UTILIZATION OF *SCYTALIDIUM THERMOPHILUM* PHENOL OXIDASE IN BIOORGANIC SYNTHESIS

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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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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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Yelda Kaptan

ABSTRACT

UTILIZATION OF *SCYTALIDIUM THERMOPHILUM* PHENOL OXIDASE IN BIOORGANIC SYNTHESES

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In this study, the ultimate aim was to utilize phenol oxidases of *Scytalidium thermophilum* in bioorganic syntheses. For this purpose, studies were conducted towards enhancing the production of phenol oxidases by *Scytalidium thermophilum*, developing a suitable method for laccase activity assays, analyzing the effects of organic solvents on phenol oxidase activity and analysis of the biotransformation of a number of organic substrates by phenol oxidases of *Scytalidium thermophilum*. In order to enhance the production of phenol oxidases, induction experiments were carried out with gallic acid, syringaldazine and chlorogenic acid. Gallic acid was found as the most effective inducer for phenol oxidase production. Inductive effect of edible mushroom *Agaricus bisporus* was also assayed, however, the phenolic compounds released by mushroom did not represent any induction for phenol oxidase activity of *Scytalidium thermophilum*. Different substrates were tested and

catechol was determined as the most suitable substrate rather than syringaldazine and ABTS. Molar extinction coefficient (ε) of catechol was calculated as 3450 M^{-1} cm^{-1} and 3700 M^{-1} cm^{-1} by using "substrate blank" and "enzyme blank" respectively at 420 nm. Kinetic parameters, K_m and V_{max} for the enzymatic reactions in which catechol was used as substrate were calculated as 52.03 mM and 0.253 U/ml respectively from Lineweaver-Burk plot and as 41.25 mM and 0.2055 U/ml from Hanes-Woolf plot. Effect of some organic solvents on phenol oxidases of *Scytalidium thermophilum* was assayed and DMSO was found as an appropriate solvent for the organic substrates. Phenol oxidase containing culture supernatant could oxidize benzoin, hydrobenzoin and benzoyl benzoin.

Keywords: Phenol oxidase, *Scytalidium thermophilum*, *Humicola insolens*, laccase, bioorganic synthesis

ÖZ

SCYTALIDIUM THERMOPHILUM FENOL OKSİDAZININ BİYOORGANİK SENTEZLERDE KULLANILMASI

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Bu çalışmada temel amac, *Scytalidium thermophilum* fenol oksidazlarının biyoorganik sentezlerde kullanılmasıdır. Bu amaçla yapılan çalışmalar, *Scytalidium thermophilum* fenol oksidazının üretiminin arttırılması, lakkaz aktivitesi tayin yöntemlerinin geliştirilmesi, organik çözgenlerin fenol oksidaz aktivitesi üzerine etkilerinin analiz edilmesi ve *Scytalidium thermophilum* fenol oksidazının bir çok organik substratın biyotransformayonundaki rolünün analiz edilmesine yöneliktir. Fenol oksidaz üretiminin arttırılması amacıyla, gallik asit, klorojenik asit ve siringaldazinin indükleyici etkileri araştırılmış ve gallik asit fenol oksidaz üretimi için en uygun indüktör olarak belirlenmiştir. Yenilebilir mantar *Agaricus bisporus*' un *Scytalidium thermophilum* fenol oksidazı üzerine etkisi araştırılmış ancak herhangi bir indükleyici etki saptanmamıştır. Aktivite ölçümlerinde katekol, ABTS ve siringaldazin substratları denenmiş ve katekol en uygun substrat olarak saptanmıştır. Katekolün 420 nm deki molar ekstinksiyon katsayısı (ε), "substrat

koru" kullanılarak 3450 M^{-1} cm^{-1}, "enzim koru" kullanılarak 3700 M^{-1} cm^{-1} olarak hesaplanmıştır. Kinetik parametreler, K_m ve V_{max} değerleri, katekolün substrat olarak kullanıldığı enzimatik deneyler için, Lineweaver-Burk grafiğinden sırasıyla 52.03 mM ve 0.253 U/ml olarak, Hanes-Woolf grafiğinden ise sırasıyla 41.25 mM ve 0.2055 U/ml olarak bulunmuştur. Bazı organik çözgenlerin bu enzimler üzerine etkisi incelenmiş ve DMSO, enzim üzerindeki aktivator etkisi sebebiyle organik substratlar için daha uygun çözgen olarak saptanmıştır. Kültür ortamında bulunan Fenol oksidazların benzoin, hidrobenzoin ve benzoil benzoini oksitleyebildikleri saptanmıştır.

Anahtar kelimeler: Fenol oksidaz, *Scytalidium thermophilum*, *Humicola insolens*, lakkaz, biyoorganik sentez

To my father, mother and sister

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Finally, and most of all I want to express my deepest gratitude and love to my parents Mürüvvet- Nazım Kaptan and my sister and brother-in-law Yeşim –Muzaffer Özakça for their patience, encouragement, tolerance and support all through the study.

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LIST OF ABBREVIATIONS

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

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

APS : Ammonium persulfate

DHN : Dihydroxy naphtalene

DMSO : Dimethyl sulfoxide

DOPA : 3,4- dihydroxy Phenylalanine

ee: Enantiomeric excess

ε_{max} : Molar Extinction coefficient ($M^{-1}cm^{-1}$)

GC : Gas Chromatography

H. i. : Humicola insolens

H_2O_2 : Hydrogen peroxide

HPLC : High Performance Liquid Chromatography

K_m : Michaelis constant of phenol oxidase

PMSF: Phenyl Methyl Sulfonyl Fluoride

PO: Phenol Oxidase

R_f : Retention factor

SDS : Sodium dodecyl sulfate

SDS-PAGE : Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

S.t. : Scytalidium thermophilum

T. t. : Torula thermophila

*t*BC : 4-*tert*-butylcatechol

TEMED : N,N-tetramethylene-ethylenediamine

TLC: Thin Layer Chromatography

YpSs agar : Yeast peptone, soluble starch agar

U : Enzyme activity unit

UV: Ultra Violet

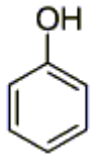
V_{max} : Maximum rate for the *p*-benzoquinone formation in phenol oxidase reaction

CHAPTER 1

INTRODUCTION

1.1 Phenols

Phenols are compounds having a hydroxyl group bonded directly to a benzene or benzenoid ring. The parent compound of this group, C_6H_5OH, called simply *phenol,* is an important industrial chemical. Many of the properties of phenols are analogous to those of alcohols, but this similarity is something of an oversimplification. Phenols are difunctional compounds; the hydroxyl group and the aromatic ring interact strongly, affecting reactivity of each other. This interaction leads to some novel and useful properties of phenols.



Phenol

Figure 1.1 Simplest structure of phenols

The three dihydroxy derivatives of benzene are 1,2-, 1,3-, and 1,4-benzenediol, but each is more familiarly known by their common names as pyrocatechol, resorcinol and hydroquinone respectively.

Figure 1.2 Common permissible IUPAC names of diphenols

Catechol is used as a topical antiseptic, reagent, antifungal preservative on seed potato pieces, photographic developer, and developer in fur dyes. Catechol is also used as an antioxidant in many industries including rubber, chemical, dye, photographic, pharmaceutical, fat, cosmetics, and oil.

The uses of Resorcinol and its derivatives are numerous. Being an antiseptic agent, Resorcinol is used in cosmetic preparations. Although it has been used internally as an intestinal antiseptic, it is chiefly applied externally in the form of pastes and ointments in the treatment of skin diseases. The derivatives of resorcinol are, in general, less toxic than resorcinol itself and many of them are useful in pharmaceutical preparations. It is employed superficial fungus infections. Many important dyes are based on resorcinol, particularly resin dyes and a number of azo dyes. In combination with pyrocatechol, amines, and an oxidising agent resorcinol is used in the dyeing of furs.

Hydroquinone and its derivatives are used principally in photographic dye chemicals, in medicine, as an antioxidant, and in paints, varnishes, and motor fuels and oils. Hydroquinone and certain derivatives are also used as polymerization inhibitors by direct reacting with peroxy-free radical to tie up free radicals. Hydroquinone is also used in cosmetics and medical preparations.

1.1.1 Oxidation of Phenols to Quinones

Phenols are more easily oxidized than alcohols, and a large number of inorganic oxidizing agents have been used for this purpose. The most use of phenol oxidations in organic chemistry are those involving derivatives of 1,2-benzendiol (pyrocatechol) and 1,4-benzendiol (hydroquinone).

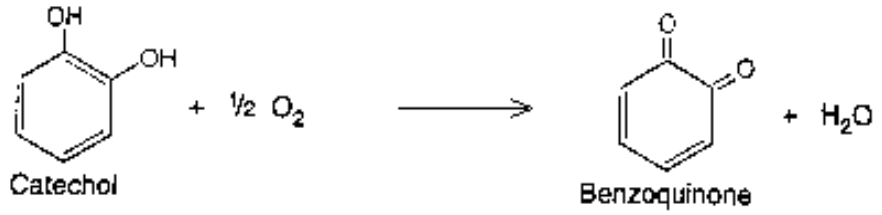


Figure 1.3 Oxidation of catechol to *o*-benzoquinone by Phenol Oxidase activity (Hublik and Schinner, 2000)

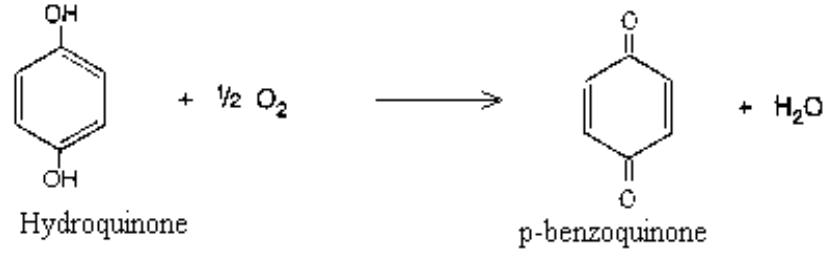


Figure 1.4 Oxidation of hydroquinone to p-benzoquinone by Phenol Oxidase activity (Hublik and Schinner, 2000)

1.2 Quinones

Natural products having a benzoquinone structure show biologically important properties such as cardiovascular, antitumour, antibacterial, antigerminative and antiprotozoa activities. Furthermore, benzoquinones are important fine-chemicals in industry and are useful dienophiles in chemical transformations (Saladino *et al*., 2002).

p-Quinones are used as a fungicide and to make dyes and other agrochemicals. It is also an inhibitor especially for vinyl monomers and unsaturated polyester resins. They are cross-linking agents.

There are a lot of quinonoids in the natural world such as coenzyme Q_{10}, Vitamin K_2, Shikonin, Pyrrolo Quinoline Quinon (PQQ), etc., which are all sharing important roles in organic bodies.

Coenzyme Q_{10}, which is a coenzyme widely distributed within any organic bodies, is well in use for pharmaceutical products and raw materials of food supplements as it holds the function of supplying energy as well as the antioxidation property.

Vitamin K_2 is known to coagulate blood and to improve the decrease of bone mass and also finds its application in pharmaceutical products and raw materials of food supplements.

Shikonin is a vegetable pigment of reddish purple extracted from the root of wandering Jew and has the property of antibacterial action and anti-inflammatory action. This substance is used as an additive to cosmetic products such as lipsticks. PQQ is one of the coenzymes for the essential oxidation-reduction enzymes in the energy acquiring systems of organic bodies.

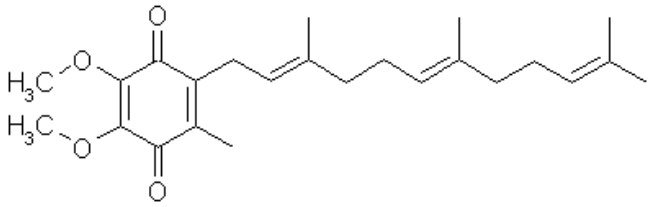


Figure 1.5 Molecular structure of Ubiquinone (Coenzyme Q_{10})

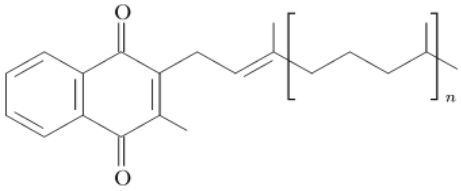


Figure 1.6 Molecular structure of Vitamin K_2

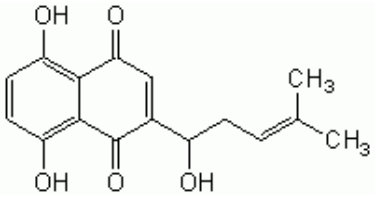


Figure 1.7 Molecular structure of Shikonin

1.3 Phenol Oxidases

The general terms, phenoloxidase, phenolase or polyphenol oxidases are used to describe enzymes which catalyse the oxidation of aromatic compounds by molecular oxygen which is also substrate for phenol oxidases. Since a wide range of substrates are used by these enzymes, there is confusion with respect to terminology and identification of the substrates of particular enzymes (Griffith, G.W., 1994).

Phenol Oxidases represent a major group of enzymes involved in secondary metabolic activity, most commonly being associated with the production of melanins and other pigments.

Although Phenol Oxidases do not have a specific, exact classification, belonging to the Oxidoreductases, they are generally divided into three classes:

1. Monophenol oxidases
2. Diphenol oxidases
3. Polyphenol oxidases

1.3.1 Monophenol Oxidases

The enzymes in this group are able to oxidize both monophenols and diphenols. The oxidation reactions involve hydroxylation of monophenols to give *o*-diphenols and the removal of hydrogens from *o*-diphenol to give an *o*-quinone. When the substrate is a monophenolic compound, there is a delay of quinone formation due to *o*-diphenol production. Cresolase and Tyrosinase are the common examples.

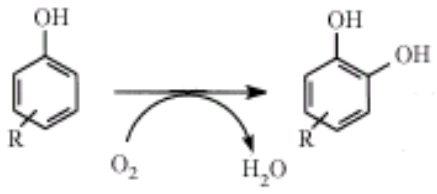


Figure 1.8 Cresolase activity of Tyrosinase (Jolivet *et al.*, 1998)

1.3.2 Diphenol Oxidases

The enzymes with E.C 1.10 refers to oxidoreductases acting on diphenols and related substances as donors. When the acceptor is oxygen, then the diphenol oxidases have E.C 1.10.3 number. Diphenol Oxidases are not capable of oxidizing monophenols. Therefore this property is very helpful to distinguish monophenol and diphenol oxidases. Laccase, Catechol oxidase and Urishiol oxidase are the most common examples of this group.

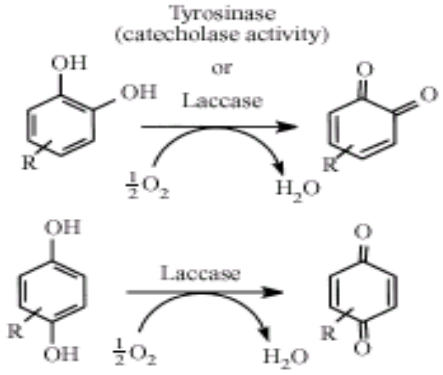


Figure 1.9 Diphenolase activities of Tyrosinase and Laccase (Jolivet *et al*., 1998)

1.3.2.1 Laccase

Laccase, (E.C. 1.10.3.2, p-benzenediol: oxygen oxidoreductase) is a copper containing enzyme belonging to oxidoreductases, and catalyzes the oxidation of various aromatic compounds, particularly phenols, by reducing molecular oxygen to water. In general, laccases have four copper atoms in different binding sites, which play important role in the enzyme catalytic mechanism.

In a typical laccase reaction, the phenolic compound is subjected to a one-electron oxidation giving rise to an aryloxyradical. This active species can be converted to a quinone in the second stage of the oxidation. The quinone as well as the free radical product undergoes non-enzymatic coupling reactions leading to polymerization (Higuchi T., 1989).

Laccase is widely distributed in higher plants, in fungi and in some bacterial strains of *Azospirillum lipoferum* and *Alteromonas* sp. Very recently, it has been reported that laccases are widespread in bacteria. (Duran *et al*. , 2002)

All of the plant laccases are extracellular monomeric proteins with 22-45 % glycosylation. Generally they function in lignin biodegradation and also lignin biosynthesis together with peroxidases (Solomon *et al*.,1996).

Most plant laccases are capable of oxidatively coupling monolignols to dimers and trimers, while peroxidase has a much greater activity toward higher oligomers, leading to the proposal that Laccase catalyzes the initial polymerization of monolignols into oligolignols, while peroxidases then synthesize the extended polymeric lignin from oligolignols (Dean *et al*., 1993).

On the other hand, it has been showed that *Rhus vernicifera* laccase is not involved in lignin biosynthesis, since it cannot oxidize monolignols (Nakamura, W., 1967).

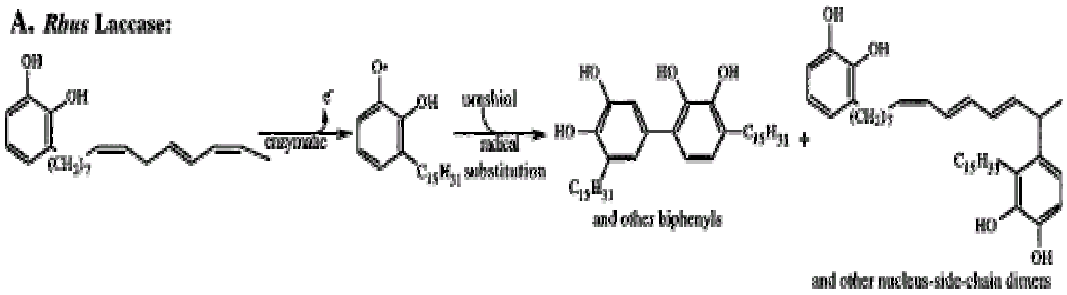


Figure 1.10 Reaction mechanism of laccase from *Rhus vernificera*(Solomon *et al*. 1998)

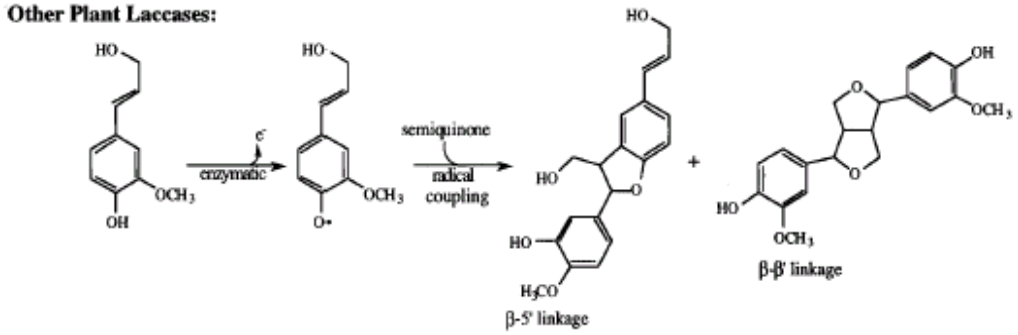


Figure 1.11 Reactions catalyzed by plant laccases (Solomon *et al*. 1998)

All fungal laccases are monomers or homodimers. Like the plant proteins, they are glycosylated but generally to a lesser extend (10-25%). Most, but not all, are extracellular. There are essentially three possible functions of fungal laccases: pigment formation, lignin degradation, detoxification. The metal stoichiometry is, for all well-characterized enzymes, one T1 copper and a T2/T3 trinuclear cluster.

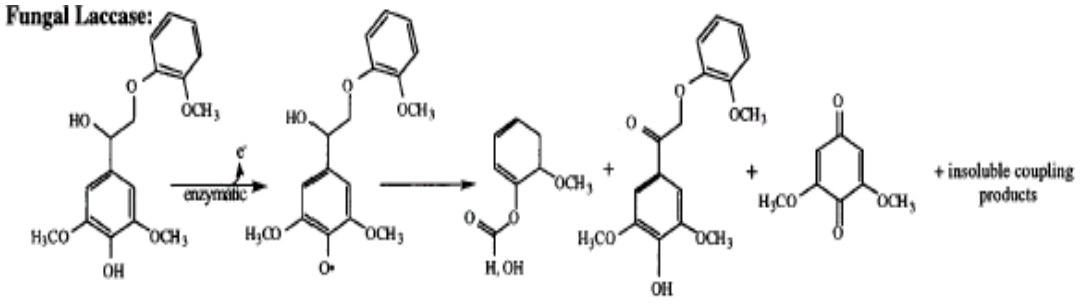


Figure 1.12 Reactions catalyzed by fungal laccases (Solomon *et al*. 1998)

Among fungal laccases, a great variability is observed in the induction mechanism, degree of polymorphism, and physicochemical (molecular mass, isoelectric point, carbohydrate content) and kinetic properties. In some fungal species, the addition of inducers to the culture medium results in the biosynthesis of new extracellular forms.

Intracellular laccases have been seperated from their counterparts on the basis of pH optimum, isoelectric point and/or molecular mass but all appear to have similar substrate ranges.

Laccase contains four copper atoms that have been classified according to their electron paramagnetic resonance (EPR) features: Type 1, responsible for blue color of the enzyme is primary electron acceptor during oxidation of a substrate. Next, electrons are transferred to the two-electron-acceptor,the T2-T3 center (Ducros et al., 1998).

Type 2 and 3 centers combine to function as a trinuclear copper cluster with respect to exogenous ligand interaction including reaction with dioxygen. The Type 2 center is 3-coordinate with two histidine ligands and water as ligands.

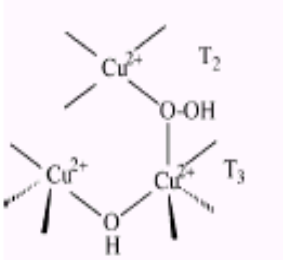


Figure 1.13 Bridging between Type 2 and one of Type 3 copper (Duran *et al*. 2002)

The Type 3 coppers are each 4-coordinate, having three histidines ligands and bridging hydroxide. The structural model of bridging between the Type 2 and 3 has provided insight into the catalytic reduction of oxygen to water. It has been elucidated that the Type 2 copper is required for the reduction of oxygen since bridging to this center is involved in the stabilization of the peroxide intermediate.

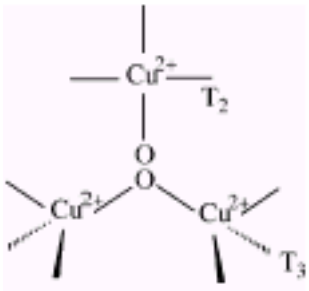


Figure 1.14 Bridging between all three copper (Duran *et al*. 2002)

It is clear that the Type 2 Cu is required for dioxygen reactivity in laccase and that dioxygen reduction occurs in the absence of the Type 1 Cu. This demonstrates that the Type 2/3 trinuclear Cu site represents the active site for the binding and multielectron reduction of dioxygen (Duran *et al*. , 2002).

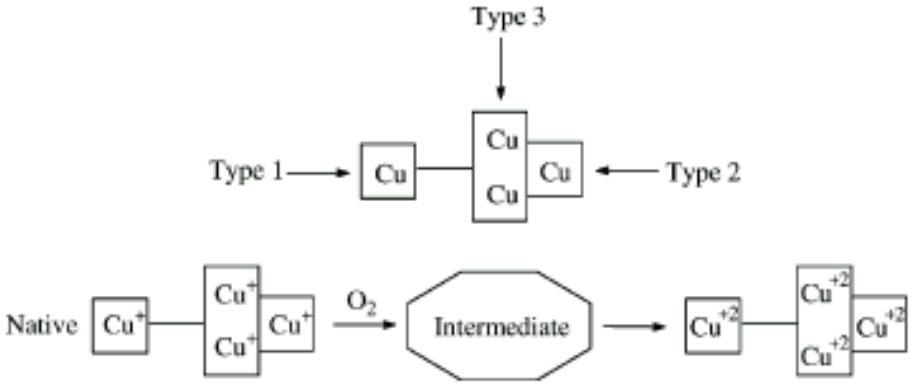


Figure 1.15 Reactivity of laccase derivatives with oxygen (Duran *et al*. 2002)

1.3.2.2 Tyrosinase

Tyrosinase (E.C. 1.14.18.1, monophenol monoxygenase) is widely distributed throughout the phylogenetic scale from bacteria to mammals and even presents different characteristics in different organs of the same organisms, such as in roots and leaves of higher plants.

Tyrosinase (EC 1.14.18.1) is the enzyme that starts the melanin biosynthetic pathway and its regulation has been related with the therapy of malignant melanoma, and the effect of skin depigmenting pharmaceuticals and cosmetics. Furthermore, tyrosinase has industrial applications such as its use as biosensor for oxygen and phenols, the stereospecific synthesis of quinones, phenols and phenolic polymers, and in the bioremediation of wastewater containing phenolic pollutants (Fenoll *et al*.,2002).

Tyrosinase catalyzes two different oxygen-dependent reactions that occur consequently: the o-hydroxylation of monophenols to yield o-diphenols (cresolase activity) and the subsequent oxidation of o-diphenols to o-quinones (catecholase activity).

Chemical and spectroscopic studies of tyrosinase have shown that the active site contains a coupled binuclear copper complex. Tyrosinase exhibits a type 3 copper center as shown in Figure 1.16.

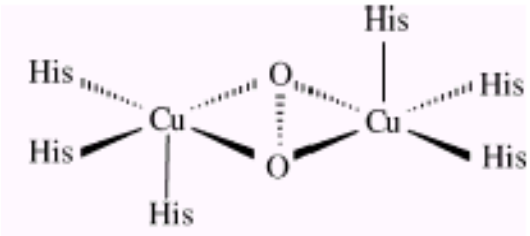
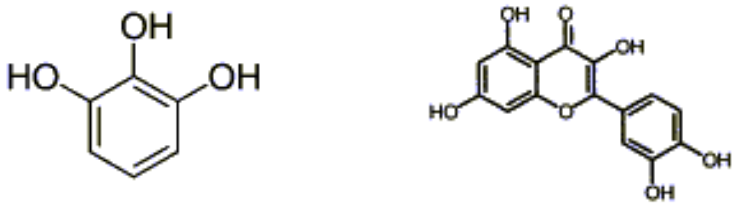


Figure 1.16 Copper centers of tyrosinase (Duran *et al*. 2002)

These three copper states in the active site of tyrosinase suggest a structural model for the reaction mechanism involved in the o-hydroxylation of monophenols and oxidation of the resulting diphenols.

1.3.3 Polyphenol Oxidases

The name Polyphenol oxidase generally refers to Tyrosinase (E.C 1.14.18.1) however the other phenol oxidases such as cresolase (E.C 1.14.18.1), catechol oxidase (E.C 1.10.3.1), laccase (E.C 1.10.3.2) can also be classified in this group. The term polyphenol oxidase is also used for the enzymes that can catalyze polyphenolic substances such as quercetin (pentaphenolic compound) and pyrogallol (triphenolic compound).



a) Pyrogallol b) Quercetin

Figure 1.17 Polyphenolic substrates Pyrogallol (a) and Quercetin (b)

1.4 Phenol Oxidases in Living Systems

Phenol Oxidases are present in a wide range of living systems- microorganisms, plants, animals and human.

In microorganisms, they act as defensive/offensive mechanism. It is important for detoxification of toxic phenolic compounds, melanin pigmentation in some fungi and lignin degradation (Cambria *et al*., 2000).

In plants, phenol oxidase is probably present but its concentration is particularly high in mushroom, potato tubers, apples, peaches, bananas, tea leaves and coffee beans (Whitaker, 1994). It is predominantly located in chloroplast thylakoid membrane and acts as a defence enzyme and is important in lignin biosynthesis and degradation (Tagger *et al*., 1998). It is responsible for browning reactions in fruits and leaves.

In insects, PPO is important for exoskeleton formation (Fenoll *et al*., 2002).

In mammalian, PPO is important for skin, hair and eye pigmentation by acting role in melanin synthesis (Whitaker, 1994).

1.5 Industrial Applications of Polyphenol Oxidases

Phenol oxidases are important industrial enzymes finding a wide range of usage area in industry particularly for the removal of toxic and environmentally hazardous phenolic compounds. Although polyphenols and derivatives have environmental impact, they can be used as by-products of agriculture after a detoxification treatment with phenol oxidases including laccase. Industrial functions of phenol oxidases are not limited with agriculture. Furthermore, phenol oxidases find also application in medical treatments.

Table 1.1 Industrial Applications of Phenol oxidases

Industry	Function	Reference
Beverage industry	Clarification of juice including lignin residues	Kirk *et al*., 1999
Textile industry	Bleaching	Kirk *et al*., 1999
Pulp industry	Bleaching of wood pulp and lignin degradation	Kaichang, 1998
Waste water treatment	Removal of toxic phenolic compounds	Atlow *et al*., 1984
Wine industry	Removal of phenolic compounds coming from grape	Croser, 2000
Tea and coffee industry	In fermentation and curing processes to get dark brown color	Camarero *et al*., 1999
In medicine	Treatment in Parkinson's disease and myocardium following neurogenic injury Therapy of malignant melanoma	Klibanov *et al.* Raju *et al.* 1993 Fenoll *et al*., 2002
Pharmaceutical and cosmetic industry	Function in skin depigmenting lotions	Fenoll *et al*., 2002

1.6 Fungal Phenol Oxidases in Melanin Biosynthesis

Melanins are a large group of diverse substances having some common properties (reviewed in Bell and Wheeler, 1986; Butler and Day, 1998; Butler *et al*., 2001; Henson *et al*., 1999; Jacobson, 2000; Wheeler and Bell, 1988). 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. Melanins are also hydrophobic and negatively charged.

In fungi several different types of melanin have been identified to date. The two most important types are DHN-melanin (named for one of the pathway intermediates, 1,8-dihydroxynaphthalene) and DOPA-melanin (named for one of the precursors, L-3,4-dihydroxyphenylalanine). Both types of melanin have been implicated in pathogenesis (Hamilton and Gomez,2002; Jacobson, 2000;Kwon-Chung *et al*.,1982;Perfect *et al.,*1998; Wheeler and Bell,1988).

A number of possible functions have been postulated for fungal melanins in general, 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 (reviewed in Butler and Day, 1998; Jacobson, 2000).

Fungal DHN-melanin biosynthetic pathway is shown in Figure 1.18 (adapted from Butler and Day, 1998; Tsai *et al*., 1999; Wheeler and Bell, 1988). Probable reaction types are indicated in Figure 1.18 ([O], oxidation;[H], reduction; –H2O, dehydration). Step 1 is catalyzed by a polyketide synthase**,** steps 2 and 4 by a reductase and steps 3 and 5 by a dehydratase enzyme. The polymerisation of 1,8-DHN (step 6) is thought to be catalysed by a laccase enzyme.

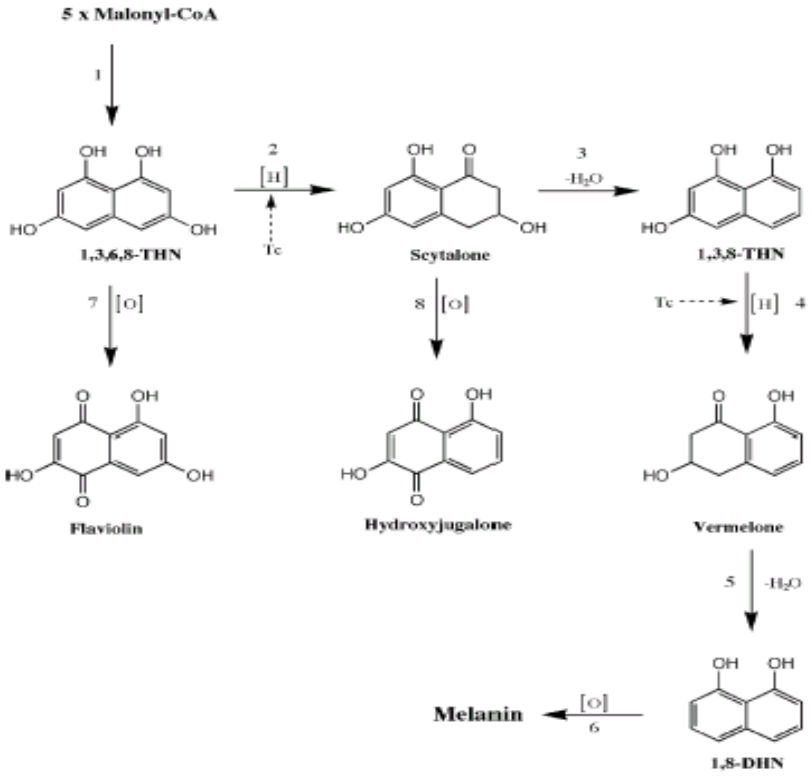


Figure 1.18 Fungal DHN-melanin biosynthetic pathway (Langfelder *et al*., 2002)

Fungal DOPA-melanin biosynthetic pathway is shown in figure 1.19. Essentially, this synthetic pathway strongly resembles the pathway found in mammalian cells, though some of the details may differ. Tyrosinase or laccase catalyses the hydroxylation of L-tyrosine to dopaquinone (reactions 1-3), or the L-DOPA oxidation to dopaquinone (reaction 3). For both these reactions L-DOPA is an essential cofactor (Pomerantz and Warner,1967). Dopaquinone is a highly reactive intermediate. In reaction 4, cyclysation of dopaquinone results in leucodopachrome which is oxidized by reaction with dopaquinone to produce dopachrome. Reaction 6 is an aromatisation reaction (also called tautomerisation). DOPA- melanin is thought to be produced bu simultaneous oxidation and polymerisation. Tyrosinases are type 3 copper proteins. Histidine and cysteine residues of Tyrosinase are essential for copper binding. These residues are essential for enzyme activity and are conserved in almost all fungal tyrosinases. As is the case for fungal tyrosinases, conserved histidine

residues are thought to complex the active site copper ions in laccase enzymes (Messerschmidt and Huber,1990;Williamson,1994).

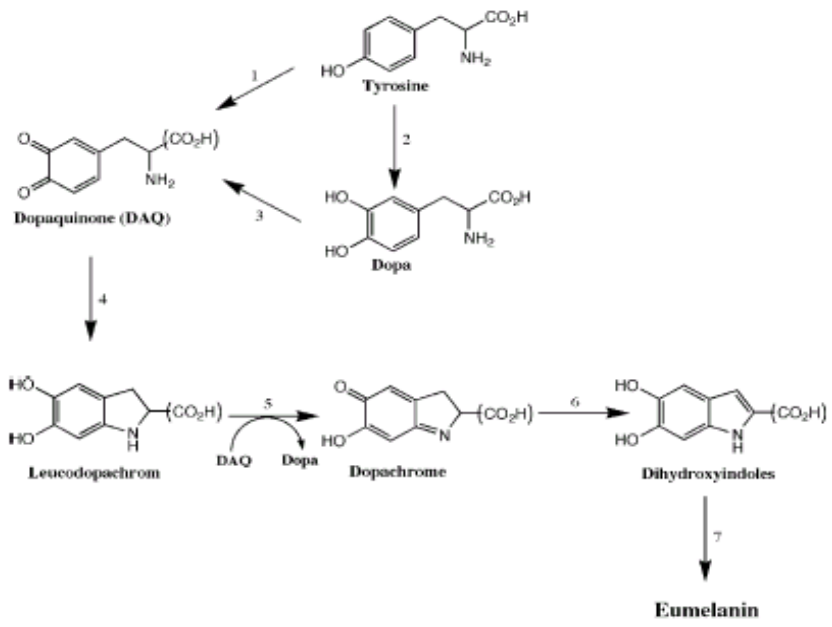


Figure 1.19 Dihydroxyphenylalanine (DOPA)-melanin biosynthetic pathway in fungi (Langfelder *et al*., 2002)

1.7 Thermophilic Fungi

Thermophilic and thermotolerant fungi are of central importance as a source of thermostable enzymes, which are important for industrial utilization because of the possible economic benefits of being able to degrade plant residues at elevated temperatures.

1.7.1 *Scytalidium thermophilum*

Scytalidium thermophilum is a member of *Deuteromycetes* (Fungi imperfecti) which have no known sexual state in their life cycle. *S. thermophilum* plays an important role in determining selectivity of compost produced for growing *Agaricus bisporu*s. The effects of this fungus on the growth of the mushroom mycelium have been described at three distinct levels. First, this fungus decreases the concentration of ammonia in the compost, which otherwise would counteract the growth of the mushroom mycelium. Second, it immobilizes nutrients in a form that apparently is available to the mushroom mycelium. And third, it may have a growth-promoting effect on the mushroom mycelium, as has been demonstrated for *Scytalidium thermophilum* and for several other thermophilic fungi (Wiegant, W. M.).

Straatsma & Samson (1993) examined a large number of isolates assigned to *Scytalidium thermophilum*, namely *Torula thermophila*, *Humicola insolens* and *Humicola grisea* var. *thermoidea* as members of the *Torula-Humicola* complex (Emerson 1968, Ellis & Griffiths 1976), identifying two extreme cultural types within the complex. Type 1 isolates had single very dark spores borne on short lateral hyphal branches (as shown in Figure 1.20) and were designated as H. *grisea* var. *thermoidea* type. Type 2 isolates developed intercalary, slightly pigmented spores in chains, typical of S. *thermophilum* or its basionym, *Torula thermophila* (Mouchacca 1997). They also identified isolates which were intermediate between types 1 and 2, suggesting that these isolates could only be recognised when cultures were young. After aging they could not be separated from the common type 2 isolates due to growing longer chains (Lyons *et al*.,2000).

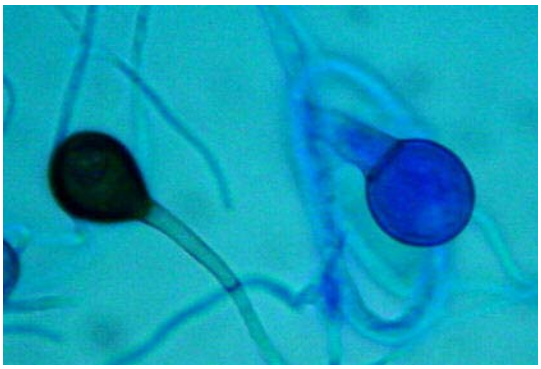


Figure 1.20 *Humicola insolens* spores (type 1) under light microscope (Hamilton, 2002)

1. 8 Effect of Different Inducers on the Production of Phenol Oxidases

Inducer of an enzyme is the compound that triggers its production generally being either the substrate or a substrate analogoue. Various phenolic substrates have potential to be used as inducers for polyphenol oxidases. P-anisidine, 2,5-xylidine, gallic acid, tannic acid, caffeic acid, chlorogenic acid, ferulic acid, syringaldazine are the most commonly used inducers for fungal polyphenol oxidases.

It was also reported that veratryl alcohol (3,4-dimethoxybenzyl alcohol) and ethanol have great induction on laccase of *Trametes versicolor* (Haars and Hüttermann, 2000).

1.9 Utilization of Phenol Oxidases in Biotransformation

The presence of phenols in industrial residues is an area of environmental concern, since toxic phenols enter the environment in wastewater streams, released by numerous industries, including pulp and paper mills, coal and steel works, wood preservation plants, and various chemical and petrochemical industries. (Luke *et al*.).

Numerous conventional methods for dephenolisation of industrial wastewaters have been proposed and, although effective, most suffer from serious drawbacks such as high cost, incompleteness of purification, formation of hazardous byproducts, and applicability to a limited phenol concentration range (Atlow *et al*.). More recently, biologic oxidation has been considered for the treatment of phenol-containing wastewaters (Shishido *et al*.).

Immobilization of the phenol oxidase producing microorganisms on the suitable supports is an effective system for application in the conversion of the toxic phenolic pollutants and considerable potential for application to biotransformation of phenolic and other aromatic compounds.

1.10 Utilization of Phenol Oxidases in Bioorganic Syntheses

Many enzymes, including phenol oxidases, are used as biocatalysts in the bioorganic synthesis of relevant compounds. Furthermore, enzymes are also promising potentials used as in-situ catalysts for the purpose of medical treatments of some diseases.

L-DOPA (3,4-dihydroxy phenyl L-alanine) is a useful drug in the treatment of Parkinson's disease and myocardium following neurogenic injury (Raju *et al*.,1993). L-DOPA is produced from L-tyrosine by a one-step oxidation reaction by submerged fermentation. The key enzyme responsible for biosynthesis of L-DOPA is 'tyrosinase '. In microorganisms tyrosinase activity is generally very weak and L-tyrosine and L-DOPA are rapidly decomposed to other metabolites. Thus, stoichiometric formation of L-DOPA is difficult to achieve. The mycelial activity of *Aspergillus oryzae* or *A.flavus* catalysing L-tyrosine to L-DOPA was achieved in the acidic range below pH 5.0 , so stable L-DOPA production is possible for the use in the drugs of Parkinson's disease (Singh,1999; Haq *et al*.,2000).

A number of tyrosinase dependent prodrug strategies have been reported for the treatment of melanoma. For example, non-toxic phenol and catechol prodrugs

have been oxidised by tyrosinase to afford toxic quinones within the vicinity of melanoma tumours. In addition, it has been recently reported a novel tyrosinase meditated drug delivery system which utilises tyrosinase to mediate the release of cytotoxic drugs, from prodrugs, via a cyclisation/delivery system (Jordan *et al*., 2002).

trans-Resveratrol (3,5,4'-trihydroxystilbene) is one of the phenolic compounds present in wine that could be responsible for the decrease in coronary heart disease observed among wine drinkers (French paradox). Growing evidence suggest that resveratrol plays a role in the prevention of carcinogenesis. It is also antimicrobial, anti-HIV, anti-inflammatory and is also reported to be a potentially important cancer chemoprotective agents. Synthesis of resveratrol by laccase from *Myceliophtora thermophyla* and from *Trametes pubescens* was achieved by Nicotra *et al.* (2004).

Polyaniline is one of the most important conducting polymers, which may be used as active component of organic lightweight batteries, micro-electronics, optical display, for anticorrosive protection, in bioanalysis, etc. due to its good electrical and optical properties as well as high environmental stability. Laccase isolated from *Coriolus hirsutus* is potentially used in the synthesis of water-soluble conducting polyaniline (Karamyshev *et al*., 2003).

Aromatic aldehydes can be prepared in aqueous medium by oxidation of the corresponding methyl aromatic compounds in the presence of oxygen, the enzyme laccase from *Trametes versicolor* and catalytic amounts of various N-hydroxy compounds. Allylic alcohols also gave the corresponding aldehydes in good yield. (Langhals *et al*., 1998).

1.11 Phenol Oxidases of *Scytalidium thermophilum*

Scytalidium thermophilum is a well-studied fungus and some of its strains are known as good producers of industrial cellulases while some of them are potential

trehalase producers (Maheshwari *et al*., 2000). Phenol oxidase production of *S. thermophilum* is limited with only a few articles in literature.

One of the studies about phenol oxidase of *Scytalidium thermophilum* relates to a DNA construct containing a nucleic aid sequence encoding laccase and to an isolated laccase encoded by the nucleic acid sequence. This study is an invention with patent of Berka *et al*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the nucleic acid fragment of the invention, or progeny thereof, under conditions suitable for expression of the laccase protein, and recovering the laccase protein from the culture. The preferred host cell, for heterologous expression was *Aspergillus oryzae*. Heterologous laccase was produced with a yield of 0,6 g per 1200 ml fermentor broth. Purified laccase had a molecular weight of 75-80 kDa approximately and it had a pI of 5,1. The laccase is more stable at neutral to alkaline pH than at acidic pH. The pH profiles of laccase activity had optimal pH of 7 and 4, for syringaldazine and ABTS oxidation, respectively.

Production and partial characterization of *Scytalidium thermophilum* polyphenol oxidase has been studied in our laboratory (Mete, S., 2003). In this study, among different complex lignocellulosic and phenolic waste materials, grape pomace was found to be the best substrate for enzyme production in the presence of glucose as co-substrate. 3 mM gallic acid in glucose based medium greatly stimulated extracellular polyphenol oxidase production. The type of polyphenol oxidase was determined as laccase. The optimum temperature and pH of the enzyme were determined as 65 ^{o}C and pH 7,5. The molecular weight of laccase was determined to be 83 kDa and pI was found as 5,4.

1.12 Scope of the Study

The aim of this study was the utilization of phenol oxidase of *Scytalidium thermophilum* in bioorganic synthesis. Towards this aim, enhancement of the phenol oxidase production by *Scytalidium thermophilum* with potential inducers, optimisation of assay methods in terms of determination of molar extinction

coefficient (ε_{max}) and the determination of some kinetic parameters such as K_m and V_{max}, enzyme activity assays with syringaldazine and ABTS as the substrates other than catechol and utilization of the enzyme in bioorganic synthesis with a number of organic substrates were carried out. For the enhancement of the phenol oxidase activity, effects of the different inducers including gallic acid, syringaldazine, chlorogenic acid and edible mushroom *Agaricus bisporus* were investigated. Identification of phenol oxidases was conducted by activity staining assays on agar plates and SDS-polyacrylamide gel. Effects of some organic solvents on phenol oxidase activity were assayed due to their use in biotransformation experiments. Conversion of a number of phenolic compounds into their oxidation products was screened by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Yeast extract was purchased from Merck Ltd., Acrylamide and bisacrylamide were obtained from AppliChem Ltd. Catechol, syringaldazine, Gallic acid, ABTS and all other chemicals were of analytical grade and purchased from Sigma-Aldrich Ltd.

2.1.2 Microorganisms

Torula thermophila strain 3A was isolated from mushroom compost (Ögel *et al*., 1998). The industrial strain of *Humicola insolens* was provided by Dr. Mehmet Batum from ORBA Inc.

2.2. Methods

2.2.1 Microbial Cultivations

Both Scytalidium thermophilum type cultures were inoculated onto YpSs agar slants and incubated at 45 ^{o}C for 4-5 days until complete sporulation and stored at room temperature for maximum 2 months to use as stock cultures.

The ingredients of the YpSs agar were as follows; granulated yeast extract, 4,0g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; soluble starch, 15.0 g; agar, 20.0 g in 1 L distilled water. (Cooney and Emerson,1964).

Spores from the stock culture were inoculated into liquid preculture which consisted of YpSs medium without agar and containing glucose instead of starch as the carbon source. Different types of modified YpSs broth media have been evaluated to improve the phenol oxidase production. (Appendix A)

The preculture was used for the vegetative growth of the spores before scale up. After 24 hours incubation at 45 ^{o}C, preculture was transferred into the main culture which could be one of different types of modified YpSs broth. Preculture volume was 2 % of the main culture volume. All the cultures were incubated in a shaker incubator at 45 ^{o}C with 155 rpm shaking rate.

The quantitative phenol oxidase activity assays were daily carried out for 8-9 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 filter paper or centifugation at 6000xg rpm for 10 minutes.

2.2.2 Enzyme Assays

2.2.2.1 Enzyme Assays with Culture Supernatant

Extracellular phenol oxidase activity was determined by a spectrophotometrical method. The change in the A_{420} per minute was calculated with respect to appearance of quinones from diphenols at 60 ^{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 60 ^{o}C before the activity measurement.

Extracellular enzyme activity measurement was implemented by optimizing the assay medium in two trials. In one trial, the reaction mixture contained 0.5 ml of 100 mM catechol solution in 0.1 M phosphate buffer, pH 7.0, 0.5 ml of culture supernatant and 1 ml of buffer solution. For the second trial, the reaction mixture consisted of 1 ml of distilled water, 0.5 ml of 100 mM catechol solution in 0.1 M

phosphate buffer at pH 7.0 and 0.5 ml of culture supernatant. However, in the former trial, higher enzyme activity results were recorded, therefore all activity measurements were performed under these assay conditions. The substrate blank cuvette contained 500 µl buffer solution instead of enzyme in some of the assays while in extinction coefficient and kinetic studies enzyme blank was used. In enzyme blank cuvette contained 1,5 ml of phosphate buffer solution and 500 µl of culture supernatant rather than catechol solution. 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 towards to calculate 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 $\varepsilon_{420} = 3450\ M^{-1}\ cm^{-1}$. The following equation was used to calculate phenol oxidase activity in the kinetic assays in section 3.5.

$$\text{Enzyme Activity (U/ml)} = (\Delta OD/\Delta t)\ (1/\varepsilon)\ (1000)\ (2)\ (2)$$

2.2.2.2 Determination of Intracellular Enzyme Activities

Scytalidium thermophilum mycelia were extracted by SANYO Soniprep 650 sonicator. After removal of the supernatant by filtration, remaining mycelia were washed with distilled water and dried on a filter paper. 2 gr of mycelium was suspended in extraction buffer (Appendix B) and the suspension was sonicated for 10 seconds three times with 30 seconds intervals.

Intracellular phenol oxidase activity was determined by agar spot test and activity staining on SDS-Polyacrylamide gel electrophoresis.

2.2.2.3 Agar Spot Test for Qualitative Enzyme Assays

Qualitative enzyme activity assays were carried out for both extracellular and intracellular phenol oxidases of *Humicola insolens* and *Torula thermophila* type cultures. Laccase, tyrosinase and peroxidase enzymes were tried to be identified individually in the presence of the others.

Agar spot test was performed by applying 100 µl supernatant into small halls on 2 % agar poured into petri plates. Control plate included 100 µl phosphate buffer solution at pH 7,0 instead of enzyme. Specific chromogenic substrates were used to determine enzyme specific to them. 4-amino-*N*,*N*-diethylaniline, hydrogen peroxide and 4-*tert*- butyl-catechol were the identifiers for laccase, peroxidase and tyrosinase activities, respectively. Plates were shaked with relevant substrate solution at 60 ^{o}C and 70 rpm for different minutes for each assay. Concentrations of these substrates and procedure are explained in Appendix C.

2.2.2.4 Activity Staining by SDS-PAGE

Activity staining method was used for the identification of three different phenol oxidases, laccase, tyrosinase and peroxidase activities individually in the presence of the others. This method allows differentiation of the three activities on the same polyacrylamide gel electrophoresis (Rescigno *et al*., 1997). Electrophoresis on polyacrylamide gel consisting of 10% separating gel and 4 % stacking gel was carried out for 4 hours at 80 v. After the electrophoretic run was completed, the gel was rinsed with 0.1 M phosphate buffer at pH 7.0 and immersed into freshly prepared solutions of 40 mM 4-amino-N-N diethylaniline (ADA), then of 10mM H_2O_2 and then of 25 mM 4-*tert*-butyl catechol for laccase, peroxidase and tyrosinase activities, respectively. Elecrophoretic assay procedure is explained in Appendix D.

2.2.2.5 Enzyme Assays with Different Organic Substrates

A number of organic compounds used as substrates for phenol oxidases in the culture supernatant has been assayed at four different trials. The three substrates, benzoin, benzoyl benzoin and hydrobenzoin were assayed at each run at different concentrations while 3,4,5-trimetoxy benzoic acid, chromanone and thiochromanone were assayed for two runs at different concentrations.

All reaction tubes were left at 60 ^{o}C throughout the duration of the reactions given at the reaction time row of the Table 2.1. In the first two trials, ethanol was used to dissolved the six substrates given in Appendix E. Culture supernatant was taken from the flask on the 2nd day of the culture for first trial while it was taken on the fourth day of the same cell culture for the second set of the experiment. Concentrations of all substrates at each run are shown in Appendix E.

Experimental conditions with respect to organic solvent used to dissolve phenolic substrates, the day of the cell culture when culture supernatant was collected, phenol oxidase activities in the culture supernatant used in the reactions and the reaction times of each trial are given in Table 2.1. Product formation was screened by thin layer chromatography (TLC) at every 24 hours. Extraction of the products at the end of the reactions was immediately carried out to analyze the product by high performance liquid chromatography (HPLC) or gas chromatography (GC). The final products were extracted into ethyl acetate phase and stored at this solvent until the chromatographic analysis.

Table 2.1. Assay conditions of the experiments of organic substrates

	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**
Solvent	Ethanol	Ethanol	DMSO	DMSO
Day of culture supernatant	From the second day of the cell culture	From the fourth day of the cell culture	From the fifth day of the culture	From the fifth day of the culture
Enzyme Activity at the begining of the reaction	11.6 U/ml	14.4 U/ml	23.6 U/ml	24 U/ml
Reaction time	156 hours	144 hours	216 hours	262 hours

2.2.2.6 Monitoring Product Formation by Thin Layer Chromatography (TLC)

All samples were loaded onto TLC silica plates purchased from Sigma-Aldrich at suitable size by capillary tubes. The diameter of the loaded spots were not more than a few millimetres. The TLC plates were cut at the size of 5 cm width and 6 cm height. The mobile phase-the running solvent- was ethyl acetate: distilled hexane solvent system at different ratios (v : v) depending upon the type of substrate. These ratios according to the type of the substrate are shown in Appendix F. The volume of the solvent was as low as 100 microliters since only the very bottom of the plate was in the liquid.

After running in the solvent system was completed, thin layer plates were left for drying and were then monitored under UV light. When the product formation was detected by UV light, the product was extracted by ethyl acetate or chloroform from

enzymatic reaction mixture, and HPLC method was applied to analyse the product in the solvent.

TLC analyses were carried out after all four different trials. Benzoin, hydrobenzoin and benzoyl benzoin was assayed in all trials. The substrates were dissolved in ethanol in two trials and dissolved in DMSO in the other two trials. Information about the trials was given in detailed in Table 2.1.

2.2.2.7 High Performance Liquid Chromatography (HPLC) Analyses of Products

HPLC from Thermo Seperation with P2000 vacuum pump and UV1000 UV dedector was used to analyse organic products. Chromquest was the controller program for retention time, peak width, and peak area. Detection was performed at 254 nm. OD chiral column was used to analyse all fractions and the solvent system consisted of 10% of isopropanol and 90% of hexane at HPLC grade.

2.2.2.8 Gas Chromatography (GC) Analyses of Products

GC Analyses were performed by Thermo Seperation Products. Samples were injected into autosampler from which the samples were then passed through 50 m capillary column in the oven at 250 ^{o}C.

HPLC and GC analyses were carried out by Çiğdem İğdir and Olcay Mert in organic chemistry laboratory of Prof. Dr. Ayhan Sıtkı Demir.

CHAPTER 3

RESULTS AND DISCUSSION

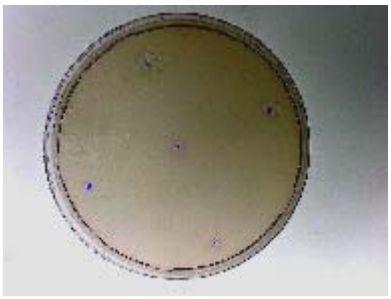
3.1 Comparison of the Phenol Oxidases Produced by *Scytalidium thermophilum* type cultures *Humicola insolens* and *Torula thermophila*

Scytalidium thermophilum consists of the type cultures *Humicola insolens* and *Humicola grisea* var. *thermoidea* (type 1) and *Torula thermophila* (type 2). In this study, phenol oxidase activities of *Humicola insolens* and *Torula thermophila* were compared in order to find the most eligible phenol oxidase and the most appropriate type culture of *Scytalidium thermophilum* for further studies. After a number of experiments, *Humicola insolens* was selected as the most suitable type culture for further studies.

3.1.1 Qualitative Determination of Intracellular and Extracellular Phenol Oxidase Activities of *Torula thermophila* and *Humicola insolens*

Phenol oxidases of two type cultures of *Scytalidium thermophilum* were determined by agar spot test, following the instructions given in section 2.2.3.2 for the identification of the phenol oxidase types which were produced intracellularly and which were secreted into the culture media extracellularly. The term "extracellular phenol oxidase" refers to enzyme in culture supernatants while the term "intracellular phenol oxidase" refers to cell lysate obtained by sonication.

Laccase activity was investigated by using 4-amino-*N*,*N*, diethyl aniline (ADA) which yields pink color formation in the presence of laccase.



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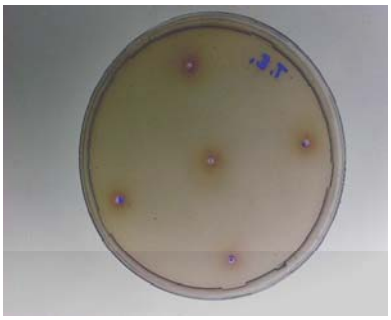

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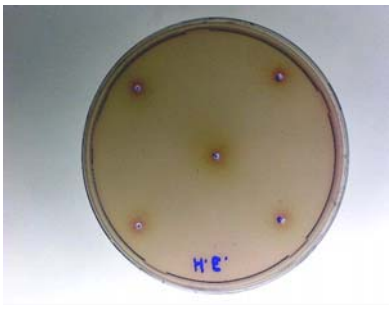

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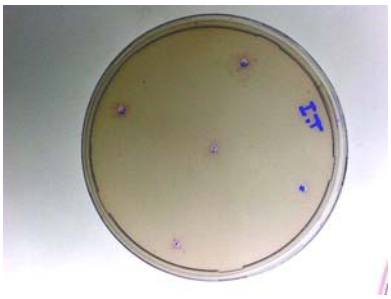

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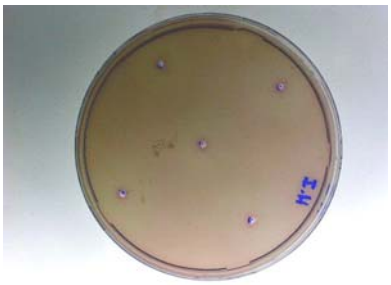


e

Figure 3.1 Activity staining for laccase production by *S. thermophilum* a; Control plate with phosphate buffer instead of supernatant, b; Extracellular laccase test for type culture *Torula thermophila*, c; Extracellular laccase test for type culture *Humicola insolens*, d; Intracellular laccase test for type culture *Torula thermophila*, e; Intracellular laccase test for type culture *Humicola insolens*

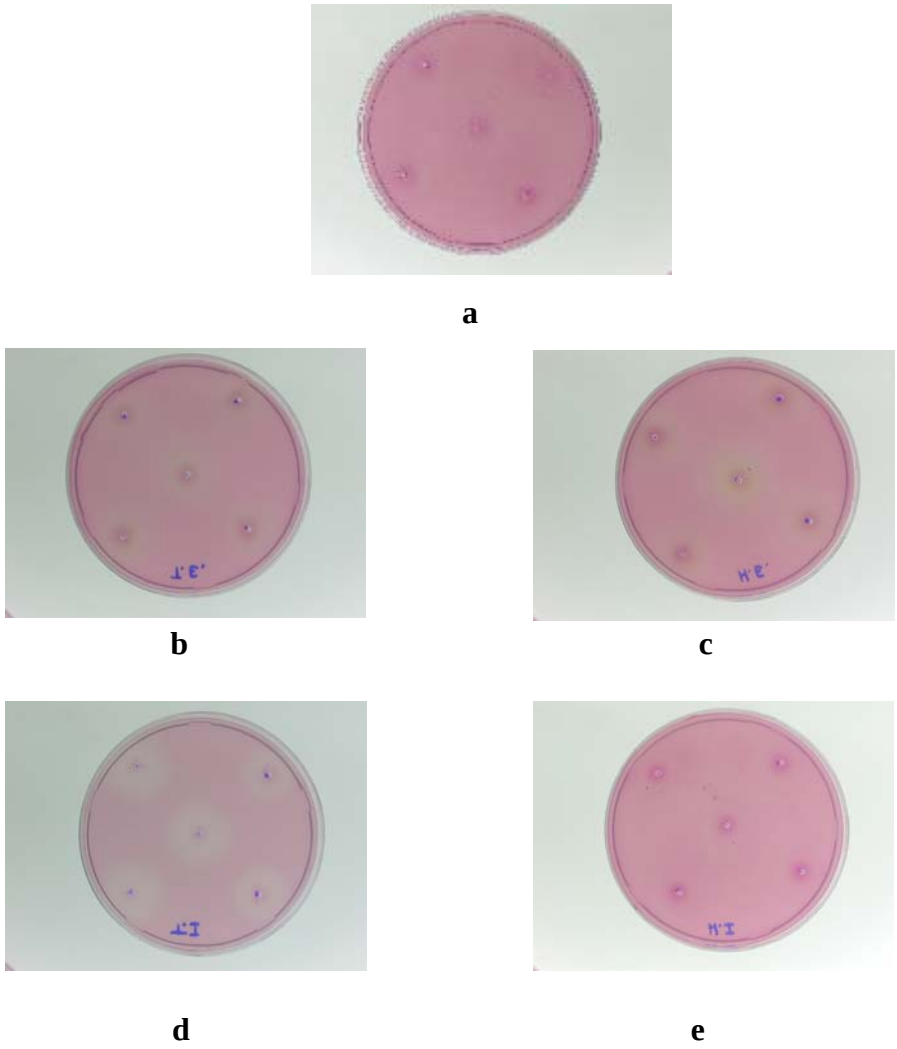


Figure 3.2 Activity staining for peroxidase production by *S. thermophilum* a; Control plate with phosphate buffer instead of supernatant, b; Extracellular peroxidase test for type culture *Torula thermophila*, c; Extracellular peroxidase test for type culture *Humicola insolens*, d; Intracellular peroxidase test for type culture *Torula thermophila*, e; Intracellular peroxidase test for type culture *Humicola insolens*

Pink color formation is due to the semiquinoid cation formation from ADA by oxidation with laccase. Pink color formation was observed only in the plates containing culture supernatants of *Torula thermophila* and *Humicola insolens* (as can be seen in Figure 3.1.b and Figure 3.1.c). Cell lysate containing plates did not exhibit any laccase activity by ADA.

Peroxidase activity was assayed by the addition of H_2O_2 onto ADA-containing plate. Peroxidase is activated by H_2O_2 and therefore, activity is not observed only in the presence of ADA. However, if pink coloration has already been observed in the presence of ADA, H_2O_2 addition may be expected to yield a darker pink color by the presence of peroxidase. Accordingly, peroxidase activity was observed only in intracellular samples of *Humicola insolens* was observed among all plates (Figure 3.2.e). Interestingly, on test plates of extracellular and intracellular peroxidase activities of *Torula thermophila* (Figure 3.2.b and figure 3.2.d) and on the extracellular peroxidase test plate of *Humicola insolens* (Figure 3.2.c), clear zone formations were observed. Dark pink color formation is the characteristic of the peroxidase activity rather than clear zone. Due to these unpredicted clear zones and difficulty in distinguishing laccase and peroxidase activities, it was decided to perform the same activity staining procedure on SDS-polyacrylamide gel electrophoresis.

Tyrosinase activity was determined by using 4-*tert*-butyl catechol in the presence of acetic acid. Quinone form of 4-*tert*-butyl catechol oxidized by tyrosinase - 4-*tert*-butyl-1,2-benzoquinone- reacts with ADA used for laccase determination on the same plate and blue colored stable product is formed. According to the results shown in Figure 3.3, expected blue coloration was detected clearly only from the intracellular samples of *H. insolens* (Figure 3.3.e). Since laccase activity was not observed on cell lysates of *H. insolens*, the observed blue coloration could be attributed to tyrosinase. Otherwise, it would not be possible to distinguish between the two enzyme activities by the agar spot test.

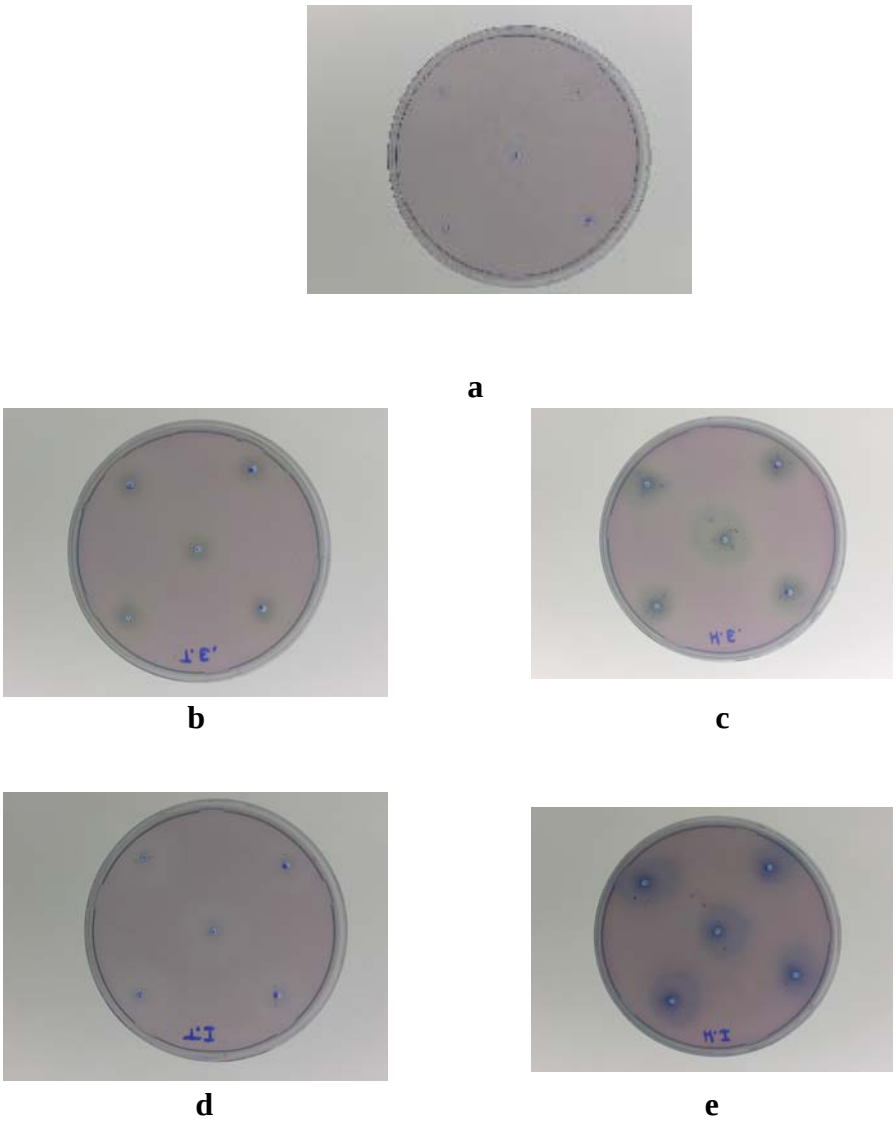


Figure 3.3 Activity staining for tyrosinase production by *S. thermophilum* on agar plates a; Control plate with phosphate buffer instead of enzyme, b; Extracellular tyrosinase test for type culture *Torula thermophila*, c; Extracellular tyrosinase test for type culture *Humicola insolens*, d; Intracellular tyrosinase test for type culture *Torula thermophila*, e; Intracellular tyrosinase test for type culture *Humicola insolens*

3.1.2 Comparison of Intracellular and Extracellular Phenol Oxidase Production of *T. thermophila* and *H. insolens* by Activity Staining on Polyacrylamide Gel

Activity staining of intracellular and extracellular phenol oxidases on SDS-Polyacrylamide Gel was carried out to control and to prove the components of phenol oxidases found in cell lysate and in culture supernatant of two type cultures of *S. thermophilum* as described in section 3.1.1. Intracellular and extracellular samples of *Torula thermophila* and *Humicola insolens* collected on the fifth day of the cell cultures grown in YpSs broth with gallic acid were eight times concentrated and loaded on different wells on the same gel (Figure 3.4).

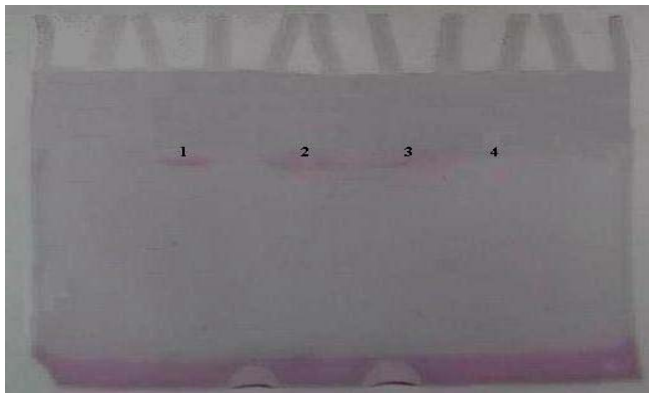


Figure 3.4 Activity Staining on SDS- PAGE Gel

Lane 1 : Culture supernatant of *H .insolens*
Lane 2: Culture supernatant of *T. thermophila*
Lane 3: Cell lysate of *H. insolens*
Lane 4: Cell lysate of *T. thermophila*

In Figure 3.4, in the lanes of extracellular enzyme samples of *H. insolens* and *T. thermophila* exact laccase bands were observed while there was a sligth band on

the lane of intracellular enzyme sample of *H. insolens*. However there was no laccase band in the intracellular enzyme sample of *T. thermophila.*

The intensity of the pink color increased after the addition of hydrogen peroxide to the gel. However, additional bands were not observed after hydrogen peroxide and *t*BC treatments suggesting absence of peroxidase and tyrosinase activities.

Apart from the gel containing 4 different samples, another gel was fully loaded only with two times concentrated culture supernatant taken from the fifth day of the growth medium of *Humicola insolens* (Figure 3.5).

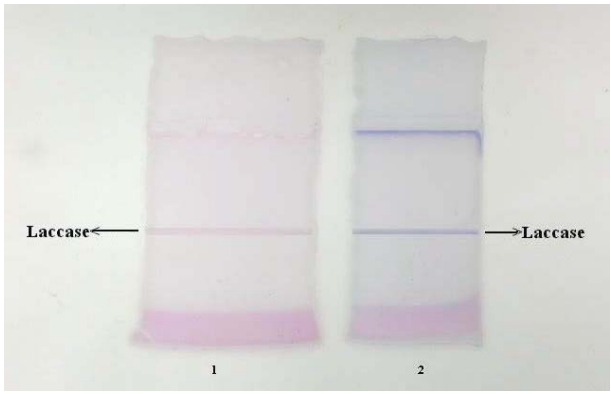


Figure 3.5 Activity staining applied on *H. insolens* culture supernatant, 1: one piece of the gel washed only with ADA; 2: the rest piece of the same gel washed with ADA, H_2O_2 and *t*BC in this order

In figure 3.5, the two gels are the pieces of the one same gel. The whole gel was cut into two pieces after the electrophoretic run. The part number 1 (on the left) was washed only with ADA while the other (on the right) was washed with ADA, hydrogen peroxide and 4-*tert*-butyl catechol one after the other. The band on the first gel part (on the left), was the proof of the laccase enzyme in the culture

supernatant of *H. insolens*. However, on the second gel (on the right), there was not any additional band although it was washed with the substrates of peroxidase and tyrosinase enzymes. Therefore, it was concluded that there were not any extracellular peroxidase and extracellular tyrosinase activities in the culture supernatant of *H. insolens.* A confusion comes from the gel photo that there are two bands in the gel, however, this does not represents the any band corresponding to another enzyme due to first appearing in pink color then turning into blue simultaneously. The reason for these two bands in different places of the gel may be a partial damage out of the active site of the some enzyme molecules due to electrical field without preventing the enzyme activity while the other molecules could remain whole. Therefore these slightly different molecules represented enzymatic activity in different lines of the gel.

In comparison of phenol oxidases produced by two type cultures of *S. thermophilum* according to the results of the qualitative agar spot test and SDS-polyacrylamide gel electrophoresis, extracellular laccase production in the culture supernatant media of both *Torula thermophila* and *Humicola insolens* was proved by two assay methods. However, the presence of peroxidase and tyrosinase activities in cell lysate of *Humicola insolens* and the presence of tyrosinase in the culture supernatant media of two type cultures observed by the agar spot test (figures 3.2 and 3.3) did not appear on the SDS-polyacrylamide gel. Culture supernatant of *Humicola insolens* also showed the highest activity with catechol at 420 nm. By using these data, culture supernatant of *Humicola insolens* was chosen as the most appropriate agent to use in future analyses.

3.2 Analysis of Phenol Oxidase Induction by Edible Mushroom *Agaricus bisporus*

In previous studies it was shown that gallic acid, chlorogenic acid, caffeic acid, syringaldazine and grape pomace are able to induce phenol oxidase production by *S. thermophilum*. Due to the stimulating effect of *Scytalidium thermophilum* on

the growth of the edible mushroom *Agaricus bisporus*, it was also of interest to analyse the effect of the phenolic chemicals of mushroom on phenol oxidase production by *Scytalidium thermophilum*. Extracellular phenol oxidase activities were analysed for both type cultures of *Scytalidium thermophilum*, grown in medium containing mushroom pieces and in control media.

3.2.1 Effect of Mushroom Containing Medium on Phenol Oxidase Production by *Torula thermophila*

Agaricus bisporus (217.6 g/L) was cut into small pieces and added into 4% glucose containing YpSs medium just before sterilization of the medium. Extracellular enzyme activity was assayed every 24 hours starting from the second day until the seventh day of the cultivation. As can be seen in Figure 3.6, the presence of mushroom in the growth medium did not have any inductive effect on phenol oxidase production.

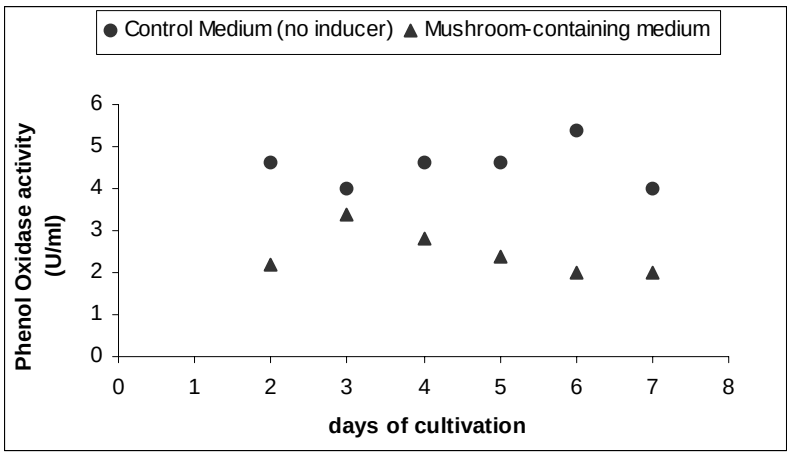


Figure3.6 Extracellular phenol oxidase production by *T. thermophila* in the presence and absence of the mushroom *Agaricus bisporus*.

3.2.2 Effect of Mushroom Containing Medium on Phenol Oxidase Production by *Humicola insolens*

Same experiments described in section 3.2.1 was repeated for *H. insolens*. Enzyme activity was assayed daily, starting from the first day to the ninth day of the cultivation. According to Figure 3.7, the presence of mushroom in the growth medium of *H. insolens* did not have any inductive effect on phenol oxidase production.

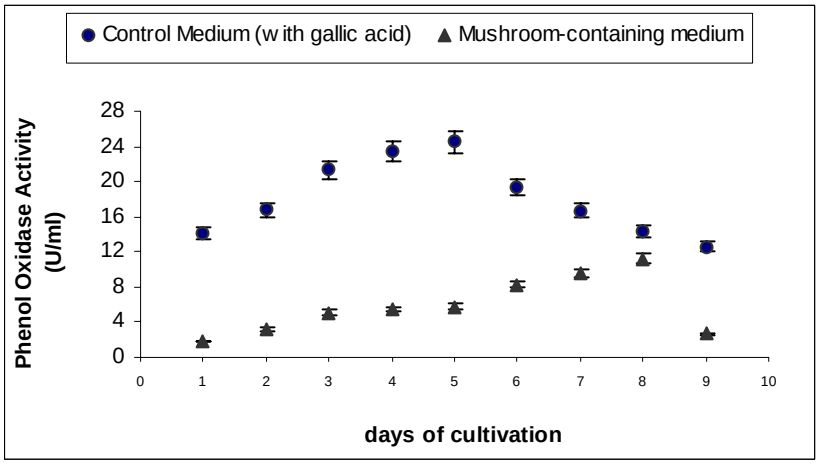
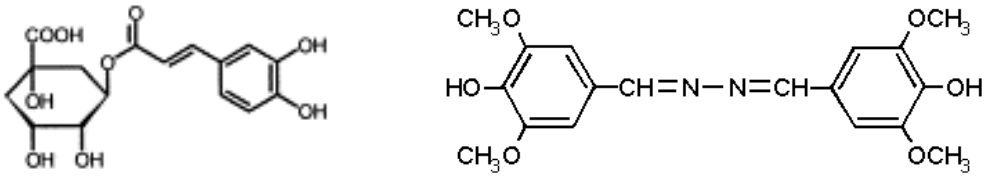


Figure 3.7 Extracellular phenol oxidase production by *H. insolens* in the presence and absence of the mushroom *Agaricus bisporus*.

3.3 Analysis of Phenol Oxidase Induction by Syringaldazine and Chlorogenic Acid

Previous studies have shown that gallic acid is the best inducer of phenol oxidase production (S. Mete, 2003). This has also been confirmed in this study. However, a dark brown color is formed in the culture medium in the presence of gallic acid owing to the possible polymerization of gallic acid. Due to this brown

color, problems arisen in enzyme assays, which are based on the change in color of the substrate, due to oxidation by phenoloxidase. Especially, substrates like syringaldazine, which are specific to laccase, as apposed to catechol, did not show any activity. It was therefore of interest to investigate induction effects of chlorogenic acid and syringaldazine instead of gallic acid in this study. Although these inducers are not as efficient as gallic acid, if dark brown coloration could be prevented, then it might be possible to detect activity of the putative *S. thermophilum* laccase on more specific substrates, like syringaldazine and ABTS.



a b

Figure 3.8 Structural formulae of Chlorogenic acid (a) and Syringaldazine (b)

Supernatants from 1mM chlorogenic acid and 1mM syringaldazine containing culture media were collected and activities were measured by using 100 mM catechol, 4 mM ABTS and 0.28 mM syringaldazine solutions. Final concentrations in the assay mixture were 25 mM catechol, 0.027 mM ABTS and 0.02 mM syringaldazine. Figure 3.9 shows the extracellular phenol oxidase activity from *Scytalidium thermophilum* grown in medium containing 1 mM chlorogenic acid. Accordingly, phenol oxidase produced in chlorogenic acid medium showed the highest activity with catechol, and represented very slight activity with syringaldazine, and no activity was recorded with ABTS. Assays with final 0.025 mM, 0.050 mM, 0.075 mM, 0.1 mM, 0.125 mM, 0.15 mM, 0.175 mM and 0.5 mM ABTS in gallic acid medium and with 0.002 mM, 0.008 mM, 0.009 mM, 0.01 mM, 0.02 mM, 0.025 mM, 0.07 mM and 0.125 mM syringaldazine in gallic acid medium were also carried out however, in none of these concentrations, phenol oxidase from culture supernatant represented any enzymatic activity.

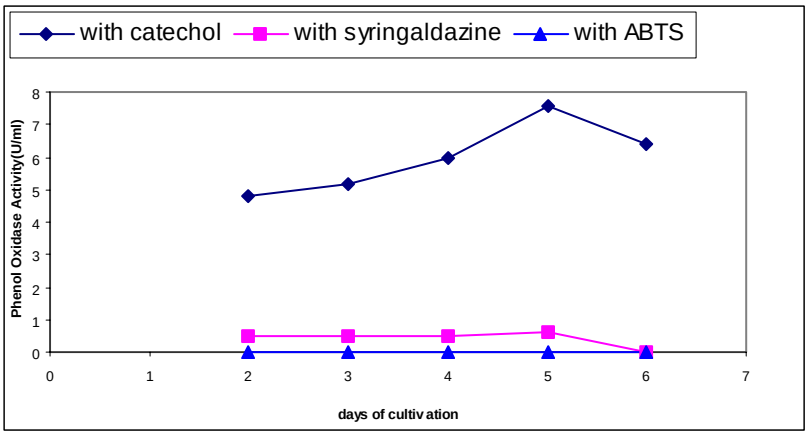


Figure 3.9 Phenol oxidase production induced by chlorogenic acid, and activities determined with assay substrates catechol, ABTS and syringaldazine

Figure 3.10 shows the phenol oxidase activity induced by 1mM syringaldazine in the growth medium of *Scytalidium thermophilum*. Phenol oxidase activity in syringaldazine medium with catechol was higher than that in chlorogenic acid medium, however, again no activity was observed with syringaldazine and ABTS as assay substrates.

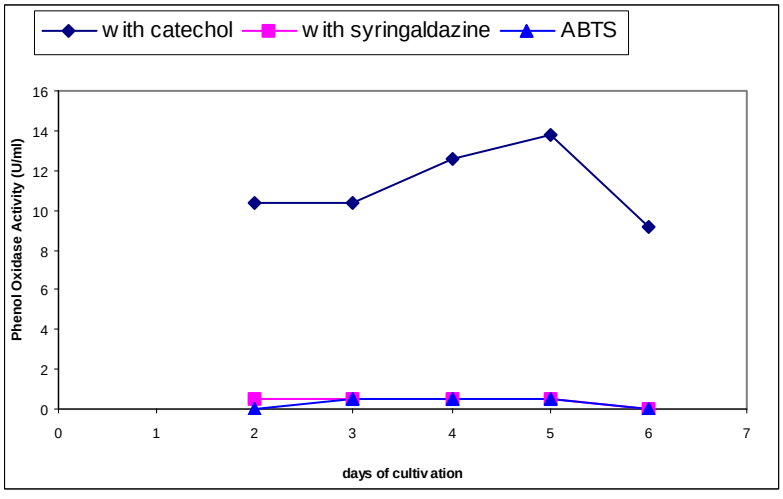


Figure 3.10 Phenol oxidase production induced by syringaldazine, and activities determined with assay substrates catechol, ABTS and syringaldazine

3.4 Determination of the Extinction Coefficient of Catechol

Extinction coefficient (ε) of the oxidation product of catechol was calculated at 420 nm from reaction endpoint peak absorbance. Calculation by this method can only be achieved with fulfilled conditions such as high enzyme activity, complete conversion of the substrate to product and stable product formation (J. Herbert Waite, 1976).

Peak Absorbance determination of each substrate concentration was assayed as duplicate. Primarily, all absorbance values were plotted versus time. The time course of the reactions resulted in hyperbolic curves in these graphs. The endpoints of the reactions, in other words, the starting points of the plateau regions of the hyperbolic curves were utilized for Molar extinction coefficient calculation. ε_{max} of catechol was found directly from slope of the peak absorbance versus substrate concentration graph explained by Beer- Bouguer-Lambert Law:

$$A = \varepsilon cl$$

A: absorbance at specified wavelenght

ε : Molar extinction coefficient

c : concentration (moles/liter)

l : pathlenght of light through the cuvette (1 cm)

Extracellular phenol oxidase activity of *S. thermophilum* with catechol was assayed at 420 nm, 60 ^{o}C and pH 7,0. Since no information was found in the literature about the extinction coefficient (ε_{max}) of catechol under these conditions, it was necessary to calculate the extinction coefficient of catechol under these optimal assay conditions.

Calculation of extinction coefficient was carried out by two different sets of experiments. In the first set, sample cuvette contained 0,5 ml culture supernatant, 1 ml phosphate buffer at pH 7.0, and 0.5 ml of catechol solution. Concentrations of

catechol solutions varied between 2 x 10^{-6} M and 1 x 10^{-4} M (Figure 3.10). In the blank cuvette, instead of 0.5 ml culture supernatant, 0.5 ml of buffer existed (substrate blank). In the second set, sample cuvette preparation was the same but blank cuvette consisted of 1.5 ml buffer and 0.5 ml of culture supernatant (enzyme blank), and the range of substrate concentration was from 1x10^{-6} M to 5x10^{-6} M. Supernatants used in these experiments were collected from showing highest phenol oxidase activity under optimal growth conditions.

The term "peak absorbance" refers to the maximum absorbance of oxidized catechol which remains constant after this value. The slope of the plot of peak absorbance versus catechol concentration gives the ε_{max} of catechol at defined conditions according to Beer-Bouguer-Lambert Law. Figure 3.11 is the plot of experimental set 1 which was assayed with "substrate blank".

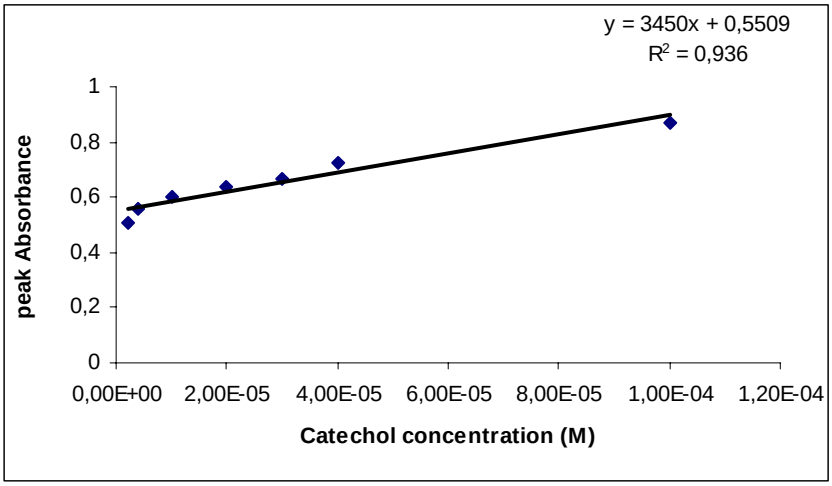


Figure 3.11 Peak Absorbance versus catechol concentration (M) using "substrate blank"

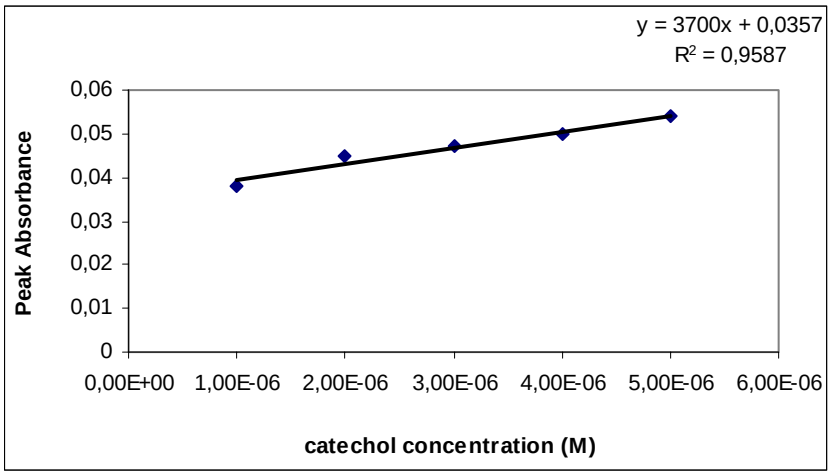


Figure 3.12 Peak Absorbance versus catechol concentration (M) using "enzyme blank"

Plot of the second experimental set assayed with enzyme blank is shown in Figure 3.12. According to the graphs shown in Figure 3.11 and Figure 3.12, maximum molar extinction coefficients (ε_{max}) of catechol were found as 3450 M^{-1} cm^{-1} and 3700 M^{-1} cm^{-1} from the plots when "substrate blank" and "enzyme blank" were used in the assays respectively. ε_{max} of catechol was used to calculate enzyme activities in the determination assays of kinetic parameters, K_m and V_{max}.

3.5 Kinetic Analysis of Extracellular Phenol Oxidase

To determine the kinetic parameters of extracellular phenol oxidase taken from culture supernatant of *Scytalidium thermophilum*, initial reaction rates at different catechol concentrations, ranging from 0 to 500 mM were determined. Results obeyed Michaelis-Menten kinetics. Experimental data of Figure 3.13 is given in Table 3.1. There was a substrate inhibition at and over 200 mM catechol concentration. Therefore, for Lineweaver-Burk and Hanes-Woolf plots, the last two data points corresponding to substrate inhibition were neglected. Phenol oxidase activities were calculated by using the equation given in section 2.2.2.1 and extinction coefficient of catechol (ε) took place in this equation was used as 3450 M^{-1} cm^{-1}.

Michaelis- Menten equation:

$$V = V_{max} [S] / (K_m + [S])$$

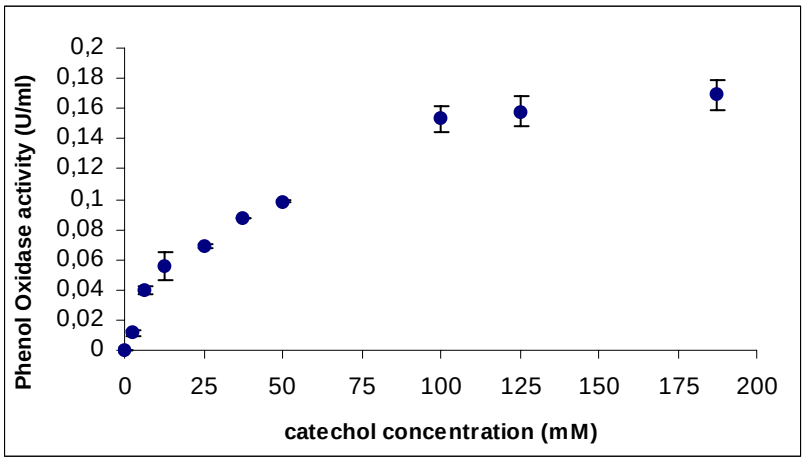


Figure 3.13 Phenol oxidase activity of *S. thermophilum* versus substrate concentration

Since Michaelis- Menten curve represents a hyperbolic graph, it is difficult to calculate K_m and V_{max} parameters from the plot of its equation with high accuracy. There are several equations such as Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee that give straight-line relationships by the rearrangement of Michaelis-Menten equation to calculate these parameters.

Table 3.1 Data used in Michaelis-Menten Curve: s.d; standard deviation, SEM; Standard Error of Mean

Catechol Conc.(mM)	PO Activity1	PO Activity2	Mean	± s.d.	SEM
2.5	0.097	0.0129	0.0113	0.0016	0.00113
6.25	0.037	0.042	0.0395	0.0025	0.00177
12.5	0.065	0.046	0.055	0.0095	0.00671
25	0.068	0.07	0.069	0.001	0.00071
37.5	0.087	0.088	0.0875	0.0005	0.00035
50	0.098	0.099	0.0985	0.0005	0.00035
100	0.145	0.161	0.153	0.008	0.00566
125	0.148	0.168	0.158	0.01	0.00707
187.5	0.159	0.179	0.169	0.01	0.00707

Although Lineweaver- Burk reciprocal plot is the most commonly used one in general, it has the disadvantage as the high concentration points tend to be compressed towards the 1/v axis, since the lowest points represent the highest rates and substrate concentrations as shown in Figure 3.14. In this study, K_m and V_{max} of *S. thermophilum* phenol oxidase for catechol were calculated both by Lineweaver- Burk and by Hanes- Woolf plots.

Lineweaver-Burk plot is shown in Figure 3.14. Accordingly, K_m and V_{max} were calculated as 52.03 mM and 0.253 U/ml respectively.

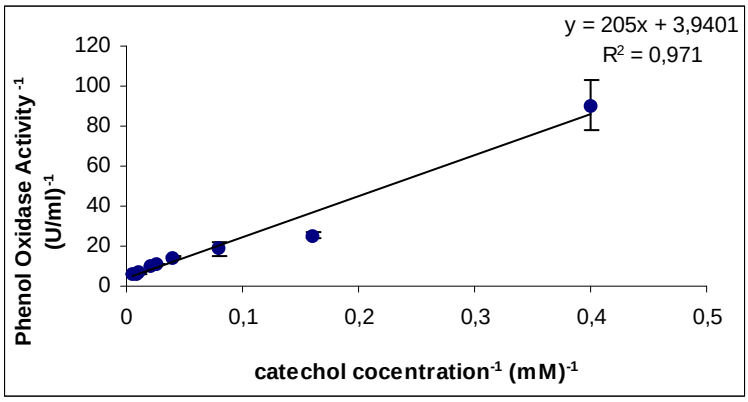


Figure 3.14 Lineweaver-Burk Plot of *S. thermophilum* phenol oxidase

Lineweaver-Burk equation:

$$1/V = (K_m/V_{max})(1 /[S]) + 1/V_{max}$$

K_m and V_{max} values were calculated from Hanes-Woolf plot shown in Figure 3.15 as 41.25 mM and 0.206 U/ml respectively.

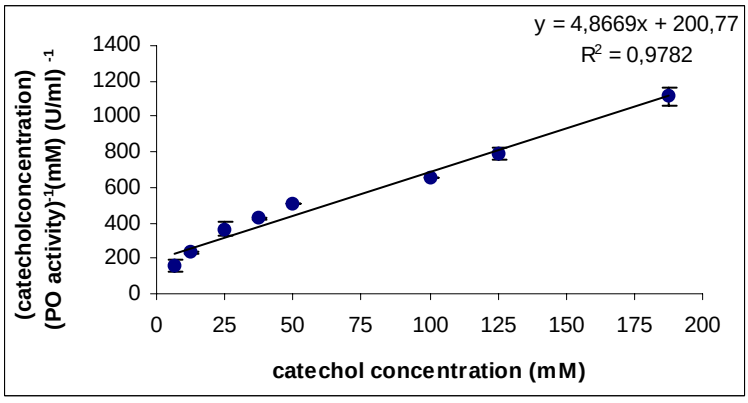


Figure 3.15 Hanes- Woolf Plot of *S. thermophilum* phenol oxidase

Hanes-Woolf equation:

$$[S]/V= [S]/V_{max}+ Km/V_{max}$$

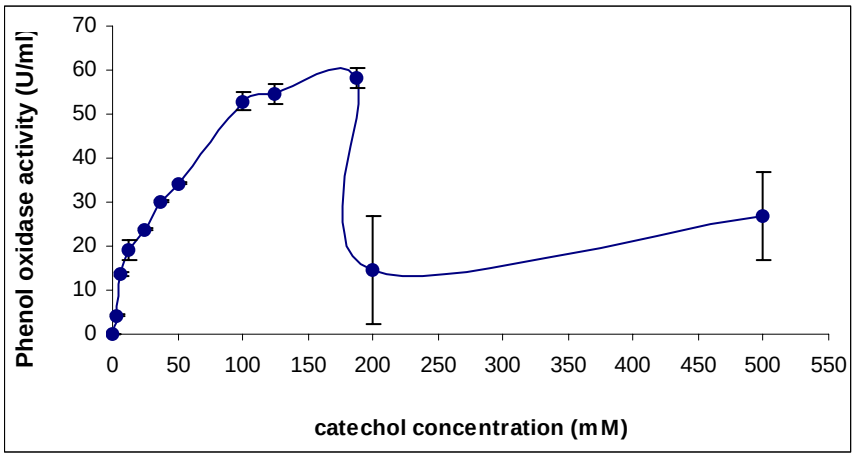


Figure 3.16 Substrate inhibition of *S. thermophilum* phenol oxidase

Inhibition of *S. thermophilum* phenol oxidase by high concentrations of catechol is seen in Figure 3.16. This inhibition is most probably due to the denaturation of the phenol oxidase by catechol. Since catechol represents high tendency for autooxidation by air, it is difficult to measure the phenol oxidase activity at higher catechol concentrations with high accuracy. The recorded low activities at high concentrations may be due to the autooxidation of catechol instead of enzyme activity. Observation of the higher activity at 500 mM catechol concentration than that of at 200 mM catechol concentration strenghtens this probability. Activities recorded at 200 mM and 500 mM concentrations are not consistent, therefore, the standard error of mean (SEM) and standard deviation for these concentrations are too high to be acceptable. In Table 3.2, several examples were given from literature about kinetic parameters of phenol oxidases from different fungal sources. However, there is no information about K_m and V_{max} values of *Scytalidium thermophilum* phenol oxidase with catechol and any other substrates in literature. Therefore, the K_m and V_{max} parameters found in this study could not be compared.

Table 3.2 Kinetic parameters of phenol oxidases from different fungal sources

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)

3.6 Utilization of *Scytalidium thermophilum* in Bioorganic Synthesis

3.6.1 Effect of Organic Solvents on *S. thermophilum* Phenol Oxidase Activity

In this study, it was aimed to analyse activity of phenol oxidase on a number of organic compounds. However, since these organic compounds are insoluble in water, it was necessary to dissolve them in appropriate organic solvents. In enzyme assays, since enzymes can be inactivated easily by such organic solvents, it was necessary to investigate the effects of the organic solvents used to dissolve organic compounds on *S. thermophilum* phenol oxidase activity.

Effects of the organic solvents on the enzyme activity were screened spectrophotometrically by the same procedure with the quantitative extracellular enzyme activity measurement. Ethanol, acetone and DMSO were selected as the organic solvents.

Ethanol, one of the most commonly used solvent, was investigated whether it has any effect on enzyme activity. Extracellular phenol oxidase activity was measured spectrophotometrically in the presence of ethanol solutions at different final concentrations ranging from 7.5 % to 25 % (v/v).

Ethanol effect on the extracellular enzyme activity was assayed on the fifth day of cultivation in YpSs medium containing gallic acid as inducer. Sample cuvette consisted of 0,5 ml of phosphate buffer at pH 7.0, 0.5 ml supernatant, 0.5 ml of 25 mM catechol and 0.5 ml of ethanol solution at relevant concentration. Ethanol enhanced phenol oxidase activity at all concentrations, but the highest activity was achieved at 17.5 % final ethanol concentration. Effect of ethanol on extracellular *S. thermophilum* phenol oxidase is shown in Figure 3.17.

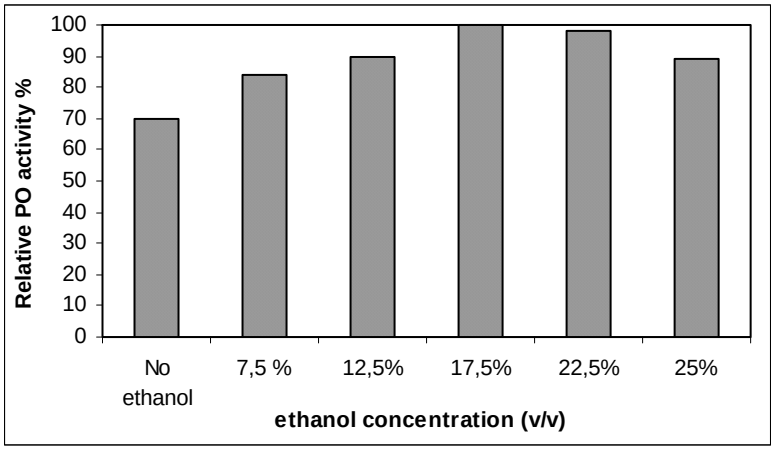


Figure 3.17 Effect of ethanol on extracellular phenol oxidase activity

Acetone is a clear, colorless, low boiling, flamable, volatile liquid, characterized by rapid evaporation and a faintly aromatic, sweetish odour. It is readily miscible in most organic solvents and completely miscible, in all proportions, in distilled water. Acetone effect on phenol oxidase activity was assayed using the fifth day culture supernatant prepared as described for the assay with ethanol above. Acetone concentrations varied between 2.5 % and 15 %. The reason for choice of the maximum acetone concentration as 15 % was the corrosive action of acetone on the

spectrophotometer cuvette over this concentration. However, even the lowest acetone concentration caused inhibition on phenol oxidase activity. Effect of acetone at different concentrations on extracellular *S. thermophilum* phenol oxidase is shown in Figure 3.18.

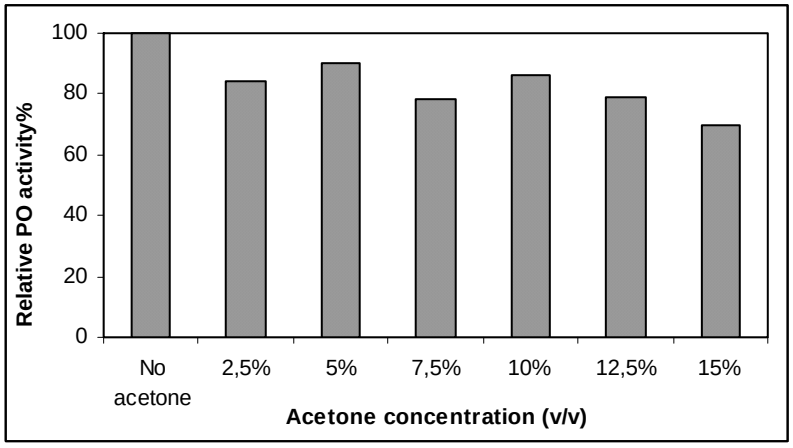


Figure 3.18 Effect of acetone on extracellular phenol oxidase activity

DMSO (Dimethyl sulfoxide) is an effective solvent used for seperation of aromatic compounds from aliphatic ones. Since it is nontoxic, soluble in water, completely capable of solving wide range of organic substrates, DMSO is likely the ideal and the most suitable solvent for the preparation of substrate solution. 12.5 % and 25 % DMSO final concentrations in the assay mixture were tested for their effects on phenol oxidase activity. It was even better solvent than ethanol in enhancing phenol oxidase activity. Enzyme activity was analysed throughout a five day incubation starting from the third day up to the seventh day of the cultivation. Activation at 25 % final concentration of DMSO was the highest. Activation of phenol oxidase by changing DMSO concentrations is shown in Figure 3.19 by means of relative activity. Effect of the DMSO on *Scytalidium thermophilum* extracellular phenol oxidase on day basis of the cultivation is shown in Figure 3.20. Rise in the phenol oxidase activity by DMSO at two different concentrations is obviously observed in Figure 3.02 with respect to days of the cultivation.

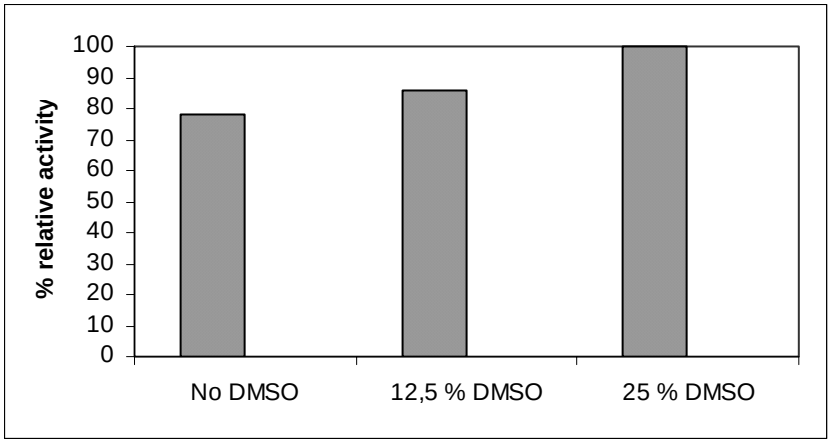


Figure 3.19 Activator effect of DMSO on extracellular phenol oxidase activity. Supernatant from fifth day of the culture was used in the assay.

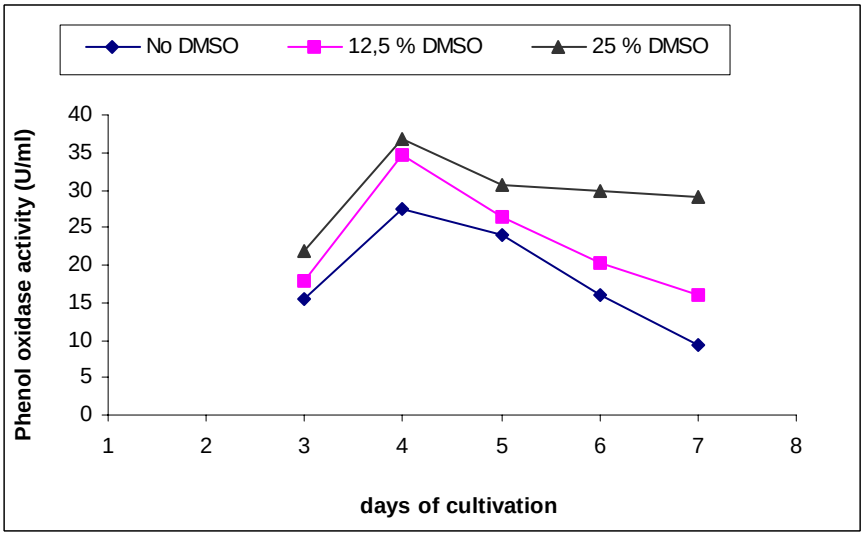


Figure 3.20 Effect of DMSO on extracellular phenol oxidase activity on day basis of the cultivation

3.6.2 Enzyme Assays with Different Organic Substrates

Thermophilic phenol oxidases are likely to be efficient biocatalysts that can be utilized for biotransformation of some chiral organic compounds in racemic mixtures or in *meso*- forms. Particularly, the organic compounds having pharmaceutical and industrial importance were chosen as the substrates for phenol oxidases.

Benzoin, hydrobenzoin and benzoyl benzoin were three compounds analysed in all trials. The others, 3,4,5-trimethoxy benzoic acid, chromanone, thiochromanone, ADA, ABTS, gallic acid and tannic acid were assayed in some of the trials. Molecular formulae of these organic compounds is shown in Figure 3.21.

ABTS is used in combination with laccase in pulp industry as pretreatment before soda cooking of wheat straw to improve characteristics of crude or refined pulp (Duarte, J.C. ,1997). Benzoin is used as an intermediate, photopolymerization catalyst and ingredient of inhalants and internally as an expectorant and topically in various preparations as an antiseptic and protective agent. It is a common ingredient in vaporizer fluids marketed for inhalation. Its synonyms are -Hydroxybenzyl phenyl ketone and 2-Hydroxy-2-phenylacetophenone.

Gallic acid is widely found in gallnuts, sumach, oak bark, and many other plants, both in its free state and as part of the tannin molecule. Gallic acid, and its catechin derivatives are also present as one of the main phenolic components of both black and green tea. Esters of gallic acid have a diverse range of industrial uses, as antioxidants in food, in cosmetics and in the pharmaceutical industry. In addition, gallic acid is employed as a source material for inks, paints and colour developers. Studies utilising this compound have found it to possess many potential therapeutic properties including anti-cancer and antimicrobial properties (Stupans, 2003).

Tannins are water-soluble polyphenolic compounds having wide prevalence in plants. They are believed to be a glucoside in which each of the five hydroxyl groups of the glucose molecule is esterified with a molecule of digallic acid. Tannin is used

in tanning animal skins to make leather; it transforms certain proteins of animal tissue into compounds that resist decomposition. It is also used in manufacturing inks, as a mordant in dyeing, and is also applied to burns to help the healing and to cuts to reduce bleeding.

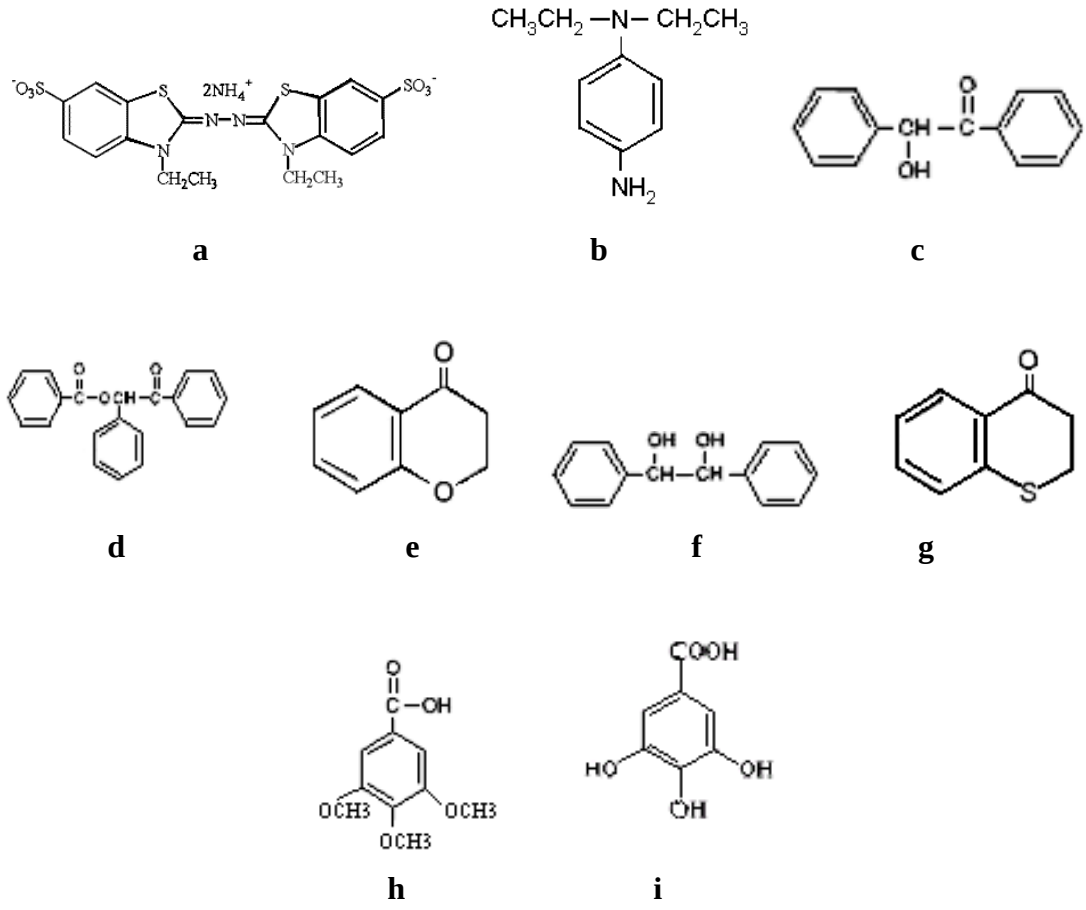


Figure 3.21 Molecular structures of organic substrates, a: ABTS (2 ,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), b: ADA (4- amino- *N*,*N*-diethylaniline), c: benzoin (2-Hydroxy-2-phenylacetophenone), d: benzoyl benzoin, e: chromanone (2,3-Dihydro-1-benzopyran-4-one), f: hydrobenzoin (1,2-diphenyl-1,2-ethandiol), g: thiochromanone, h: 3,4,5-trimethoxy benzoic acid, i: gallic acid

All reactions with the above mentioned organic substrates were carried out at 60 ^{o}C and at pH 7.0. The substrates were dissolved both in ethanol and in DMSO. It was of interest to analyse the followings:

- Aryl coupling on *p*-methoxy groups was investigated for 3,4,5-trimethoxy benzoic acid.
- Regioselectivity of the oxidation of racemic benzoin was analysed.
- Regioselectivity of the oxidation of hydrobenzoin in meso form was analysed.
- Oxidation of the protected benzoin on benzoyl benzoin after hydrolysis was analysed using the racemic mixture of benzoyl benzoin as the starting material.
- Ability of phenol oxidase to oxidize chromanone and thiochromanone was investigated.
- The product of ADA that was used as the laccase specific substrate in activity staining (section 3.1.1) was investigated by HPLC and GC analyses.
- One of the most frequently used substrates for laccase activity, ABTS, was assayed for enzyme activity measurement and its end product was screened by HPLC.
- Gallic acid was used as an inducer more than as a substrate, however, it is also likely that this compound is used as a substrate by phenol oxidases in the culture medium, possibly yielding dark coloured polymers. Therefore, the end-product was detected by HPLC.

All the results of assays with these organic substrates has been shown in Table 3.3.

Table 3.3 : Results of the biotransformation experiments with extracellular phenol oxidase

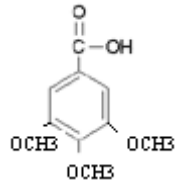
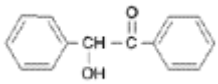
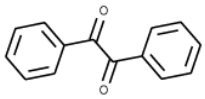
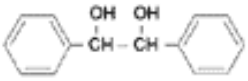
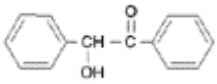
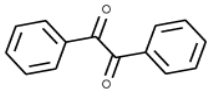
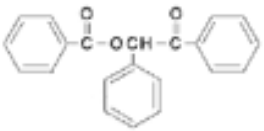
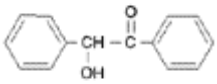
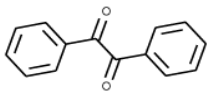
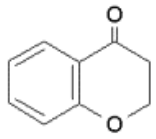
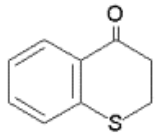
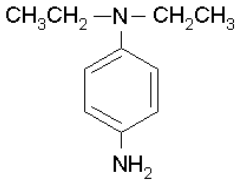
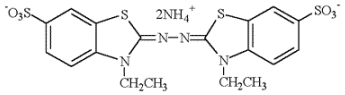
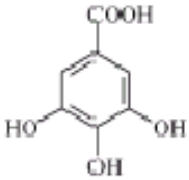
Substrate	Product	Remarks
A.  3,4,5-trimethoxy benzoic acid	No product formation was detected by HPLC	
B.  Benzoin (2-Hydroxy-2-phenylacetophenone)	 Benzil (Diphenylethanedione, Dibenzoyl)	At all four trials, racemic benzoin was oxidized into benzil by PO .
C.  Hydrobenzoin	 Benzoin  Benzil	At 4 trials, hydrobenzoin in meso form was oxidized into first benzoin then into benzil.
D.  Benzoyl benzoin	 Benzoin  Benzil	Racemic benzoin was firstly hydrolyzed into benzoin then oxidized into benzil in 3 runs. In the frst run, the solvent was ethanol, which is a poor solvent for benzoyl benzoin. This is possibly due to the weak oxidation imto benzil in the first run.

Table 3.3 : Results of the biotransformation experiments with extracellular phenol oxidase (Continued)

E.  Chromanone	No product formation was detected by HPLC	
F.  Thiochromanone	No product formation was detected by HPLC	
G.  ADA	No product formation was detected by HPLC	
H.  ABTS	No product formation was detected by GC	
I.  Gallic acid	No product formation was detected by GC	
J. Tannic acid (polymeric material)	**N**o product formation was detected by GC	

3.6.3 Results of Thin Layer Chromatography (TLC)

In all plates, there were three sample loading points at the bottom. The row on the left was for substrate solution without enzyme. The point in the middle consisted of samples both from substrate solution without enzyme and from enzyme-substrate reaction mixture while the row on the right was the lane for enzymatic reaction solution. TLC results of the substrates converted into the products were shown in Figure 3.22.

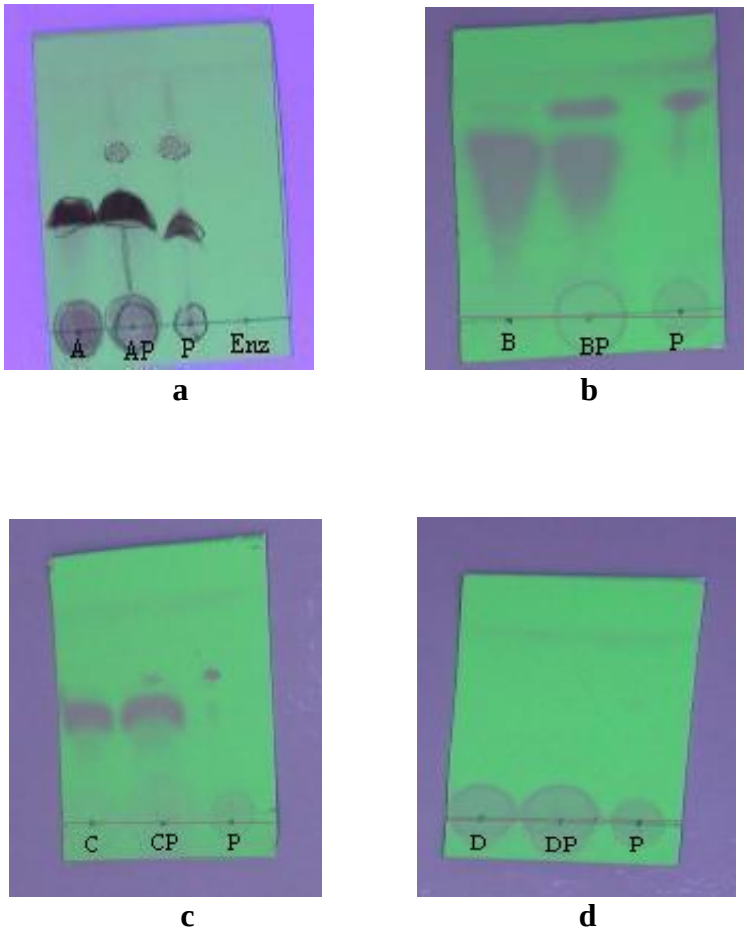


Figure 3.22 TLC results of four organic substrates of laccase under UV light a: ADA, b: benzoin, c: benzoyl benzoin, d: hydrobenzoin

In Figure 3.22.a, TLC plate of ADA, product bands are observed on the lanes of solution of enzymatic reaction. The spot on the right with the mark “Enz” belongs to enzyme solution from culture supernatant to express no interference coming from enzyme solution.

TLC plate belonging to benzoin substrate shows product formation on the right and in the middle rows while substrate lane on the left had very slight product band which might be due to autooxidation of the substrate (Figure 3.22.b). On the right lane, the substrate spot had completely disappeared since all substrate was converted to product.

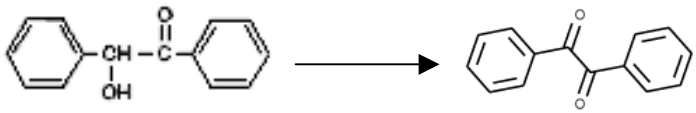
TLC plate belonging to benzoyl benzoin, had product band on the lane of only enzyme sample and also in the middle lane while substrate row on the left had no additional product band (Figure 3.22.c).

TLC plate of hydrobenzoin had no running spot due to low hexane concentration in the running solvent system, whereas product was detected by HPLC in the extract of this solution (Figure 3.22.d).

3.6.4 Results of HPLC (High Performance Liquid Chromatography)

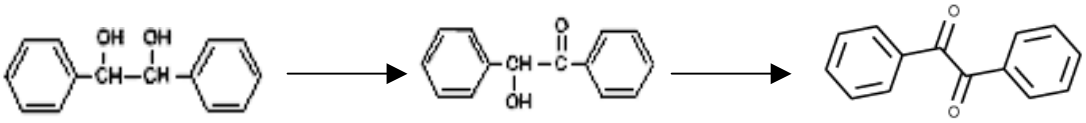
In the bioorganic synthesis experiments, ten different organic substrates were assayed against *S. thermophilum* culture supernatant containing phenol oxidase. Product formation was first analyzed by TLC and later the chemistry of the products were analysed by HPLC and GC. According to the results of the chromatographic analyses, only benzoin, hydrobenzoin and benzoyl benzoin were converted into detectable products. These three substrates were dissolved in ethanol and DMSO, therefore the HPLC results of them with respect to the solvent used were slightly different.

HPLC was performed for benzoin, benzoyl benzoin, hydrobenzoin and ADA. Although product formation was observed on TLC with ADA, no product was detected by HPLC analysis. Benzoin was converted into benzil directly by phenol oxidase. Hydrobenzoin and benzoyl benzoin were first oxidized into benzoin, then into benzil by the enzyme of interest.



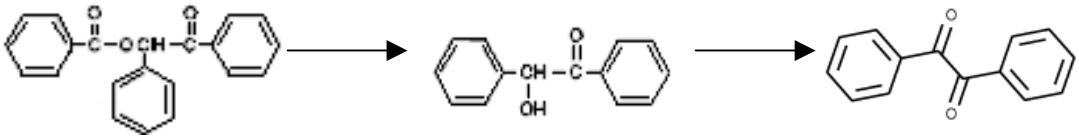
Benzoin Benzil

Figure 3.23 Oxidation of benzoin to benzil by *S. thermophilum* phenol oxidase



Hydrobenzoin Benzoin Benzil

Figure 3.24 Oxidation of hydrobenzoin first to benzoin and then to benzil by *S. thermophilum* phenol oxidase



Benzoylbenzoin Benzoin Benzil

Figure 3.25 Oxidation of benzoylbenzoin first to benzoin and then to benzil by *S. thermophilum* phenol oxidase

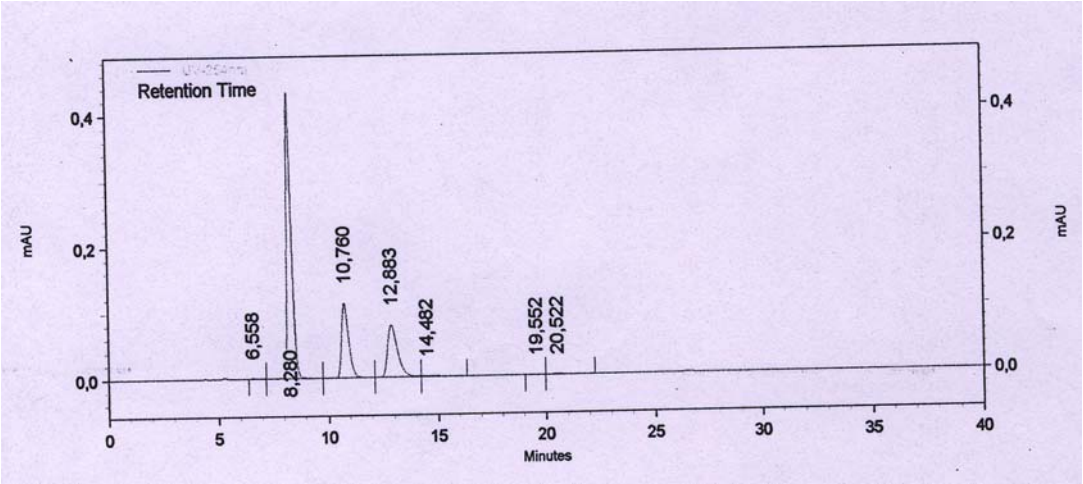


Figure 3.26 Chromatogram of benzoin dissolved in absolute ethanol

Figure 3.26 illustrates the retention times of the compound eluted from chiral column. The peak at 8.2 retention time belongs to benzil which is the oxidation product of benzoin with 56.2 area percent. The starting material, benzoin, could not be detected in the eluent since all substrate, benzoin, was turned into product, benzil.

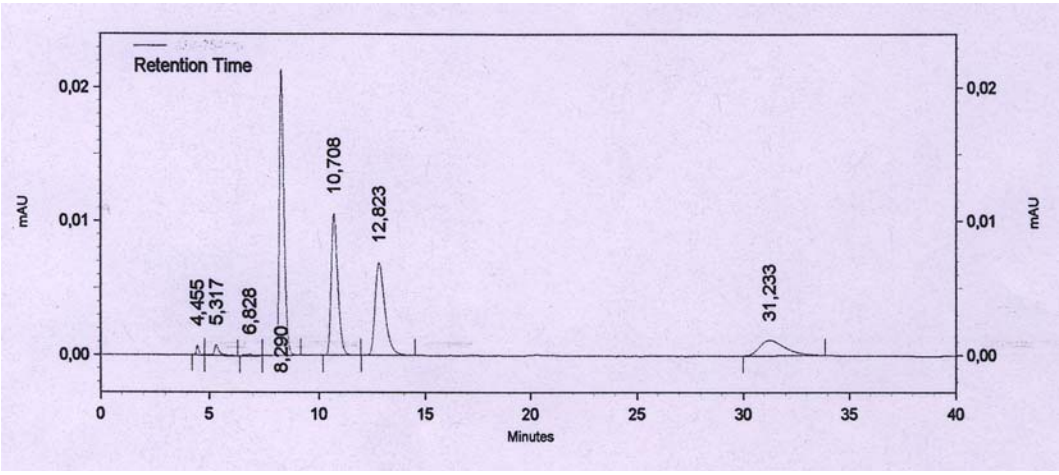


Figure 3.27 Chromatogram of hydrobenzoin dissolve in absolute ethanol

As shown in Figure 3.27, benzil formation was detected at 8.2 retention time with 35.6 area percent. The non-oxidized hydrobenzoin has a peak at 31.2 retention time. The presence of the other peaks except the ones belong to benzil and hydrobenzoin can be explained as impurity.

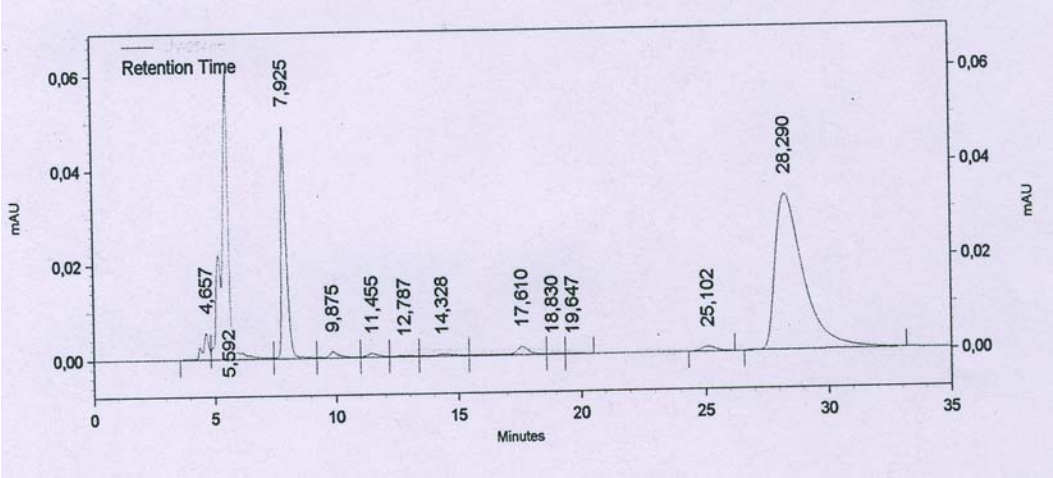


Figure 3.28 Chromatogram of hydrobenzoin dissolved in DMSO

In the chromatogram of hydrobenzoin dissolved in DMSO shown in Figure 3.28, benzil, benzoin and hydrobenzoin peaks are observed. 7.9 retention time belongs to benzil and 28.2 retention time with 53.4 area percent belongs to hydrobenzoin while benzoin has two peaks at 17.6 and 25.1 minutes. Two different peaks of benzoin explains the formation of R and S enantiomers in the enzymatic reaction mixture due to oxidation of hydrobenzoin into benzoin before it was oxidized into benzil. The intermediate, benzoin, could be achieved by the oxidation of hydrobenzoin in meso form regioselectively by the enzyme. R-benzoin was excess in the ratio of 65:35 percent enantiomeric excess (ee). It should be investigated that whether excess of R enantiomer of benzoin could be increased by changing some assay conditions.

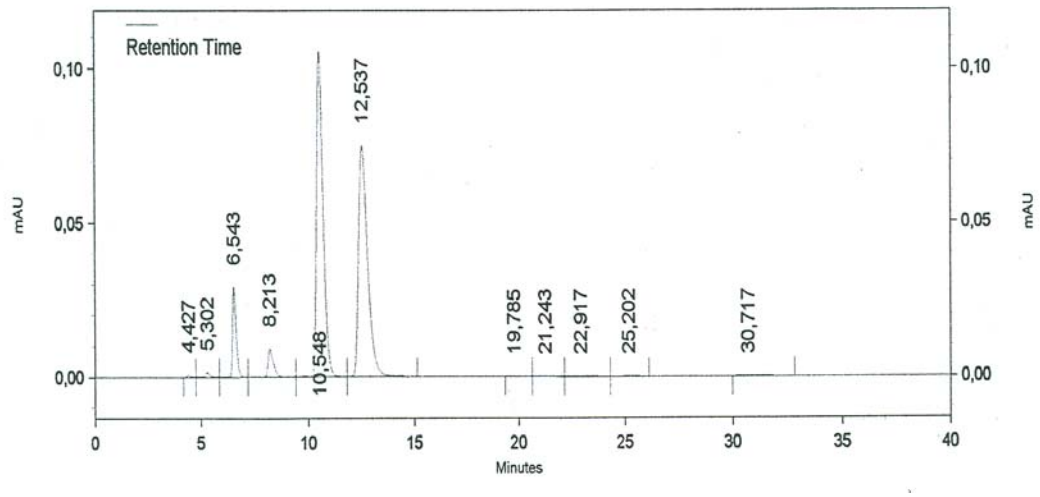


Figure 3.29 Chromatogram of benzoyl benzoin dissolved in absolute ethanol

When benzoyl benzoin was dissolved in ethanol, benzil formation was slightly observed with respect to 3.1 area percent. However, since DMSO is a better solvent than ethanol, area percent of benzil peak is greater as it is 34.8. Figure 3.29 demonstrates the chromatogram of benzoyl benzoin when ethanol was used as the solvent. The peak of benzil was detected at 8.2 retention time. The next two peaks in the chromatogram are the peaks of R and S enantiomers of benzoyl benzoin that is poorly oxidized when dissolved in ethanol. Due to the same reason, benzoin peaks are not observed in the chromatogram although benzoyl benzoin is oxidized into benzoin before its oxidation into benzil. In the oxidation of benzoyl benzoin into benzoin which is intermediate before oxidation into benzil, the regioselectivity of the phenol oxidase was not observed as it was in the oxidation of hydrobenzoin.

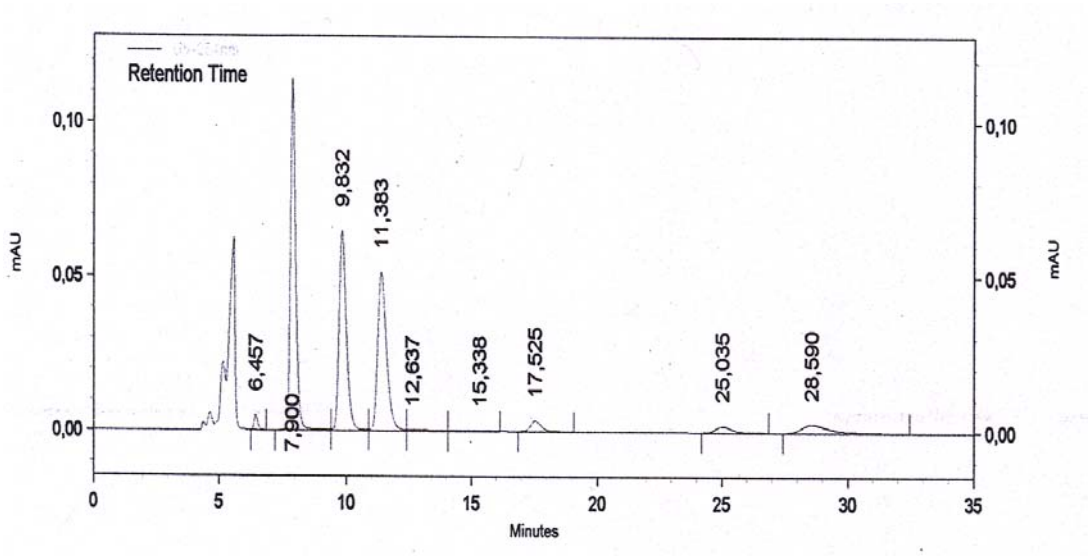


Figure 3.30 Chromatogram of benzoyl benzoin dissolved in DMSO

In Figure 3.30, peaks of benzoin enantiomers, are obviously observed when benzoyl benzoin was dissolved in DMSO. Retention time of benzil peak is at 7.9. 9.8 and 11.3 are the retention times belonging to benzoyl benzoin enantiomers. Benzoin peaks are at 17.5 and 25.0 retention times, while these peaks are absent in Figure 3.29. The rest of the peaks can be defined as impurities.

Autoxidation of benzoin, hydrobenzoin and benzoyl benzoin were screened by control experiments. In these set of experiments, reactions were let in the same conditions with respect to temperature, period of time, substrate concentrations etc. with phosphate buffer at pH 7.0 instead of culture supernatant. According to these control experiment without enzyme, autooxidations of these three substrates were detected by HPLC. However these oxidation peaks were negligible when they were compared with the peaks of enzymatic reactions. In addition, none of the oxidation into benzoin was regioselectively since all enantiomeric excess (ee) ratios were zero.

Table 3.4 gives a summary of the HPLC results including the retention times and the area percents of these three organic substrates (benzoin, hydrobenzoin and

benzoyl benzoin) used in the bioorganic synthesis experiments with respect to the solvent used to dissolve them and the oxidation products.

Table 3.4 HPLC results of benzoin, hydrobenzoin and benzoyl benzoin

Organic substrate	**Solvent used to dissolve**	**Compounds detected in eluent**	**Retention time (min)**	**Area percent**
Benzoin	Ethanol	Benzil	8.2	56.2
Hydrobenzoin	Ethanol	Benzil	8.2	35.6
		Hydrobenzoin	31.2	11.1
	DMSO	Benzil	7.9	14.3
		Benzoin	17.6	1.4
			25.1	0.8
		Hydrobenzoin	28.2	53.4
Benzoyl benzoin	Ethanol	Benzil	8.2	3.1
		Benzoyl benzoin	10.5	44.4
			12.5	44.3
	DMSO	Benzil	7.9	34.7
		Benzoyl benzoin	9.8	27.3
			11.3	27.1
		Benzoin	17.5	2.2
			25.0	2.0

3.6.5 GC (Gas Chromatography) Results

GC method was applied for ABTS, Chromanone, thiochromanone, gallic acid, tannic acid and 3,4,5-trimethoxy benzoic acid. None of these substrates was turned into any product by extracellular phenol oxidase from culture supernatant of *Humicola insolens*.

CHAPTER 4

CONCLUSIONS

In this study, assay of extracellular phenol oxidase activity with different organic compounds and basic kinetic studies of phenol oxidase in culture supernatant of *Scytalidium thermophilum* were carried out for the purpose of finding out possible application potentials.

Towards the enhancement of the phenol oxidase production, different inducers were investigated and gallic acid was found as more appropriate inducer rather than syringaldazine and chlorogenic acid. Experiments with *Torula thermophila* and *Humicola insolens* have shown that there was not any stimulant effect of edible mushroom *Agaricus bisporus* on the phenol oxidase activity of these type cultures.

Activity staining and SDS-polyacrylamide gel electrophoresis experiments of extracellular phenol oxidase produced by *Scytalidium thermophilum* identified the enzyme as laccase. Extracellular laccase activity was assayed with catechol as substrate in most of the experiments. ε_{max} of catechol at 420 nm, 60 ^{o}C and pH 7,0 was calculated as 3450 M^{-1} cm^{-1} with "substrate blank" and as 3700 M^{-1} cm^{-1} with "enzyme blank". Phenol oxidase activity with syringaldazine and ABTS as the substrates other than catechol was also assayed since these compounds are the specific substrates of laccase. Significant activity was not detected even in culture supernatants concentrated eight times by ultrafiltration, suggesting, the enzyme might in fact be a catechol oxidase.

DMSO was used to dissolve water-insoluble organic compounds which were used in the experiments of bioorganic syntheses by phenol oxidases. Utilization of phenol oxidases in bioorganic synthesis was investigated with ten different organic substrates. In particular, the three substrates having pharmaceutical importance,

benzoin, hydrobenzoin and benzoyl benzoin were found as good substrates for laccase which could be a valuable potential enzyme in pharmaceutical industry, while the rest of the assayed substrates were not. Benzil biosynthesis from benzoin, hydrobenzoin and benzoyl benzoin by laccase was achieved. In the oxidation of hydrobenzoin, benzoin was first generated before it was oxidized into benzil. Benzoin product was in the ratio of 65:35 enantiomeric excess (ee), R enantiomer of benzoin was the excess one. This promising result is particularly important in pharmaceutical chemistry since benzil is a valuable drug intermediate in pharmaceutical industry. Further studies should be towards increasimg this ee.

In kinetic studies, K_m and V_{max} were calculated in two different ways. K_m and V_{max} were calculated as 52.03 mM and 0.253 U/ml by using the Lineweaver-Burk plot respectively while these were calculated as 41.25 mM and 0.2055 U/ml from Hanes-Woolf plot.

For further studies, at least partial purification of the phenol oxidase of *Scytalidium thermophilum* is probably indispensable. In addition, bioorganic synthesis of numerous organic compounds which are very important for pharmaceutical, chemical or for relevant other industries can be continued using either pure enzyme or culture supernatants.

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APPENDIX A

MEDIUM COMPOSITIONS

Modified YpSs Broth Media to improve Phenol oxidase production

Yeast extract..... 4.0 g/L

K_2HPO_4..... 1.0 g/L

$MgSO_4.7H_2O$..... 5.0 g/L

$CuSO_4.5H_2O$..... 0.1 g/L

Glucose monohydrate..... 44.0 g/L

Gallic acid..... 0.17 g/L

OR

Syringaldazine..... 0.088 g/L

OR

Chlorogenic acid..... 0.44 g/L

Preparation of Mushroom (*Agaricus bisporus*) Containing Media

Yeast extract..... 4.0 g/L

K_2HPO_4..... 1.0 g/L

$MgSO_4.7H_2O$..... 5.0 g/L

$CuSO_4.5H_2O$..... 0.1 g/L

Glucose monohydrate..... 44.0 g/L

Edible mushroom……208.76 g /L (for *Humicola insolens*)

217.6 g/L (for *Torula thermophila*)

APPENDIX B

EXTRACTION OF INTRACELLULAR PHENOL OXIDASES

Buffer for Extraction of Intracellular Polyphenol Oxidases

200 μl of 1 mM EDTA solution

100 μl of 1.4 mM β-mercaptoethanol

500 μl of 1 mM PMSF

in 100 ml 0.1 M potassium phospate buffer at pH: 7.0

Extraction Procedure

2 gr of mycelia were weighed and suspended in extraction buffer by shaking with a glass stick to obtain nearly homogenous distribution of the cells in the buffer. Then, obtained suspension was sonicated for 10 seconds three times with 30 seconds intervals.

APPENDIX C

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

Agar concentration in the plates

%2 agar

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 into this solution.

B. Hyrdogen peroxide (H_2O_2) Solution

100 µl hydrogen peroxide was added into 100 ml of distilled water.

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

0.34 g 4-*tert*-butyl catechol was dissolved in 100 ml distilled water and 57 µl acetic acid was added into *t*BC solution

D. 0.1 M Sodium phosphate buffer, pH: 7.0

Stock Solutions

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

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

39 ml of x + 61 ml of y are mixed and diluted to a total volume of 200 ml with distilled water.

* Reagents A, B and C were prepared freshly

Procedure

All plates were washed with buffer solution and stained with ADA, H_2O_2 and *t*BC solutions by the following procedure given in table B.

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

Step	Reagent	Time	Assay
1	Sodium phosphate Buffer pH:7,0	5 min	Plates were first washed with buffer at 60^{o}C by shaking at 70 rpm
2	ADA	20 min	Buffer was poured of and ADA solution was added and shaked at 60^{o}C and 70 rpm
3	Sodium phosphate Buffer pH:7,0	5 min	ADA was poured and buffer was added to wash the plates
4	H_2O_2	10 min	After pouring the buffer, H_2O_2 was added and plates were shaked at 60^{o}C and 70 rpm
5	Sodium phosphate Buffer pH:7,0	5 min	H_2O_2 was removed and buffer was added to wash the plates
6	*t*BC	10 min	After removal of the buffer solution, *t*BC was added and plates were shaked at 60^{o}C and 70 rpm

APPENDIX D

REAGENTS AND GEL PREPARATION FOR PHENOL OXIDASE ACTIVITY STAINING OF SDS-PAGE SLAB GEL

Stock Solutions

A. Acrylamide/bis (30% T, 2.67 % C)

87.6 g acrylamide (29,2 g/ 100 ml)

2.4 g *N'N'*-bis-methylene-acrylamide (0.8 g/ 100 ml)

Make to 300 ml with distilled water. Filter and store at 4 ^{o}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 8.8 with 1 N HCl. Make to 100 ml with distilled water and store at 4 ^{o}C.

C. 0.5 M Tris-HCl, pH 6.8

6 g Tris base

~60 ml distilled water

Adjust to pH 6.8 with 1 N HCl. Make to 100 ml with distilled water and store at 4^{o}C.

D. Sample Buffer (SDS reducing buffer) (store at room temperature)

Distilled water	6.0 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Gycerol	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
SDS	3.0 g

Bring to 600 ml with distilled water. Store at 4^{o}C. Warm to 37 ^{o}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 distilled water in an eppendorf by vortexing. This solution should be prepared fresh daily.

G. TEMED (*N,N*-tetramethylene-ethylenediamine)

Use TEMED near from the bottle.

Procedure

A. Preliminary Preparation

Clean the glasses and spacers with ethanol. Assemble the gel sandwich a clean surface. Lay the longer rectangular glass plate down first, then 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 D.1: Preparation of 5% separating gel for activity staining

Monomer Concentration (30%T, 2.67% C)	5%
Acrylamide/bis (30% T, 2.67% C Stock)	1.67 ml
Distilled water	5.78 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
0.5 M Tris-HCl, pH 6.8	-
10% Ammonium persulfate (fresh)	50 µl
TEMED (*N*,*N*-tetramethylene-ethylenediamine)	5 µl

Prepare the monomer solution by combining all reagents except ammonium persulfate and TEMED. Deaerate 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 D.2: Preparation of 4% stacking gel for activity staining

Monomer concentration (30%T, 2.67% C)	4%
Acrylamide/bis (30% T, 2.67% C Stock)	1.3 ml
Distilled water	6.2 ml
1.5 M Tris-HCl, pH 8.8	-
0.5 M Tris-HCl, pH 6.8	2.5 ml
10% Ammonium persulfate (fresh)	50 µl
TEMED (*N*,*N*-tetramethylene-ethylenediamine)	10 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^{o}) angle. This will prevent air from being trapped under the comb teeth while the monomer solutions are poured. Allow the gel to polymerise 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 samples (diluted 1:2 with sample buffer) into the wells in an order.

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 200 volts, constant voltage setting. The usual run time is approximately 45 minutes. This electrophoresis cell is for rapid separation and is not recommended for runs over 60 minutes long. When run finishes, excrude gels very carefully. Carry out the modified activity staining method of Rescigno *et al*. (1997)

APPENDIX E

PREPARATION OF THE ORGANIC SUBSTRATES UTILIZED IN BIOTRANSFORMATION

A: 3,4,5-trimethoxy benzoic acid

B: Benzoin (2-Hydroxy-2-phenylacetophenone)

C: Hydrobenzoin (1,2-diphenyl-1,2-ethandiol)

D: Benzoylbenzoin

E: Chromanone (2,3-Dihydro-1-benzopyran-4-one)

F: Thiochromanone

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

H: ADA (4- amino- *N*,*N*-diethylaniline)

I: Gallic acid

J: Tannic acid

Table E.1: Concentrations of the organic substrates utilized in biotransformation

Substrate	Trial # 1	Trial # 2	Trial # 3	Trial # 4	TLC results
A	0.0519 mM	0.0523 mM	0.1080 mM	0.0955 mM	No product Formation detected in TLC and HPLC
B	0.0353 mM	0.0447 mM	0.0476 mM	0.0537 mM	First product observed in 18 hours in ethanol, in 24 hrs in DMSO

C	0.0350 mM	0.0611 mM	0.0877 mM	0.0844 mM	First product observed in 96 hrs in DMSO, slightly observed in 112 hrs in ethanol
D	0.0378 mM	0.0252 mM	0.0358 mM	0.0276 mM	Product observed in 59 hours in ethanol, in 42 hrs in DMSO
E	0.0331 mM	0.0317 mM	0.0533 mM	-	No product formation observed in both solvent in TLC
F	0.1 % (v/v)	0.1 % (v/v)	0.2 % (v/v)	-	Additional bands were observed but they were not product
G	-	-	-	0.1084 mM	Product observed in 21 hrs in DMSO (only assayed in DMSO)
H	-	-	-	0.182 mM	No product formation observed in TLC
I	-	-	-	0.1339 mM	No product formation observed in TLC
J	-	-	-	3.1 % (w/v)	No product formation observed in TLC

APPENDIX F

PREPARATION OF THE TLC PLATES AND RATIO OF THE SOLVENT SYSTEM USED FOR ORGANIC COMPOUNDS

Procedure

The TLC plates were cut at the size of 5 cm width and 6 cm height. A small quantity of a solution of the mixture to be analyzed is deposited as a small spot on a TLC plate sheet. The sheet is then placed in a beaker containing a small amount of solvent, which is the mobile phase and a watch glass in suitable size is closed on the top of the beaker. The solvent gradually moves up the plate via capillary action, and it carries the deposited substances along with it at different rates. The mobile phase - the running solvent- was ethyl acetate: distilled hexane solvent system at different ratios (v : v) depending upon the type of substrate as shown in Table F.1.

After running in the solvent system was completed, thin layer plates were left for drying and were then monitored under UV light. When the product formation was detected by UV light, the product was extracted by ethyl acetate or chloroform from enzymatic reaction mixture, and HPLC method was applied to the organic solvent containing organic product.

Table F.1: Ratio of the organic solvent system with respect to the organic substrate

Organic substrate	**Ethyl acetate: distilled hexane ratio (v: v)**
3,4,5-trimethoxy benzoic acid	2:1
Benzoin	1:2
Hydrobenzoin	1:5
Benzoyl benzoin	1:5
Chromanone	1:4
Thiochromanone	1:4
ABTS	5:1
ADA	5:1
Gallic acid	5:1
Tannic acid	5:1