MORPHINE BIOTRANSFORMATIONS BY MICROBIAL PHENOL OXIDASES

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Approval of the Graduate School of Natural and Applied Science

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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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ABSTRACT

MORPHINE BIOTRANSFORMATIONS BY MICROBIAL
PHENOL OXIDASES

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The objective of this study is to perform morphine biotransformation by using phenol oxidases. *Syctalidium thermophilum, Thermomyces lanuginosus* and *Phanerochaete chrysosporium* cells and culture fluid were used as microbial intracellular and extracellular phenol oxidases. Besides the phenol oxidases produced in laboratory, commercial pure phenol oxidases, *A. bisporus* tyrosinase and laccase, *T. versicolor* laccase and horseradish peroxidase, were also used in the morphine biotransformation reactions. Morphine biotransformation to pseudo-morphine was achieved by using pure *T.* versicolor laccase, *A.bisporous* tyrosinase and laccase. Before utilization of phenol oxidases in morphine biotransformations, the time course of microbial phenol oxidase productions were followed. Maximum phenol oxidase activity of *S. thermophilum* were detected on the 5th day of cultivation as

0.17 U/ml and the 4th day of cultivation as 0.072 U/ml, respectively. On the other hand, maximum laccase activity of *P. chrysosporium* was detected on the 8th day of cultivation as 78.5 U/ml. Although phenol oxidases which were obtained from *S. thermophilum* or *T. lanuginosus* could not catalyze morphine biotransformation, phenol oxidases including a peroxidase of *P. chrysosporium* transformed morphine to pseudo-morphine and an unknown product.

Keywords: Phenol oxidases, morphine, pseudo-morphine, biotransformation

ÖZ

MİKROBİYAL FENOL OKSİDAZLAR İLE MORFİN BİYO- DÖNÜŞÜMLERİ

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Ocak 2006, 96 sayfa

Bu çalışmanın amacı, fenol oksidazlar kullanılarak, morfin biyo-dönüşümlerinin gerçekleştirilmesidir. Bu amaçla, *Syctalidium thermophilum, Thermomyces lanuginosus* ve *Phanerochaete chrysosporium* adlı küflerin hücreleri ve kültür sıvıları, hücre içi ve hücre dışı mikrobiyal fenol oksidazlar olarak kullanılmıştır. Laboratuar ortamında değişik küflerden elde edilen fenol oksidazların yanında saf olarak satın alınmış olan *Agaricus bisporus* tirosinaz ve lakkaz, *Tramates versicolor* lakkaz ve peroksidaz, enzimleri de kullanılmıştır. Saf *T. versicolor* lakkazı, *A. bisporus* tirosinazı ve lakkazı kullanılarak morfinden pseudo-morfin biyo-dönüşümü gerçekleştirilmiştir. Küflerden elde edilen fenol oksidazlar biyo-dönüşüm reaksiyonlarında kullanılmadan önce, küflerin ürettikleri fenol oksidaz miktarları günlere göre incelenmiştir. *S. thermophilum* ve *T. lanuginosus*'un ürettikleri maksimum fenol oksidaz aktiviteleri sırasıyla, 0,17 U/ml

olarak kültürleme işleminin 5. gününde ve 0,072 U/ml olarak kültürleme işleminin 4. günde gözlemlenmiştir. Diğer taraftan, *P. chrysosporium*'un ürettiği maksimum fenol oksidaz aktivitesi 8. günde 78.5 U/ml olarak kaydedilmiştir. *S. thermophilum* ve *T. lanuginosus* kullanılarak elde edilen fenol oksidazlar, morfin biyo-dönüşüm reaksiyonunu gerçekleştiremediği halde, *P. chrysosporium* kullanılarak elde edilen, peroksidaz da içeren, fenol oksidazlar morfinin pseudo-morfine ve bilinmeyen başka bir ürüne dönüşmesini sağlamışlardır.

Anahtar kelimeler: Fenol oksidazlar, morfin, pseudo-morfin, biyo-dönüşüm

To My father and mother

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ABBREVIATION

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

BSA : Bovine serum albumin

H. i. : Humicola insolens

H_2O_2 : Hydrogen peroxide

HPLC : High Performance Liquid Chromatography

HRP : Horseradish peroxidase

L-DOPA : L-3,4-dihydroxyphenylalanine

LiP : Lignin peroxidase

MnP : Manganese peroxidase

PO: Phenol Oxidase

PPO : Polyphenol oxidase

PVPP : Polyvinyl polypyrolidone

R_f : Retention factor

TLC: Thin Layer Chromatography

U : Enzyme activity unit

UV: Ultra Violet

YpSs agar : Yeast peptone, soluble starch agar

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

CHAPTER 1

INTRODUCTION

Biotransformations involve the use of biological agents, in the form of whole cells or isolated enzymes, to catalyze chemical reactions. Biotransformation systems may be used for environmentally benign biocatalysis of synthetic reactions, bioremediation of pollutants, or waste beneficiation. Biotransformations offer a number of advantages over conventional chemical processes. Chemical preparation of compounds may be difficult to achieve on a large scale owing to complexity of chemical structures, cost and environmental issues such as toxic waste streams, with high energy consumption. However, in biotransformation reactions, the specifity of enzyme-catalyzed reactions allows the stereospecific transformation of specific functional groups, without the necessity for the protection of other labile groups.

Phenol oxidases are increasingly becoming recognized as valuable oxidasing biocatalysts for systhesis of chemical compounds which are significant for pharmaceutical, nutraceutical and food industries because of their broad substrate specifity as mentioned below.

In this study, morphine biotransformation was focused on to obtain useful semisynthetic morphine derivatives for pharmaceutical industry by using microbial phenol oxidases. Morphine alkaloids and their derivatives have important pharmacological activity. Pharmaceutical compounds derived from morphine alkaloids can be used as the analgesics (pain relief drugs), examples being morphine, codeine, dihydrocodeine, diacetylmorphine (heroin), hydromorphone, and oxymorphine; the antitussives (cough suppressants) of which codeine, hydrocodone, oxycodone and pholcodeine are examples; and the narcotic antagonists, such as naloxone and naltrexone, which are used in the treatment of opiate overdose and alcohol addiction (Boonstra, *et al.* 2001).

1.1 Phenol Oxidases

Phenol oxidases catalayse oxidation of aromatic compounds by direct activation of oxygen. The terminology used for naming these enzymes in the literature is confused because of wide range of substrates that used by these enzymes (Burton, 2003).

Phenol oxidases involved in secondary metabolic activity, most commonly being associated with the production of melanins and other pigments. These enzymes are also related to changes in the properties of cell walls such as increasing impermeability and hydrostatic strength (Griffith, 1994).

Phenol oxidases can be separated into four groups:

1. Laccases
2. Polyphenol oxidases
3. Peroxidases
4. Catechol oxidases

1.1.1 Laccases

Laccases are multi-copper oxidases. Laccase (E.C. 1.10.3.2, p-benzenediol:oxygen oxidoreductase) able to catalyze the oxidation of various aromatic compounds (particularly phenols) with reducing of dioxygen to water (Duran et. al., 2002).

Laccases have 4 copper ions which are coordinated at the active site of each molecule or functional unit. Copper atoms are distributed in different binding sites and are classified in three types, according to specific spectroscopic and functional characteristics. These copper ions are coordinated to form Type 1, Type 2 and Type 3 copper site. Type 1 copper site is responsible for primary electron acceptor during oxidation of a substrate. Type 2 and Type 3 sites cluster combine to occur a trinuclear copper cluster to react with dioxygen (Burton, 2003). In Figure 1.1, model of the laccase trinuclear oxygen binding side is shown.

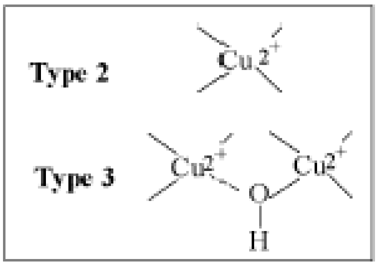


Fig 1.1 Model of the laccase trinuclear oxygen binding site (Burton, 2003)

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).

The laccase catalytic cycle involves binding of the reducing substrate, followed by binding of the second substrate, oxygen. This allows intramolecular electron transfer which results in oxygen reduction, and then proton transfer which leads to release of the reaction products (Burton, 2003).

Laccase is widely distributed in higher plants, in fungi and in some bacterial strains like *Azospirillum lipoferum* and *Alteromonas* sp. (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). On the other hand *Rhus vernicifera* is not involved in lignin biosynthesis, since it cannot oxidize monolignols which is the first step of polymerization of monolignols into oligolignols. In *R. vernicifera,* it is known that laccase catalyzes polymerization of urushiol (an oily toxic irritant) into lacquer (spirit varnish). Although the role of this process is unclear, it is proposed that this reaction occurs in wound-healing. In

Figures 1.2 and 1.3, reaction mechanisms of laccase from *R. vernicifera* and other plants are shown, respectively.

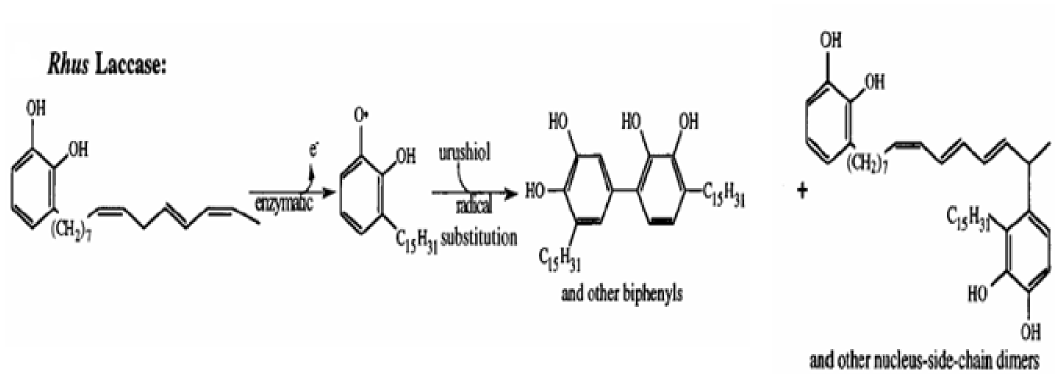


Figure 1.2 Reaction mechanism of laccase from *Rhus vernificera* (Solomon *et al*. 1998).

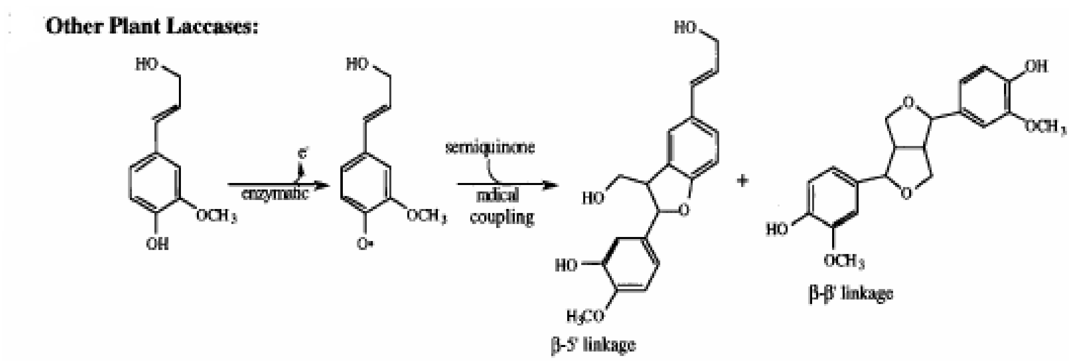


Figure 1.3 Reaction mechanism of other plant laccase (Solomon *et al*. 1998).

Like plant laccases fungal laccase proteins are glycosylated, but generally to a lesser extent (10-25 %). Most but not all, extracellular, and given species may produce isoenzyms both extra and intracellular types. There essentially three possible functions which have been ascribed to fungal laccases: (1) pigment formation, (2) lignin degradation, and (3) detoxification.

1.1.1.1 Laccase Catalysed Reactions

Laccases catalyse the oxidation of variety organic compounds including methoxyphenols, phenols, o-, and p-diphenols, aminophenols, polyphenols, polyamines and lignin-related molecules as seen in Figure 1.4.

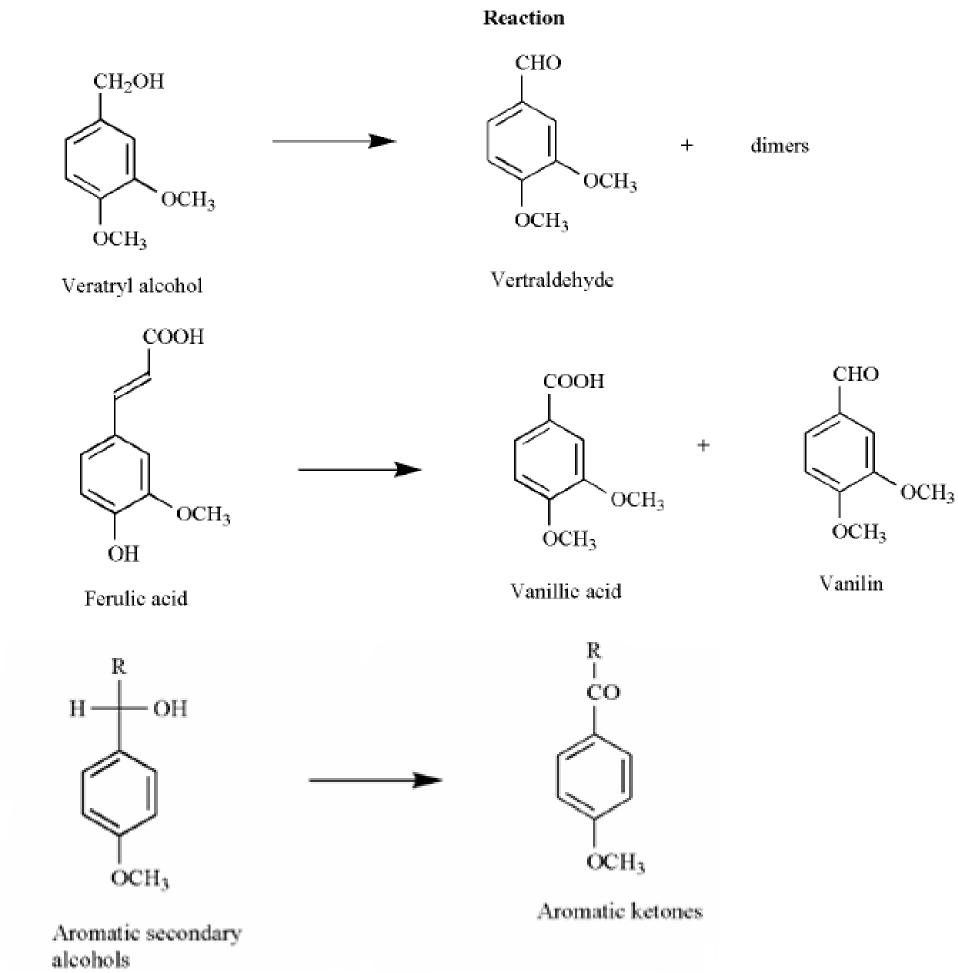


Figure 1.4 Examples of typical laccase-catalysed biotransformation reactions (Burton, 2003).

One of the most important usage of laccases is their use in synthesis of products which have pharmaceutical importance. For example the synthesis of the actinocin from 4-methyl-3hydroxyanthranilic gave the chromophore of actinomycinantibiotics as shown in Figure 1.5 (Osiadacz, *et al.,*1999). Similarly, the conversion of alkaloids yield biologically active products (Burton, 2003).

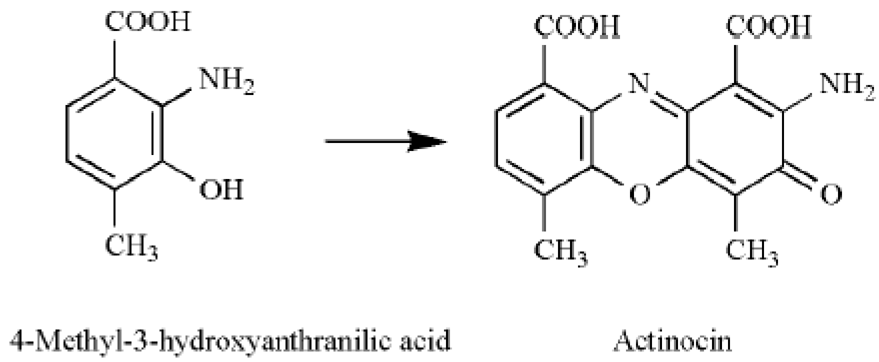


Figure 1.5 Sythesis of actinomycin chromophore using laccase (Osiadacz, *et al.,*1999).

1.1.2 Polyphenol Oxidase (PPO)

Polyphenol oxidase (1,2-benzenediol:oxygen oxidureductase; EC 1.10.3.1) is frequently called tyrosinase, polyphenolase, phenolase, cresolase, or catecholase (Whitaker, 1972).

Polyphenol oxidase (PPO) differs from the laccases, in that it contains only two copper ions at one reaction site in each functional unit of the enzyme. The copper ions are coordinated at the same site acting cooperatively in the catalytic reaction (Burton, 2003).

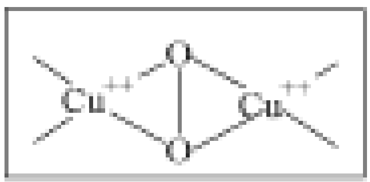
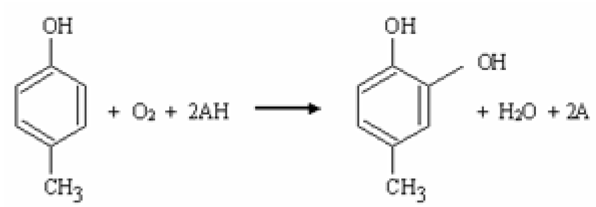
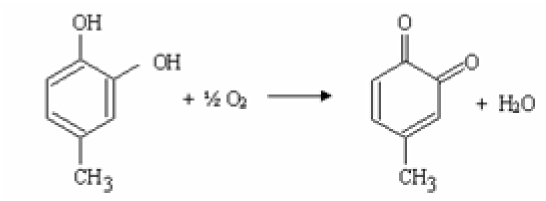


Figure 1.6 The polyphenol oxidase copper site

The copper ions are responsible for binding of both molecular oxygen and the reducing substrate, which is always either phenol or a dihydric phenol (catehcol). Two reactions can be catalysed by polyphenol oxidase, hydroxylation of phenols (sometimes called cresolase activity) and oxidation of catechols to o-quinones (catecholase activity) as in seen in Figure 1.7.



p-Cresol 4-Methylcatechol



Catechol o-Benzoquinone

Figure 1.7 Phenol oxidase catalyzed reactions (Whitaker, 1972).

PPO and laccases can be differentiated by inability of PPO to covert p-diphenols. In Figure 1.8 differences of activities of tyrosinase and laccase is seen. Laccases are also not inhibited by carbon monoxide, while PPOs are.

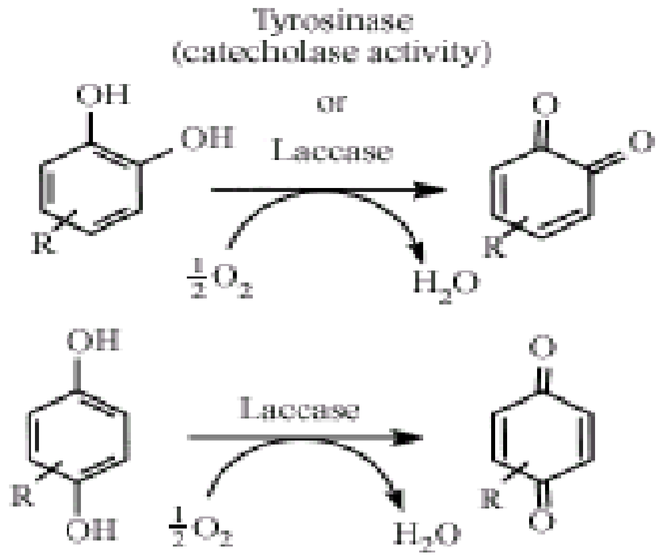


Figure 1.8 Activities of tyrosinase and laccase ((Jolivet *et al.*, 1998)

1.1.2.1 Applications Using PPO as Biocatalyst

The potential value of using PPO to produce catechols has been recognized since ortho-hydroxylation of phenols is not easily achieved by chemical synthesis. PPO has been investigated for the production of L-dihydroxyphenylalanine (L-DOPA) which has been used to treat Parkinson's disease (Burton, 2003). Hydroxylation of phenolic compounds was developed production o-dihydroxy products from bicyclic and tricyclic phenols, like tetralins and chromanamines.

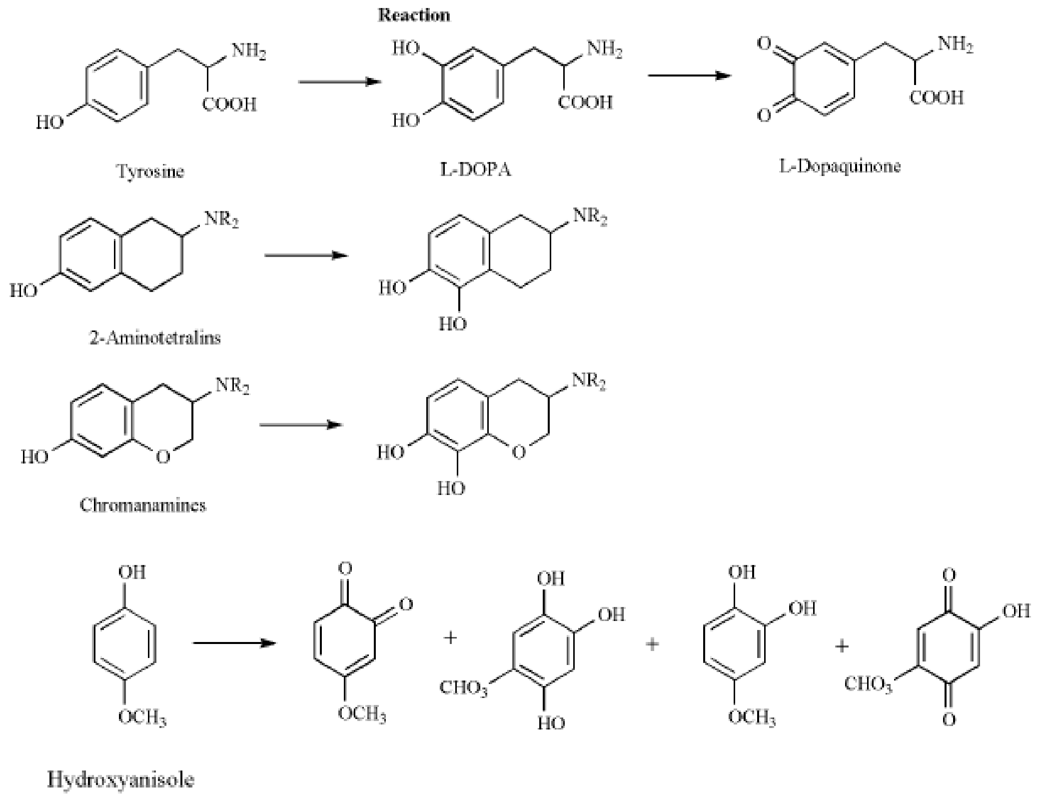


Figure 1.9 Examples of polyphenol-catalysed biotransformation (Burton, 2003).

On the other hand, PPOs are utilized for production of biphenyl products from relatively complex substrate such as flavanols (any of various hydroxy derivatives of flavone), due to addition reactions occurring after oxidation (Burton, 2003). Such reactions are interesting because of their potential use for production of antioxidant and colored products.

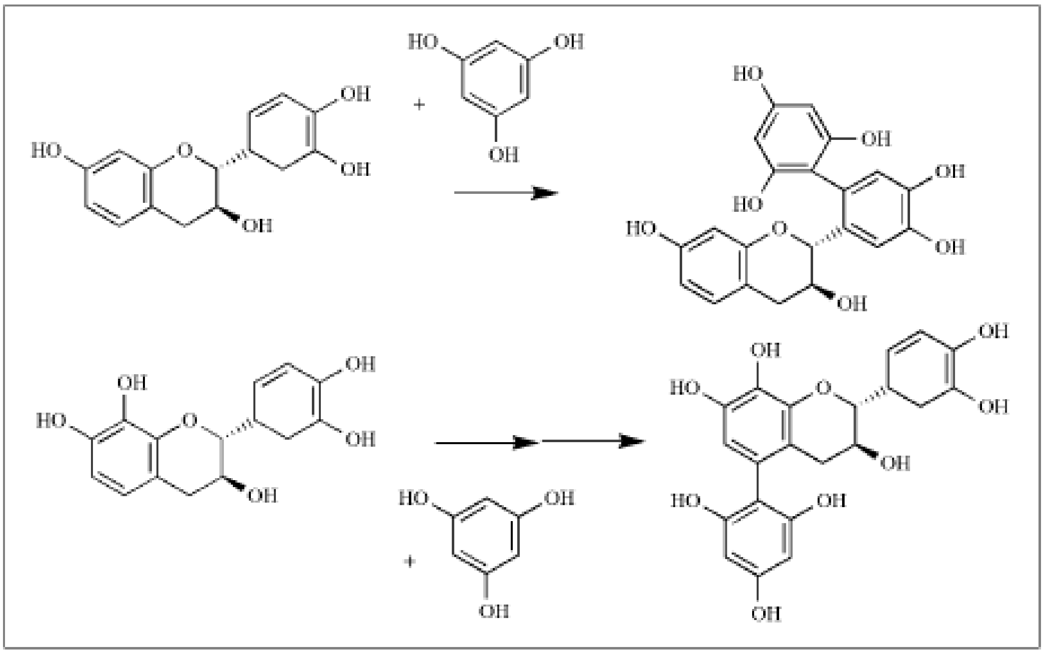


Figure 1.10 Biphenyl products from PPO-catalysed biotransformation of flavonols (Burton, 2003)

1.1.2.2 Tyrosinase

Tyrosinase (E.C.1.14.18.1), monophenol monoxygenese) is widely distributed from bacteria to mammals and present different characteristics in different organs of the some organisms, such as in roots and leaves of higher plants (Durán *et al.*, 2002).

Tyrosinase is responsible of darkening of mushrooms, potatoes, apples, and many other plants and plant products on injury to the tissue. Darkening occurs result of the enzymatic oxidation of certain monohydric and o-dihydric phenols. The two activities of the mushroom enzyme have come to be known as the ‘’cresolase’’ and ‘’catecholase’’ activities (Jolivet *et al.*, 1998). In Figures 1.11 and 1.12 cresolase and catecholase activities are shown respectively.

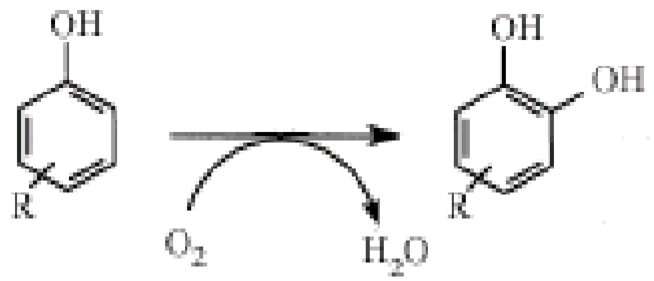


Figure 1.11 Cresolase activity of tyrosinase (Jolivet *et al.*, 1998)

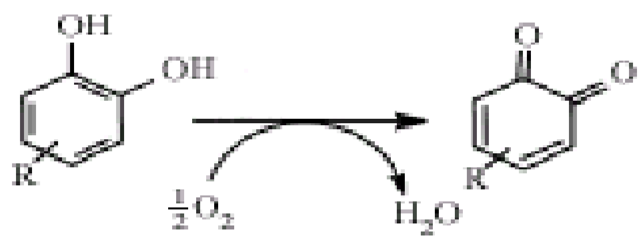


Figure 1.12 Catecholase activity of tyrosinase (Jolivet *et al.*, 1998)

Tyrosinase has potential applications in degradation of phenols and amines, removal of xenobiotic compounds, oxidation of catechol and polymerization of phenolic coumpounds (Durán and Esposito*,* 2000)

1.1.3 Peroxidases

Peroxidases are differentiated from laccases and PPOs because peroxidases require the presence of hydrogen peroxide for activity. Peroxidases are hemoproteins, produced mainly by a number of microorganisms and plant sources (Durán and Esposito*,* 2000). Peroxidases catalyze oxidations utilizing hydrogen peroxide, and generally producing free radicals which can subsequently be coupled or reacted further. In Figure 1.13, examples of reactions which are catalyzed by peroxidase are shown.

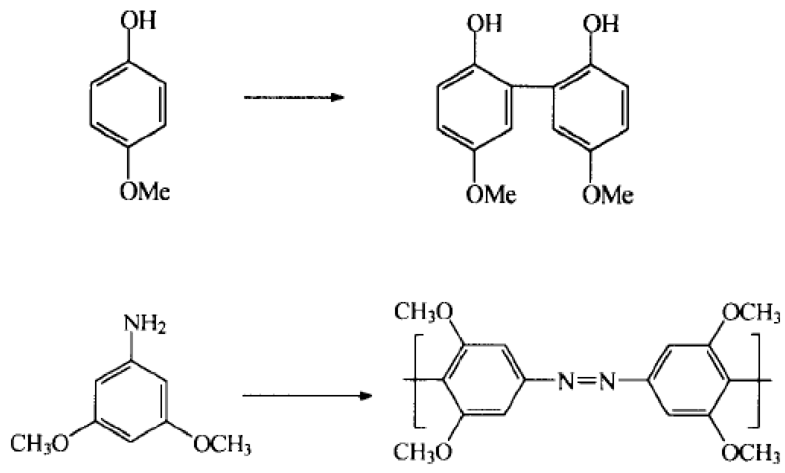


Figure 1.13 Peroxidase-catalyzed reactions (Burton, 2001).

1.1.3.1 Horseradish Peroxidase (HRP)

HRP can catalyze the oxidation of phenols, biphenols, anilines, benzidines and related heteroaromatic compounds. HRP is suitable for wastewater treatment because it retains its activity over a broad pH and temperature range (Durán and Esposito, 2000).

In literature, HRP is used for polymerization of humic acid, degradation of aniline, phenol, dewatering of slimes, degration of chlorophenol, decontamination of black liquor (Durán and Esposito, 2000).

1.1.3.2 Lignin Peroxidase (LiP)

LiP's are quite well known, especially those coming from the basidiomycete as *Phanerochaete chrysosporium* and few in ascomycetes. LiP molecular mass ranges from 38 to 43 kDa. LiP from different sources was shown to mineralize a variety of recalcitrant aromatic compounds and to oxidize a number of polycyclic aromatic and phenolic compounds (Durán and Esposito, 2000).

In lignin peroxidase catalytic cycle the primary reaction product of LiP with H_2O_2 is the two-electron oxidized state compound I, LiPI. LiPI is reduced back to the native enzyme via two single-electron steps with compound II, LiPII, as an intermediate. In the process, the aromatic reducing substrate is oxidized to an aryl cation radical ($Ar.^+$) (Gold and Alic, 1993)

$$LiP + H_2O_2 \longrightarrow LiPI + H_2O$$
$$LiPI + Ar \longrightarrow LiPII + Ar.^+$$
$$LiPII + Ar \longrightarrow LiP + Ar.^+ + H_2O$$

LiP catalyzes the H_2O_2-dependent oxidation of a wide variety of nonphenolic lignin model compounds and aromatic pollutants, including synthetic lignin. These reactions include benzylic alcohol oxidations, ring-opening reactions, demethoxylations, and oxidative dechlorinations. All of these reactions are consistent with a mechanism involving the initial one electron oxidation of aromatic nuclei by an oxidized enzyme intermediate to form a substrate aryl cation radical (Gold and Alic, 1993).

In literature LiP from *P. chrysosporium* utilized for degration of aromatic compounds, degration of phenolic materials, remediation of effluent Kraft briefly LiP used for waste water treatment in industry (Durán and Esposito, 2000).

1.1.3.3 Manganese Peroxidase (MnP)

MnP catalyzes the oxidation of several monoaromatic phenols and aromatic compounds. In the free form, MnP acts on phenols and dyes (Durán and Esposito, 2000).

The oxidation of lignin and other phenols by MnP is dependent on free manganous ion. As shown below, the primary reducing substrate in the MnP catalytic cycle is Mn(II), which efficiently reduces both compound I (MnPI) and compound II (MnPII), generating Mn(III), which subsequently oxidizes the organic substrate by diffuses from the enzyme surface and in turn oxidizes the phenolic substrate (Gold and Alic, 1993) .

$$MnP + H_2O_2 \longrightarrow MnPI + H_2O$$
$$MnPI + Mn(II) \longrightarrow MnPII + Mn(III)$$
$$MnPII + Mn(II) \longrightarrow MnP + Mn(III) + H_2O_2$$

MnP from *P. chrysosporium* used for degradation of phenols and lignins, degradation of pentachlorophenol and dyes degradation (Durán and Esposito, 2000).

1.1.4 Catechol Oxidases

Catechol oxidases have recently been recognized as a group of type three copper enzymes whose active sites differ from PPO and laccase, but where the mechanism for catechol hydroxylation is very similar. These enzymes act as catechol oxidases but did not show phenol hydroxylation activity (Burton, 2003).

1.2 Production of Phenol Oxidases

Phenol oxidases are found in many plant tissues, in some higher animals, including insects and humans and in some fungi (especially those that produce brown filaments). In higher plants, the enzyme protects the plant against insects and microorganisms. In humans phenol oxidase responsible for pigmentation of the skin, hair and eye. In insects, it is involved in sclerotization of exoskeleton. In fungi, they are important for melanin biosynthesis, and lignin decomposition.

In industrial applications, fungi which are readily cultureable, genetically stable and nonpathogenic, are used as enzyme sources.

1.2.1 Thermophilic Fungi

In this study phenol oxidase production was obtained from thermophilic fungi, *Syctalidium thermophilum* and *Thermomyces lanuginosus.*

Thermophilic fungi have a growth temperature minimum at or above 20°C and a growth temperature maximum at or above 50°C. The most important advantage of this process is prevention of production of unwanted other microorganisms caused contamination during cultivation. Enzymes from thermophilic fungi are resistant to high temperatures which provide usage of this enzymes in industry more efficiently at high temperatures. Thermostable enzymes are applied in industrial high-temperature bioprocesses and their physicochemical properties studied for bio-engineering applications (Maheshwari *et al.,* 2000)

1.2.1.1 *Thermomyces lanuginosus*

T. lanuginosus grows optimally between 48-52°C, but can be cultured in temperatures ranging from 30-55°C. This thermophilic fungus will tolerate brief exposure to temperatures as high as 68°C and its spores can survive being boiled for up to 5 minutes. It is frequently found in organic soils, decomposing vegetable matter, composts and animal excrement throughout the world.

T. lanuginosus secretes a variety of degradative thermozymes including: α-amylase, glucoamylase, hemicellulases, lipase, phytase, serine-protease and xylanases. Other interesting enzymes isolated from *T. lanuginosus* include: α-galactosidase, β-galactosidase, trehalase, malate dehydrogenase and invertase.

T. lanuginosus xylanases have significant current and potential uses for several industries including paper and pulp, food, and biofuel. For the biofuel industry, xylanases can be used to aid in the conversion of lignocellulose to fermentable sugars (e.g., xylose). (Damaso, *et al.,*2004).

1.2.1.2 *Syctalidium thermophilum*

S. thermophilum is a thermophilic soft rot fungus belonging to subdivision *Deuteromycetes*. *S. thermophilum* is also known as *Humicola insolens, Torula thermophila* and *Humicola grisea* var. *thermoida. S. thermophilum* grows optimally between 40-58°C. In nature it grows readily in self-heating heaps, manure, mushroom composts, guayule pets (Maheshwari *et al.,* 2000).

S. thermophilum is an important thermophilic fungus in the production of mushroom compost. Thermophilic fungi grow massively during the last phase of the composting process, form spores that have survived the pasteurization process. They are believed to contribute significantly to the quality of the compost. The effect of these fungi on the growth of mushroom mycelium have been described at three distinct levels. First, these fungi decrease the concentration of ammonia in the compost, which otherwise would counteract the growth of the mushroom mycelium. Second, they immobilize nutrients in a form that apparently is available to the growth of mushroom mycelium. And third, they may have a growth promoting effect on the mushroom mycelium, as has been demonstrated for *S. thermophilum* and for other thermophilic fungi (Wiegant, 1992)

1.2.2 *Phanerochaete chrysosporium*

P. chrysosporium is a secondary decomposer of both hardwood and softwood branches/logs, and can be found in temperate forests throughout North America, Europe and Iran. This species is the best-studied representative of the white-rot fungi which are so named for their ability to degrade the lignin component in wood and effectively bleach out the brown pigmentation associated with this biopolymer (Broda, *et al*., 1996). This ability has generated much interest in the paper and pulp industry as an environmentally benign alternative to the chemical bleaching of pulp (Sigoillot, *et al*., 2001). The lack of subtrate specificity of the peroxidase-based ligninolytic system of *P. chrysosporium* can be exploited for the bioremediation of numerous recalcitrant organic waste byproducts (Paszczynski and Crawford, 1995)

The extracellular ligninolytic system of *P. chrysosporium* comprises a myriad of interesting enzymes and biochemical intermediates. These include: lignin peroxidases (LiP), manganese peroxidases (MnP), cellobiohydrolases, endoglucanases, β-glucosidases, glyoxal oxidase, xylanase, xylosidases, α-galactosidase, pyranose 2-oxidase, superoxide dismutases and mannose-6-phosphatases and laccase. Several enzymes, including glucose oxidase and glyoxal oxidase, have been reported H_2O_2 production in lignin-degrading cultures of *P. chrysosporium* (Boominathan, *et al.,*1990). Both lignin peroxidases and manganese peroxidases and H_2O_2 –generating enzymes are produced during secondary metabolism, in response to nitrogen starvation (Boominathan, *et al.,*1990). Laccase production by *P. chrysosporium* is reported as a result of culture conditions. *P. chrysosporium* strain ME446 produced laccase when grown in a defined medium (Tien and Kirk, 1988) with high nitrogen source ammonium tartrate (24mM), glucose, acetate buffer (pH 4.5) and high copper (0.4 mM $CuSO_4$). Copper appered to serve as a inducer (Dittmer *et al.,* 1997). Addition to this, *P. chrysosporium* BKM-F1767 produced extracellular laccase in a defined medium (Tien and Kirk, 1988) containing dimethylsuccinate buffer (pH 4.2), ammonium tartrate (24 mM) as nitrogen source, and cellulose powder (10g/liter) replacing glucose as the carbon source (Srinivasan *et al.,* 1995).

1.3 Alkaloids

Alkaloid can be defined as a complex molecular structure. It has a nitrogen atom as part of heterocyclic system and it has a significant pharmacological properties and whose origin from the plant kingdom (Bruce, 1998).

Plants have the ability to produce tens of thousands of highly complex secondary metabolites to assist their survival in the environment, many of which protect the plant from predators. Man has used these compounds of self-defence as sources of medicinal agents, poisons and potions for thousands of years.

Extracts derived from the opium poppy, *Papaver somniferum* is notable example for utilization of plants for their pharmacological properties (Bruce, 1998).

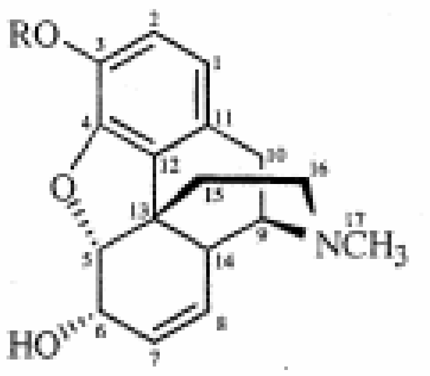
The opium poppy, *P. somniferum,* probably originated in Asia Minor and there are strong indications that the Sumerians cultivated it to extract opium at least three thousands years before Christ (Blakemore and White, 2002).

1.3.1 Morphine

Morphine is structurally classified into alkaloid, based on the presence of nitrogen in its molecule. The morphine alkaloids are found in opium, which is the dried latex material which exudes from cut seed capsules of the opium poppy, *P. somniferum.* Morphine is the major alkaloid component of opium, making up approximately 42% of total alkaloid content. Other opiate constituents are noscapine (21%), papverine (18%), codeine (12%) and thebaine (6.5%) (Boonstra, *et al.,* 2001).

Morphine was first isolated in 1803 by the German pharmacist Friedrich Wilhelm Adam Serturner, who named it "morphium" after Morpheus, the Greek god of dreams (Blakemore and White, 2002). Morphine continues to be useful in medicine both as an analgesics and as an anesthetic.

Morphine is called by 7,8-Didehydro-4,5-epoxy-17-Methyl-morphinan-3,6-diol, its empirical formula is $C_{17}H_{19}NO_3$ and its molecular weight is 285.33. Structure of morphine is illustrated below.

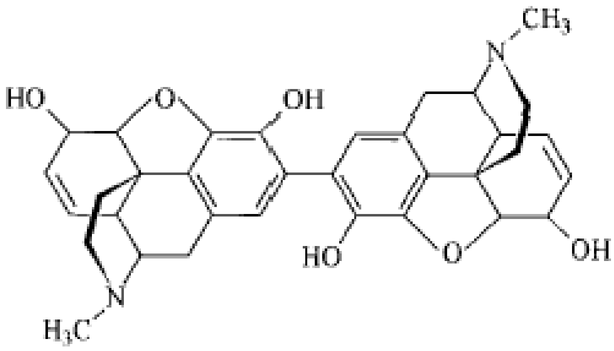


Morphine, R = H

1.3.2 Pseudo-morphine

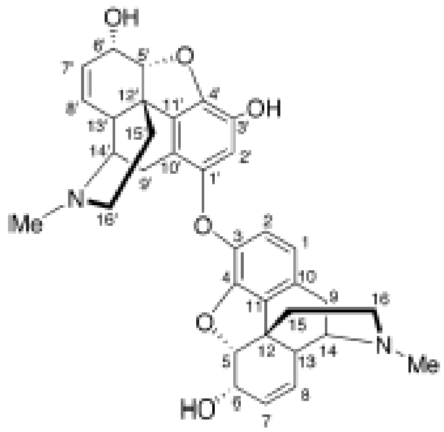
Plants synthesize broad range of secondary metabolites, including alkaloids and terpenoids, that are toxic to herbivores and pathogen and so are believed to acts as defense compounds (Wittstock and Gershenzon, 2002)

In response to mechanical damage, morphine immediately undergoes oxidation by bismorphine-forming peroxidase (BFP) and is metabolized into the dimer of morphine, bismorphine (pseudo-morphine). Biochemical characterization of bismorphine demonstrated that the two amino groups of this alkaloid ionically bind to the carboxyl groups of galacturonic acid residues of cell wall polysaccharides pectins, resulting in cross-linking of pectins to each other. Binding of bismorphine to pectins, significantly contributes to their resistance to hydrolysis by pectinase (Morimoto *et al.*, 2001)



Pseudo- morphine

On the other hand, together with bismorphine, a new product bismorphine B was identifed in wounded capsules of *P. somniferum* in which two morphine units are coupled through a biphenyl ether bond. When the physiological function of bismorphine B in opium poppy was investigated, it was evident that this alkaloid more effectively cross-links cell wall polysaccharide pectins than bismorphine and that such cross-linking reaction leads to resistance against hydrolysis by pectinase (Morimoto *et al.* , 2003). Structure of bismorphine B is shown below.



bis morphine B

1.4 Enzymatic and Microbial Conversion of Morphine Alkaloids

The important pharmacological activity of morphine has spurred chemist to make many derivatives of this natural compound. There are three main therapeutic categories into which the pharmaceutical compounds derived from morphine alkaloids can be grouped: the analgesics (pain relief drugs), examples being morphine, codeine, dihydrocodeine, diacetylmorphine (heroin), hydromorphone, and oxymorphine; the antitussives (cough suppressants) of which codeine, hydrocodone, oxycodone and pholcodeine are examples; and the narcotic antagonists, such as naloxone and naltrexone, which are used in the treatment of opiate overdose and alcohol addiction (Boonstra *et al.,* 2001). In

Figure 1.14 structures of natural morphine alkaloids and their semisynthetic derivatives is shown.

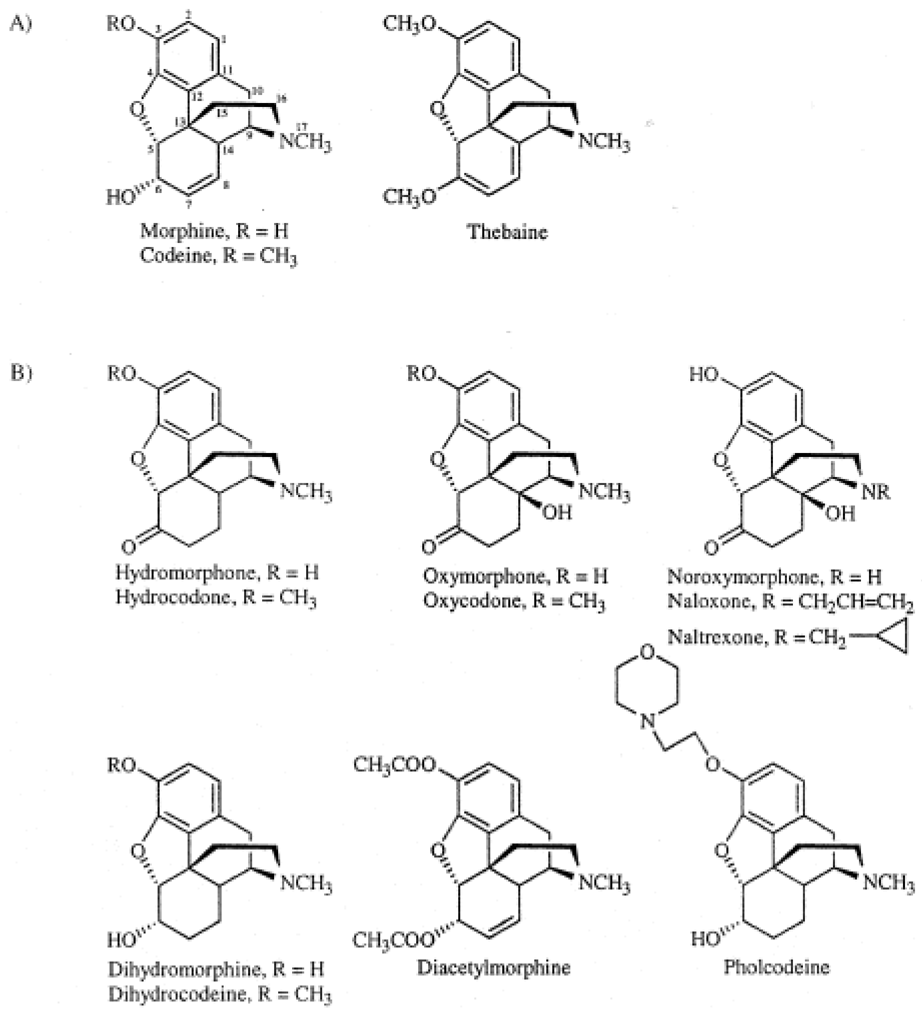


Figure 1.14 : Morphine alkaloids. Naturally occurring morphine alkaloids (A) and examples of their semisynthetic derivatives (Boonstra *et al.,* 2001).

Semisynthetic opiates are commercially prepared by chemical modifications of morphine, codeine and thebiane which are isolated from opium poppy. However, the synthesis of these compounds is often difficult to achieve on a commercial scale due to chemical complexity of starting material, the abundance of functional groups in these compounds, cost and environmental concerns (such as toxic waste streams and energy consumption). Biotransformations can offer a number of advantages over conventional chemical processes. The specificity of the enzyme-catalyzed reactions allows the stereospecific transformation of special functional groups, without the necessity for the protection of other labile groups (Bruce *et al.,* 1995).

1.4.1 Microbial Transformations of Morphine Alkaloids

From 1960s, transformation of morphine alkaloid transformations were realized to produce new compounds with improved pharmacological activities.

1.4.1.1 Microbial Transformations by Fungi

Early working in 1960 and 1962 identified the ability of the basidiomycete *Trametes sanguinea* on transformation of thebaine by allycil oxygenation and demethylation to 14-hydroxy-codeinone and subsequent reduction to 14-hydroxy-codeine (Boonstra *et al.*, 2001).

Cunninghamella bainieri (Gibson et al, 1984) and *Mucus piriformis* were found to *N*-demethylate codeine to norcodeine (Madyastha and Reddy, 1994).

In 1998, oxidation of morphine to 2,2’-bimorphine (pseudo-morphine) was achieved by *Cylindrocarpon didymium.* The oxidation of morphine by whole-cell suspensions and cell extracts of *C. didymium* gave rise the formation of pseudo-morphine. Biotransformation of morphine was observed by HPLC and TLC. The identity of pseudo-morphine confirmed by mass spectrometry and H nuclear magnetic resonance spectroscopy. *C. didymium* also displayed activity with the morphine analogs hydromorphone, 6-acetylmorphine, and dihydromorphine, but not codeine or diamorphine (Stabler and Bruce, 1998).

The degradation of morphine in aqueous solution was extensively studied. It is known the degradation process is mainly dependent on the pH of the solution and the presences of atmospheric oxygen. In morphine, the oxidation of free phenolic group at C-3 leads to formation of pseudomorphine as the main product at pH 7.4 (Stabler and Bruce, 1998). In 2003, the oxidation mechanism of morphine to pseudo-morphine was clarified as explained below (Garrido, *et al.,* 2003).

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Considering the oxidation mechanism for phenol, the occurrence of dimer structure can be explained. It is well-known that the oxidation of phenolic group gives rise to semiquinone that exist in equilibrium with the corresponding quione. It is this quinone that undergoes coupling with protonated or free base morphine resulting in the formation of the dimer pseudomorphine as shown in Figure 1.15 (Garrido, *et al,* 2003).

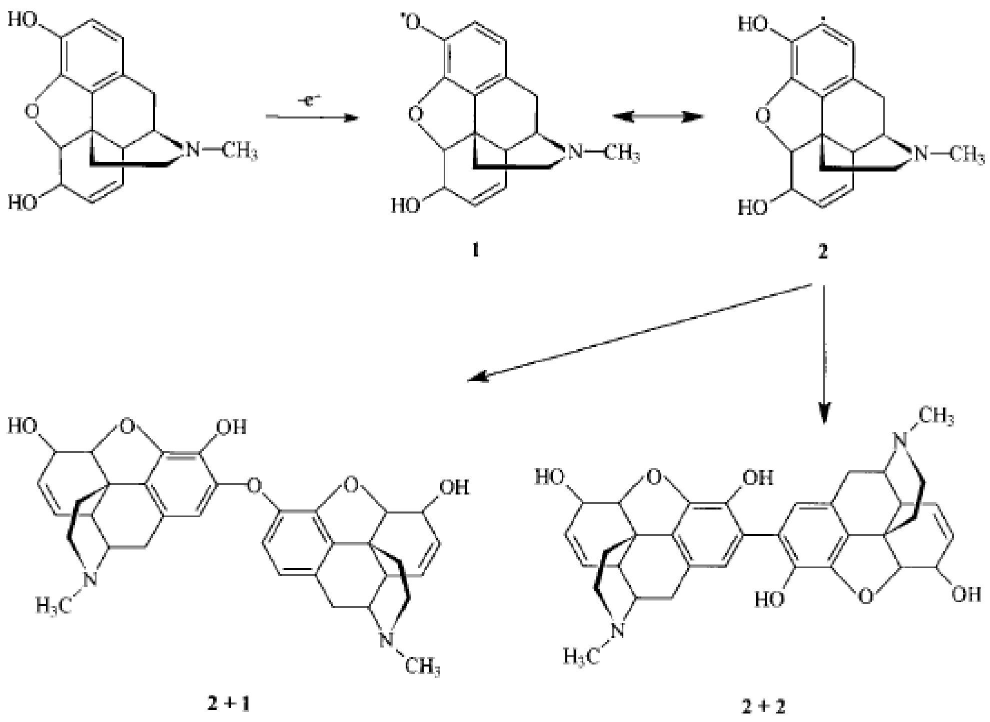


Figure 1.15: Oxidation of morphine to pseudo-morphine (Garrido, *et al.,* 2003)

In 2001, again whole-cell suspension of *Cylindrocarpon didymium* was observed to transform 2,2'-bimorphine to the compounds 10-α-S-monohydroxy-2,2'-bimorphine and 10, 10' -α,α'-S,S'-dihydroxy-2,2'-bimorphine at pH 4 as shown in Figure 1.16. This reaction was hydroxylation reaction. Biotransformation of products were observed by HPLC and TLC systems. Mass spectrometry and H nuclear magnetic resonance spectroscopy confirmed the identities of these new morphine alkaloids (Stabler *et al.,* 2001).

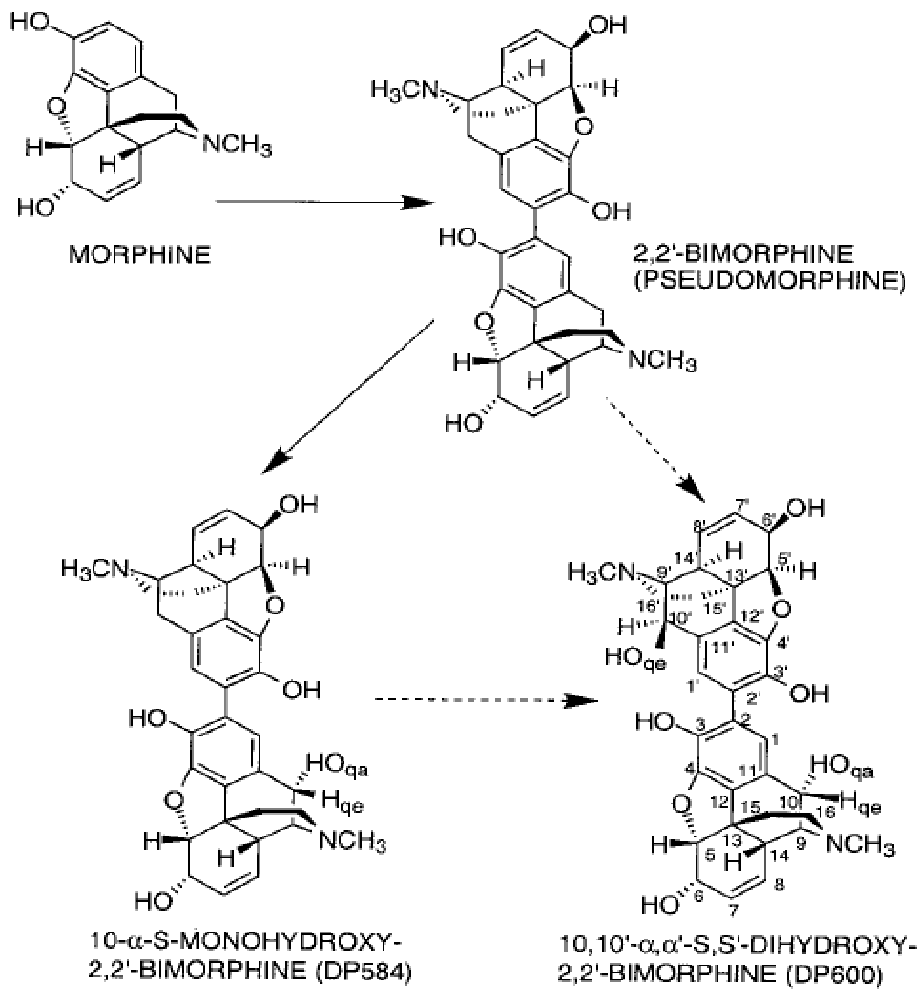


Figure 1.16 : Transformation of morphine by *C. didymum* (Stabler, *et al.* , 2001)

1.4.1.2 Microbial Transformation of Morphine Alkaloids by Bacteria

Similar transformations have also been described for the bacteria. Morphine was transformed into 14-hydroxymorphine by resting cells of an *Arthrobacter* species by unknown enzymatic hydroxylation which can be inhibited by ethylenediaminetetraacetic acid, Zn^{2+}, and Cu^{2+} and stimulated by Fe^{2+}, NADH , NADPH (nicotinamide adenine dinucleotide phosphate). Reaction mixtures were observed by thin-layer-chromatography (TLC) and gas chromatography (Liras and Umbreit, 1975).

In 1981, it has been found that addition of a 14-hydroxy group to the morphine alkaloid structure increased its analgesic potency (Bruce, 1998).

Streptomyces griseus performed both N-demetylation and 14-hydroxylation of codeine to pruduce 14β-hydroxycodeine and N-norcodeine (Kunz *et al*, 1985).

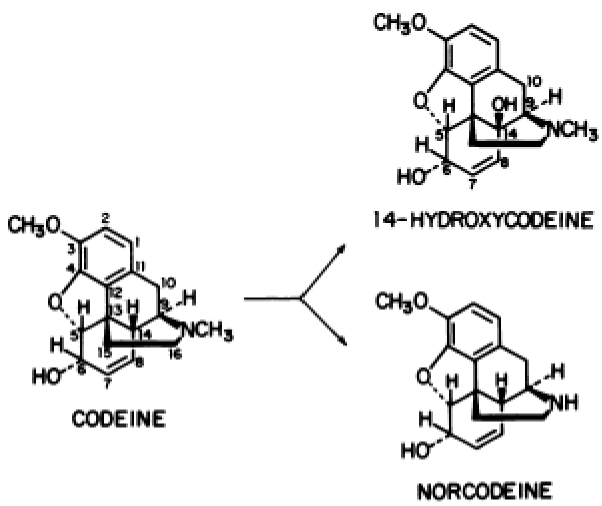


Figure 1.17: Biotransformation products of codeine oxidation by *S.griseus* ATCC 10137 (Kunz *et al*, 1985).

The bacterium *Pseudomonas putida* M10 which was isolated from industrial-waste liquors, utilized morphine as a carbon source. Experiments with whole cells showed that both morphine and codeine, but not thebaine could be utilized. A novel NADP-dependent dehydrogenase, morphine dehydrogenase, was purified from crude cell extracts which catalyses the oxidation of the C-6 hydroxy group of morphine, yielding NADPH and oxidized morphine to morphinone and oxidized codeine to codeinone (Bruce *et al.*, 1990).

In 1991, purification and characterization of morphine dehydroganse from *Pseudomonoas putida* M10 was done. Morphine dehydrogenase was found as highly specific with regard to substrates, oxidizing only the C-6 hydroxy group of morphine and codeine. Its optimum pH was found as 9.5 (Bruce *et al.*, 1991).

In 1993, the morphine alkaloid hydromorphine (dihydromorphinone) was obtained by degradation of morphine by *Pseudomonas putida* M10. Morphine was used as a carbon source for cultures of *P. putida* M10. NADH-dependent morphinone reductase performed this reaction by catalyzing the reduction of the 7,8-unsaturated bond of morphinone and codeinone, yielding hydromorphone and hydrocodone, respectively. These products have important industrial applications: hydromorphone is an important analgesic compound, some five to seven times more potent than morphine while hydrocodone is used generally as an antitussive. HPLC analysis was used for observation of morphine-metabolizing cultures. The structures have been identified by H nuclear magnetic resonance (H-NMR) and mass spectrometry (Hailes and Bruce, 1993). As shown in Figure 1.18.

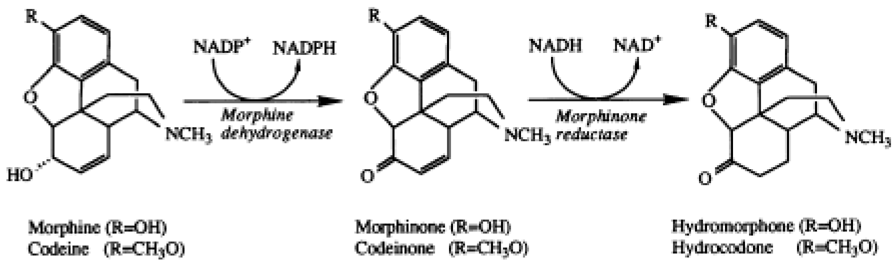


Figure 1.18: Initial products of morphine and codeine metabolism in P. putida M10 (Hailes and Bruce, 1993).

In 1995 washed-cell of *Pseudomonas putida* M10 was incubated with morphine and oxymorphone and this reactions gave large number of transformation products including hydromorphine, 14β-hydroxymorphine, 14β-hydroxymorphinone and dihydromorphine. As shown in Figure 19, similarly, in incubations with oxymorphone as substrate, the major transformation product was identified as oxymorphol. The identities of all these products were done by mass spectrometry and H-NMR (Long *et al.*, 1995).

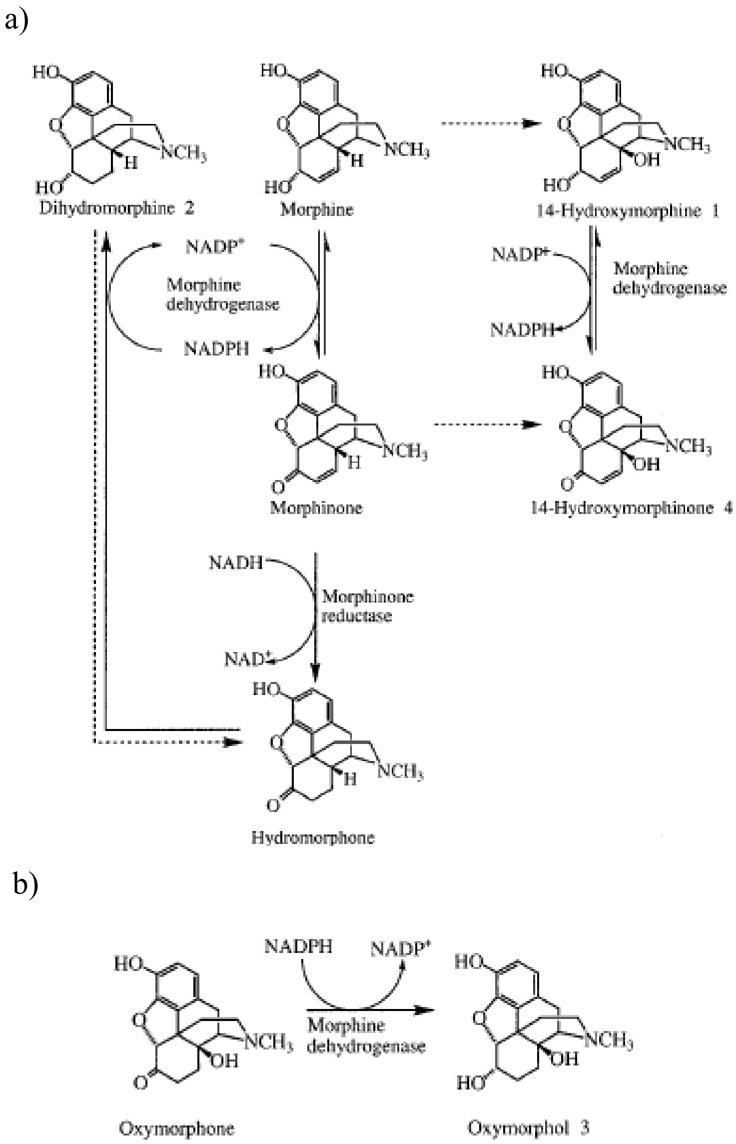


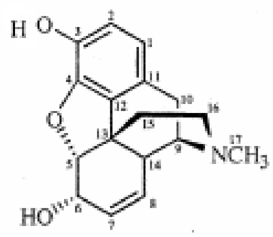
Figure 1.19: Products of transformation of morphine (a) and oxymorphone (b) by *P. putida* M10 (Long *et al.*, 1995).

In 1999, biotransformation of codeine to morphine was performed by *Spirulina plantesis.* Biotransformation was started by adding fitler-sterilized codeine in absolute ethanol at 1.5 mM level to 20 ml of 15-day-old *S. plantesis.* In another experiment, codeine at the same concentration was fed to 20 ml of immobilized *Spirulina* cultures on day zero. Maximum conversion to morphine was observed in 105 h and in 96h with freely suspended and immobilized *Spirulina* cells, respectively (Rao *et al*., 1999).

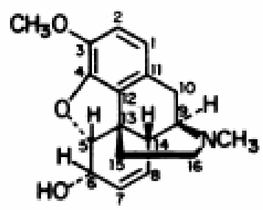
1.4.2 Enzymatic Transformations of Morphine Alkaloids

The interaction of morphine with an enzymatic oxidation reduction system was investigated. After incubating [^{14}C]morphine with horseradish peroxidase (HRP) and H_2O_2 two radioactive compounds were detected by TLC. Spectral analysis (u.v., i.r., fluorescent spectroscopy) were used to identify compounds which were morphine and pseudo-morphine. On the other hand, by using compounds structurally related to morphine (like normorphine, 6-acetylmorphine, nalorphine, dihydromorphinone, naloxone, naltrexone, oxymorphone) it was detected that;

(1) A free phenolic hydroxyl group in position 3 was necessary for the enzymatic oxidation of morphine to pseudomorphine (Roerig, *et al.,* 1976). For example codeine is lack of free phenolic hydroxyl group in position 3 as shown below:

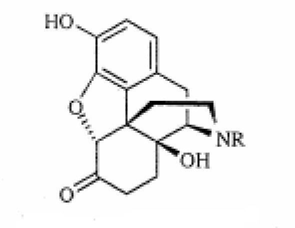


Morphine

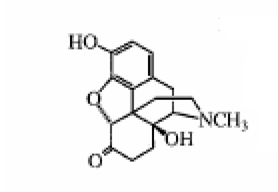


CODEINE

(2) a carbonyl group in position 6 of the morphinan ring prevents the formation of the dimer (Roerig, *et al.*, 1976). For example naloxone, naltrexone, oxymorphone as shown below:



Naloxone, R = $CH_2CH{=}CH_2$
Naltrexone, R = CH_2—◁



Oxymorphone

(3) substitution of other functional groups on the morphine do not effect the peroxidase catalyzed dimerization (Roerig, *et al.*, 1976).

Enzymatic transformation of morphine to pseudo morphine was realized by HRP with using p-coumaric acid as activator. Pseudo-morphine was detected in few seconds by TLC (Vágúıfalvı and Stifter, 1981). In this study HRP could act on morphine to form free radical. Because in general, peroxidase catalyzes a transfer of one reducing equivalent from the substrate to H_2O_2, forming a free radical, can then dimerize, disproportionate or undergo further oxidation. Phenols such as homovanillic acid, or p-hydroxyphenyl acatic acid dimerize to a product forming a covelent bond between the aromatic rings at a position *ortho* to the phenolic hydroxyl group (Roerig *et al.*, 1976).

On the other hand, morphine dehydrogenase and laccase were used as enzyme sensors for morphine and codeine. The first enzyme sensor for morphine and codeine was based on immobilizing morphine dehydrogenase (MDH) and salicylate hydroxylase (SHL) on top of a Clark oxygen electrode. The second enzyme sensor for morphine was based on laccase (LACC) and PQQ-dependent glucose dehydrogenase (GDH) immobilized at a Clark oxygen electrode. Principles of enzyme sensors were based on oxidations of morphine and codeine. First sensor can measure morphine and codeine directly using a coupled sequence of MDH and SHL. Morphine or codeine were oxidized by MDH with reduction of cofactor $NADP^+$. The cofactor reoxidized by SHL. SHL consume oxygen and changes can be seen on Clark electrode. The second sensor measures morphine.

Laccase oxidizes morphine and consumes oxygen which was indicated by a clark electrode. PQQ-dependent glucose dehydrogenase regenerates morphine. This substrate cycling sensor does not respond to codeine (Bauer *et al*., 1999)

1.5 The Aim of the Study

The objective of this study is to perform morphine biotransformations by using phenol oxidases.

Syctalidium thermophilum, Thermomyces lanuginosus and white rot fungus *Phanerochaete chrysosporium* cells and culture fluid were used as microbial intracellular and extracellular phenol oxidases. Besides the phenol oxidases produced in laboratory, commercial pure phenol oxidases, *A. bisporus* tyrosinase and laccase, *T. versicolor* laccase and horseradish peroxidase, were also used in the morphine biotransformation reactions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Potato dextrose agar (Merck) , ABTS [2,2'-azinobis(3-ethylbenzathiazoline -6-sulfonic acid), *Agaricus bisporus* tyrosinase and laccase, *Tramates versicolor* laccase, horseradish peroxidase and all other chemicals were analytical grade and purchased from Sigma-Aldrich Ltd.

2.1.2 Microorganisms

Phanerochaete chrysosporium was provided by Prof. Dr. Nazif Kolonkaya from Haccettepe University. Thermophilic fungi *Scytalidium thermophilum* (type culture *Humicola insolens*) (ORBA Inc.) and *Thermomyces lanuginosus* IMI-84400 (Peter Biely, Slovak Acedemy of Sciences) were provided by Prof. Dr. Zümrüt Ögel.

2.2 Methods

2.2.1 Analytical Methods

2.2.1.1 Enzyme Activity Assays

The extracellular phenol oxidase activities in culture supernatants were checked daily. Samples were withdrawn every 24 hours and supernatant was separated from fungal biomass by filtration through a filter paper and phenol oxidase activity was determined.

2.2.1.1.1 Phenol Oxidase Activity Assay

Extracellular phenol oxidase activities of *S. thermophillum* and *T. lanuginosus* were measured by using catechol as the substrate. Cathecol was prepared in 0.1 M phosphate buffer at pH 7. The enzyme and substrate solutions were preincubated for 5 minutes at 60 ^{0}C or 50 ^{0}C for *S. thermophilium* and *T. lanuginosus*, respectivily. The reaction mixtures contained 0.5 ml of 0.1 M cathecol solution, 1ml 0.1 M phosphate buffer and 0.5 ml enzyme solution. Blank solution contained 0.5 ml 0.1 M phosphate buffer instead of culture fluid. The reaction was monitored by measuring the change in 420 nm for 5 minute by 30 second intervals. The initial straight line of the progress curve was used to express enzyme activity. On the other hand, 4-methyl catechol was used as the substrate for *A. bisporus* extract. Crude extract was diluted 5 times by using sodium phosphate buffer, pH 7. Reaction mixture contained 100 mM 4-methyl cathecol in 0.2 M phosphate buffer at pH 7 as substrate (1ml) and 5 times-diluted extract (1 ml). Absorbance data were collected with a time interval of 5 seconds for 30 seconds at room temperature at 410 nm in a spectrophotometer. Initial reaction rates were calculated from initial linear part (Sutay, 2003).

The enzyme activity was expressed in units defined as 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 3450 M-1 cm-1 (Kaptan, 2004).

2.2.1.1.2 Laccase Activity Assays

2.2.1.1.2.1 Qualitative Plate Assay

Agar solution (0.5 %) in 15 ml 50 mM glycine-HCl buffer (pH 3) containing 14 μmol af ABTS was boiled and placed in a petri dish. Three evenly spaced 5-mm-diameter wells were prepared in the gel. Then 20 μl of 500X-concentrated extracellular fluid was pipetted into the first well. As a negative and positive controls 20 μl of the same extracellular fluid boiled for five minutes and *T. versicolor* laccase solution (2 mg/ml of glycine-HCl buffer) were pipetted into the second and third wells respectively. was placed in the third well. The development of bluish green color around the wells was considered as a result of laccase activity.

2.2.1.1.2.2 Quantitative Activity Assay

The extracellular laccase activity of *P. chrysosporium* culture supernatant was observed from 4^{th} day to 10^{th} day of cultivation. Samples were withdrawn every 24 hours and supernatant was separated from fungal biomass by filtration through a filter paper.

All the assays were done at room temperature and pH 3, the optimum pH of *P. chrysosporium* laccase by using 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as the substrate. The laccase reaction mixture (in total volume 1.05 ml) contained 50 μl of extracellular culture fluid, 0.5 ml of 14 μmol of ABTS in 50 mM glycine-HCl (pH 3) and 0.5 ml of glycine-HCl (pH 3) buffer. Blank solution contained 0.5 μl of glycine-HCl (pH 3) buffer instead of culture fluid. The reaction was monitored spectrophotometrically by measuring the change in 436 nm for 2 minute by 10 second intervals. The enzyme activity was expressed in units defined as 1 U = 1 nanomole of substrate oxidized in one second by 1 ml enzyme solution. The extinction coefficient (ε) of 29,300 $M^{-1}cm^{-1}$ was used for oxidized ABTS (Srinivasan *et*. al., 1995).

2.2.1.2 Protein Analysis

Protein concentration was determined by using Bradford Method (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard protein. Composition of reagents, procedure and standard curve are given in Appendices B and C, sequentially.

2.2.1.3 Thin Layer Chromatographic Analysis

TLC was carried out on silica gel F_{254} gel plates (Merck). Plates were cut at the size of 5 cm width and 5 cm height. Samples (5 µl) were loaded on the plates and developed in dichloromethane:methanol:ammonia (840:140:20 [vol/vol/vol]). The solvent system information was kindly given by Afyon Alkaloid Plant. After running, morphine and product spots were detected under UV light and their retention factors were determined. The retention factor, or R_f, is defined as the distance traveled by the compound divided by the distance traveled by the solvent.

$$R_{\mathrm{f}} = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent front}}$$

When the product formation and/or morphine consumption were detected, samples were analyzed by HPLC.

2.2.1.4 High Performance Liquid Chromatography

Samples were dried by using vacuum evaporator before analyzed by Varian ProStar HPLC system. Samples then solved in 200 µl mobile phase and diluted 20X for HPLC analyzes.

Morphine and product analysis were carried out using Microsorb-MV 100 C18 column (250 mm x 4.6 i.d, 5 µm pore size) (Catalog no: R0086200C5, Varian). The solvent system consists of 0.05 M (pH 3.6) sodium acetate solution:acetonitrile :ethanol:tetrahydrofuran (THF) (1700:200:60:40 [vol/vol/vol/vol]) and final pH again adjusted to 3.6 with glacial acetic acid at a flow rate of 0.5 ml/min. ProStar 330 PDA detector was used at 280 nm. The HPLC solvent system was also prepared by using suggestions given by Afyon Alkaloid Plant.

2.2.2 Enzyme Preparation

In this study, four different types of fungi were utilized to obtain phenol oxidases to be use in morphine biotransformation.

2.2.2.1 Enzyme Production by *Phanerochaete chrysosporium*

P. chrysosporium was grown on potato dextrose agar petri dishes and incubated at 35 ^{0}C for 4-5 days until sporulation and stored at 4 ^{0}C for maximum 2 weeks to use as stock culture. Spors from stock culture were inoculated to liquid medium. Different types of liquid culture media were screened for production of phenol oxidase as described in Appendix A. Liquid media contained, 10 g/l cotton waste or glucose as carbon source and 24 mM ammonium tartrate or 0.12 g/l NH_4Cl as nitrogen source. Cultures were grown in 2 lt Erlenmeyer flasks, containing 80 ml of medium at 30 ^{0}C without shaking or were grown in 500 lt Erlenmeyer flasks, containing 100 ml of medium at 35 ^{0}C with shaking at 155 rpm.

2.2.2.2 Enzyme Production by *Scytalidium thermophilum*

S. thermophilium was grown on standart YpSs agar plate (App. A) at 47 ^{0}C until sporulation and stored at room temperature for maximum 3 weeks to use as stock culture. Stock cultures were used for the inoculation of pre-culture. Spores collected from stck cultures were used o inoculate preculture medium, which consisted of YpSs medium containing 1% glucose as the carbon source instead of starch. Preculture volume was 2% of the main culture. After 24 hours incubation, precultures were used to inculate 250 ml main cultures in 500 ml flasks. The cultures were incubated in a shaker incubator at 155 rpm and 47 ^{0}C (Mete, 2003).

2.2.2.3 Enzyme Production by *Thermomyces lanuginosus*

T. lanuginosus was grown on standart YpSs agar plate at 47 ^{0}C until sporulation and stored at room temperature for maximum 3 weeks to use as stock culture. Spores from stock cultures were used for the inoculation of pre-culture (App A). Precultures were grown in 50 ml Erlenmeyer flasks, containing 10 ml of preculture medium. The cultures were incubated in a shaker incubator at 47 ^{0}C and 155 rpm. After 24 hours incubation, precultures were used to inoculate the main cultures. Cultures were grown in 250 ml Erlenmeyer flasks, containing 100 ml of main culture medium. Main culture medium incubated as preculture media (Astarcı, 2003).

2.2.2.4 Preparation of Phenol Oxidase Extract from *Agaricus bisporus*

Fruit bodies of *Agaricus bisporus* were obtained from a local supermarket. After removing the stalks, the caps were washed. 15 g of caps were homogenized in 200 ml sodium phosphate buffer at pH 7 with 2.5 g polyvinyl polypyrolodone (PVPP) to precipitate phenols which can react with oxygen in the presence of phenol oxidase at 4°C in a blender (ARCELIK Rollo K-1350) for 4 x 15 sec. Extracts were centrifuged at 10,000xg for 10 minutes (SIGMA) and filtered (Sutay, 2003). The supernatants were used as the crude extracts.

2.2.2.5 Enzyme Enrichment

2.2.2.5.1 Enrichment of *P. chrysosporium* Culture Fluid

Two different systems a membrane concentrator (Vivapore) and/or ultrafiltration (Amicon system) were used to concentrated phenol oxidases in extracellular culture fluid of *P. chrysosporium.*

Phenol oxidases in extracellular culture of *P. chrysosporium* were concentrated 50 X by using Vivapore membrane concentrator with molecular weight cut-off of 7.5 kDa. By using the membrane concentrator, 5 ml of culture fluid (crude enzyme) was concentrated to 100 µl. Then, 50 X-concentrated culture fluid was used in laccase activity assay and morphine biotransformation.

Ultrafiltration was performed in two steps, in 50 ml and 10 ml (Amicon) strirred cells, at room temperature. In the first step, volume of culture fluid was decreased from 50 ml to 10 ml by using 10 kDa cut-off polyethersulfone (diameter with 44.5 mm) membrane. Then 5x concentrated culture fluid volume was decreased from 10 ml to 1ml by using again polyethersulfone membrane (diameter with 25 mm) in 10 ml strirred cell. Pressure applied was 1.5 bar in each step. Then, ultrafiltrated crude extract volume reduced from 1 ml to 100 µl by using a membrane concentrator as defined above. Finally 500X-concentrated culture fluid was obtained to detect presence of laccase in extracellular culture fluid on plate assay and to use in morphine biotransformation reactions.

After each step, specific activities, yields and purification folds were calculated with using following equations:

$$\text{Specific Activity} = \frac{\text{Total activity of concentrated enzyme (U)}}{\text{Total protein (mg)}}$$

$$\text{Yield (\%)} = \frac{\text{Total activity of concentarted enzyme (U)}}{\text{Total activity of crude enzyme (U)}} \text{ x } 100$$

$$\text{Purification Fold} = \frac{\text{Specific activity of concentrated enzyme (U/mg)}}{\text{Specific activity of crude enzyme (U/mg)}}$$

2.2.2.5.2 Enrichment of *A. bisporus* Extracts

Phenol oxidases found in *A. bisporus* extracts was concentrated by using either ammonium sulphate (AS) precipitation or ultrafiltration.

A. bisporus crude extract (150 ml) was precipitated by using various ammonium sulfate (AS) concentration using solid AS salt at 4 ^{0}C. AS concentrations were tested from 65 to 90 %. AS and enzyme extract mixture was stirred for at least 1 hour and then the extract was centrifuged at 18 000 X g for 20 min at 25 ^{0}C and the pellet obtained was dissolved in a minimum amount of phosphate buffer (pH 7) and dialyzed overnight against the same buffer.

Ultrafiltration was done in two steps, in 350 ml and 50 ml (Amicon) stirred cells, at room temperature. In first step, volume of culture fluid was decreased from 350 ml to 50 ml by using 100 kDa cut-off cellulose (diameter with 44.5 mm). Then concentrate extract volume was decreased from 50 ml to 10 ml by using same membrane (diameter with 25 mm) in 10 ml stirred cell. Pressure applied was 1.5 bar in each step. Crude extracts concentrated 35X by reducing the initial volume from 350ml to 50 ml by ultrafiltration.

2.2.3 Biotransformation of Morphine

Morphine biotransformations were performed by using both the phenol oxidases produced in the laboratory and commercial pure phenol oxidases (sigma).

2.2.3.1 Use of Commercial Peroxidases Laccase and Tyrosinase in Morphine Biotransformation Reactions

Enzymatic transformation of morphine was attempted by using commercial horseradish peroxidase (HRP) *A. bisporus* laccase, *A. bisporus* tyrosinase and *Tramates versicolor* laccase. The reaction mixtures contained 4 mg morphine.HCl in 8 ml 0.2 M phosphate buffer (pH 7), 400 µl 3 mM p-coumaric acid, 0.1 mg of enzyme in 400 µl H_2O. Blank solutions contained 400 µl of H_2O instead of enzyme solution. Reaction mixtures were followed by TLC, at 24 h intervals until morphine spot became invisible or a new product was formed. Then, samples were analyzed by HPLC.

2.2.3.2 Utilization of *A. bisporus* Extract in Morphine Biotransformation Reactions

35X-concentrated extract reacted with morphine solutions in different pHs (6-8). Reaction mixtures contained 4 µmol morphine HCl solved in 200 µl of 0.2 M phosphate buffer (pHs 6-8), 300 µl of 35X-concentrated extract, 10 µl of 3 mM p-coumaric acid solution. Blank solutions contained 300 µl of phosphate buffer instead of extract. Reaction and blank mixtures incubated at room temperature. Samples (5 µl) were removed and analyzed by thin-layer chromatography (TLC) at 24 h intervals.

2.2.3.3 Utilization of Thermophilic Fungi Culture Fluid in Morphine Biotransformations

Thermophilic fungi, *S. thermophilum* (type culture *Humicola insolens*) and *T. lanuginosus* IMI-84400 culture fluid were used as enzyme source for morphine biotransformation reactions. Culture fluid of *S. thermophilum* was taken from 5th day of cultivation and culture fluid of *T. lanuginosus* was taken from 4th day of cultivation. Morphine biotransformation reactions took place at different morphine concentrations and at 45-50-60 ^{0}C. Concentrations of morphine.HCl and enzyme solution in biotransformation reactions are shown in Table 2.1.

Table 2.1 Biotransformation reaction conditions used to test *S. thermophilum* and *T. lanuginosus* phenol oxidases

Morphine concentration in phosphate buffer and p-coumaric acid solution(3mM) (mg/ml)	Enzyme ratio in reaction mixture (ml/ml)
0.5	0.5
0.8	0.2
3.3	0.6
5.5	0.9
25	9

2.2.3.4 Utilization of *P. chrysosporium* Culture Fluid in Morphine Biotransformation Reaction

500X-concentrated extracellular culture fluid which was taken from 8th day of cultivation and used as enzyme source in morphine biotransformation reactions. Reaction mixtures were prepared with and without hydrogen peroxidase (H_2O_2). Reaction mixtures contained 4 μmol morphine HCl dissolved in 200 μl of 50 mM glycine-HCl buffer (pH 3), 300 μl of 500X-concentrated extracellular fluid, 10 μl of 3 mM p-coumaric acid solution and (10 μl of 0.32 mM H_2O_2 solution). Blank solutions contained 300 μl of glycine-HCl (pH 3) buffer instead of culture fluid. Reaction and blank mixtures incubated at room temperature. Samples (5 μl) were removed and analyzed by thin-layer chromatography (TLC) by 24 h intervals until morphine spot became invisible or a new product spot was formed. Then samples were analyzed by high-performance liquid chromatography (HPLC).

2.2.3.5 Utilization of Thermophilic Fungi Culture Cells in Morphine Biotransformations

After sample supernatant was separated from fungal biomass by filtration through a filter paper, spoonful cells put into 4ml phosphate buffer containing morphine. Reaction mixtures were incubated in a shaker at 155 rpm and at 47 ^{0}C or at room temperature without shaking. Samples which were taken from reaction mixtures was followed by TLC.

On the other hand, considering the possibility of biotransformation of morphine during cultivation of the microorganisms, morphine, at 1 mg/ ml which is the minimum concentration that could be observed on TLC plate, was added to the growth medium and daily analysis was performed by TLC.

CHAPTER 3

RESULTS AND DISCUSSION

Making modifications on the chemical structures of morphine alkaloids, produces semisynthetic morphine alkaloids. These semisynthetic alkaloids have important pharmacological activity. Chemical preparation of these compounds is often difficult due to their structural complexity, cost and environmental issues. On the other hand, biotransformation reactions have advantages over chemical processes because of specificity of enzyme-catalyzed reactions. The microbial transformation of the morphine alkaloids is used significantly to produce more effective analgesic compounds.

Enzymatic oxidations in alkaloid biosynthesis were previously assigned to enzymes such as copper-containing phenol oxidases (Chou and Kutchan, 1998). In opium poppy (*Papaver somniferum),* biosynthesis of morphine begins with formation of dopamine by hydroxylation of $_{L}$- tyrosine by tyrosinase which is a type of phenol oxidase (Lorenz *et al.,* 1988). In addition, opium poppy synthesize pseudo-morphine from morphine in response to mechanical damage. In opium poppy, biosynthesis of pseudo-morphine forms by oxidation of morphine by peroxidase, which is another type of phenol oxidase (Morimoto *et al.*, 2001).

Phenol oxidases are present in various types of organisms. In higher plants, the enzyme protects the plant against insects and microorganisms. In humans, phenol oxidase is responsible for pigmentation of the skin, hair and eye. In insects, phenol oxidase is important in formation of the exoskeleton. Phenol oxidases are also present in some fungi especially which produce brown filaments (Whitaker, 1972).

In this study, morphine biotransformation reactions were performed by using both the phenol oxidases produced in the laboratory and the ones bought as commercial pure phenol oxidases.

Phenol oxidases which were produced in the laboratory were from *Syctalidium thermophilum*, *Thermomyces lanuginosus* and *Phanerochaete chrysosporium*. On the other hand, the commercial phenol oxidases were *Tramates versicolor* laccase, *Agaricus bisporus* laccase and tyrosinase and horseradish peroxidase (HRP).

3.1 Utilization of Commercial Phenol Oxidases in Morphine Biotransformation

In this study, commercial laccase from *T. versicolor,* commercial tyrosinase and laccase from *A. bisporus* and horseradish peroxidase (HRP) have been used in morphine biotransformation reactions. Except for HRP, the biotransformation of morphine with any of the abovementioned enzymes has not been reported in literature.

Samples taken from the biotransformation mixtures containing morphine and phenol oxidases were analyzed by TLC and HPLC.

3.1.1 Utilization of HRP in Morphine Biotransformation

In literature, pseudo-morphine formation from morphine was accomplished by using commercial HRP in the presence of p-coumaric acid as an activator at room temperature and the pseudo-morphine formation was detected by TLC in a few seconds (Vágúıfalvı and Petz -Stifter, 1982).

In this study, in order to achieve a positive control, this experiment was repeated and pseudo-morphine formation was detected by TLC in a few seconds as stated in the literature.

Thin layer chromatography was used to follow the biotransformation reactions of morphine with phenol oxidases. Samples were taken from reaction mixtures and morphine solution at specified time intervals depending on the rate of reactions. Morphine and reaction products were analyzed by TLC on gel F_{254} plates (Merck) and developed in dichloromethane:methanol:ammonia (840:140:20 [vol/vol/vol]).

In Figure 3.1, the positions of the spots are seen as viewed under 254 nm UV light after the run. As seen in the Figure, the morphine spot became undetectable under UV light after a few seconds and the new product appeared. New product was defined as pseudo-morphine because it had an R_f value of 0.15 on TLC, which coincided with that of pseudo-morphine standard shown in Figure 3.2.

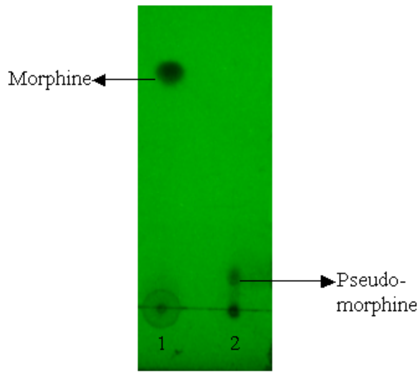


Figure 3.1 TLC results of the sample taken from the HRP-morphine mixture. (1) morphine solution without enzyme (2) reaction mixture

3.1.2 Utilization of *T. versicolor* Laccase in Morphine Biotransformation

Commercial *T. versicolor* laccase was used in morphinebiotransformation. Reaction was performed at room temperature in phosphate buffer (pH 7).

In Figure 3.2, TLC results of pure morphine, reaction product and pseudo-morphine standard, which was kindly given from Afyon alkaloid plant, are shown. Pseudo-morphine and morphine R_f values were found to be 0.15 and 0.52, respectively. No product was detected by TLC 1 hour later the reaction started. The reaction mixture containing 0.14 mM p-coumaric acid as activator. Pseudo-morphine formation was observed in the samples withdrawn 6 hours later but, morphine did not convert to pseudo-morphine completely even after 72 hours, since morphine spot was still visible. So, *T. versicolor* laccase can catalyze biotransformation of morphine to pseudo-morphine however not as efficiently as HRP.

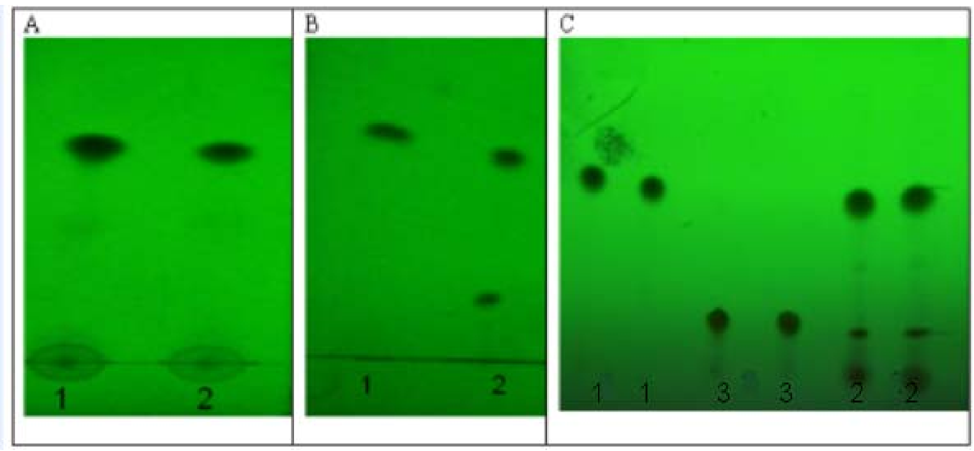


Figure 3.2 TLC results of samples taken from morphine biotransformation reaction using *T. versicolor* laccase. (1) Morphine solution without enzyme (2) Reaction mixture (3) Pseudo-morphine standard. (A) 1st hour of reaction (B) 6th hour of reaction (C) 72nd hour of reaction.

Morphine biotransformation analysis of the 7th day sample was also carried out using HPLC with C18 column which separates compounds according to their polarities.

Figure 3.3 illustrates the retention times of morphine and product pseudomorphine eluted from C18 column. Black chromatogram belongs to reaction mixture withdrawn at 7th day. Chromatogram of pure morphine (pink chromatogram) was given for control purposes. As observed from the Figure, morphine is converted to pseudomorphine but not completely even after 7 days. However, we have to keep in mind the possible deactivation of the enzyme during biotransformation reaction at room temperature up to 7 days. The peak eluted at 2.96 min belongs to morphine, which is the substrate of reactions, and the peak eluted at 2.36 min should belong pseudo-morphine which is the oxidation product of the morphine. Pseudo-morphine standard could not be run because of difficulties in purchasing and obtaining the alkaloid substances. Relative peak areas of pseudomorphine and morphine were determined as 72 and 26 %, respectively, showing about 70 % conversion. Computer outputs of the chromatograms are given in Appendix E.

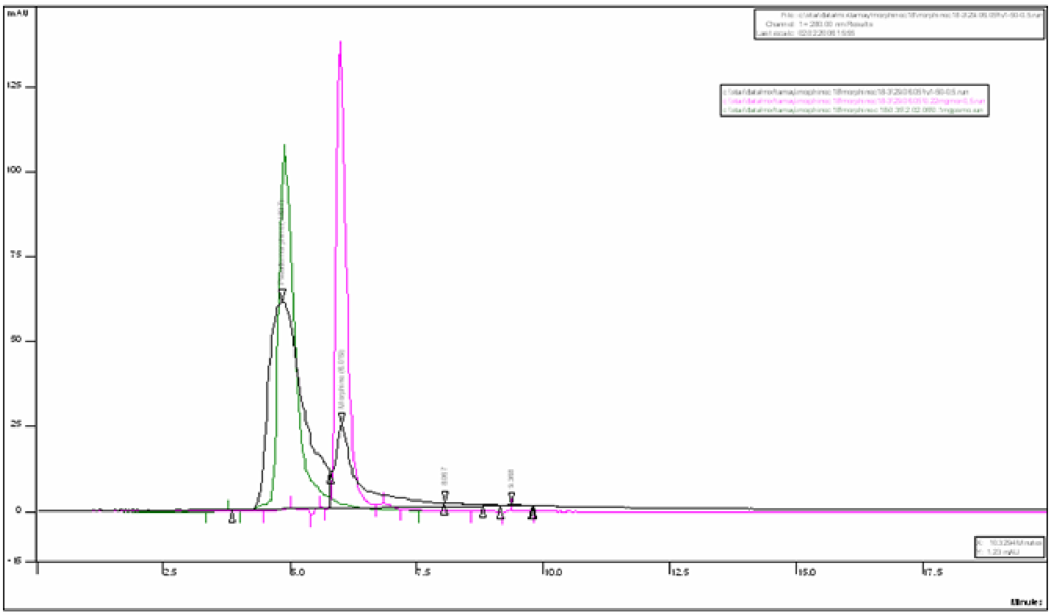


Figure 3.3 HPLC Chromatogram of morphine biotransformation with commercial *T. versicolor* laccase (T = 30 ^{0}C, Flow rate 1 ml/min). Sample was withdrawn at 7th day of the biotransformation reaction. Pink: Morphine Standard, Black: Reaction mixture, Green: Pseudo-morphine standard.

3.1.3 Utilization of *A. bisporus* Laccase in Morphine Biotransformation

Commercial pure *A. bisporus* laccase was also used in morphine biotransformation. The reaction was followed by TLC and then HPLC.

Samples which were taken from the biotransformation reaction and morphine standard solution, followed by TLC as done previously. Again, no product was observed by TLC after 1 hour. The product which was observed after 6 hour was determined as pseudo-morphine since its R_f value was consistent with standard pseudo-morphine. However morphine did not converted to pseudo-morphine, completely even after 72 hours.

In Figure 3.4 HPLC chromatogram of this reaction is shown. Black chromatogram belongs to the reaction mixture withdrawn at 7th day. Pure morphine (pink chromatogram) and pure pseudo-morphine (green chromatogram) were given to column for control purposes. As observed from the Figure, morphine is converted to pseudomorphine but not completely even after 7 days. The peak at 2.97 min belongs to morphine and the peak at 2.40 min should belong to pseudo-morphine. Relative peak areas of pseudomorphine and morphine were determined as 77 and 16 %, respectively. *A. bisporus* laccase seems slightly more efficient than *T. versicolor* laccase in morphine biotransformation.

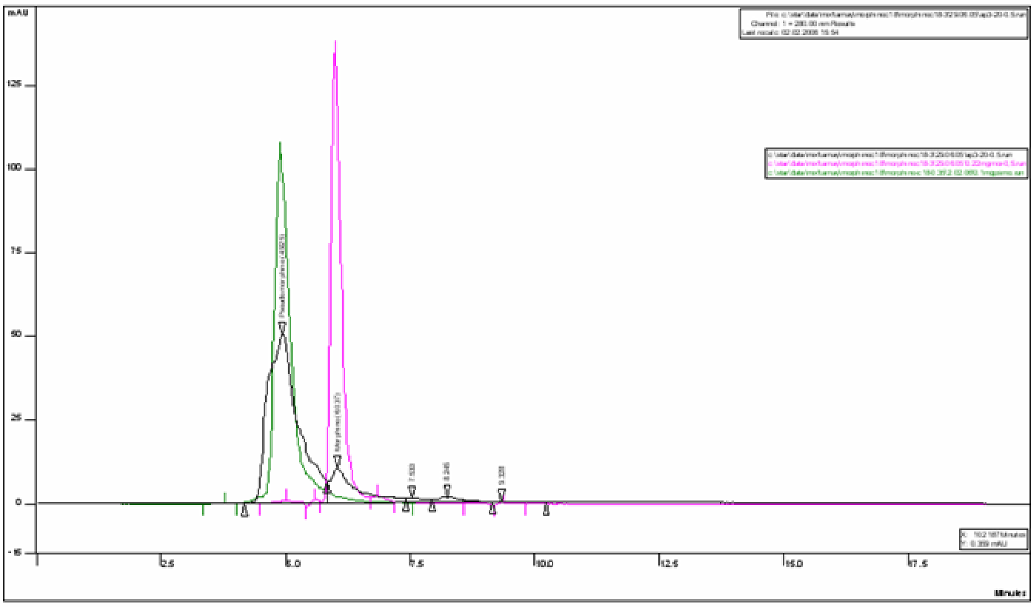


Figure 3.4 HPLC Chromatogram of morphine biotransformation by using *A. bisporus* laccase (T = 30 ^{0}C, Flow rate 1 ml/min). Sample was withdrawn at 7th day of the biotransformation reaction. Pink: Morphine standard, Black: Reaction mixture. Green: Pseudo-morphine standard

3.1.4 Utilization of *A. bisporus* Tyrosinase in Morphine Biotransformation

Commercial pure *A. bisporus* tyrosinase was used in morphine biotransformation. The reaction was followed by TLC and then HPLC as done previously.

In Figure 3.5, TLC results of morphine biotransformation was given at different time intervals.

As observed from the Figure, no morphine biotransformation was observed after one hour. Formation of pseudo-morphine was observed after 4 hours. The reaction had been already completed at 72^{nd} hour since no morphine spot was detected.

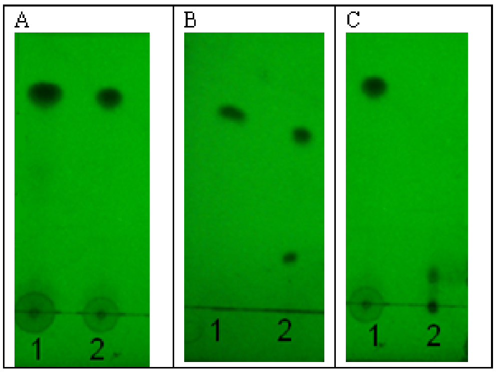


Figure 3.5 TLC results of samples taken from morphine biotransformation reaction using *A. bisporus* tyrosinase. (1) morphine without enzyme (2) Reaction mixture (A) 1^{st} hour of reaction (B) 4^{th} hour of reaction (C) 72^{nd} hour of reaction

Biotransformation reaction was also followed by using HPLC analysis on C18 column. In Figure 3.6, The HPLC chromatogram of a sample withdrawn at 7^{th} day was given. The pink peak belongs to morphine standard given to column.

The blue peak belongs to 7th day sample. As seen from the Figure, only one peak, pseudo-morphine peak, present in the reaction mixture. Morphine could not be detected in HPLC as on TLC, since all the morphine was converted to pseudo-morphine by commercial *A. bisporus* tyrosinase. Accordingly, *A. bisporus* tyrosinase is more efficient in morphine biotransformation to pseudo-morphine than *T. versicolor* and *A. bisporus* laccases.

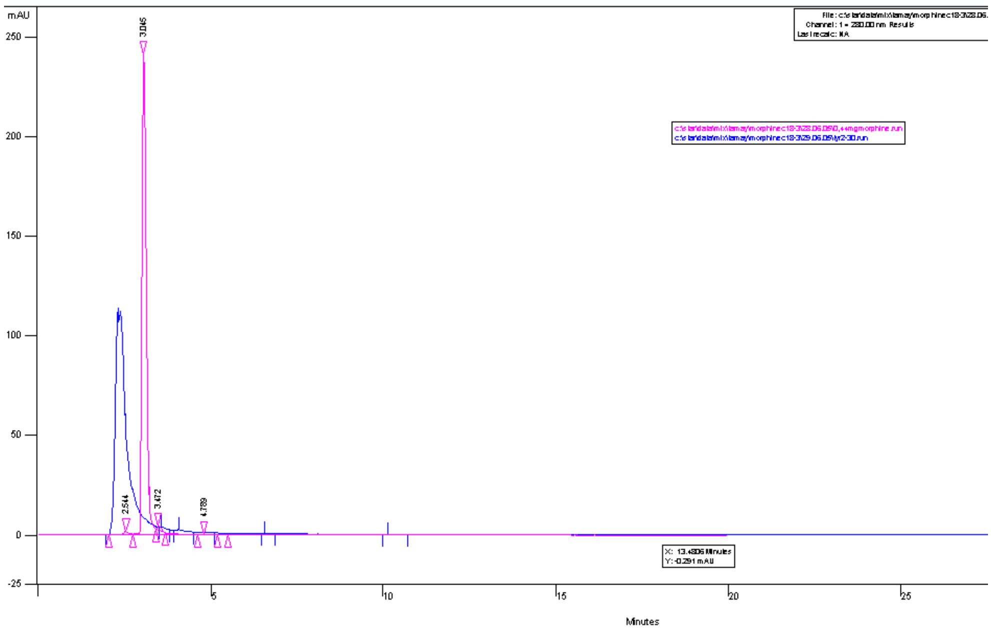


Figure 3.6 HPLC chromatogram of morphine biotransformation with *A. bisporus* tyrosinase (T = 30 ^{0}C, Flow rate 1 ml/min). Sample was withdrawn at the 7th day of the biotransformation reaction. Pink: Morphine standard, Blue: Reaction mixture.

3.1.4.1 Preparation of Tyrosinase and Laccase from *A. bisporus*

Commercial pure *A. bisporus* laccase and tyrosinase converted morphine to pseudo-morphine. However, these enzymes are too expensive to use both in laboratory and industry. Therefore, preperation and use of tyrosinase and laccase from the *A. bisporus* and utilization in unpurified form in biotransformation reactions were attempted in the laboratory.

Phenol oxidases were extracted from fruit bodies of *A. bisporus* in phosphate buffer at pH 7 with polyvinyl polypyrolidone (PVPP). PVPP was used to precipitate phenols which can react with oxygen in the presence of phenol oxidase. PVPP concentration of 12.5 mg/ml was used during extraction which had been optimized previously in our laboratory (Sutay, 2003). However, biotransformation of morphine could not be achieved by using phenol oxidases extracted from *A. bisporus*. Thus, phenol oxidase activity was concentrated by ultrafiltration and ammonium sulfate (AS) precipitation.

3.1.4.2 Enrichment of Phenol Oxidases by AS Precipitation

A. bisporus crude extract (150 ml) was precipitated by various ranges of ammonium sulfate fraction using solid AS salt at 4 ^{0}C. AS levels were tested from 65 to 90 %. Maximum phenol oxidase activity was precipitated by using 65 % AS. However, as observed in Table 3.1, specific activity was lower in this case. The maximum specific activity was at 60 % AS saturation. The purification fold was about 2 in this step. The specific activity was 38 U/ mg protein and % recovery was 67% (Table 3.1). At AS concentrations more than 65% saturation, lower activities were recorded probably because of difficulties in removing ammonium sulphate.

Table 3.1 Phenol oxidase activity changes after AS precipitation

Step	Vol (ml)	Activity (U/ml)	Total Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (u/mg)	Fold	Recovery
Crude Extract	290	1.8	522	0.1	28	18.7	1	100
60 % AS Precipitate after dialysis	11	31.8	350	0.8	9.2	38.2	2.1	67
65 % AS Precipitate after dialysis	8	52.9	418	1.9	14.7	28.4	1.5	80

A. bisporus concentrate obtained by 60% AS precipitation was used as the enzyme source for morphine biotransformation reaction. By using precipitation, enzyme concentration increased 18 times. But no new product was observed on TLC.

3.1.4.3 Concentration of Phenol Oxidase by Ultrafiltration

The *A. bisporus* crude extract was concentrated by using Amicon stirred cells ultrafiltration system using 100 and 30 kDa cut-off cellulose membranes. Crude extract was concentrated 35 times by reducing initial volume from 350 ml to 10 ml. The concentrate had a specific activity of 47 U/mg protein, activity of 25.6 U/ml, 68 % recovery and 2.35 fold purification was achieved. The enzyme concentrate was purer but more dilute than the concentrate prepared by AS precipitation. However, no new product formation could be observed on TLC.

We were expecting a positive result –formation of pseudo-morphine- by using concentrated enzymes since commercial laccase and tyrosinase from *A. bisporus* converted morphine to pseudo-morphine. However, concentrating the enzyme did not resulted in morphine biotransformation. The reason of this surprising result was considered to be because of inhibition of the enzyme activity against morphine because of the presence of other molecules.

3.1.4.4 Investigation of Possible Inhibitory Effects of Concentrated Phenol Oxidase Preparation

To investigate possible inhibitory effects of the concentrated *A. bisporus* enzyme, a test was conducted. In this test, *A. bisporus* enzyme concentrate was added to the reaction mixture containing commercial *A. bisporus* tyrosinase. A reaction mixture containing only commercial *A. bisporus* tyrosinase was also conduction as the positive control.

The results of the reactions after 1 and 72 hours are given in Figure 3.7. As observed, no pseudo-morphine formation was observed in the reaction mixture containing *A. bisporus* enzyme concentrate. However, pseudo-morphine formed in the reaction mixture containing only the pure enzyme. The reaction mixture containing commercial tyrosinase and *A. bisporus* extract was followed up to 2 months and no product formation was observed. This test confirmed the presence of inhibitory molecules in the enzyme concentrate. So, purification is necessary to use the prepared enzyme concentrate in morphine biotransformation reactions.

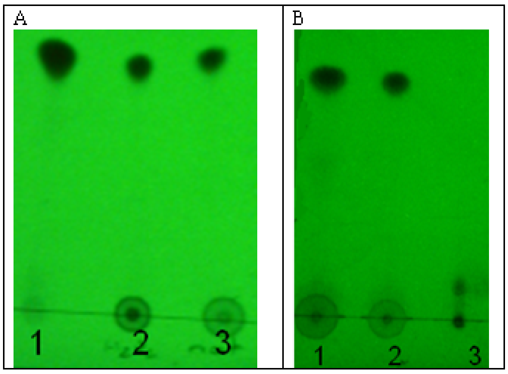


Figure 3.7 TLC results of samples taken from reaction mixture containing commercial tyrosinase and commercial tyrosinase-*A. bisporus* enzyme concentrate mixture. 1) morphine without enzyme 2) commercial tyrosinase and A. bisporus concentrate 3) commercial tyrosinase. A) 1st hour of reaction B) 72nd hour of reaction

3.2 Production of Microbial Phenol Oxidases and Utilization in Morphine Biotransformation

In this study, phenol oxidases production from *S. thermophilum*, *T. lanuginosus* and *P. chrysosporium* were performed and their phenol oxidases were used in morphine biotransformation reactions.

In general, enzymes can be produced intracellularly, membrane-bound or extracellularly. Intracellular and membrane bound enzymes are not secreted to the medium by the cells. However, extracellular enzymes are secreted. Therefore, to screen all the possible forms of phenol oxidases, both cells and culture fluid were used separately in biotransformation reactions, after separation of the cells from the culture fluid by filtration. In addition, considering the possibility of biotransformation of morphine during cultivation of the microorganism, morphine, at 1 mg/ ml which is the minimum concentration that could be observed on TLC plate, was added to the growth medium and daily analysis was performed by TLC.

3.2.1 Production of *Scytalidium thermophilum* Phenol Oxidases and Utilization in Morphine Biotransformation

S. thermophilum culture medium and cultivation conditions were used as given in the literature (Mete, 2003) to obtain extracellular phenol oxidases.

Daily samples which were taken from extracellular culture fluid of *S. thermophilum* was assayed spectrophotometrically for the production of phenol oxidases. The time course of phenol oxidase activity is shown in Figure 3.8.

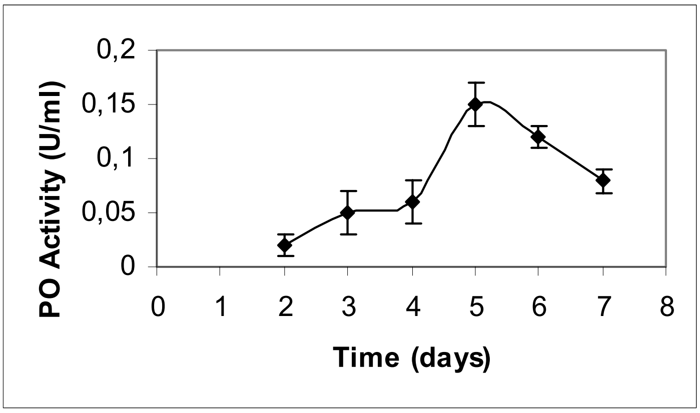


Figure 3.8 Extracellular phenol oxidase activity of *S. thermophilum.* Fermentation was performed at 47 ^{0}C at 155 rpm in a shaker incubator.

As seen from the Figure, maximum phenol oxidase activity was observed at 5^{th} day of cultivation. Therefore, extracellular culture fluid and cells taken from the 5^{th} day of cultivation reacted with morphine at 45-50-60 ^{0}C. In addition, considering the possibility of biotransformation of morphine during cultivation of the microorganism, morphine was added to the growth medium and daily analysis was performed by TLC.

S. thermophilum extracellular culture fluid and cells were used in morphine biotransformation reactions. Samples which were taken from reaction mixtures were analyzed by TLC up to 2.5 months.

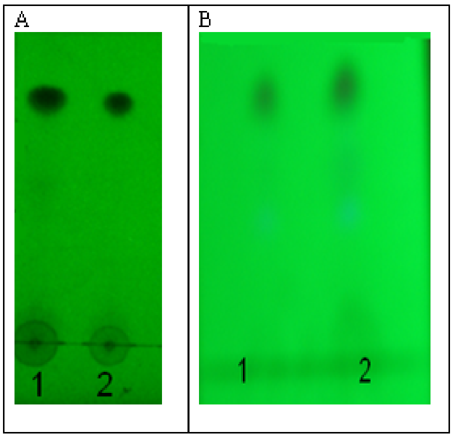


Figure 3.9 TLC results of reaction mixtures of morphine with *S. thermophilum* extracellular culture fluid at 45-50-60 ^{0}C. 1) morphine without enzyme 2)Reaction mixture. A) 1^{st} day of reaction B) After 2.5 months of reaction started

No new product formation was observed by using extracellular enzyme even after 2.5 months as seen from Figure 3.9. Besides, no positive result was obtained by using cells or addition of morphine to the culture medium. Although horseradish peroxidase, *T. versicolor* laccase, *A. bisporus* laccase and tyrosinase, catalyzed morphine biotransformation to pseudo-morphine as abovementioned, neither *S. thermophilum* extracellular culture fluid nor its cells performed morphine biotransformation. This result may be related with insufficient concentration of phenol oxidases present. Therefore, biotransformation of morphine with *S. thermophilum* culture fluid may be repeated by using concentrated culture fluid and more cells. On the other hand, the substrate selectivity of the intracellular and extracellular phenol oxidases of *S. thermophilum* may not be suitable for morphine biotransformation. Phenol oxidases of *S. thermophilum* was reported as a novel enzyme due to its catalytic properties resembling mainly catechol oxidase, but displaying some features of laccases at the same time (Ögel *et al.*, 2005).

Reactions were performed at high temperatures because *S. thermophilum* was thermophilic fungi and its enzymes' optimal temperatures was determined as 60 ^{0}C, in literature (Mete, 2003).

3.2.2 Production of *Thermomyses lanuginosus* Phenol Oxidases and Utilization in Morphine Biotransformation

T. lanuginosus culture medium and cultivation conditions were used as given in the literature (Astarcı, 2003) to obtain extracellular phenol oxidases.

Daily samples taken from culture fluid of *T. lanuginosus* was assayed spectrophotometrically. The time course of phenol oxidase activity is shown in Figure 3.10.

Optimized growth and assay conditions (Astarcı, 2003) were used to produce and assay phenol oxidases. Phenol oxidase production first appeared on 2nd day of cultivation, reached its peak on day 4 as 0.072 U / ml.

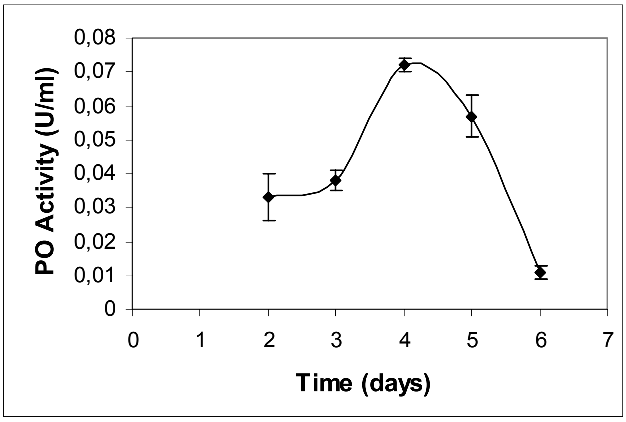


Figure 3.10 Extracellular phenol oxidase activity of *T. lanuginosus.* The fermentations were performed at 47 ^{0}C at 155 rpm in a shaker incubator.

Extracellular culture fluid and cells of *T. lanuginosus* taken from the 4th day of cultivation used in morphine biotransformation reaction. Daily samples which were taken from reaction mixtures up to 2.5 months followed by TLC.

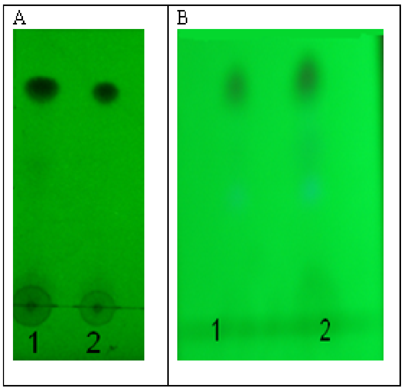


Figure 3.11: TLC results of biotransformation reaction with *T. lanuginosus* extracellular culture fluid at 45-50-60 ^{0}C. 1) morphine without enzyme 2)Reaction mixture. A) 1st day of reaction B) After 2.5 months of reaction started

No new product formation was observed on TLC even after 2.5 months as seen from Figure 3.11. In addition, morphine was added to the growth medium and daily analysis was performed by TLC but morphine during cultivation of the microorganism were not used by *T. lanuginosus*. The biotransformation reaction using cells also did not give any product. Reactions were performed at high temperatures because *T. lanuginosus* was a thermophilic fungus and its phenol oxidase optimal temperature was determined as 50 ^{0}C, in the our laboratory (Astarcı, 2003).

3.2.3 Production of *Phanerochaete chrysosporium* Phenol Oxidases and Utilization in Morphine Biotransformation

P. chrysosporium produces three major classes of enzymes, namely lignin peroxidase (LIP), manganese dependent peroxiadase (MNPs), and laccases, which play an important role in fungal degration of lignin.

It was reported that, *P. chrysosporium* laccase activity was present when cellulose was used as sole carbon source but absent when it was substituted by glucose (Srinivasan *et al.,*1995). Therefore, cotton stalk, a lingo-cellulosic material, the part of the cotton plant that is left after harvesting period, was used as the only carbon source and phenol oxidase inducer, considering its high content of cellulose and lignin (a phenolic polymer).

Three different types of culture media were screened for production of phenol oxidases to use in morphine biotransformation. Table 3.2 shows these different culture media and the results of morphine biotransformation.

Table 3.2 Growth media composition and the result of biotransformation reaction.

Growth medium	Result of biotransformation reaction
Glucose 10g/ l Ammonium tartrate 24 mM Thiamine 1.2g/l 400mM veratryl alch 1ml/l 0,2 M sodiun acetate buf (pH 4,5)100ml/l Basal medium 100 ml/l Trace element 50 ml/l (Tien and Kırk, 1988)	No product
Glucose 10g/l NH_4Cl 0.12 g/l $CaCl_2 . 2H_2O$ 0.132 g/l $MgSO_4 . 7H_2O$ 1.025 g/l KH_2PO_4 2 g/l Thiamin 10 mg/l	No product
Cotton waste 10 g/l Ammonium tartrate 24 mM Thiamine 1.2g/l 400mM veratryl alch 1ml/l 0,2 M sodiun acetate buf (pH 4,5)100ml/l Basal medium 100 ml/l Trace element 50 ml/l	Product formation was observed

A plate assay was used to determine extracellular laccase activity in culture fluid of *P. chrysosporium* qualitatively. Presence of laccase activity was observed by using 2,2'-azinobis(3-ethylbenzathiazoline-6-sulfonic acid (ABTS) which is considered one of the most sensitive substrates for laccase assay. ABTS gave bluish green color when reacted with laccase. Culture fluid was concentrated 500X by using ultrafiltration and membrane concentrator as reported in literature

(Srinivasan *et al.*, 1995). The results of the laccase plate assay showed the presence of laccase activity in the concentrated extracellular culture fluid of *P. chrysosporium* (Fig 3.12). *T. versicolor* laccase and heat-treated extracellular culture fluid of *P. chrysosporium* were used as positive and negative controls, respectively.

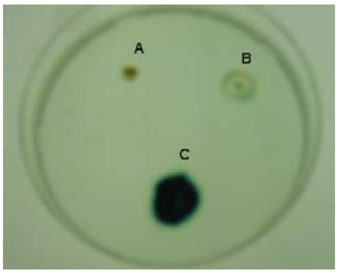


Figure 3.12 Qualitative assay for laccase production by *P. chrysosporium* in the presence of ABTS .(A) 20 µl of 500X-concentrated extracellular fluid boiled for 5 min, as a negative control. (B) 20 µl of 500X-concentrated extracellular fluid. (C) commercial *T. versicolor* laccase solution (2 mg/ml in glycine-HCl buffer) as positive control.

Laccase activity could be observed in 50X-concentrated culture fluid. However, it was not detected in culture fluid without making concentration. This was also reported in literature. Laccase activity detected in 40X-concetrated extracellular fluid of *P. chrysosporium* by using cellulose powder was used as carbon source (Srinivasan *et al.*, 1995).

The time course of laccase production in cotton stalk medium is shown in Figure 3.13. Laccase activity first appeared on 5^{th} day of cultivation. Maximum activity was observed at 7^{th}-8^{th} day of cultivation at 30^0C without shaking. Laccase activity was determined spectrophotometrically at 436 nm with ABTS as the substrate. All assays were done at pH 3, the optimum pH for laccase of *P. chrysosporium* with ABTS as the substrate (Srinivasan *et al*., 1995).

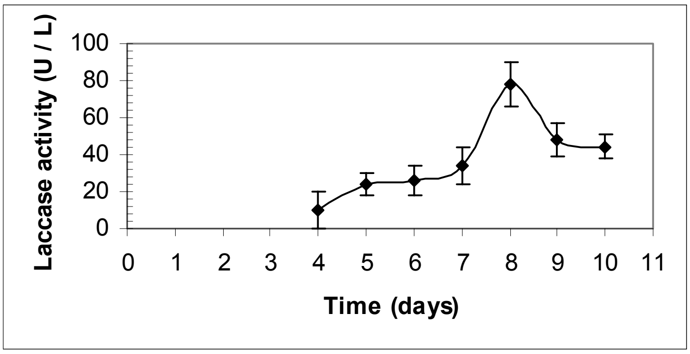


Figure 3.13 Extracellular laccase production of *P. chrysosporium.* Cultures were grown in cotton stalk medium under static conditions at 30 ^{0}C. Culture fluids taken from 4 th day to 10 th day of cultivation and 50 X -concentrated used in laccase activity assay.

In literature, maximum laccase activity in 40X concentrated extracellular fluid of *P. chrysosporium* grown in cellulose powder medium at 25 ^{0}C was reported as 55 U/L at 6th day of cultivation by using same enzyme assay method.

P. chrysosporium extracellular culture fluid and cells used in morphine biotransformation at room temperature and at pH 3. Reaction mixtures were prepared with and without hydrogen peroxide (H_2O_2). *P. chrysosporium* is known as good producer for peroxidase and peroxidases require the presence of H_2O_2 for activity. Therefore, H_2O_2 was put in reaction mixture to utilize peroxidase of *P. chrysosporium* in morphine biotransformation.

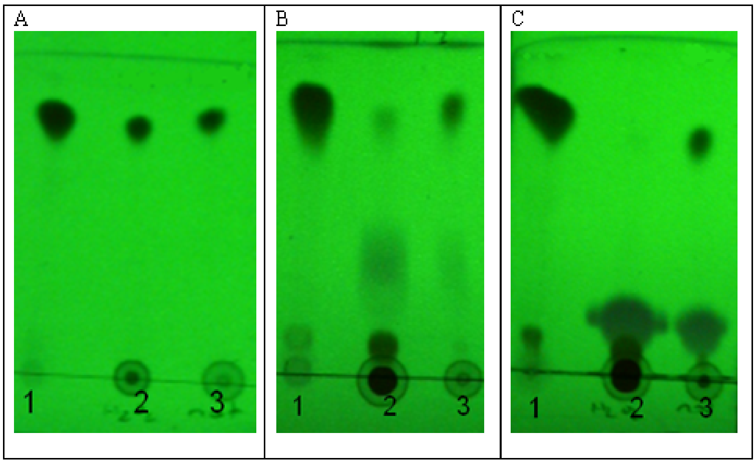


Figure 3.14 TLC results of biotransformation reactions with *P. chrysosporium* extracellular culture fluid in the presence and absence of H_2O_2 at room temperature. 1) Morphine solution without enzyme 2) Reaction mixture with H_2O_2 3) Reaction mixture without H_2O_2 A) 1st day of reaction B) 20th day of reaction C) 30th day of reaction.

P. chrysosporium extracellular culture fluid taken from 8th day of cultivation was concentrated 500X and used in morphine biotransformation.

As observed from Figure 3.14, after 25 days a new product formation was observed in reaction mixture contained H_2O_2 and 5 days later morphine spot became invisible in reaction mixture. Morphine was converted to new product with extracellular peroxidases. On the other hand, the same product was also observed on TLC, in the sample without H_2O_2. Laccases which were produced by *P. chrysosporium* can be responsible for this conversion.

Figure 3.15, 3.16, 3.17 shows the retention times of morphine and products eluted from C18 column.

Pink peak in Figure 3.15 is morphine standard (0.532 mg/ml) which was given to column as control. Morphine was detect at 5.66th minute. Green peaks belong to reaction mixture without enzyme. This solution was used as negative control. As seen from the figure there are two black peaks the first one eluted at 4.67th min and the second one, eluted at 5.66th min. The first peak at should belong to pseudo-morphine. Quantitatively pseudo-morphine and morphine areas were determined as 8.86 and 88.5 % for solution containing morphine solution without enzyme. Showing some degradation of morphine at pH 3 in the absence of enzyme solution.

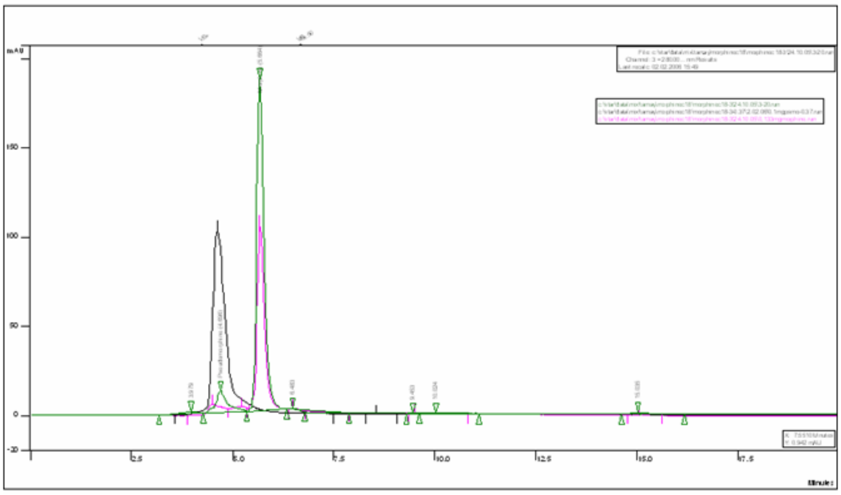


Figure 3.15 Chromatogram of reaction mixture contain morphine without enzyme solution. (T = 30 ^{0}C, flow rate 0.5 ml /min). Green : Reaction mixture, Pink: Morphine standard, Black: Pseudo-morphine standard.

In Figure 3.16, green peaks shows the reaction mixture containing extracellular culture fluid of *P. chrysosporium* with H_2O_2. Pink peak belongs to morphine standard. The peaks eluted at 5.66th min is morphine and at 4.67 min is pseudo-morphine. For the reaction mixture relative pseudo-morphine and morphine area were analyzed as 94.7 and 2.5 %, respectively.

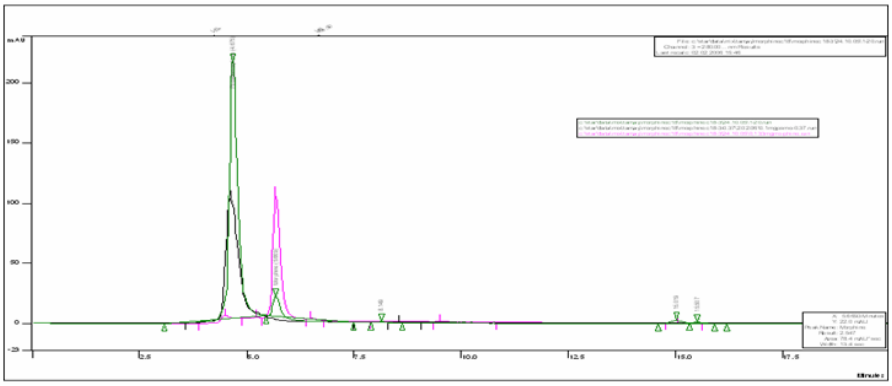


Figure 3.16 Chromatogram of reaction mixture containing 500X concentrated culture fluid of *P. chrysosporium* with H_2O_2. (T = 30 ^{0}C, flow rate 0.5 ml /min). Green: Reaction mixture with H_2O_2, Pink: Morphine standard, Black: Pseudo-morphine standard.

In Figure 3.17 green line shows reaction mixture containing *P. chrysosporium* without H_2O_2 and the pink peak belongs to morphine standard. The peaks eluted at 5.66 min is morphine, the peak at 4.67 min is pseudo-morphine. Another peak is eluted at 15th min belongs to an unknown product. Finally relative morphine, pseudo-morhine and unknown product areas were determined as 26.3, 12.2 and 61.3, respectively.

The unknown product detected during HPLC analysis of the reaction mixture without H_2O_2, had not been observed on TLC as seen in Figure 3.14 probably because of its low concentration. So, the sample prepared for HPLC analysis, dried by using vacuum evaporator and solved in 300 µl of HPLC mobile phase, was re–analyzed on TLC and the spot belonging to unknown product was observed as seen in Figure 3.18.

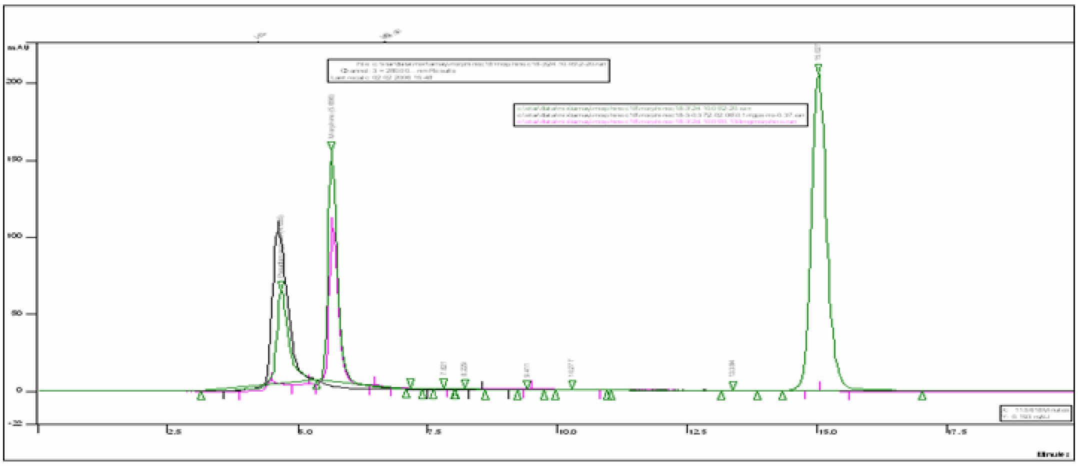


Figure 3.17 Chromatogram of reaction mixture containing 500X concentrated culture fluid of *P. chrysosporium* without H_2O_2. (T = 30 ^{0}C, flow rate 0.5 ml /min). Green: Morphine standard, Black: Reaction mixture with enzyme without H_2O_2

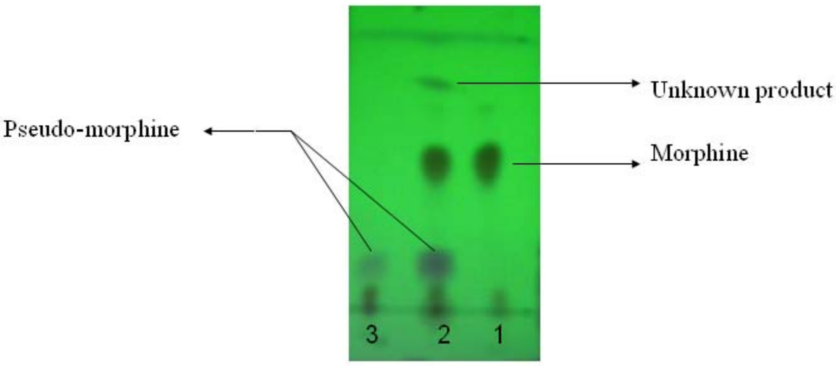


Figure 3.18 TLC result of samples which were dried by using vacuum evaporator and then solved in 300 µl of HPLC mobile phase 1) Reaction mixture with H_2O_2 2) Reaction mixture without H_2O_2 3) Morphine solution without enzyme solution.

Unknown product will be identified by either using mass spectrometry or ^1H nuclear magnetic resonance spectrometry. For this reason, this reaction was repeated by using more morphine and enzyme solution.

Table 3.3 gives HPLC results of samples taken from morphine biotransformation by using commercial phenol oxidases. In literature morphine to pseudo-morphine biotransformation was reported by using commercial horseradish peroxidase in presence of H_2O_2 and p-coumaric acid as activator. In this study we have determined *A. bisporus* tyrosinase and laccase and *T. versicolor* laccase as enzymes having catalytic activity for this reaction with p-coumaric acid as activator.

Table 3.4 gives HPLC results of samples taken from morphine biotransformation by using microbial phenol oxidases obtaining from thermophilic fungi and *P. chrysosporium* in the laboratory. Biotransformation of morphine was achieved by using *P. chrysosporium* phenol oxidases.

Table 3.3 HPLC results of samples taken from morphine biotransformation by using commercial phenol oxidases.

Enzyme source	**Reaction pH**	**Substrate**	**Product**	**HPLC Analyses**		
				Retention time (min)	**Flow rate (ml/min)**	**Area Percent**
Agaricus bisporus tyrosinase	7	Morphine	Pseudo-morphine	2.35	1	100
Tramates versicolor laccase	7	Morphine	Morphine	2.95-5.98	1-0.5	27
			Pseudo-morphine	2.36-4.83	1-0.5	73
Agaricus bisporus laccase	7	Morphine	Morphine	2.97-5.99	1-0.5	16
			Pseudo-morphine	2.40-4.92	1-0.5	77

Table 3.4 HPLC results of samples taken from morphine biotransformation by using microbial phenol oxidases

Enzyme source	**pH**	**Substrate**	**Product**	**HPLC Analyses**		
				Retention time (min)	**Flow rate (ml/min)**	**Area Percent**
Thermomyces lanuginosus culture fluid	7	Morphine	No Product			
Scytalidium thermophilum culture fluid	7	Morphine	No Product			
Thermomyces lanuginosus cells	7	Morphine	No Product			
Scytalidium thermophilum cells	7	Morphine	No Product			
500X-concentrated *Phanerochaete chrysosporium* culture fluid	3	Morphine	Morphine	5.6	0.5	26
			Pseudo-morphine	4.7	0.5	12.3
			Unknown product	15	0.5	61.3
500X-concentrated *Phanerochaete chrysosporium* culture fluid with H_2O_2	3	Morphine	Morphine	5.7	0.5	2.5
			Pseudo-morphine	4.7	0.5	94.7
Control of non-enzymatic formation of pseudo-morphine at pH 3	3	Morphine	Morphine	5.7	0.5	88
			Pseudo-morphine	4.7	0.5	9
Use of morphine during cutivation by *T. lanuginosus* and *S. thermophilum*	7	Morphine	No Product			

CHAPTER 4

CONCLUSIONS

In this study, various microbial phenol oxidases were screened for morphine biotransformation. For this purpose, phenol oxidases were obtained commercially or were produced microbiologically in the laboratory.

Commercial *T.* versicolor laccase, *A.bisporous* (edible mushroom) tyrosinase and laccase catalyzed morphine biotransformation to pseudo-morphine. However, when tyrosinase and laccase were obtained in the laboratory by extraction from *A.bisporous*, morphine biotransformation could not be achieved because of inhibition by impurities. So, purification is necessary for the biotransformation reactions.

In the laboratory, phenol oxidases from *S. thermophilum, T. lanuginosus* and white rot fungus *P. chrysosporium* were produced. Both extracellular and intercellular phenol oxidases, were screened for morphine biotransformation.

No product formation was observed by using either culture fluid or cells of *S. thermophilum* and *T. lanuginosus*. This result might be because of insufficient enzyme concentration. So the experiments can be repeated by using concentrated culture fluids.

P. chrysosporium peroxidases catalyzed the transformation of morphine to pseudo-morphine, and laccase converted morphine to pseudo-morphine and an unknown product.

For further studies, characterization of this unknown product will be performed. Identification of unknown product can be achieved by H nuclear magnetic resonance or mass spectrometry. Afterwards if the unknown product is valuable, the enzyme responsible for the biotransformation will be determined and further studies will be performed for characterization and production of the enzyme.

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APPENDIX A

MEDIUM COMPOSITIONS

Modified YpSs Agar for *Syctalidium thermophilum* (Mete, 2003)

Yeast Extract .... 4 g/L
K_2HPO_4 .... 1 g/L
$MgSO_4 . 7H_2O$ .... 0.5 g/L
Agar .... 2 g/L
Soluble starch .... 15 g/L

Preculture medium for *Syctalidium thermophilum* (Mete, 2003 and Kaptan, 2004)

Yeast Extract .... 4 g/L
K_2HPO_4 .... 1 g/L
$MgSO_4 . 7H_2O$ .... 0.5 g/L
Glucose .... 10 g/L

Modified YpSs Broth Medium for *Syctalidium thermophilum* (Mete, 2003 and Kaptan, 2004)

Yeast Extract .... 4 g/L
K_2HPO_4 .... 1 g/L
$MgSO_4 . 7H_2O$ .... 0.5 g/L
$Cu. SO_4 . 5H_2O$ .... 0.1 g/L
Gallic acid .... 0.17 g/L
Glucose .... 40 g/L

Modified YpSs Agar for *Thermomyces lanuginosus* (Astarcı, 2003)

Yeast Extract .... 4 g/L
K_2HPO_4 .... 1 g/L
$MgSO_4 . 7H_2O$ .... 5 g/L
Avicel .... 10 g/L
Agar .... 2 g/L

Preculture medium for *Thermomyces lanuginosus* (Astarcı, 2003)

Yeast Extract .... 4 g/L
K_2HPO_4 .... 10 g/L
$MgSO_4 . 7H_2O$ .... 0.5 g/L
Glucose .... 7.5 g/L

Broth Medium for *Thermomyces lanuginosus* (Astarcı, 2003)

Yeast Extract .... 15 g/L
K_2HPO_4 .... 10 g/L
$MgSO_4 . 7H_2O$ .... 3 g/L
$CuSO_4$ .... 0.03 g/L
Gallic acid 0.03

Growth medium for *Phanerocheate chrysosporium*

Cotton stalk .... 10 g/L
Ammonium Tartarate .... 4.42 g/L
0.2 M Sodium Acetate buffer (pH 4.5) .... 1 ml/L
Thiamine (100 mg/L stock)................ .... 1 ml/L
0.4 M Veratyl alcohol......................... .... 100 ml/L
Trace Element .... 60 ml/L
Bassal Medium................................. .... 100 ml/L

Stock Reagents

1. Bassal Medium

KH_2PO_4 .... 20g/L
$MgSO_4$ .... 5 g/L
$CaCl_2$ .... 1 g/L

2. Trace element solution

$MgSO_4$ .... 3 g/L
$MnSO_4$ .... 0.5 g/L
NaCl .... 1 g/L
$FeSO_4 . 7H_2O$ .... 0.1 g/L
$CoCl_2$ .... 0.1 g/L
$ZnSO_4 . 7H_2O$ .... 0.1 g/L
$CuSO_4$ .... 0.1 g/L
$AlK(SO_4)_2 . 12H_2O$ .... 10 mg/L
H_3BO_3 .... 10 mg/L
$Na_2MoO_4 . 2H_2O$ .... 10 mg/L
Nitrilotriacetate* .... 1.5 g /L

*Firstly Nitrilotriacetate was dissolved in 800 ml H_2O and pH was adjusted to ˜6.5 with 1 N KOH and then each component was added and the volume was brought to 1 liter when trace element solution was prepared.

APPENDIX B

BRADFORD METHOD

Bradford Assay is a rapid and accurate method commonly used to determine the total protein concentration of a sample. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

Chemicals needed to prepare concentrated stock reagent solution (5x stock) were given in table B.1. These chemicals were mixed and diluted to 1 L with distilled water to prepare 5x concentrated stock reagent solution. The stock solution was stored at 4 ^{0}C. To prepared diluted (1x) reagent solution 1 volume concentrate was mixed with 4 volumes of distilled water. This solution was well mixed and filtered. Bradford reagent should wait at least 24 hours at room temperature before use.

Table B.1 Bradford Reagent Content

Chemical	Amount
85 % Ortho-phosphoric acid	500 ml
95% Ethanol	250 ml
Brillant Blue G-250 dye	500 mg

Bovine serum albumim (BSA) was used as protein standard. To prepare 1 mg/ml stock BSA solution, 25 mg BSA was dissolved in 25 ml of pH 7 sodium phosphate buffer. This stock solution was diluted at different ratios given in table B.2

Table B.2 BSA Dilution Ratios for Bradford Method

Protein (mg/ml)	0	0.01	0.02	0.03	0.04	0.05
BSA stock (ml)	0	0.1	0.2	0.3	0.4	0.5
Buffer (ml)	10	9.9	9.8	9.7	9.6	9.5

After preparation of diluted BSA samples, 0.5 ml BSA sample and 5 ml of Bradford reagent were mixed in glass tube. Ten minutes later absorbance at 595 nm was measured by using a spectrophotometer.

APPENDIX C

BSA STANDARD CURVE FOR BRADFORD METHOD

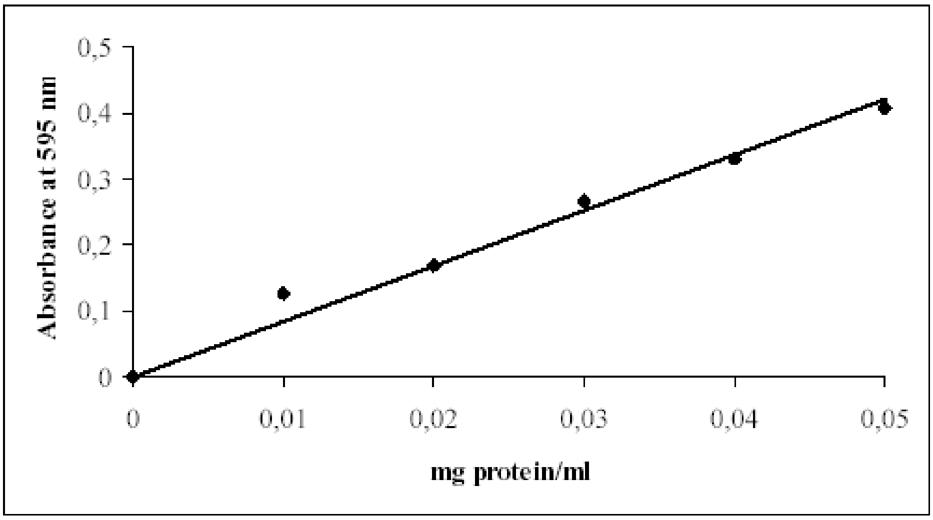

Figure C.1 BSA standard curve for Bradford Method

APPENDIX D

STANDARD CURVE FOR MORPHINE

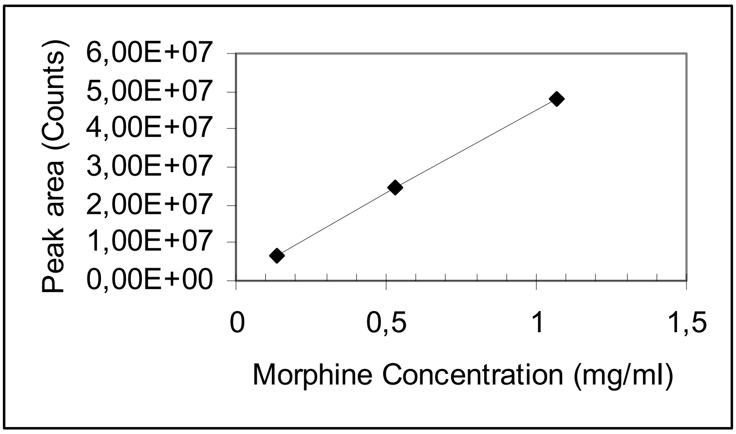

Figure D.1 Standard curve for morphine

APPENDIX E

COMPUTER OUTPUTS OF THE CHROMATOGRAMS

HPLC Result of Sample Containing Pure *T. versicolor* Laccase and Morphine (pH 7)

Flow rate : 1ml/min

Peak No	Peak Name	Result ()	Ret Time (min)	Time Offset (min)	Peak Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	Status Codes
1		0.0153	1.709	0.000	2336	0.00	BP	8.0	
2		72.9452	2.368	0.000	11149850	0.00	PV	21.3	
3	Morphine	26.9958	2.960	0.003	4126378	0.00	VB	12.0	
4		0.0437	4.573	0.000	6685	0.00	TS	0.0	
	Totals	**100.0000**		**0.003**	**15285249**				

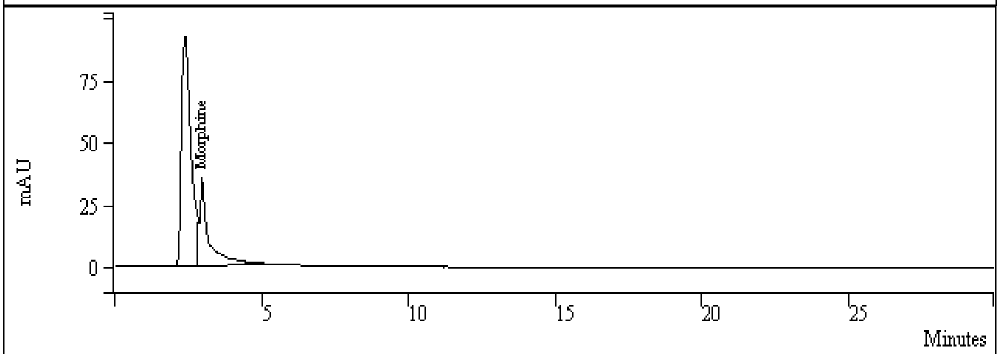

Figure E.1 HPLC Result of Sample Containing Pure *T. versicolor* Laccase and Morphine

Flow rate: 0.5 ml/min

Peak No	Peak Name	Result ()	Ret Time (min)	Time Offset (min)	Peak Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	at od
1		76.3963	4.837	0.000	14842690	0.00	BV	42.6	
2	Morphine	23.4139	6.019	0.032	4548982	0.00	VB	20.5	
3		0.0656	8.067	0.000	12751	0.00	TS	0.0	
4		0.1242	9.368	0.000	24126	0.00	TS	0.0	
	Totals	**100.0000**		**0.032**	**19428550**				

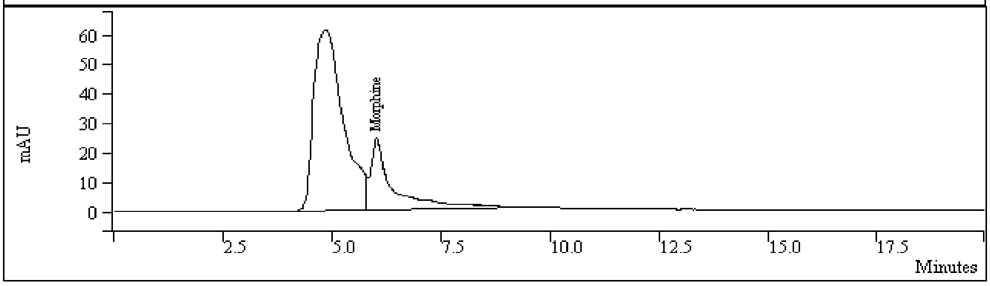

Figure E.2 HPLC Result of Sample Containing Pure *T. versicolor* Laccase and Morphine

HPLC Result of Sample Containing Pure *A. bisporus* Laccase and Morphine (pH 7)

Flow rate 1ml/min

Peak No	Peak Name	Result ()	Ret Time (min)	Time Offset (min)	Peak Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	Status Codes
1		77.6603	2.405	0.000	9210747	0.00	BV	19.2	
2	Morphine	16.1782	2.973	0.000	1918779	0.00	VV	20.2	
3		4.2772	4.080	0.000	507286	0.00	VV	34.9	
4		1.8423	5.107	0.000	218499	0.00	VB	0.0	
5		0.0421	8.013	0.000	4992	0.00	BB	8.2	
	Totals	**100.0001**		**0.000**	**11860303**				

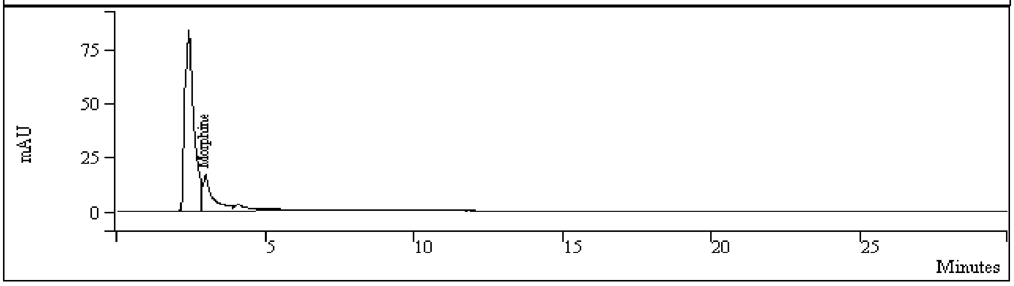

Figure E.3 HPLC Result of Sample Containing Pure *A. bisporus* Laccase and Morphine (pH 7)

HPLC Result of Sample Containing Pure *A. bisporus* Tyrosinase and Morphine (pH 7)

Flow rate 1ml/min

Peak No	Peak Name	Result ()	Ret Time (min)	Time Offset (min)	Peak Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	Status Codes
1		99.4630	2.349	0.000	14283415	0.00	BB	19.0	
2		0.1487	3.547	0.000	21360	0.00	TS	0.0	
3		0.3087	4.064	0.000	44332	0.00	TS	0.0	
4		0.0252	6.560	0.000	3621	0.00	BB	5.9	
5		0.0544	10.149	0.000	7806	0.00	BB	13.0	
	Totals	**100.0000**		**0.000**	**14360534**				

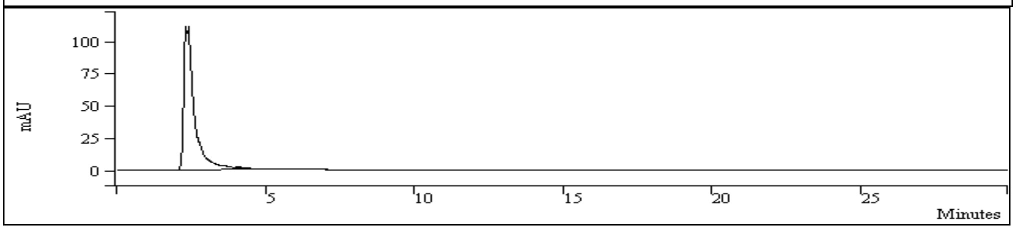

Figure E.4 HPLC Result of Sample Containing Pure *A. bisporus* Tyrosinase and Morphine (pH 7)

HPLC Result of Sample Containing Morphine without enzyme solution (pH 3)

Flow rate: 0.5 ml/min

Peak No	Peak Name	Result ()	Ret Time (min)	Time Offset (min)	Peak Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	at od
1		0.8918	3.979	0.000	123646	0.00	BB	30.7	
2		8.8611	4.696	0.000	1228529	0.00	BB	15.4	
3	Morphine	88.4748	5.664	-0.011	12266422	0.00	BB	11.5	
4		0.5176	6.483	0.000	71756	0.00	VB	0.0	
5		0.1187	9.453	0.000	16458	0.00	BV	11.2	
6		0.4729	10.024	0.000	65564	0.00	VB	45.0	
7		0.6631	15.035	0.000	91939	0.00	BB	17.9	
	Totals	**100.0000**		**-0.011**	**13864314**				

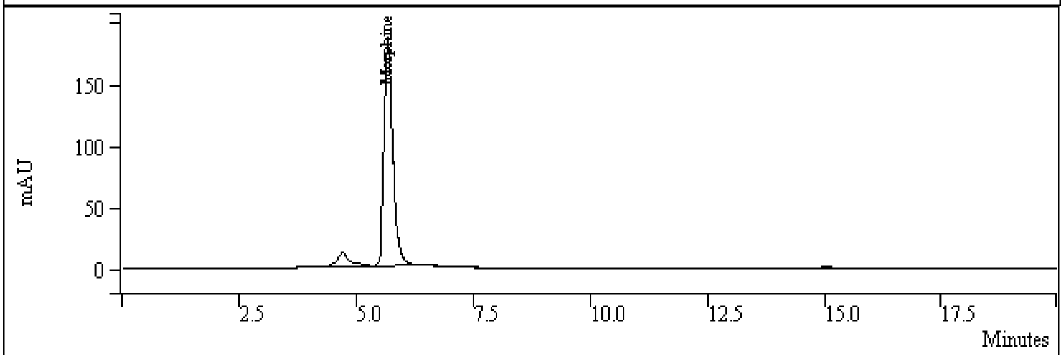

Figure E.5 HPLC Result of Sample Containing Morphine without enzyme solution (pH 3)

HPLC Result of Sample Containing containing 500X concentrated culture fluid of *P. chrysosporium* with H_2O_2 and morphine (pH 3)

Flow rate: 0.5 ml/min

Peak No	Peak Name	Result ()	Ret Time (min)	Time Offset (min)	Peak Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	:at od
1		1.5146	3.315	0.000	3337865	0.00	BP	16.5	
2		91.5827	4.744	0.000	201826752	0.00	PB	16.5	
3	Morphine	3.3148	5.512	-0.163	7305133	0.00	VB	0.0	
4		0.7054	6.283	0.000	1554633	0.00	VB	0.0	
5		0.4176	8.248	0.000	920306	0.00	BB	12.8	
6		0.1033	9.557	0.000	227597	0.00	BB	11.7	
7		0.0540	12.411	0.000	119037	0.00	BB	17.1	
8		0.0136	13.051	0.000	29886	0.00	TS	0.0	
9		1.3826	15.141	0.000	3046885	0.00	BV	18.6	
10		0.8883	15.603	0.000	1957584	0.00	VB	28.0	
11		0.0129	17.403	0.000	28366	0.00	BB	15.8	
12		0.0047	18.773	0.000	10283	0.00	BB	17.3	
13		0.0055	19.653	0.000	12110	0.00	BB	16.6	
	Totals	**100.0000**		**-0.163**	**220376464**				

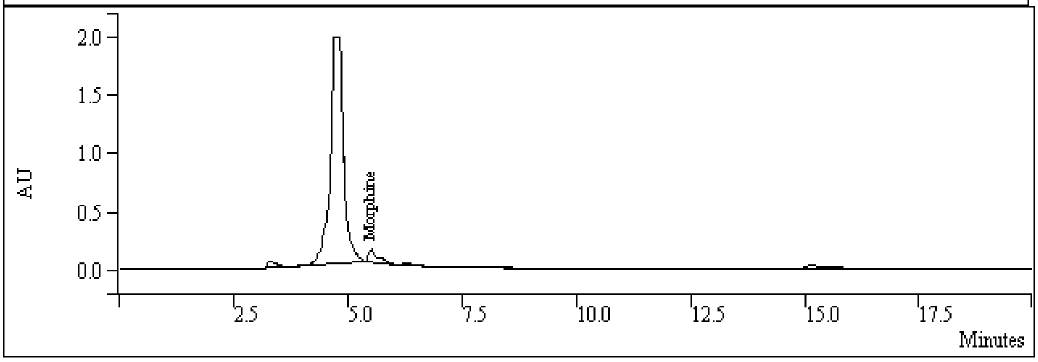

Figure E.6 HPLC Result of Sample Containing containing 500X concentrated culture fluid of *P. chrysosporium* with H_2O_2 and morphine (pH 3)

HPLC Result of Sample Containing containing 500X concentrated culture fluid of *P. chrysosporium* without H_2O_2 and morphine (pH 3)

Flow rate: 0.5 ml/min

Peak No	Peak Name	Result ()	Ret Time (min)	Time Offset (min)	Peak Area (counts)	Rel Ret Time	Sep. Code	Width 1/2 (sec)	at od
1		12.2704	4.685	0.000	4311152	0.00	BB	13.8	
2	Morphine	26.2734	5.656	-0.019	9231055	0.00	BP	11.4	
3		0.0505	7.821	0.000	17731	0.00	PV	0.0	
4		0.0171	8.229	0.000	6015	0.00	VP	0.0	
5		0.0130	9.411	0.000	4579	0.00	PV	0.0	
6		0.0647	10.277	0.000	22736	0.00	VB	0.0	
7		0.0264	13.384	0.000	9273	0.00	BB	14.2	
8		61.2845	15.027	0.000	21532024	0.00	BB	18.4	
	Totals	**100.0000**		**-0.019**	**35134564**				

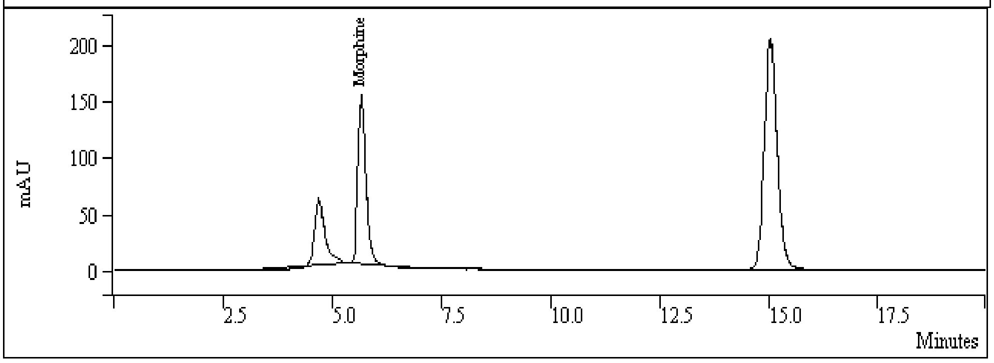

Figure E.7 HPLC Result of Sample Containing containing 500X concentrated culture fluid of *P. chrysosporium* without H_2O_2 and morphine (pH 3)

APPENDIX F

HPLC Results of Samples which were Analyzed in Afyon Alkaloid Plant

Column: µ Bondapak C18 (300 mm X 3.9 i.d, 10 µm pore size, Waters, USA)

Solvent system: 2.2 g Sodium 1-heptanesulfonate is dissolved in 500 ml water. 360 ml acetonitrile, 20 ml glacial acetic acid are added to solution and final volume completed to 2 liter.

HPLC Result of Sample Containing Pure *T. versicolor* Laccase and Morphine (pH 7)

Flow rate: 1.5 ml/min

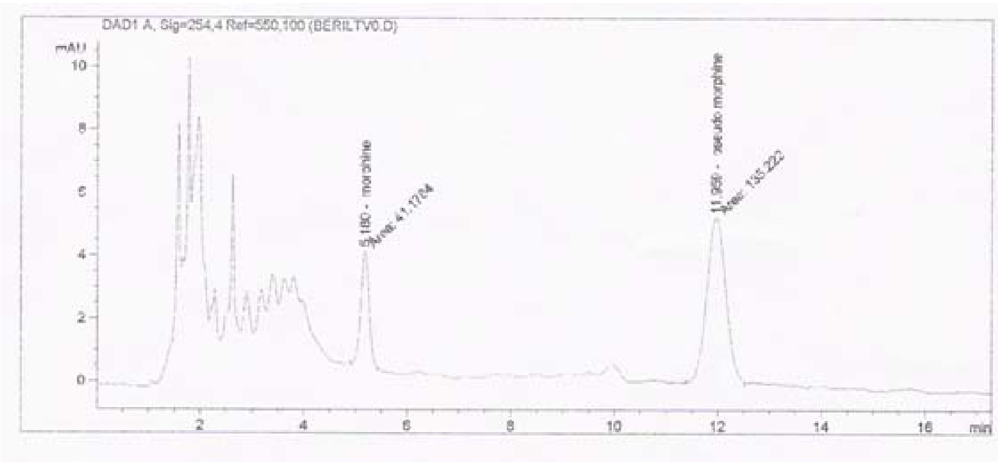

Figure F.1 HPLC Result of Sample Containing Pure *T. versicolor* Laccase and Morphine (pH 7)

External Standard Report

Sorted By : Signal
Calib. Data Modified : 29.05.2005 14:21:06
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=254,4 Ref=550,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [mg/ml]	Grp	Name
5.180	MM	41.17842	3.58745e-4	1.47726e-2		morphine
11.959	MM	135.22235	5.34662e-5	7.22983e-3		pseudo morphine

Totals : 2.20024e-2

HPLC Result of Sample Containing Pure *A. bisporus* Laccase and Morphine (pH 7)

Flow rate: 1.5ml/min

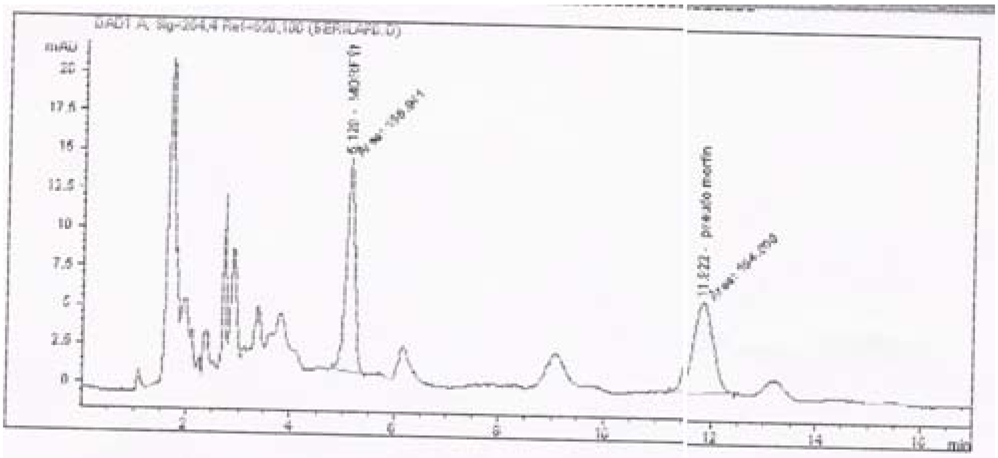

Figure F.2 HPLC Result of Sample Containing Pure *A. bisporus* Laccase and Morphine (pH 7)

Sorted By : Signal
Calib. Data Modified : 29.05.2005 14:00:01
Multiplier : 20.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=254,4 Ref=550,100

RetTime (min)	Type	Area (mAU*s)	Amt/Area	Amount (ug/ml)	Grp	Name
5.120	MM	156.94051	3.58745e-4	1.12603		MORFYN
11.822	MM	154.60934	5.34662e-5	1.65328e-1		pseudo morfin
Totals :				1.29136		

HPLC Result of Sample Containing Morphine without enzyme solution (pH 3)

Flow rate: 1.5 ml/min

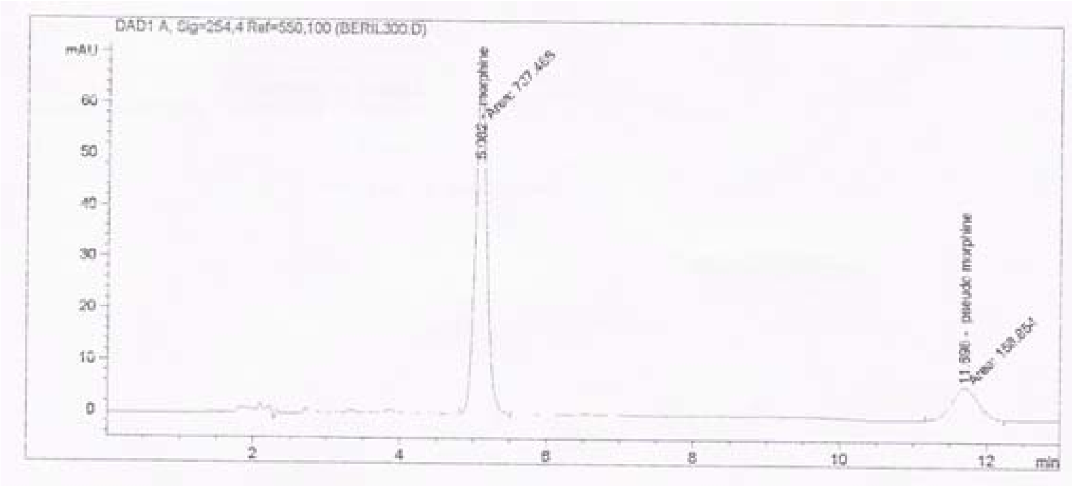

Figure F.3 HPLC Result of Sample Containing Morphine without enzyme solution (pH 3)

Sorted By : Signal
Calib. Data Modified : 29.05.2005 14:47:16
Multiplier : 20.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=254,4 Ref=550,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [mg/ml]	Grp	Name
5.082	MM	737.48621	3.58745e-4	5.29139		morphine
11.698	MM	158.85378	5.34662e-5	1.69866e-1		pseudo morphine
Totals :				5.46126		

HPLC Result of Sample Containing containing 500X concentrated culture fluid of *P. chrysosporium* with H_2O_2 and morphine (pH 3)

Flow rate: 1.5 ml/min

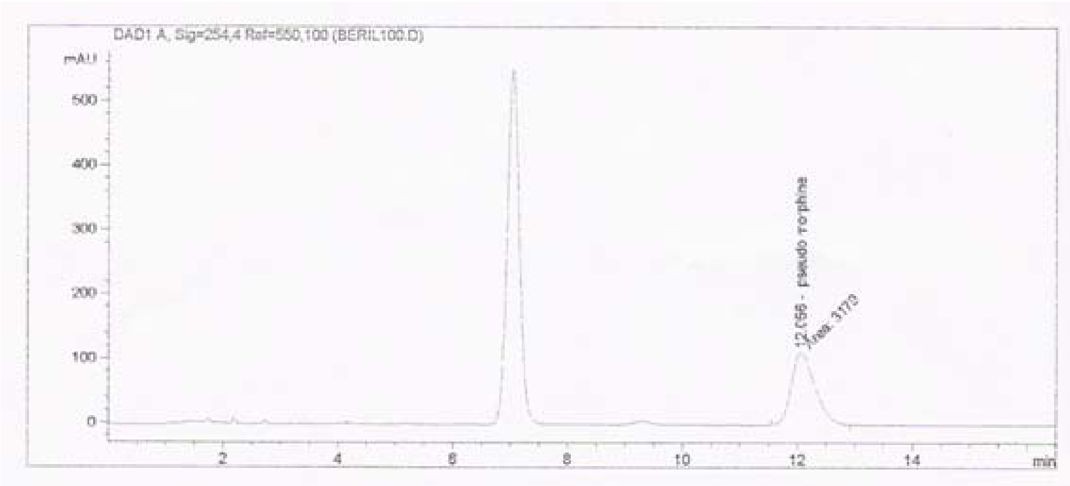

Figure F. 4 HPLC Result of Sample Containing containing 500X concentrated culture fluid of *P. chrysosporium* with H_2O_2 and morphine (pH 3)

Sorted By : Signal
Calib. Data Modified : 29.05.2005 14:42:54
Multiplier : 20.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=254,4 Ref=550,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [mg/ml]	Grp	Name
5.094		-	-	-		morphine
12.056	MM	3178.99561	5.34662e-5	3.39938		pseudo morphine
Totals :				3.39938		

HPLC Result of Sample Containing containing 500X concentrated culture fluid of *P. chrysosporium* without H_2O_2 and morphine (pH 3)

Flow rate: 1.5 ml/min

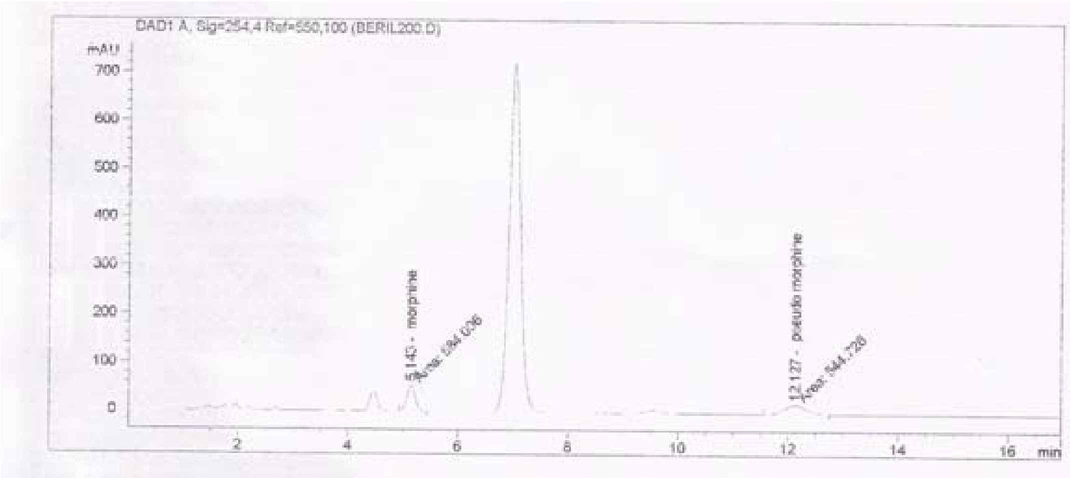

Figure F. 5 HPLC Result of Sample Containing containing 500X concentrated culture fluid of *P. chrysosporium* without H_2O_2 and morphine (pH 3)

Sorted By : Signal
Calib. Data Modified : 29.05.2005 14:42:54
Multiplier : 20.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=254,4 Ref=550,100

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [mg/ml]	Grp	Name
5.143	MM	584.00641	3.58745e-4	4.19019		morphine
12.127	MM	544.72644	5.34662e-5	5.82490e-1		pseudo morphine
Totals :				4.77268		