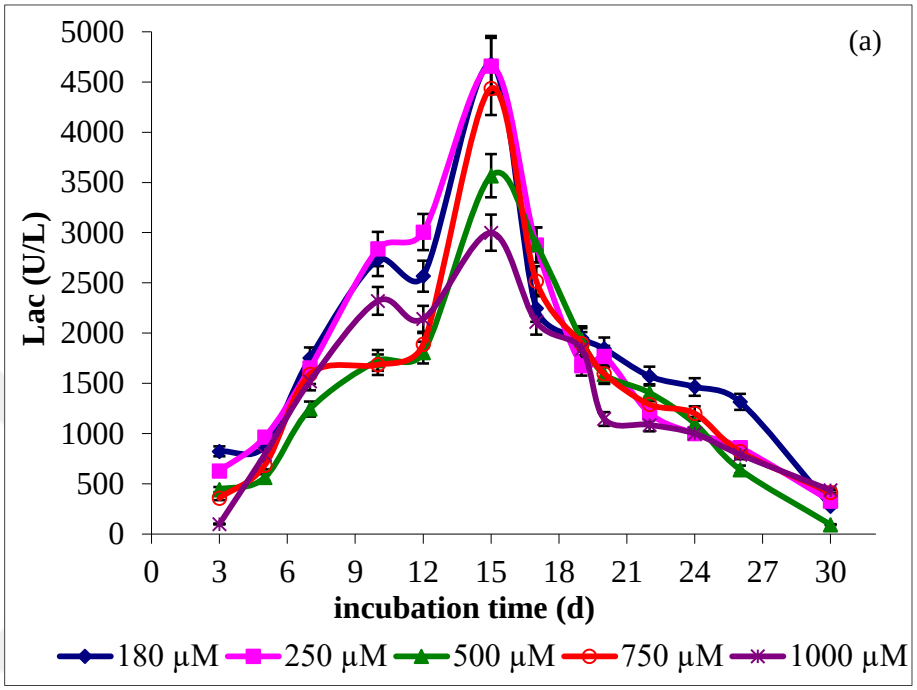
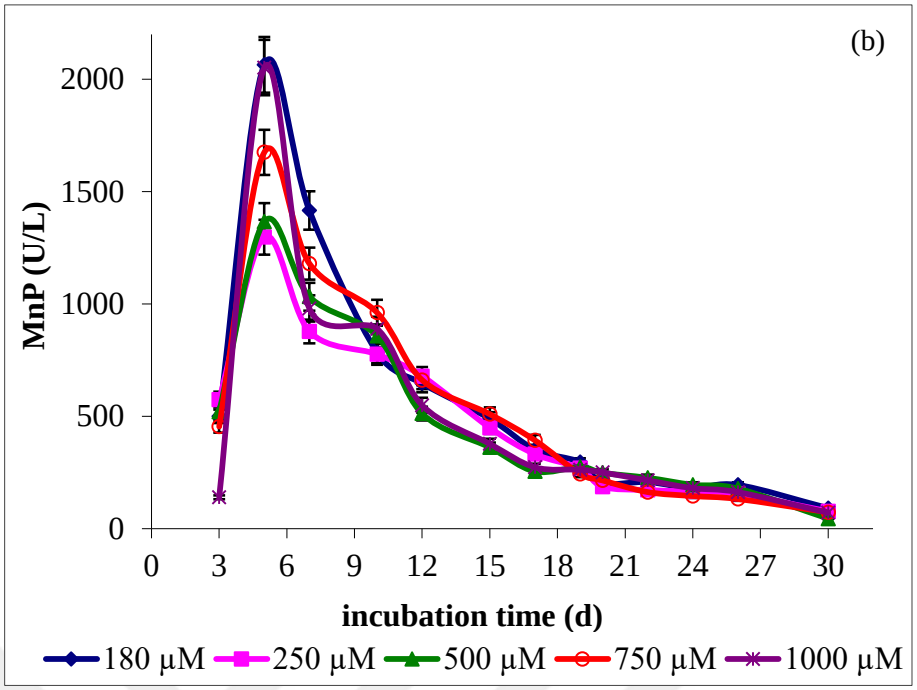


Figure 3.22 The influence of manganese concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments



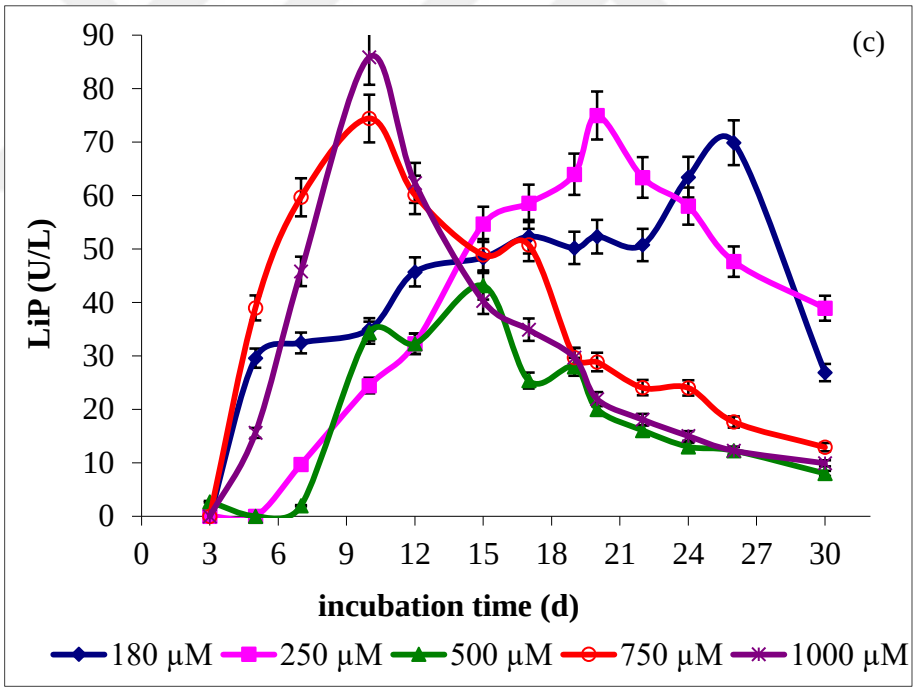


Figure 3.22 The influence of manganese concentrations on ligninolytic enzymes activities (Lac (a), MnP (b), LiP (c)) on SSF cultures using cherry wastes throughout incubation time. The values are the mean ±SD for experiments of three separate experiments (continued)

3.2.6.2 Effect of Different Manganese Concentrations on Hydrolytic Enzymes Activities

Figure 3.23 illustrated the role of Mn^{2+} concentration in hydrolytic enzyme activities of *P.eryngii* of peach cultures time-dependent. Results of experiments implied that enhancement of Mn^{2+} concentration played significant roles in enhancing the hydrolytic enzyme productions. Among the tested concentrations of Mn^{2+}, supplementation of 500 µM Mn^{2+} induced the CMCase activity of about 12.2±0.09 U/mL on the 3 day, but the activity from the culture including 180 µM Mn^{2+} was obtained as 11.7±0.09 U/mL (Figure 3.23 (a)).The CMCase activities were increased by rising Mn^{2+} conditions up to 500 µM, and then decreased rapidly above concentrations of 500 µM. On the other hand, the additions of Mn^{2+} except 750 µM to culture media, were increased the xylanase activities compared to the activities in the media including 180 µM Mn^{2+}, but the changes activities among these concentrations were unimportant. The maximum activity of xylanase was recorded as 3.48±0.02 U/mL in 1000 µM Mn^{2+}, while the maximum BGLA activity was attained as 34.78±2.5 U/L in 250 µM of Mn^{2+} (Figures 3.23 (b) and (d)). The maximal exoglucanase activities under all optimized conditions were determined on the same day of incubation, and there were insignificant differences between the highest activities in 500, 750, 1000 µM Mn^{2+} ($p>0.05$) (Figure 3.24 (c)). These experimental results emphasized that Mn^{2+} was the suitable inducer for hydrolytic enzyme productions in *P.eryngii*.

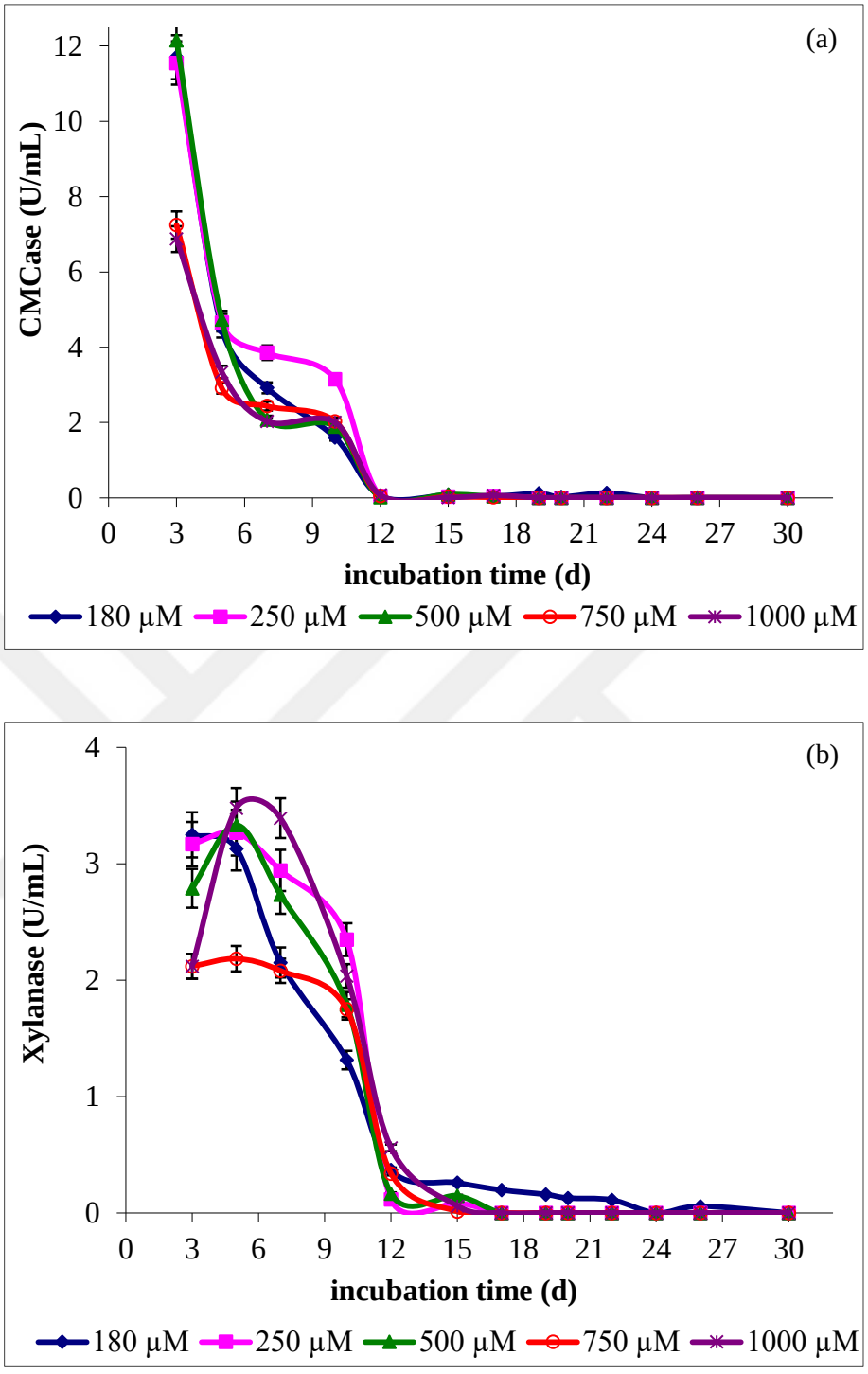
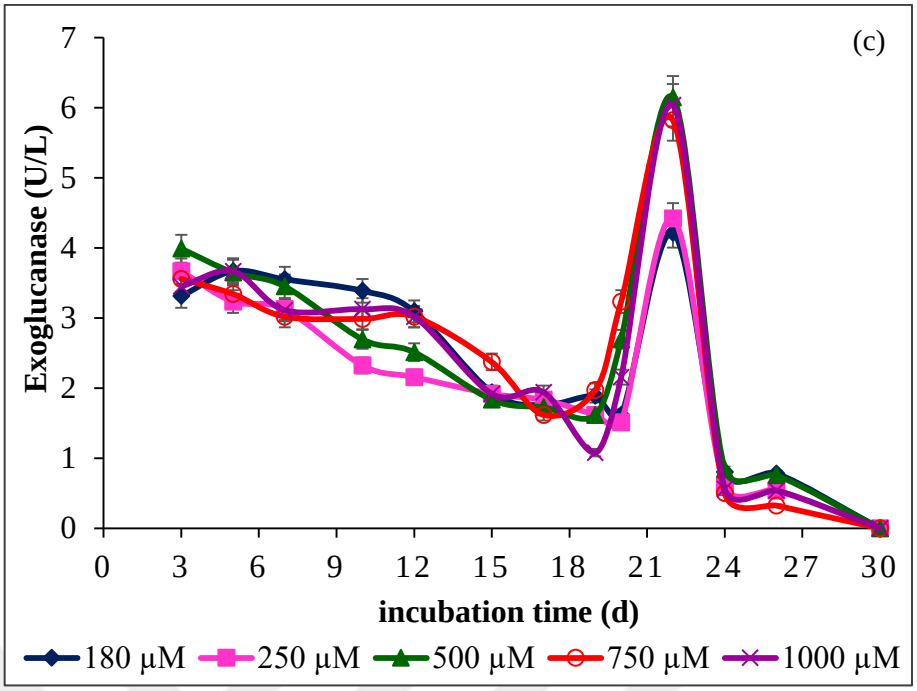


Figure 3.23 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on manganese concentrations. The values are the mean ±SD for experiments of three separate experiments



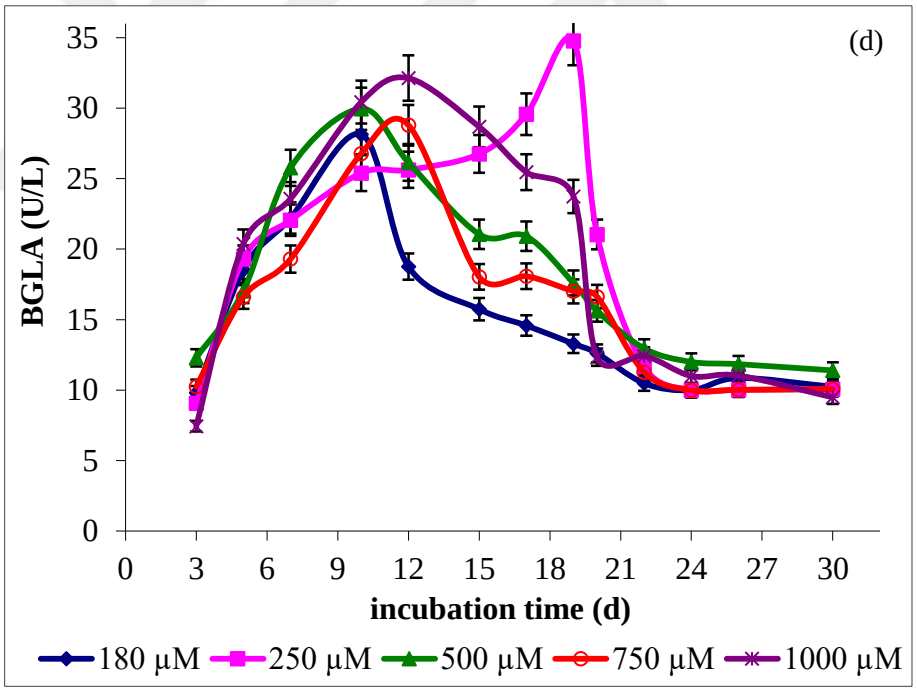


Figure 3.23 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using peach wastes depending on manganese concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

Five different concentrations of Mn^{2+} were tested for their effects on hydrolytic enzymes activities of *P.eryngii* under SSF cherry cultures as seen in Figure 3.24. In all

experiments, Mn^{2+} induced CMCase, xylanase and exoglucanase activities, but not BGLA activities. There were insignificantly differences among the CMCase activities in control (180 µM) and supplemented with Mn^{2+} except 750 and 1000 µM ($p>0.05$), the highest activity was recorded as 1.73±0.01 U/mL in supplemented 1000 µM Mn^{2+} culture (Figure 3.24 (a)). Also, the maximal xylanase and BGLA activities were determined as 3.73±0.02 U/mL and 66.95±3.7 U/L in the presence of 1000 and 180 µM Mn^{2+}, respectively, as can be seen in Figures 3.24 (b) and (d)). The maximum exoglucanase activity was recorded as 18.87±0.9 U/L in 1000 µM Mn^{2+} (Figure 3.24 (c)).

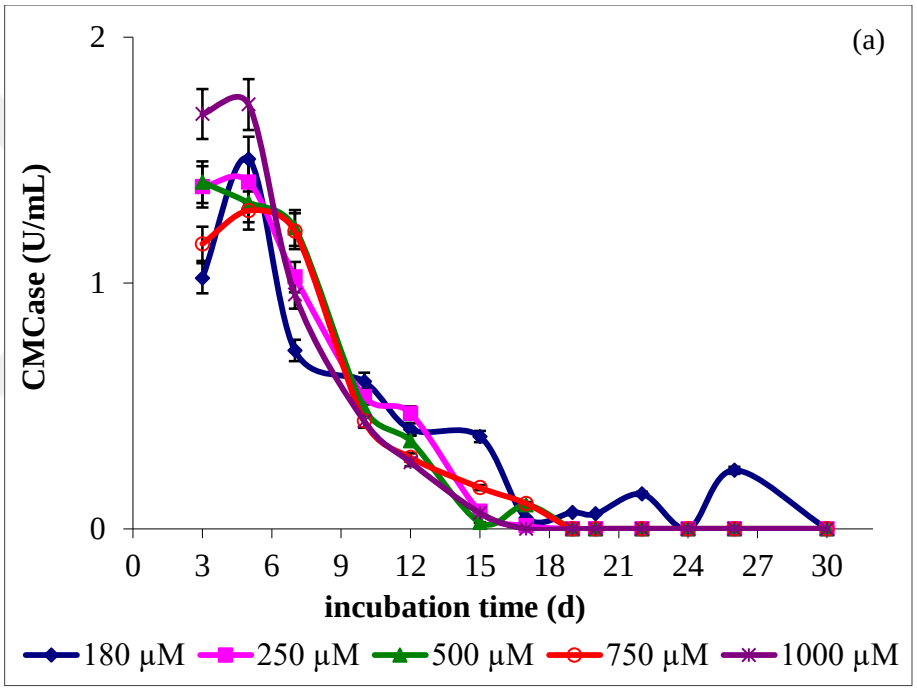
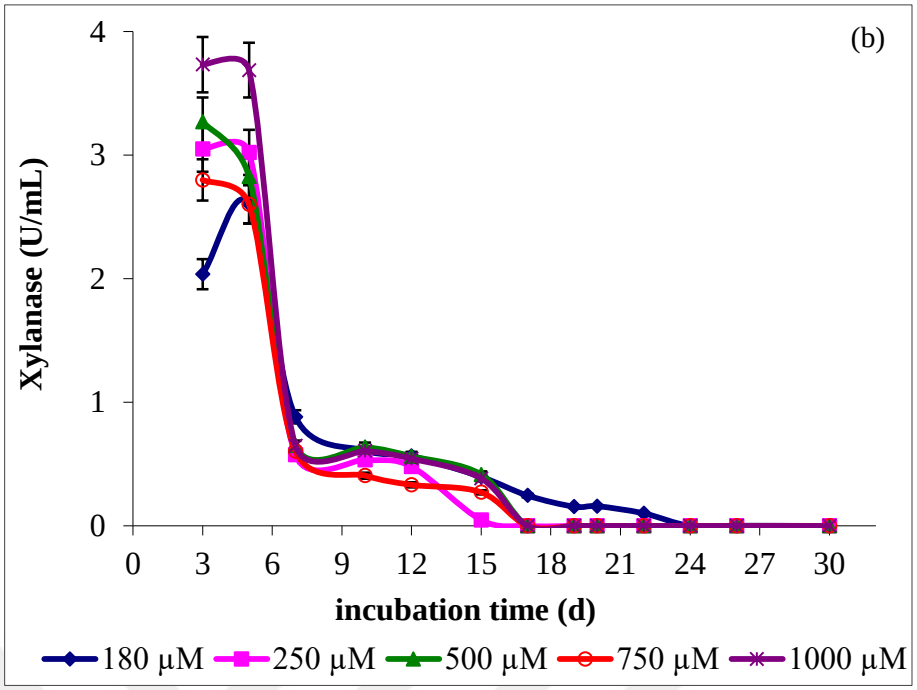


Figure 3.24 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on manganese concentrations. The values are the mean ±SD for experiments of three separate experiments



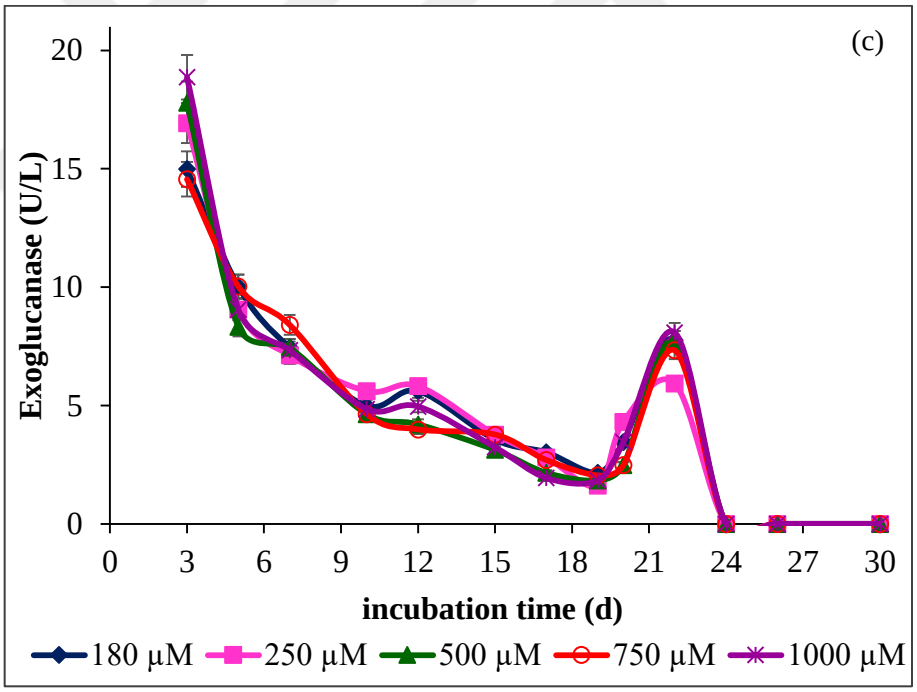


Figure 3.24 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on manganese concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

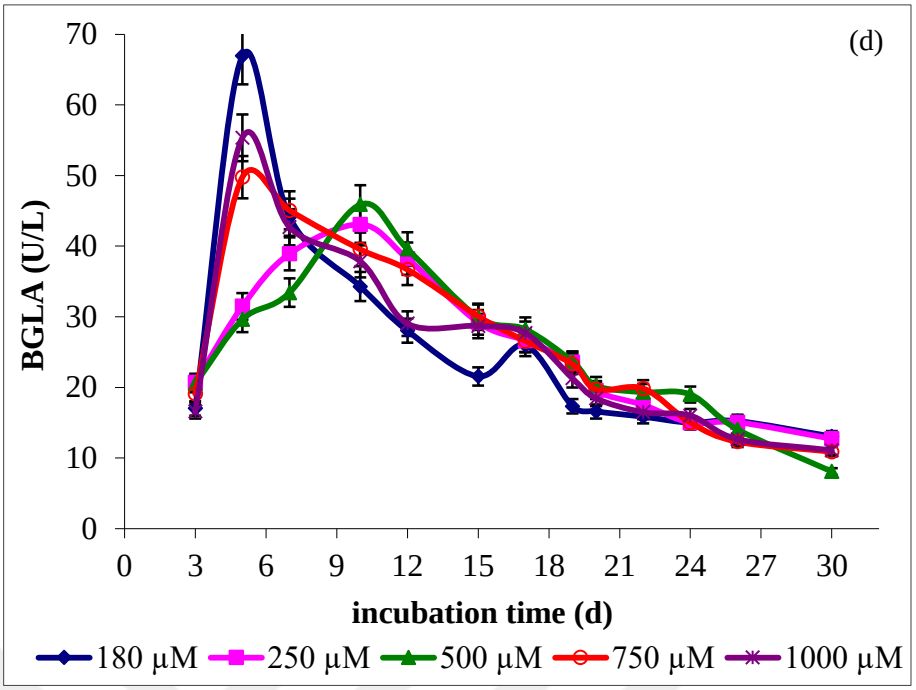


Figure 3.24 Variations of hydrolytic enzymes activities (CMCase (a), xylanase (b), exoglucanase (c), BGLA (d)) on SSF cultures using cherry wastes depending on manganese concentrations. The values are the mean ±SD for experiments of three separate experiments (continued)

Some divalent cations are necessary for ligninolytic and hydrolytic enzyme synthesis, activities or stabilities by *P. eryngii* under SSF. Mn^{2+} are important of them, especially MnP. Because it is served as a cofactor in its catalytic cycle. For this purpose, the effects of varying concentrations of Mn^{2+} on these enzyme activities were investigated during incubation. The maximal ligninolytic activities of peach cultures were determined on the same day of incubation (20 day). The Mn^{2+} concentrations above 180 µM except 500 µM did not stimulate LiP activities, but the Lac, MnP, exoglucanase and BGLA activities were increased by adding Mn^{2+}. The changes in CMCase activities were similar to each other when the culture was enriched by different Mn^{2+} concentrations, 180, 250, 500 µM. Their activities in 750 and 1000 µM were lower than those. The xylanase activities were generally not induced by the incerements in Mn^{2+}. In contrast to peach culture, the high concentration of Mn^{2+} suppressed the Lac, MnP, CMCase and BGLA activities from cherry cultures and xylanase activities were induced by the supplementation of Mn^{2+} to this culture. There were small changes in exoglucanase activities with Mn^{2+} increments. Thus, optimum Mn^{2+} concentrations were determined as 250 and 180 µM for peach and cherry

cultures, respectively when the ligninolytic enzymes, especially Lac were chosen as reference enzymes.

Table 3.4 demonstrated the optimal values for both cultures in terms of higher yield ligninolytic and hydrolytic enzymes productions by *P. eryngii* under SSF conditions.

Table 3.4 Optimization conditions of both cultures for high yield ligninolytic and hydrolytic enzymes activities

Optimization parameter	Non-optimized culture	Optimized peach culture	Optimized cherry culture
Pretreatment	--------	No required	No required
Cu^{2+}	70 µM	70 µM	1000 µM
Fe^{2+}	18 µM	18 µM	1000 µM
Tween 80	--------	0.025% (v/v)	--------
NH_4NO_3	2.0 g/L	4.0 g/L	2.0 g/L
Mn^{2+}	180 µM	250 µM	180 µM

The maximum ligninolytic and hydrolytic enzymes activities on both cultures before and after optimization were demonstrated in Table 3.5. Generally, all enzymes activities except exoglucanases in peach and cherry cultures were increased after optimizations.

Table 3.5 The maximum ligninolytic and hydrolytic enzymes activities on both cultures before and after optimization

	Peach Culture		**Cherry Culture**	
	Non-Optimized Condition	**Optimized Condition**	**Non-Optimized Condition**	**Optimized Condition**
Lac (U/L)	2193.06±50.4	39313.75±2815	1297.22±34.6	4677.26±75
MnP (U/L)	732.73±19.8	4768.04±36	430.91±8.6	2064.68±35
LiP (U/L)	94.09±4.1	895.80±7.6	37.63±1.1	69.89±2.5
CMCase (U/mL)	3.64±0.02	11.55±0.09	1.30±0.01	1.50±0.01
Xylanase (U/mL)	3.08±0.02	3.27±0.02	1.16±0.01	2.60±0.01
Exoglucanase (U/L)	4.31±0.2	4.42±0.01	15.09±0.03	14.99±0.02
BGLA (U/L)	12.72±0.03	34.77±0.09	23.39±0.05	66.95±3.7

Zinat et al. (2013) researched the conversion of superfluous jute plants and jute stick into upgraded animal feed by SSF using a cellulolytic fungus, *P. sajor-caju*, and they found the highest CMCase activity as 1.21 U/mL. Ashger et al. (2016) revealed that studies on ligninolytic and hydrolytic enzymes by *P. eryngii* are still lacking. In their study, various lignocellulosic substrates were used for the production of lignocellulose-degrading enzymes by *P. eryngii*. Among all substrates utilized, the SSF of banana stalk favored the higher production of crude ligninolytic extract and CMCase, exoglucanase and BGLA activities were 0.198, 0.098 and 0.128 U/mL, respectively from banana stalk on 6th day of fermentation. The CMCase activity was lower than the obtaining values from both cultures in this thesis. The ligninolytic and hydrolytic enzymes were simultaneously produced in their study in contrast to those of this thesis. In another study, *P. ostreatus* cultures in 1 L Erlenmeyer flasks containing 500 mL of medium with 1% microcrystalline cellulose were performed and the cellulase activity reached a maximum value of 3.19 U/mL after 9 days of fermentation (Liguori et al., 2015). Akpinar & Ozturk Urek (2014) also found that the highest Lac activity was obtained as 1618.5 U/L on day 12 of cultivation using apricot. The highest MnP activity was attained as 570.82 U/L on day 17 in pomegranate culture and about the same as apricot culture. The maximum LiP value was 16.13 U/L in apricot cultures in that study. On the other hand, An et al. (2016) investigated the ligninolytic and hydrolytic enzyme production (CMCase, xylanase, and Lac) by *P. ostreatus* using various substrates and found CMCase, xylanase, Lac activities as 3152, 3064, 543 U/L, respectively. When compared to literature, *P. eryngii* can be named as powerful fungus because of its capability of ligninolytic and hydrolytic enzyme productions with high yield. And also, the effective degradation of lignocellulose could be performed by this organism. Tables 3.6 and 3.7 summarized the comparison of maximum ligninolytic and hydrolytic enzymes activities under optimal conditions of both cultures in this thesis with literature. The increased values of the maximum ligninolytic and hydrolytic enzymes activities under optimal conditions of both cultures values in this thesis compared with the literature are shown in the parentheses.

Table 3.6 Comparison of maximum ligninolytic and hydrolytic enzymes activities from peach cultures after optimization with literature

Results in Thesis	**Enzyme Activities**	**Microorganism**	**Reference**
Lac activity 39 313.75 U/L	1618.5 U/L (24.29↑)	*P. eryngii*	Akpinar & Ozturk Urek (2014)
	543 U/L (72.39↑)	*P.ostreatus*	An et al. (2016)
	4540 U/L (8.66↑)	*Trametes* sp.	Daâssi et al. (2013)
MnP activity 4768.04 U/L	570.82 U/L (8.36↑)	*P. eryngii*	Akpinar & Ozturk Urek (2014)
LiP activity 895.80 U/L	16.13 U/L (55.93↑)	*P. eryngii*	Akpinar & Ozturk Urek (2014)
	51.615 U/L (17.55↑)	*P. eryngii*	Akpinar & Ozturk Urek (2012)
CMCase activity 11.55 U/mL	1.21 U/mL (9.55↑)	*P. sajor-caju*	Zinat et al. (2013)
	0.198 U/mL (58.08↑)	*P. eryngii*	Ashger et al. (2016)
	3.152 U/mL (3.66↑)	*P. ostreatus*	An et al. (2016)
Xylanase activity 3.27 U/mL	3.064 U/mL (1.07↑)	*P. ostreatus*	An et al. (2016)

Table 3.7 Comparison of maximum ligninolytic and hydrolytic enzymes activities from cherry cultures after optimization with literature

Results in Thesis	**Enzyme Activities**	**Microorganism**	**Reference**
Lac activity 4677.26 U/L	1618.5 U/L (2.89↑)	*P. eryngii*	Akpinar & Ozturk Urek (2014)
	543 U/L (8.61↑)	*P. ostreatus*	An et al. (2016)
	4540 U/L (1.03↑)	*Trametes* sp.	Daâssi et al. (2013)
MnP activity 2064.68 U/L	570.82 U/L (3.62↑)	*P. eryngii*	Akpinar & Ozturk Urek (2014)
LiP activity 69.89 U/L	16.13 U/L (4.33↑)	*P. eryngii*	Akpinar & Ozturk Urek (2014)
	51.615 U/L (1.35↑)	*P. eryngii*	Akpinar & Ozturk Urek (2012)
CMCase activity 1.50 U/mL	1.21 U/mL (1.24↑)	*P. sajor-caju*	Zinat et al. (2013)
	0.198 U/mL (7.58↑)	*P. eryngii*	Ashger et al. (2016)

3.3 The Variations of Reducing Sugar and Nitrogen Concentrations under All Optimized Conditions for Cherry and Peach Cultures

The reducing sugar and nitrogen levels in all growth media for both cultures decreased rapidly up to 3rd day of cultivation, but not depleted fully by *P.eryngii*, so that the cultivation was reached to carbon and nitrogen-limited conditions. Thus, significantly ligninolytic and hydrolytic enzymes secretion by *P.eryngii* started. In addition, good colonization on these wastes with all conditions was achieved with *P.eryngii*, fungal growth being observed from the second day of the fermentation, and complete colonization of fungus was observed within 30 days of cultivation. The observation of fungal growth, protein production and the consumption of glucose and nitrogen by *P.eryngii* could be proofs that these cherry and peach wastes generated by food processing industries were good substrates for producing ligninolytic and hydrolytic enzymes. These results were supported by Akpinar & Ozturk Urek (2014). Also, the uses of these wastes for economically production of these enzymes in this thesis have an importance and novel with regards to literature, since to date most agroindustrial wastes used to produce ligninolytic and hydrolytic enzymes by WRF have not been published.

3.4 Purifications and Partial Characterizations of Enzymes from Peach and Cherry Cultures

In this thesis, the ligninolytic and hydrolytic enzyme secretions by *P. eryngii* were evaluated together under SSF conditions using both peach and cherry wastes, but the reference enzymes were chosen as Lacs for optimizing growth conditions due to the decolarization studies using crude and purified these enzymes. The Lacs are preferred for biotechnological applications when compared to other enzymes. Gnanamani, Jayaprakashvel, Arulmani & Sadulla (2006) reported that oxidative enzymes had an advantage over peroxidases in utilizing oxygen as cofactor, which is cheap and readily available instead of H_2O_2.

In the initial purification steps which were carried out with ethanol as an organic solvent and ammonium sulphate, in order to fractionation of proteins by precipitation, the crude Lacs extract of *P. eryngii* which was concentrated with 10 kDa ultrafiltrate was used similar to the study in which the Lacs were concentrated by ultrafiltrate with a PM-10 membrane (10 kDa, Amicon) before enzyme loading to the anion exchange column (Gnanamani et al., 2006). Initial volume activity, protein amount and spesific activity of the concentrated extract from peach cultures were 39.314 U/mL, 0.068 mg/mL, 578.15 U/mg, while they from other cultures were 4.68 U/mL, 0.155 mg/mL and 30.19 U/mg, respectively.

3.4.1 Ammonium Sulfate Precipitation

The proteins in the crude Lac extracts of *P. eryngii* from peach and cherry cultures were precipitated by fractionation with organic solvent, ethanol. Proteins are maintained in a solution by the interaction of the surface hydrophilic groups with the water solvent. Consequently, if the polarity of the solvent is reduced by the addition of an organic solvent less polar than water, the protein will tend to become less soluble (Taqi, 2012). To minimize the denaturation of the protein in the less polar solvent, solvent fractionation performed at low temperature is usually necessary.

The ratio between enzyme supernant and solvent volumes was adjusted at 1:1, 1:2 and 1:3, v/v, respectively. Lac activity alterations in the supernatant and pellet phases were determined. The pellets were firstly dissolved by sodium acetate buffer (20 mM, pH 4.5), and then the activities of these samples were monitored. The enzymes were mainly found in pellet phases. As the result of ethanol precipitation of proteins in the *P. eryngii* crude Lac extracts from peach and cherry cultures, the best purification yield was found as 17.46% by the ratio of enzyme supernatant of peach culture and solvent volumes at 1:3 and this result were similarly with the other enzyme from cherry culture (data not shown).

The fractionation of proteins by using neutral salts depending on their solubility differences is a widespread method for the first step of purification. One of the neutral

salts used for this purpose is ammonium sulphate. With this method, many of proteins are not denatured and activity is recovered with dissolving pellet in a suitable buffer.

In this research, crude Lacs extracts of *P. eryngii* from peach and cherry cultures were separately saturated to 35, 45, 55, 65 and 85%, w/v, ammonium sulphate (Figure 3.25). The supernatant and pellet phases were seperated with centrifuge and specific activity alterations were determined. After the ammonium sulfate precipitation, the Lac assay regarding peach cultures revealed that the most of the enzymes were precipitated at 55% saturations with an approximately 1.01-fold of purification factor, while the purification factor of precipitated Lac from cherry cultures was determined as 1.15-fold at the same saturation. This was the best concentration of ammonium sulfate saturation for Lac precipitations of both cultures.

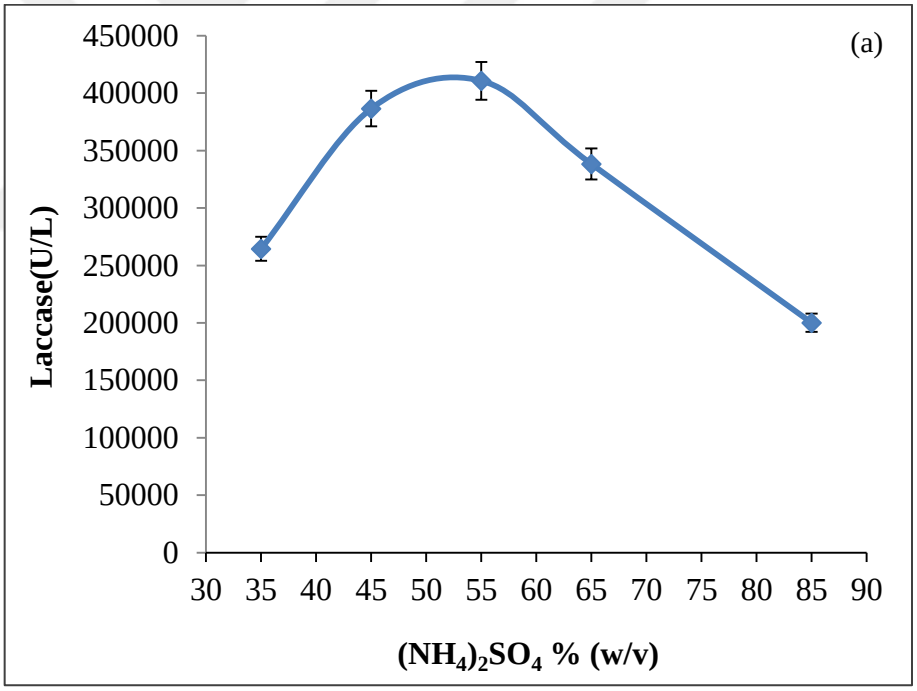


Figure 3.25 Lacs volume activity distributions in the bottom phases from peach (a) and cherry (b) cultures depending on ammonium sulphate concentrations

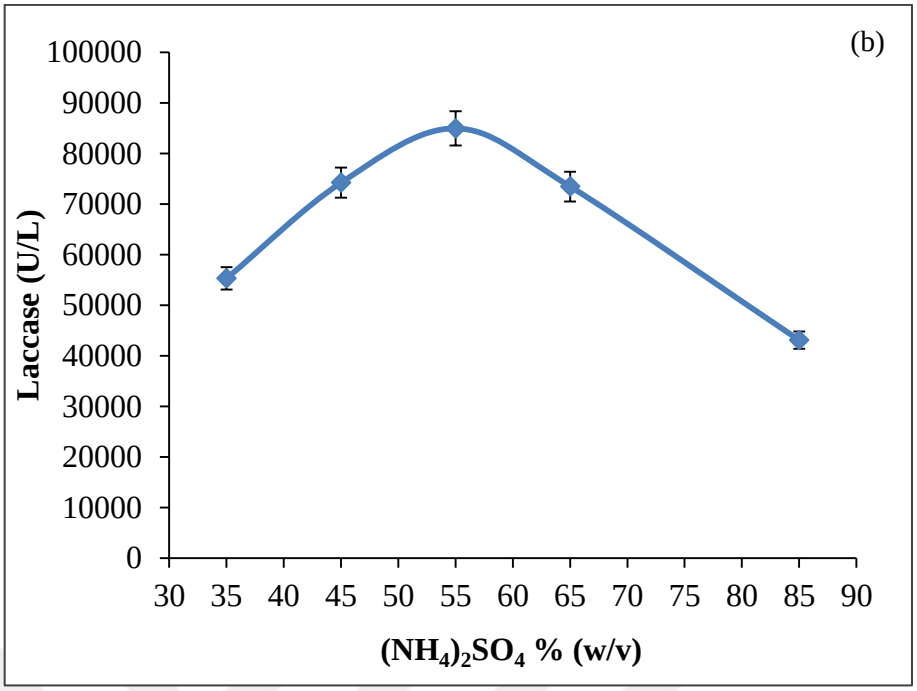


Figure 3.25 Lacs volume activity distributions in the bottom phases from peach (a) and cherry (b) cultures depending on ammonium sulphate concentrations (continued)

El-Batal et al. (2015) investigated the Lac production by *P. ostreatus* and its application in synthesis of gold nanoparticles. In that study, the partial purification of Lac was carried out by adding ammonium sulfate of 80% saturation to the cell free filtrate. After precipitation of the enzyme using ammonium sulfate, total activity decreased from 450 to 414.67 U/mL, but the specific activity increased from 112.5 to 204 U/mg. That enzyme kept 90% of activity at pH 6.0. The efficiency of this results was higher than that in this thesis.

Purification folds found as a result of precipitation with ethanol were so low when compared to ones obtained from precipitation with ammonium sulfate, so it was decided that precipitation with ethanol is not suitable method for Lac fractionation from extract of both peach and cherry cultures by *P. eryngii* as pre-purification step. The precipitation with organic solvents is not necessarily an alternative to ammonium sulfate, but it can be used as an additional step (Taqi, 2012).

3.4.2 Chromatographic Applications for the Purification of Laccases

As a second purification step, the fractions of 55% $(NH_4)_2SO_4$ from cherry and peach cultures were applied to the DEAE-Sepharose ion exchange chromatography column based on the charge differentiations of proteins depending on the variations of ionic strength as shown in Figures 3.26.

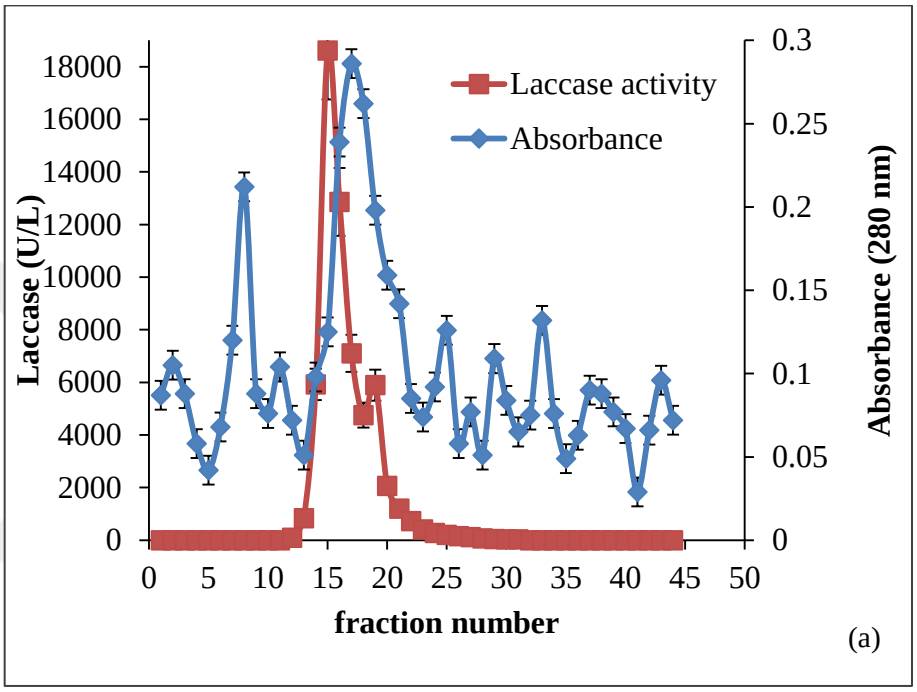


Figure 3.26 Elution profile of *P. eryngii* Lacs from peach (a) and cherry (b) cultures on DEAE-Sepharose chromatography

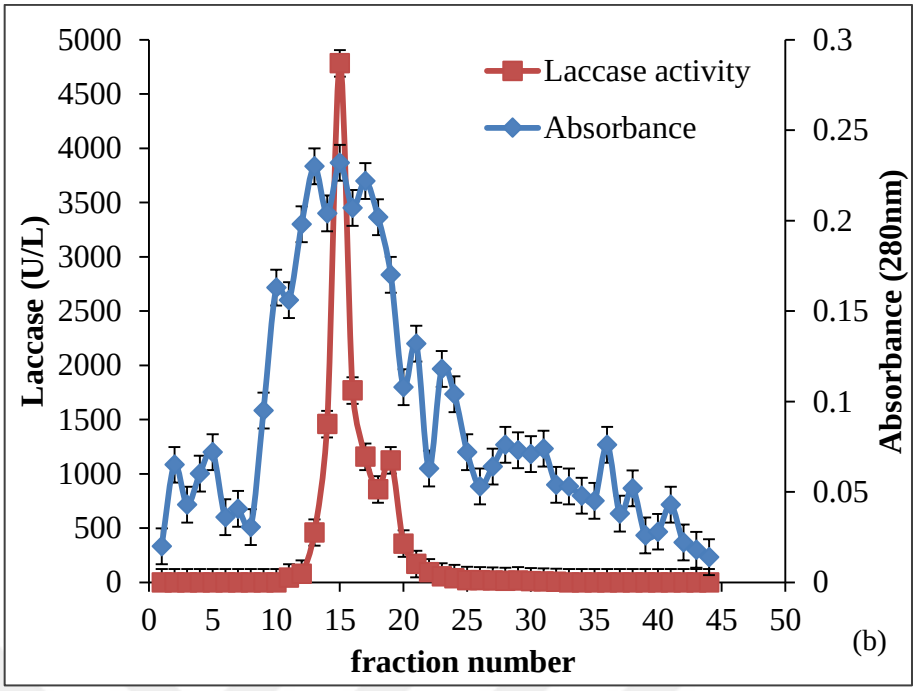


Figure 3.26 Elution profile of *P. eryngii* Lacs from peach (a) and cherry (b) cultures on DEAE-Sepharose chromatography (continued)

Lac activities from peach cultures were obtained in among 13th-23rd numbered fractions with ranging values from 840.502 to 18 614.64 U/L, whereas they from cherry culture were observed in among 12th-22nd numbered fractions with ranging values from 44.44 to 4783.62 U/L. The highest Lac activities were determined the same numbered fractions for both cultures, the activity values of the fractions were low by comparison with 15th fraction. After chromatography on DEAE-Sepharose, the Lacs from peach and cherry cultures were respectively eluted with other proteins achieving purification factors 3.88 and 3.48, thus the major Lacs were separated.

The active Lac fractions obtained from DEAE-Sepharose ion exchange chromatography was applied to Sephadex G-100 gel filtration column. The protein levels and Lac activity distributions obtained from gel filtration chromatography were shown in Figure 3.27. The highest activity with 1161.3± U/L was found in among 19th-33rd fraction for peach cultures. Negligible level of activities in 19th, 29th, 30th, 31st, 32nd, 33rd fractions were observed. On the other hand, the highest activity with 460.04 U/L was found in among 18th-29th fraction for cherry cultures. Negligible level of activities in 18th, 27th, 28th and 29th fractions were observed. The highest Lac activity

was detected in the 21st and 22rd fractions for peach and cheery cultures, respectively. As the result of Sephadex G-100 gel filtration chromatography, 3.95-fold purification was obtained for peach culture, while it was 5.15 for cherry culture.

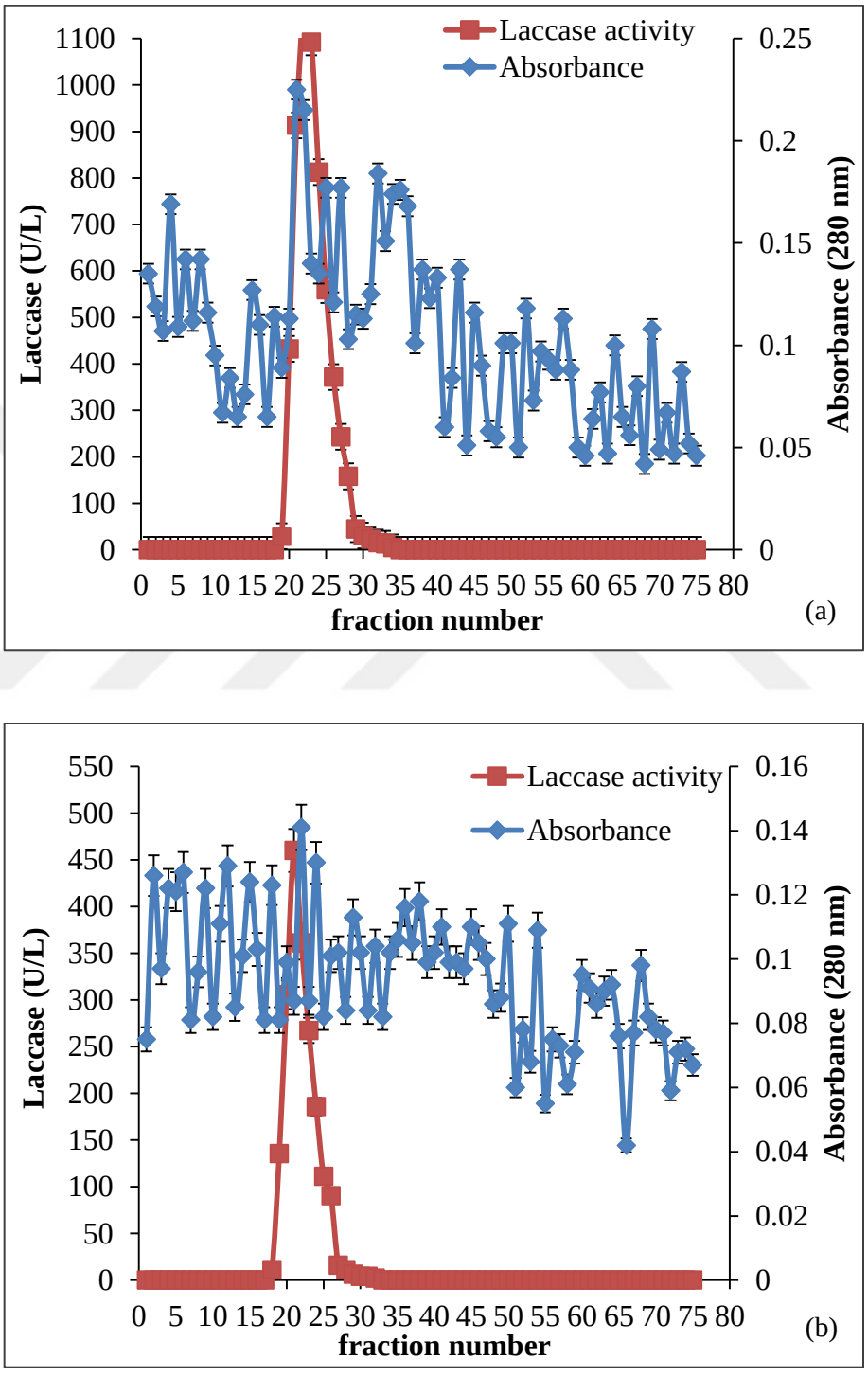


Figure 3.27 Elution profile of *P. eryngii* Lacs from peach (a) and cherry (b) cultures on Sephadex G-100 chromatography

In terms of determining the molecular weight of purified Lacs by using Sephadex G-100 gel filtration chromatography, standard protein mixture being molecular weight range with 6500-66 500 Da was applied to the gel filtration column under same conditions.

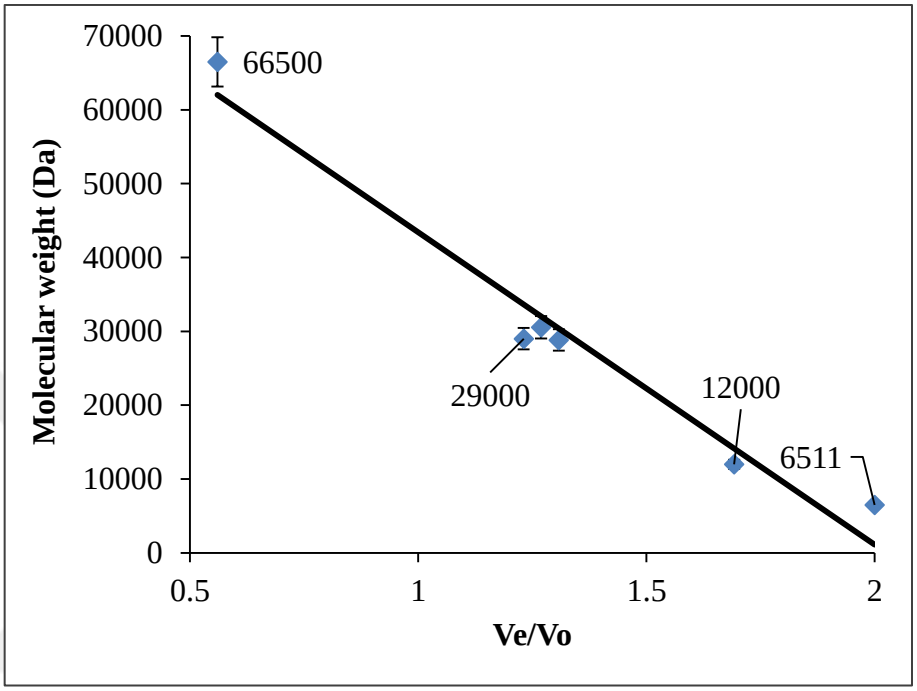


Figure 3.28 Calibration curve for the determination of the purified Lacs from peach and cherry cultures by *P. eryngii*. Marker protein used for calibration: Bovine serum albumin (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), cytochrome c from horse heart (12.4 kDa), aprotinin from bovine lung (6.5 kDa), ($y=-42318x+85747$; $R^2=0,97$)

As seen from Figure 3.28, molecular weight of purified Lac for peach cultures was found as 30±1 kDa, whereas it was 29±1 kDa for cherry culture. These findings supported by Camassola, da Rosa, Calloni, Gaio & Dillon (2013). In their study, the molecular mass of laccase by *Pleurotus* spp. was detected as 30 kDa by SDS-PAGE electrophoresis.

The Lacs from peach and cherry culture filtrates were purified to homogeneity by ammonium sulphate precipication, anion exchange chromatography and gel filtration. The steps used for purification of these enzymes were summarized in Tables 3.8 and 3.9, respectively.

Table 3.8 Purification of Lac from peach culture

	Protein (mg/mL)	**Volume activity (U/mL)**	**Total activity (U)**	**Spesific activity (U/mg)**	**Activity Yield (%)**	**Purification factor**
Culture supernatant	0.068	39.31	5307.39	578.15	100	1.0
Ammonium sulphate (%55) precipication	0.175	102.69	1540.34	586.79	29.02	1.01
DEAE-Sepharose chromatography	0.029	65.01	422.59	2241.72	7.96	3.88
Sephadex G100	0.047	107.4	289.98	2285.11	5.46	3.95

Table 3.9 Purification of Lac from cherry culture

	Protein (mg/mL)	**Volume activity (U/mL)**	**Total activity (U)**	**Spesific activity (U/mg)**	**Activity Yield (%)**	**Purification factor**
Culture supernatant	0.155	4.68	1166.75	30.19	100	1.0
Ammonium sulphate (%55) precipication	0.610	21.25	531.13	34.84	45.52	1.15
DEAE-Sepharose chromatography	0.114	11.97	83.82	105.0	7.18	3.48
Sephadex G100	0.027	4.20	52.04	155.56	4.46	5.15

At the end of the purification steps, the purification procedure yielded 2285.11 U/mg of pure enzyme from peach culture and the recovery of total Lac activity was 5.46%. On the other hand, the purification procedure yielded 155.56 U/mg of pure enzyme from cherry culture and the recovery of total Lac activity was 4.46%. The extracellular Lac from *P. florida* was purified after ammonium sulfate precipitation in two steps, DEAE-Sephacel chromatography, gel filtration with Sephadex G-50, and had a spesific acitivity of 52.6 U/mg (Sathishkumar & Palvannan, 2013). This value lower than the spesific activities obtaining from this thesis. The Lac of *Trametes trogii*

was purified 7.7-fold to a specific activity of 148 U/mg protein after ammonium sulfate precipitation and chromatographic assays (Ai, Wang, & Huang, 2015). The spesific activities of purified Lacs from peach and cherry were higher than that of it. The Lac purification of *Pleurotus ferulae* was performed by ammonium sulfate precipitation, Mono Q strong anion exchange and Superdex 75 columns with 4.85-fold to a specific activity of 105.6 U/mg protein (Ding et al., 2014). The Lac of *Shiraia* sp. was purified 2.14 fold after cation exchange and Superdex G-200 chromatography (Yang, Ding, Liao & Cai, 2013). The extracellular Lac produced by *Daedalea flavida* was partially purified by ammonium sulfate precipitation and gel filtration chromatography to improve the specific activity, from the initial value of 1.16 to 63.63 U/mg of total soluble protein (Singha & Panda, 2015). Liu et al. (2009) purified Lac from *P. ostreatus* using ammonium sulfate (35% w/v), DEAE-Sepharose, and Sephadex G-100 column chromatography with a spesific activity of 1077.28 U/mg of protein and a 3.67 purification fold. W. Huang, Tai, Hseu & C. Huang (2011) purified Lac by Q-Sepharaose XL column with a 2.8-purification fold and gel filtration column using Sephacryl S-200 with 3.8-purification fold; and then spesific activity were finally determined as 59.27 U/mg.

3.4.3 SDS-PAGE Gel Electrophoresis

Lacs are glycoproteins with molecular masses of generally 32.18-120 kDa, depending of the strain (Manavalan et al., 2015). In this thesis, Lacs from both cultures were resolved on into one peak after gel filtration column. Also, Flurkey (2003) reported that the molecular weight of Lac ranges from 40 to 100 kDa, however the common size ranges from 60 to 80 kDa.

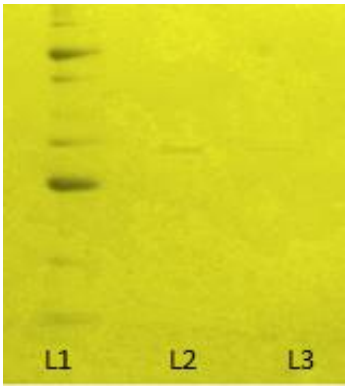


Figure 3.29 SDS-PAGE electropherogram of Lacs from *P. eryngii*, consisting of protein standards: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa). L1 demonstrated the molecular markers weights (6.5-66 kDa), L2 was Lac enzyme from peach culture, L3 was Lac enzyme from cherry culture

The results illustrated the electrophoretic analysis of the purified Lac from both peach and cherry cultures resulted in a single band of protein, and their molecular masses were estimated to be approximately 30±1 kDa (Figure 3.29), indicating that the Lacs of *P. eryngii* has a monomeric structure. These mean these molecular masses disagree with the values reported for other fungal Lacs (around 50-80 kDa) (Yuan et al., 2016). For example, Gnanamani et al. (2006) reported the effect of inducers and culturing processes on Lac synthesis in *P. chrysosporium* NCIM 1197 and the partially purification of the enzyme. In their results, the SDS-PAGE intense band with reference to 62 kDa of standart protein marker suggests molecular weight of the partially purified Lac. However; the molecular weight of Lac from *P. eryngii* was indicated as 34 kDa with a single band in a study summarizing the molecular weights of different fungal Lacs (Taqi, 2012). Also, Castaño, Cruz & Torres (2015) investigated the optimization of production, purification and characterization of a Lac from the fungus *Xylaria* sp. The purification steps were comprised of diafiltration, DEAE-sepharose and size exclusion chromatography using Sephadex G-100. After purification, the molecular mass of Lac was found as 38 kDa by electrophoresis. In another study, *P. eryngii* had a single isoform of Lac with molecular weight of 34 kDa (Wang & Ng, 2007).

3.5 Enzymatic Characterization of Purified Laccases

The effects of some agents, EDTA, PMSF, iodoacetamide, β-mercaptoethanol, N-ethyl-5-phenylisoazolium-3'-sulfonate, 1-bromo-2,5-pyrolidinedione, on the purified Lac activities from peach and cherry cultures were investigated. These chemicals were added to assay mixture, and then differences of Lac activities were analyzed. According to results, the Lac activities were partially or completely decreased after all of the above-mentioned chemicals were added separately. The main purpose of these analyzes was to estimate the amino acids that played a role in the active cites of Lacs and/or affected the activities. Thus, the main amino acids in these purified enzymes were Ser, Thr, His, Cys. After the reactions performed, the amino acids found in the enzyme structure were demonstrated in Table 3.10. Cys amino acid was found to be important for the active center and structure of the both enzymes. Because, copper type 1 (Cu T1) is located in domain 3, while the trinuclear center is integrated between domains 1 and 3; both domains provide residues for copper coordination. The structure is stabilized by two disulfide bonds; the first bond located between domains one and three and the other one between domains one and two. In addition, their redox sites and copper coordination are highly conserved the eight ligands of His in the trinuclear cluster T2/T3 show a highly conserved pattern with four His-X-His motifs. The X motif is a Cys that binds Cu T1, while the adjacent His bind each a Cu T3 site (Rivera-Hoyos et al., 2013). β-mercaptoethanol, a protein denaturant, dramatically reduced the Lac activity, which can be explained by its stimulation of the reduction of sulfhydryl groups in the presence of this compound or by the oxidation of disulfide bridges, exposing their native structure in the catalytic site.

Table 3.10 The amino acids found in active sites of purified Lacs by *P. eryngii*

	Lac, peach culture	**Lac, cherry culture**
PMSF	Ser, Thr	Ser, Thr
iodoacetamide	Met, His, Cys	Met, His, Cys
β-mercaptoethanol	Cys	Cys
***N*-ethyl-5-phenylisoazolium-3'-sulfonate**	Asp, Cys	Asp, Cys
1-bromo-2,5-pyrolidinedione	His, Tyr	His, Tyr

The significant reduction in Lac activities from peach and cherry cultures could be due to the key roles of the metal-binding active sites of chelating agent EDTA on Lac activity. EDTA functions as a non-competitive inhibitor of metalloenzymes by removing the metal ion cofactors like Mg^{2+} and Ca^{2+} (Devasena, 2010).The Lac enzyme of *Xylaria* sp. was slightly inhibited by EDTA, but strongly inhibited by sodium azide, dithiothreitol in another study (Castaño et al., 2015). In other study, different inhibitors (PMSF, EDTA) were evaluated to check the inhibition properties of Lac from *Marasmius* sp. (Vantamuri & Kaliwal, 2016). It was observed that PMSF inhibits the little enzyme activity, and this is non-Ser and non-metallo Lac in contrast to the findings in this thesis. On the other hand; PMSF was known as a Ser-protease inhibitor due to the mechanism between the inhibitor and –OH groups in Ser (Hamada, Kubota & Sagisaka, 2017), thus it blocked the Ser or Thr amino acids of Lac structure or catalytic center and affected the enzyme activity. Panwar, Srivastava & Kapoor (2014) reserached production, extraction and characterization of alkaline xylanase from *Bacillus* sp. PKD-9 with potential for poultry feed in which sulfhydryl reagents such as iodoacetamide and iodoacetic acid had very little effect on activity of xylanase, suggesting that either enzyme may not contain important Cys residues with a free thiol group in its active site for catalytic activity or if present were inaccessible to these reagents. Also, cysteines were alkylated by iodoacetamide (Karp et al., 2012). The decrements in Lac activities can be explained that the iodoacetamide agents could inhibited the Cys residues in the enzyme. In summary, these agents are suggested to affect the Lac activity by chelating the Cu (II) atoms or by modifying amino acid residues or by causing conformational change in the glycoprotein and they except EDTA completely inhibited Lac activity.

These purified enzymes were a typical blue Lacs according to their UV-visible spectrum, which showed an intense band around approximately 600 nm, corresponding to the type-1 copper site (data not shown). UV studies and EDTA inhibition proved the presence of copper in the Lac structure. Most of the studies reported so far are on blue Lacs. Yellow/white Lacs differs from blue Lacs in two respects. Yellow/white Lacs lack absorption band around 610 nm always found in blue Lacs and yellow/white Lacs oxidize non-phenolic substrates in absence of mediator

molecules which are required in cases of blue Lacs (Chaurasia, Bharati & Singh, 2013).

3.6 Kinetic Parameters of Purified Laccases

Using different ABTS concentrations (1.0; 2.0; 3.0; 4.0; 5.0 mM), Table 3.11 shows the K_m and V_{max} values for the laccases from the concentrated purified enzymatic extract, calculated from the Lineweaver–Burk plots (Figure 3.30). The catalytic efficiency was determined as the ratio of V_{max} to K_m, and it was defined as k_{cat}.

Table 3.11 Kinetic parameters of *P. eryngii* Lacs from peach and cherry cultures

	V_{max} (mmol/min)	K_m (mmol)	k_{cat} (min^{-1})
Lac, peach culture	1.694	1.1381	1.488
Lac, cherry culture	0.052	0.329	0.16

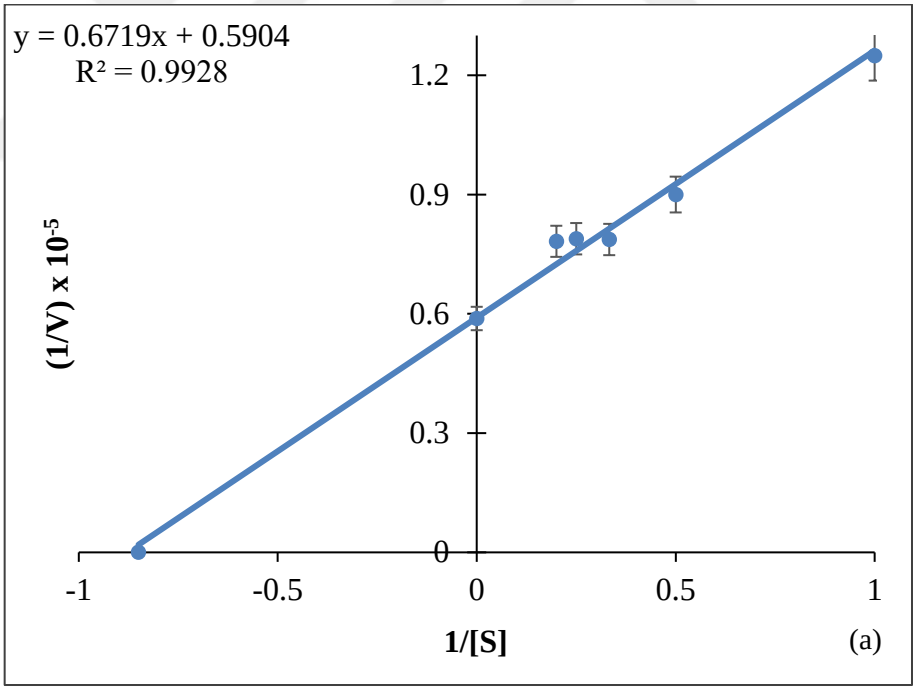


Figure 3.30 Lineweaver-Burk kinetic curves of purified Lacs from peach (a) and cherry (b) cultures for determining their kinetic parameters

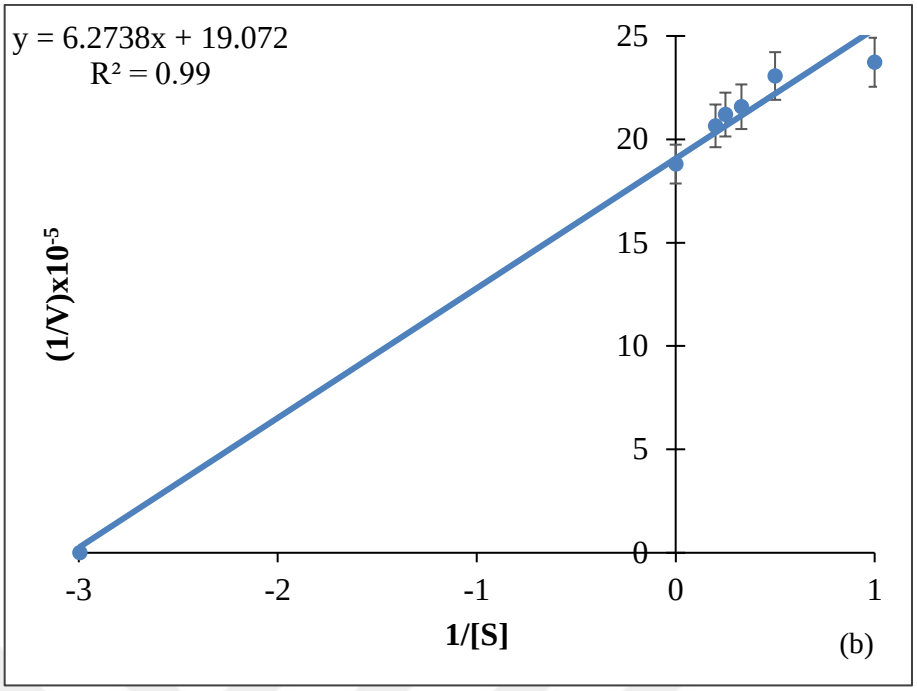


Figure 3.30 Lineweaver-Burk kinetic curves of purified Lacs from peach (a) and cherry (b) cultures for determining their kinetic parameters (continued)

Songulashvili et al. (2016) reported that the relationship between *Coriolopsis gallica* 1184 Lac and substrate concentration using Michaelis-Menten curve with V_{max} and K_m for ABTS were 142 µM/min and 17 µM and another K_m values were detected as 20, 40, 66 for the substrates, guaiacol, syringaldazine, 2,6-DMP, respectively. Castaño et al. (2015) found that the values of K_m and V_{max} were of 297 µM and 581.4 µM/min from studying Lac activity of *Xylaria* sp. when assessing the effect of different ABTS concentrations on the reaction rate. Yang et al. (2013) found that the K_m value for ABTS was 0.19 mM. The calculated K_m values of the purified Lac from peach and cherry cultures were higher than reported K_m values against the same substrate for Lac from *Trametes* sp. and *Cladosporium cladasporiodies* (Daâssi et al., 2013). According to results shown in Table 3.8, both Lacs showed the high affinity on ABTS and these findings supported by Martínez et al. (2013). The K_m value for ABTS was so high and detected as 23 mM. Values of K_m of different Lacs widely vary for the same substrate. In agreement with the results in this thesis, the affinity (K_m value) of Lac from *P. ostreatus* HP-1 was calculated as 46.51 mM (Patel, S. Gupte, Gahlout & A. Gupte, 2014). Kinetic constant, K_m, of purified Lac1 and Lac2 isoforms isolated from *T. versicolor* HEMIM-9 grown in SSF on oak sawdust were respectively determined as

12 and 45 mM for ABTS substrate (Martínez-Morales et al., 2015). The interaction of enzyme with its substrate was indicated through K_m values and a lower K_m value reflect high affinity of enzyme for its substrate and higher V_{max} indicated that small amount of enzyme can convert substrate into the product (Asgher, Shahid, Kamal & Iqbal, 2014). Purified Lac from *T. pubescens* displayed high activity toward a wide range of substrates, including phenolic and non phenolic substrates. The enzyme activity to the various substrates was ranked as follows: ABTS > 2,6-DMP > guaiacol > syringaldazine > ferulic acid > veratryl alcohol > hydroquinone > catechol > pyrogallol > phenol (Si, Peng & Cui, 2013). The low K_m value for ABTS also indicated a high affinity of the enzyme toward this substrate supported by the researches of Si et al. (2013) and above mentioned studies.

The effects of inhibitor, sodium azide, on Lac activities were studied at different concentrations (0.001; 0.005; 0.01 mM) using different substrate concentrations (1.0-5.0 mM) (Table 3.12). A control test was conducted in parallel in the absence of the inhibitor. Daâssi et al. (2013) assayed the effect of different concentrations (0.1, 1.0 and 10.0 mM) of sodium azide on Lac activity of the white rot fungus *Trametes* sp. and sodium azide of 0.1 mM was found to completely inhibit Lac activity. The sodium azide caused complete loss of Lac activity at 0.1 mM concentration in another research (Ai et al., 2015). These kinetic results evidenced that the sodium azide inhibited the purified Lac activities of both cultures. As can be illustrated in Figure 3.31, the inhibition of Lacs from both cultures by sodium azide has been shown to closely resemble competitive inhibition model. The values of both the competitive inhibition constants were calculated for Lacs from peach and cherry cultures, by following equation (3.1):

$$V = \frac{Vmax\,.\,[S]}{Km\left(1 + \frac{[I]}{Ki}\right) + [S]} \qquad (3.1)$$

Table 3.12 Inhibitory effect of different concentrations of sodium azide on the purified Lacs obtained from peach and cherry cultures, using ABTS as a substrate

Sodium azide concentration (mM)	**K_i values (mM), for peach cultures**	**K_i values (mM), for cherry cultures**
0.001	3.137×10^{-4}	1.188×10^{-4}
0.005	2.728×10^{-4}	0.365×10^{-4}
0.01	2.566×10^{-4}	0.279×10^{-4}

In otherwords, Lineweaver–Burk plots for Lac enzyme activities from peach and cherry cultures with varying substrate concentration and inhibitör concentrations. These graphs (Figure 3.31) indicate types of competitive inhibition. K_i was determined from the slope, which is equal to the following equation:

$$slope = \frac{Km}{Vmax}\left(1 + \frac{[I]}{Ki}\right) \quad (3.2)$$

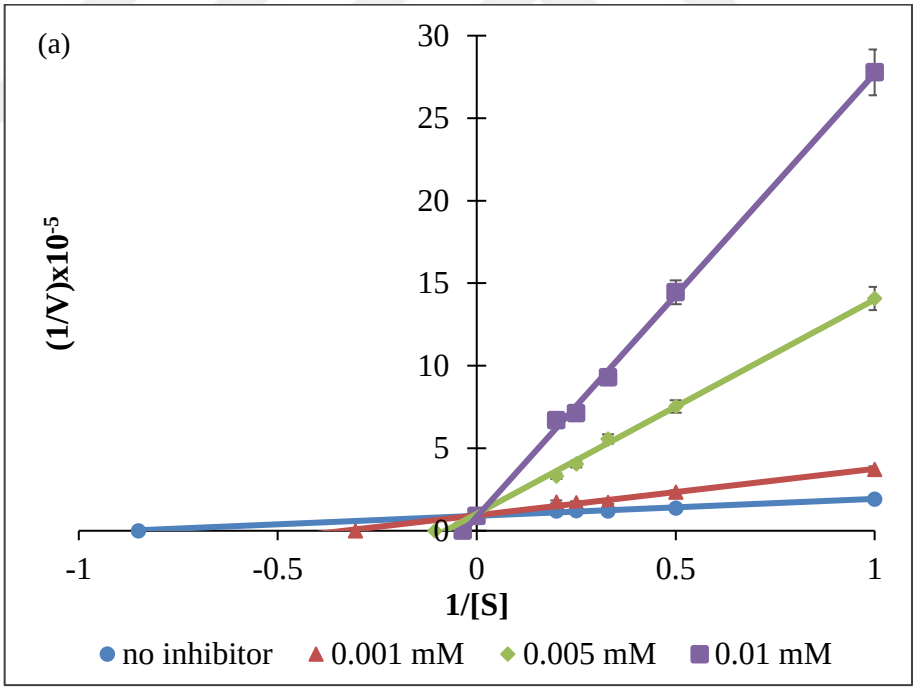


Figure 3.31 Lineweaver-Burk plots of the activities of Lacs from *P. eryngii* by peach (a) and cherry (b) cultures depending on different sodium azide concentrations (0.001; 0.005; 0.01 mM)

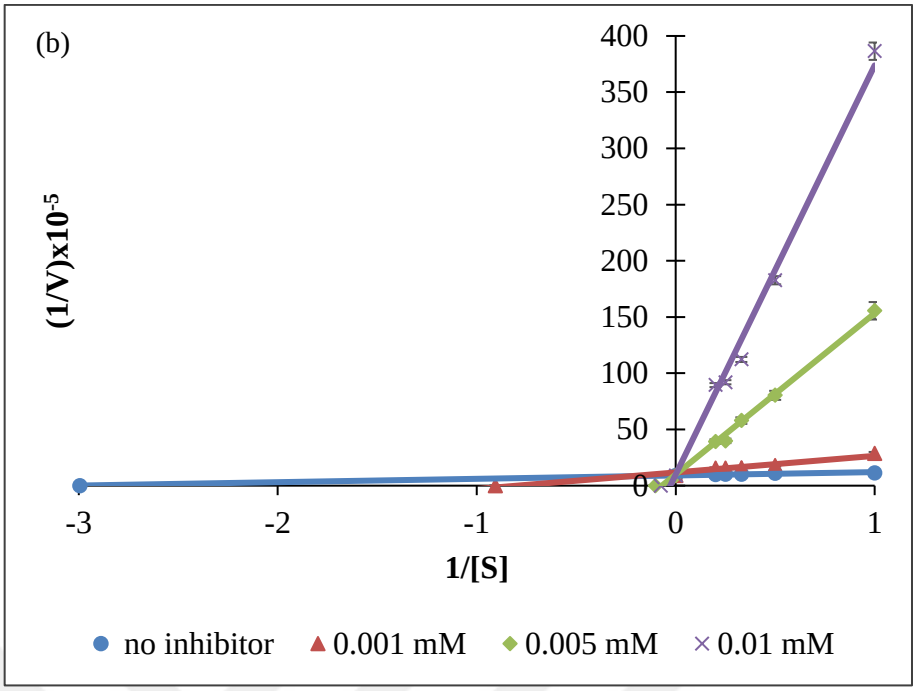


Figure 3.31 Lineweaver-Burk plots of the activities of Lacs from *P. eryngii* by peach (a) and cherry (b) cultures depending on different sodium azide concentrations (0.001; 0.005; 0.01 mM) (continued)

Several inhibitory ions such as azide, halides cyanide and hydroxide generally affect Lac activity (El-Batal et al., 2015; Patel et al., 2014). For example, the inhibitory effects of pH and chloride on the catalysis of Lac from *T. versicolor* were demonstrated by studying the alteration of inhibition characteristics of sodium chloride at different pHs for the oxidation of ABTS (Raseda, Hong, Kwon & Ryu, 2014). These ions can bind to the type 2 and type 3 copper atoms, resulting in the interruption of internal electron transfer with the subsequent inhibition activity. Namely, the inhibitory binds to the active site of the enzyme to unfortunately prevent binding of the substrate, ABTS.

Patel et al. (2014) reported that the sodium azide was an inhibitor of metallo-enzymes showed 'non-competitive' inhibition, but this observation was not consistent with the findings of Heinzkill, Bech, Halkier, Schneider & Anke (1998) and this thesis. These studies noted competitive inhibition with sodium azide for Lac from different species of fungi.

3.7 Dye Applications

3.7.1 Decolorizations of Some Dyes with Crude Extracts from Peach and Cherry Cultures

Approximately, 10,000 different dyes and pigments, mainly for use in the printing, color photography, textile dyeing, pharmaceutical, food, cosmetics industries are produced annually worldwide. Synthetic dyes are chemically diverse, with those intended for industrial use divided into azo, triphenylmethane and heterocyclic/polymeric structures and the amount of dye lost in the effluents is dependent on the class of dyes used (Jagiasi & Patel, 2015). Azo dyes, characterized by nitrogen to nitrogen double bonds, are synthetic organic compounds widely used in dyeing industries, accounting for up to 60-70% of all textile dyestuffs produced. Besides the conventional physicochemical methods, microbial degradation or their extracellular enzymes of azo dyes has been attracted significant attention (Akpinar & Ozturk Urek, 2017). Ligninolytic enzymes, especially Lacs, participate in the decolorization and degradation of the azo dyes. The release of azo dyes into the environment in effluent has become a major concern in wastewater treatment, since they are highly recalcitrant to conventional wastewater treatment processes. It has also been investigated whether all concentrations of different dyes affect Lac and MnP enzyme activities from both cultures. According to results, no inhibition of all enzyme activities was observed.

In this thesis, synthetic dyes such as MO, TT, RR and RB were treated with crude and purified enzyme extracts (obtained from peach cultures after 20 days and cherry culture after 15 days). As can be seen in Figure 3.32, the highest decolorization was performed with MO (50 mg/L) as 43% after 5 min of treatment by the ligninolytic enzymes obtained from peach culture, while it was carried out with MO (100 mg/L) as 12.6% after 30 min of treatment by the ligninolytic enzymes from cherry culture. These are an extremely short period to achieve azo dye decolorization without any mediators. When the decolorization was performed with changing mediator, ABTS, concentrations under the same conditions before mentioned, crude extracts including

enzyme mixture from peach and cherry cultures directly reacted with ABTS for investigated every concentration, as a result any of decolorization reactions was performed. On the other hand, the dye decolorization of MO with the purified Lac was higher (53%; 5 min) than those obtained with the crude enzymes from peach culture, whereas the decolorization of it with the purified Lac was similar than those obtained with the crude enzymes from cherry culture. The reason why decolorization was carried out with purified Lac was that this enzyme was predominant in this process when compared to other ligninolytics (Levin, Papinutti & Forchiassin, 2004). The decolorizations of other dyes by crude extracts including ligninolytic activities were too lower. These can be explained by the recalcitrance of the dyes to decolorization with their high redox potential or steric hindrances limiting accessibility of the enzymes to functional groups in dyes. Decolorization activities of the enzymes can be increased by making it in the presence of possible mediators. On the other hand, enzyme and dye samples were treated for 1 h; and Lac and MnP activities were measured at intervals of 10 min. According to results, there were no changes in enzyme activities. It could not therefore be said that these dyes adversely affected enzymes stabilities and activities. The direct blue 71 was decolorized by the purified Lac in the absence of redox mediators; the enzyme decolorized 30% of the dyes in 30 min (Martínez-Morales et al., 2015). Lac from *P. florida* produced under SSF conditions, was used for the decolorization of Remazol Brilliant Blue R (RBBR) of 50 ppm. This dye was decolorized up to 46% by *P. florida* Lac alone in 10 min (Sathishkumar & Palvannan, 2013). These results were comparable to findings in the thesis. In another study, dye decolorizing ability of the fungus *Ganoderma lucidum* was demonstrated for recalcitrant dyes; RBBR and RB (Murugesan, Nam, Kim & Chang, 2007). Acoording to their results, there were no decolorization with crude enzyme alone produced during SSF of wheat bran. Crude enzymes obtaining from cultures of *Pleurotus flabellatus* on wheat bran were tested for Direct Blue 14, and the decolorization of the dye using crude enzyme was approximately 46% in the first 60 min (Singh, Vishwakarma & Srivastava, 2013). The other dyes were not oxidized or only partly oxidized by Lacs from both cultures. Because these dyes could be too large to penetrate into the Lac active site or have a particularly high redox potential.

Manavalan et al. (2015) suggested that the decolorization performance of recalcitrant dyes by Lac might be increased using a suitable mediator.

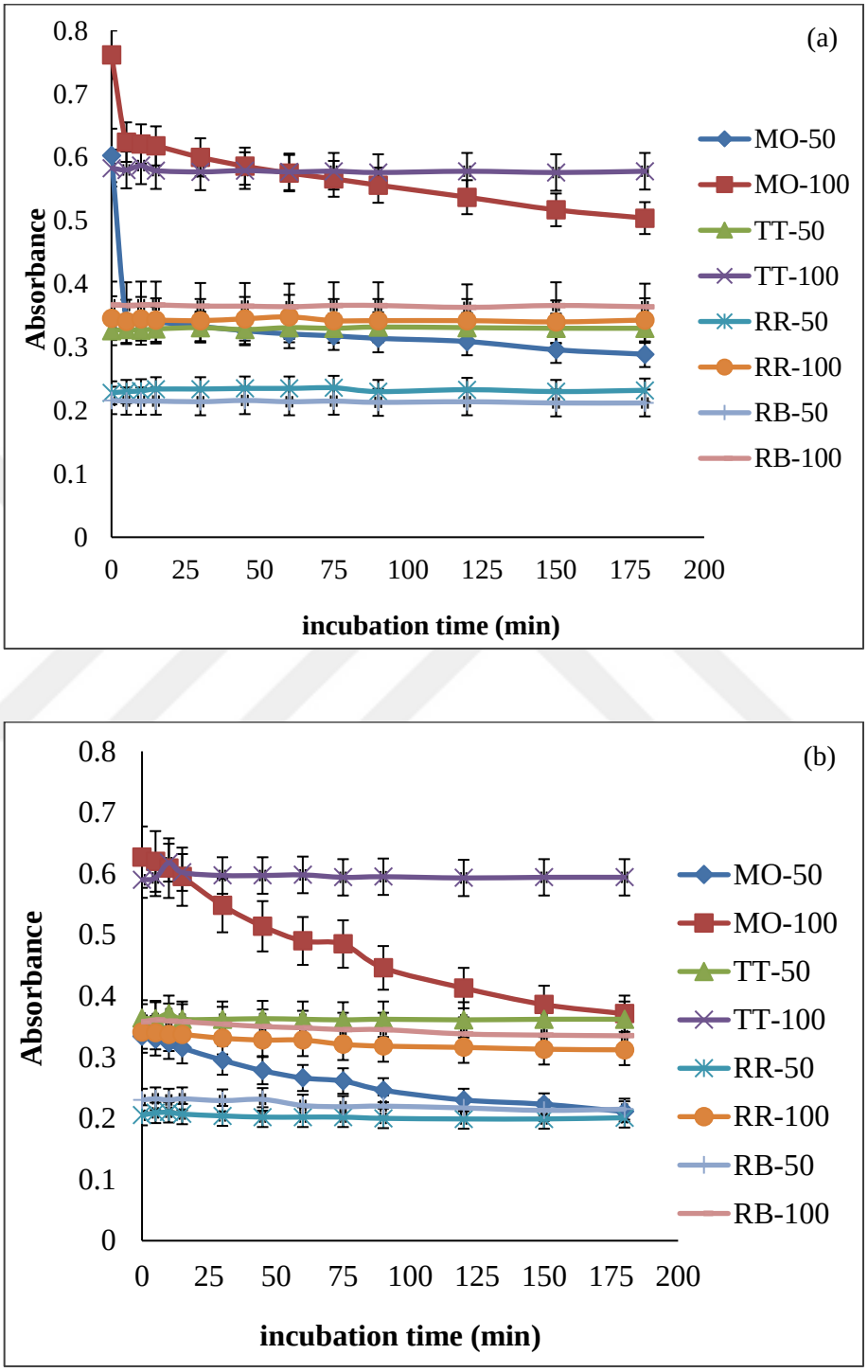


Figure 3.32 The decolorization performances of some azo dyes with crude enzyme extracts from peach (a) and cherry (b) cultures. The values are the mean ±SD for experiments of three separate experiments

The chemical changes of dye structures during decolorization by crude peach and cherry extracts were partially characterized using FTIR spectroscopy. The FTIR sprectra were attained for only MO dyes (50 ppm), since the highest degrees of decolorization by Lacs from both cultures were achieved with this dye. Firstly, the effects of different sodium azide concentrations (0.5, 1.0, 2.0, 2.5, 5.0 mM) on Lac and MnP activities from peach and cherry cultures were investigated and the optimal concentration of this compound of 1.0 mM completely inhibited these enzymes of both cultures. After that, the decolorization reaction was stopped by adding sodium azide of 1.0 mM to the analysis medium. It was reported that it was a good strategy in which the decolorization was completely inhibited by the Lac inhibitor sodium azide (0.5 mM) (Murugesan et al., 2007). The chemical structure of MO was shown in Figure 3.33. FTIR analysis of MO (Figure 3.34) and metabolites obtained after its decolorization by ligninolytic extracts from peach and cherry cultures (Figure 3.35). FTIR is an active tool for identifying types of functional groups that are responsible for entrapping the molecules of dye. Comparison of FTIR spectrum of control dye with metabolites formed after decolorization at 10 min intervals during fifty min indicated the biodecolorization of the parent dye compound by *P.eryngii* of peach and cherry cultures. FTIR spectra of control MO display peak at 2915 cm^{-1} for asymmetric $–CH_3$ stretching vibrations, peak at 1599 cm^{-1} for –N=N– stretching, peak at 1441 cm^{-1} for C–H in plane C–H bend, peak at 1364 cm^{-1} for S=O stretching vibrations confirming the sulfonic nature of dye, peak at 1120 cm^{-1} –C–N for the azo nature, peaks at 1005 cm^{-1}, 943 cm^{-1} and 849 cm^{-1} for ring vibrations, peak at 820 cm^{-1} for disubstituted benzene ring, peaks at 694 cm^{-1}, 621 cm^{-1} and 576 cm^{-1} for –C–S– stretching vibrations. These findings supported by (Parshetti, Telke, Kalyani & Govindwar, 2010). In the comparison of the control MO solution and those subjected to the enzyme treatment, spectral changes were found after contact with the enzyme extracts from both cultures of the fungus *P. eryngii*. Similar results of FTIR analyzed of MO after enzyme treatments from both cultures were observed and there were a differences at peak 2342 cm^{-1}. The spectra of MO treated by both enzyme extracts from both cultures showed vibrations at 3343-3325 cm^{-1} for implying the azo dye biodecolorization. Also, the Figure 3.35 illustrated that FTIR spectra displayed peak at 2342 cm^{-1} for NH_2 antisymmetric stretch, peaks at 2179-2125 cm^{-1} for C=C vibrations, peaks at 1636-

1632 cm^{-1} for –C=C or $–NH_2$ bending, peaks at 900-640 cm^{-1} for aromatic =C–H bending (Ayed, Chaieb, Cheref & Bakhrouf, 2010; Enayatizamir et al.; 2011; Parshetti et al.; 2010; Sathishkumar & Palvannan; 2013).

There were little studies FTIR analyzes for dyes after fungal biodecolorization. J.P Jadhav, Phugare, Dhanve & S.B Jadhav (2010) carried out some analytical studies for the biodecolorization of Direct Orange 39 dye by *Pseudomonas aeruginosa* and formation of new metabolites, and they found that the some oxidoreductases played the significant roles in biodecolorization of Direct Orange 39. Biodecolorization of some triphenylmethane dyes were performed by bacterium *Staphylococcus epidermidis*, and the metabolites produced during the dyes biodecolorization were monitored using FTIR spectroscopy (Ayed et al., 2010). Almeida & Corso (2014) investigated the comparison of the toxicity of azo dye Procion Red MX-5B following biosorption treatments with the fungi *A. niger* and *Aspergillus terreus*, and FTIR spectral analyzes of the dye were carried out. Nowadays, the methods of the removal from dye pulluants base on the chemical methods such as adsorption, coagulation, membrane filtration (Zhou, Wu, Lei & Negulescu, 2014), but these methods are difficult, expensive and time-consuming (Forgacs, Cserháti & Oros, 2004). Thus, biological methods are great importance for this purpose. The microbial decolorization of azo dye, MO, was researched by Jagiasi & Patel (2015) and Parshetti et al. (2010). They obtained similar results in comparison with the analysis of FTIR in this thesis. In otherwords, the changes in peaks approximately at 1600 cm^{-1} and at 3330 cm^{-1} were demonstrated that the MO dye decolorizated. In addition, the FTIR spectra of samples after decolorization did not display the peak at 1364 cm^{-1} for S=O stretching vibrations. This could indicate the destruction of the sulfonic group of dye by enzyme treatments and explain the decolorization of MO chromophore by enzymes (Sathishkumar & Palvannan; 2013). Parshetti et al. (2010) also showed the proposal pathway for decolorization of MO by azo reductase. In that study, the initial step in decolorization of MO (the concentration of 50 ppm) was catalyzed via symmetric cleavage of azo bond, and the formation of sulfonated aromatic amines from the dye showed. Jagiasi & Patel (2015) found the FTIR spectrum indicating the loss of azo bond. Telke, Kadam, Jagtap, Jadhav & Govindwar (2010) had reported the similar results for

disappearance of peaks during study of biochemical characterization and potential for textile dye decolorization of laccase from *Aspergillus ochraceus* NCIM-1146.

Overall findings in this thesis suggested the abilities of Lac enzymes of *P. eryngii* economically produced from peach and cherry solid state cultures for the efficiently decolorization of MO of azo dyes. These observations has established that the white rot fungus, *P. eryngii*, can decolorize dye contaminants via their ligninolytic systems. Further studies should clarify the *in vivo* dye decolorization ability of the fungus, and potential of this strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors.

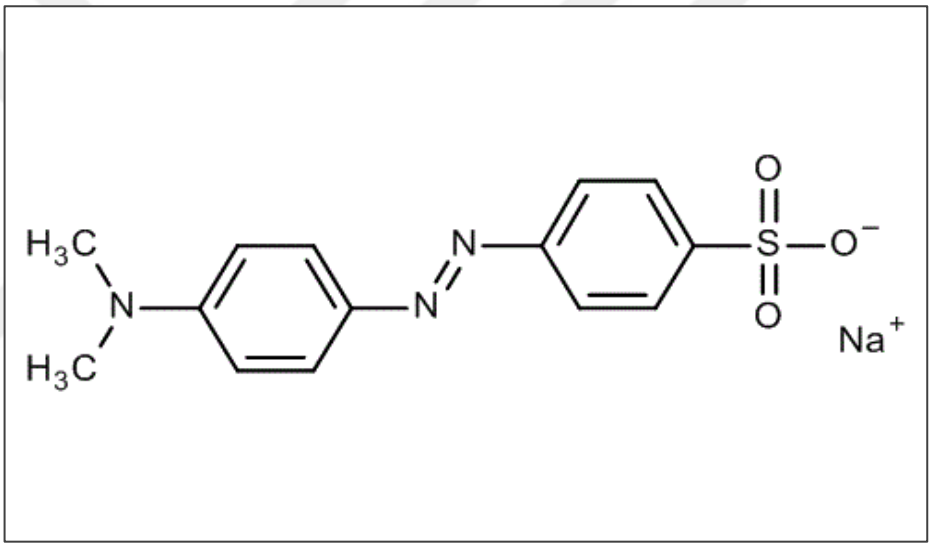


Figure 3.33 The chemical structure of MO.

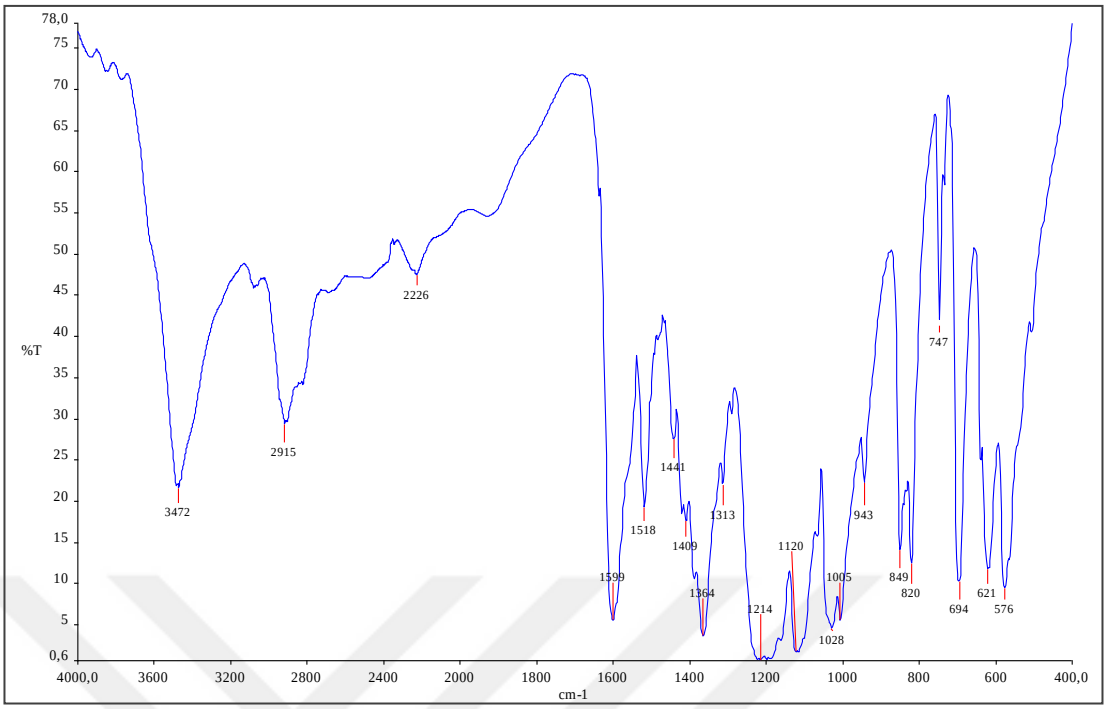


Figure 3.34 FTIR spectra of MO (control dye)

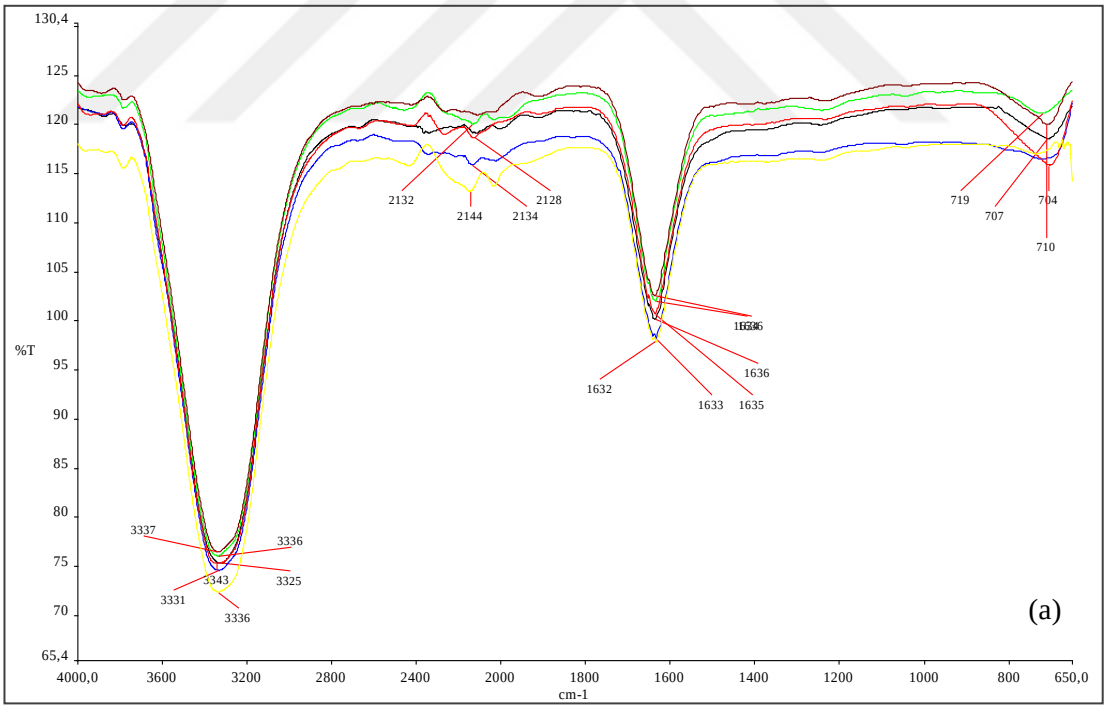


Figure 3.35 FTIR spectra of MO after enzyme treatment from peach (a) and cherry (b) cultures of *P. eryngii* (black line; initial time, blue line; 10 min after; red line; 20 min after; green line; 30 min after; brown line; 40 min after; yellow line; 50 min after)

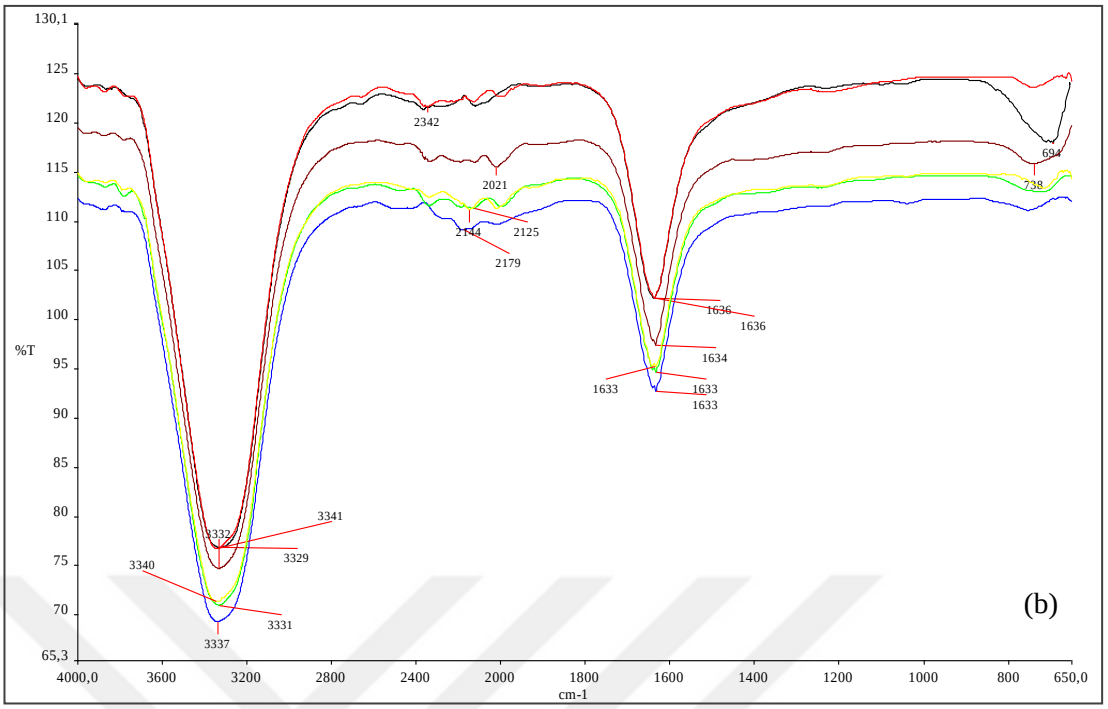


Figure 3.35 FTIR spectra of MO after enzyme treatment from peach (a) and cherry (b) cultures of *P. eryngii* (black line; initial time, blue line; 10 min after; red line; 20 min after; green line; 30 min after; brown line; 40 min after; yellow line; 50 min after) (continued)

CHAPTER FOUR
CONCLUSIONS

The use of abundant and renewable lignocellulosic wastes such as corn stover, wheat straw, sugarcane bagasse and rice straw have received global attention as an alternative source for lignocellulose biotechnology. In Turkey, large quantities of fruit wastes, called pomace, are generated from fruit and juice industries. These wastes, causing environmental problems, are abundant, renewable and inexpensive energy sources. They can be utilized to produce value-added products such as biotechnological enzymes.

The aim is to investigate the ligninolytic and hydrolytic enzyme productions by *P. eryngii* with SSF using peach and cherry wastes from fruit juice industry. To our knowledge, this is the first report that describes potential of the peach and cherry wastes obtained from fruit juice industry for production of ligninolytic and hydrolytic enzymes by *P. eryngii*, cultivated in SSF, a condition under which the fungus grew closer as found in nature. In the first stage of this study, the chemical compositions of these wastes were determined. The experimental findings showed that peach and cherry wastes could be lignocellulosic wastes due to their chemical composition. The lignocellulosic wastes were analyzed in terms of cellulose, lignin, total carbohydrate, reducing sugar, protein, total nitrogen, some metal contents. The lignin concentrations in both wastes were higher when compared to other lignocellulosic wastes; while the cellulose concentrations in peach and cherry wastes were lower.

The peach and cherry wastes with/without pretreatment were further used as subtrates for ligninolytic and hydrolytic enzymes productions by *P. eryngii* under SSF conditions. The both peach and cherry wastes were pretreated with dilute acid and dilute base solutions, hot water. Also, the main components of these wastes were analyzed to investigation their changes during all pretreatments and the effects of the changes on ligninolytic and hydrolytic enzymes productions by *P. eryngii.* After all pretreatments, the chemical compositions of the wastes changed. Generally, the lignin contents decreased, while the total carbohydrate concentrations increased. The protein

contents in all conditions were approximately same with each other. The highest decreases in cellulose contents of both wastes with base-pretreated were observed followed by acid-pretreated. Also, the higher ligninolytic and hydrolytic enzymes activities were obtained by using untreated lignocellulosic wastes of peach and cherry. Therefore, the further studies were carried out untreated peach and cherry wastes for ligninolytic and hydrolytic enzymes production by *P. eryngii*. After that, some parameters; different concentrations of Cu^{2+}, Fe^{2+}, Tween 80, ammonium nitrate, Mn^{2+}, were optimized for high yield of enzyme production on both SSF conditions. The investigated inducers played a major role in the productions of the extracellular enzymes especially Lac. At the end of the optimization studies, the optimum concentrations of Cu^{2+}, Fe^{2+}, Tween 80, ammonium nitrate and Mn^{2+} were 70 μM, 18 μM, 0.025% (v/v), 4.0 g/L, 250 μM for peach cultures, respectively. Tween 80 was no added to cherry cultures because of which this compound did generally not induce the extracellular ligninolytic and hydrolytic enzymes activities. The optimum concentrations of Cu^{2+}, Fe^{2+}, ammonium nitrate and Mn^{2+} were 1000 μM, 1000 μM, 2.0 g/L, 180 μM for cherry cultures, respectively. The changes in maximum ligninolytic and hydrolytic activities after were shown in Table 3.5 after optimization of culture medium. Under optimized conditions, the all ligninolytic enzymes and CMCase, BGLA activities were extremely increased, while only exoglucanase activities were decreased on peach cultures of *P. eryngii*. In similar to peach cultures, the all ligninolytic and hydrolytic enzymes activities were extremely increased and there is no significant changes in exoglucanase enzyme activities when compared to control's values. Generally, *P. eryngii* did not exhibit exoglucanase and BGLA production because of unfavorable culturing conditions and lacking of the genetic machinery. According to literature, *P. eryngii* did not produce LiP, but results in this thesis demonstrated that this organism was able to secrete LiP under stress provided by environmental conditions and in the presence of some potent inducers. Namely, results of our study implied that inducers played a significance role in enhancing the production and activity of LiP. According to this thesis, it can be concluded that *P. eryngii* is able to secrete high levels of ligninolytic and hydrolytic enzymes of biotechnological importance in economic culture medium by SSF. Peach and cherry wastes have an enormous potential as ligninolytic and hydrolytic enzymes inducer in

SSF of *P. eryngii* and as a substrate, since the fungus was able to metabolize part of the carbohydrates contained in it.

In the second part of the thesis, the purifications of Lacs were carried out by ammonium sulfate precipitation, anion exchange chromatography, gel filtration respectively due to their biotechnological and industrial importance. The Lacs fractions with high yield and high purification factor were subjected to electrophoretic analysis as well as enzymatic characterizations; their structural and kinetic characteristics. Initial volume activity, protein amount and spesific activity of the concentrated extract from peach cultures were 39.31 U/mL, 0.068 mg/mL, 578.15 U/mg, while they from other cultures were 4.68 U/mL, 0.155 mg/mL and 30.19 U/mg, respectively. As the result of Sephadex G-100 gel filtration chromatography, 3.94-fold purification was obtained for peach culture, while it was 5.34 for cherry culture. Also, the production efficiencies of Lacs from peach and cherry cultures after purification were calculated as 0.025 and 0.067 mg protein/g solid waste, respectively.

Lacs are glycoproteins with molecular masses of generally 32.18-120 kDa, depending of the strain. In this thesis, Lacs from both cultures were resolved on into one peak after gel filtration column. The results illustrated the electrophoresis analysis of the purified Lacs from both peach and cherry cultures resulted in a single band of protein, and their molecular masses were estimated to be approximately 30±1 kDa. The K_m values for ABTS substrate were detected as 1.1381 and 0.329 mM in terms of Lacs activities from peach and cherry cultures, respectively. The effects of selected chemical agents such as EDTA, PMSF, iodoacetamide, β-mercaptoethanol and sodium azide, on the purified Lac activities were also investigated. These compounds partially or completely inhibited the Lac activities of both cultures. Thus, Cys and His amino acids were found to be important for the active center and structure of the both enzymes. Also, the UV studies and EDTA treatments of enzymes demontrated that these enzymes contained Cu^{2+} atoms in their active cites.

In the third part of the thesis, the decolorization of some azo dyes was carried out by crude extracts and purified Lacs from both cultures, and the effects on the dyes on

Lac enzymes stabilities were investigated in addition to optimization of productions, purification and characterization studies. Finally, FTIR analyzes were carried out to determine whether there were the chemical changes of MO dyes. The extracellular liquid produced in SSF performed decolorization of the synthetic four azo dyes; MO, TT, RR and RB. The maximal percentage of MO decolorization was obtained as 43% after 5 min treatment. Also, these dyes could not cause the Lac and MnP denaturations in the playing major roles in decolorization. Lastly, the changes in peaks approximately at 1600 cm^{-1} and at 3330 cm^{-1} were demonstrated that MO could be biologically decolorized.

As a consequence of this thesis, this study proved that peach and cherry wastes can be good choice as substrates for the economic and eco-friendly production of ligninolytic and hydrolytic enzymes by *P.eryngii*, considering that significant enzyme activity levels were achieved. When the optimization of cultivation conditions, culture medium components and their concentrations are performed, these enzymes can be efficiently used in various industries (pulp and paper, textile, and bioremediation of industrial pollutants). Also, the analyzes carried out in the presence of the dye can be evidence of this.

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