PRODUCTION AND BIOCHEMICAL CHARACTERIZATION OF POLYPHENOL OXIDASE FROM *THERMOMYCES LANUGINOSUS*

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ABSTRACT

PRODUCTION AND BIOCHEMICAL CHARACTERIZATION OF POLYPHENOL OXIDASE FROM *THERMOMYCES LANUGINOSUS*

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Polyphenol oxidases are enzymes that catalyze the oxidation of certain phenolic substrates to quinones in the presence of molecular oxygen. Polyphenol oxidases are widely used in several applications. In food industry, they are used for enhancement of flavor in coffee, tea and cocoa production, and determination of food quality. In medicine, they have several uses in treatments of Parkinson's disease, phenlyketonurea and leukemia. In wastewater treatment, they are used for the removal of phenolic pollutants from wastewaters. In pharmaceutical industry,

differentiation of morphine from codeine is possible by means of polyphenol oxidase immobilized electrodes.

In this study, a thermophilic fungus, *Thermomyces lanuginosus* was evaluated in terms of poyphenol oxidase production. The effect of different nutrient sources, inducers and fermentation parameters on enzyme production were investigated and maximum PPO activity of 97 U/ml was observed in bioreactor experiments at 50°C, 400 rpm and pH 8.0 in a fermentation medium containing 1.4% yeast extract, 0.3% $MgSO_4$, 1% KH_2PO_4, 0.003% $CuSO_4$, 0.032% gallic acid. Type of polyphenol oxidase produced by *Thermomyces lanuginosus* was determined as laccase.

For biochemical characterization studies, the enzyme was enriched by electrophoresis. Temperature and pH optima for the enzyme were determined as 60°C and 8.0, respectively. Enzyme retained 67% activity after 1 h incubation at 80°C and retained 87% of its activity after 1 hour of incubation at pH 9.0 at room temperature. The enzyme obeys Michealis-Menten kinetics with K_m and V_{max} values being 5 mg /ml catechol and 38 U/ml, respectively. Molecular weight of the enzyme was determined as 29 kDa and isoelectric point of enzyme was found to be approximately 6.0.

Key words: Polyphenol oxidase, laccase, thermophilic fungus, *Thermomyces lanuginosus*, enzyme production, biochemical characterization.

ÖZ

POLİFENOL OKSİDAZ'IN *THERMOMYCES LANUGINOSUS* İLE ELDESİ VE BİYOKİMYASAL KARAKTERİZASYONU

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Polifenol oksidazlar fenollerin kinonlara dönüşmesini oksijen varlığında katalizleyen enzim sistemleridir. Polifenol oksidaz enzimleri çeşitli uygulamalarda kullanılmaktadır. Çay, kahve ve kakao üretiminde aroma arttırıcı olarak ayrıca diğer bazı meyve ve sebzelerde kalitenin tayini amacı ile kullanılmaktadırlar. Tıpta Parkinson, lösemi ve fenilketonüri gibi çeşitli hastalıkların tedavilerinde, atık su arıtımında, zararlı fenolik maddelerin giderilmesi amacı ile kullanılmaktadırlar.

Ayrıca farmasötik endüstrisinde, immobilize polifenol oksidaz enzimi içeren elektrotlar ile morfinin kodeinden ayrılması mümkün olmaktadır.

Bu çalışmada termofilik bir küf olan *Thermomyces lanigunosus*'un hücre dışı polifenol oksidaz üretimi incelenmiş olup, çeşitli besin kaynaklarının, indükleyici bazı maddelerin ve değişik fermantasyon parametrelerinin enzim üretimi üzerine etkisi araştırılmıştır. En yüksek enzim aktivitesi (97 U/ml), bioreaktör ile 50°C, 400 rpm ve pH 8.0 de yapılan fermantasyonlarda %1.4 yeast extract, %0.3 $MgSO_4$, %1 KH_2PO_4, %0.003 $CuSO_4$ ve %0.032 gallik asit içeren ortamda görülmüştür. *Thermomyces lanigunosus* tarafından üretilen polifenol oksidaz tipinin lakkaz olduğu belirlenmiştir.

Biyokimyasal karakterizasyon için, enzim elektroforez ile saflaştırılmıştır. Enzim için en uygun sıcaklık ve pH değerleri sırası ile 60°C ve 8.0 olarak belirlenmiştir. Enzim 80°C de 1 saatlik inkübasyon sonrasında aktivitesinin %67 sini , pH 9 ve oda sıcaklığında 1 saatlik inkübasyon sonrasında da aktivitesinin %87 sini koruduğu gözlenmiştir. Enzimin Michealis-Menten kinetiğine uyduğu görülmüş, K_m ve V_{max} değerleri sırası ile 5 mg /ml katekol 38 U/ml bulunmuştur. Enzimin moleküler ağırlığı 29 kDa ve izoelektrik noktasıda 6.0 civarında tespit edilmiştir.

Anahtar Kelimeler: Polifenol oksidaz, lakkaz, termofilik fungus, *Thermomyces lanigunosus*, biyokimyasal karakterizasyon, enzim üretimi.

To my mother and father

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ABBREVIATIONS

ADA	4-amino-N,N-diethylaniline
APS	Ammonium persulfate
BSA	Bovine serum albumin
IEF	Isoelectric Focusing
MW	Molecular Weight
MWCO	Molecular Weight Cut Off
OD	Optical Density
PPO	Polyphenol Oxidase
R_f	Relative mobility
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis

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INTRODUCTION

1.1 Structure of Phenols

Phenols comprise a class of aromatic organic compounds having at least one hydroxyl group attached directly to the benzene ring.

Phenol was isolated for the first time from coal tar, in 1834. At room temperature, it is a white, crystalline material that melts at 41°C. Initially, its disinfecting properties were discovered and used to treat wounds. Soon thereafter, the synthetic potential of phenol was realized and a vast number of derivatives were synthesized, preparing the ground for today's high-quality, high-value phenol derived products. Phenols and their ethers are ubiquotus in nature. Some derivatives have medical and herbicidal applications. Aqueous solutions of phenols are used as disinfectants. However main use of phenol is production of phenolic polymers also called phenolic resins which are used in insulation, laminating, plywood and moulding compounds. Substituted phenols such as benzenediols and

benzenetriols have uses in photography, dyeing and tanning industries. The compound bisphenol-A is an important monomer in materials widely employed in the manufacture ofdurable plastics, food packaging and dental sealants and coating inside beverage cans.

The presence of hydroxyl groups in the molecules of phenols means that phenols are like alcohols in being able to form strong intermolecular hydrogen bonds. This hydrogen bonding causes phenols to be associated and therefore to have higher boiling points than hydrocarbons of the same molecular weight. The ability to form strong hydrogen bonds to molecules of water confers on phenols a modest solubility in water (Vollhardt and Schore, 1998)

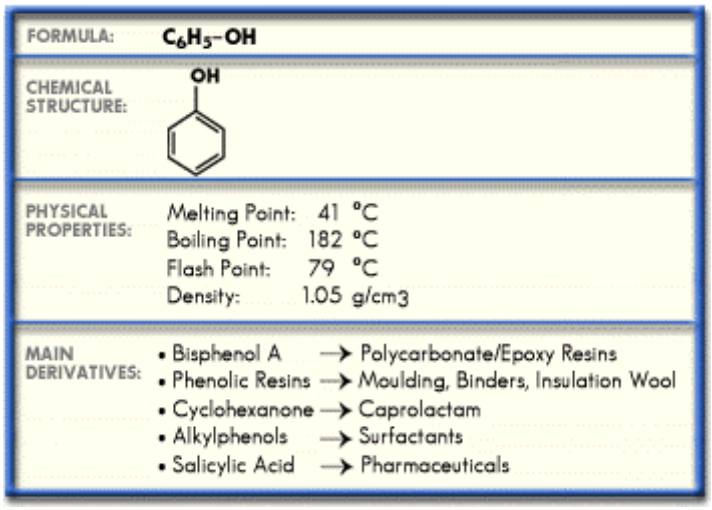

Figure 1.1 Structure, physical properties and main derivatives of phenols

1.2 Polyphenols in Nature

With over 8,000 variations having been identified, polyphenols are common substances throughout the plant kingdom. They provide an important role in plant metabolism, provide some defense against predators (by their astringency), form the brilliant colors in many fruits and vegetables, and prevent premature seed germination. Being so ubiquitous, polyphenols naturally form an integral role in human and animal diets. Until recently, their role had been classified by animal nutritionists as "anti-nutrients" due to the adverse affect on protein and carbohydrate digestibility of tannin, which is itself a compound polyphenol.

Current research has focused on another class of plant phenolics, the flavonoids, which exhibit considerable antioxidant properties by scavenging free radicals and other reactive oxygen species formed during normal metabolism. Left unattended, free radicals attack DNA and eventually accelerate the aging process and likelihood of cancer. Flavonoids are responsible for reducing the risk of cardiovascular diseases, inhibiting LDL cholesterol oxidation (Waterhouse *et al*.,1996) and modulating the immune functions (Sanbongi *et al*., 1997)

On the other hand, phenolic compounds in the aquatic environment can arise from natural substance degradation, from agricultural practices and industrial activities (e.g. pulp and paper industry, petrochemical works). So

far, attention has primarily been drawn to nitrophenols (Wiggins and Alexander 1988) and chlorinated phenols (Svenson and Zhang 1995, Boyd *et al*., 2001) as priority pollutants. Phenols, cresols and dimethylphenols have been considered of lower environmental hazard as they are relatively easily biodegradable in activated sludge systems (Brenner *et al*., 1992, Kahru *et al*., 2000, Orupõld *et al*., 2001). However, in recent scientific literature, the short-chained alkylated phenols as potential environmental pollutants have received attention as there is increasing evidence on the contamination of groundwater by these compounds (Licha *et al*., 2001). Phenol, cresols, dimethylphenols and resorcinols (further referred to as 'phenols') have been considered as major pollutants in the oil-shale semi-coke dump leachates (Sooba *et al*., 1997, Kahru *et al*., 1998 and 1999) making up 9–32% of the total chemical oxygen demand (COD) of these leachates (Orupõld *et al*., 1997 and 2000). The analysis of the leachates containing 24–195 mg phenols/L showed that their toxicity (EC50) for *Daphnia* and photobacteria was 1–6% (Kahru *et al*., 2000), whereas the phenolic compounds caused 4–50% of the net toxicity of these leachates (Kahru *et al*., 1999). The total amount of leachates can reach 8000 m^3 /day; they contaminate the surrounding soils and are a potential threat to the groundwater that is the source of drinking water in this region. Previous studies showed that the soils and semi-coke solid wastes from the oil-shale region were polluted with PAHs (up to 434 mg/kg) and oil products (up to 7231 mg/kg). However, the

amount of water-extractable phenols (measured after 24 h of the shaking of soils with water) was very low (up to 0.7 mg/kg, Põllumaa *et al*., 2001).

1.3 Polyphenol Oxidases

In literature three types of polyphenol oxidases which were classified according to their ability to oxidase different types of phenolic compounds.

Tyrosinase (monophenol monooxygenase E.C. 1.14.18.1) oxidizes monophenols such as tyrosine, p-cresol and p-coumaric acid but not diphenols and triphenols (Figure 1.2) . Tyrosinase is mosty found in animals including humans and it is important for hair, skin and eye pigmentation and a number of diseases result from insufficient or too much activity (Whitaker, 1994).

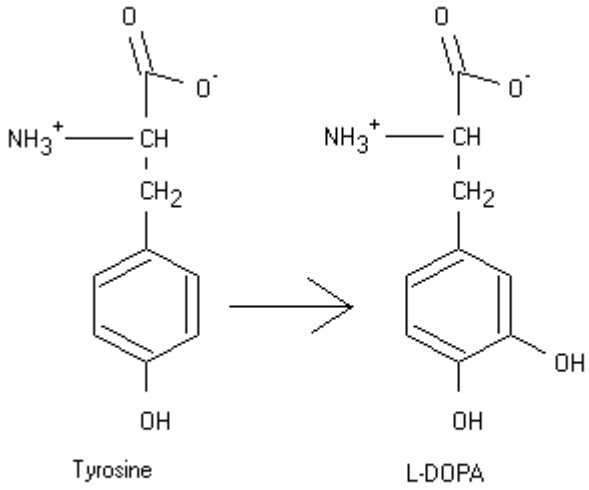

Figure 1.2 Reaction of Tyrosinase

Second type of polyphenol oxidase (1,2- benzenediol: oxygen oxidoreductase; E.C 1.10.3.1) is also known as polyphenolase, phenolase, catechol oxidase, cresolase, or catecholase mostly found in higher plants especially mushroom, apple, peach, tobacco and tea leaves (Whitaker, 1994). These enzymes in higher plants and fungi oxidize a great variety of monophenolic and o-diphenolic compounds and catalyze two types of reactions. First reaction involves the hydroxylation of a monophenol to give a diphenol (Fig. 1.3) and second reaction involves the removal of hydrogens from diphenol to give quinone (Fig 1.4).

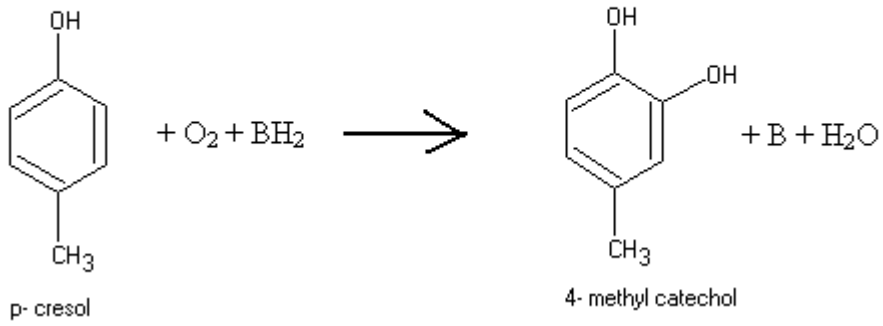

Figure 1.3 Hydroxylation reaction by catechol oxidase

There is another enzyme, laccase (E.C. 1.10.3.2) most often found in fungi, catalyzes the oxidation of monophenols, o- and p-diphenols, aminophenols and diaminoaromatic compounds. Different from other types, laccase is not inhibited by carbon monoxide and it has the largest substrate

specificity among all polyphenol oxidase enzymes (Mayer, 1987). In fungi laccase is responsible for lignin degradation, mycelial morphogenesis, detoxification of toxic compounds and pigment formation. Their function varies according to the environmental factors.

Polyphenol oxidases are found in almost all living organisms including plants, animals, bacteria and fungi.

In plants, polyphenol oxidase is involved in defense mechanism. When a plant gets a bruise or cut, certain phenolic compounds are oxidized in the presence of oxygen to form a polymer structure in case of oxygen penetration or microbial contamination. In insects polyphenol oxidase is responsible for exoskeleton formation. In animals it is important for pigmentation and as mentioned above, function in fungi depends on the environmental factors (Whitaker, 1994).

In terms of molecular weight of polyphenol oxidases, little is known about the overall diversity. Molecular weights of different polyphenol oxidases vary according to the source of the enzyme. Molecular weights of plant polyphenol oxidases are approximately 144,000. The generally accepted molecular weight of mushroom polyphenol oxidase is 128,000 Da . Whereas other fungi polyphenol oxidases have molecular weights varying from 46,000 to 88,000 Da.

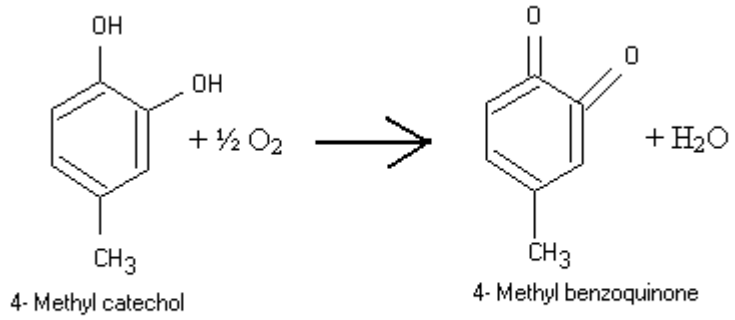

Figure 1.4 Dehydrogenation reaction by catechol oxidase

For example *Neurospora crassa* polyphenol oxidase has a molecular weight of 46,000. In *Alternaria* it is 88,000. In bacteria, *Streptomyces glaucescens* and *Streptomyces antibioticus* polyphenol oxidases have molecular weights of 30,900 and 30,736, respectively and. In mammals human polyphenol oxidase is about 62,000 and rat polyphenol oxidase is 58,000 however there is little homology between these two mammalian polyphenol oxidase (Whitaker, 1994).

Isoelectric point of different polyphenol oxidase can vary according to the nature of the habitat. The pI values of *Basidiomycete* laccase vary in the range of 3.0-5.0. The pI of *Cynodon hirsutus* laccase is 4.0 (Skrobogat'ko *et al*., 1998).

In terms of temperature, optimum value for the activity of the polyphenol oxidases varies in a wide range. Among all organisms fungi

polyphenol oxidases are considered to be the most active and stable enzymes. The highest optimum temperature for fungal polyphenol oxidases was observed in *Thermoascus aurantiacus* which can optimally perform its function at 70 – 80 °C. This enzyme also has a significant thermostability between 50-100°C (Machuca *et al*., 1998).

Different substrates oxidized by polyphenol oxidases are shown in table 1.1

Table 1.1 Substrates of polyphenol oxidases

Di- or triphenolic compounds	Catechol
	4-methyl catechol
	d-catechin
	Chlorogenic acid
	Caffeic acid
	Protocatechuic acid
	3,4-Dihydroxy-L-phenylalanine
	Dopamine
	Gallic acid
	Pyrogallol
Monophenolic compounds	*p*-Cresol
	p-Coumaric acid

Optimum temperature for *Aspergillus nidulans* polyphenol oxidase is 40°C (Bull *et al*., 1972). Another polyphenol oxidase from *Chaetomium thermophile* has an optimum temperature of 50°C.

Optimum pH values of polyphenol oxidases also vary denpending on the source organism. For example optimum pH level for *T.aurantiacus* polyphenol oxidase was found to be 2.8 and above pH 5.0 it was found to be inactive and other ten fungal laccases have been found to perform their activities in a pH range of acidic region. However *Phizoctina praticola* laccase is mostly active between pH 6.5-7.5. *Chaetomium thermophile* polyphenol oxidase is active in the pH range of 4.5 to 5.5 and this enzyme was also found very stable between pH 4.0 to 11.0. On the other hand, *Coprinus cinerus* laccase pH optima have been found to vary depending on the substrate. Laccase of this organism can oxidize 2,2 azinobis at pH 4.0 and syringaldazine at pH 6.5 (Schneider *et al*., 1999). Besides, Aspergillus nidulans polyphenol oxidase can be given as an example of active polyphenol oxidase in alkaline region. It was found to be active at pH 6.0 – 8.5 (Bull *et al*., 1972).

1.4 Production of Polyphenol Oxidase

1.4.1 Use of Thermophilic Fungi

Polyphenol oxidases are found in almost all fungal strains and they are considered to be excellent sources for industrial polyphenol oxidase

production. Thermophilic fungi have an advantage particularly in fermentation conditions. Higher temperatures used in fermentations reduce the risk of contamination. Especially in industrial scale production, sterilization is an expensive parameter and fermentation at higher temperatures prevents the growth of undesired contaminants. From another point of view, fermentations performed at higher temperatures increase the cost however, thermophilic organisms produce thermostable products.

Today, thermophilic organisms as producers of certain desired products are under interest due to their resistance to heat, denaturants solvents and proteolytic enzymes with respect to their counterparts from mesophiles (Fontana, 1988). For instance *Chaetomium thermophile* polyphenol oxidase was purified and optimum pH and temperature value for the enzyme was found to be 5.0 and 55°C, respectively. This temperature is higher than the optimum temperature of *Alternaria tenius*, coco beans, tea leaves. Also polyphenol oxidase from *Chaetomium thermophile* is stable between pH 4.0-11.0 and temperatures up to 55°C (Ishigami *et al*., 1986). However the enzyme purified from *Alternaria tenius* was found to be active only at pH 4.0 – 5.5 and temperatures up to 40°C (Motoda, 1979a).

1.4.2 *Thermomyces lanuginosus*

In this study polyphenol oxidase production was performed by using *T. lanuginosus* and the enzyme was characterized to determine possible application areas.

Thermomyces lanuginosus is considered to be a producer of several enzymes in industry. For instance it is used for the production of Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8) catalyzing the hydrolysis of phytic acid (*myo*-inositol hexakisphosphate) to the mono-, di-, tri-, tetra-, and pentaphosphates of *myo*-inositol and inorganic phosphate and these phosphates are essential nutrients of animal feeds (Randy *et al*., 1998).

Hemicellulases and xylanases are also produces by using *T. lanuginosus*. These enzymes are extensively used in paper and pulp industries (Singh *et al*., 2003).

A thermotolerant β–galactosidase is also produces by *T. lanuginosus* (Fischer *et al*., 1995).

1.4.3 Optimization of Polyphenol Oxidase Production

For production of polyphenol oxidase, all of the parameters such as temperature, pH, medium composition must be optimized. Also production of the enzyme is greatly influenced with the addition of some inducers.

In 1992, Assaving *et al.,* studied the effect of carbon sources on polyphenol oxidase production by *Aspergillus*. In this study, lignin hydrolyzate (waste product of paper and pulp industry) was added into the medium and during growth lignin was degraded with the formation of soluble aromatic products and accumulation of these products leads to 60-100 fold increase in tyrosinase production.

In another study, nutritional requirements of *Alternaria tenius* for polyphenol oxidase production were investigated. Glucose, sucrose and fructose were found to be excellent carbon sources for enzyme production and $CuSO_4$ was the most effective salt source. Ammonium salts appeared to be good nitrogen sources for enzyme production, while organic nitrogen sources seemed to be ineffective. Also adjusting the pH of the medium during cultivation was found to be useful for enzyme production (Motoda *et al.*, 1971).

In 1978, Motoda studied the effect of yeast extract and tea extract on polyphenol oxidase production by *Alternaria* tenius. In this study, polyphenol oxidase was produced in the filtrate of cultures shaken at 30°C in the medium containing either yeast extract or tea extract and metal ions. However, the production was repressed in the presence of both yeast extract and tea extract. In medium without tea extract, the enzyme activity increased with the increase in the amount of yeast extract added, but the addition of both Cu^{2+} and Mo^{6+} had no effect on enzyme production despite the effect seen in medium containing tea extract. The optimum concentration of yeast extract for the enzyme production was 1%, and lower rates of aeration led to an increase in the yield of enzyme.

Trametes pubescens known as an efficient polyphenol oxidase producer was studied and different carbon sources were used for improving

the polyphenol oxidase production. Glucose and cellobiose were found to be the most effective carbon sources which were also utilized well by the organism (Galhaup *et al., 2002*).

In another study, effect of different nitrogen sources on polyphenol oxidase production by *Trametes pubescens* was studied. Peptone from meat was found to be the best nitrogen sources for enzyme production. (Galhaup *et al.*, 2002).

In another study done by Machuca, *Thermoascus aurantiacus* polyphenol oxidase production increased 2-10 fold with the addition of an aromatic compound (p-anisidine) as an inducer. (Machuca, 1998).

Different *Basidiomycetes, Ascomycetes* and *Deuteromycetes* species were studied about whether 2,5,xylidine (phenolic compound) had an inductive effect in terms of polyphenol oxidase production and inductive effect was observed only in *Basidiomycetes*. Whereas 2,5-xylidine had no inductive effect on other fungi (Bollag and Leonowicz, 1984).

Laccase production from *Botyris cinerea* have been studied and found that laccase was produced when fungus was grown on malt with wine juice. However enzyme was not produced in the absence of malt juice. Afterwards, researchers tried to promote the enzyme formation by Cu^{+2} and

cycloheximide. However no further induction could be observed. Then different phenolic compounds such as gallic acid, tannic acid, 2,5 xylidine, caffeic acid, ferulic acid and 4-methyl catechol was tried and gallic acid was found to be the most effective inducer for enzyme production. Tannic acid was also found as an effective inducer. On the other hand caffeic acid exhibited an induction at lower extent with respect to the gallic and tannic acid. Other phenolic compounds showed no induction. (Gigi *et al.,* 1980).

In another study, influence of copper on laccase production of *Neurospora ccrassa* was studied (Schilling *et al*., 1992). Due to the reason that polyphenol oxidase enzymes are copper containing enzymes, copper concentration must be taken into account as an important parameter for the optimization of polyphenol oxidase production in microbial processes. The results revealed that copper strongly influenced the production of both laccase and tyrosinase during fungal growth.

1.5 Applications of Polyphenol Oxidases

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

In food processes, polyphenol oxidases have been mainly used for enhancement of the flavor in tea, coffee and cocoa (Motoda, 1979b). Tea is manufactured in a process, during the main chemical damages in the tea leaves take place. In the process, polyphenols abundantly present in tea

leaves are oxidized by the action of polyphenol oxidase contained in the leaves themselves, and the oxidation products in the black tea contribute to color, strength and flavor in liquors. Therefore, polyphenol oxidase activity of tea leaves and the oxidation products play an important role in the formation of black tea.

On the other hand, in some food processes, polyphenol oxidase activity is undesirable and plays an important role in determination of food quality. Many fruits including peaches, apricots, apples, grapes, bananas, strawberries, and the vegetables potatoes, lettuce, mushrooms and eggplants are lost due to the action of polyphenol oxidase activity because activity in mechanically damaged foods leads to a browning reaction in the presence of O_2, and this causes a nutritional loss and being unacceptable for the consumer. In this respect, the controlled PPO activity is supposed to be important in control of the quality (Whitaker, 1994).

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

the polyphenol oxidase in strecker degradation decreases the cost of the process and saves the producer from the heating process (Motoda, 1979b).

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

According to the recent studies, attachment of the *Streptococcus* to the tooth surface was inhibited by the water extract of potato polyphenol oxidase (Cowan *et al*., 200).

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

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

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

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

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

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

1.6 The Aim of the Work

The objective of the work is to produce thermostable polyphenol oxidase from *Thermomyces lanuginosus* and characterization of the polyphenol oxidase.

Study was initiated in shake flasks in order to determine the optimum nutritional requirements of the organism such as carbon, nitrogen, salt sources and and inducers for enzyme production and also to determine the optimum fermentation temperature.

Fermentation in shake flask experiments were followed by bioreactor experiments carried out to determine the optimum pH and dissolved oxygen levels for enzyme production.

Afterwards, enzyme characterization studies were performed for determination of several parameters such as optimum temperature and pH for enzyme activity and stability, K_m and V_{max} values, molecular weight and isoelectric point of the enzyme in order to designate the possible utilization areas of the enzyme.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Catechol and gallic acid were obtained from Sigma Chem Ltd., (USA). All other chemicals used were analytical grade from Sigma (USA), Merck (Germany) or Carlo-Erba (Spain).

2.2 Microorganism

In this study, a thermophilic white-rod fungus *Thermomyces lanuginosus* IMI-84400 strain kindly provided by Peter Biely (Slovak Academy of Sciences) was used for polyphenol oxidase production.

2.3 Maintenance and Cultivation

Thermomyces lanuginosus was grown on standard YpSs (Yih *et al.,* 2000) agar plate at 45°C until sporulation and stored at 20°C in an incubator. Stock

cultures were subcultured every 3 weeks and were used for the inoculation of pre-cultures. Different liquid media were tested to find the best medium for the isolate to grow and produce polyphenol oxidase. The compositions of all the culture media are given in the Appendix A.

2.4 Determination of Optimum Culture Conditions

Fermentations were performed in either shake flasks (250 ml) or in a 3 L bioreactor (Probiotem). Optimization of medium composition, and fermentation temperature studies were performed in shake flask experiments. In shake flask fermentations, *T.lanuginosus* strain IMI-84400 was cultivated in 50 ml erlenmayer flasks containing 10 ml of preculture medium containing 0.4% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$ and 1.5% glucose at 50°C, 155 rpm in a shaker incubator (Minitron). These precultures (10 ml) were used to inoculate 100 ml different enzyme production media in 250 ml flasks. Incubations were performed in a shaker incubator at 50°C and 155 rpm for 4-5 days. Optimization of pH and dissolved oxygen levels were performed in 3 L bioreactor having both pH and dissolved oxygen control units (Probiotem). In bioreactor experiments the fungus was cultivated in 250 ml erlenmayer flask containing 100 ml of preculture medium containing 0.4% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 1.5% glucose. This preculture (100 ml) was used to inoculate 2 L enzyme production medium in 3 L bioractor containing 1.4% yeast extract, 0.3% $MgSO_4$, 1% KH_2PO_4, 0.003% $CuSO_4$ and 0.032% gallic acid. Fermentations were performed

at 50°C, 400 rpm and different pH and dissolved oxygen levels. Daily samples were taken from the fermentation medium to determine polyphenol oxidase activity until polyphenol oxidase activities started to decrease.

2.4.1 Cell Growth Studies

Growth measurements were performed by weighing dried cell mass. Cells were harvested by filtration of the production medium through previously dried and tared Whatman No.1 filter paper and dried in either 60°C incubator or desiccator until constant weight was reached.

2.4.2 Polyphenol Oxidase Assay

Polyphenol oxidase activity was measured specrophotometrically. Culture supernatant was used as crude enzyme source. 100 mM cathecol in 0.2 M Phosphate buffer at pH 7.0 was used as substrate. To prepare the substrate, 0.11 g catechol was mixed with 10 ml 0.2 M phosphate buffer (pH 7.0) which was previously heated to 50°C and then substrate mixture was vortexed to dissolve catechol.

The reaction mixture contained 1ml 0.2 M phosphate buffer (pH 7.0), 0.5 ml culture supernatant and 0.5 ml substrate solution (100 mM) incubated at 50°C for 3 minutes. The reference cuvette contained buffer instead of enzyme and

change in absorbance was followed at 410 nm and initial reaction rate was used to determine the enzyme activity (Maria *et al* ., 1981).

One unit of enzyme (U) is defined as the amount of the enzyme required to obtain 0.01 O.D change at 410 nm at 50 °C at pH 7.0.

2.5. Protein Determination

Protein concentration was measured according to the modified Bradford method by using bovine serum albumin (BSA) as a standard (Bradford, 1976). The composition of the reagents, preparation of the standard curve and the protocol are given in appendix B, C and D.

2.6 Optimization of Growth Conditions in Shake Flask Experiments

2.6.1 Influence of Carbon Sources on Growth and Enzyme Production

Different carbon sources were added into the fermentation medium in order to induce the polyphenol oxidase production and growth. For this purpose 3% starch, glucose, fructose, cellulose, avicel (microgranulated cellulose for column chromatography), xylose and xylan were used in the fermentation medium. Each carbohydrate was tested at least in two flasks. Samples were taken at regular intervals to follow the polyphenol oxidase activity and growth was determined by weighing the dried cell mass.

2.6.2 Effect of Nitrogen Sources on Growth and Enzyme Production

The effect of different organic (yeast extract, casein + peptone) and inorganic nitrogen sources (sodium nitrate, ammonium sulphate) on enzyme production were determined. The concentrations of organic and inorganic nitrogen sources were selected as 1.5% and 2%, respectively, by considering the higher efficiency of organic sources than inorganic ones. Samples were taken at regular intervals to follow the polyphenol oxidase oxidase activity and growth was determined by weighing the dried cell mass.

2.6.3. Optimization of Copper Concentration

As it has been mentioned in the first chapter, polyphenol oxidases are extremely dependent on copper for performing their activities (Lerch, 1983). Due to this reason, concentration of copper in the production medium was also studied. For this purpose, $CuSO_4$ at different concentrations varying from 0 to 80 ppm were used in fermentation media. Activity measurements were carried out at regular intervals in order to determine the enzyme production.

2.6.4 Effect of Phenolic Compounds on Enzyme Production

Also different phenolic compounds were used in a concentration varying from 1 to 3 mM in order to induce the enzyme production. Gallic acid, caffeic

acid, coumaric acid, cresol and L-DOPA were used phenolic compounds. In addition 2% tea extract and olive oil process waste were used as phenol source in order to increase the enzyme production. Activity measurements were carried out at regular intervals in order to determine the enzyme production.

2.6.5. Effect of Cultivation Temperature on Enzyme Production

Fermentations were carried out at different temperatures varying from 40 to 55 °C in order to determine the optimum temperature for the maximum enzyme production.

2.7. Optimization of Fermentation Parameters in Bioreactor

2.7.1 Effect of Fermentation pH on Enzyme Production

Fermentations were carried out at pH levels varying from 6.0 to 9.0 in bioreactor in order to determine the optimum pH value for the maximum enzyme. production. Since the organism always tends to decrease the pH of the culture medium, only NaOH was used to control the pH of culture medium. Activity measurements were carried out at regular intervals to follow the enzyme production.

2.7.2 Effect of Dissolved Oxygen on Polyphenol Oxidase Production

Fermentations were performed in a 3 L bioreactor containing 2 L enzyme production medium at 70, 80 and 90% dissolved oxygen levels in order to determine the optimum dissolved oxygen level for enzyme production.

2.8 Concentration and Partial Purification of Polyphenol Oxidase

2.8.1 Crude Extract Preparation

All steps were performed at room temperature. After 24 h of growth under the conditions described above, the cells were filtered through Whatman No 1 filter paper and then insoluble materials were removed by centrifugation in a Sigma centrifuge at 14000 x g for 16 min at 25°C Then supernatant was used as the crude extract.

2.8.2 Ultrafiltration

The crude extract from the 3rd or 4th day of the cultivation was concentrated by using 50 ml Amicon stirred cells ultrafiltration apparatus. Ultrafiltration was conducted with Sigma Milipore disk membrane with NMWCs of 10,000 Da by applying a gauge pressure of 2.5 bar with nitrogen tube. A volume of 50 ml crude extract was concentrated to 10 ml. Then a further

concentration by using a vivapore concentrator (Sigma) with a NMWC of 7500 was carried out before electrophoretic studies.

2.8.3 Ammonium Sulfate Fractionation

Ammonium sulfate were added slowly to the 50 ml crude extract to give required saturation and stirred gently for at least 1 h at room temperature after all ammonium sulfate completely dissolved. Then the solution was centrifuged at 14000 g for 16 min. Thereafter the pellet was dissolved in a minimum amount of 50 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer overnight at room temperature to remove ammonium sulfate and other small molecules present in the medium.

2.8.4 Concentration by Sucrose

Concentration with sucrose was also studied in order to evaluate whether it was useful for concentration of the enzyme. For this purpose crude extract in a dialysis tube (50 ml) was put in a saturated sucrose solution and gently stirred until the volume of the crude extract reached half of the volume at the beginning. Thereafter the solution was dialyzed against the 50 mM phosphate buffer (pH 7.0) overnight at room temperature.

2.8.5 Enrichment of Polyphenol Oxidase by Electrophoresis

An analytical zymogram technique allowing the differentiation of laccase, peroxidase and catechol oxidase enzymes on the same polyacrylamide gel electrophoresis was used to visualize polyphenol oxidase bands (Rescigno *et al*., 1997). In this method samples were concentrated 10 fold by Vivapore concentrators (Sigma) having 7.500 NMWC and enzyme remained active under non-denaturing conditions even in the presence of 0.2% (w/v) sodium dodecylsulfate (SDS).

Vertical slab gel electrophoresis was performed with the addition of 0.2 % SDS and β- mercaptoethanol was excluded from sample application buffer and samples were not denaturated before application by heating . The gel had a monomer concentration of 12% polyacrylamide, thickness of 1 cm. Length and width of the gel were 10 and 8 cm, respectively. . Electrophoresis was carried out for 4-5 h at 80 V and room temperature. After electrophoresis, the gel was cut and one piece was first soaked in 0.1 M phosphate buffer (pH 7.0) for 5 min at room temperature. Then, freshly prepared 25 mM 4 amino- N-N diethylalanine (ADA) in 10 mM HCl was added to the gel and shaken gently in a shaker incubator at 50 °C in order to observe the corresponding laccase activity. After 5 min, the excess solution was poured off, and the gel was rapidly washed with the same buffer above. For observing the peroxidase activity, the gel was soaked in 10 mM H_2O_2 and gently shaken in a shaker incubator at 50 °C for 5 minutes. After washing

with the same buffer, the gel was treated with 25 mM 4 tert butyl-catechol (tBC) dissolved in 10 mM acetic acid for determining the corresponding cathecol oxidase activity.

After the activity staining was applied to one part of the gel, the stained side was lined up along the edges of the unstained gel and used as a guide to cut out the band of interest from the unstained gel and a narrow longitudinal strip was cut out from unstained gel. Then, this thin band was cut into pieces and these pieces were inserted in a small dialysis bag with 3 ml 0.1 M sodium phosphate buffer pH 7.0 and 0.1% SDS in order to increase the protein recovery. Dialysis bag was then placed on the platform of gel electrophoresis chamber and electrophoresis tank was filled with enough electrode buffer and 100 mA constant current was applied for 2-3 hours to elute the protein from the gel. Afterwards, the buffer recovered from the dialysis bag contained the protein of interest and acrylamide was removed by centrifugation at 1000 x g for 10 minutes. Pure enzyme obtained after this step was used for characterization studies.

2.9 Characterization of Polyphenol Oxidase

Characterization studies were performed with both pure enzyme obtained from gel and crude extract.

2.9.1. Kinetic Analysis of Polyphenol Oxidase

Initial reaction rates of polyphenol oxidase activity were determined at different substrate concentrations ranging from 0,25 to 20 mg catechol/ml. Reaction rates were plotted against substrate concentration to determine whether the enzyme obeys Michealis-Menten kinetics. Kinetic constants were determined from Lineweaver-Burk plot.

2.9.2 Effect of Temperature on Polyphenol Oxidase Activity and Stability

Polyphenol oxidase was assayed at different temperatures varying from 50 to 80 °C to find the optimum temperature of the enzyme.

For determining the thermostability, both crude (0.25 mg/ml) and pure (0.02 mg/ml) enzyme solutions in 0.2 M phosphate buffer at pH 7.0 were incubated for 1 h at different temperatures ranging from 40 to 80 °C and then cooled prior to measuring residual activity under standard assay conditions at 50°C.

2.9.3 Effect of pH on Polyphenol Oxidase Activity and Stability

The optimum pH of polyphenol oxidase was measured by using buffers at different pH ranging from 4 to 10. Three different buffers having 0.2 M ionic

strength were used to cover the pH range, which were acetate buffer (pH 4-5.5), sodium phosphate buffer (pH 6.0-8.0), and glycine-NaOH buffer (pH 8.5-10.0).

The stability of the enzyme in a pH range from 4.0 to 10.0 was tested for both 1 h and 18 h treatments. Crude and purified enzyme solutions were incubated at six different pHs for both 1 and 18 h at room temperature, and then their pH were changed to 7.0 again by using 1 ml vivaspin concentrators having 7,500 MW cutoff membrane (Sartorius).

$$\% \text{ Residual Activity} = \frac{\text{Total enzyme activity after pH treatment}}{\text{Total enzyme activity before pH treatment}} \times 100$$

2.9.4 Electrophoretic Analysis

2.9.4.1 SDS Polyacrylamide Gel Electrophoresis

The purified polyphenol oxidase isolated from the gel was concentrated to 0.1 ml from 3 ml by using vivapore concentrator and used for molecular weight and isoelectric point determination. The SDS-PAGE was conducted in 4% stacking and 10% separating gels according to the Laemmli (1970). Samples for

SDS-PAGE containing 10 μg protein were mixed with the sample application buffer containing 2%(w/v) SDS, 5% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8) and heated in boiling water. The molecular weight standards used were the Fermentas Protein Molecular Weight Marker containing 116 kDa, 66 kDa, 45 kDa, 35 kDa, 25 kDa, 18 kDa and 14 kDa protein markers. Separation was achieved at constant voltage 50 V at stacking and 80 V at separating gels at room temperature.

Solutions used in this procedure and their methods of preparation were given in Appendix E and F.

2.9.4.2 Isoelectric Focusing

Analytical isoelectric focusing was performed over the pH range 3.0-10.0, by using ampholines from Biochemica. Standards for isoelectric focusing were all from Serva. Electrophoresis was carried out by BioRad isoelectric focusing device. Solutions used in this procedure and their methods of preparation were given in appendix G and H. Marker and enzyme were applied directly onto gel in a volume of 3 μl. Then the gel was conducted at constant 100 V for the first 15 min, followed by 200 V for 15 min and finally 300 V for 30 minutes.

2.10 Gel Processing

2.10.1 Comasssie Brilliant Blue Staining

SDS-PAGE gels were stained according to comassie brillant blue staining. SDS-PAGE gels were stained in a solution containing 0.1% comassie brilliant blue R-250, 40% methanol and 10% acetic acid. Destaining was achieved by a solution containing the same solution but comassie dye. Gels were kept in 7% acetic acid at 4°C.

R_f (relative mobility) values were calculated according to the following equation given below:

$$R_f = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by tracking dye}}$$

Molecular weight of polyphenol oxidase was calculated by using the standard graph constructed by plotting R_f values of the marker proteins against the logarithm of molecular weight of marker proteins.

2.10.2 Quick Stain

Isoelectric focusing gels were stained in a solution containing 3.5% perchloric acid and 0.025% Comasssie Brilliant Blue G for 1 hour. Then the gel

was soaked into 7% acetic acid solution for intensification of stain and preservation of the gel for a long time.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization of Polyphenol Oxidase Production

At the beginning of this study, the organism was grown in 300 ml Erlenmayer flasks containing 100 ml YpSs Avicel medium (Yih *et al*., 2000) at 45°C, 155 rpm for 6-7 days. The organism could produce the enzyme during first 4-5 days and the maximum enzyme activity was observed at the fourth day of inoculation.

3.1.1 Effect of Carbon Sources on Polyphenol Oxidase Production

While the organism were producing polyphenol oxidase with YpSs medium, Thermomyces medium remarkably increased the enzyme production and the latter was used in further studies. Thereafter different carbon sources, cellulose, starch, glucose, sucrose, fructose and xylose were added in a concentration of 3% to the fermentation medium containing 1.43% yeast extract,

0.3% $MgSO_4$, 1% KH_2PO_4 to enhance the polyphenol oxidase secretion for fermentations carried out in shake flasks at 50°C for 6-7 days.

Table 3.1 The effect of carbon sources on polyphenol oxidase production. Fermentation medium: 3% different carbon source, 1.43% yeast extract, 0.3% $MgSO_4$, 1% KH_2PO_4. The fermentations were performed at 45°C at 155 rpm in a shaker incubator. Cells were harvested at 4th day of fermentation.

Carbon source	**Activity (U/ml)**	**Cell Concentration (mg/ml)**	**Specific Activity (U/mg cell)**
None	12 ± 2	0.508 ±0.003	23.6
Avicel	7 ± 2	0.549 ± 0.006	12.7
Starch	6 ± 2	0.585 ± 0.015	10.2
Glucose	6.3 ± 0.9	0.589 ± 0.001	10.6
Xylan	1.1 ± 0.1	0.399 ± 0.002	2.75
Xylose	0	0.215 ± 0.005	0
Fructose	0	0.228 ± 0.004	0

As observed from Table 3.1, none of the carbon sources increased polyphenol oxidase production although some of them (avicel, starch and glucose) contributed to the growth. The maximum enzyme activity and specific activity were obtained at 4th day of incubation in a medium containing none of these carbon sources.

Similar results were obtained from a study done with *Alternaria tenius* (Motoda, 1978). In this study lactose, dextrin and fructose showed no effect on polyphenol oxidase production although they contributed to growth. On the other hand, *Alternaria tenius* polyphenol oxidase production was induced by using

sucrose, glucose and xylose which were not found to be beneficial in the case of *Thermomyces lanuginosus*.

3.1.2 Effect of Nitrogen Sources on Polyphenol Oxidase Production

The influence of different nitrogen sources was studied in order to increase polyphenol oxidase production. For this purpose, yeast extract, ammonium sulfate, sodium nitrate, casein and peptone were tested. Organic and inorganic nitrogen sources were added at 1.5 and 2%, respectively.

Maximum enzyme production was obtained when 1.5% yeast extract was used as nitrogen source on 4^{th} day of incubation (Table 3.2). Although use of sodium nitrate gave maximum specific activity, enzyme production was about 25% of the activity produced with the use of yeast extract. Following this, different concentrations of yeast extract, in the range of 0.5 - 3%, were used and enzyme production increased by increasing yeast extract concentration up to 2%. Then, a sharp decrease was observed probably because of the production or activation of the proteolytic enzymes with increasing protein concentration in the culture medium (Table 3.3). However, since the enzyme production was slightly higher at 2% than 1.5%, the latter was used in the following studies. In literature, best nitrogen sources differ from one fungus to another. For instance peptone from meat was found to be the most effective nitrogen source for *Trametes pubescens* in terms of polyphenol oxidase production (Galhaup *et* al., 2002)

However, for *Alternaria tenius*, inorganic nitrogen sources appeared to be more beneficial for this organism (Motoda, 1978).

Table 3.2 The effect of nitrogen sources on polyphenol oxidase production. Fermentation medium: Nitrogen source, 0.3% $MgSO_4$, 1% KH_2PO_4. The fermentations were performed at 45°C at 155 rpm in a shaker incubator. Cells were harvested at 4th day of fermentation.

Nitrogen source	**Activity (U/ml)**	**Cell Concentration (mg/ml)**	**Specific Activity (U/mg cell)**
Ammonium sulphate	3.4 ± 0.4	0.268 ± 0.002	12.6
Sodium nitrate	3.8 ± 0.2	0.115 ± 0.002	33.04
Yeast extract	12.75 ± 1	0.570 ± 0.01	22.3
Casein + peptone	0	0.117 ± 0.003	0

Table 3.3 The effect of yeast extract concentration on polyphenol oxidase production. Fermentation medium: 0.3% $MgSO_4$, 1% KH_2PO_4 and 0.5-3.0% yeast extract. The fermentations were performed at 50°C at 155 rpm in a shaker incubator. Cells were harvested at 4th day of fermentation.

Yeast extract concentration (%)	**Activity (U/ml)**	**Cell Concentration (mg/ml)**	**Specific Activity (U/mg cell)**
0.5	7.2 ± 0.4	0.350 ± 0.008	20.5
1.0	8.2 ± 0.4	0.498 ± 0.005	16.45
1.5	14.1 ± 0.4	0.625 ± 0.009	22.5
2.0	14.4 ± 1.6	0.771 ± 0.022	19.0
2.5	8.4 ± 0.2	0.610 ± 0.011	13.75
3.0	0.0	0.476 ± 0.005	0.0

3.1.3 Effect of Copper on Polyphenol Oxidase Production

Since all phenol oxidase enzymes are copper containing proteins and coppers are required for the active site, addition of copper was thought to be beneficial and copper sulfate concentration was optimized in order to increase polyphenol oxidase production (Huber *et al*., 1983). As observed in Fig. 3.1, addition of copper up to 30 ppm increased the enzyme production however further addition was detrimental.

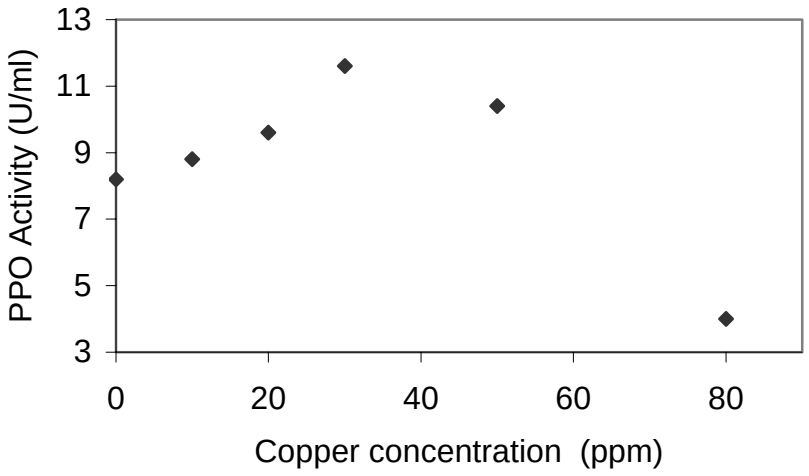

Figure 3.1 The effect of $CuSO_4$ concentration on polyphenol oxidase production. Fermentation medium: 1.43% yeast extract, 0.3% $MgSO_4$, 1% KH_2PO_4 and 10-80 ppm $CuSO_4$. The fermentations were performed at 45°C at 155 rpm in a shaker incubator.

Among fungi, copper appeared to serve as an inducer for polyphenol oxidase production. Optimum copper sulfate level for *Phanereochaete chrysosporium* was found to be 0.4 M (Edward *et al*., 1998). On the other hand, in *Alternaria tenius* polyphenol oxidase production, the formation of enzyme increased with the amount of copper sulfate and reached a maximum at a concentration of 10-15 ppm (Motoda, 1978).

3.1.4 Effect of Phenolics on Polyphenol Oxidase Production

In literature, significant increases in enzyme production were reported like 2-10 fold increase in *Thermoascus aurantiacus* polyphenol oxidase production with the addition of an aromatic compound (p-anisidine) as an inducer (Gigi *et al*., 1980). So several phenolic compounds, gallic acid, coumaric acid, caffeic acid p-cresol, L-DOPA, tea extract and oil process waste at 1 and 3 mM, were added into the culture media as inducers. However, only 3 mM gallic increased the enzyme production significantly (40%) (Fig. 3.2). The effects of other phenolics were less than 20%. Different *Basidiomycetes, Ascomycetes* and *Deuteromycetes* species were studied about whether 2,5,xylidine (phenolic compound) had an inductive effect in terms of polyphenol oxidase production and inductive effect was observed only in *Basidiomycetes*. Whereas 2,5-xylidine had no inductive effect on other fungi (Machuca *et al*., 1998).

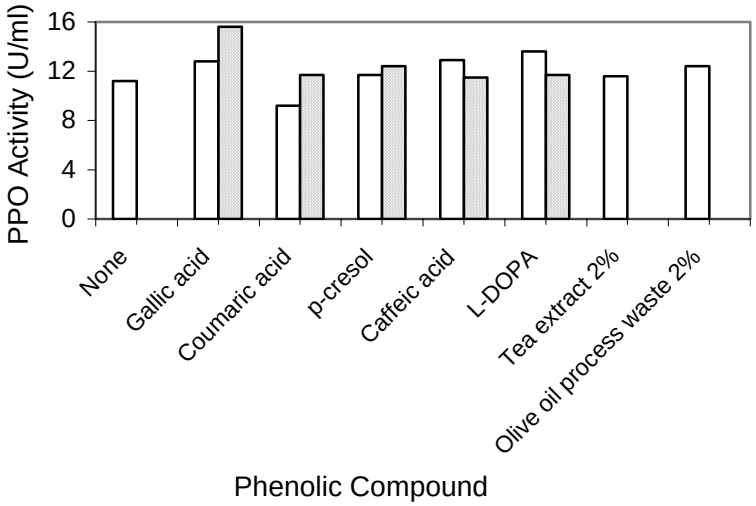

Figure 3.2 The effect of phenolics on polyphenol oxidase production. Fermentation medium: 1.4% yeast extract, 0.3% $MgSO_4$, 0.003% $CuSO_4$, 1% KH_2PO_4. Fermentations were performed in a shaker incubator at 45°C at 155 rpm for 4 days. [1mM (□), 3 mM (▒)]

3.1.5 Effect of Fermentation Temperature on Enzyme Production

The effect of temperature on enzyme production was investigated in shake-flask cultures. Four different temperatures, 40, 45, 50 and 55°C were tried and the best enzyme production was obtained at 50°C (Fig. 3.3).

3.1.6 Effect of Fermentation pH on Enzyme Production

To determine the optimum pH for enzyme production, fermentations were performed at different pH values. Firstly, pH was uncontrolled in order to determine the pH profile of the fermentation .

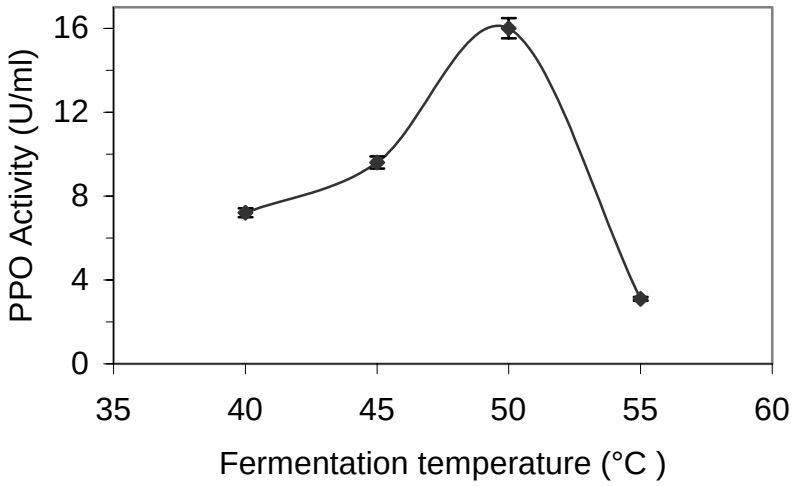

Figure 3.3 Effect of fermentation temperature on enzyme production. Fermentation medium: 1.43% yeast extract, 0.3% $MgSO_4$, 1% KH_2PO_4, 0.003% $CuSO_4$. Fermentations were carried out in a shaker incubator at 155 rpm. Cells were harvested at 4th day.

Afterwards, pH control was performed at pH 7.0, 8.0, 8.5 and 9.0. As shown in Fig. 3.4, maximum enzyme production was achieved at pH 8.0. At pH 8.5 enzyme production rate was almost the same with pH 8.0 until 2nd day. However a subsequent decrease was observed after 2nd day of fermentation.

Generally, fungal species like *Aspergillus oryzae, Trametes sp Alternaria tenuis* (Oyashiki *et al*., 1990), (Motoda, 1998). So *Thermomyces lanuginosus* has an advantage that it can be used at alkaline required applications.

Phanereochaete chryososporium produce polyphenol oxidase at low pHs varying from neutral to acidic (Edward *et al*., 1998), (Motoda, 1978),

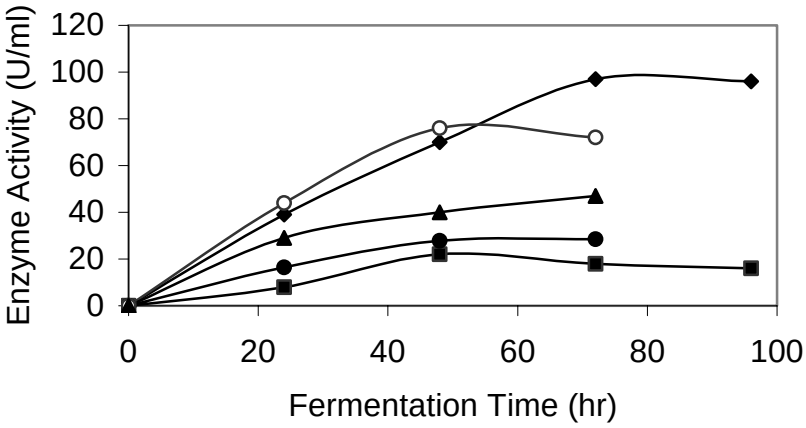

Figure 3.4 The effect of fermentation pH on PPO production. Fermentation medium: 1.4% yeast extract, 0.3% $MgSO_4$, 1% KH_2PO_4, 0.003% $CuSO_4$, 0.032% gallic acid. Fermentations were performed in a 3 L bioreactor at 50°C, 400 rpm, 80% dissolved oxygen at varying pH levels (pH 7.0 (●), 8.0 (♦), 8.5 (○), 9.0 (▲) and uncontrolled (■).

3.1.7 Effect of Dissolved Oxygen on Polyphenol Oxidase Production

For optimization of dissolved oxygen, different dissolved oxygen levels in the range of 70- 90% were tried. Although maximum enzyme production was observed at 80% dissolved oxygen, the difference between 80% and 90% was not significant (Fig 3.5). However, further increase in dissolved oxygen from 80 to 90% was not beneficial for the enzyme production.

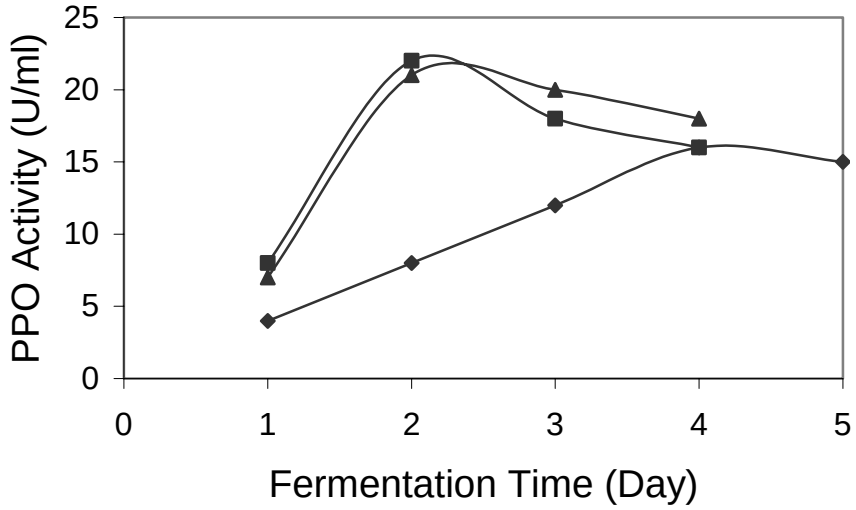

Figure 3.5 The effect of dissolved oxygen on polyphenol oxidase production. Fermentation medium: 1.4% yeast extract, 0.3% $MgSO_4$, 1% KH_2PO_4, 0.003% $CuSO_4$, 0.032% gallic acid. Fermentations were performed in a bioreactor at 50°C, at 400 rpm, pH 7.0, dissolved oxygen [70% (◆), 80% (■), 90% (▲)].

3.2 Concentration and Enrichment of Polyphenol Oxidase

3.2.1 Crude Extract Preparation

After 3-4 day cultivation in *Thermomyces* medium at 50°C, 155 rpm in a shaker incubator, the culture broth was filtered through Whatman No.1 filter paper to remove cells and insoluble materials were removed by centrifugation at 14.000 g at 25 °C for 16 min. Supernatant was used for further concentration studies. The specific activity of polyphenol oxidase in crude extract was 25.3 U/mg protein. For concentration of polyphenol oxidase, three different methods

were used which were ammonium sulfate fractionation, sucrose concentration and ultrafiltration.

3.2.2 Ammonium Sulfate Fractionation of Protein

Total of 100 ml of crude extract was subjected to various ranges of ammonium sulfate fractionation using solid ammonium sulfate salt at room temperature. Several saturation levels varying from 50 to 80% saturation were tested to measure the fractionation range of polyphenol oxidase. After the desired saturation was achieved, solution was stirred at least for 1 hour and then culture extract was centrifuged at 14.000 x g for 16 min at 25 °C and pellet was dissolved in a minimum amount of phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer. The maximum activity was obtained at 80% saturation. The purification fold was 3.88 in this step. The specific activity of the polyphenol oxidase after this step was 97.5 U/mg protein.

3.2.3 Concentration by Sucrose

Concentration with sucrose was also studied in order to evaluate whether it was useful for concentration of the enzyme. For this purpose 25 ml of crude extract (0.25 mg protein/ml) in a dialysis tube was put in a saturated sucrose solution and gently stirred until the volume of the crude extract reached half of the volume of the beginning. Thereafter the solution was dialyzed against the 50 mM

phosphate buffer (pH 7.0) overnight. The activity obtained after this step was 80 U/mg protein. The purification fold and recovery were 2.5, and 40%, respectively. (Table 3.4)

Table 3.4 Concentration of polyphenol oxidase from crude extract

Step	Vol (ml)	Activity (U/ml)	Total activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (u/mg)	Fold	Recovery
Crude extract	100	9.6	960	0.378	37.80	25	1.00	100.00
70% AS precipitate after dialysis	5	0.8	4	0.078	0.312	12	0.48	0.04
80% AS precipitate after dialysis	20	12.0	240	0.123	2.46	97	3.88	25.00

3.2.4 Ultrafiltration

The crude extract from the 3rd or 4th day of the cultivation was concentrated by using Amicon stirred cells ultrafiltration apparatus. Ultrafiltration was conducted with Sigma Milipore disk membrane with NMWCs of 10,000 Da by applying a gauge pressure of 2.5 bar. Crude extract (0.65 mg protein/ml) was concentrated 5 times. The concentrate obtained after this step had an activity of 71 U/mg protein and 3.12 fold purification was achieved.

3.2.5 Enrichment of Polyphenol Oxidase from SDS- Polyacrylamide Gel

The nature of the phenol oxidase activity was determined by activity staining. For this purpose differential screening technique allowing the

differentiation of laccase, peroxidase and catechol oxidase was used (Rescigno *et al*., 1997). Both laccase and peroxidase activities were observed but catechol oxidase activity was not present (Fig 3.6). Laccase was electrophoretically enriched and used in characterization studies. Peroxidase was not studied in detail. After the activity staining was applied to one part of the gel, the stained side was lined up along the edges of the unstained gel and used as a guide to cut out the band of interest from the unstained gel and a narrow longitudinal strip was cut out from unstained gel. Then, this thin band was cut into pieces and these pieces were sealed in a small dialysis bag with 3 ml 0.1 M sodium phosphate buffer pH 7.0 and 0.1% SDS in order to increase the protein recovery. Dialysis bag was then placed on the platform of gel electrophoresis chamber and electrophoresis tank was filled with enough electrode buffer and 100 mA constant current was applied for 2-3 hours to elute the protein from the gel. Afterwards, the buffer recovered from the dialysis bag contained the protein of interest and acrylamide was removed by centrifugation at 1000 x g for 10 minutes. Pure enzyme obtained after this step was used for characterization studies.

3.3 Characterization of Polyphenol Oxidase

3.3.1. Determination of Kinetic Parameters of Polyphenol Oxidase

Initial reaction rates of polyphenol oxidase activity were determined at different substrate concentrations ranging from 0.25 to 20 mg catechol/ml. Reaction rates were plotted against substrate concentration in order to determine

whether the enzyme obeys Michealis-Menten kinetics. Kinetic constants were determined from Lineweaver-Burk plot.

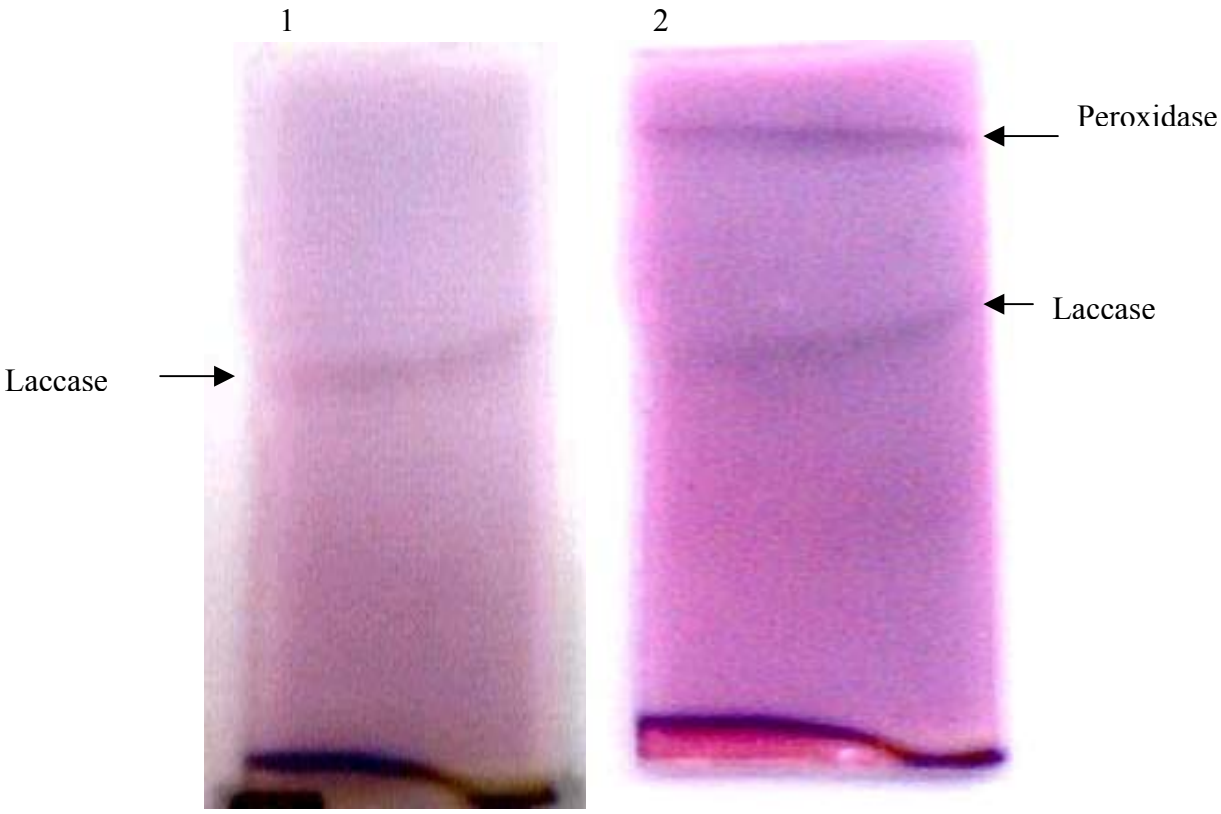

Figure 3.6 Laccase and peroxidase activities on polyacrylamide gel. (1) gel after the treatment with ADA, (2) gel after the treatment with H_2O_2.

Reaction rate at different catechol concentrations showed that polyphenol oxidase obeys Michealis-Menten kinetics. K_m and V_{max} values were determined as 5 mg /ml catechol and 38 U/ml respectively by using Lineweaver-Burk plot (Fig. 3.8).

In comparison with the other polyphenol oxidases from different organisms, polyphenol oxidase from *T.lanuginosus* has lower affinity .

Streptomyces glaucescens polyphenol oxidase has a K_m value of 0.5 mg/ ml catechol (Whitaker, 1994). K_m values for *Trametes sp.* P_1 and P_2 were found as 0.05 and 0.2 mg/ ml catechin, respectively (Khan, 1990). *Alternaria* polyphenol oxidase has a K_m of 0.12 mg/ ml catechin (Motoda, 1978). In plants *Nicotiana tobaccum* and *Jerusalem atichole* laccases have K_m values of 0.8 and 0.6 mg/ ml catechol, respectively (Ziyan and Pekyardimci, 2003), (Chunhua *et al*., 2001).

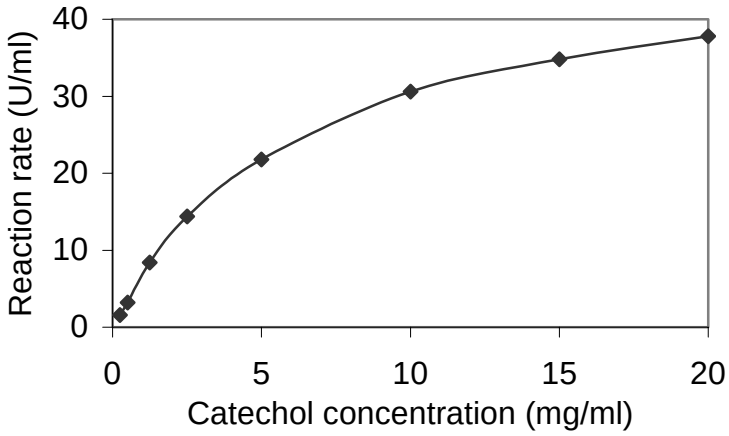

Figure 3.7 Michaelis-Menten plot for polyphenol oxidase

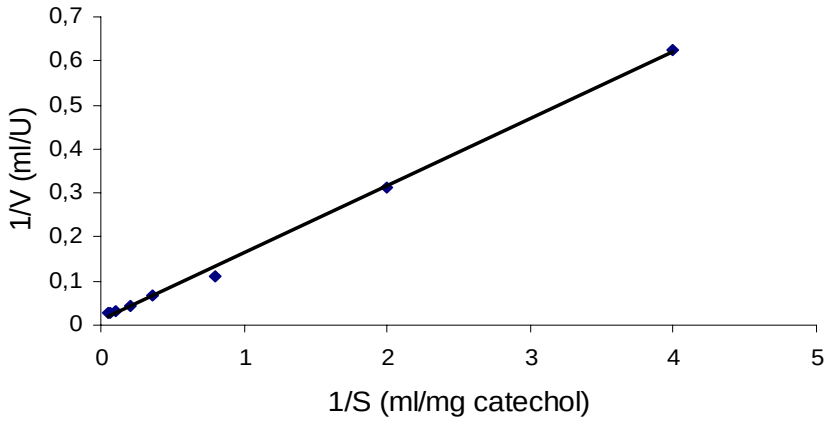

Figure 3.8 Lineweaver-Burk plot for polyphenol oxidase

3.3.2 Effect of Temperature on Enzyme Activity

Enzyme activity measurements were performed at different temperatures varying from 40 to 80°C. The experiments were performed by using both the crude and pure enzyme preparations.

As observed from Figure 3.9 the optimum temperature was 60°C.

The temperature profile of *T.lanuginosus* polyphenol oxidase can be regarded as moderately high within the range determined by other thermophilic fungi laccase. A thermophilic fungus *Chaetomium thermophile*, polyphenol oxidase optimum temperature was found to be 55°C (Ishigami and Yamada, 1988). Laccase of other thermophilic fungus *Thermoascus aurantiacus* polyphenol oxidase has an optimum temperature about 70-80°C (Machuca *et al.*, 1998)

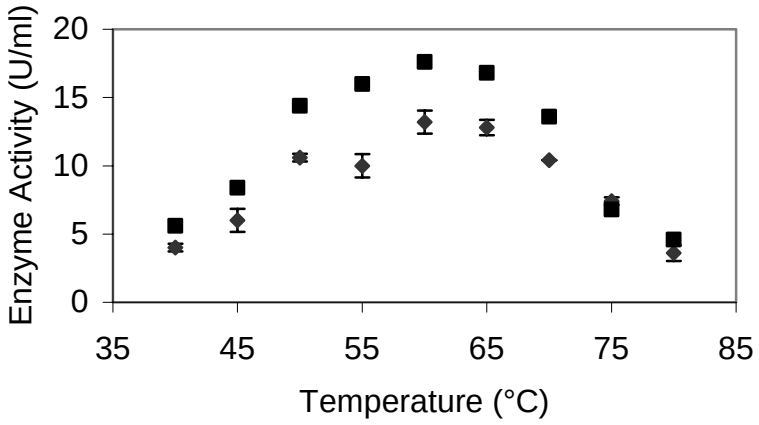

Figure 3.9 The effect of temperature on the activity of polyphenol oxidase. (♦) crude extract, (■) pure enzyme.

3.3.3 Thermostability of Polyphenol Oxidase

To determine thermostability, experiments were performed by incubating the enzyme solutions at various temperatures for 60 minutes. For both crude and pure enzymes retained 97% of their activities at 50°C.The crude enzyme(0.25 mg/ml) was more thermostable than pure enzyme (0.02 mg/ml) because it was in a more concentrated solution. Both enzymes retained about 78% of their activities at 60°C. However, pure enzyme lost 70% of the original activity at 80°C. For crude enzyme the activity loss in 1 h at 80°C was 60% (Fig. 3.10).

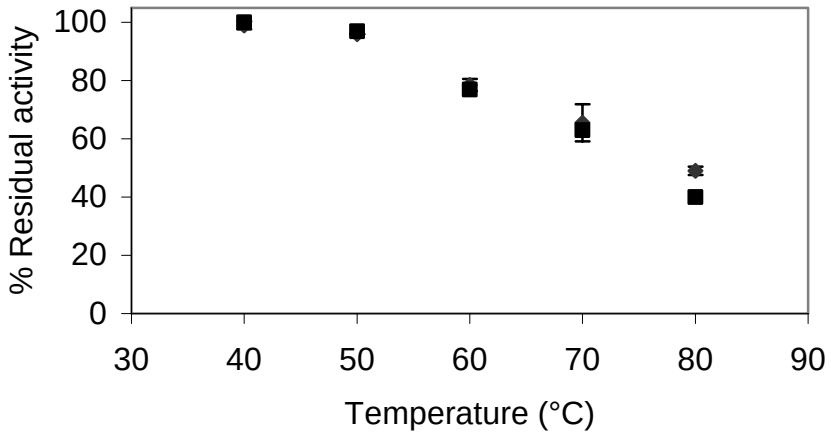

Figure 3.10 Thermostability of polyphenol oxidase. Relative activity was defined as percentage of residual activity compared to maximum activity remained. (♦) crude extract, (■) pure enzyme.

The optimum temperature *Thermoascus aurantiacus* polyphenol oxidase was in the range of 70 – 80 °C and the enzyme has a significant thermostability between 50-100°C (Machuca *et al.*,1998). Optimum temperature for *Aspergillus nidulans* polyphenol oxidase is 40°C (Bull *et al*., 1973). *Trametes versicolor* polyphenol oxidase has an optimum temperature of 55°C and stable up to 60°C (Khan, 1990). Another polyphenol oxidase from *Chaetomium thermophile* has an optimum temperature of 50°C. In comparison to other fungal strains *Thermomyces lanuginosus* polyphenol oxidase can be considered as moderately thermostable.

3.3.4 Effect of pH on Enzyme Activity

The activities of polyphenol oxidase at various pH ranges were measured by using catechol as the substrate. The reaction pHs were adjusted from 4.0 to 10.0 by using 0.2 M acetate buffer (pH 4-5.5), sodium phosphate buffer (pH 6.0-8.0), and glycine-NaOH buffer (pH 8.5-10.0).

The optimum pH of polyphenol oxidase was determined as 8.0 as shown in Figure 3.14. Enzyme solutions exhibited no activity at pH levels above 9.0 and below 5.0.

The optimum pH of *T.lanuginosus* laccase in the range of neutral to alkaline is an exception among fungal laccases, which are generally unstable at higher pH values. Thermophilic fungi *Chaetomium thermophile* (Ishigami and

Yamada, 1986) and *Thermoascus aurantiacus* (Machuca *et al*., 1998) polyphenol oxidases had the highest activity at pH 5.0 and 2.8, respectively. On the other hand, among 11 fungi studied by Bollag *et al*. (1984) only the optimum temperature of *Phizoctina praticola* laccase was in the neutral region (pH 7.2), whereas the optima of the all other extracellular laccases were significantly lower (between pH 3.0 – 7.0)

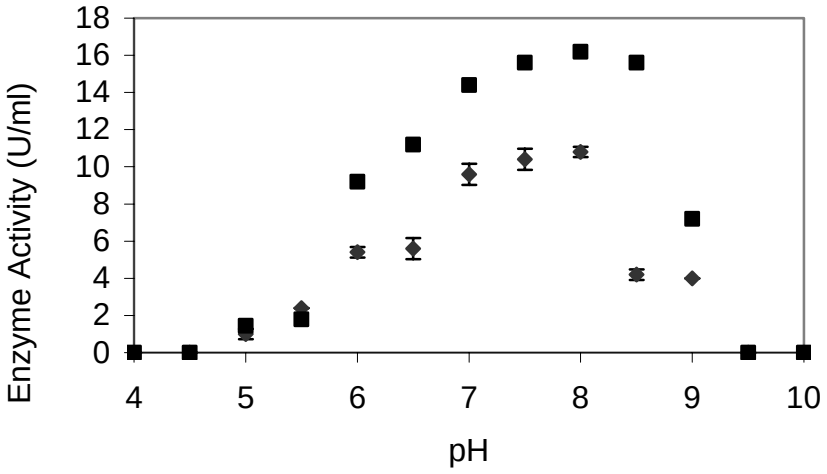

Figure 3.11 Effect of pH on the activity of polyphenol oxidase. (♦) crude extract, (■) pure enzyme. Enzyme experiments were performed at 50°C.

3.3.5 Effect of pH on the Stability of Polyphenol Oxidase

The pH stability of the enzyme was measured as follow: the enzyme solutions were held in various 0.2 M buffers whose pH were between 5.0 to 9.0 at 25°C for 1 h, and residual activity was measured under standard assay conditions

at 50°C pH 7.0. As shown in figure 3.12 The crude enzyme (0.25 mg/ml) was more stable than pure enzyme (0.02 mg/ml) due to its being in a more concentrated solution. In 1 hr pH treatment, both crude and pure enzymes lost not more than 20% their activities (Fig 3.11), so it was very stable. However, at pH 7.0, 97% activity was retained

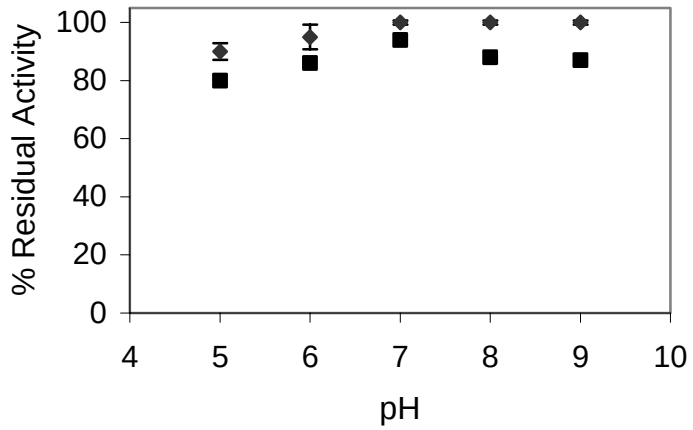

Figure 3.12 The effect of pH on stability of polyphenol oxidase. The enzyme solutions were incubated at different pHs for 1 h. (♦) crude extract, (■) pure enzyme.

3.3.6 Long-term pH Stability

The long-term pH stability of the enzyme solutions were measured after 18 h incubation. The effect of pH on the long term enzyme stability was illustrated in Figure 3.13.

As observed from figure 3.13, In 18 hr treatments both enzyme retained more than 80% activity at pH 7.0, 8.0 and 9.0. However they lost more than 60%

of their activities at pH 5.0 and 6.0 Optimum pH values of polyphenol oxidases also vary depending on the source organism. For example optimum pH level for *T.aurantiacus* polyphenol oxidase was found to be 2.8 and above pH 5.0 it was found to be inactive and other ten fungal laccases have been found to perform their activities in a pH range of acidic region (Machuca *et al*., 1998). *Chaetomium thermophile* polyphenol oxidase is active in the pH range of 4.5 to 5.5 and this enzyme was also found very stable between pH 4.0 to 11.0 (Edward *et al*., 1998). On the other hand *Coprinus cinerus* laccase pH optimum have been found to vary depending on the substrate . However, *Aspergillus nidulans* polyphenol oxidase can be given as an example of active polyphenol oxidase in alkaline region. It was found to be active at pH 6.0 – 8.5 (Bull *et al*., 1973).

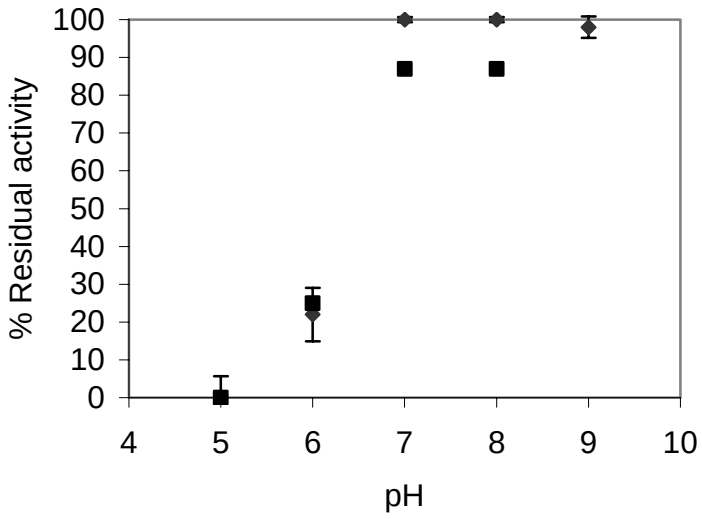

Figure 3.13 Long-term pH stability of the polyphenol oxidase. The enzyme solutions were incubated at different pHs for 18 h at room temperature. (♦) crude extract, (■) pure enzyme.

Unlike most of fungal polyphenol oxidases *T. lanuginosus* polyphenol oxidase was found to be active from neutral to alkaline pH range and it can be used under alkaline conditions.

3.3.7 Determination of the Molecular Weight of Polyphenol Oxidase

For molecular weight determination of polyphenol oxidase enriched enzyme from gel was 100 fold concentrated by using Vivapore concentrators and SDS-PAGE was performed. After SDS-PAGE was conducted, comassie brilliant blue staining was performed in order to visualize protein bands. The molecular weight determined from the relative mobility of standard proteins on SDS-PAGE was about 29 kDa (Figure 3.14)

Molecular weight of the laccase (29 kDa) purified from *T.lanuginosus* can be regarded as low molecular weight polyphenol oxidase according to the other organisms as well as fungi. Molecular weight of different polyphenol oxidase vary according to the source of the enzyme. Molecular weights of plant polyphenol oxidases are approximately 144,000. The generally accepted molecular weight of mushroom polyphenol oxidase is 128,000 Da. Whereas other fungi polyphenol oxidases have molecular weights varying from 46.000 to 88,000 Da. For example *Neurospora crassa* polyphenol oxidase has a molecular weight of 46,000. In *Alternaria* it is 88,000. In bacteria, *Streptomyces glaucescens* and *Streptomyces antibioticus* polyphenol oxidases have molecular weights of 30,900 and 30,736,

respectively and. In mammals human polyphenol oxidase is about 62,000 and rat poyphenol oxidase is 58,000 however there is little homology between these two mammalian polyphenol oxidases (Whitaker, 1994).

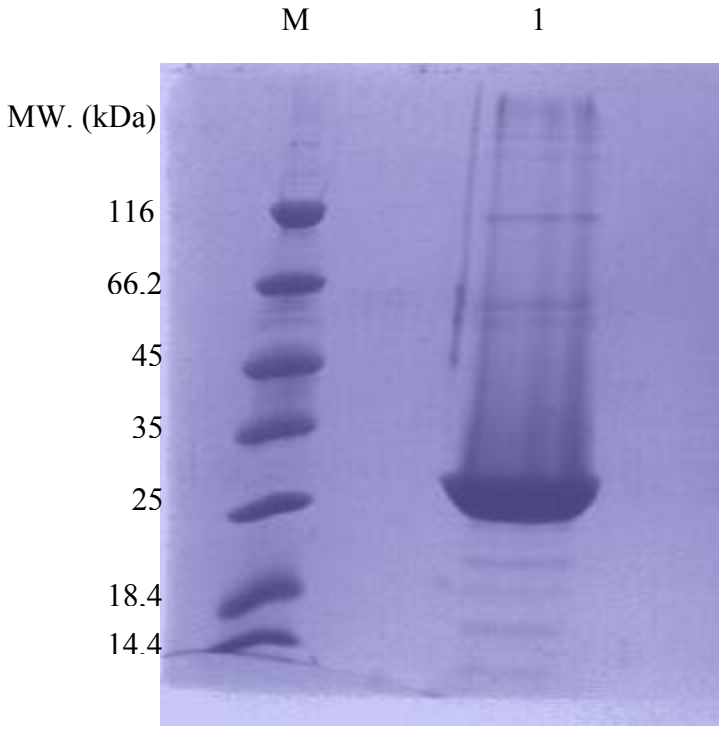

Figure 3.14 SDS-PAGE of polyphenol oxidase (12% Monomer concentration). M: marker proteins, 1: Purified polyphenol oxidase (0.2 mg/ml).

3.3.8 Determination of Isoelectric Point of Polyphenol Oxidase

Isoelectric focusing electropheresis (IEF) was performed on purified polyphenol oxidase. Isoelectric point of polyphenol oxidase was tried to be

evaluated with respect to the marker proteins and Isoelectric point of polyphenol oxidase was determined as 6.0. (Figure 3.15). Isoelectric point of different polyphenol oxidase can vary according to the nature of the habitat. The pI values of *Basidiomycete* laccase vary in the range of 3.0-5.0. the pI of *C.hirsutus* laccase is 4.0 (Skrobogat'ko *et al*., 1998).

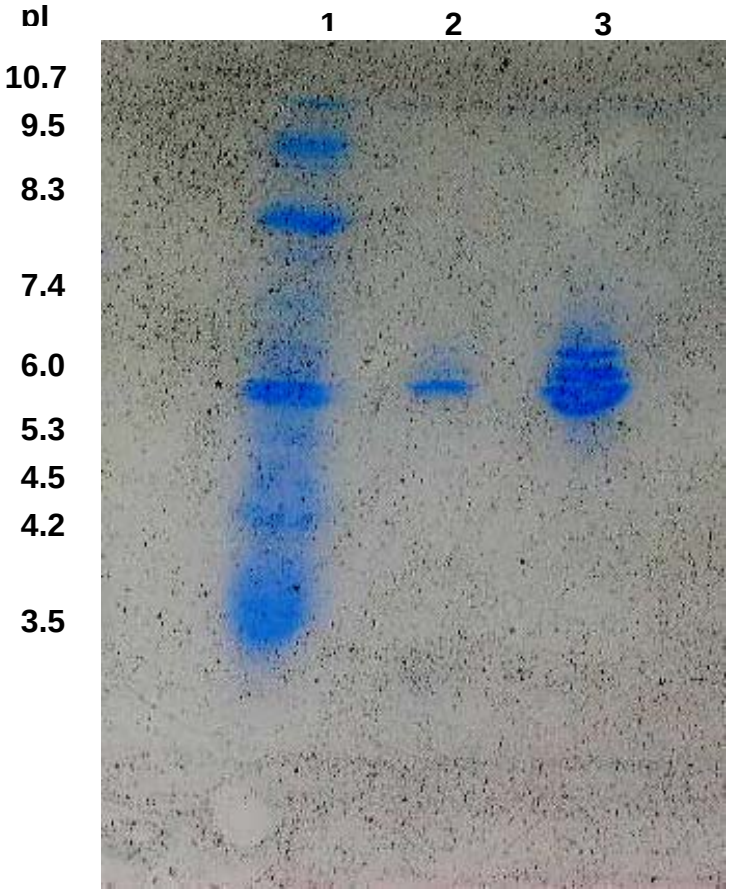

Figure 3.15 . IEF of polyphenol oxidase M: marker proteins 1 and 2: Purified polyphenol oxidase at different concentrations (0.02 and 0.2 mg/ml, respectively)

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

The aim of the study was to produce polyphenol oxidase from a thermophilic fungus *Thermomyces lanuginosus* and to characterize the thermostable polyphenol oxidase.

The highest polyphenol oxidase activity, 98 U/ml was measured in a medium containing 1.5% yeast extract, 0.3% $MgSO_4$, 1% KH_2PO_4 and 0.096% gallic acid at pH 8.0, 50°C, 400rpm, 80% dissolved oxygen in a bioreactor.

The enzyme obeys Michealis-Menten kinetics and the K_m and V_{max} values were calculated as 5 mg catechol/ml and 15 U/ml, respectively. The optimum temperature and pH of the enzyme were determined as 60°C and pH 8.0, respectively. About 80% of the activity was retained when enzyme solutions were incubated at pH 7.0-9.0 for 18 h at room temperature. Enzyme solutions retained 50% activity at 80 °C for 1 h incubation. The molecular weight of the

enzyme was estimated to be around 29 kDa by means of 12% SDS-PAGE results. The isoelectric point of polyphenol oxidase was measured as approximately 6.0.

In conclusion, *T. Lanuginosus* produces a thermostable and low molecular weight polyphenol oxidase in a shorter fermentation period in comparison with other fungi. Low molecular weight proteins have the advantage that they are more stable than that of their counterparts produced by other organisms. In addition production in shorter time provides advantage by decreasing the cost of fermentations in several applications.

In the future, polyphenol oxidase production by *T. Lanuginosus* could be increased by applying different genetic manipulations. Enzyme can be immobilized for the preparation of PPO electrode and membrane bioreactors. Characterization of the enzyme could be studied in more detail, amino acid sequence and three dimensional structure of the enzyme can be identified by sequencing and NMR techniques.

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APPENDIX A

MEDIUM COMPOSITIONS

Modified YpSs Agar (Yih *et al*., 2000)

4.0 g/L Yeast extract

1.0 g/L K_2HPO_4

5.0 g/L $MgSO_4.7.H_2O$

10.0 g/L Avicel

2.0 g/L Agar

Preculture Medium (Yih *et al*., 2000)

4.0 g/L Yeast extract

10.0 g/L K_2HPO_4

0,5 g/L $MgSO_47.H_2O$

7.5 g/L Glucose

Modified *Thermomyces* Medium

15.0 g/L Yeast extract

3 g/L $MgSO_4 7.H_2O$

10 g/L KH_2PO_4

0.03 g/L $CuSO_4$

0,034 g/L Gallic acid

APPENDIX B

COMPOSITION OF BRADFORD REAGENT

5X Reagent

500 mg (SERVA) Brilliant Blue G

250 mg 95% Ethanol

500 mg 85% Phosphoric acid

After mixing, the volume is completed to 1000 ml by adding distilled water and stored at 4°C. This reagent is used as stock solution. When it is needed, dilute stock 1:4 with distilled water and filter through normal filter paper. Before use wait for at least 24 h at 25°C. The reagent is stored in a dark bottle because of its light sensitivity (Bradford, 1976).

APPENDIX C

STANDART CURVE PREPARATION TABLE FOR BRADFORD METHOD

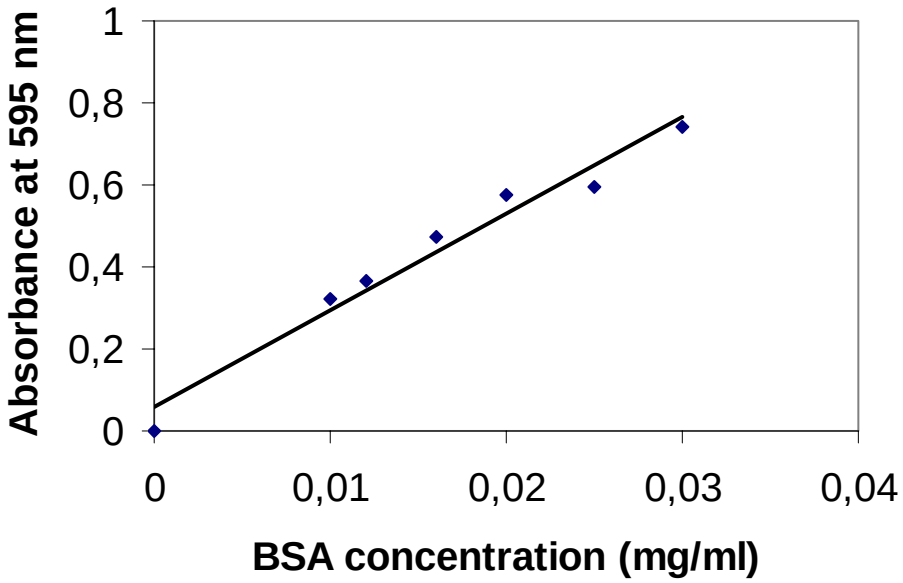
Tube#	**BSA (μl)**	**DH_2O (μl)**	**Bradford's Reagent (ml)**
1	0	2000	2
2	200	1800	2
3	240	1760	2
4	320	1680	2
5	400	1600	2
6	500	2500	2
7	600	1400	2

- 10 mg/ml BSA stock solution is prepared
- 10 mg/ml BSA stock solution is diluted to 100 μg/ml BSA solution

- Six standarts in the range of 10 to 30 μg protein solutions are prepared
- The reagent is added to all tubes. Then they are vortexed. After waiting for 10 min, optical density is read at 595 nm (Bradford, 1976).

APPENDIX D

STANDART CURVE FOR BRADFORD METHOD



APPENDIX E

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Reagents:

A) 30% Acrylamide/bis solution

Since acrylamide is a nerve toxin precautions should be taken by wearing gloves and mask during preparation of this solution (Laemmli, 1970).

- Weigh 58.4 g acrylamide in a 500 ml beaker
- Add 1.6 g N,N-bis-methylene-acrylamide
- Pour 150 ml dH_2O into the beaker (due to the light sensitivity cover the beaker with aluminum foil and mix on a magnetic stirrer.
- When it is completely dissolved, complete the volume to 200 ml by adding distilled water
- Store the solution in dark bottle at 4°C.

B) 10% SDS Solution:

- Dissolve 10 g SDS in 100 ml distilled water with gentle stirring to prevent foaming

C) 1.5 M Tris-HCl, pH 8.8 Running Buffer

- Weigh 54.45 g Tris base and dissolve in 150 ml distilled water
- Stir to dissolve and adjust to pH 8.8 with 2M HCl
- Complete the volume to 300 ml with distilled water and store at 4°C

D) 0.5 M Tris-HCl, pH 6.8 Stacking Buffer

- Weigh 6 g Tris base and dissolve in 60 ml distilled water
- Adjust the pH to 6.8 with 2M HCl
- Complete the volume to 150 ml with distilled water and store at 4°C.

E) 10% Ammonium persulfate (APS)

Dissolve 100 mg APS in 1 ml distilled water in an Eppendorf tube by vortexing. It should be prepared fresh daily.

F) Sample Buffer

1 ml of 0.5M Tris-HCl, pH 6.8, 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml β-mercaptoethanol, 0.4 ml 0.05% (W/v) bromophenol blue (in water) and 3.8 ml distilled water are mixed together.

G) 5x Stock Electrode Buffer (25mM Tris, 192mM glycine, 0.1% SDS)

Weigh 15 g Tris base, 72 g glycine and dissolve in 1000 ml distilled water. Store at 4°C.

Before use, pour 600 ml of stock electrode buffer solution into the electrophoresis tank and dilute it to 3 L total volume with distilled water. Stir with magnetic stirrer and add 3 g SDS. Place the tank in cold room and complete stirring gently (Laemmli, 1970).

I) Fixer Solution for Silver Staining

- Pour 150 ml ethanol in a 500 ml graduated cylinder
- Add 36 ml acetic acid and 150µl 37% formaldehyde
- Complete the volume to 300 ml with distilled water and pour into a plastic container

APPENDIX F

PROTOCOL FOR SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

A) Preliminary Preparation

Clean the surface of the glasses with pure ethanol, align the spacers at the edges of the bigger glass and place the small glass on it. Adhust the bottom edges very carefully in order not to have leakege. Install the clamps and fasten the screws.

Place the sandwich on the base and check that the bottoms of glasses are properly sealed.

B) Preparation of SDS-PAGE gel solution (10% slab gel)

Seperating gel:

Add the followings into an Erlenmayer flask and shake gently:

- 33.3 ml of 30% acrylamide/bis

- 40.5 ml distilled water
- 25 ml of 1.5 M Tris-HCl, pH 8.8
- 1 ml of 10% SDS solution
- 500 μl of 10% APS
- 50 μl TEMED

Immediately collect the solution into a glass pipette using a pump and discharge it in the space between the glass (very gently not to form bubbles). Fill the space up to 5 cm below the upper edge of the small glass. With a Pasteur pipette pour minimum amount of water-saturated n-butanol in order to cut off the contact of the gels with air, which will prevent the polymerization. Allow standing to complete the polymerization.

Stacking gel:

Dry the upper part of the gel by the help of a filter paper and place the comb between the glass sandwiches.

Add the following into a small beaker (4% gel)

- 1.3 ml of 30% acrylamide/bis
- 6.1 ml of distilled water

- 2.5 ml of 0.5M Tris-HCl, pH 6.8
- 100 μl of 10% (w/v) SDS
- 50 μl 10% APS (fresh)
- 10 μl TEMED

Immediately pour the gel solution between the glasses and allow to stand for polypmerization. After polymerization is completed take off the comb and fill the wells with 1x loading buffer.

Load the standard and samples into wells in an order and keep note for them.

Install the slab gel sandwiches to the cooling core.

Fill the upper chamber of the core with 1x electrode buffer. Gently place the cooling core into the electrophoresis tank. Be sure that there are no bubbles trapped on the upper side of the glass sandwich. If they are present, using a glass rod and remove them. This is necessary because trapped air bubbles can act as an insulator.

Perform the separation at 4°C using 10mA constant current for the firs 30 min. and then 25 mA per gel for the rest of the run.

When the run is over extrude the gels very carefully

Immerse the gels in fixing solution containing 50% mrthanol, 12% acetic acid and 0.5 ml of 37% formaldehyde/L and perform shaking on a platform shaker for at least 1 hour (gels can be kept in this solution overnisght).

The gels are then silver stained using the procedure of Blum *et al.,* (1987)

For SDS-PAGE molecular weight markers are given below:

Proteins	**Molecular weight**
β-galactosidase	116.2
Bovine serum albumin	66.2
Ovalbumin	45.0
Lactate dehydrogenase	35.0
Restriction endonuclease	25.0
B-lactoglobulin	18.4
Lysozyme	14.4

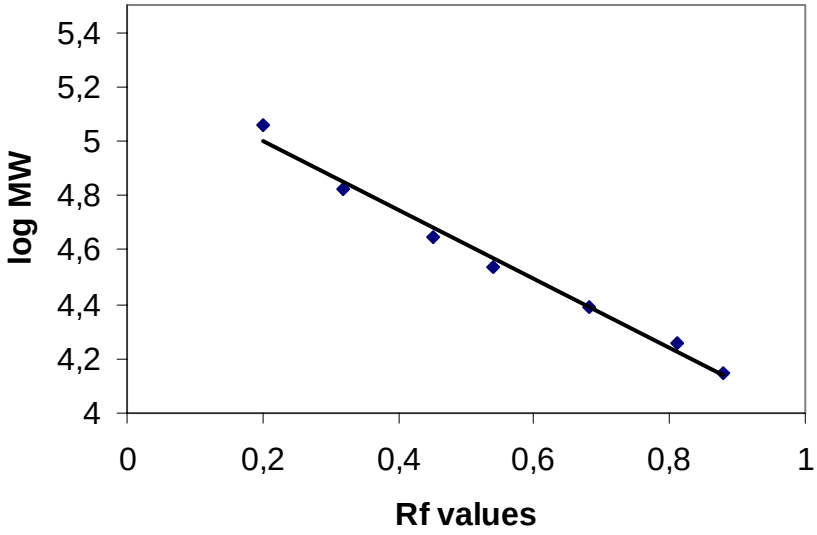


Figure F.1. Standard curve for SDS-PAGE

APPENDIX G

ISOELECTRIC FOCUSING GEL ELECTROPHORESIS

Reagents:

A) Monomer-Ampholyte solution

Distilled water	2.25 ml
Monomer solution	1 ml (25% T, 3% c)
Ampholyte	0.25 ml
Glycerol	1 ml

B) Catalyst solution

10% APS	30 μl
FMN	50 μl
TEMED	6 μl

APPENDIX H

PROTOCOL FOR ISOELECTRIC FOCUSING GEL ELECTROPHORESIS

Procedure:

Use of Gel Support Film for Polyacrylamide

- Bead a drop of water on the hydrophilic surface of Gel Support Film for Polyacrylamide and spread on the hydrophilic surface
- Pipet a few drops of water onto clean glass plate
- Place the hydrophobic side of the gel support film against the plate
- Roll the gel support film flat with a test tube or similar object to force out excess water and air bubbles
- Wipe or blot off any excess liquids at the edges

Casting Polyacrylamide Gels

1. With the gel support film facing down, place the glass plate on the casting tray so that it rests on the spacer bars

2. prepare the monomer-ampholyte solution. Degas the solution for 5 minutes under vacuum
3. Prepare the catalyst solutions to the degasses monomer and swirl gently
4. Add the catalyst solution between the glass plate and the casting tray
5. Pipet the solution between the glass plate and casting tray
6. Position a photopolymerization light over the tray
7. Irradiate the solution for 45 minutes
8. To lift the gel from casting tray:
    - Lift one corner with a flat spatula inserted between the gel and casting tray
    - When air appears under the gel, gently lift the plate free from the casting tray
9. Flip the plate, glass side down, onto the casting tray and further irradiate for 15 minutes to eliminate unpolymerized monomer on the gel surface

Sample Application

- Place a sample template on the top of the polymerized gel and allow 1 cm at both the top and bottom of the gel for the gel to contact the electrodes
- Apply samples using a pipettor
- Allow samples to diffuse into gel for 5 minutes
- Remove the template from the gel

Running the Gel

Set Up Procedure

- Slide the lid of IEF Cell toward the elctrode plugs to remove it
- Lightly moisten the graphite electrodes with water. Turn the gel with the absorbed samples upside down and place it directly on top of the electrodes
- Plug the power cables of the cell into a power supply

Run Conditions

- Focusing is carried out under constant valtage conditions in a stepped fashion. Begin focusing at 100 V for 15 minutes
- Increase the voltage to 200 V for 15 minutes
- Finally, increase the voltage to 300 V for an additional 60 minutes

- As the focusing nears completion, the current will decrease. This is a general indication that the focusing is near completion.

Removing the Gel

- After electrofocusing is complete, turn off the power supply and unplug the power cables
- Slide the protective lid from the cell and remove the gel from the electrodes. At this point separate the gel /gel support film from the glass plate

Band Detection

Comassie Blue G-250 "Quick Stain"

This technique does not require destaining and will not stain ampholytes. It cannot be used in the presence of detergents except urea.(Bio-Rad Ins. Man.)

3.5% perchloric acid

0.025% Comassie blue G-250

Immerse the gels in this soution for 1 hour. Place in 7% (v/v) acetic acid for intensification and presevation.