ANALYSIS OF POLYPHENOL OXIDASE PRODUCTION BY SOUTHERN PINE BEETLE ASSOCIATED FUNGI

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FOR
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IN
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Approval of the Graduate School of Natural and Applied Sciences.

_____

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

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Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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BY
SOUTHERN PINE BEETLE ASSOCIATED FUNGI

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In this study, two symbiotic fungi of Southern Pine Beetle (SPB), *Entomocorticium peryii* and *Entomocorticium* sp.A were evaluated in terms of polyphenol oxidase (PPO) production. The effect of different inhibitors, inducers and assay parameters such as temperature and pH on enzyme activity were investigated and maximum PPO activity was observed at 30°C, pH 8.0 and when tannic acid was used as an inducer. Copper-chelator salicyl hydroxamic acid (SHAM) and *p*-coumaric acid, both indicated as inhibitors of tyrosinase and catechol oxidase significantly reduced the activity.

For biochemical characterization studies, the enzyme was concentrated by ultrafiltration. To determine type of the enzyme, activity staining after Native-PAGE was carried out. Type of polyphenol oxidase produced by *E. peryii* and *E.* sp.A was determined as catechol oxidase by activity staining. However higher activity was observed on hydroquinone (*p*-diphenol) rather than catechol (*o*-diphenol).

The enzyme obeys Michealis-Menten kinetics with K_m and V_{max} values being 10.72 mM hydroquinone and 59.44 U/ml for *E. peryii* and 8.55 mM hydroquinone and 73.72 U/ml for *E.* sp.A respectively..

Keywords: Polyphenol oxidase, catechol oxidase, symbiotic fungi, Southern Pine Beetle (SPB), enzyme production.

ÖZ

ÇAM BÖCEĞİ İLE ORTAK YAŞAM SÜREN FUNGUSLARDA POLİFENOL OKSİDAZ ENZİMİ ÜRETİMİ ANALİZİ

Valiev, Abduvali
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Bu çalışmada 'Southern Pine Beetle' olarak anılan bir tür çam böceği ile ortak yaşam süren funguslarda polifenol oksidaz enzimi üretimi incelenmiştir. Farklı enzim inhibitörlerinin, indükleyici bileşiklerin ve sıcaklık, pH gibi deney parametrelerinin enzim aktivitesi üzerine olan etkisi incelenmiş ve en yüksek polifenol oksidaz aktivitesi 30°C'de , pH 8.0'de ve enzim indükleyicisi olarak tannik asit kullanıldığında gözlemlenmiştir. Katekol oksidaz ve tyrosinaz inhibitörü olarak bilinen salisil hidroksamik asit ve *p*-kumarik asitleri aktiviteyi önemli ölçüde azaltmıştır.

Biyokimyasal karakterizasyon için enzim önce ultrafiltrasyon ile konsantre edilmiş ve elektroforezdan sonra aktivite boyama çalışmaları yapılmıştır. *E. peryii* ve *E.* sp.A için polifenol oksidaz enzim türü aktivite boyaması ile katekol oksidaz olarak belirlenmiştir. Diğer yandan, enzimin hidrokinona (*p*-difenol) karşı aktivitesinin katekoldan (*o*-difenol) daha fazla olduğu gözlenmiştir.

Enzimin Michealis-Menten kinetiğine uyduğu görülmüş, ve *E. peryii* için K_m ve V_{max} değerleri sırasıyla 10.72 mM hidrokinon ve 59.44 U/ml, *E*. sp.A için ise sırasıyla 8.55 mM hidrokinon ve 73.72 U/ml olarak hesaplanmıştır.

Anahtar Sözcükler: Polifenol oksidaz, katekol oksidaz, simbiyotik fungus , çam böceği, enzim üretimi.

DEDICATED
TO
MY BELOVED PARENTS

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

SPB : Southern pine beetle

PPO : Polyphenol oxidase

CO : Catechol oxidase

ADA : 4-amino-*N*,*N*-diethylaniline

APS : Ammonium persulfate

H_2O_2 : Hydrogen peroxide

L-DOPA : L-3,4-dihydroxyphenylalanine

PVP : Polyvinyl-pyrrolidone

PCA : *p*- coumaric acid

SHAM : Salicyl hydroxamic acid

CTAB : Cetyltrimethylammonium bromide

NATIVE-PAGE : Native (non denaturing) -Polyacrylamide gel electrophoresis

*t*BC : 4-tert-butyl catechol

TEMED : *N*,*N*-tetramethylene-ethylenediamine

OD : Optical density

rpm: Revolutions per minute

U : Enzyme activity unit

ABTS : 2 ,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)

ε : Molar Extinction coefficient (M^{-1} cm^{-1})

YpSs broth : Yeast peptone, soluble starch broth

MEA : Malt extract agar

PDA : Potato dextrose agar

UF : Ultra filtration

NMWC : Nominal molecular weight cutoff

UV: Ultra violet

CHAPTER 1

INTRODUCTION

"If you knew what you were doing, it wouldn't be called research, would it ?"

Albert Einstein

Polyphenol oxidase (EC 1. 14. 18. 1) is a copper containing enzyme, widely distributed throughout microorganism, plants and animals. It has crucial role in such processes as animal skin pigmentation and browning of fruits and vegetables (Prota, 1988). The enzyme uses the copper ion as a prosthetic group which participates in several redox reactions. The structure of the active site of the enzyme, in which copper is bound by six or seven histidine residues and a single cysteine residue is highly conserved. The enzyme seems to be of almost universal distribution in animals, plants, fungi and bacteria. Oxygen is required for the catalytic processes that lead to hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and oxidation of *o*-diphenols to highly reactive *o*-quinones (diphenolase activity) (Mayer & Harel, 1979). The *o*-quinones polymerize to form melanin through a series of subsequent enzymatic and nonenzymatic reactions. Although the physiological function of polyphenol oxidase (PPO) in fungi is not yet understood, melanin synthesis is correlated with spore formation, virulence of pathogenic fungi, and tissue protection after injury (Miranda et al., 1997). An interesting function of fungal PPO comes from an examination of fungal interactions (Score et al., 1997). In experiments in which different species of fungi were allowed to grow together either in pure or mixed cultures on Petri dishes. The formation of peroxidase, laccase and tyrosinase was followed in the medium. In addition, PPO is responsible for the undesired enzymatic browning of mushrooms that takes place during senescence or damage during post-harvest handling (García-Carmona, 2001).

1.1 Phenols

Phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) attached to an aromatic hydrocarbon group. The simplest of the class is phenol (C_6H_5OH) which is shown in Figure 1.1.

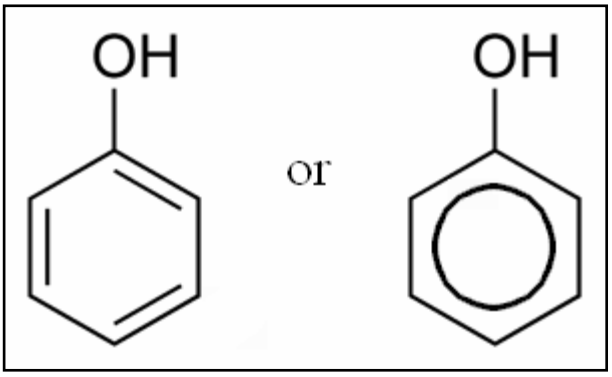


Figure 1.1 : Simplest structure of phenol

Although similar to alcohols, phenols have unique properties and are not classified as alcohols (since the hydroxyl group is not bonded to a saturated carbon atom). They have relatively higher acidities due to the aromatic ring tightly coupling with the oxygen and a relatively loose bond between the oxygen and hydrogen. The acidity of the hydroxyl group in phenols is commonly intermediate between that of aliphatic alcohols and carboxylic acids. Loss of a positive hydrogen ion (H+) from the hydroxyl group of a phenol forms a negative phenolate ion.

Phenolics can be very large in size, such as condensed tannins. Different phenolics having relevant roles in food products include oleuropein, hydroxyl benzoic acid derivatives, cinnamates, isoflavones, lignans, stilbenes, anthocyanins, flavanones, chalcones and dihydrochalcones, flovanols, flavones, flavonols, proanthocyanidins, tannin like compounds, ellagitannins and miscellaneous phenols. These phenolic compounds having different chemical structures have different biological functions. Of particular note are their antioxidant, anti-inflammatory, antitumorial and oestrogenic activities, which might suggest their potential in the prevention of coronary heart disease and cancer. Phenolic compounds play an important role in food visual appereance. Anthocyanin pigments are responsible for most of the blue, purple, red and intermediate color of plant-derived foods and appear black in some commodities. Phenolic compounds are also relevant in terms of food flavour, as they can play a role in the bitter, sweet, pungent or astringent taste of some products and can also contribute to aroma (Tomás-Barberán and Espin, 2001). Some phenols are germicidal and are used in formulating disinfectants. Others possess estrogenic or endocrine disrupting activity.

1.2 Phenol Oxidative Enzymes

We can categorize phenol oxidative enzymes in two major groups as summarized in Figure 1.2. These are peroxidases and polyphenol oxidases.
Since peroxidases are another topic, only some general information about PPOs given on following pages.

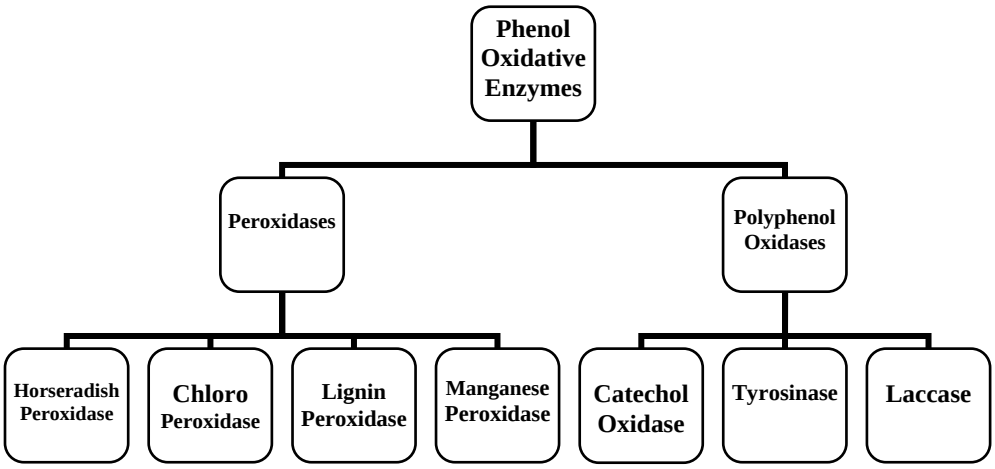


Figure 1.2: General classification of phenol oxidases

1.2.1 Tyrosinase

Tyrosinases (monophenol, *o*-diphenol oxidoreductase) belong to a larger group of proteins named type-3 copper proteins, which include the cresolase from plants and the oxygen-carrier haemocyanins from molluscs and arthropods (Gerdemann *et al.* 2002). Tyrosinases are involved in the melanin pathway and are especially responsible for the first steps of melanin synthesis from L-tyrosine leading to the formation of L-dopaquinone and L-dopachrome (Sanchez-Ferrer *et al.* 1995). The particularity of tyrosinases is to catalyse the *o*-hydroxylation of monophenols (cresolase activity or "monophenolase") and the subsequent oxidation of the resulting *o*-diphenols into reactive *o*-quinones (catecholase activity or "diphenolase"), both reactions using molecular oxygen. Subsequently, the *o*-quinones undergo non-enzymatic reactions with various nucleophiles, producing intermediates, which associate spontaneously in dark brown pigments (Soler-Rivas *et al.* 1999).

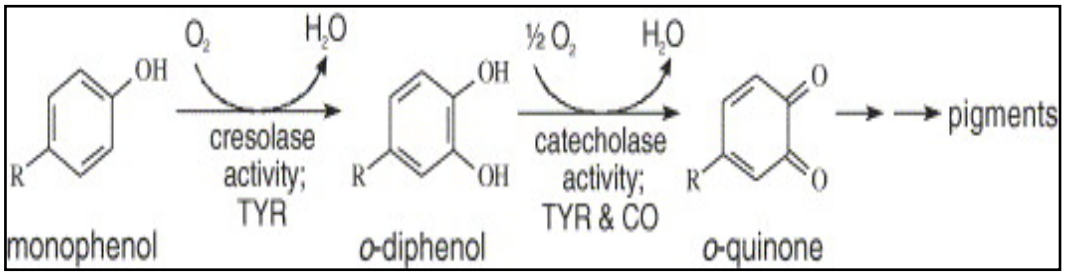


Figure 1.3: Tyrosinase catalysed reactions

Tyrosinases are present in mammals, invertebrates, plants, and microorganisms in which they are involved in several biological functions and have considerable heterogeneity (Van Gelder *et al.* 1997). Tyrosinases were first characterized in mammals for their role in the development of melanomas, and for their implication in pigmentation troubles such as albinism and vitiligo (Riley 1997). In fungi, tyrosinases are mainly associated with browning and pigmentation (Soler-Rivas *et al.* 1999). Melanins in the fungal cell-walls are derived from L-tyrosine, γ-glutaminyl-3,4-dihyroxybenzene (GDHB), or catechol in the case of Basidiomycotina, and from 1,8-dihydroxynaphthalene (DHN) in the case of Ascomycotina (Bell and Wheeler 1986). Melanins constitute a mechanism of defence and resistance to stress such as UV radiations, free radicals, gamma rays, dehydration and extreme temperatures, and contribute to the fungal cell-wall resistance against hydrolytic enzymes in avoiding cellular lysis (Bell and Wheeler 1986). Fungal pigments are also involved in the formation and stability of spores (Mayer and Harel 1979), in the defence and virulence mechanisms (Jacobson 2000). Fungal tyrosinases were firstly characterized from the edible mushroom *Agaricus bisporus* (Nakamura *et al.* 1966) because of enzymatic browning during development and postharvest storage, which particularly decreases the commercial value of the product (Jolivet *et al.* 1998). Further studies on tyrosinases from other fungi such as *Neurospora crassa* (Kupper *et al.* 1989),

Lentinula edodes (Kanda *et al.* 1996), *Aspergillus oryzae* (Nakamura *et al.* 2000), and *Pycnoporus sanguineus* (Halaouili *et al.* 2005) gave new insights into mushroom-tyrosinase biochemical and molecular characteristics.

1.2.2 Laccase

Laccases, EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase, are part of a larger group of enzymes termed the multicopper enzymes, which includes among others ascorbic acid oxidase and ceruloplasmin. Laccase was first described by Yoshida (1883), and was characterized as a metal containing oxidase by Bertrand (1985). This makes it one of the oldest enzymes ever described. Laccases can be roughly divided into two major groups which show clear differences, i.e. those from higher plants and those from fungi (Mayer and Harel, 1979). The presence of laccase–like enzymes has been reported in bacteria (Givaudan et al., 1993) as well as in insects (Hopkins and Kramer, 1992).

1.2.2.1 Fungal Laccases

In contrast to the rather sparse characterization of the laccases in higher plants, there is an abundance of reports in fungi. Almost every fungus in which a laccase has been reported appears to contain several laccases. Sometimes these are referred to as laccase 1 and laccase 2. Often they differ in molecular weight, and frequently additional forms are present as in *Cryphonectria* (Kim et al., 1995). Four different laccases have been detected in *Rhizoctonia solani* (Wahleithner et al., 1996) and *Fusarium proliferatum* (Kwon and Anderson, 2001), and there appear to be at least three laccases in *Botrytis cinerea* (Marbach et al., 1984). While the laccases of *Botrytis cinerea* are predominantly extracellular, laccase activity has been detected in

purified extracellular matrix (Doss; Gil and Schouten), the fungus also contains a constitutive, intracellular laccase (Mayer,1997).

The genes for numerous laccases have been cloned and the sequences deposited in the appropriate gene register. In many fungal species the presence of both constitutive and inducible laccases have been reported. Usually the enzyme originates in the cytoplasm, but many instances of secretion of laccases have been reported. Relatively little attention has been paid to the sub-cellular location of the enzyme or to the mechanism of secretion. Although the structure of the active site seems to be conserved in all the fungal laccases, there is great diversity in the rest of the protein structure and in the sugar moiety of the enzyme.

1.2.2.2 Laccase Catalysis

The range of substrates which various laccases can attack is very wide. Basically any substrate with characteristics similar to a *p*-diphenol will be oxidized by laccases. In addition at least some of the fungal laccases can also oxidize monophenols such as cresol and some are able to oxidize ascorbic acid. At the same time it must be remembered that frequently inadequate experimentation has been carried out to differentiate between laccases and peroxidases and laccases and other polyphenoloxidases. An indication of the complexity of this problem is provided by the work of Scherer and Fischer (1998). These authors purified a laccase II from *Aspergillus nidulans*. Sequencing of the enzyme, despite its substrate specificity as a laccase, and the high degree of purification, indicated more similarity to peroxidase than to laccase. It has also been shown in a ground-breaking paper by Xu et al. (1998) that the catalytic properties of a laccase from the fungus *Myceliophthora thermophilica* could be appreciably changed by site-directed mutagenesis. In a triple mutant, catalytic properties of the enzyme as well as inhibition were markedly altered.

Thus it is clear that the properties of laccases can be readily changed, either by changes in their amino acid sequences or in composition of the carbohydrate moiety.

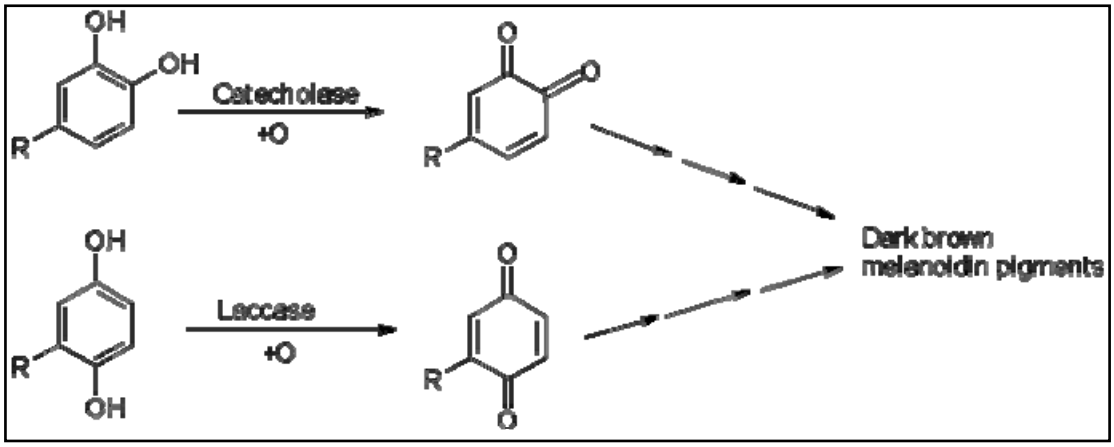
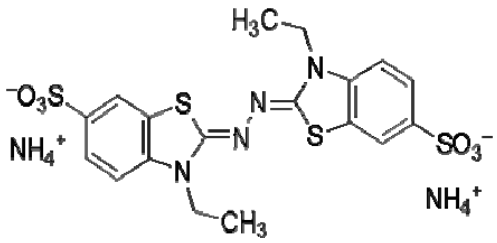


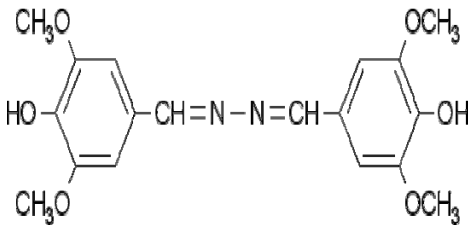
Figure 1.4: Catalytic activity of laccase and it's differance from catecholase

Physical methodology such as circular dichroism (CD) and electron paramagnetic resonance (EPR) have been used to study the metal centers of fungal laccases, showing that they are relatively stable compared to the secondary structure of the protein (Bonomo et al., 2001). The latter can undergo changes thereby altering substrate specificity. Numerous substrates have been used to study laccase activity. The only conclusive proof of laccase activity is that it is able to oxidize quinol as determined by oxygen uptake no matter what other substrates are attacked. The use of syringaldazine as a substrate can be flawed, since this substrate is also oxidized by peroxidase. Special precautions are needed for its use, including the removal of

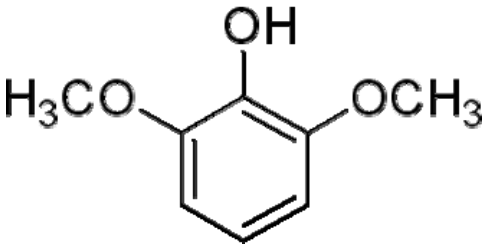
hydrogen peroxide, and this is frequently omitted in work in which the presence of laccase is supposedly shown. Another substrate frequently used is 2,2-azobis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) which is shown in figure 1.5. This must also be used with care since it is also oxidized by peroxidase. To differentiate between laccase and peroxidase on the one hand and between laccase and polyphenol oxidase on the other requires painstaking experiments. One of the difficulties is the fact that the immediate product of laccase activity is a semi-quinone i.e. a free radical. These are extremely reactive and are able to interact with numerous compounds which are present particularly in inadequately purified enzyme preparations. The complexity of such reactions has been shown by Ferrari et al. (1997).



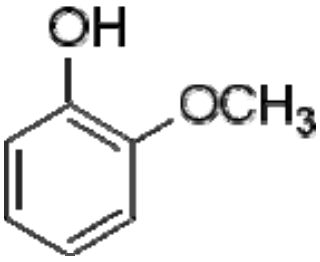
ABTS



Syringaldazine



2,6 - dimethoxyphenol



Guaiacol

Figure 1.5 : Structures of substrates for laccase

New laccases are often discovered when some of the known ones are inactivated using knock out techniques (Kim et al., 1995). The reports on the molecular weight, pH optima, substrate specificity and other properties of the laccases show extreme diversity (Thurston, 1994). To what extent this diversity is the result of isolation and purification procedures is unknown. However, the sugar moiety is clearly different among the laccases both in amount and composition.

1.2.3 Catechol Oxidase

Catechol oxidases (CO) refer to metalloenzymes catalyzing the oxidation of *o*-diphenols to the corresponding *o*-quinones (catecholase or diphenolase activity) (Figure 1.6) . They are moderately glycosylated copper-binding proteins using molecular oxygen as a cofactor and exhibiting two copper-binding domains. Together with tyrosinase and hemocyanin, they are present in eukaryotes and prokaryotes.

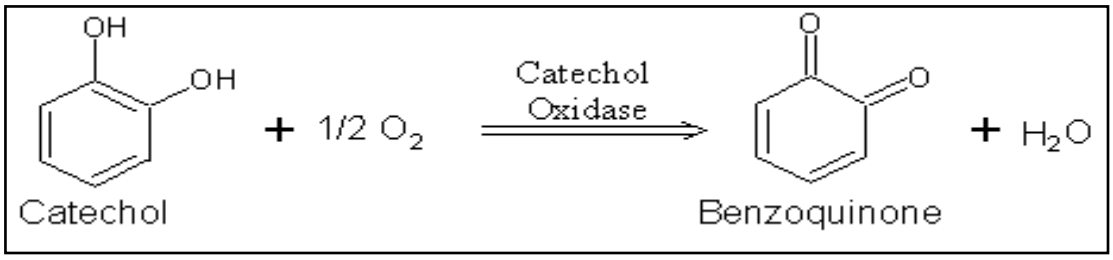


Figure 1.6: Reaction catalysed by catechol oxidase

Above reaction has important implications in food managing and processing: upon browning, significant postharvest losses of several crops do occur. In potato, catechol oxidases are also related to blackspot, an internal damage of tuber observable during their storage and resulting from impact injuries (McGarry et al). During storage, CO activity increases in the outer parts of potato tubers, and both the rate and the extent of the browning of tubers are cultivar-dependent (Vamos-Vigyazo, 1981). Despite its technological relevance, the molecular basis of this increase is not fully understood. Moreover, a close connection between the expression of catechol oxidases and potato dormancy has been suggested in several studies.

1.2.3.1 Similarity between Catechol oxidase, Tyrosinase and Hemocyanin at the Molecular Level

Catechol oxidase is strongly related to both tyrosinase and hemocyanin. All these enzymes have a dinuclear copper center with histidine ligands at the active site (Inlow, Flurkey and Marusek, 2005). In the case of hemocyanin there are three histidine ligands for each copper. Catechol oxidase also has six histidines, but one of them is covalently linked to a cysteine (Figure 1.7: b, c, and d).

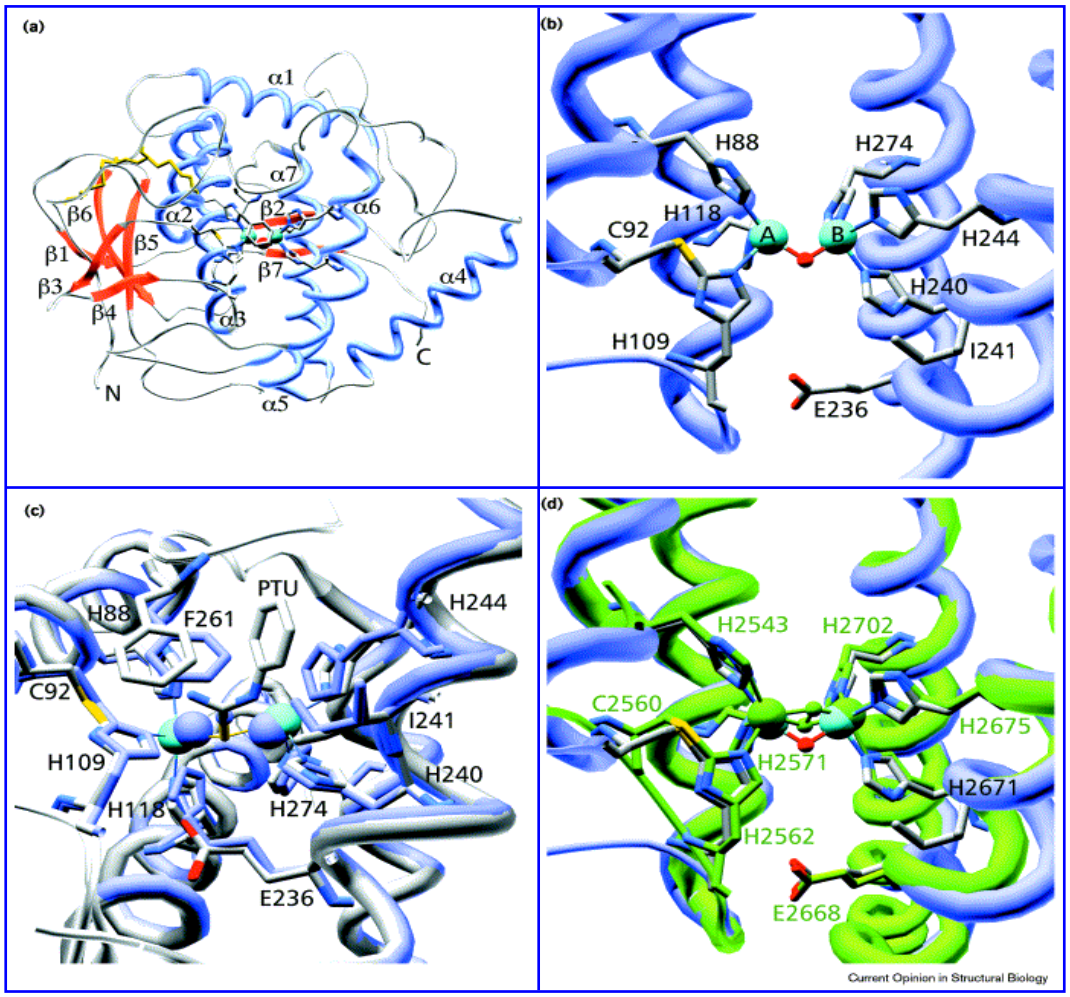


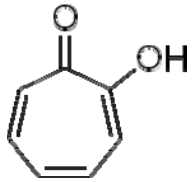
Figure 1.7: 3D Structure of catechol oxidase from *Ipomoea batatas* (Sweet Potato)
(a: The front view of 30 kDa CO; b,c &d : The active site views from different points).
(Eicken, Krebs and Sacchettini, 1999)

The structure of the monomeric 39 kDa CO from sweet potatoes was solved and refined to 2.5 Å in the dicupric Cu(II)–Cu(II) state (Eicken, Krebs and Sacchettini, 1999). The enzyme is ellipsoid in shape, with dimensions of approximately 55 by 45 by 45 Å (Figure 1.7a). The secondary structure is primarily a-helical, with the core of the enzyme formed by a four-helix bundle composed of

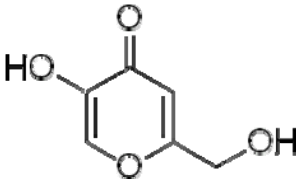
helices a2, a3, a6 and a7. The helix bundle accommodates the catalytic dinuclear copper center and is surrounded by two a helices, a1 and a4, and several short b strands. Two disulfide bridges (C11 to C28 and C27 to C89) constrain the loop-rich N-terminal region of the protein (residues 1–50) to helix a2. Both of the two active site coppers were clearly visible in the electron density maps and each is coordinated by three histidine residues contributed by the helix bundle.

1.3 Polyphenol Oxidase Inhibitors

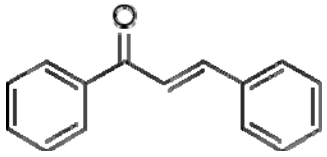
Because of the importance of browning caused by PPO in the food industry (Vamos-Vigyazo, 1995) and its great significance in melanogenesis (Seo et al., 2003), potential inhibitory compounds of polyphenol oxidase enzyme studied very much. An inhibitor, often used as a reference compound is kojic acid (5-hydroxy-2(hydroxyl-methyl)-4*H*-pyran-4-one) (Kahn, 1995), which is effective at 10–50 μM. One of the more promising compounds, whose use is permitted in foods (Vamos-Vigyazo, 1995), is hexylresorcinol, which is active at around 100 μM, and this compound has proven to be useful in differentiating between laccase and PPO (Dawley and Flurkey, 2003).



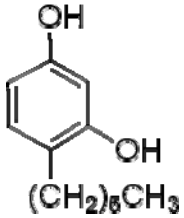
Tropolone



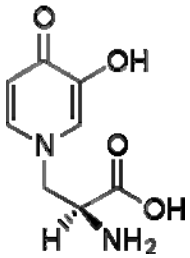
Kojic acid



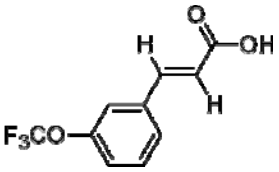
trans-Chalcone



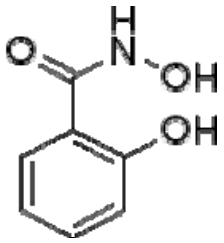
4-Hexylresorcinol



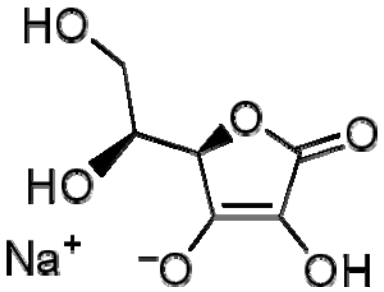
L-
Mimosine



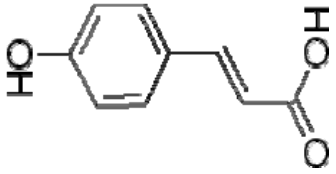
Cinnamic acid



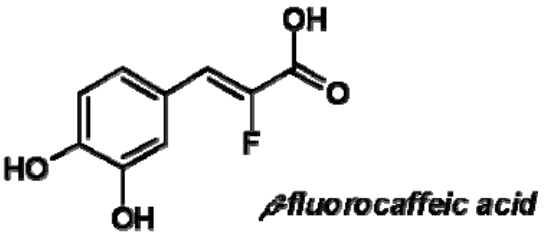
Salicylhydroxamic acid (SHAM)



Sodium L-ascorbate



p-Coumaric acid



p-fluorocaffeic acid

Figure 1.8: Structures of some polyphenol oxidase inhibitors

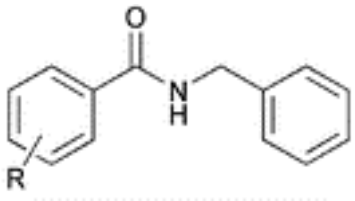
1.3.1 Inhibitors related to phenolic compounds

The search for naturally occurring inhibitors has led to the discovery of a number of active compounds. Among the more interesting ones discovered were chalcones and related compounds (Nerya et al., 2004). The most active compounds were glabridin, effective at around 1 μM and isoliquiritigenin, active at 8 μM (Nerya et al., 2003). A study of chalcone derivatives, related to these compounds (Nerya et al., 2004 and Khatib et al., 2005), showed that the number and position of hydroxyl groups in the A and B rings of chalcones was important in determining their inhibitory activity. The precise mechanism of action of these chalcone derivatives is not entirely clear. The possibility of actually using such compounds in preventing browning of intact mushrooms was examined and was found to be unsuccessful (Nerya et al., 2006).

Some flavanols active in the 50–100 μM, range have been found, acting perhaps as copper chelators (Kubo et al., 2000). More recently, flavanones isolated from *Garcinia subelliptica* were found to inhibit tyrosinase (Masuda et al., 2005). Among the most interesting recent reports is that procyanidins can inhibit PPO from apples (Le Bourvellec et al., 2004). The most active of these compounds, ProAV, with a degree of polymerisation of 80, was inhibitory at 0.02 g/l. Translated into molarity, assuming a molecular weight of about 300 for the monomer, this implies a very high inhibitory activity in the micromolar range. Other very active inhibitory compounds were the oxidation products of caffeoylquinic acid, also at very low concentrations.

1.3.2 New classes of inhibitors

New classes of inhibitors are tetraketones (Khan et al., 2006) active at 2.06 μM ,decanoate derivatives, which inhibited tyrosinase irreversibly at 96.5 μM (Qiu et al., 2005) and substituted 1,3,4-oxadiazole analogues (Khan et al., 2005). *N*-Benzylbenzamides (Figure 1.9) have been shown to be active as tyrosinase inhibitors, in which the hydroxylation pattern of the B ring was the most important in determining activity (Cho et al., 2006). Competitive inhibition by hydroxystilbenes has also been reported, although activity was not very great (Song et al., 2006). The *cis*-isomer of 3,5-dihydroxy stilbene form was about twice as effective as the *trans*-isomer and this has some implications with regards to the interaction between the inhibitor and the enzyme.



***N*-Benzylbenzamides**

Figure 1.9 : *N*-Benzylbenzamides, tyrosinase inhibitor

Chitosans have been reported to extend post-harvest life of fruits and it has been claimed that they have a direct inhibitory effect on PPO. In the case of longan (*Dimocarpus longan*) fruit increases in PPO during storage were delayed when the fruit was treated with chitosan, but the effects were probably due to secondary

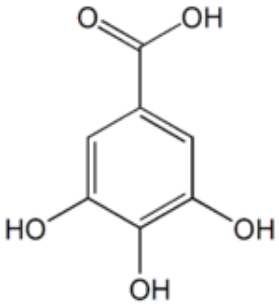
changes, such as the protective coating by chitosan, and not a direct inhibition of PPO (Jiang and Li, 2001).

1.4 Polyphenol Oxidase Inducers

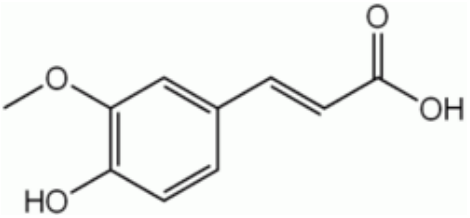
Inducers play a major role in the production of polyphenol oxidase enzyme. Different aromatic compounds gallic acid, ferulic acid, xylidine, guaiacol, syringaldazine, veratryl alcohol (Figure 1.10) and inorganic copper sulphate have been widely used to stimulate polyphenol oxidase production .

In *P. chrysosporium* copper in the form of copper sulphate at 30 mM accelerated the extracellular laccase production at 3.5-fold increase compared to control (Gnanamani, 2005). Also in another study supplementing the culture medium of *Trametes versicolor* with veratryl alcohol or xylidine improved laccase activity levels. The latter gave the best results, showing activity values about 1700 U/l (M.Angeles Sanroman,2002).

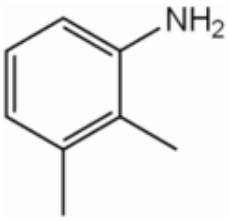
Also in this study, 1mM gallic acid used as an inducer and significant increase in catechol oxidase production was observed.



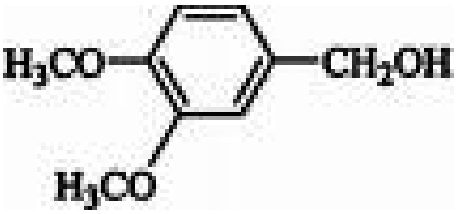
Gallic acid



Ferulic acid



Xylidine



Veratryl alcohol

Figure 1.10: Structures of some polyphenol oxidase inducers

1.5 Applications of Polyphenol Oxidases

Polyphenol oxidase enzymes have significant applications in many areas such as food, medicine, and industry.

In food processes, polyphenol oxidases have been mainly used for enhancement of the flavor in tea, coffee and cocoa (Motoda, 1979). Tea is manufactured in a process, during the main chemical damages in the tea leaves take place. In the process, polyphenols abundantly present in tea leaves are oxidized by the action of polyphenol oxidase contained in the leaves themselves, and the oxidation products in the black tea contribute to color, strength and flavor in liquors. Therefore,

polyphenol oxidase activity of tea leaves and the oxidation products play an important role in the formation of black tea.

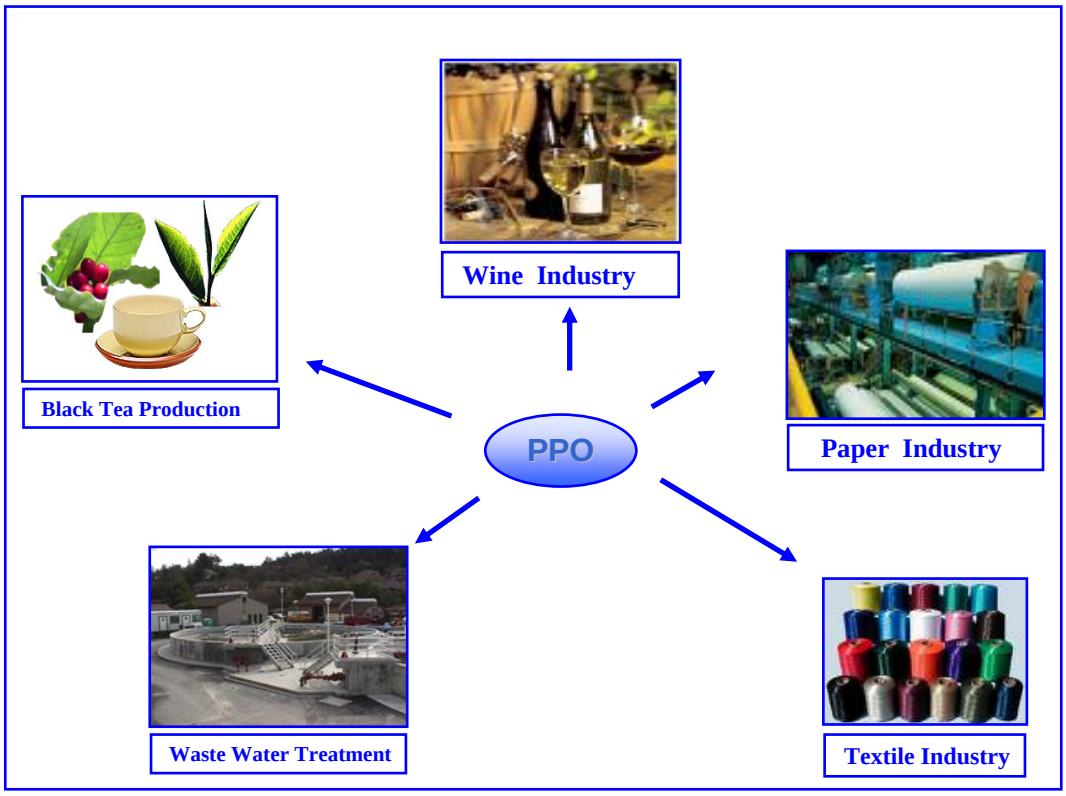


Figure 1.11: Industrial applications of polyphenol oxidase (Croser, 2000)

On the other hand, in some food processes, polyphenol oxidase activity is undesirable and plays an important role in determination of food quality. Many fruits including peaches, apricots, apples, grapes, bananas, strawberries, and the vegetables potatoes, lettuce, mushrooms and eggplants are lost due to the action of polyphenol oxidase activity because activity in mechanically damaged foods leads to a browning reaction in the presence of O_2, and this causes a nutritional loss and being

unacceptable for the consumer. In this respect, the controlled PPO activity is supposed to be important in control of the quality (Whitaker, 1994).

One of the most important reactions concerned with the development of flavor in foods is the Strecker degradation, by which amino acids and dicarbonyl compounds react to give ammonia, carbondioxide and aldehydes with one less atom than the amino acids. These aldehydes contribute to flavor. Aldehydes are formed by the oxidative degradation of amino acids not only during the interaction of amino acids and sugars at high temperatures but also during the interaction of amino acids and polyphenols in the presence of polyphenol oxidase at normal temperatures. So action of the polyphenol oxidase in Strecker degradation decreases the cost of the process and saves the producer from the heating process (Motoda, 1979).

In medicine, polyphenol oxidases are used for prevention of bacterial adhesion, treatment of Parkinson's disease and control of melanin synthesis.

According to the recent studies, attachment of *Streptococcus* to the tooth surface was inhibited by the water extract of potato polyphenol oxidase (Cowan *et al*., 2000).

In Parkinson's disease treatment, polyphenol oxidase activity is exploited for the conversion of L-tyrosine ester to L-DOPA which is an important neurotransmitter and is found insufficient in Parkinson's patients (Xu *et al*., 1998).

According to the recent investigations melanin is insufficient in Phneylketonurea patients, and can be used as a sun blocker when applied to the skin. Melanin synthesis can be achieved with polyphenol oxidase in the presence of tyrosine (Pietz *et al*., 1998) .

In industrial applications, polyphenol oxidases are mainly used for the elimination of toxic wastes. The toxicity and hazardous nature of some phenols and their associated derivatives, and their increasing presence in a number of industrial

wastewaters have been well documented. Recent interest in the enzymatic methods for phenol removal has focused primarily on polyphenol oxidase and peroxidase to treat low concentration phenol containing wastewaters. The use of peroxidase has the disadvantage that stoichiometric amounts of H_2O_2 are the oxidant whereas polyphenol oxidase requires O_2 (Edwards *et al*., 1997).

The o-quinones produced by the action of polyphenol oxidases undergo spontaneous nonenzymatic polymerization in water, eventually forming water-insoluble polymers which can be separated from solution by filtration.

Phenol concentration in several solutions can be measured by means of electrodes containing immobilized polyphenol oxidase (Bauer *et al.,* 1999) and electrodes having laccase provide differentiation of codeine from morphine (Pencreach *et al*., 2002).

1.6 Wood Decaying Fungi

Wood-inhabiting fungi are one of the most important components of forest ecosystem, and play an important role in degrading the wood in forest ecosystem. The major species of these fungi include the groups of Aphyllophorales (Basidiomycota), Discomycetes (Ascomycota) and some imperfect fungi. They have the ability to degrade cellulose, hemicelluloses and lignin of wood. The three types of wood decaying fungi are white rot, brown rot and soft rot fungi. Many other organisms of forest ecosystem have symbiotic relationship with wood-decaying fungi. Wood-inhabiting fungi could offer the nutrition for many insects and birds, and spores of many wood-rotting species are spread by some insects. The high biodiversity of wood-decaying fungi is one of the important factors for the health of forest ecosystem.

1.6.1 Role of Polyphenol Oxidases in Wood Biodegradation

Wood and other lignocellulosic materials are formed by three main polymeric constituents, cellulose, lignin, and hemicelluloses (Figure 1.12). Cellulose is a linear and highly ordered (often crystalline) polymer of cellobiose (D-glucopyranosyl-β-1,4-D-glucopyranose) that represents over 50% of wood weight. By contrast, lignin is a three-dimensional network built up of dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (*p*-hydroxyphenyl, H) phenylpropanoid units, derived from the corresponding *p*-hydroxycinnamyl alcohols, which give rise to a variety of subunits including different ether and C—C bonds.

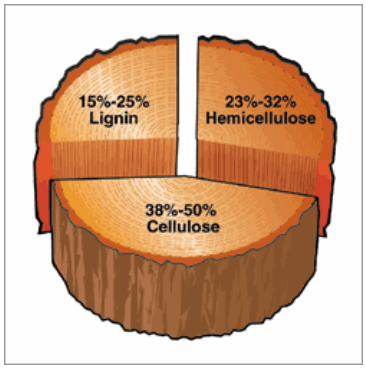


Figure 1.12: Polymeric constituents of wood (Ullrich, 1996)

Basidiomycetes are the main wood rotters due to their ability to degrade or modify lignin, an enzymatic process that originated in paralel with the evolution of vascular plants. Wood-rotting basidiomycetes are classified as white-rot and brown-rot fungi based mainly on macroscopic aspects. Basidiomycetes can overcome difficulties in wood decay, including the low nitrogen content of wood and the

presence of toxic and antibiotic compounds. Extracellular oxidative enzymes (oxidoreductases) mainly polyphenol oxidases and peroxidases secreted by fungi are involved in degradation of cell-wall components .White-rot basidiomycetes, the most frequent wood-rotting organisms, are characterized by their ability to degrade lignin, hemicelluloses, and cellulose, often giving rise to a cellulose- enriched white material. Due to the ability of white-rot basidiomycetes to degrade lignin selectively or simultaneously with cellulose, two white-rot patterns have been described in different types of wood, namely selective delignification, also called sequential decay.

1.6.2 Role of PPO in Fungal Pathogenicity and Fungal Defence Reactions

Induction of PPO in fungi has been researched much less than induction of plant PPO. Infection of the mushroom *A.bisporus* with *Pseudomonas tolaasii*, causes discolouration of the cap. This discolouration is accompanied by an induction of the fungal PPO (Soler-Rivas et al., 2000). The same effect could be induced by treatment of mushrooms with extracts containing the bacterial toxin tolaasin or with purified tolaasin. The induction of PPO activity could be ascribed to the conversion of a latent PPO, Mr 67 kDa to an active form with Mr of 43 kDa as a result of treatment with partially purified extracts, while addition of pure tolaasin induced the transcription of a gene coding for PPO. Thus treatment with the bacterial extract had a dual effect, post-translational modification of PPO and induction of mRNA formation. At this stage it is not clear whether the raised levels of PPO in the mushroom, following infection, is part of a defense reaction, or is simply the result of disruption of cellular metabolism in the host. The similarities with processes of induction of PPO in plants are clear, but further studies are needed.

1.6.3 Role of PPO in Melanin Biosynthesis

Melanins are a large group of diverse substances having some common properties In general, melanins are macromolecules formed by the oxidative polymerization of phenolic or indolic compounds. Often the resulting pigments are brown or black in color but many other colors have also been observed.

The enzymatic browning of fruits and vegetables is mostly related to the oxidation of endogenous phenolic containing compounds catalyzed by polyphenol oxidase. Tyrosinase also is responsible for melanization in animals, and is the key enzyme for the regulation of melanogenesis in mammals. Melanin is synthesized in epidermal melanocytes, and then is transferred into epidermal keratinocytes *via* the melanocytes' dendrites. Melanogenesis is the process by which melanin is produced (and subsequently distributed) by melanocytes within the skin and hair follicles.

In fungi, there is little doubt that PPO is active in the formation of melanin. In addition to the protective role of melanin, it apparently is needed in the cell walls of the appressorium in order to allow them to develop the osmotic potential needed to breach host cell walls. Support for this idea comes from the observation that mutants of *Magnaporthe grisea*, lacking melanin, were unable to develop high osmotic potentials.

Also number of possible functions have been postulated for fungal melanins , based on the properties of melanin. However, it is difficult to know which of these (or other) properties of fungal melanin are of survival value without extensive knowledge of the ecology of the individual fungus (Butler and Day,1998). The proposed functions of fungal melanins include protection against UV irradiation, enzymatic lysis, oxidants, and in some instances extremes of temperatures. Also, melanins have been shown to bind metals, function as a physiological redox buffer,

thereby possibly acting as a sink for harmful unpaired electrons, provide structural rigidity to cell walls, and store water and ions, thus helping to prevent desiccation (Jacobson, 2000).

1.6.4 Southern Pine Beetle (SPB) and It's Relationship with Wood Decaying Fungi

Southern pine beetle (SPB) (Figure 1.13) *Dendroctonus frontalis* is among the most damaging of North American forest insects (Thatcher et al., 1980). The SPB is considered a primary bark beetle, in that it is essentially an obligate parasite (Raffa et al., 1993) that attacks and kills healthy living trees through mass colonization by conspecifics (Paine et al., 1997). Reproductive female beetles initiate attacks on host trees by boring entrance holes through the rough outer bark of southern pines, creating a nuptual chamber and releasing a pheromone to attract more beetles to the tree (Kinzer et al., 1969). The host tree attempts to repel the attack primarily through the release of preformed (constitutive) resin (Hodges et al., 1979). If enough SPB attack the tree in this manner, the tree's resin system is overcome, the beetles are able to complete development and the tree dies (essentially from disruption of water flow within the vascular system). Once the female beetle has mated, she begins chewing ovipositional (egg) galleries within the inner bark and phloem of the tree (Thatcher, 1960). As she does so, the female SPB inoculates several fungi into the phloem tissue (Bramble and Holst, 1940).

Figure 1.13: Southern pine beetle (Moser, 1999)

Although many fungi have been associated with galleries of SPB in pine phloem, three have been the focus of most SPB-fungal research, and appear to have the most significant impacts on the SPB life cycle: *Ophiostoma minus* ,*Ceratocystiopsis ranaculosus* and *Entomocorticium* sp. A (an undescribed basidiomycete, formerly referred to in the literature as isolate SJB122), microscopic views are shown in Figure :1.14.

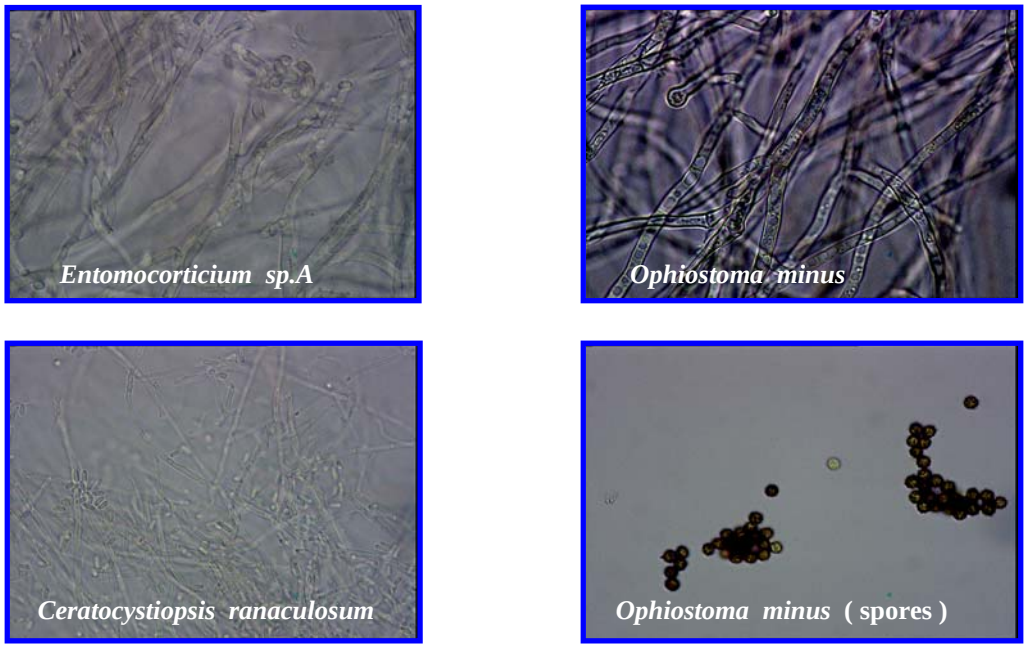


Figure 1.14: Microscopic views of SPB associated fungi

Ophiostoma minus, the causal agent of the "blue stain", often found in the xylem and phloem of SPB infested wood is an ascomycetous fungus carried phoretically on the SPB exoskeleton (Rumbold, 1931) and by phoretic mites (Bridges and Moser, 1983). Early research into the SPB-fungi system focused on the putative role of *O. minus* as a tree killing pathogen (Basham, 1970). However, the fungus is apparently not necessary for tree death to occur (Bridges, 1985). Although artificial inoculations of southern pines with *O. minus* do cause resinosis and tissue damage, they do not result in mortality of mature trees (Popp et al., 1995). It seems probable that *O. minus*, in concert with SPB tunneling, hastens tree death (Paine et al., 1997). The benefits of this relationship to the fungus are clearer. Bark beetles and their arthropod associates serve as the only effective means by which stain fungi gain access to new host tissue (Dowding, 1969). Thus, at the early stages of attack, the SPB-*O. minus* relationship may be categorized as mutualistic, although the frequency with which these

organisms are associated does not necessarily imply this (Harrington, 1993). Subsequent research has focused on the impacts of *O. minus* on SPB larval development. As SPB eggs hatch within the niches the female has created in the pine phloem, the fungi she inoculated begin growing and colonizing the tissue as well. Within this community of organisms patches of *O. minus* develop. When these areas of heavy colonization by the blue stain fungus overlap areas within which the developing larvae are feeding, the SPB almost always suffers. Although much of the evidence has been circumstantial, higher levels of phloem colonization with *O. minus* are correlated with reduced developmental success - inhibited egg production, slower larval growth and development, even larval mortality (Barras, 1970). In addition, overall levels of *O. minus* within SPB infestations have been negatively correlated with SPB population increase (Bridges, 1985). The relationship here seems simple. The more blue stain that is present, the less SPB reproductive success will occur (Lombardero et al., 2000). At the time of larval development, *O. minus* appears to be a competitor and antagonist of SPB (Barras, 1970). The mechanism of this antagonism, however, has remained unclear. Some have speculated that *O. minus* leaves the phloem nutrient impoverished and deprives the developing larvae of necessary sustenance (Hodges et al., 1968). As such, it has also been suggested that the beneficial roles of the two other major fungal associates of SPB consists largely of outgrowing or outcompeting *O. minus* and keeping this blue stain fungus out of SPB larval galleries (Bridges and Perry, 1985).

The antagonism of SPB larvae by *O. minus*, which at first seemed contradictory to the pattern seen between *O. minus* and attacking SPB adults, may be partially explained when the interactions of SPB with its two other significant fungal associates are examined. Each female SPB possesses a prothoracic structure specialized for transporting fungi. This mycangium consists of paired invaginations of the exoskeleton each of which has one pore-like ventral opening and contains two types of secretory cells (Barras and Perry, 1972). Within each side of the mycangium, the female SPB is able to maintain a pure culture of either *C. ranaculosus*

(a hyaline ascomycete) (Barras and Taylor, 1973) or *Entomocorticium* sp. A, formerly referred to in the literature as SJB122 (Barras and Perry 1972). This slow growing fungus is an amber colored basidiomycete, which appears to belong to genus *Entomocorticium* (Hsiau, 1996). Each female may carry either one (rarely both) of the two fungi, or no fungi, in either of the two mycangial pouches (Bridges, 1985). Although it seems likely that the majority of inoculation of mycangial fungi into pine phloem occurs later (Barras, 1975) perhaps during oviposition, the relative virulence of these two fungi in healthy trees has also been investigated. Inoculations of both *C. ranaculosus* and *Entomocorticium* sp. A invariably result in smaller amounts of tree damage (Cook and Hain, 1985). However, both mycangial fungi do cause reactions, especially at the tissue and cellular level, that differ from those seen in response to mere mechanical wounding. SPB mycangial fungi do not appear to be highly virulent in their pine hosts nor do they seem to assist in any meaningful way in tree killing. It seems more likely that the proper window in time to evaluate the role of the mycangial fungi in the SPB life cycle is post-mass attack. Once the tree's resistance has been overcome, as the female SPB deposits her eggs within the pine phloem, she may inoculate the area immediately surrounding the eggs with the contents of her mycangium. As the eggs hatch the early instar larvae begin feeding, constructing fine, sinuous galleries as they go (Payne, 1983). Eventually, the larvae cease moving forward and begin enlarging their feeding area to an obovate shape. It is within these "feeding chambers" that one can find luxuriant growth of either of the two mycangial fungi. It is assumed that the mid to late instar larvae feed on fungal hyphae and spores, although due, in part, to difficulties in artificially rearing SPB, it has never been explicitly demonstrated. It appears extremely likely that larval SPB get the majority of their nutrition from the fungal growth within their feeding chambers rather than from the phloem itself. The mycangial fungi may, in fact, provide their most substantial benefits to SPB by concentrating dietary N for larvae (Ayres et al., 2000). For the fungi, again, the advantages of association with SPB are clear. The fungi obtain a selective medium within which to grow as they are borne,

protected and pure, to the next available resource (Happ et al., 1971). The benefits to the beetle from these fungi appear obvious as well. Beetles containing *Entomocorticium* sp. A are more fecund, heavier, and have higher lipid contents, than those containing *C. ranaculosus*. In turn, beetles containing *C. ranaculosus* tend to be more fit than those whose mycangia contain no fungi (Bridges, 1985). Thus, the two mycangial fungi can be considered to be nutritional mutualists of SPB.

1.6.5 Fungal competition

The three major SPB associated fungi, *O. minus*, *Entomocorticium* sp. A, and *C. ranaculosus*, compete for the rare and ephemeral resource of uncolonized pine phloem (Klepzig and Wilkens, 1997). It is likely that, in doing so, these fungi complete for primary resource capture, followed by direct interaction, which can lead to defense, and/or secondary resource capture. Likewise, the degree to which these fungi differentially compete can be quantified. The de Wit replacement series has been used extensively to study plant competition, and is being increasingly accepted as an analytical tool for microbial competition (Adee et al., 1990). In using this technique with microbes, varying proportions of inoculum of potentially competing microbes are introduced onto a substrate. In the case of competing fungal hyphae, this may consist of inoculating substrate (e.g., agar medium, pine billets) with varying numbers of agar disks colonized with hyphae of one fungal species. The initial inoculum of one species is increased with each replicate as the initial inoculum of the other species is decreased. The population size (in the case of fungal hyphae, the area colonized) is determined at the end of the experiment as a function of initial population size (in this case, the percentage of each species in the original population). If no differential competition is occurring between the two species, it is expected that there will be a close to one-to-one linear relationship between proportion of the

fungus in the initial inoculum and its representation (area colonized) in the final population (Wilson and Lindow, 1994). Differential competition between two fungi is indicated when there is a significant positive deviation from linearity for one and a significant negative deviation from linearity for the other. To determine this, the areas colonized by each fungus at the end of the experiment are recorded, and the means are calculated and log transformed. An ANOVA is performed on the transformed means to test for deviations from linearity in the relationships between final area colonized and initial inoculum proportion, for each fungal species. Specifically, pairwise competitions between *O. minus*, *Entomocorticium* sp.A., and *C. ranaculosus* conducted to determine the degree to which differential competition occurs among these frequently co-occurring fungi vectored by the same beetle. From laboratory experiments, it is absolutely clear that differential competition occurs amongst these three fungi (Klepzig and Wilkens, 1997). In all three pairwise comparisons, there were significant deviations from linearity in the relationships between initial and final population representation in the competing fungi. The clearly superior competitor, at least on the artificial media used, was *O. minus* whose rapid growth rate and aggressive resource capture tactics overwhelmed the two mycangial fungi at even the lowest levels of *O. minus* inoculum. The mycangial fungi were rapidly outcompeted by *O. minus* for the available substrate. *Entomocorticium* sp. A. and *C. ranaculosus*, however, were very similar in their relative competitive abilities. Beyond the determination of the existence of competition with SPB associated fungi, is the question of the outcomes of competition among these fungi. Which of the SPB fungi is best able to hold on to colonized substrate in the face of a concerted secondary resource capture effort by another fungal species? When the three SPB associated fungi are forced to compete one-on-one on both artificial medium (malt extract agar) and natural substrate (loblolly pine billets), *O. minus* invariably comes out the victor in primary resource capture. Due to its relatively rapid growth rate, *O. minus* can quickly colonize and gain control of substantially more of the available territory (uncolonized agar as well as pine phloem) than can either of the two mycangial fungi.

The two mycangial fungi are approximately equal competitors for the capture of primary resource. However, due to its higher growth rate, *C. ranaculosus* is significantly able to outcompete *Entomocorticium* sp. A.

Once the primary resource capture phase of the one-on-one competition is over, however, and the direct confrontations begin, the SPB associated fungi differ in their competitive abilities in interesting ways. When *O. minus* grows into the same area of substrate as *C. ranaculosus*, aerial hyphae begin developing at the colony margins of both species. Within a short while (about 48 hours), however, the heavily melanized hyphae of *O. minus* have grown over the margins of the hyaline *C. ranaculosus* colonies and begun the process of secondary resource capture. By the 11th day of competition between these two fungi, *C. ranaculosus* colonies have most often been completely overgrown by *O. minus*. The competition between the fast growing *O. minus* and the slow growing, amber colored, floccose basidiomycete, *Entomocorticium* sp. A unfolds in a much different fashion. Although, due to the slow growth rate of *Entomocorticium* sp. A, *O. minus* is able to capture a great deal of uncolonized substrate before it reaches the basidiomycete, the direct interaction of these two fungi slows *O. minus* drastically. Very slightly before the growing hyphae of *O. minus* reach the *Entomocorticium* sp. A colony margins, they slow in growth rate. There is little to no development of the aerial hyphae seen in the *O. minus*/*C. ranaculosus* interaction. The *O. minus* colony, if it grows further, grows around the *Entomocorticium* sp. A colony, never growing over the basidiomycete and never accomplishing any secondary resource capture. This dramatic limitation on the growth and further spread of *O. minus* suggests either very close range diffusion of antibiotics from *Entomocorticium* sp. A to *O. minus*, or localized nutrient depletion by *Entomocorticium* sp. A such that *O. minus* cannot develop further in substrate which has been colonized by *Entomocorticium* sp. A. These same patterns of competitive interactions also hold true within loblolly pine billets.

Environmental (abiotic) factors may also alter the intensity and nature of competitive interactions (Callaway and Walker 1997). Temperature drastically affects growth rates in all three SPB associated fungi. Of particular note are the differences in the manner in which the three fungi respond to varying temperatures. *Ophiostoma minus* seems particularly adaptable to a range of temperatures; its range of optimal temperatures for growth is wider, and its minimum growth temperature lower, than are the same variables for either of the two mycangial fungi. This may be due, in part, to the protected manner in which the mycangial fungi are transported (within a mycangium) and cultivated (within the galleries of successful SPB) relative to *O. minus* (which is transported on beetle and mite exoskeletons, and inoculated by SPB attacking living trees). Nutrient levels within phloem may also impact growth and competitive interactions among SPB fungi.

Several implications for SPB and its pine host arise from the interactions described above. It is apparent that *O. minus* is best equipped to capitalize on the uncolonized phloem available in the early stages of SPB attack in pines. Not only does this aggressive fungus grow more rapidly than the two mycangial fungi, it is also more tolerant of pine allelochemicals than *Entomocorticium* sp. A (Bridges, 1987). This, of course, may be advantageous to the beetle and, especially if *O. minus* does assist in killing the tree, disadvantageous to the tree. As tree resistance is overcome, and the female beetles begin inoculating the mycangial fungi into the phloem, the aggressive saprophytic (for the tree is essentially dead at this point) characteristics of *O. minus* become a disadvantage for SPB. At this point, the female needs to establish colonies of either *Entomocorticium* sp. A or *C. ranaculosus* in the vicinity of the larvae, and far enough away from growth of *O. minus* for the fungi to become established and serve as a larval food source. At this point the differences between the two mycangial fungi come into focus. One of the fungi, *C. ranaculosus*, grows marginally faster than the other, but once *O. minus* reaches it does not seem capable of defending this territory enough to allow larval development (Klepzig and Wilkens, 1997). This fungus, especially when considered with its apparent relative inferiority

as a larval nutritional substrate (Bridges, 1983; Goldhammer et al., 1990; Coppedge et al., 1995) would seem to be of less value as a symbiont than *E.*sp.A. *Entomocorticium* sp. A, while slower growing than *C. ranaculosus*, is definitely capable of growing and providing nutrition for SPB larvae, even when surrounded by *O. minus*. The key to larval success, then, may be establishing a thriving culture of *Entomocorticium* sp. A soon enough, or far enough away, that it can grow without interference from *O. minus*. In this sense, as well as in the nutritional sense, *Entomocorticium* sp. A is apparently the superior of the two mycangial fungi.

1.7 Scope of the Study

The main objective of the study was to produce polyphenol oxidase from Southern Pine Beetle (SPB) associated fungi and its characterization. Ezyme production by SPB associated fungi is largely unexplored. Extracellular PPO is produced by most wood-decaying fungi because of the presence of lignin and other phenolic substances on wood. It was therefore of interest to investigate the ability of these fungi to produce PPO.

For this purpose at the beginning of the study five SPB associated fungi were screened for polyphenol oxidase production on agar medium. Two of those fungi namely *E. peryii* and *E.* sp.A were polyphenol oxidase positive. For the enhancement of the polyphenol oxidase activity in shake flask batch cultures, different inducers including gallic acid, tannic acid, ADA and caffeic acid were directly added into culture medium. Identification of phenol oxidases was conducted by activity staining on native-polyacrylamide gel.

PPO from culture supernatant was characterized in terms of kinetic parameters, temperature and pH dependencies.In addition, effects of some PPO inhibitors were

investigated. Also to determine the substrate specificity of the enzyme, different PPO substrates were tested.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Catechol and gallic acid were obtained from Sigma Chem Ltd., (USA). Yeast extract was purchased from Merck Ltd., Acrylamide and bisacrylamide were obtained from AppliChem Ltd. All other chemicals used were analytical grade from Sigma (USA) and Merck (Germany) .

2.1.2 Microorganism

In this study, Southern Pine Beetle associated fungi *Entomocorticium peryii*, *Ophiostoma minus*, *Ophiostoma ranaculosum*, *Ophiostoma nigrocarpum* and *Entomocorticium* sp.A were kindly provided by Dr. Kier D. Klepzig (USDA Forest Service, Southern Research Station, Pineville, USA) was used for polyphenol oxidase production.

2.2 Methods

2.2.1 Microbial Cultivations

All cultures were inoculated onto MEA or PDA slants and incubated at 30 ^{o}C for 6-7 days until complete growth and stored at + 4^{o}C temperature for maximum 1 month to use as stock cultures.

The ingredients of the MEA were as follows; 30.0 g malt extract, 3.0 g peptone from soymeal, 15.0 g agar, in 1 L distilled water.

The ingredients of the PDA were as follows; 39.0 g PDA mixture in 1.0 L distilled water.

Spores from the stock culture were inoculated into liquid preculture which consisted of YpSs broth (Appendix A) containing glucose instead of starch as the carbon source.

The preculture was used for the vegetative growth of the spores before scale up. After 24 hours incubation at 30 ^{o}C, preculture was transferred into the main culture (modified YpSs broth). Preculture volume was 5 % of the main culture volume. All the cultures were incubated in a shaker incubator at 30 ^{o}C at 155 rpm.

The quantitative phenol oxidase activity assays were daily carried out for 9 days. Every 24 hours, samples were collected by decantation and cell biomass was separated from the liquid medium containing the enzyme sample by filtration through a Whatmann No.1 filter paper. Separated cell biomass was dried 24 h at 60 ^{o}C incubator for growth curve measurements and supernatant further centrifuged at 2500 rpm for 10 min.

2.2.2 Enzyme Assays

2.2.2.1 Qualitative Polyphenol Oxidase Test

For this purpose modified Bavendamm (Bavendamm, 1928) test for extracellular oxidases was used **.** Bavendamm's medium contained 2 % Difco malt extract, 2 % Difco bacto agar, amended with either 0.5 % tannic acid or 0.5 % gallic acid, adjusted to pH 4.5 with 1N sodium hydroxide before autoclaving.

Plates were inoculated with test isolates from stock culture, here *Scytalidium thermophilum* type culture *Humicola insolens* was used as a positive control and Bavendamm's medium without gallic or tannic acid used as a negative control. Plates were incubated at 30^0C until fungal growth covered the non-amended control. Any change in color due to phenol oxidase reaction or gallic / tannic acid oxidation and it's effect on fungal growth were recorded. The tests were performed in duplicate.

2.2.2.2 Quantitative Polyphenol Oxidase Assays with Culture Supernatant

Extracellular polyphenol oxidase activity was determined by a spectrophotometric method. For this purpose SHIMADZU UV-1700 spectrophotometer (Figure 2.1) which was connected to PC for continuous data storage. The change in the A_{420} per minute was calculated with respect to appearance of quinones from diphenols at 30 ^{o}C. The substrate solution was prepared freshly just before use, due to rapid autooxidation of catechol with molecular oxygen in the air. Therefore, buffer solution, used to dissolve catechol was preincubated instead of preincubation of catechol solution. Both buffer and enzyme solutions were preincubated for 5 minutes at 30 ^{o}C before the activity measurement.

Extracellular enzyme activity measurements were performed using both enzyme blank (no substrate only supernatant) and substrate blank (buffer and catechol, no culture supernatant). As a result, all activity masurements were performed under enzyme blank conditions, because of obtaining more accurate results. Phenol oxidase activities were calculated in two different ways. One unit of enzyme activity was defined as a change in optical density at 420 nm of 0,01 per minute under the stated assay conditions except for the activity calculations of kinetic analysis. In the kinetic studies to determine K_m and V_{max} values, the enzyme activity was expressed in units defined as 1 U = 1 μmole of substrate oxidised (or 1 μmole product formed) in

one minute by 1 ml of culture supernatant. The experimentally determined extinction coefficient of catechol at 420 nm was taken as ε_{420} = 3450 M^{-1} cm^{-1} (Z.B.Ögel et al, 2006). The following equation was used to calculate phenol oxidase activity in the kinetic assays in results and discussion part.

Enzyme Activity (U/L) = (ΔOD/Δt) (1/ε) (1.000.000) (2) (2)

Figure 2.1: SHIMADZU UV-1700 spectrophotometer

2.2.3 Cell Growth Studies

Growth measurements were performed by weighing dried cell mass. Cells were harvested by filtering the growth medium through previously dried and tared Whatmann No.1 filter paper and dried 24 hour in 60°C incubator and then waited 15 minute in desiccator until constant weight was reached.

2.2.4 Effect of Temperature and pH on Polyphenol Oxidase Activity

Polyphenol oxidase was assayed at different temperatures varying from 20 to 45 °C by 5^0C increments to find the optimum temperature of the enzyme assay.

The optimum pH of polyphenol oxidase was measured by using acetate and sodium phosphate buffers at different pH ranging from 4.0 to 8.5. Sodium phosphate buffer having 0.1 M ionic strength were used to cover the pH range of 6.5-8.5, and 0.1M acetate buffer used to cover 4.0-6.5 pH range.

2.2.5 Concentration of Polyphenol Oxidase

2.2.5.1 Crude Extract Preparation

Cultures from 5^{th} day of growth were filtered through Whatman No. 1 filter paper and then insoluble materials were further removed by centrifugation in a MSE Mistral 1000 centrifuge at 2500 rpm for 20 min at room temperature. Then supernatant was used as the crude extract.

2.2.5.2 Ultrafiltration

The crude extracts from the 5^{th} day of the cultivation were concentrated by using 180 ml Amicon Stirred Cell Ultrafiltration System (Figure 2.2). Ultrafiltration was conducted with Sigma Milipore disk membrane with a 10 kDa nominal molecular weight cutoff (NMWC) by applying a gauge pressure of 2.0 bar with nitrogen tube. A volume of 180 ml crude extract was concentrated to 12 ml. Then a further concentration by using vacuum concentrator (Eppendorf Concentrator 5301) was carried out before electrophoretic studies.

Figure 2.2 : Amicon ultrafiltration equipment

2.2.6. Electrophoretic Analysis

Electrophoretic analysis involved non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) and activity staining studies. Experimental setup, Serva BlueLine Electrophoresis Equipment, is shown in Figure 2.3.

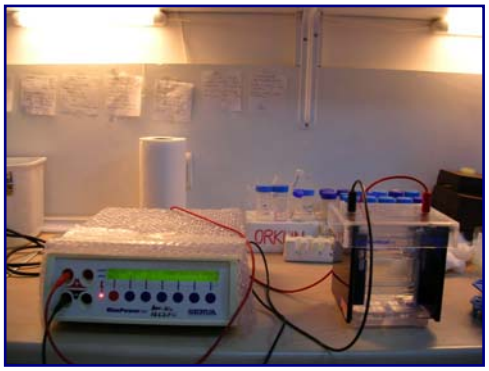


Figure 2.3: Serva BlueLine electrophoresis equipment

2.2.6.1. Activity Staining

Supernatant concentrated by ultrafiltration and vacuum concentrator was run on polyacrylamide gel to separate proteins. For that purpose, electrophoresis was carried out by using 4 % polyacrylamide stacking gel and 7.5 % polyacrylamide separating gels containing no anionic detergent, SDS (Appendix B). 0.5 ml protein sample was mixed with 0.5 ml sample buffer. Sample was loaded on the gel in four wells without denaturation. Separation was performed at a constant voltage of 80 V both in stacking and separating gels.

After electrophoretic run, the gel was stained for different phenol oxidase activities depending on the modified procedure of Rescigno *et al.* (1997). The procedure consisted of 3 steps including staining with 4-amino-*N,N*-diethylaniline (ADA) for laccase activity, with hydrogen peroxide (H_2O_2) for peroxidase activity and with 4-tert-butyl catechol (*t*BC) for tyrosinase /catechol oxidase (CO) activity . Preparation of reagents and procedure is given in Appendix C.

2.2.7 Substrate Specificity Analysis for Polyphenol Oxidase

Substrates other than catechol were tested in order to see affinity of enzyme to other phenolics. Phenolics include caffeic acid , L-dopa, L-tyrosine, *p*-hydroquinone, ABTS, gallic acid and tannic acid. Optical densities were measured at suitable wavelengths, these were 475 nm for L- tyrosine, 440 nm for *p*-hydroquinone and 420 nm for other substrates (Ögel et al., 2006).

2.2.8 Effect of Different Inducers on Polyphenol Oxidase Activity

Some organic phenolic compounds were directly added to growth medium to see their inducing potential for polyphenol oxidase production. For this aim 0.02 % (w/v) gallic acid, 0.02 % (w/v) caffeic acid, 0.02 % (w/v) ADA and 0.06 % (w/v) tannic acid were used.Growth medium without inducer was used as a control.

2.2.9 Effect of Different Inhibitors on Polyphenol Oxidase Activity

Some selected polyphenol oxidase inhibitors were added into assay medium to see their effect. For this purpose 25 mM PVP, PCA, CTAB and SHAM were used (Open names are given on page xv, list of abbreviations part).

2.2.10 Kinetic Analysis of Polyphenol Oxidase

Initial reaction rates of polyphenol oxidase activity were determined at different substrate concentrations ranging from 1.0 to 150 mM *p*-hydroquinone. Reaction rates were plotted against substrate concentration to determine whether the enzyme obeys Michealis-Menten kinetics. The graphical method of Lineneweaver and Burk was used to determine K_m and V_{max} values.

2.2.11 Catalase Activity Test

Catalase may also show phenoloxidase activity, for that reason we also checked the ability of our enzyme to decompose H_2O_2 into H_2O and O_2. For this purpose enzyme assay (www.sigmaaldrich.com/img/assets/18160/catalase.pdf) in special quartz cuvettes (Hellma) were carried out at 240 nm UV spectrum. 0.036 % (w/w)

Hydrogen peroxide solution (H_2O_2) in 50 mM Potassium Phosphate Buffer pH 7.0 was used as a substrate.Details of the experiment is given on Appendix D.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Qualitative Polyphenol Oxidase Assay on Agar Plates

For this purpose modified Bavendamm test (Bavendamm, 1928) for extracellular oxidases was used . Bavendamm's medium contained either 0.5 % tannic acid or 0.5 % gallic acid were inoculated with test isolates from stock culture, here *Scytalidium thermophilum* type culture *Humicola insolens* was used as a positive control and Bavendamm's medium without gallic or tannic acid used as a negative control. Plates were incubated at 30^0C until fungal growth covered the entire control medium. Results of change in color due to gallic acid oxidation are shown in Figure 3.1. No fungal growth were observed on plates containing tannic acid.

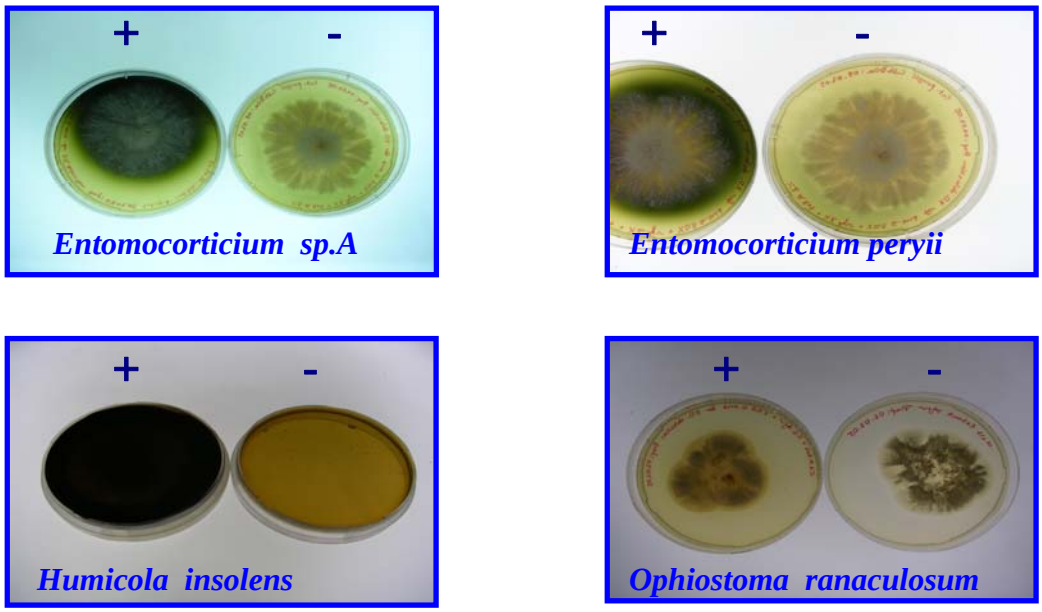


Figure 3.1 Results of qualitative polyphenol oxidase assay,
(+): medium with gallic acid, (-): medium without gallic acid

From the results it was concluded that only *E. peryii* & *E.* sp.A are potential producers of polyphenol oxidase enzyme.

Table 3.1: Qualitative PPO assay results

Fungal species	PPO activity
Entomocorticium peryii	+
Entomocorticium sp.A	+
Ophiostoma minus	-
Ophiostoma ranaculosum	-
Ophiostoma nigrocarpum	-

3.2 Time Course of Phenol Oxidase Production

E. peryii and *E.* sp.A extracellular phenol oxidase production and fungal biomass generation, over 9 days of cultivation on 2% glucose-containing modified Ypss medium (Materials and Methods 2.2.1)with gallic acid as an inducer in batch culture, is shown in Figures 3.2 & 3.3. The exponential phase lasted up to day five for *E. peryii* and four for *E.* sp.A, followed by stationary phase until day six and death phase. Phenol oxidase production was simultaneous to microbial growth, indicating growth-associated production of the enzyme, rather than a secondary metabolite.

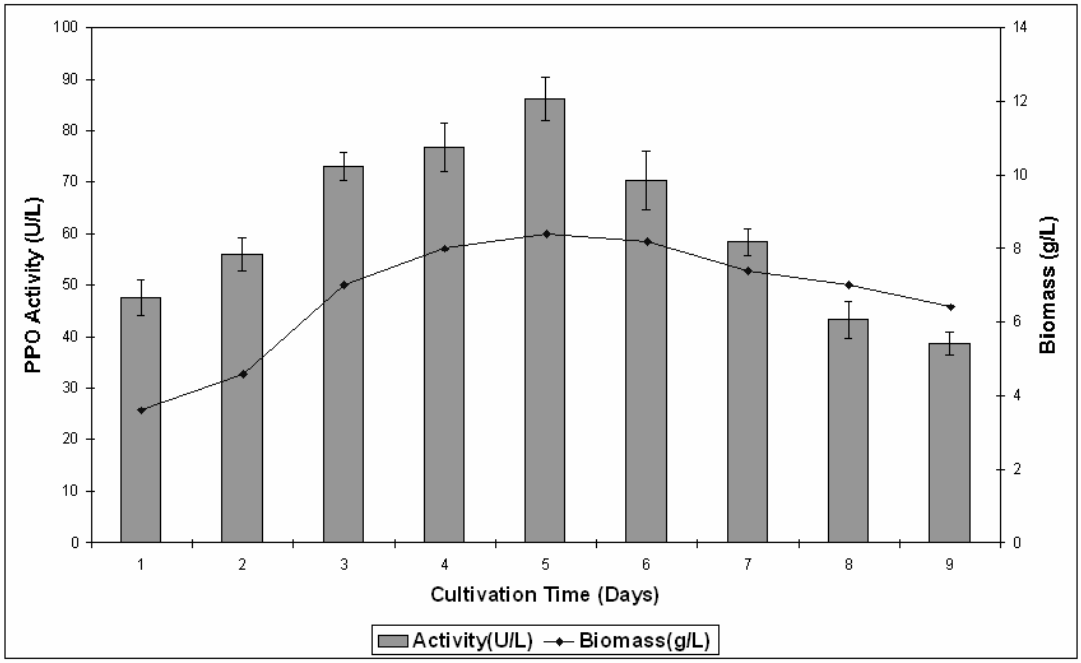


Figure 3.2: Time course of *E. peryii* PPO production with respect to biomass Generation

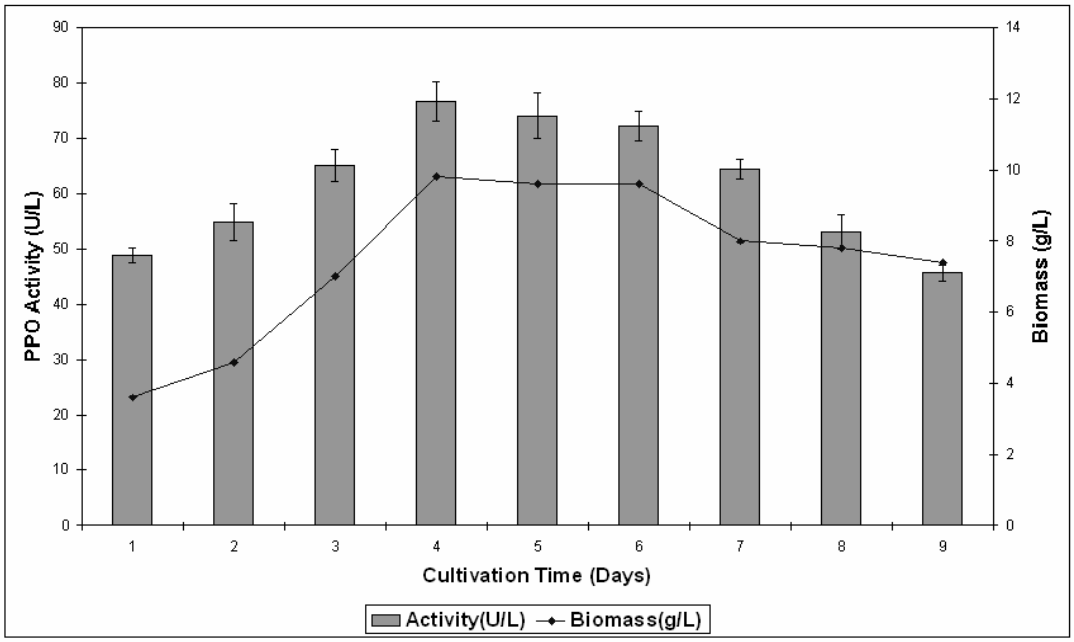


Figure 3.3: Time course of *E*.sp.A PPO production with respect to biomass generation

3.3 Effect of Temperature on Polyphenol Oxidase Activity

Enzyme activity measurements were performed at different temperatures varying from 20 to 45°C. The experiments were performed by using the culture supernatant.

As shown in Figures 3.4 & 3.5 the optimum temperature was 30°C for both fungi.

The temperature profiles of *Entomocorticium peryii* and *Entomocorticium* sp.A polyphenol oxidase can be regarded as significantly low within the range determined by other mesophilic fungi polyphenol oxidases. A mesophilic white rot fungus, *Trametes versicolor* polyphenol oxidase optimum temperature was found to be 45°C (Martines and Duvnjak, 2006), although it's optimum growth temperature is 28°C.

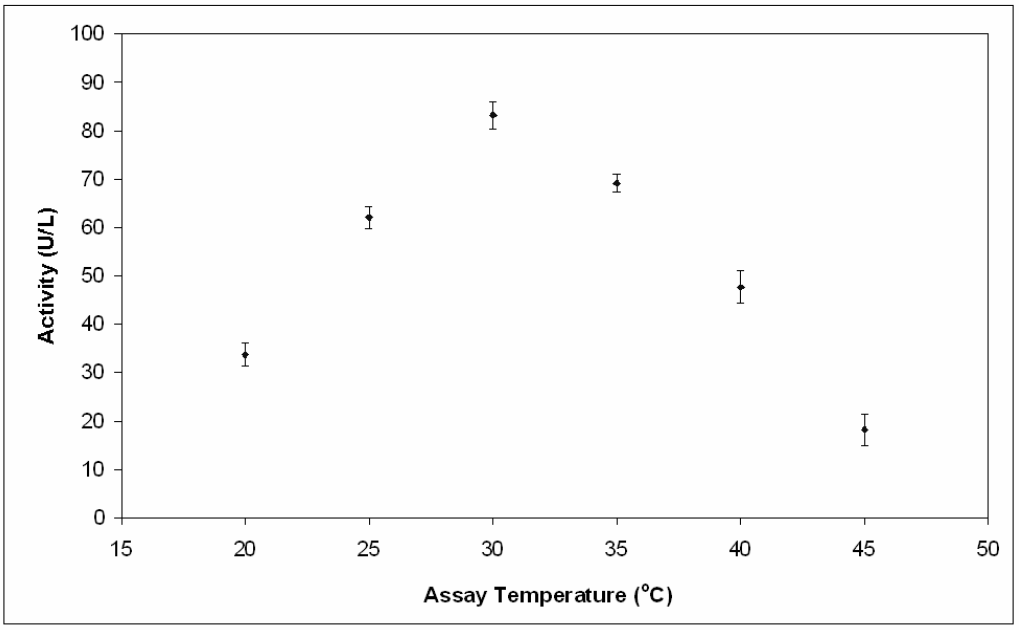


Figure 3.4: Effect of temperature on PPO activity of *E.peryii*

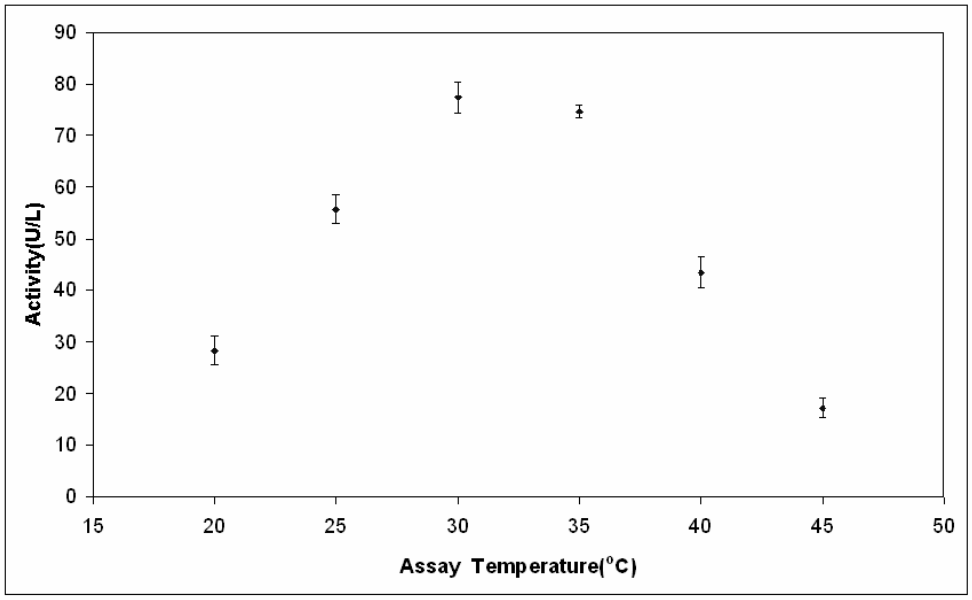


Figure 3.5: Effect of temperature on PPO activity of *E.*sp.A

3.4 Effect of pH on Polyphenol Oxidase Activity

The activities of polyphenol oxidase at various pH ranges were measured by using catechol as the substrate. The reaction pH's were adjusted from 4.0 to 6.0 by using 0.1 M acetate buffer and from 6.5 to 8.5 by 0.1M sodium phosphate buffer.

The optimum pH of polyphenol oxidase was determined as 8.0 for both fungi, as shown in Figures 3.6 & 3.7. Enzyme solutions exhibited no activity at pH levels below 6.5 (data is not shown on the figures 3.6 & 3.7).

The optimum pH of *E.peryii & E.*sp.A polyphenol oxidase in the range of neutral to alkaline is an exception among fungal polyphenol oxidases, which are generally unstable at higher pH values. Among 11 fungi studied by Bollag *et al.* (1984) only the optimum temperature of R*hizoctonia praticola* polyphenol oxidase was in the neutral region (pH 7.2), whereas the optima of the all other extracellular

polyphenol oxidases were significantly lower (between pH 3.0 – 7.0). One of the few exceptions appears to be the laccase of Acreonium murorum, which has shown optimum activity at pH 9.0 for syringaldezine oxidation (Gouka et al.,2001)

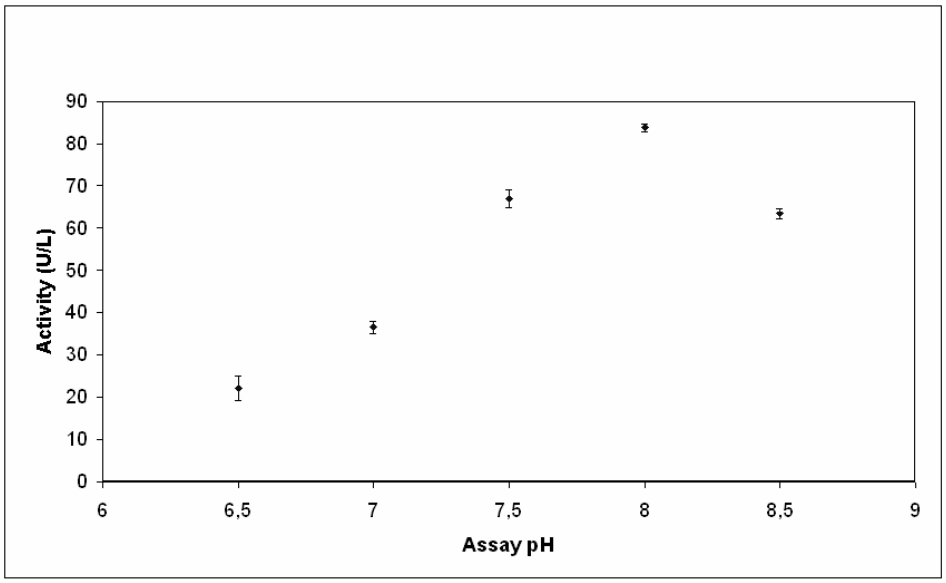


Figure 3.6: Effect of pH on PPO activity of *E. peryii*

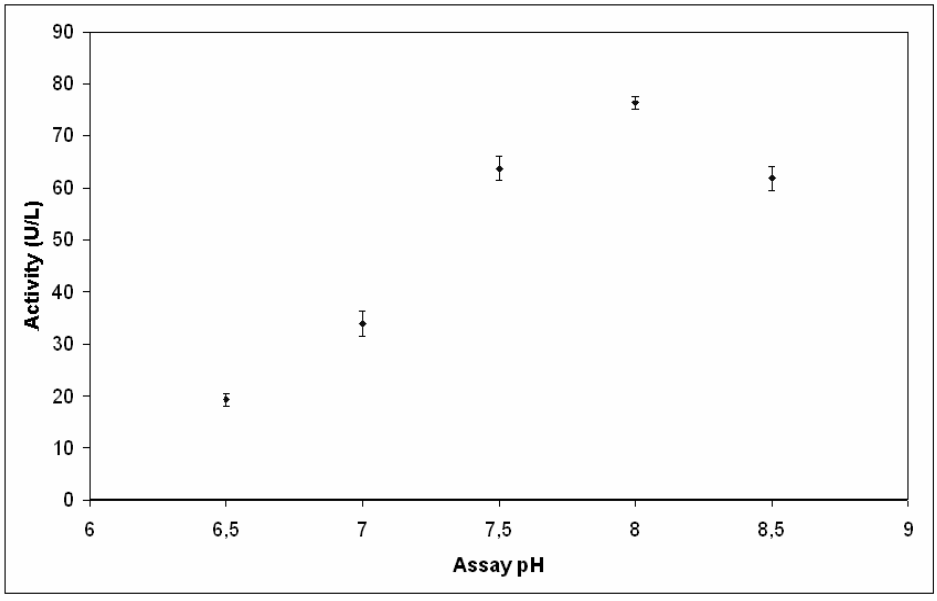


Figure 3.7: Effect of pH on PPO activity of *E.* sp.A

3.5 Effect of inducers on Polyphenol Oxidase Production

In order to study the effect of some phenolic compounds for their ability to induce phenol oxidase production, a number of phenolic and aromatic compounds were added into the growth medium. As shown in Figure 3.8, the addition of gallic acid and tannic acid showed the most pronounced effect on phenol oxidase production.

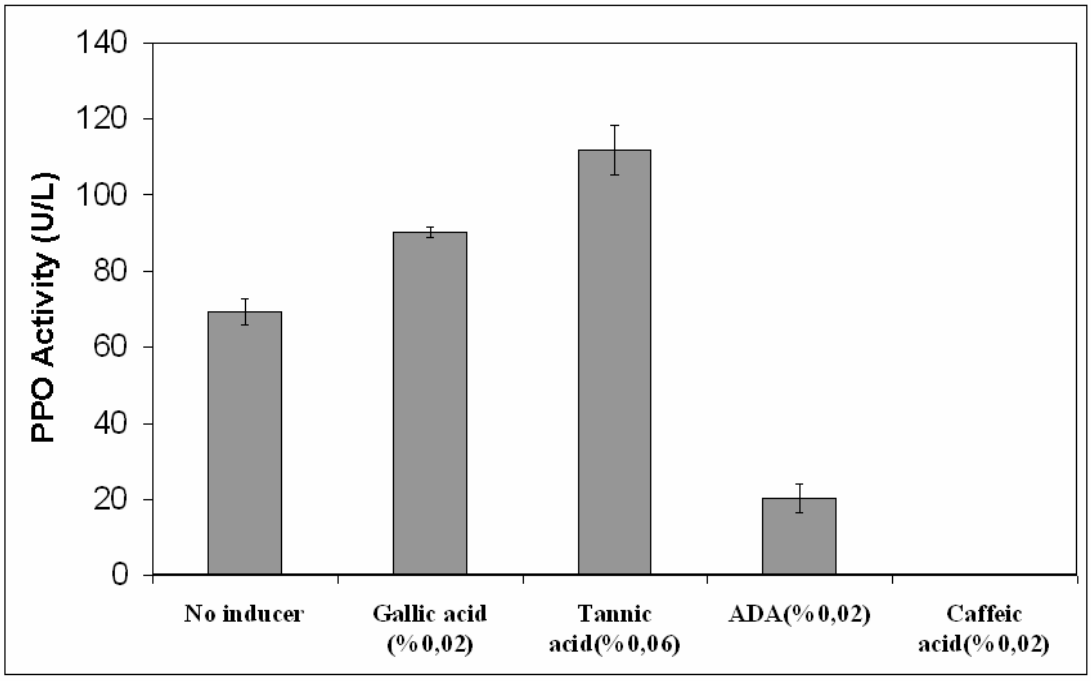


Figure 3.8: Effect of inducers on PPO of *E. peryii*

3.6 Effect of Inhibitors on PPO Production

The effect of a number of phenol oxidase inhibitors on *E. peryii* and *E.* sp.A was analysed with catechol as the substrate. Inhibitors were selected according to information in the literature (Walker and McCallion 1980). Results presented in Figures 3.9, 3.10 show that there is inhibition in the presence of the copper-chelator salicyl hydroxamic acid (SHAM) and *p*-coumaric acid, both indicated as inhibitors of the polyphenol oxidases tyrosinase and catechol oxidase.

Activity was enhanced by the non-ionic detergent cetyltrimethylammonium bromide (CTAB), reported as an inhibitor of laccase, also polyvinylpyrrolidone (PVP), reported as an inhibitor of *o*-diphenol oxidase (Walker and McCallion *1980*) showed very little inhibition.

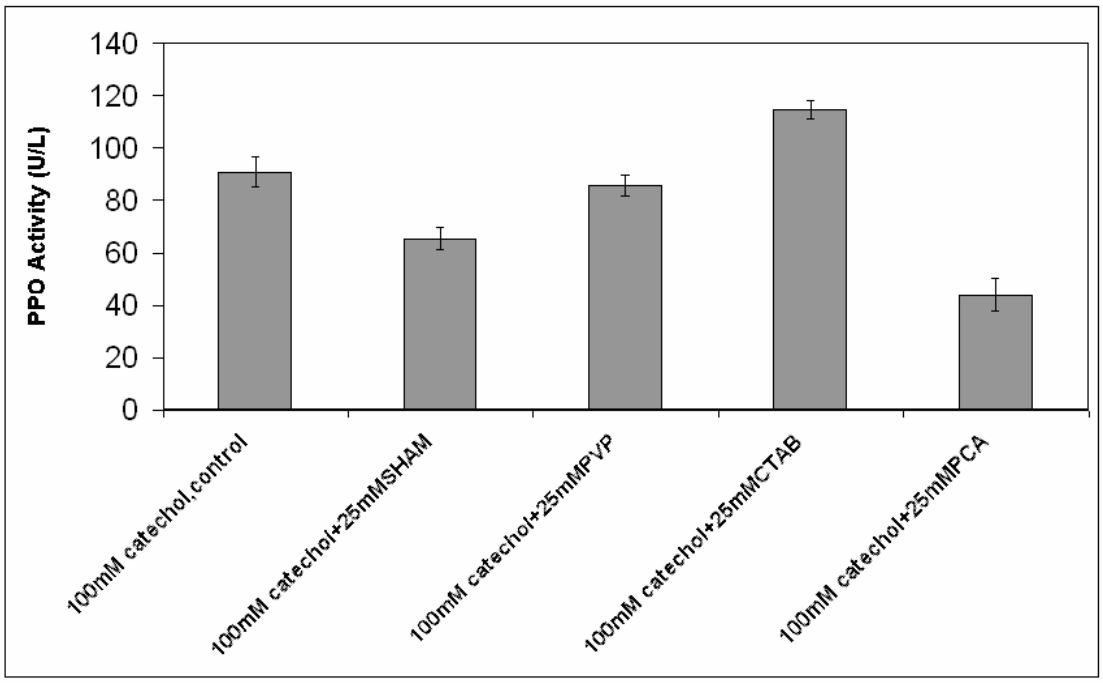


Figure 3.9: Effect of Different Inhibitors on *E. peryii* PPO production
SHAM: salicyl hydroxamic acid, PCA: *p*-coumaric acid,
CTAB:cetyltrimethylammonium bromide, PVP: polyvinylpyrrolidone

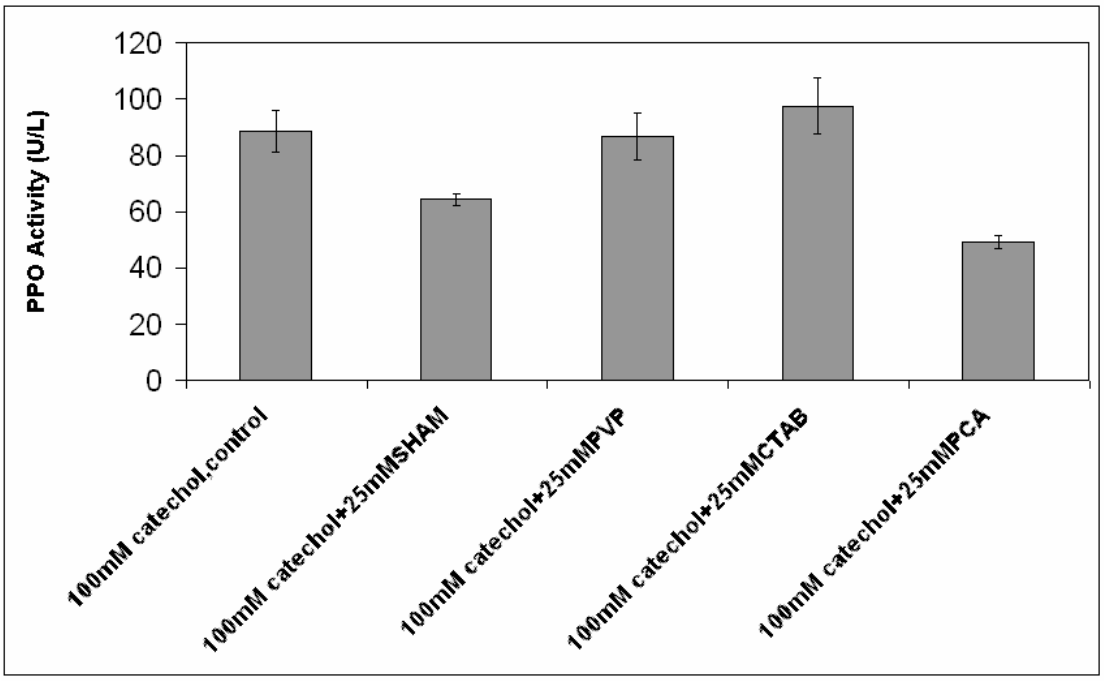


Figure 3.10: Effect of Different Inhibitors on *E.* sp.A PPO production

No significant difference between results of *E. peryii* and *E.* sp.A were observed.In both culture supernatants SHAM and PCA reduced the activity nearly to the half of the control and surprisingly CTAB slightly increased the activity.

3.7 Substrate specificity

In this study, it was of interest to analyze some of the catalytic properties of *E. peryii* and *E.* sp.A directly in culture supernatants. Activity was determined against a number of different substrates. According to the results presented in Tables 3.2 and 3.3, there is high activity on catechol, 3,4-dihydroxy-L-phenylalanine (L-DOPA) and hydroquinone. ABTS and L-tyrosine were not oxidised. There is only

very low activity on tannic acid and caffeic acid. These results strengthen the previous opinion that the enzyme is catechol oxidase.However enzymes of both fungi showed high affinity to hydroquinone (*p*-diphenol).

Results of activity staining supports the results of substrate specificity in which there was no any activity against ABTS which is a laccase specific substrate but the activity with catechol was higher than other substrates except hydroquinone.

Table 3.2: Substrate specificity of *E. peryii*

Substrate	Activity (U/L)
100mM Hydroquinone	89,97
100mM Catechol	78,84
10mM Gallic acid	51,71
50mM L-Dopa	44,52
50mM Caffeic acid	8,8
1mM Tannic acid	0,93
5mM L-Tyrosine	No activity
5mM ABTS	No activity

Table 3.3: Substrat specificity of *E.* sp.A

Substrate	Activity(U/L)
100mM Hydroquinone	97,62
100mM Catechol	83,48
10mM Gallic acid	50,32
50mM L-Dopa	35,01
50mM Caffeic acid	6,03
1mM Tannic acid	No activity
5mM L-Tyrosine	No activity
5mM ABTS	No activity

3.8 Determination of the Type of Polyphenol Oxidase(s) by Activity Staining on Polyacrylamide Gel

After native-PAGE (see section 2.2.6) 15x and 30x concentrated [by using ultrafiltration with10 kDa nominal molecular weight cutoff (NMWC) membrane] crude extracts obtained from gallic acid induced Ypss medium of *E. peryii* was electrophorised (samples loaded only to 3,4,6 and 7 numbered wells) and the gel was stained for different types of PPO activities using the modified procedure of Rescigno *et al.* (1997) (Appendix C). The procedure consists of 3 steps including staining with 4-amino-*N*,*N*-diethylaniline (ADA) for laccase activity, with hydrogen peroxide (H_2O_2) for peroxidase activity and with 4-tert-butyl catechol (*t*BC) for tyrosinase/catechol oxidase activity.

After staining with ADA, no visible band appeared. Since ADA is a specific substrate for laccase, this result suggests that the observed PPO activity is not due to laccase. Also by adding H_2O_2 no bands appeared , indicating absence of peroxidase activity . By staining the gel further with *t*BC, a visible blue band (Figure 3.11) appeared showing the presence of tyrosinase or catechol oxidase activity.

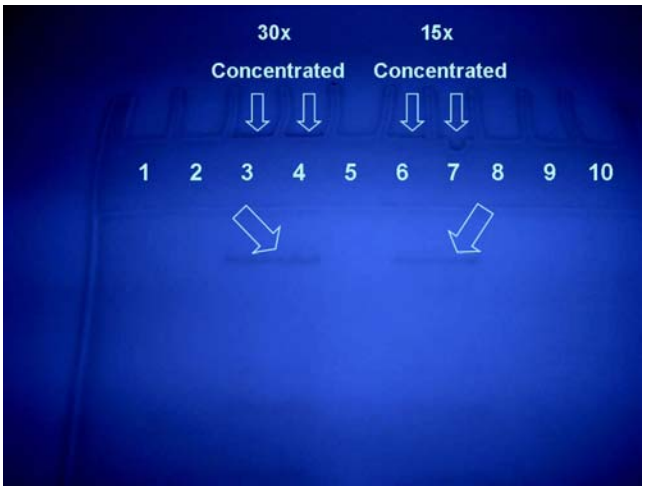


Figure 3.11: Activity staining result, indicating catechol oxidase activity

3.9 Kinetic Analysis of Extracellular Polyphenol Oxidase

To determine the kinetic parameters of extracellular phenol oxidase taken from culture supernatant of *E. peryii* and *E.* sp.A, initial reaction rates at different hydroquinone concentrations, ranging from 1 to 250 mM were determined. Hydroquinone was used because of yielding the highest activity (Table 3.2, 3.3) as a substrate is high activity results with this substrate during substrate specificity experiments.Results obeyed Michaelis-Menten kinetics. There was a substrate inhibition at and above 200 mM hydroquinone concentration. Therefore, for Lineweaver-Burk plot the last two data points corresponding to substrate inhibition were neglected.

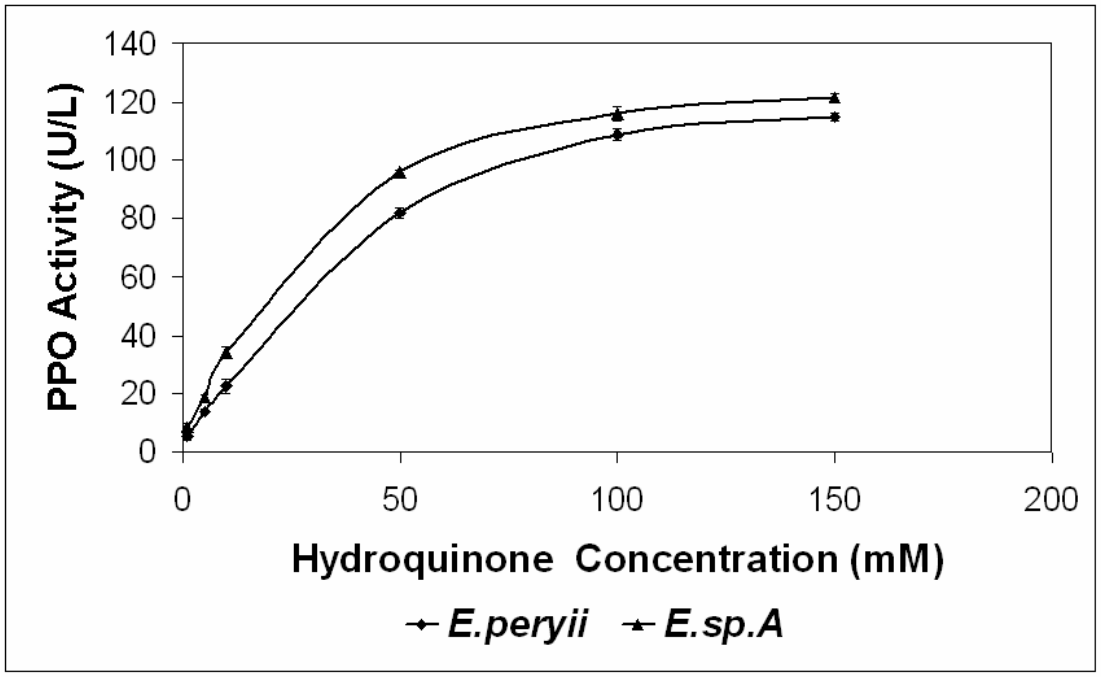


Figure 3.12: Polyphenol oxidase activity of *E. peryii and E.* sp.A versus substrate concentration

Lineweaver-Burk plot for *E. peryii* is shown in Figure 3.13. K_m and V_{max} were calculated as 10.72 mM hydroquinone and 59.44 U/ml respectively.

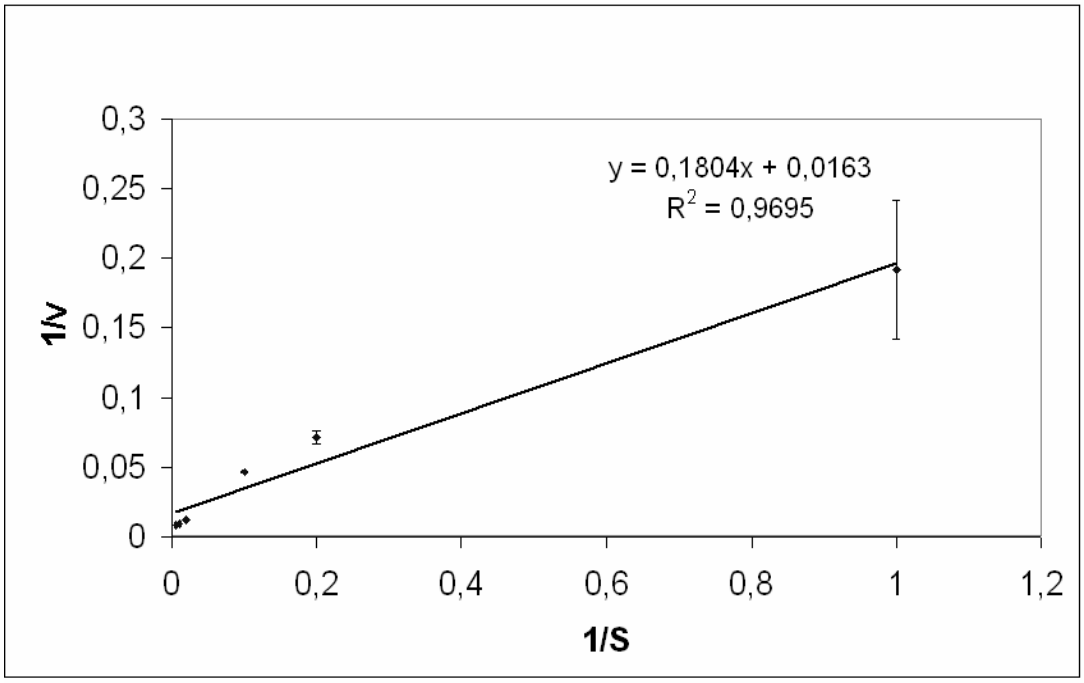


Figure 3.13: Lineweaver-Burk Plot of *E. peryii* polyphenol oxidase

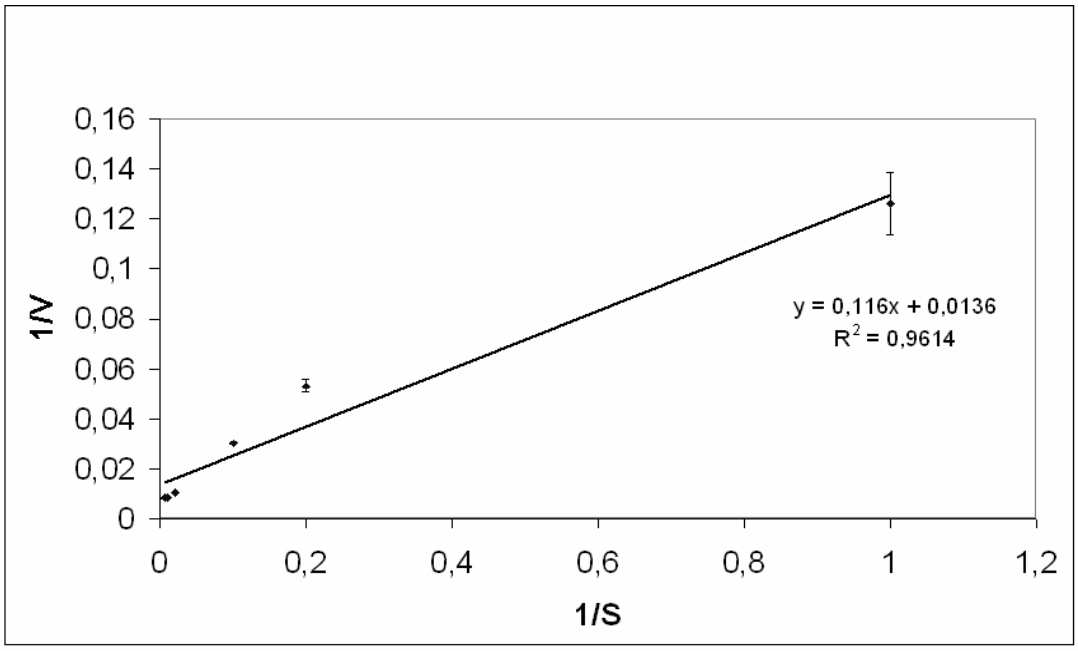


Figure 3.14: Lineweaver-Burk Plot of *E.* sp.A phenol oxidase

K_m and V_{max} values for *E*.sp.A are 8.55 mM hydroquinone and 73.72 U/ml respectively.

Several examples are given in literature about kinetic parameters of phenol oxidases from different fungal sources.

Table 3.4: Kinetic parameters of polyphenol oxidase from different fungi with different substrates (Y.Kaptan, M.S. Thesis, 2004)

Source	K_m (mM)	V_{max}	Substrate	Reference
Mushroom (cap flesh)	5 9	- -	Catechol L-DOPA	Zhang *et al*. (1999)
Mushroom (cap skin)	2.1	0.15 U	Catechol	Zhang and Flurkey (1997)
Mushroom	0.7±0.04 2.36±0.4	- -	Monophenol 4-methyl catechol	Fenoll *et al.* (2002)
Polyporus pinsitus	0.1±0.02	-	ABTS	Xu F. (1996)
Aspergillus oryzae	0.82	-	L-tyrosine	Nakamura *et al*. (2000)
Pycnoporus sanguineus	0.13 0.083	- -	ABTS Syringaldazine	Bar M. (2001)
Coprinus micaceus	0.022 0.42	- -	ABTS Syringaldazine	Bar M. (2001)
Trametes versicolor	70.75 g m_3^-	0.247 g O_2 m^{-3} min^{-1}	Catechol	Aktaş *et al.* (2003)

Comparing Km values of *E. peryii* and *E.* sp.A with above table values although substrate is different, higher Km values of *E. peryii* and *E*. sp.A indicating low affinity to substrate hydroquinone.

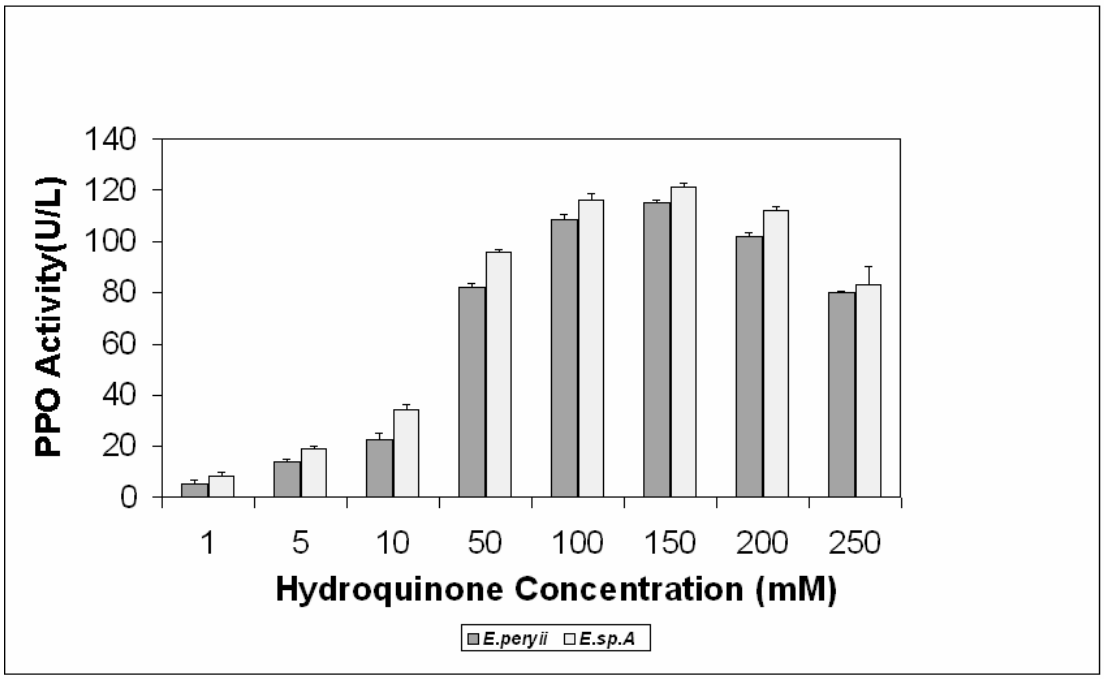


Figure 3.15: Product inhibition of *E. peryii* and *E.* sp.A polyphenol oxidase

Inhibition of *E. peryii* and *E.* sp.A polyphenol oxidase by high concentrations of hydroquinone is seen in Figure 3.15. Probably high product concentrations causing the partial inhibition of an enzyme.

3.10 Catalase Activity Test

According to recent studies some catalases also show phenol oxidase activity (H.N.Kirkman,2006) .Likewise some catechol oxidases may show catalase activity. In one study, one of the two catechol oxidase isozyme from sweet potatoes (*Ipomoea batatas*) show catalase-like activity when H_2O_2 was used as a substrate (Bernt Krebs, 2001). For that reason , in this study, culture supernatant was analysed for its ability to decompose H_2O_2 into H_2O and O_2.

Experiment was performed according to the procedure given in Appendix D and *Scytalidium thermophilum* type culture *Humicola insolens* supernatant was used as a positive conrol.

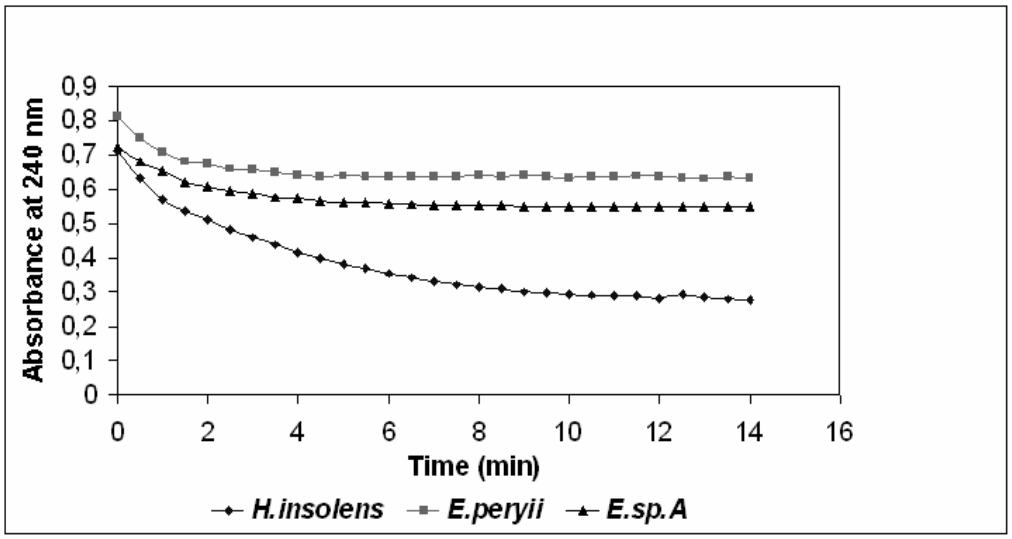


Figure 3.16: Catalase activity test results of *E.peryii* and *E.*sp.A catechol oxidase

From slopes of the above graph catalase activity of *Humicola insolens* was calculated as 46.70 Units/ml supernatant (See Appendix D for calculation details), whereas only slight catalase activities were detected for *E. peryii* and *E.* sp.A. Due to such low activities it was concluded that the observed PPO activity in culture supernatants is not due to catalase.The observed low activity could even be a result of slight catalase activity of catechol oxidase.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

The main objectives of the study were to analyse polyphenol oxidase production of Southern Pine Beetle (SPB) associated fungi and to characterize the nature of the PPO enzyme(s). Five SPB associated fungi were screened for polyphenol oxidase production and two of those fungi namely *E. peryii* and *E.* sp.A were shown to produce PPO in the presence of gallic acid.

Enhancement of the polyphenol oxidase production with different inducers were investigated and tannic acid and gallic acid were to be the best.

Activity staining and Native-polyacrylamide gel electrophoresis experiments of extracellular phenol oxidase produced by *E. peryii* and *E*. sp.A suggested a single enzyme with catechol oxidase activity. Extracellular catechol oxidase activity was assayed with catechol as substrate in most of the experiments. Polyphenol oxidase activity with different substrates were also assayed in order to obtain some information about substrate specificity of enzyme. No activity was observed against ABTS and L-tyrosine indicating that enzyme is different from laccase and lacks the cresolase activity of tyrosinase.

In kinetic studies, K_m and V_{max} values were calculated using hydroquinone as a substrate. K_m and V_{max} were calculated as 10.72 mM and 59.44 U/ml for *E.peryii* and 8.55 mM, 73.72 U/ml for *E*.sp.A by using the Lineweaver-Burk plot .

In the future studies characterization of the enzymes from *Entomocorticium peryii* & *Entomocorticium* sp.A can be studied in more detail, amino acid sequence and three dimensional structure of the enzyme can be identified by sequencing and X-Ray Crystallography. Further, the biological role of PPO production as part of symbiosis with SPB can be studied.

REFERENCES

Aktaş, N., and Tanyolaç, A. (2003). Kinetics of Laccase-catalyzed Oxidative Polymerization of Catechol. *Journal of Molecular Catalysis B: Enzymatic*, 22, 61-69.

Assavanig, A., Amornkitticharoen, B., Ekpaisal, N., Meevootisom, V and Flegel, T.W. (1992). Isolation, characterisation and function of laccase from *Trichoderma. Applied Microbiology and Biotechology* , 38, 198–202 .

Astarcı E. (2003). Production and Biochemical Characterization of Polyphenol Oxidase from *Thermomyces lanuginosus.* MS Thesis, METU, Ankara, TURKEY.

Atlow, C. S., Bonadonna-Aparo, L., Klibanov, M. A. (1984). Dephenolization of Industrial Wastewaters Catalysed by Polyphenol oxidase. *Biotechnology and Bioengineering*, 26, 599-603 .

Bruce, A and King, B. (1983). Biological control of wood decay by *Lentinus lepideus* (Fr.) produced by *Scytalidium* and *Trichoderma* residues. *Materials und Organismen* , 18 ,171–181.

Burke, R. M., Cairney, J. W. G. (2002). Laccases and other polyohenol oxidases in ecto- and ericoid mycorrhizal fungi. *Mycorrhiza,* 12, 105-116.

Call, H.P., and Mucke, I. (1997). History, overview and applications of mediated ligninolytic system, especially laccase mediator system. *Journal of Biotechnol*ogy, 53, 163-202.

Cambria, M., T., Cambria, A., Ragusa, S., and Rizzarelli, E. (2000). Production, Purification, and Properties of an Extracellular Laccase from *Rigidoporus lignosus*. *Protein Expression and Purification,* 18, 141-147.

Chazarra, S., Garciá-Carmona, F., Cabanes, J. (1999). Characterization of monophenolase activity of polyphenol oxidase from iceberg lettuce. *Journal of Agricultural and Food Chemistry*, 47, 1422-1426.

Chefetz, B., Chen, Y., and Hadar, Y. (1998). Purification and Characterization of Laccase from *Chaetomium thermophilium* and its role in Humification. *Applied and Environmental Microbiology,* 3175-3179.

Choi, S.W. , and Sapers, G.M. (1994). Purpling reaction of sinapic acid model systems containing L-DOPA and mushroom tyrosinase. *Journal of Agriculture and Food Science*, 42 , 1183–1189.

Coll, P.M., Tabernero, C., Santamaría, R., and Pérez,P. (1993). Characterization and structural analysis of the laccase I gene from the newly isolated ligninolytic Basidiomycete PM1 (CECT 2971). *Applied Environmental Microbiology*, 59, 4129–4135 .

Couto, S.R., Gundin, M. Lorenzo, M., and Sanroman, M.A. (2002). Screening of supports and inducers for laccase production by *Trametes versicolor* in semi-solid state conditions. *Process Biochemistry,* 38, 249–255.

Dean, J.F.D., and Eriksson, K.-E.L. (1994). Laccase and the deposition of lignin in vascular plants. *Holzforschung* , 48 , 21–33.

DeVries, O.M.H., Kooistra, W.H.C.F., and Wessels, J.G.H. (1986). Formation of an extracellular laccase by a *Schizophyllum commune* dikaryon. *Journal of General Microbiology* , 132 , 2817–2826.

Dincer, B., Colak, A., Aydın, N., Kadioglu, A., Güner, S. (2002). Characterization of polyphenol oxidase from medlar fruits (*Mespilus germanica* L., Rosaceae). *Food Chemistry*, 77, 1-7.

Dittmer, J.K., Patel, N.J., Dhawale, S.W., and Dhawale, S.S. (1997). Production of multiple laccase isoforms by *Phanerochaete chrysosporium* grown under nutrient sufficiency. *FEMS Microbiology Letters*, 149, 65–70.

Durán, N., Esposito, E. (2000). Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Applied Catalysis B: Environmental*, 28, 83-99.

Durán, N., Rosa, M.A., Annibale, A.D., Gianfreda, L. (2002). Application of laccases and tyrosinases (phenol oxidases) immobilized on different supports: a review. *Enzyme and Microbial Technology*, 31, 907-931.

Eggert, C.B. , Temp U. and Eriksson, K.-E. (1996). The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Applied Environmental Microbiology*, 62,1151–58.

Farnet, A.M., Tagger, S. and Le Petit, J. (1999). Effects of copper and aromatic inducers on the laccase of the white-rot fungus *Marasmius quercophilus*. *C. R. Acad. Sci. Paris, Life Sciences*, 322, 499–503.

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

Garzillo M. A., Paolo, S., Burla G. and Buonocore V. (1992). Differently Induced Extracellular Phenol Oxidases from *Pleurotus ostreatus*. *Biochemistry,* 69, 209-215.

Gigi O., Marbach I., Mayer A. (1980). Induction of laccase formation in *Botrytis. Pytochemistry*, 19, 2273-2275.

Gramss, G., Gunther, TH., and Fritsche, W. (1998). Spot tests for oxidative enzymes in ectomycrrhizal, wood and litter decaying fungi. *Mycological Research*, 102, 67-72.

Griffith, G. W., (1994). Phenoloxidases. *Progress in Industrial Microbiology,* 29, 763-788 .

Guillen F, Martinez AT, Martinez MJ. (1992). Substrate specifity and properties of the aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*. *European Journal of Biochemistry* , 209 ,603–611.

Halaouili, S., Asther, M., Sigoillot, J.-C., Hamdi, M., and Lomascolo, A. (2006). Fungal tryrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. *Journal of Applied Microbiology*, 100 ,219–232.

Huber, M., Lerch, K. (1987). The Influence of Copper on the Induction of Tyrosinase and Laccase in *Neurospora crassa*. *FEBS*, 219, 335-338.

Kaptan,Y. (2004). Utilization of *Scytalidium thermophilum* Phenol Oxidase in Bioorganic Synthesis. MS Thesis, METU, Ankara, TURKEY.

Klepzig K. D. & Wilkens, R. T. (1997). Competitive interactions among symbiotic fungi of the southern pine beetle. *Applied and Environmental Microbiology* , 63 , 621–627.

Klepzig K.D. (2006). Melanin and the ecology of southern pine beetle associated fungi. *Symbiosis*, 40, 137-140.

Klepzig, K. D. (1998). Competition between a biological control fungus, *Ophiostoma piliferum*, and symbionts of the southern pine beetle.
Mycologia , 90 , 69–75.

Langfelder, K., Streibel, M., Jahn, B., Haase, G., and Brakhage, A. A. (2003). Biosynthesis of fungal melanins and their importance for human pathogenic fungi. *Fungal Genetics and Biology* , 38, 143-158.

Li, C.Y. (1981). Phenoloxidase and peroxidase activities in zone lines of *Phellinus weirii*. *Mycologia* , 73 , 811–821.

Luterek J, Gianfreda L, Wojtas-Wasilewska M, Rogalski J, Jastek M, Malarczzyk E, et al. (1997). Screening of the wood-rotting fungi for laccase production: Induction by ferulic acid, partial purification, and immobilization of laccase from the high laccase-producing strain *Cerrana unicolor*. *Acta Microbioligica Polonica* , 46 ,297– 311.

Marusek, C.M., Trobough, N.M., Flurkey, W.H. and Inlow, J.K. (2006). Comparative analysis of polyphenol oxidase from plant and fungal species. *Journal of Inorganic Chemistry,* 100 , 108–123.

Mayer, A.M. (2004). Resistance to herbivores and fungal pathogens: variations on a common theme. *Journal of Plant Sciences,* 52, 279–292.

Mayer, Alfred M. (2006). Polyphenol oxidases in plants and fungi : Going places? A review . *Phytochemistry*, 67, 2318-2331.

Motoda, S. (1978). Effect of yeast extract on production of polyphenol oxidase by *Alternaria tenuis*. *Journal of Fermaentation Technology*, 56,543 -545.

Motoda, S. (1998). Purification and characterization of polyphenol oxidase from *Trametes sp. Journal of Bioscience and Bioengineering*, 87, 137-143.

Motoda, S., Shibata, Y., Fukimbara, T. (1971). Studies on microbial polyphenol oxidase. *Journal of Fermentation Technology*, 49, 997-1004. *Phytochemistry*, 19, 2273-2275.

Poppe,J., and Welvaert, W. (1983). Identification of Hymenomycetes in pure-culture by characterization of their mycelia and trials for artificial fructification. *Med. Fac. Landbouww. Rijksuniversity Gent.*, 48 , 901–912.

Ramirez, E.C., Whitaker, J.R., and Virador, V.M. (2004). Polyphenol oxidase. In: J.R. Whitaker, A.G.J. Voragen and D.W.S. Wong, Editors, *Handbook of food enzymology*, Marcel Dekker, New York, 509–522.

Rast, D.M., Baumgarten, D., Mayer, C., and Hollenstein, G.O. (2003). Cell wall-associated enzymes in fungi. *Phytochemistry* , 64, 339–366.

Rayner, A.D.M. , Griffith, G.S. and Wildman, H.G. (1994). Elicitors in plant and microbial metabolism—Induction of metabolic and morphogenetic changes during mycelial interactions among species of higher fungi. *Biochemistry Society Transactions*, 22 , 389–394.

Rescigno, A., Sollai, F., Rinaldi, A.C., Soddu, G., Sanjust, E. (1997). "Polyphenol oxidase activity staining in polyacrylamide electrophoresis gels. *Journal of Biochemical and Biophysical methods*, 34, 155-159.

Richardson, A., McDougall, G.J. (1997). A laccase-type polyphenol oxidase from lignifying xylem of tobacco. *Phytochemistry*, 44, 229-235.

Rodriguez Couto S, Gundin M, Lorenzo M, Sanroman MA. (2002). Screening of supports and inducers for laccase production by *Trametes versicolor* in semi-solid state conditions. *Process Biochemistry* , 38 , 249–55.

Rosei, M.A., Mosca, L., and De Marco, C. (1992). Melanins production from enkephalins by tyrosinase. *Biochemistry and Biophysical Research Communications* , 184 , 190–196.

Saladino, R., Neri, V., Mincione, E., and Philippone, P. (2002). Selective Oxidation of Phenol and Anisole Derivatives to Quinones with Hydrogen Peroxide and Polymer-supported Methylrhenium Trioxide Systems. *Tetrahedron,* 58, 8493-8500.

Sanchez-Amat, A., Lucas-Elio, P., Fernandez E., Garcia-Borron, J. C., and Solano, F. (2001). Molecular Clonning and Functional Characterization of a Unique Multipotent Polyphenol Oxidase from *Marinomonas mediterranea*. *Biochimica et Biophysica Acta*, 1547, 104-116.

Sanchez-Ferrer, A., Bru, R., Cabanes, J., Garcia-Carmona, F. (1988). Characterization of catecholase and cresolase activities of monastrell grape polyphenol oxidase. *Phytochemistry*, 27, 319-321.

Schneider, P., Caspersen, M., Mondorf, K., Halkier, T., Skov, L., Ostergaard, P. R., Brown, K. M., Brown S. H., and Xu, F. (1999). Characterization of a *Coprinus cinereus* Laccase. *Enzyme and Microbial Technology,* 25, 502-508 .

Score, A.J. and Palfreyman, J.W. (1994). Biological control of the dry rot fungus *Serpula lacrymans* by *Trichoderma* spp.: The effects of complex and synthetic media on interaction and hyphal extension rates. *International Biodeterioration & Biodegradation* , 33 , 115–128.

Score, A.J., Palfreyman, J.W. and White, N.A. (1997). Extracellular phenoloxidase and peroxidase enzyme production during interspecific fungal interactions. *Int. Biodeterioration & Biodegredation*, 39 , 225–233.

Score, J. A., Palfreyman, J. W., and White, N. A. (1997). Extracellular Phenoloxidase and Peroxidase Enzyme Production During Interspecific Fungal Interactions. *International Biodeterioration and Biodegradation,* 39, 225-233.

Sheptovitsky, Y.G., Brudvig, G.W. (1996). Isolation and characterization of spinach photosystem II membrane-associated catalase and polyphenol oxidase. *Biochemistry*, 35, 16255-16263.

Shi, C., Dai, Y., Xia, B., Xu, X., Xie, Y., Liu, Q. (2001).The purification and spectral properties of polyphenol oxidase I from *Nicotiana tabacum*. *Plant Molecular Biology Reporter*, 19, 381a-381h.

Sigoillot, C., Record, E., Belle V., Robert, J. L., Levasseur, A., Punt, P. J., Van den Hondel, C.A.M. J. J., Fournel, A., Sigoillot, J. C., and Asther, M. (2004). Natural and Recombinant Fungal Laccases for Paper Pulp Bleaching. *Applied Microbiology and Biotechnology*, 64, 346-352 .

Skorobogatko, K. O., Stepanova, V. E., Gavrilova, P. V., Morozova, O., Lubimova, N, Dzchafarova, N, Aida., Iaropolov, I. A., and Makower A. (1998). Purification and characterization of the constitutive form of laccase from the basidiomycetes *Coriolus hirsutus* and effect of inducers on laccase synthesis. *Biotechnology and Applied Biochemistry*, 28, 47-54.

Solomon, E. I., Sundaram,, U. M., and Machonkin, T. E. (1996). Multicopper Oxidases and Oxygenases. *Chemical Reviews,* 96, 2563-2605 .

Suresh, K., and Subramanyam, C. (1998). Polyphenols are involved in copper binding to cell walls of *Neurospora crassa*. *Journal of Inorganic Biochemistry,* 69, 209-215

Thurston, C.F. (1994). The structure and function of fungal laccases. *Microbiology* ,140 , 19–26.

Tipton K.F. (2002). Principles of enzyme assay and kinetic studies. In: R. Eisenthal and M.J. Danson, Editors, *Enzyme assays*, Oxford University Press, Oxford , 20-30.

Velazhahan, R and Vidhyasekaran, P. (1994). Role of phenolic compounds, peroxidase and polyphenol oxidase in resistance of groundnut to rust. *Acta Phytopathologica et Eutomologica Hungarica*, 29 , 23–29.

White, N.A., and Boddy, L. (1992). Extracellular enzyme localization during interspecific fungal interactions. *FEMS Microbiology Letters* , 98 , 75–80.

Wood, D.A. (1985). Production and roles of extracellular enzymes during morphogenesis of Basidiomycete fungi. In: D. Moore, L.A. Casselton, D.A. Wood and J.C. Frankland, Editors, *Developmental Biology of Higher Fungi*, Cambridge University Press, Cambridge, 375–387.

Zhang, X., Flurkey, W.H. (1997). Phenoloxidases in *portabella* mushrooms. *Journal of Food Science*, 62, 97-100.

Zhang, X., Leeuwen, J., Wichers, H.J., Flurkey, W.H. (1999). Characterization of tyrosinase from the cap flesh of portabella mushrooms. *Journal of Agricultural and Food Chemistry*, 47, 374-378.

APPENDIX A

MEDIUM COMPOSITIONS

Modified YpSs Broth for Polyphenol Oxidase Production

Main Culture

Yeast extract..... 4.0 g

K_2HPO_4..... 1.0 g

$MgSO_4.7H_2O$..... 0.5 g

$CuSO_4.5H_2O$..... 0.04 g

Glucose monohydrate..... 20.0 g

Gallic acid..... 0.172 g

Distilled water.....1.0 L

Preculture for 1 L [% 5 (v/v)]

Yeast extract..... 0.2 g

K_2HPO_4..... 0.05g

$MgSO_4.7H_2O$..... 0.025 g

Glucose monohydrate..... 1.0 g

Distilled water.....50 ml

Malt Extract Agar (MEA) for Stock Culture

Malt extract.....30.0 g

Peptone from soymeal.....3.0 g

Agar.....15.0 g

Distilled water.....1.0 L

Potato Dextrose Agar (PDA) for Stock Culture

PDA mixture.....39 g

Distilled water.....1.0 L

APPENDIX B

REAGENTS AND GEL PREPARATION FOR POLYPHENOL OXIDASE ACTIVITY STAINING OF SLAB GEL

Stock Solutions

A. Acrylamide/bis (30% T, 2.67% C)
87.6 g acrylamide (29.2g/100 ml)
2.4 g N'N'-bis-methylene-acrylamide (0.8g/100 ml)
Make to 300 ml with distilled water. Filter and store at 4°C in the dark (30 days maximum). Since acrylamide is a neuro toxin, precautions should be taken by wearing gloves and mask during preparation of this solution.

B. 1.5 M Tris-HCl, pH 8.8
27.23 g Tris base
~80 ml distilled water
Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.

C. 0.5 M Tris-HCl, pH 6.8
6 g Tris base
~60 ml distilled water
Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.

D. Sample Buffer (store at room temperature)

Distilled water	6.0 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
0.05% (w/v) bromophenol blue	0.2 ml
	8.0 ml

E. 5X Electrode (Running) Buffer, pH 8.3 (enough for 10 runs)
Tris base 9.0 g
Glycine 43.2 g

Bring to 600 ml with distilled water. Store at 4°C. Warm to 37°C before use if precipitation occurs. Dilute 60 ml 5X stock with 240 ml distilled water for one electrophoretic run.

F. 10% Ammonium Persulfate (APS)
Dissolve 100 mg APS in 1 ml of distilled water in an eppendorf by vortexing. This solution should be prepared fresh daily.

G. TEMED (*N*,*N*-tetramethylene-ethylenediamine)
Use TEMED neat from the bottle.

Procedure

A. Preliminary Preparation

Clean the glasses and spacers with ethanol. Assemble the gel sandwich on a clean surface. Lay the longer rectangular glass plate down first, the place two spacers of equal thickness along the short edges of rectangular plate. Next, place the shorter glass plate on top of the spacers. Install the clamps and fasten the screws. Transfer the clamp assembly to one of the casting stand. To check whether the glasses are properly sealed, pour distilled water between glasses. If water level does not decrease, glasses are properly sealed. Then, pour out the water and let the glasses to dry.

B. Preparation of Gel Solution

Separating Gel

Add the followings into a small beaker.

Table B.1 : Preparation of %7.5 separating gel for activity staining

Monomer Concentration (30% T, 2.67% C)	%7.5
Acryamide/bis (30% T, 2.67% C Stock)	1.25 ml
Distilled water	2.5 ml
1.5 M Tris-HCl, pH 8.8	1.25 ml
0.5 M Tris-HCl, pH 6.8	-
10% Ammonium persulfate (fresh)	25 µl
TEMED (*N*,*N*-tetramethylene-ethylenediamine)	2.5 µl

Prepare the monomer solution by combining all reagents except ammonium persulfate and TEMED. Dearate the solution under vacuum for at least 15 minutes. Add the two catalysts just prior to casting the gels.

After adding two catalysts immediately pour the solution between glasses up to 5 cm below the upper edge of the small glass. In order to avoid air contact, pour distilled water onto gel. Allow to stand to complete the polymerization.

Stacking Gel

Add the followings into a small beaker.

Table B.2 : Preparation of %4 stacking gel for activity staining

Monomer Concentration(%T, 2.67% C)	%4
Acryamide/bis (30% T, 2.67% C Stock)	0.65 ml
Distilled water	3.05 ml
1.5 M Tris-HCl, pH 8.8	-
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% Ammonium persulfate (fresh)	25 µl
TEMED	5 µl

Dry the area above the separating gel with filter paper before pouring the stacking gel. Immediately pour the gel solution between glasses. Place a comb in the gel sandwich and tilt it so that the teeth are at a slight (~10°) angle. This will prevent air from being trapped under the comb teeth while the monomer solutions are poured. Allow the gel to polymerize 30-45 minutes.

After polymerization is completed remove the comb by pulling it straight up slowly and gently and fill the wells with 1x loading buffer. Load the the samples (diluted 1:1 with sample buffer) into the wells in an order and keep note for them.

After the gels are cast, the clamp assemblies are snapped onto the inner cooling core to form the upper buffer chamber. The upper buffer is in direct contact with the inner glass plate of the gel sandwich to provide even heat distribution over the entire gel length, preventing thermal band distortion during electrophoretic separations. Fill the chamber with 1x loading buffer. Gently place the cooling core into the electrophoresis tank.

Place the lid on top of the buffer chamber to fully enclose the cell. Attach the electrical leads to a suitable power supply with the proper polarity. The recommended power condition for optimal resolution with minimal thermal band distortion is 80 volts, constant voltage setting. The usual run time is approximately 2 hours. When run finishes, extrude gels very carefully. Carry out the modified activity staining method of Rescigno *et al.* (1997).

APPENDIX C
MODIFIED ACTIVITY STAINING METHOD OF RESCIGNO *et al.* (1997)

Reagents

A. 4-amino-*N*,*N*-diethylaniline (ADA) Solution

0.655 g 4-amino-*N*,*N*-diethylaniline was dissolved in 100 ml of distilled water. 98 µl HCl was also added. This solution should always be prepared freshly.

B. Hydrogen peroxide (H_2O_2) Solution

100 µl hydrogen peroxide was mixed with 100 ml of distilled water.

C. 4-tert-butyl catechol Solution (*t*BC) Solution

0.34 g 4-tert-butyl catechol was dissolved by stirring in 100 ml of distilled water. 57 µl acetic acid was also added.

E. Sodium Phosphate Buffer, pH 8.0

Stock Solutions

a: 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml of distilled water)

b: 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml of distilled water)

5.30 ml of **a** + 94.70 ml of **b** are mixed and diluted to a total volume of 200 ml with distilled water.

Procedure

A small portion of the gel was stained with ADA, H_2O_2 and *t*-BC by the procedure given in the following table:

Table C.1 : Modified activity staining procedure of Rescigno *et al.* (1997)

Step	Reagent	Time	Assay
1	Sodium phosphate buffer, pH 8.0	5 min	Gel was first soaked in buffer at 30^0C by shaking at 100 rpm in Shaker Incubator
2	ADA Solution	20 min	Buffer was poured off and ADA solution was added and shaked at 30^0C at 100 rpm
3	Sodium phosphate buffer, pH 8.0	5 min	ADA was poured off and gel was soaked in buffer
4	H_2O_2 Solution	10 min	Buffer was poured off and H_2O_2 solution was added and shaked at 30^0C at 100 rpm
5	Sodium phosphate buffer, pH 8.0	5 min	H_2O_2 solution was poured off and gel was soaked in buffer
6	*t*BC Solution	20 min	Buffer was poured off and *t*BC was added and shaked at 30^0C at 100 rpm. Deep blue spots were observed corresponding to catechol oxidase (polyphenol oxidase activity)

APPENDIX D

Enzymatic Assay of CATALASE

REAGENTS:

A. 50 mM Potassium Phosphate Buffer pH 7.0 at 25^0C (Prepare 200 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No, P-5379. Adjust to pH 7.0 at 25^0C using 1 M KOH.)

B. 0.036% (w/w) Hydrogen Peroxide Solution (H_2O_2) (Substrate Solution)
(Prepare in Reagent A using Hydrogen Peroxide, 30% (w/w),
Sigma Prod. No. H-1009. Determine the A240nm of this solution using Reagent A as a blank. The A240nm should be between 0.550 and 0.520 absorbance units. Add hydrogen peroxide to increase the absorbance and Reagent A to decrease the absorbance.)

C. Supernatant
(Immediately before use prepare a solution containing supernatant in cold Reagent A.)

PROCEDURE:
Pipette (in milliliters) the following reagents into
suitable cuvettes.

	Test	Blank
Reagent A	------	3.0
Reagent B (Substrate Solution)	2.9	------

Equilibrate to 25^0C. Monitor the A240nm until constant and record the A240nm using a suitably thermostatted spectrophotometer. Then add:

	Test	Blank
Reagent C (Supernatant)	0.1	------

Immediately mix by inversion and record the time required for the A240nm to decrease from 0.45 to 0.40 absorbance units.

CALCULATION:

$$\text{Units/ml enzyme} = \frac{(3.45)\,(df)}{(min)\,(0.1)}$$

3.45 = Corresponds to the decomposition of 3.45 micromoles of hydrogen peroxide in a 3.0 ml reaction mixture producing a decrease in the A240nm from 0.45 to 0.40 absorbance units.

df = Dilution factor

min = Time in minutes required for the A240nm to decrease from 0.45 to 0.40 absorbance units

0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will decompose 1.0 μmole of H_2O_2 per minute at pH 7.0 at 25^0C, The rate of disappearance of H_2O_2 is followed by observing the rate of decrease in the absorbance at 240 nm.