PARTIAL PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE FROM THERMOPHILIC *Bacillus* sp.

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İZMİR

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ABSTRACT

PARTIAL PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE FROM THERMOPHILIC *Bacillus* sp.

Polyphenol oxidases are enzymes that catalyze the oxidation of phenolic compounds using molecular oxygen. The ability of polyphenol oxidases to act on phenolic compounds makes them highly useful biocatalysts for various biotechnological applications. They are commonly found in animals, plants and fungi. Recent genome analysis have shown that polyphenol oxidases are also widespread in bacterial species.

In this study, detection, partial purification and characterization of polyphenol oxidase from thermophilic *Bacillus* sp., which was isolated from a geothermal region was achieved. The samples from bacterial culture were boiled and compared with not boiled ones in order to prove the existence of enzyme in bacterium. The existence was also supported with the appearance of dark bands on polyacrylamide gel after staining with catechol solution. Results of activity staining and activity measurements of samples from intracellular and extracellular extract revealed that the enzyme was intracellular. Partial purification was performed by acetone precipitation and gel filtration chromatography with 35% yield and 1.24 purification fold.

Characterization studies indicated that the enzyme showed highest activity at pH 7.0 and 60ºC, was stable at temperatures between 30 and 60ºC and more than 80% of activity was retained in the pH range of 5-8. The results of agent and metal ion effect on enzyme activity revealed that the enzyme was totally inhibited in the presence of DTT and sodium diethyldithiocarbamate and highly activated with copper ions whereas other agents or metal ions did not have significant effect on activity. K_m and V_{max} values for the enzyme were determined as 91mM and 2.25 Δabs/min/ml, respectively.

ÖZET

POLİFENOL OKSİDAZ ENZİMİNİN TERMOFİLİK *Bacillus* sp.' den KISMİ OLARAK SAFLAŞTIRILMASI VE KARAKTERİZASYONU

Polifenol oksidazlar, moleküler oksijen varlığında fenolik bileşiklerin oksidasyonunu katalizleyen enzimlerdir. Polifenol oksidazların fenolik bileşikler üzerine etki edebilme yetenekleri, onları çok sayıda biyoteknolojik uygulamada kullanışlı birer biyokatalizör yapar. Bu enzimler çoğunlukla hayvanlar, bitkiler ve funguslarda yaygın olarak bulunurlar. Yakın zamandaki genom analizleri, polifenol oksidazların bakteriyel türlerde de yaygın olduğunu göstermiştir.

Bu çalışmada, polifenol oksidazın jeotermal bir bölgeden izole edilmiş olan termofilik *Bacillus* suşundan bulunması, kısmi saflaştırılması ve karakterizasyonu gerçekleştirilmiştir. Polifenol oksidaz enziminin termofilik *Bacillus* suşundaki varlığını kanıtlamak amacıyla, bakteri kültüründen alınan örnekler kaynatılmış ve kaynatılmamış olanlar ile karşılaştırılmıştır. Enzimin bakterideki varlığı, poliakrilamit jel üzerinde katekol solüsyonu ile boyama sonrası koyu renkli bantların belirmesi ile de desteklenmiştir. Hücre içi ekstraktan ve bakterinin büyüme ortamından alınan örneklerin spektrofotometrik aktivite ölçüm ve poliakrilamit jelde aktivite boyama sonuçları, enzimin hücre içi olduğunu ortaya çıkarmıştır. Enzimin kısmi saflaştırılması, aseton çöktürmesi ve jel filtrasyon kromatografisi ile iki basamakta gerçekleştirilmiştir. Saflaştırma sonrası verim ve saflık katsayısı sırasıyla %35 ve 1.24' tür.

Katekol substratı kullanılarak yapılan karakterizasyon çalışmaları enzimin pH 7.0 ve 60ºC' de en yüksek aktiviteye sahip olduğunu göstermiştir. Enzim, 30 ve 60ºC arası sıcaklıklarda stabildir ve pH 5-8 aralığında aktivitesinin %80' inden fazlasını korumuştur. Enzim aktivitesi üzerine bazı etmenlerin ve metal iyonlarının etkisi incelendiğinde; sodyum dietilditiyokarbamat ve DTT'nin varlığında aktivite tamamen inhibe olmuş ve bakır iyonları ile yüksek derecede stimüle olmuşken diğer iyonların ve etmenlerin aktivite üzerinde önemli bir etkisi görülmemiştir. Enzimin kinetik parametleri olan K_m ve V_{max} değerleri sırasıyla 91mM ve 2.25 Δabs/min/ml olarak bulunmuştur.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphobic acid)
APS	Ammonium persulfate
BSA	Bovine serum albumin
CBB	Commasie brilliant blue
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
L-DOPA	3,4-dihydroxyphenylalanine
PAGE	Polyacrylamide gel electrophoresis
PPO	Polyphenol oxidase
SDS	Sodium dodecyl sulfate
SGZ	Syringaldazine
TEMED	Tetramethylethylenediamine
2xYT	Yeast extract tryptone

CHAPTER 1

INTRODUCTION

1.1. Phenols

Phenols are a class of aromatic compounds that have a hydroxyl group directly attached to a benzene or a benzonoid ring and the simplest member of this class, C_6H_5OH, is phenol (Carey 1996).

The presence of hydroxyl group highly effects the physical properties of phenols. The hydroxyl group enables to form strong hydrogen bonds with other phenol molecules and with water, therefore phenols have higher boiling points and more solubility in water than hydrocarbons of the same molecular weight (Carey 1996, Solomons 1990).

Phenols and its derivatives are widespread in nature. Phenol itself was isolated for the first time from coal tar in the early nineteenth century (Carey 1996). Tyrosine, an amino acid, is found in proteins. Methyl salicylate is found in oil of wintergreen and eugenol is found in oil of cloves. Thymol and vanillin are important components of thyme and vanilla beans. Estradiol is a female sex hormone and tetracylines are significant antibiotics (Solomons 1990).

Phenols are important medicinal and industrial chemicals. They are used as the raw material in the production of a large number of commercial products like aspirin, dyes, adhesives and plastics (Carey 1996). They were found to act as antiseptic and disinfectant. At the same time they are found in some commercial products such as deodorants, soaps, disinfectant sprays and oinments, first aid sprays, gargles, lozenges and muscle rubs because of their antiseptic and anesthetic activities. However the medical use of phenol is limited due to its skin irritaton and toxic properties. In food and cosmetics phenols are used as antioxidants since they are oxidized instead of the protected substance. Also some phenols are used in photography as photographic developers (Bailey 1995).

1.2. Polyphenol Oxidases

Polyphenol oxidases (PPOs) are enzymes, belonging to a group of copper-containing metalloproteins and are members of oxidoreductases, that catalyze the oxidation of a wide range of phenolic compounds by utilizing molecular oxygen (Queiroz, et al. 2008, Simsek and Yemenicioglu 2007). There are mainly three types of polyphenol oxidases classified according to their substrate specificities and mechanism of actions. These are; tyrosinase, catechol oxidase and laccase.

1.2.1. Tyrosinase

Tyrosinases (E.C. 1.14.18.1) are copper containing monooxygenases that use molecular oxygen to catalyze two different reactions: the *o*-hydroxylation of monophenols to *o*-diphenols which is referred to as monophenolase or cresolase activity and the subsequent oxidation of *o*-diphenols to *o*-quinones which is referred to as diphenolase or catecholase activity (Figure 1.1) (Claus and Decker 2006).

Tyrosinases are mainly involved in the biosynthesis of melanin and are responsible for the first steps of melanin synthesis from L-tyrosine leading to the formation of L-dopaquinone and L-dopachrome (Halaouli, et al. 2006).

Chemical and spectroscopic studies of tyrosinases have revealed that their active site consists of a pair of coupled copper ions (Duran, et al. 2002). These copper ions, CuA and CuB, each of which are coordinated by three histidine residues (His) constitutes the site where interaction of tyrosinase with both molecular oxygen and its phenolic substrate takes place (VanGelder, et al. 1997).

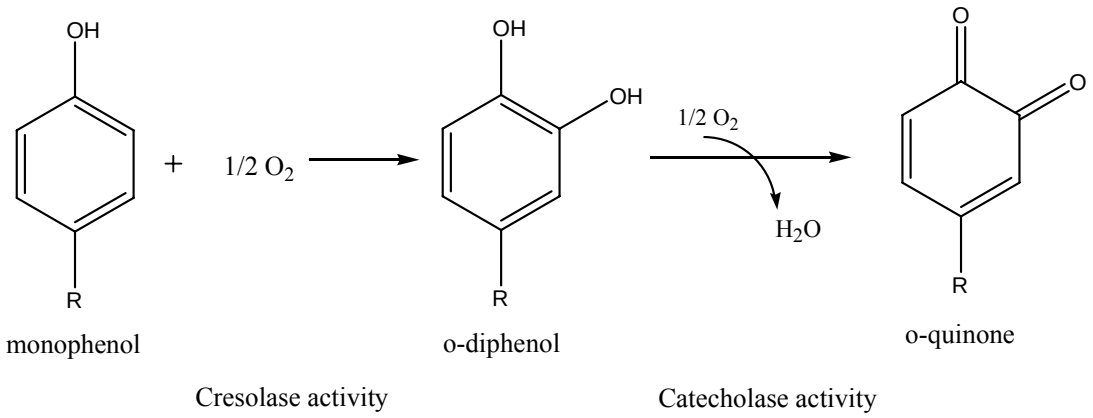

Figure 1.1. Reaction mechanism of tyrosinase

1.2.2. Catechol Oxidase

Catechol oxidases (E.C. 1.10.3.1), which are also known as *o*-diphenol oxidases, catalyze the oxidation of *o*-diphenols to the corresponding *o*-quinones by molecular oxygen (Figure 1.2). Similar to tyrosinases, the active site of catechol oxidases contains a pair of coupled copper ions and this feature makes catechol oxidases belong to the group of type-3 copper proteins which also includes tyrosinases and heamocyanins. Unlike tyrosinases, these enzymes lack hydroxylase activity hence can not use monophenols (like tyrosine and cresol) as substrate (Guell and Siegbahn 2007).

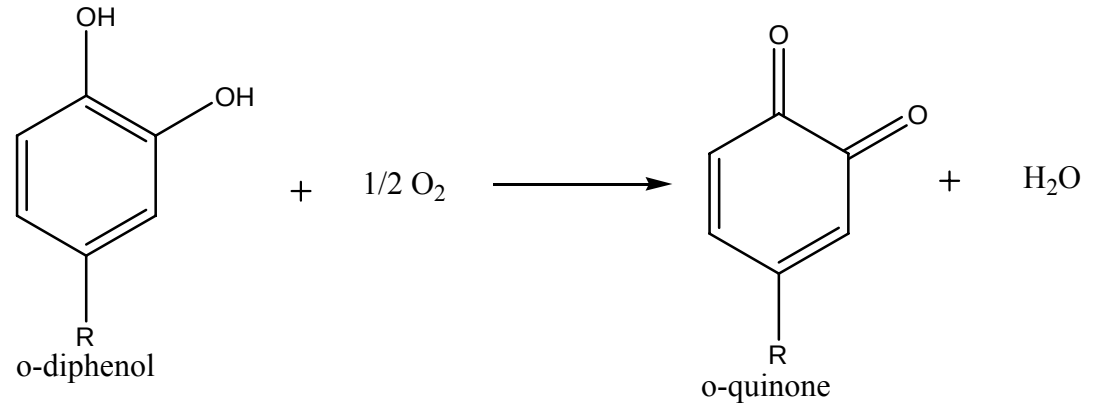

Figure 1.2. Reaction mechanism of catechol oxidase

1.2.3. Laccase

Laccases (E.C. 1.10.3.2) are multicopper enzymes that are capable of oxidizing various aromatic compounds by a radical catalyzed reaction mechanism in the presence of oxygen (Figure 1.3). They are a family of blue-multicopper oxidases which comprise, beside laccase, plant ascorbate oxidase, the mammalian plasma protein ceruloplasmin and bilirubin oxidase (Polaina and MacCabe 2007).

Laccases contain four copper atoms in the active site which play an important role in catalytic activity. These copper atoms are classified in three types according to their spectroscopic and functional characteristics (Duran, et al. 2002); type 1 (or blue), type 2 (or normal) and type 3 (or coupled binuclear) (Alexandre and Zhulin 2000).

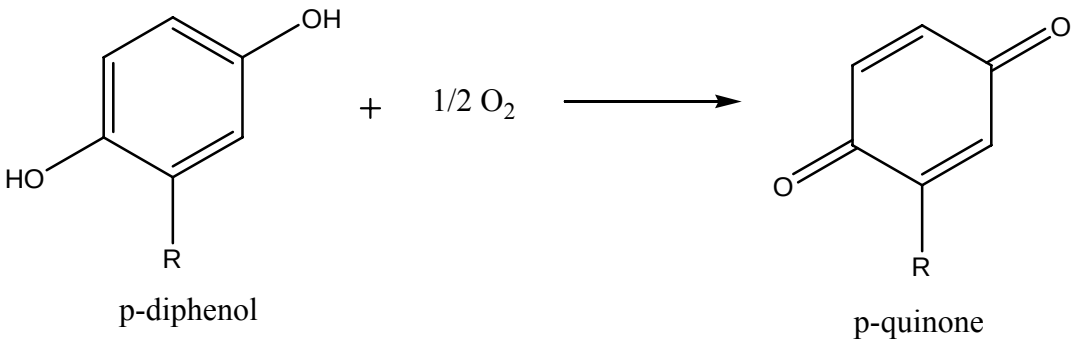

Figure 1.3. Reaction mechanism of laccase

In literature; the name polyphenoloxidase is used to summarize tyrosinases, catechol oxidases and laccases. Although these three types of enzymes have the ability to oxidize an overlapping range of phenolic compounds, they are differentiated on the basis of their substrate specificities, polypeptidic copper binding sites and sensitivity to inhibitors. Concerning substrate specificity; the main difference between these enzymes is that only tyrosinase can show cresolase activity and oxidize monophenols like L-tyrosine and only laccase have the ability to oxidize methoxy-activated phenols such as syringaldazine and 2,6-dimethoxyphenol (Sanchez-Amat, et al. 2001). Also laccase shows more affinity for the oxidation of *p*-diphenols like hydroquinone than *o*-diphenols like catechol (Solano, et al. 1997). Concerning copper binding sites; while the active site of tyrosinase and catechol oxidase contains a pair of coupled copper ions

(type-3), the active site of laccase contains three different types of four copper ions (one type-1, one type-2 and a pair of type-3) (Hernandez-Romero, et al. 2006) . Concerning inhibition, tyrosinase and catechol oxidase activity is inhibited by tropolone, cinnamic acid and salicylhydroxamic acid (Fernandez, et al. 1999). However the laccase activity is not effected much by these chemicals, instead it is strongly inhibited by azide, cyanide, thiocyanide, flouride and sulfhydryl reagents (Polaina and MacCabe 2007). Some phenolic substrates that are oxidized by polyphenol oxidases are shown in Figure 1.4

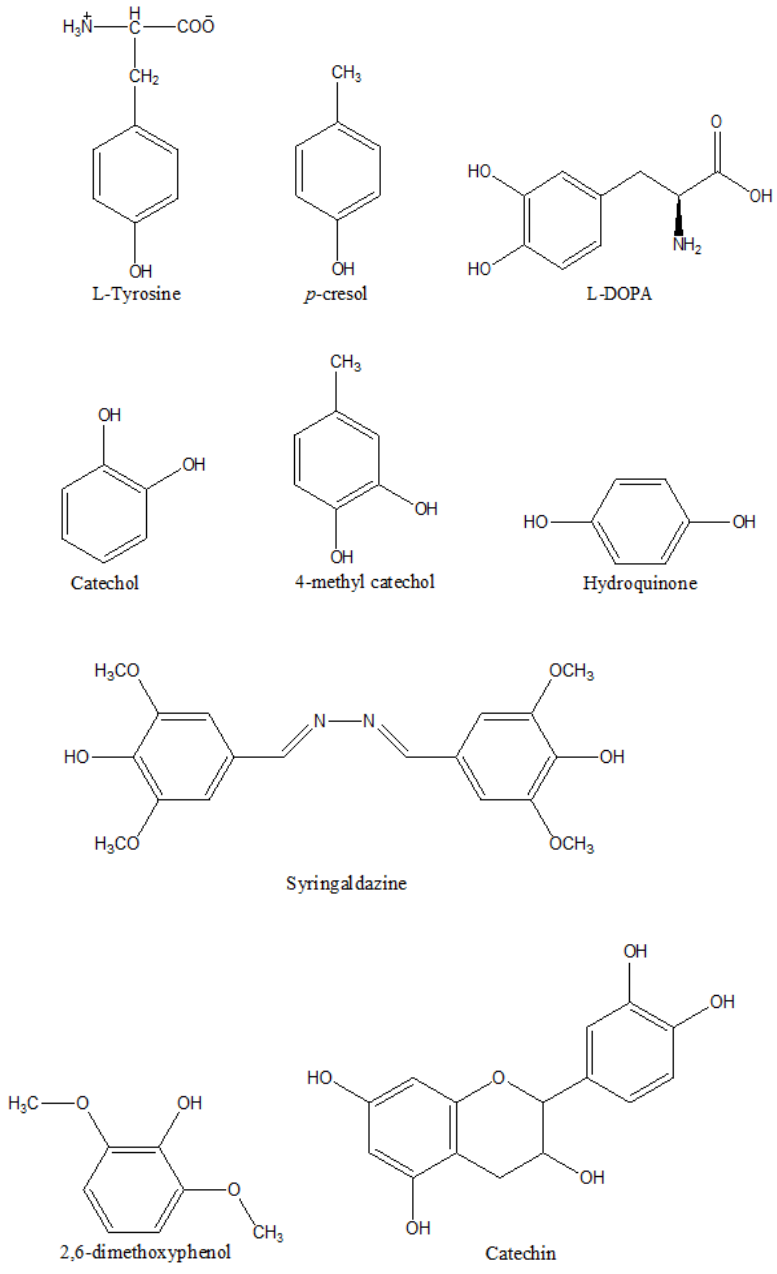

Figure 1.4. Some substrates of polyphenol oxidase

1.3. Biological Function and Distribution of Polyphenol Oxidases

Polyphenol oxidases are widely distributed in nature. They can be found in almost all living organisms including animals, plants, fungi and bacteria.

In animals including humans, tyrosinase is mostly found and involves in the pigmentation of skin, hair and eye by melanin synthesis. In humans, the too much or insufficient activity of this enzyme causes a number of important diseases such as albinism, vitiligo and melanoma. Polyphenol oxidase activity is also found in insects where they are responsible for the exoskeleton formation (Halaouli, et al. 2006).

In plants, all types of polyphenol oxidases are found. Tyrosinase activity has important roles in metabolism of plants including wound healing system (Kong, et al. 2000). When a plant gets a bruise, cut or damage, the enzyme oxidizes some phenolic compounds to form a polymer structure in order to protect the plant against insects or microorganisms (VanGelder, et al. 1997) and causes enzymatic browning of fruits and vegetables which is most cases undesirable as it causes loss of quality. Besides tyrosinases, also laccases have important roles in plants. They participate in lignification process of plant cell walls since they are able to cross-link phenolic monomers (Claus 2004).

In fungi polyphenol oxidases, particularly laccases play role in lignin degradation, fungal spore formation and pigmentation, detoxification of toxic compounds, pathogenesis (fungal virulence factors) and fungal morphogenesis (Ruijssenaars and Hartmans 2004).

To date polyphenol oxidases were mostly identified and studied from eukaryotic sources. Nevertheless, homology searches in protein databases and experimental data have shown that polyphenol oxidases are also widespread in bacteria (Ruijssenaars and Hartmans 2004). On the other hand little attention has been paid to bacterial polyphenol oxidases and the function of the enzyme in these organisms is not fully understood. The best documented function of polyphenol oxidase in bacteria is the formation of melanins. Melanin, a polyphenolic pigment, protects the bacterial spores and cells against oxidants, free radicals and UV radiation (Claus and Decker 2006, Dalfard, et al. 2006).

1.3.1. Bacterial Polyphenol Oxidases

With the discovery of polyphenol oxidases in bacterial species, the studies of them from these new sources have picked up in order to find more species that exhibit polyphenol oxidase activity.

Until now relevant genes have been found in both gram-positive and gram-negative bacteria. The first bacterial laccase was reported from *Azospirillum lipoferum* (Diamantidis, et al. 2000) as a multimeric enzyme. Laccase was also found in bacterial species living in extreme environments e.g. *Aquifex aeolicus* (Deckert, et al. 1998). Bacterial tyrosinases were first purified from *Streptomyces* species (Claus and Decker 2006). *Streptomyces* tyrosinases are well characterized and studied. Besides, polyphenol oxidases are found in a number of *Bacillus* species. Some biochemical characteristics and activity of polyphenol oxidases from different bacteria can be seen in Table 1.1

Table 1.1. Biochemical characteristics of polyphenol oxidases from different bacteria

Species	Type of PPO	Optimum pH	Optimum temperature (ºC)	Molecular mass (kDa)	References
Aquifex aeolicus	Laccase	nd	nd	59.3	(Deckert, et al. 1998)
Azospirillum lipoferum	Laccase	6.0	nd	48.9, 97.8, and 179.3 (Multimeric)	(Diamantidis, et al. 2000)
Bacillus halodurans	Laccase	7.5-8	nd	56	(Ruijssenaars and Hartmans 2004)
Bacillus licheniformis	Laccase	4.2	85	65	(Koschorreck, et al. 2008)
Bacillus subtilis	Laccase	3.0 (ABTS) 7.0 (SGZ)	75	65	(Martins, et al. 2002)
Bacillus sphaericus	Laccase	nd	nd	nd	(Claus and Filip 1997)
Bacillus spp.	Tyrosinase	nd	40	nd	(Mayende, et al. 2006)
Bacillus sp. HR03	Tyrosinase Laccase	5.0 5.5	55 55	50 50	(Dalfard, et al. 2006)
Bacillus thuringiensis	Tyrosinase	9.0	75	14	(Liu, et al. 2004)

(cont. on next page)

Table 1.1 (cont.) Biochemical characteristics of polyphenol oxidases from different bacteria

Escherichia coli	Laccase	6.5	55	54	(Kim, et al. 2001, Roberts, et al. 2002)
Marinomonas mediterranea	Tyrosinase Laccase	5.0 6.5	nd nd	nd 54.3	(Sanchez-Amat, et al. 2001, Sanchez-Amat and Solano 1997)
γ-proteobacterium JB	Laccase	6.5	55	120	(Bains, et al. 2003, Singh, et al. 2007)
Pseudomonas putida	Tyrosinase Laccase	7.0 7.0	30 30	39 59	(McMahon, et al. 2007)
Sinorhizobium meliloti	Tyrosinase Laccase	nd 5.0	nd nd	54.1 95	(Castro-Sowinski, et al. 2002, Mercadoblanco, et al. 1993)
Streptomyces antibioticus	Tyrosinase	nd	nd	29.5	(Bernan, et al. 1985)
Streptomyces glaucescens	Tyrosinase	6.8	nd	29.1	(Lerch and Ettinger 1972)
Streptomyces griseus	Laccase	6.5	40	114 (Homotrimer)	(Endo, et al. 2003)
Streptomyces lavendulae REN-7	Laccase	4.5	50	73	(Suzuki, et al. 2003)
Streptomyces michiganensis	Tyrosinase	7.0	33	32 and 34.5	(Philipp, et al. 1991)
Streptomyces sp. KY-453	Tyrosinase	6.8	nd	29	(Yoshimoto, et al. 1985)
Thermomicrobium roseum	Tyrosinase	9.5	70	43 (90-two subunits	(Kong, et al. 2000)
Thermus thermophilus	Laccase	4.5 (ABTS) 5.5 (SGZ)	92	53	(Miyazaki 2005)
Vibrio tyrosinaticus	Tyrosinase	6.6-7.8	nd	38.5 41	(Pomerant.Sh and Murthy 1974)

nd: not determined

1.4. Industrial Applications of Polyphenol Oxidases

In recent years polyphenol oxidases have garnered significant interest because of their high capacity for oxidizing aromatic compounds. This feature makes the use of polyphenol oxidases very suitable for some biotechnological applications in food industry, pulp and paper industry, textile industry, medicine and environmental technology.

In food industry, although polyphenol oxidases are undesirable for their browning effects, they can be applied for various beneficial purposes. They can be used in beverage processing for the elimination of phenolics which are responsible for browning, haze formation and turbidity development in beer, wine and fruit juice. Polyphenol oxidases, particularly laccases are currently of interest in baking since they are able to cross-link biopolymers (Rodriguez Couto and Toca Herrera 2006). They can be also used for the biosynthesis of antioxidants and food colorants (Simsek and Yemenicioglu 2007). Applications of polphenol oxidases in different aspects of food industry includes color formation and flavor enhancement of tea, cocoa and coffee, ascorbic acid determination, sugar beet pectin gelation and as a biosensor **(**Polaina and MacCabe 2007).

In pulp and paper industry, the industrial preparation of paper requires the removal of lignin from woody tissues and pulp bleaching. Conventionally pulp bleaching is carried out by using chlorine-based agents which causes environmental concerns because they lead to the release of toxic contaminants. The applications of laccases for the purposes of delignification and biobleaching were found to be succesful since they provide cleaner and milder strategies (Rodriguez Couto and Toca Herrera 2006).

In environmental technology, the presence of hazardous phenolic compounds and their derivatives in industrial wastewaters from coal conversion, petroleum refining, wood preservation, textile, paper, food and chemical industries constitues a big problem. Government legislation is becoming more stringent in developed countries for the removal of the toxic compounds from wastewaters before they are discharged into the environment. Recent interest has focused on the use of peroxidases and polyphenol oxidases as an enzymatic approach for the removal of phenolics from industrial

effluents. Peroxidases have the ability to treat phenolic compounds over wide ranges of pH and temperature but requires stoichiometric amounts of hydrogen peroxide. In this aspect polyphenol oxidases appear to be more advantageous because they require only molecular oxygen as oxidant to work (Edwards, et al. 1999).

In medical area, according to a recent research, polyphenol oxidases were found to inhibit the adhesion of *Streptococcus sobrinus*, a bacteria responsible from oral cavity formation, on tooth surface (Cowan, et al. 2000). Moreover, polyphenol oxidases can be used for the treatment of Parkinson's disease. By the action of polyphenol oxidase, L-tyrosine is converted to L-DOPA that is used to supplement the insufficient amount of dopamine in Parkinson's disease (Asanuma, et al. 2003, Xu, et al. 1998). Polyphenol oxidases are also of interest in clinical applications as a marker of vitiligo which is an autoimmune disease, as a prodrug therapy agent and as a tumor-suppressing (Seo, et al. 2003).

Polyphenol oxidases find additional applications in other fields of industry. They can be used in the development of biosensors for immunoassays, for the detection of phenols and phenolic compounds in wastewaters, food and beverage (Duran and Esposito 2000), for the detection of morphine, codeine and catecholamines. In cosmetics, some hair dyes and dermatological skin lightning preparations are based on laccase. In textile industry, they are used for the purposes of denim bleaching and dye decolorization (Rodriguez Couto and Toca Herrera 2006).

1.5. Thermophiles

Temperature is an important environmental factor and constrains all living organisms. Thus classification of living organisms according to their relation to temperature is essential for biological systematics (Kristjansson 1992). Microorganisms are therefore classified into three groups on the basis of their optimum growth temperatures; psycrophiles (below 20ºC), mesophiles (moderate temperatures) and thermophiles (high temperatures) (Turner, et al. 2007). Thermophiles, the microorganisms that love heat, are further subdivided into three categories according to their minimal and maximal growth temperatures as follows: moderate thermophiles (35-

70ºC) , extreme thermophiles (55-85ºC) and hyperthermophiles (75-113ºC) (Baker, et al. 2001).

After the discovery of a thermophilic microorganism, *Thermus aquaticus* from Yellow Stone National Park by Brock and his colleagues, the research on thermophiles have gained significant interest (Kristjansson 1989). In this respect the search for new microorganisms have been carried out and up to date the representatives of these organisms have been isolated from high temperature water containing terrestrial and marine habitats. The most common habitats are geothermally and volcanically heated hydrothermal systems such as solfataric fields, neutral hot springs and submarine saline hot vents (Horikoshi 1998).

By using 16S rRNA sequence comparisons, the universal phylogenetic tree, which can be seen in Figure 1.5, has been constructed with a tripartite division of the living world consisting of the domains Eucarya, Bacteria and Archaea (Andrade, et al. 1999).

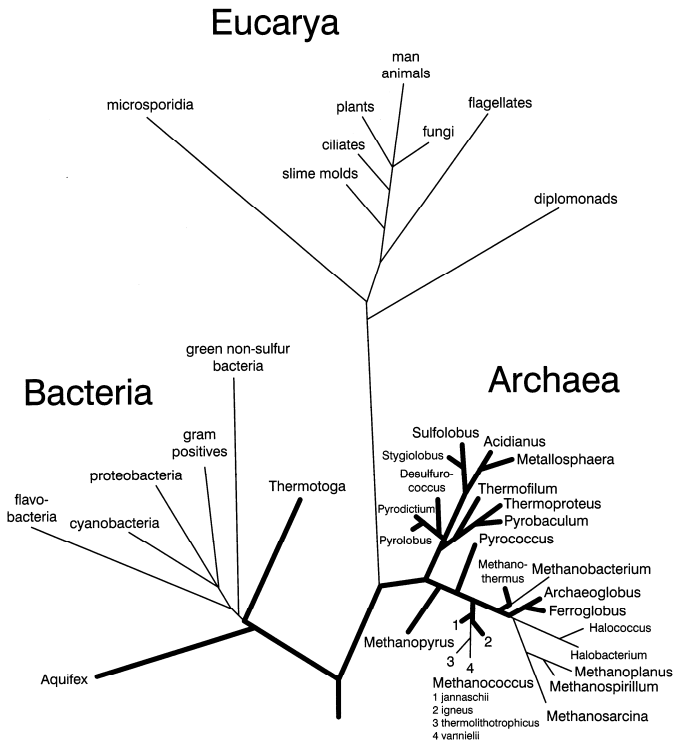

Figure 1.5. The universal phylogenetic tree. Bold lines represent thermophiles. (Source: Huber and Stetter 1998)

Thermophiles are present within Bacteria and Archaea. While most of the thermophilic bacteria characterized today grow below the hyperthermophilic boundary, hyperthermophilic species are primarily archaea (Turner, et al. 2007).

In Table 1.2, bacterial and archaeal thermophiles can be seen.

Surprisingly hyperthermophiles represent all the deep short branches within the phylogenetic tree, including the genera *Aquifex* and *Thermotoga* within the Bacteria and *Pyrodictium*, *Pyrolobus*, *Pyrobaculum*, *Desulfurococcus*, *Sulfolobus*, *Methanopyrus*, *Thermococcus*, *Methanothermus* and *Archaeoglobus* within the Archaea with the conclusion that they are the most primitive organisms still existing (Huber and Stetter 1998).

Table 1.2**.** Thermophiles and their environments
(Source: Hough and Danson 1999)

Phenotype	Environment	Typical genera
Thermophilic	55-80 ºC	*Methanobacterium*, *Thermoplasma*, *Thermus**, some *Bacillus** species
Hyperthermophilic	80-113 ºC	*Aquifex**, *Archaeoglobus*, *Hydrogenobacter**, *Methanothermus*, *Pyrococcus*, *Pyrodictium*, *Pyrolobus*, *Sulfolobus*, *Themococcus*, *Thermoproteus*, *Thermotoga**

* Genera of the domain Bacteria; all others are Archaea.

1.5.1. Thermophilic Enzymes

It is known that living organisms and their cell components such as nucleic acids, proteins/enzymes and lipids are very sensitive to heat. Therefore they are killed or denatured by heating. On the other hand the discovery of thermophiles, which can live and survive in extremes of temperature, made them very attractive and interesting field of study since not only these microbes themselves have the ability to thrive at elevated temperatures and withstand heat but also their components have.

As it is stated, cellular components of thermophilic microorganisms have to be thermostable in order to maintain viability. It was reported that the DNA of thermophiles have a reverse DNA gyrase, a unique type I DNA topoisomerase, which produces positive supercoils in the DNA and increases the melting point of DNA thus contributes to stability at elevated temperatures. The cell membranes of thermophiles consist of saturated fatty acids that provide a hydrophobic environment for the cell and are responsible for the rigidity of the membrane at high temperatures. In contrast, Archaea, which includes most of the hyperthermophiles, contains lipids linked with ether on the cell wall. This structure possesses a greater heat resistance than a membrane composed of fatty acids. These organisms also produce specialized proteins called chaperonins. Chaperonins help to refold the proteins to their native form after denaturation and maintain their functions (Haki and Rakshit 2003).

The enzymes synthesized by thermophiles and hyperthermophiles are known as thermozymes (Li, et al. 2005). The properties of these biocatalysts have adapted to nonstandart conditions, where their producers live optimally, in order to be active and maintain cellular functions. Thus it is important to study and investigate the bases of protein adaptation to high temperature so as to understand protein folding, protein structure-function relationship, the design of high temperature biocatalysts and the history of life on this planet (D'Auria, et al. 2000).

The enzymes having similar functions from mesophilic and thermophilic organisms were compared to figure out the question of how thermozymes differ from their mesophilic counterparts. According to the results of comparison studies, it was reported that the homologous thermophilic and mesophilic enzymes are highly similar with their: (i) amino acid sequences (40 to 85% similarity), (ii) superimposable three-dimensional structures, and (iii) catalytic mechanisms (Vieille and Zeikus 2001). The increasing number of three dimensional structures of thermozymes has shed some light on their thermostability strategies. These strategies include; additional intermolecular interactions (i.e. hydrogen bonds, electrostatic interactions, hydrophobic interactions, disulfide bonds, metal binding), good general conformational structure (i.e. more rigid, compact packing, reduced entropy of unfolding, conformational strain release, stability of a-helix) and replacement of some amino acids (Iyer and Ananthanarayan 2008).

It was reported that the stability of a protein is enhanced by increasing the number of hydrogen bonds and salt bridges in thermophilic organisms. Also it was believed that disulfide bridges stabilize proteins (Vieille and Zeikus 2001) thus the

effect of them on stability was examined by mutagenesis studies. In these studies, Cys residues were introduced in subtilisin E and an increase in the halflife and melting temperature of the mutant enzyme was observed (Takagi, et al. 1990).

It is known that some metals stabilize and activate enzymes. According to a study of xylose isomerase from *Bacillus licheniforms*, the major stabilizing forces were found to be related with the presence of metal ions and the stability of the enzyme was improved (Vieille, et al. 2001).

Thermophilic proteins may contain more hydrophobic cores than their mesophilic counterparts (Gerday 2007). In molecular folding and thermostability, hydrophobic interactions are the main contributors and thermostability of a protein is directly correlated with its total hydrophobicity (Li, et al. 2005).

Thermozymes are more rigid than their mesophilic cousins, particularly at room temperature. Although increased rigidity correlates with the increased thermostability, for enzyme catalysis flexibility is necessary (Gerday 2007). For that reason thermozymes, which are highly stable and active at elevated temperatures, are catalytically inactive at moderate temperatures (D'Auria, et al. 2000). However the rigidity of those enzymes at room temperature should not be overemphasized since they may be optimally flexible at the temperatures that their producers live (Gerday 2007).

Thermophilic enzymes require specific amino acid compositions. They generally prefer charged residues such as Glu, Arg and Lys that have the ability to provide an increase in the formation of ion pairs and their networks (Gerday 2007). It was reported that the most frequent amino acid exchange occuring at helical segment is Gly→ Ala exchange. Since Gly is the most flexible amino acid residue and Ala is known as the best helix inducer among all amino acids, it is not surprising that this exchange occurs frequently as a stabilizing strategy in thermozymes. Also the content of Pro, the most rigid amino acid, of these unusual enzymes is greater than their mesophilic relatives (Fontana, et al. 1998).

Other important factors in the stabilization of thermozymes are: (i) increasing molecular compactness by shortening one or more loops that have increased mobility at high temperatures; (ii) increasing the number of buried atoms in the molecule and elimination of unnecessary cavities; (iii) decreasing the entropy of unfolding (Vieille and Zeikus 2001).

1.5.2. Applications of Thermophiles in Biotechnology and Industry

The importance and use of enzymes in many processes has been well established for a long time. Although to date more than 3000 enzymes have been identified and many of these are being used in industrial and biotechnological applications, the present enzymes toolbox is still not sufficient to meet all demands (van den Burg 2003). It is because, most of the enzymes originate from mesophilic organisms and they can not withstand the harsh conditions of industrial processes (Hough and Danson 1999, van den Burg 2003). Thus, characterization of thermophiles and the stable enzymes they synthesize have been of great scientific and industrial interest for several decades.

Applied interest on thermozymes is related to the fact that their ability of thermostability enables to operate the processes, in which they are used, at high temperatures and also these enzymes are resistant to common protein denaturants like detergents, proteolytic enzymes and organic solvents (Andrade, et al. 1999). The advantages of operating industrial processes at elevated temperatures are;

- higher reaction rates,
- decrease in viscosity,
- increased diffusion rates,
- better solubility of compounds (especially polymeric substrates but except gases),
- reduced risk of contamination (very important for food and pharmaceutical industry) (van den Burg 2003).

Enzymes from thermophiles with specific features have considerable potential for many industrial applications. The possible use of the thermostable properties of thermozymes in industry has been recognized for a long time ago with the characterization and analytical application of Taq DNA polymerase (from *Thermus aquaticus*) in polymerase chain reaction, but has recently started to replace their mesophilic relatives in industrial processes which require high temperature. The thermophilic enzymes that have found way into several industrial applications are; proteases, lipases, DNA-processing enzymes, polymer-degrading enzymes such as amylases, chitinases and cellulases (van den Burg 2003) (Table 1.3).

Table 1.3. Bioconversion reactions and applications of thermostable enzymes (Source: Haki and Rakshit 2003)

Enzyme	Temperature (ºC)	Bioconversions	Applications
α-Amylase (bacterial)	90-100	Starch→dextrose syrups	Starch hydrolysis,brewing, baking, detergents
α-Amylase (fungal)	50-60	Starch→dextrose syrups	Production of maltose
Pullulanase	50-60	Starch→dextrose syrups	Production of glucose syrups
Xylanase	45-65, 105	Craft pulp→xylan+lignin	Pulp and paper industry
Chitinase	65-75	Chitin→chitobiose Chitin→*N*-acetyl glucosamine *N*-acetyl glucosamine→glucosamine Chitin→chitosan	Food, cosmetics, pharmaceuticals, agrochemicals
Cellulase	45-55, 95	Cellulose→glucose	Cellulose hydrolysis, polymer degradation in detergents
Protease	65-85	Protein→amino acids and peptides	Baking, brewing, detergents, leather industry
Lipase	30-70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis	Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry
DNA polymerase	90-95	DNA amplification	Genetic engineering/PCR

1.5.3. Thermophilic *Bacillus*

The genus *Bacillus* is a large and heterogenius collection of aerobic or facultatively anaerobic, endospore-forming, rod-shaped, Gram-positive (to Gram variable) bacteria (Yavuz, et al. 2004). The genus is widely distributed in soil, water and air and reported to have low incidence of pathogenicity (Harwood 1989). This genus includes many kinds of species with thermophilic, psycrophilic, acidophilic, alkalophilic and halophilic properties (Nazina, et al. 2001).

Thermophilic bacilli, which grow optimally at high temperatures ranging from 45 to 70ºC can be isolated from thermophilic and also mesophilic environments. In 1881, the first publication on the characterization of thermophilic bacteria *Bacillus thermophilus*; aerobic, spore-forming, and able to grow at 70ºC, was carried out. These organisms have gained significant biotechnological and industrial interest due to their ability to synthesize thermostable enzymes such as; lipases, proteases, amylases, pullulanases, xylanases, glucose-isomerases and DNA restriction endonucleases (Maugeri, et al. 2001).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

L-DOPA was purchased from Fluka; L-tyrosine and Coomassie Brilliant Blue G-250 were purchased from Merck. Catechol, ABTS, Sephadex G-100 gel filtration resin and all chemicals for electrophoresis studies were purchased from Sigma Chem. Co.

2.2. Bacterial Strain and Growth Conditions

The bacterium, thermophilic *Bacillus* sp., that was used in this study, was isolated from an uncontrolled thermal leak of Balçova Geothermal Region in İzmir (Yavuz, et al. 2004). Thermophilic *Bacillus* was cultivated overnight in 2xYT media, at 55ºC and 200 rpm. 2xYT (Yeast Extract Tryptone) medium consisted of 16.0g tryptone, 10.0g yeast extract and 5.0g NaCl per liter with a final pH of 7.0.

2.3. Determination of Protein Concentration

Protein contents of the samples were determined by Bradford method using bovine serum albumin (BSA) as the standart. Bradford method is used to determine the total protein concentration of a sample. This method is based on the shift of absorbance maximum of the dye, Coomassie Brilliant Blue G-250, from 465 to 595 nm upon

protein binding. The Coomassie Brilliant Blue G-250 dye binds arginine, lysine and histidine residues of proteins.

Composition of Bradford reagent, procedure of the assay and standart curve are given in Appendices A and B.

2.4. Evidence for Polyphenol Oxidase Activity in Thermophilic *Bacillus* sp.

In order to find out whether thermophilic *Bacillus* sp. exhibits polyphenol oxidase activity or not, two sets of bacterial growth was carried out in a volume of approximately 5ml 2xYT media as in conditions described in section 2.2. Following bacterial growth, discontinuous sonication was applied to bacterial cultures for about 6 minutes in ice bath to disrupt cells and release cellular components including enzymes. Then the homogenate was centrifuged at 5000rpm, 4ºC for 20 minutes. The resulting supernatants were taken and one set was kept in boiling water for 10 minutes. The supernatant after heat treatment was centrifuged again and supernatants of both boiled or not boiled samples were used as enzyme solutions in activity measurements. The activities were determined by recording the change in absorbance at 420nm with the assay mixture containing 1.5ml supernatant (boiled or not boiled) and 1.5ml 20mM catechol, which is a specific substrate for polyphenol oxidase, (prepared with 0.1M sodium phosphate buffer, pH 7.0) at 55ºC. The reference cuvettes contained 1.5ml 2xYT media and 1.5ml 20mM catechol solution.

2.5. Determination of Enzyme Activity

All the spectrophotometric assays were performed using Shimadzu UV-VIS spectrophotometer (Model 1700) with a constant temperature water circulator. The polyphenol oxidase activity of the samples were determined at 55ºC for 20 minutes, by recording the increase in absorbance at 420nm caused by the oxidation of catechol

substrate. The assay mixture (1 ml in all cases) contained 10µl 2M catechol with a final concentration of 20mM, 955µl 0.1M sodium phosphate buffer at pH 7.0, and the reaction was initiated by the addition of 35µl enzymatic sample. The reference cuvettes had the same composition except for the enzyme. The enzyme activities were given as Unit or percent initial activity and Unit is defined as the amount of enzyme that causes 0.001 absorbance change in one minute.

2.6. Preparation of Crude Enzyme Extract

Bacterial growth was carried out in erlenmeyer flask containing 100ml of 2xYT media and in conditions as described in section 2.2. Bacterial culture was centrifuged at 5000rpm, 4ºC for 20 minutes to obtain culture supernatant and harvest bacterial cells. The cell pellet was then resuspended in 10ml of 0.05M sodium phosphate buffer, pH 7.0, and the cells were disrupted by discontinuous sonication in ice bath for 6 minutes. The homogenate was centrifuged at 7500rpm, 4ºC for 20 minutes in order to remove cell debris and the supernatant was used as crude intracellular enzyme extract.

2.7. Enzyme Purification Procedure

The supernatant obtained after sonication, which was called as crude intracellular enzyme extract, was subjected to total protein precipitation with an organic solvent; acetone. For that purpose, two volumes of cold acetone (-20ºC) was added slowly to one volume of sample, the mixture was vortexed and then incubated at -20ºC for an hour. After incubation period, the resulting precipitate was collected by centrifugation at 15000rpm, 0ºC for 30 minutes and the pellet was resuspended in 10.5ml 0.05M sodium phosphate buffer, pH 7.0.

The enzyme solution was then loaded on to gel filtration (Sephadex G-100) column (2.5cm x 50cm) which had been equilibrated with sodium phosphate buffer (0.05M, pH 7.0). The fractions of 3ml were collected by washing the column with the

same buffer using ProTeam low pressure liquid chromatography system (Model LC 320, Teledyne ISCO) (Figure 2.1) Collected fractions were assayed for their protein concentrations at 280nm and for polyphenol oxidase activity. The active fractions were pooled and stored at -20 ºC until use for further experiments.

Figure 2.1. Low pressure liquid chromatography system

2.8. Electrophoretic Studies and Activity Staining

Electrophoretic studies included sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and activity staining after native-PAGE. Both SDS-PAGE and native-PAGE were performed using acrylamide concentrations of 12% for separating and 4% for stacking gels; runned at 65V for 30 minutes and 100V for about an hour using Thermo Scientific gel electrophoresis device (Figure 2.2) at room temperature.

2.8.1. SDS-PAGE

SDS-PAGE is an electrophoretic method that separates the proteins according to their molecular sizes in an electrical field. This method is commonly used to estimate the purity and molecular weight of a protein.

The partially purified sample and the samples from former steps were applied to SDS-PAGE. While performing SDS-PAGE, samples were diluted by a volume ratio of 1:4 with sample buffer, and kept in boiling water for 4 minutes to denature proteins. 5µl of molecular weight marker and 25µl of sample-sample buffer mixture were loaded onto gel. After electrophoretic run, at conditions given above, SDS-polyacrylamide gel was stained using colloidal coomassie staining solution and incubated overnight. Then the gel was subjected to neutralization, destaining and fixation solutions for 3 minutes, 1 minute and 1 hour, respectively. At the end of these steps, the image of the gel was taken with a special camera under white light and if needed the gel was kept in 5% acetic acid solution (v/v) at 4ºC for a long time.

The procedure for preparation of gels and reagents that were used in SDS-PAGE are given in Appendix C.

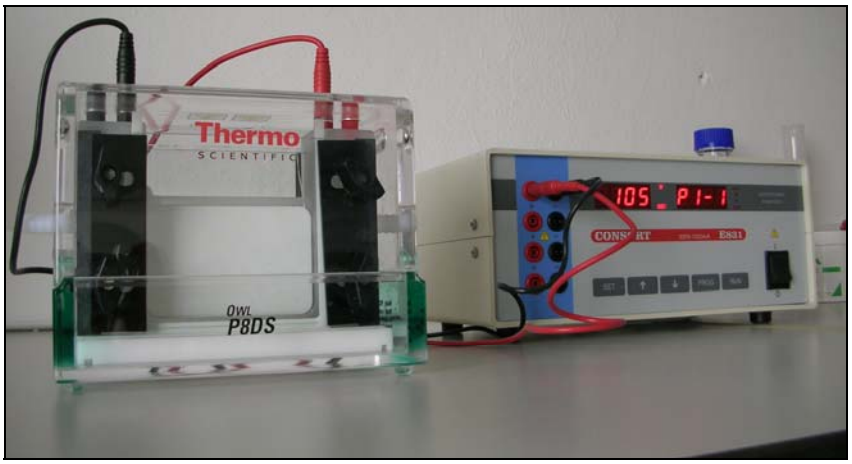

Figure 2.2. Gel electrophoresis system

2.8.2. Native-PAGE and Activity Staining

Native-PAGE is also an electrophoretic method very similar to SDS-PAGE but the main difference is that in native-PAGE the proteins are separated in non-denaturing conditions. The procedure for preparing solutions and gels for native-PAGE is based on SDS-PAGE's, but to provide non-denaturing conditions, SDS and 2-mercaptoethanol were exluded from sample buffer and SDS was also excluded in the preparation of both separating and stacking gels. Instead of these reagents, same amount of water was added. The samples were diluted with sample buffer by the ratio of 1:4 and loaded onto gel skipping the heating step.

To perform specific activity staining after electrophoretic run, native polyacrylamide gel was equilibrated by immersion in 0.1M sodium phosphate buffer, pH 7.0, for 10 minutes at room temperature. Then the gel was transferred into a freshly prepared solution of catechol (25mM, prepared in sodium phosphate buffer) and incubated overnight at 55ºC in incubator. At the end of incubation period, the gel was washed with distilled water and stored in 5% acetic acid solution at 4ºC, if needed.

2.9. Characterization Studies

2.9.1. Kinetic Analysis

To perform kinetic analysis, enzyme activity was measured at different concentrations of catechol varying from 5mM to 60mM, then the kinetic parameters of the enzyme, K_m and V_{max}, were determined by Lineweaver-Burk plot method.

2.9.2. Effect of pH on Enzyme Activity and Stability

The effect of pH on enzyme activity was investigated using 0.1M sodium phosphate buffer at different pH values. The optimum pH of the enzyme was determined under the standart assay conditions by measuring activity in the presence of buffers at different pH values ranging from 4.0 to 10.0.

To determine the pH stability of the enzyme, 35µl enzyme solution was mixed with 70µl of buffer at various pHs (pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) and then the mixture was incubated at 55ºC for 1.5 hour. The residual activity was measured under standart assay conditions using 35µl of enzyme-buffer mixture.

2.9.3. Effect of Temperature on Enzyme Activity and Stability

The effect of temperature on polyphenol oxidase activity was examined under standart assay conditions at different temperatures ranging from 30 to 90ºC and the buffer was heated to relevant temperature before the assay.

Temperature stability was assayed by incubating the enzyme solution at a range of temperatures from 30 to 80ºC for 1.5 hour and then measuring the remaining activity using standart assay procedure.

2.9.4. Effect of Metal Ions on Enzyme Activity

To examine the effect of metal ions on polyphenol oxidase function, the enzyme was incubated in the presence of 1mM metal ion at room temperature for 10 minutes. At the end of the incubation period the reaction was initiated by addition of catechol solution to give a final substrate concentration of 20mM and change in absorbance was measured under standart assay conditions. For this purpose, $CaCl_2$, $CuSO_4$, $MgSO_4$, KCl and $ZnCl_2$ were used.

2.9.5. Effect of Various Agents on Enzyme Activity

The effect of some agents such as ethylenediaminetetraacetic acid (EDTA), sodium flouride, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), sodium diethyldithiocarbamate, SDS, and Triton X-100 on enzyme activity were also examined. For this purpose, the enzyme was incubated in the presence of different agents for 10 minutes at room temperature. The concentrations of the agents were; 5% (v/v) of Triton X-100 or 1mM of other agents in 1ml assay mixture. At the end of incubation period, the reaction was initiated by addition of catechol and change in absorbance was measured under standart assay conditions.

2.9.6. Substrate Specificity of Enzyme

The substrate specificity of the enzyme was determined by measuring activity towards several monohydroxyphenol and dihydroxyphenol compounds like L-tyrosine, catechol, L-DOPA, ABTS and hydroquinone. The activities of the enzyme for this purpose were measured using solutions of these compounds prepared in 0.1M sodium phosphate buffer at concentrations of 20mM for catechol and hydroquinone, 10mM for L-DOPA, 2mM for L-tyrosine and ABTS.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Evidence for Polyphenol Oxidase Activity

In order to determine the existence of polyphenol oxidase activity in thermophilic *Bacillus* sp., two sets of bacterial growth was carried out and one set was kept in boiling water to denature the enzymes so that a comparison could be done. The results of activity measurements were given in Figure 3.1. It can be clearly seen that the sample which was not boiled (NB) showed a significant increase in absorbance whereas boiled (B) one did not exhibited that much increase. These results showed that the increase in absorbance and formation of brown color in samples when assayed with catechol was due to the existence of an enzyme, not because of a compound that exists in the growth medium of bacterium. In other words, the oxidation of catechol substrate was due to the existence of polyphenol oxidase. So it can be suggested that the responsible bacterium produces polyphenol oxidase enzyme.

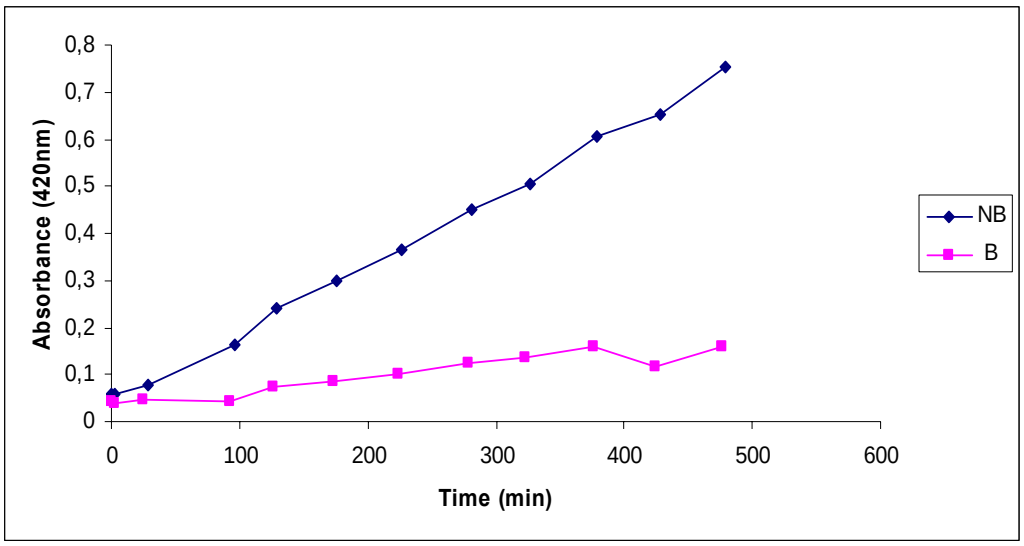

Figure 3.1. Activity measurements of boiled and not boiled samples

3.2. Partial Purification of Polyphenol Oxidase

In this study, partial purification of polyphenol oxidase from thermophilic *Bacillus* sp. was achieved by acetone precipitation and gel filtration chromatography. It should be noted that the result of activity measurement of growth medium which may contain extracellular enzymes was poor when compared with the result of intracellular extract. Also a sample from growth medium of bacterium was loaded onto native-polyacrylamide gel and subjected to activity staining with catechol solution. Although intracellular enzyme extract was stained with catechol, no dark band on the lane where growth medium of bacterium was loaded could be observed (see Figure 3.4.b for result). These results suggested that polyphenol oxidase from thermophilic *Bacillus* sp. was intracellular thus intracellular extract was used as starting material for purification.

An outline of the purification procedure is illustrated and the results are given in Table 3.1. The intracellular enzyme extract was first subjected to acetone precipitation to precipitate total protein by changing the dielectric constant of the medium and increasing the interaction of proteins. The yield and purification fold after this step was 81% and 1.19, respectively. The precipitate was then resuspended in buffer and loaded onto gel filtration column which acts as a molecular sieve and separates the proteins according to their molecular sizes. The fractions that were eluted from the column were tested for their polyphenol oxidase activities and the ones with highest activity were pooled (Figure 3.2). The resulting enzyme solution which had a specific activity of 134,9 U/mg, was purified 1.24 fold and contained 35% of the activity.

Table 3.1. Purification of polyphenol oxidase from thermophilic *Bacillus* sp.

Purification Step	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (Fold)
Crude Extract	10	7030	64,8	108,5	100	1
Acetone Precipitation	10,5	5691	43,89	129,7	81	1,19
Gel Filtration	6	2478	18,36	134,9	35	1,24

Purification fold and yield values of polyphenol oxidases that were obtained with other bacterial species are; 27 and 24% after purification of *Azospirillum lipoferum* polyphenol oxidase by acetone precipitation and hydroxyapatite chromatography (Diamantidis, et al. 2000); 21 and 9% after purification of γ-proteobacterium JB polyphenol oxidase by ammonium sulfate precipitation, ion exchange chromatography and preparative PAGE (Singh, et al. 2007); 50 and 21% after purification of *Thermomicrobium roseum* polyphenol oxidase by ion exhcange chromatography (Kong, et al. 2000); 261 and 9% after purification of *Streptomyces lavendulae* polyphenol oxidase by heat treatment, ammonium sulfate precipitation, ion exchange, hydroxyapatite and gel filtration chromatography (Suzuki, et al. 2003), respectively. Also 72% yield was obtained after purification of *Bacillus thrungiensis* polyphenol oxidase with one-step purification method using copper sulfate saturated ion exchange resin (Liu, et al. 2004). Since thermophilic *Bacillus* sp. polyphenol oxidase was partially purified, the yield and purification fold values are lower than obtained for other polyphenol oxidases from different bacterial species.

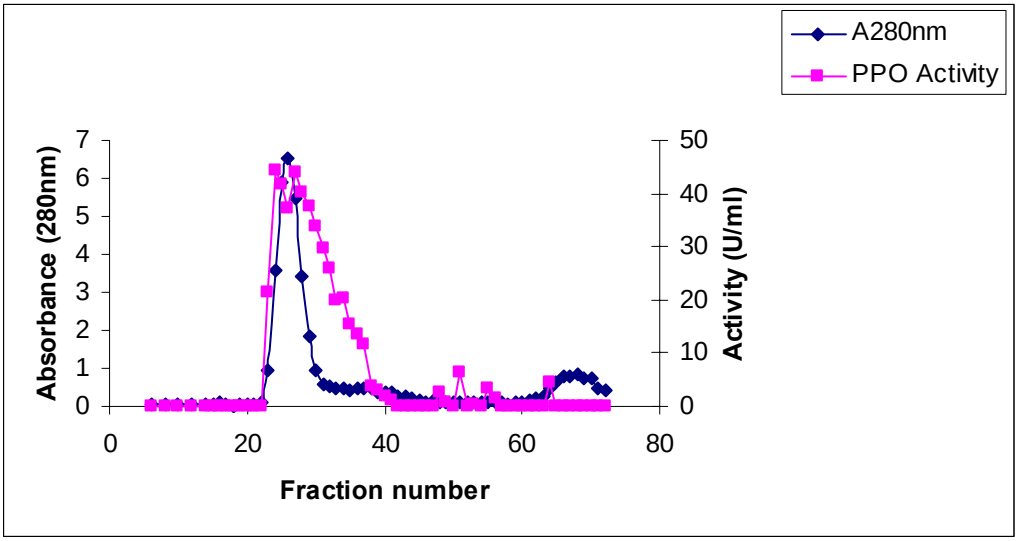

Figure 3.2. Gel filtration profile of polyphenol oxidase

3.3. Electrophoretic Studies and Activity Staining

3.3.1. SDS-PAGE

The partially purified sample and the samples from former steps were applied to SDS-PAGE. The image of the gel after colloidal coomassie staining is given in Figure 3.3. Supernatant of intracellular extract was loaded on lane 1; sample after acetone precipitation was loaded on lane 2; the mixture of polyphenol oxidase active fractions which were pooled after gel filtration column was loaded on lane 3 and a fraction which was not polyphenol oxidase active was loaded on lane 4. Since the enzyme was partially purified, the composition of the samples were very complex. Among those protein bands on gel, which band corresponds to the enzyme of interest could not be determined so did the molecular weight.

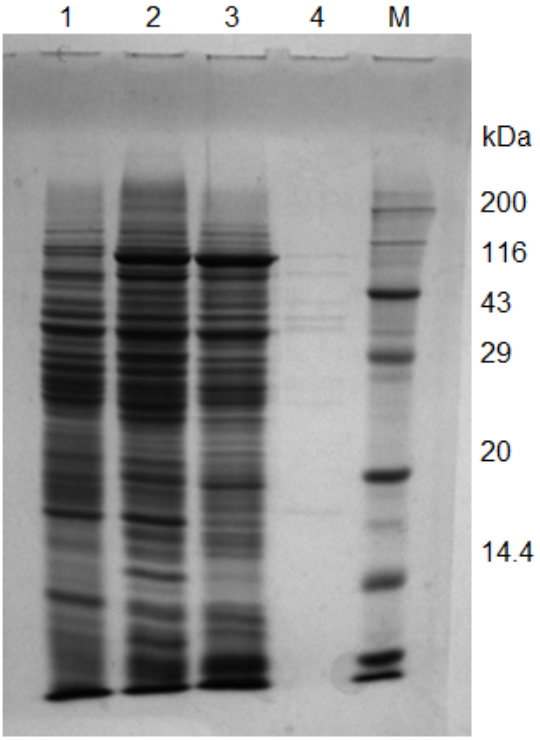

Figure 3.3. SDS-PAGE image. M: Protein marker

Molecular weight of polyhenol oxidases from other bacterial species vary from 120 to 14kDa. For example, molecular weight of polyphenol oxidase from γ-proteobacterium JB is 120kDa (Singh, et al. 2007), *Streptomyces griseus* is 114kDa (Endo, et al. 2003), *Streptomeyces lavendulae* is 73kDa (Suzuki, et al. 2003), *Bacillus subtilis* and *Bacillus licheniformis* are 65kDa (Martins, et al. 2002, Koschorreck, et al. 2008), *Thermus thermophilus* is 53kDa (Miyazaki 2005), *Streptomyces antibioticus* and *Streptomyces glaucescens* are 29kDa (Bernan, et al. 1985, Lerch and Ettinger 1972) and *Bacillus thuringiensis* is 14kDa (Liu, et al. 2004).

3.3.2. Native-PAGE and Activity Staining

Native-PAGE separates the proteins on polyacrylamide gel under non-denaturing conditions. So the enzymes loaded onto native polyacrylamide gel are not denatured and retain their catalytic activity. In the light of this knowlegde, native-PAGE of the samples was performed duplicated in same conditions. Following native-PAGE, one gel was stained using catechol substrate for the detection of polyphenol oxidase activity and the other one was stained using CBB dye to visualize all protein bands. The images of the gels under white light are given in Figure 3.4. The gels on the left side are stained with catechol solution and the right side are with CBB dye. As it can be seen from Figure 3.4.a, the appearance of dark bands indicated the existence of polyphenol oxidase in samples. Also as it was stated in section 3.2, the intracellular nature of the enzyme was evidenced with the image of the gel that can be seen in Figure 3.4.b.

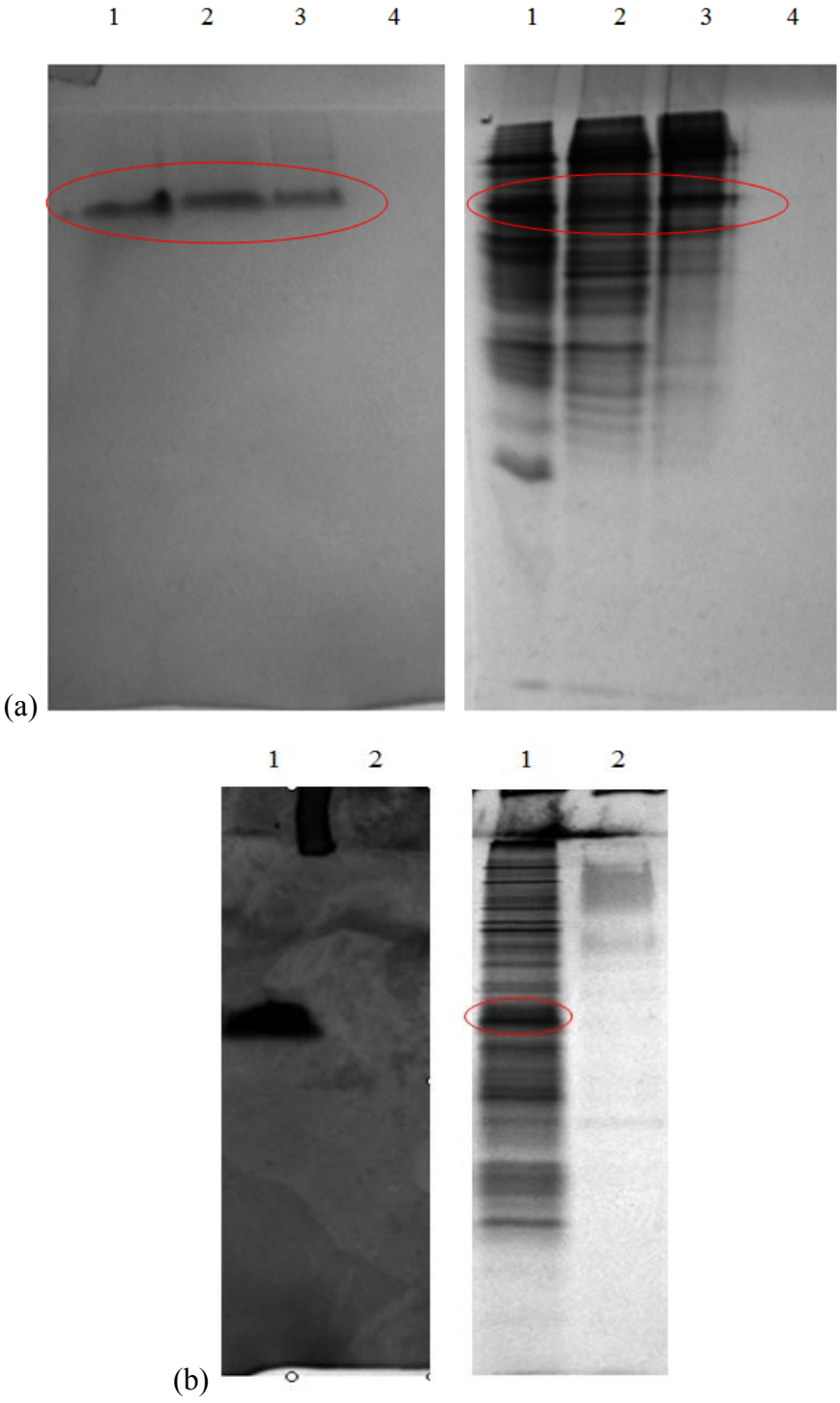

Figure 3.4. Activity and colloidal coomassie staining of polyphenol oxidase on native polyacrylamide gels. (a) Lane 1, supernatant of intracellular extract; lane 2, sample after acetone precipitation; lane 3, enzyme solution after column; lane 4 inactive fraction after column. (b) Lane 1, supernatant of intracellular extract; lane 2, growth medium of thermophilic *Bacillus* sp.

3.4. Characterization of Polyphenol Oxidase

3.4.1. Kinetic Analysis

To determine the kinetic constants, K_m and V_{max}, of thermophilic *Bacillus* polyphenol oxidase, initial reaction rates at different catechol concentrations, ranging from 5 to 60mM were measured. In order to obtain Lineweaver-Burk plot; 1/V (1/Reaction rate) values were plotted against 1/S (1/Substrate concentration) values and kinetic constants were calculated using this graph. K_m and V_{max} values of the enzyme were determined as 91mM catechol and 2.25 Δabs/min/ml, respectively.

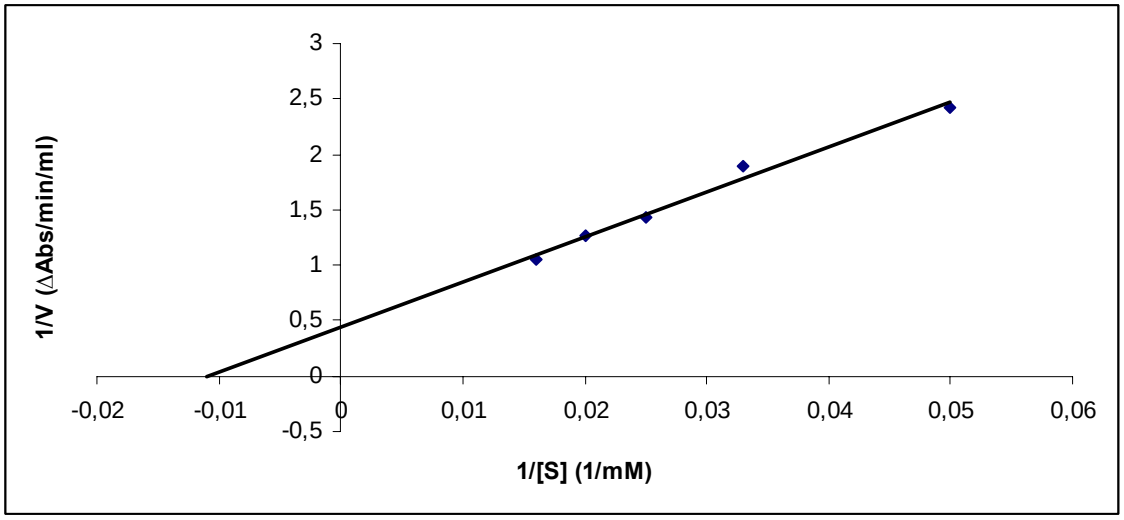

Figure 3.5. Lineweaver-Burk plot for polyphenol oxidase

Bacillus thuringiensis polyphenol oxidase has a K_m value of 34.05mM catechol (Liu, et al. 2004). Polyphenol oxidase from γ-proteobacterium JB has a K_m value of 0.055mM catechol (Singh, et al. 2007). In plants, polyphenol oxidase from apple (cv Amasya), *Ipomoea batatas* and *Amanita muscaria* have K_m values of 34mM, 2.5mM and 83mM catechol, respectively (Mueller, et al. 1996, Oktay, et al. 1995). When polyphenol oxidase from thermophilic *Bacillus* sp. was compared with polyphenol oxidases from other sources, it was seen that this enzyme has lower affinity.

3.4.2. Effect of pH on Polyphenol Oxidase Activity and Stability

The effect of pH on polyphenol oxidase activity was investigated by measuring enzyme activity at different pH values ranging from 4 to 10. The pH profile of the enzyme, which can be seen in Figure 3.6, showed a bell shaped curve with the highest activity at pH 7.0 and it was concluded that pH 7.0 was optimum pH of thermophilic *Bacillus* sp. polyphenol oxidase. A significant loss in activity was observed upon increasing or decreasing the optimum pH value even by one pH unit. The enzyme exhibited low activity at pH 5.0 and no activity at pH 4.0. On the other hand at alkaline pH values, the enzyme is not effected much as in acidic conditions and showed 30% of its activity.

Similar to thermophilic *Bacillus* sp. polyphenol oxidase, optimum pH value close to neutrality have been reported for polyphenol oxidases from other bacterial species such as; *Streptomyces michiganensis* (pH 7.0) (Philipp, et al. 1991), *Pseudomonas putida* (pH 7.0) (McMahon, et al. 2007), *Vibrio tyrosinaticus* (pH 6.6-7.8) (Pomerant.Sh and Murthy 1974), *Streptomyces glaucescens* (pH 6.8) (Lerch and Ettinger 1972), *Streptomyces griseus* (Endo, et al. 2003) and γ-proteobacterium JB (pH 6.5) (Bains, et al. 2003). Nevertheless, acidic and alkaline optimum pH values for polyphenol oxidases from bacterial species such as *Thermomicrobium roseum* (pH 9.5) (Kong, et al. 2000), *Bacillus thuringiensis* (pH 9.0) (Liu, et al. 2004) and *Bacillus licheniformis* (pH 4.2) (Koschorreck, et al. 2008) have also been observed.

The pH stability of the enzyme was examined by incubating the enzyme in various buffers for 1.5 hour. The residual activities were measured under standart assay conditions. The activity of enzyme which was not subjected to pH treatment was regarded as hundred percent, then the residual activities were calculated and plotted against pH values as in Figure 3.7. It can be clearly seen from the figure that the enzyme retained more than 80% of its activity in the pH range of 5-8, however lost 40% of its activity at pH 9. This enzyme was found to be stable as it retained most of its activity through a broad range of pH after 1.5 hour incubation period.

pH stability studies have been carried out with polyphenol oxidases from other bacteria. Kong et al. (2000) reported that *Thermomicrobium roseum* polyphenol oxidase retained more than 70% activity in the pH range of 8.5-10.0 but lost approximately 75%

of activity below pH 6.0 and above 11.0 upon incubation in various buffers at 4 ºC for 20 hours. In another study, polyphenol oxidase from *Pseudomonas putida* was incubated in various buffers for 30 minutes and retained 99% and 80% of activity acroos a broad range of pH values (pH 4-7 for monophenolase and pH 4-9 for diphenolase) (McMahon, et al. 2007).

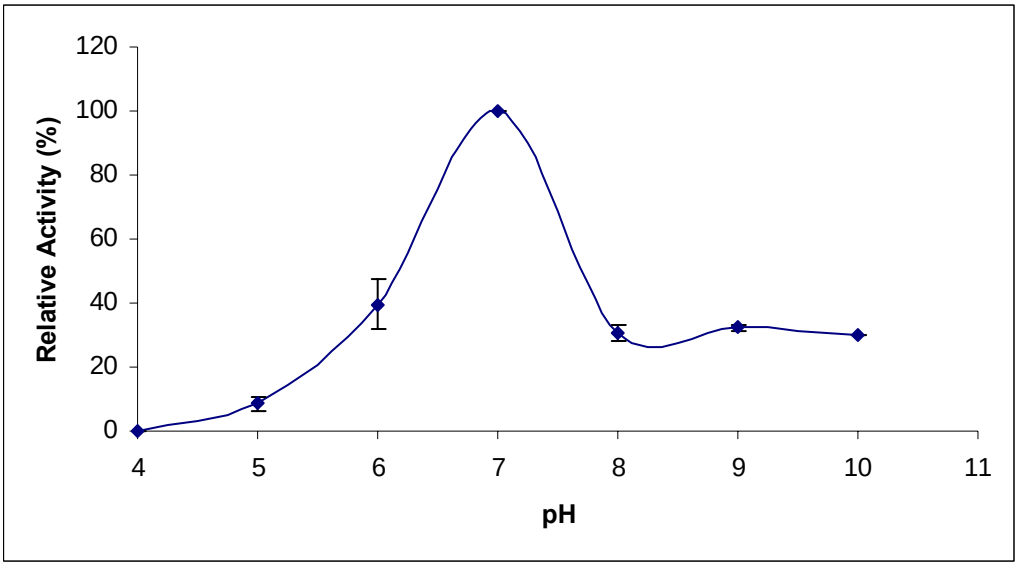

Figure 3.6. Effect of pH on polyphenol oxidase activity

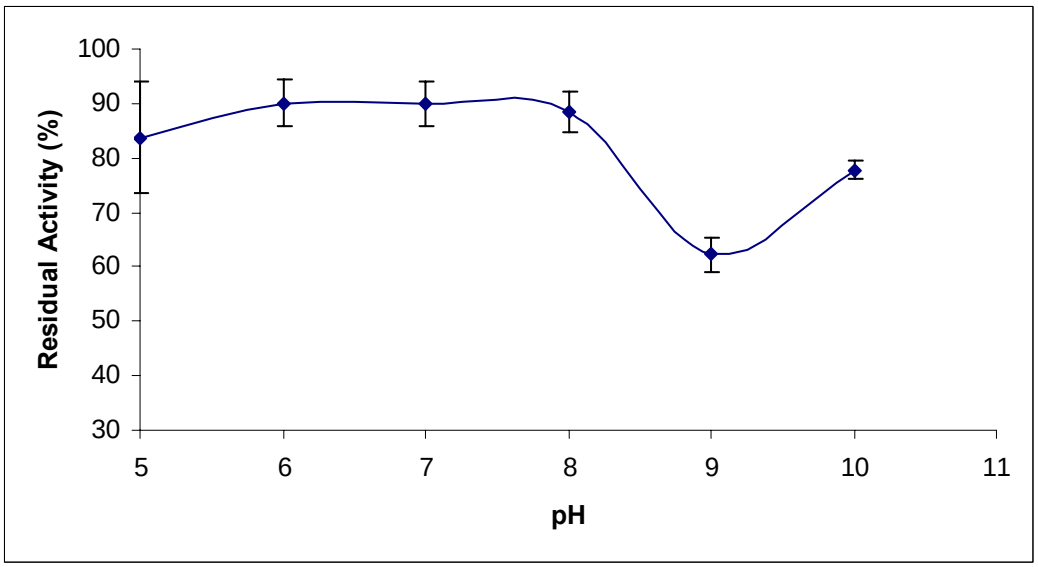

Figure 3.7. pH stability of polyphenol oxidase

3.4.3. Effect of Temperature on Polyphenol Oxidase Activity and Stability

In order to determine the effect of temperature on enzyme activity, polyphenol oxidase activities at different temperatures ranging from 30 to 90ºC were measured. The results of these measurements indicated that the enzyme showed highest activity at 60ºC. As it can be seen in Figure 3.8, the activity of the enzyme was stimulated upon heating up to 60 and 70ºC. However, at temperatures above 70ºC, a decrease in polyphenol oxidase activity was observed with 82% and 35% of the activity at 80 and 90ºC, respectively.

Such a high temperature which was determined for thermophilic *Bacillus* sp. polyphenol oxidase in this study or even higher temperatures of maximal activity were also observed for polyphenol oxidases obtained from other bacteria. The temperature maxima of 92ºC was recorded with *Thermus thermophilus* polyphenol oxidase (Miyazaki 2005), 85ºC with *Bacillus licheniformis* polyphenol oxidase (Koschorreck, et al. 2008), 75ºC with *Bacillus thuringiensis* (Liu, et al. 2004) and CotA protein of *Bacillus subtilis* (Martins, et al. 2002), 70ºC with *Thermomicrobium roseum* (Kong, et al. 2000), and 55ºC with both *Bacillus* sp. HR03 (Dalfard, et al. 2006) and γ-proteobacterium JB (Bains, et al 2003).

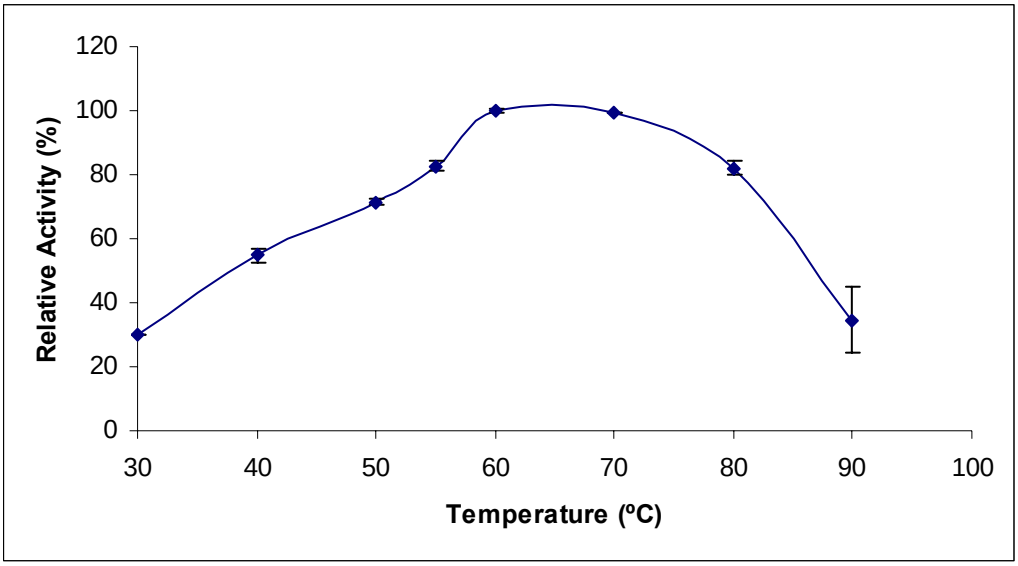

Figure 3.8. Effect of temperature on polyphenol oxidase activity

Thermal stability of polyphenol oxidase from thermophilic *Bacillus* sp. was determined by incubating the enzyme solution at different temperatures for 1.5 hour and measuring the remaining activity under standart assay conditions. The activity of the enzyme which was not subjected to temperature treatment was regarded as hundred percent. The thermal stability profile of polyphenol oxidase can be seen in Figure 3.9. These results showed that the enzyme was fairly stable for 1.5 hour at temperatures up to 60ºC. At temperatures above 60ºC, a decline in activity was observed. Although the enzyme retained nearly 70% of its activity at 70ºC; at 80ºC, the activity was completely lost upon incubation for 1.5 hour.

A hyperthermophilic polyphenol oxidase from *Thermus thermophilus* was found to be resistant to incubation at 85ºC for 10 minutes, also the enzyme retained two-thirds of its activity at 100ºC for 10 minutes (Miyazaki 2005). Polyphenol oxidase from *Bacillus thuringiensis* was most stable at 75ºC (Liu, et al. 2004). *Thermomicrobium roseum* polyphenol oxidase was very stable between 30-70ºC with 10 minutes incubation period (Kong, et al. 2000). On the other hand *Streptomyces* polyphenol oxidase had a half-life of 1-5 minutes at 60ºC (Huber and Lerch 1988). According to these results, thermophilic *Bacillus* polyphenol oxidase can be considered as thermostable with an incubation period of 1.5 hour.

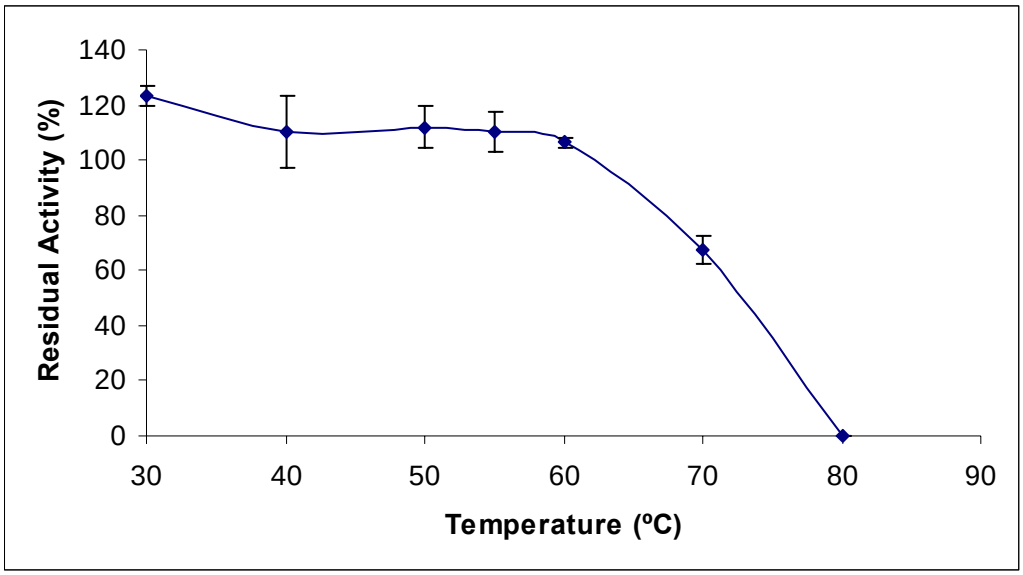

Figure 3.9. Thermal stability of polyphenol oxidase

3.4.4. Effect of Metal Ions on Polyphenol Oxidase Activity

In order to determine the effect of various metal ions on polyphenol oxidase activity, the enzyme was incubated in the presence of an ion for 10 minutes at room temperature and the activity was measured in a normal manner. The concentrations of the metal ions used in this study were all 1mM. The sample which did not contain any metal ion served as control and its activity was regarded as hundred percent. The effects of the ions on enzyme activity are shown in Figure 3.10.

According to the results, the presence of Zn^{2+} and K^{+} did not stimulate the action of polyphenol oxidase but an increase in enzyme activity was observed in the presence of Ca^{2+}, Cu^{2+} and Mg^{2+}. As it can be clearly seen from Figure 3.10, Cu^{2+} caused a significant amount of activation on polyphenol oxidase activity. This outcome is not surprising since polyphenol oxidases are copper containing enzymes and copper is essential for catalytic activity. Similar activator effect of copper on the activity of polyphenol oxidase from *Thermomicrobium roseum* (Kong, et al. 2000) and *Bacillus thuringiensis* (Liu, et al. 2004) were also reported. Also addition of copper to the growth medium of *Bacillus* (HR03) was found to increase the melanin production (Dalfard, et al. 2006).

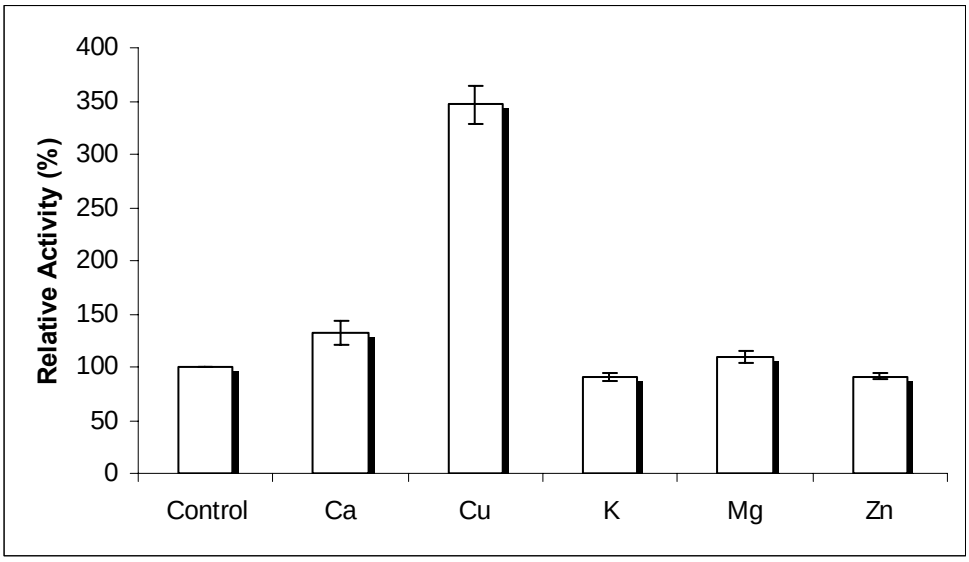

Figure 3.10. Effect of metal ions on polyphenol oxidase activity

3.4.5. Effect of Various Agents on Polyphenol Oxidase Activity

The effect of several agents, which act as inhibitor or activator on the action of polyphenol oxidase were tested. For this purpose, the enzyme was incubated in the presence of relevant agent for 10 minutes at room temperature and then the activity was measured spectrophotometrically under standart assay conditions. The activity of the sample in the absence of agent was regarded as hundred percent and this sample served as control.

The results, which can be summarized in Table 3.2, clearly indicated that DTT and sodium diethyldithiocarbamate are strong inhibitors for polyphenol oxidase from thermophilic *Bacillus* sp. Even in the presence of 1mM of these agents, the enzyme exhibited no activity under standart assay conditions. Sodium diethyldithiocarbamate is a sulfur containing compound and used as a chelating agent for transition metal ions. This agent is known as potent inhibitor of tyrosinase activity of polyphenol oxidases and it was suggested that this compound may cause inhibition by forming complexes with copper atoms in the active site (Kong, et al. 2000). Sodium fluoride, which is regarded as a typical inhibitor for laccase activity of polyphenol oxidases, did not exhibit a strong inhibitory action on polyphenol oxidase in this study. Sodium fluoride with a concentration of 5mM inhibited the polyphenol oxidase activity of *Bacillus thuringiensis* (Liu, et al 2004). Thus, higher concentrations of this agent may be required for the inhibiton of thermophilic *Bacillus* polyphenol oxidase. DMSO and some detergents such as SDS and Triton X-100 did not cause much effect on activity such that the enzyme showed approximately 97% of its activity in the presence of those detergents and 91% in the presence of DMSO. The effect of a chelating agent, EDTA, on enzyme activity was also investigated. Since the active site of polyphenol oxidase contains copper ions and they are involved in catalytic activity, chelating compounds would inhibit polyphenol oxidase activity by removing copper ions. Interestingly, the presence of 1mM EDTA barely effected the action of polyphenol oxidase and the enzyme showed 95% of its activity. However polyphenol oxidases from *Streptomyces griseus* and *Bacillus thuringiensis* showed 67% and 72% of their activity in the presence of EDTA with same concentration, respectively (Endo, et al. 2003, Liu, et al. 2004). In contrast to inhibitory effect, the activator effect of EDTA on *Bacillus thuringiensis*

polyphenol oxidase, in the concentration range of 200-400mM, have been reported (Liu, et al. 2004).

Table 3.2. Effect of various agents on polyphenol oxidase activity

Agent	Concentration	Relative activity (%)
Control	-	100
Sodium diethyldithiocarbamate	1mM	0
Sodium fluoride	1mM	89
DTT	1mM	0
EDTA	1mM	95
SDS	1mM	97
Triton X-100	5%	98
DMSO	1mM	91

3.4.6. Substrate Specificity of Polyphenol Oxidase

The substrate specificity of polyphenol oxidase from thermophilic *Bacillus* sp. was determined by measuring enzyme activity using catechol (20mM), hydroquinone (20mM), L-tyrosine (2mM), ABTS (2mM) and L-DOPA (10mM) at appropriate wavelengts. The results of this assay can be seen in Table 3.3.

Table 3.3. Substrate specificity of polyphenol oxidase

Substrate	Wavelength (nm)	Relative Activity (%)
ABTS	420	2,24
Catechol	420	100
L-DOPA	475	20
L-tyrosine	475	0,78
Hydroquinone	420	8,20

CHAPTER 4

CONCLUSION

The goal of this work was to study bacterial polyphenol oxidases. In order to do that, thermophilic *Bacillus* sp. was choosen and polyphenol oxidase obtained from this bacterium was characterized.

First of all, the activities of boiled and not boiled cultures were measured. Boiled sample which contained denatured enzymes, did not exhibit polyphenol oxidase activity whereas not boiled sample did. This result showed that increase in absorbance when assayed with catechol was due to the existence of polyphenol oxidase, not because of an oxidizing compound that exists in the growth medium of bacterium. In addition, the appearence of dark bands on native polyacrylamide gel after activity staining supported the existence of polyphenol oxidase in thermophilic *Bacillus* sp. Besides, the results of native-PAGE with samples from growth medium of bacterium and intracellular enzyme extract showed that the related enzyme is an intracellular one.

The enzyme was partially purified by acetone precipitation and gel filtration chromatograpy. The yield and purification fold after partial purification of the enzyme were 35% and 1.24, respectively.

The characterization studies indicated that polyphenol oxidase from thermophilic *Bacillus* sp. had highest activity at pH 7.0 and 60ºC. The enzyme was stable at temperatures between 30 and 60ºC and retained approximately 85% of its activity between pH 5 and 8. Also it retained 67% and 62% of its activity even after 1.5 hour incubation at 70ºC and pH 9.0, respectively. However it could not resist to incubation for 1.5 hour at 80ºC and lost all its activity. The activity of the related enzyme was highly stimulated in the presence of copper ion and totally inactivated by DTT and sodium diethyldithiocarbamate. Other agents or metal ions did not have a considerable inhibitory or stimulating effect on enzyme activity. K_m and V_{max} values for the enzyme determined from Lineweaver-Burk plot were 91mM and 2.25 Δabs/min/ml.

In conclusion, the enzyme was partially purified and some general characteristics of it were determined. To obtain higher acitivities and be applicable for industrial purposes, further purification steps may be required. Additionally, the gene responsible for polyphenol oxidase enzyme can be cloned and expressed in suitable hosts and some features of the enzyme may be altered by protein engineering techniques. The enzyme of interest can be used for the development of biosensors to detect phenolic compounds for various purposes, also the ability of polyphenol oxidase to act on phenolic compounds can be used for the degradation of phenols in industrial waste waters.

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APPENDIX A

PREPARATION OF BRADFORD REAGENT AND PROTEIN STANDARTS FOR BRADFORD ASSAY

Preparation of Bradford Reagent

- 10.0mg Coomassie Brilliant Blue G-250 (CBB G-250)
- 5ml 95% ethanol
- 10ml 85% phosphoric acid

Dissolve CBB G-250 in ethanol, add 10ml phosphoric acid. Bring to 100ml with ultra pure water and when the dye has completely dissolved, filter through Whatman No. 1 paper. Store at 4ºC.

Preparation of Protein Standarts

Bovine serum albumin (BSA) was used as protein standart. To obtain a stock solution with a concentration of 0.2 mg/ml; 0.02g BSA was dissolved in 1ml dH_2O, then 10 µl was taken from this stock and added 990µl dH_2O to give a final concentration of 0.2mg/ml.

To prepare standarts according to the table, necessary amounts of water, BSA and bradford reagent were pipetted into cuvettes respectively (Table A.1), then incubated at room temperature for 5 minutes. At the end of incubation period, absorbance was measured at 595nm using a spectrophotometer.

Table A.1. Preparation of BSA standarts

	BSA (µl)	dH_2O (µl)	Bradford Reagent (µl)
Blank	0	800	200
Standart 1- 1 µg/ml	5	795	200
Standart 2- 2 µg/ml	10	790	200
Standart 3- 4 µg/ml	20	780	200
Standart 4- 6 µg/ml	30	770	200
Standart 5- 8 µg/ml	40	760	200

APPENDIX B

STANDART CURVE FOR BRADFORD ASSAY

Table B.1. Absorbance values of BSA standarts

Concentration (µg/ml)	Absorbance (595nm)
1	0,0782
2	0,1427
4	0,2073
6	0,2806
8	0,3282

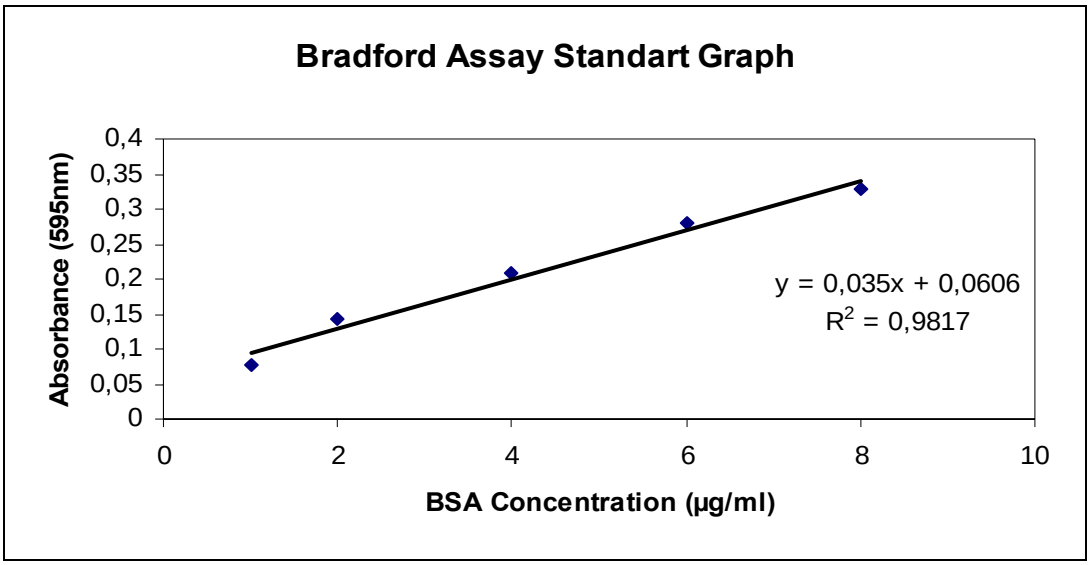

Figure B.1. BSA standart curve for Bradford assay

APPENDIX C

REAGENTS AND GEL PREPARATION FOR SDS-PAGE

Stock Solutions

A. 30% Acrylamide Mixture

- 29.2g acrylamide
- 0.8g N'N'-bis-methylene-acrylamide

Make up to 100ml with deionized water. Filter and store at 4ºC in the dark for at least one month.

B. 1.5M Tris-HCl, pH 8.8

- 18.15g Tris Base
- ~80ml deionized water

Dissolve Tris base in deionized water, adjust to pH 8.8 with HCl. Make up to 100ml with deionized water and store at 4ºC.

C. 0.5M Tris-HCl, pH 6.8

- 6g Tris Base
- ~80ml deionized water

Dissolve Tris base in deionized water, adjust to pH 6.8 with HCl. Make up to 100ml with deionized water and store at 4ºC.

D. 10% SDS

Dissolve 10g SDS in 90ml water with gentle stirring and bring to 100 ml with deionized water.

E. Sample Buffer

- 3.8ml deionized water
- 1.0ml 0.5M Tris-HCl, pH 6.8
- 0.8ml Glycerol
- 1.6ml 10% (w/v) SDS
- 0.4ml 2-mercaptoethanol
- 0.4ml 1% (w/v) bromophenol blue

F. 5X Running Buffer

- 15g Tris Base
- 72g Glycine
- 5g SDS

Dissolve Tris base, glycine and SDS in ~800ml deionized water and make up to 1L with water. Store at 4ºC. For electrophoretic run, dilute 5X stock solution to 1X with deionized water.

G. 10% Ammonium persulfate (APS)

Dissolve 0.1g APS in 1ml deionized water. This solution should be prepared fresh daily.

H. Colloidal Coomassie Staining Solution

Dissolve 40g ammonium sulfate in ~300ml water, add 8ml 85% o-phosphoric acid and add 0.5g Coomassie Brilliant Blue G-250. Make up to 400ml with water, add 100ml methanol to 500ml total volume. Store at 4ºC.

I. Neutralization Buffer

0.1M, pH 6.5 Tris-phosphate in deionized water.

J. Destaining Solution

25% (v/v) methanol solution.

K. Fixation Solution

20% (w/v) Ammonium sulfate in deionized water.

Gel Preparation

- Separating Gel

Table C.1. Preparation of 12% SDS-PAGE separating gel (for 5ml)

Deionized water	1.68ml
1.5M Tris-HCl, pH 8.8	1.25ml
10% SDS	50µl
Acrylamide/Bis (30% Stock)	2ml
10% APS	25µl
TEMED	2.5µl

- Stacking Gel

Table C.2. Preparation of 4% SDS-PAGE stacking gel (for 5ml)

Deionized water	3.05ml
0.5M Tris-HCl, pH 6.8	1.25ml
10% SDS	50µl
Acrylamide/Bis (30% Stock)	665µl
10% APS	25µl
TEMED	5µl