DOKUZ EYLUL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

ISOLATION, PURIFICATION AND CHARACTERIZATION OF CATALASE FROM *PHANEROCHAETE CHRYSOSPORIUM*

by

Berna KAVAKÇIOĞLU

January, 2012
İZMİR

ISOLATION, PURIFICATION AND CHARACTERIZATION OF CATALASE FROM *PHANEROCHAETE CHRYSOSPORIUM*

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"ISOLATION, PURIFICATION AND CHARACTERIZATION OF CATALASE FROM *PHANEROCHAETE CHRYSOSPORIUM*"** completed by **BERNA KAVAKÇIOĞLU** under supervision of **PROF. DR. LEMAN TARHAN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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To my dear father always living in my heart, Serdal KAVAKÇIOĞLU…

ISOLATION, PURIFICATION AND CHARACTERIZATION OF CATALASEFROM *PHANEROCHAETE CHRYSOSPORIUM*

ABSTRACT

Catalase is one of the antioxidant enzymes that plays a major role in the protection of the cells and the tissues from the toxic effects of hydrogen peroxide.

In this study, 129.10-fold catalase enzyme purification was obtained from white rot fungus *Phanerochaete chrysosporium* by 60 percentage ammonium sulphate precipitation, 60 percentage ethanol precipitation, DEAE-cellulose anion exchange and by applyingsephacryl-s-200 gel filtration chromatography as a final purification step. The molecular weight of purified catalase was found about 290 kDa with gel filtration chromatography. SDS-gel electrophoresis results indicated that *Phanerochaete chrysosporium* catalase consists of four apparently identical subunits, with a molecular weight of around 72 kDa. Optimum pH and the temperature values of the purified catalase was found as 7.5 and 30 celcius, respectively. It was found that purified catalase was more stable in the basic region and it lost its catalitic acitivity about 25 percentage at 60 celcius. The K_m and V_{max} values were found as 290.69 mM and 250000 U/mg respectively for the purified catalase. It was not observed that imazolyl and procymidon had inhibition effects on purified catalase. However, it was found that benomyl inhibited purified catalase as non-competitive inhibitor and K_i value was determined as 1.158 mM.

Keywords: *Phanerochaete chrysosporium*, catalase, purification.

KATALAZ ENZİMİNİN*PHANEROCHAETE CHRYSOSPORIUM*DAN İZOLASYONU, SAFLAŞTIRILMASI VE KARAKTERİZASYONU

ÖZ

Katalaz, hücre ve dokuların hidrojen peroksidin toksik etkilerinden korunmasında temel rol oynayan antioksidan enzimlerden biridir.

Çalışma kapsamında, yüzde 60 amonyum sülfat çöktürmesi, yüzde 60 etanol çöktürmesi, DEAE selüloz anyon değiştirici kromatografisi ve son saflaştırma basamağı olarak da sephacryl- s-200 jel filtrasyon kromatografisi uygulanarak beyaz çürükçül fungus *Phanerochaete chrysosporium* katalazı 129,10 kat saflaştırılmıştır. Saflaştırılmış katalazın moleküler ağırlığı jel filtrasyonu ile yaklaşık 290 kDa olarak bulunmuştur. SDS-PAGE jel elektroforezi saflaştırılmış enzimin 72 kDa ağırlığında dört eşdeğer subunitten oluştuğunu göstermiştir. Saflaştırılmış enzimin optimum pH değeri 7,5 ve optimum sıcaklığı 30 derece olarak bulunmuştur. Enzim bazik bölgede daha stabil olup 60 derece 2 saat sonunda yaklaşık yüzde 25 aktivite kaybına uğramıştır. Saflaştırılmış katalaz için K_m ve V_{max} değerleri sırasıyla 290,69 mM ve 250000 U/mg olarak bulunmuştur. İmazalil ve procymidon fungusitleri saflaştırılmış katalaz aktivitesini inhibisyona uğratmazken benomil fungusitinin yarışmasız inhibitör etkisine sahip olduğu belirlenmiş ve K_i değeri 1,158 mM olarak bulunmuştur.

Anahtar kelimeler: *Phanerochaete chrysosporium,* katalaz, saflaştırma.

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CHAPTER ONE

INTRODUCTION

1.1 Proteins

Proteins constitute a various and complex group of macromolecules performing the functions necessary for the continuance of life. The fact that approximately 50% of dry weight of a cell is made up of a large number of unique proteins shows the significance of proteins in living creatures (McKee and McKee, 2009).

Proteins are the polymers of various combinations of 20 different amino acids staying in company with covalent bounds. Only a very little part of protein structures likely to form theoretically with 20 different amino acids (650,000 – 2.10^6) can be produced by living creatures.

Basic characteristics of the proteins produced by living creatures; their structures allow plications-flexions to be performed quickly and successfully, they have specific bounding areas for one or a certain number of molecule groups, their surface characteristics are adoptable to the environment where they are (as proteins localized in membranes and hydrophobic and cytoplasmic proteins have hydrophilic structures), when they are damaged or they lose their functions, they become sensitive to degradation reactions.

Principal functions of proteins are catalysis, structural support, cellular movement, defense, regulation, transport, storage and stress response and proteins responsible for catalysis are named as enzymes.

1.1.1 Enzymes

Enzymes are biological catalysts accelerating the velocity of biochemical reactions like digestion, capturing energy and biosynthesis between 10^6 and 10^{12} times.

Enzymes differ from regular inorganic catalysts in respect of some important respects.

1. Biochemical reactions catalyzed by enzymes occur minimum a few times faster than chemical reactions catalyzed by inorganic catalysts.
2. Reactions catalyzed enzymatically may occur under moderate conditions (i.e. atmospheric pressure, neutral pH and temperature conditions under 100 ^{0}C). Contrary effective chemical catalyst requires high temperature and pressure conditions as well as extreme pH values in general.
3. Undesired side products due to reaction specificity are little if any in enzymatic reactions.
4. Catalytic activity of many enzymes can be regulated with the mechanisms like allosteric regulation, covalent modification of enzymes and control of synthesis amount.

1.1.1.1 Classification of Enzymes

Enzymes are categorized principally in 6 different classes in accordance with the type of reaction where they are catalyzed.

1. Oxireductases catalyze oxidation-reduction reactions. Dehydrogenase and reductases catalyzing redox reactions are examples for oxireductases.
2. Transferases constitute an enzyme group transferring the group received from a donor molecule to acceptor molecule. These groups cover the groups like amino, carboxyl, carbonyl, methyl, phosphoryl and acyl.

3. Enzyme class catalyzing the bounds like C-O, C-N and O-P with water addition is called as hydrolases. Esterases, phosphatases and proteases take place in this class.

4. Lyases catalyze the reactions where groups like H_2O, CO_2 and NH_3 are taken away to form a double bond or are added to a double bond. Decarboxylases, dehydratases, deaminases and synthetases are examples for lyase group enzymes.

5. Isomerases constitute a heterogenic enzyme group and catalyze many types of intra molecular re-arrangement reactions. While epimerase catalyzes inversion of asymmetric carbon atoms, mutases catalyze intra-molecular functional group transfers.

6. Enzyme group catalyzing bond generation reactions between two substrate molecules constitute ligases. DNA ligase and carboxylases are examples for ligase group enzymes.

1.1.1.2 Enzyme Kinetics

One of the most usable models in the consideration of enzymatic reaction velocity systematically is Michaelis Menten model suggested by Leonor Michaelis and Maud Menten in 1913.

According to this model, substrate, S generates an interim complex (ES) when bounded to active area of enzyme, E. During transition substrate turns to product and then product departs from enzyme. This process can be summarized as follows.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

k_1 = ES generation reaction velocity constant.

k_{-1} = ES disassociation reaction velocity constant.

k_2 = Product generation reaction and velocity constant for it to be issued from active area.

There are some assumptions in the model of Michaelis Menten:(1) k_{-1} constant is neglected beside k_1 constant. (2) Generation and destruction velocities of ES complex during reaction are same (case of steady state).

Equity derived by Michaelis and Menten is as follows.

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

V_{max}; maximum velocity reached by reaction and K_m; constant of Michaelis Menten.

$$K_m = (k_{-1} + k_2) / k_1$$

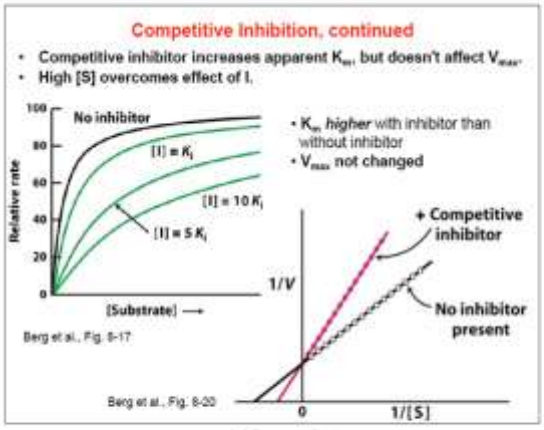
Michaelis Menten equity is very useful in defining certain characteristics of enzyme behavior. According to equity when substrate concentration, [S] becomes equal to K_m; velocity becomes equal to the half of maximum velocity. K_m value determined experimentally is a characteristic constant to enzyme and its substrate under certain conditions and indicates the relation of enzyme to substrate. When K_m is decreased, relation of enzyme to substrate increases.

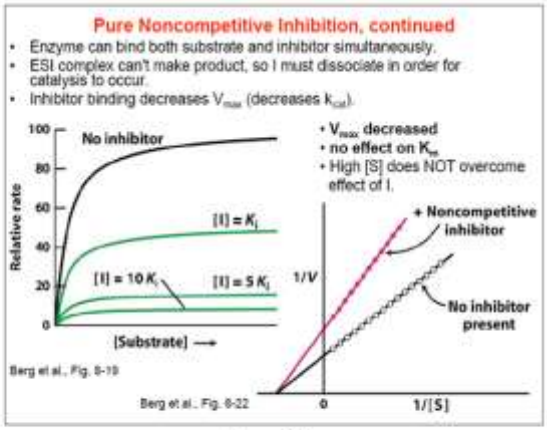
1.1.1.3 Enzyme Inhibition

Molecules decreasing enzyme activity like drugs, antibiotics and food additives are called as inhibitors. Research of enzyme inhabitation and inhibitors; understanding regulation ways of metabolic way marks are of great importance in terms of developing technique in the clarification of physical and chemical structure of enzyme as well as numerous clinical therapies and functional characteristics of enzyme (Altinordulu and Eraslan, 2009; Kashiwagi et all., 1998).

Enzyme inhibition can be carried out as reversible or non-reversible. In non-reversible inhibition; inhibitor is bounded to enzyme as covalent and effect of inhibition can't be removed with the increase of substrate concentration. Reversible inhibition is the inhibition type where inhibition effect is eliminated with the increase of substrate concentration. Reversible inhibition can come into existence in three different ways as competitive, non-competitive and limited-competitive. In competitive reversible inhibition; inhibitor and substrate compete to reach active area of enzyme. Inhibition type where inhibitor can be bounded to both free enzyme and

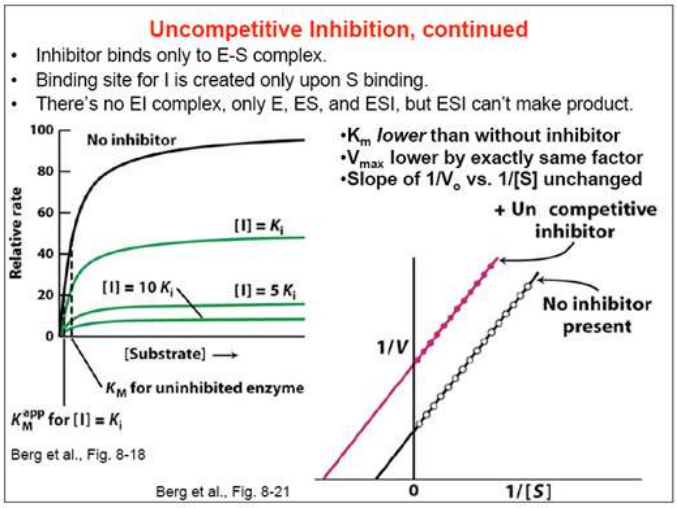
enzyme-substrate complex is non-competitive inhibition. In the type of limited competitive inhibition, inhibitor can be bounded only to enzyme-substrate complex.





$$V_0 = \frac{V_{max}[S]}{K_m(1 + \frac{[I]}{K_i}) + [S]}$$

$$v = \frac{V_{max}[S]}{\left([S] + K_m\right)\left(1 + \frac{[I]}{K_i}\right)}$$



$$1/v = \frac{K_m}{V_{max}[S]} + \frac{1 + [I]/K_i}{V_{max}}$$

1.1.1.4 Factors Affecting Enzyme Activity

Any environmental factor disordering protein structure can change enzymatic activity. Enzymes are affected by the changes in temperature and pH.

All chemical reactions are affected by temperature. Because increase in temperature will increase collision frequency in general, it will increase reaction velocity also. Velocities of the reactions with enzyme catalysis rise up with increasing temperatures. However, enzymes are the proteins denaturized in high temperatures. Optimum temperature of an enzyme is the temperature value where it operates with maximum effect (Kagawa et al., 1999; Tarhan 1991; Tarhan and Uslan, 1990; Tarhan and Telefoncu, 1990; Tarhan 1990; Goldstein, 1989).

Catalytic activity of an enzyme is related to ionic situation of active area. Change in hydrogen ion in environment may affect the ionization of functional groups in active area. For example, some enzymes need protonated state of amino group in side chain. Because amino group will lose its proton if pH of environment slides to alkali area, enzyme activity may be stressed. Ionization of substrate as well as the groups in active area of enzyme and this case can prevent it from being bounded to active area. Changes in loads of the groups which can be ionized based on pH may cause reversible structure of enzyme and enzyme denaturation. Many of enzymes can operate within a limited pH range. Thus it is important to use tampons while working with enzymes. pH value where enzymatic activity is maximum is called as optimum pH value for that enzyme.

1.1.2 Significance of Enzyme and Enzyme Technology

Especially when compared with inorganic catalysts, enzymes have extraordinary catalytic characteristics, in recent times they have been used increasingly commonly in the fields like food, pharmaceutics and chemistry industry. Advantages like high catalytic activity, absence of products with undesired side reaction and activity in moderate conditions are mostly preferred characteristics. Furthermore many different enzyme groups become usable thanks to the developments in genetics engineering and this enlarges the activity area of enzyme technology. Works in genetics engineering require purified enzyme preparations with high productivity as much as possible (Godfrey and West, 1996).

1.1.2.1 Usage of Microorganisms as Enzyme Resource in Enzyme Technology

Oldest industrial processes where enzymes in living organisms are used are fermentation process and cheese-making. Both processes has been performed centuries ago without knowing their biochemical foundations, serious works for fermentation to be understood biochemically have been performed in late 19th century and early 20th century.

Micro organisms used as target enzyme or resource of enzymes in food industry and principal reactions where enzymes are catalyzed are stated in Table 1.1(Price and Stevens, 1999).

Table 1.1 Use of organisms in food industry

Process	Organism used	Enzyme
Fermentation and wine making	Barley (*Hordeum*)	α-amylase and β-amylase, endo-1,3-β-D-glukanaz
	Saccharomyces spp	Proteolysis of oligosaccharides limited with monosaccharides; glycolytic enzymes
Cheese making	*Streptococcus lactis* *Streptococcus cremoris*	Limited lipolysis and proteolysis
	Propionobacteria spp	Conversion of lactate to propionate acetate, carbon dioxide and water
	Penicillium camemberti *Penicillium roqueforti*	limited lipolysis and proteolysis
Vinegar production	*Acetobacter*	Conversion of Ethanol to acetate

Micro organisms are used commonly also in the production of organic chemicals out of the above mentioned processes (Zindwick, 1992; Masurekar, 1992; Rambosek, 1992).

1.1.2.2 Usage of Isolated Enzymes in Enzyme Technology

Even though microorganisms are used as enzyme resource, productivity of these processes can be increased with the use of isolated enzymes. Advantages of the use of enzyme as compared with the use of an entire organism can be listed as possibility to obtain higher catalytic activity, preventability of undesired side reactions and increase of production quantity. However higher cost of the use of isolated enzyme is an independent disadvantage. Furthermore denaturation of isolated enzymes based on environment conditions is a risk also. Thus enzymes isolated from thermophilic fungi or bacteria can be a way to prevent heat denaturation.

Resource of the isolated enzymes used in industrial applications is generally fungi and bacteria. It is very easy to reproduce fungi and bacteria and thus to increase the size of process (Scawen, Atkinson, Hammond and Sherwood, 1993). In the use of microbial enzymes it is possible to increase the process productivity by modify growing conditions and adding target enzyme inductive to growing environment or works of genetic engineering.

Catalase enzyme purified within the scope of this thesis is one of the important enzymes in terms of the usability in industrial applications. It is used together with glucose oxidase in food industry in order to prevent the oxidation and degradation of foods. It is commonly used also in taking away from environment the hydrogen peroxide used in sterilization of milky products(Akertek and Tarhan, 1995; Tarhan, 1995).

1.1.3 Catalase Enzyme

1.1.3.1 General Characteristics of Catalase Enzyme

Catalase (CAT; H_2O_2:H_2O_2 oxireductase, E.C.1.11.1.6) is one of the antioxidant enzymes catalyzing decomposition of hydrogen peroxide to molecular oxygen and water and preventing hydrogen peroxide from turning to radical types and damaging cellular components (Aydemir and Kuru, 2003).It has a tetra metric structure and four equal subunits and its molecular weight is approximately 240 kDa. CAT uses substrate hydrogen peroxide as both electron receiver and electron transmitter and is localized predominantly in peroxisomes of cells.

Reaction catalyzed by CAT is two-step. In first step of reaction, hem-iron in the structure of CAT reacts with hydrogen peroxide and iron peroxide comes into existence.

$$CAT\text{-}Fe\text{-}OH + H_2O_2 \rightarrow CAT - Fe - OOH + H_2O$$

Iron peroxide interim product is called as Compound-I. When there is enough hydrogen peroxide in environment, CAT uses substrate hydrogen peroxide as both electron acceptor and electron donor and is localized predominantly in peroxisomes of cells and Compound I reacts with second hydrogen peroxide molecule turns to molecular oxygen and water.

$$CAT\text{-}Fe\text{-}OOH + H_2O_2 \rightarrow CAT - Fe - OH + H_2O + O_2$$

In the event there is not enough hydrogen peroxide in environment, compound-I is decreased by a suitable hydrogen donor and reinstated.

1.1.3.2 Chronology of the Studies on Catalase Enzyme

First studies on catalase enzyme have been made in early 19th century. In 1811 Thenard stated that H_2O_2 is hydrolyzed by a special molecule and then in 1863 this molecule was defined by Schönbein as a special ferment (Zamocky and Koller, 1999). Even though in early 1990s scientist whose name is Loew defined the enzyme degrading H_2O_2 as catalase,after 22 years Warburg indicated with cyanide inhibition experiment that active area of enzyme includes iron. In 1936 Stern stated that protoporphyrin IX is active area in all known catalases and after one year catalase crystals were obtained from cattle's liver for the first time (Sumner and Dounce, 1937).

In 1947 Chance founded the base complex coming into existence between Catalase and H_2O_2 (Compound I). Chance stated that enzyme behavior is only up to steady state concentration of hydrogen peroxide and that peroxidatic function in lower H_2O_2 concentrationsand catalytic function in higher concentrations will be dominant respectively (Chance, 1951). In 1949 Chance, Lemberg and Foulkes discovered a more advanced derivation called as Compound II independently from each other. After a few years upon the discovery of the form of inactive compound III, all of the catalase interim products were clarified.

In 1948 procaryotic catalase was obtained first by Herbert and Pinsent from Micrococcus luteus and some key residues taking place in active area were clarified and their significances in structure stabilization were discussed. In following years studies were performed on cellular localization of catalase in prokaryotes and simple eukaryotes like fungi.

First electron intensity map has been issued by Vainsthein et all in 1980. In same periods catalase of cattle's liver was obtained in accordance with X-ray. In 1984 Kirkman and Gateni notifies the existence of NADPH bounded strictly to cattle's liver catalase and determined them in X-ray analysis. Catalase data base of many different classes was established thanks to sequencing works and it was seen that this oxireductase class doesn't constitute a homogenous group. In 1989, Goldberg and Hochman divided catalases into three different groups as mono-functional catalase, catalase-peroxidase abd manganese-catalase based on physical and biochemical characteristics.

Because they are used widely in medicine and industry, catalase enzyme has been always the focus of scientific researches. First works on catalase were immobilization trials to increase structural stability and DNA manipulation strategies have been researched on catalase gens for last 10 years. Today a data base made up of 100 sequence analysis for mono-functional, catalase-peroxidase and manganese-catalase is available.

1.1.3.3 Classification of Catalases

Mono functional catalases constitute greatest sub group and are available in all aerobic creatures as prokaryotic and eukaryotic (Kagawa et al., 1999). Mono functional catalases have not been observed in archeas. Especially it has two or more isoforms in many organisms especially plants. Many of these hydroperoxidases are homo-tetramerous and their molecular weights range between 200 and 340 kDa. Emission of strong absorbance spectrums in soret band, irreversible inhibitions with 3-amino-1,2,4-triazol and easy degradation of iron hem groups with sodium

dithionite fall within its characteristic physical features. It catalyses 2-electron peroxidations of short chained aliphatic alcohols with middle velocity as well as very effective catalytic reaction velocities ($k_{cat}=4x10^{7}$ M^{-1} s^{-1}). Gens coding monofunctional catalase were isolated from the organisms more than 75 and sequence analysis has been made. In some cases cofactors like NADPH bounded strictly to subunits have been determined.

Catalase-peroxidases constitute second group peroxidases and are available in all creatures. However they have been observed only in fungi from the class of eukaryotic creatures to catalase peroxidases. Their molecular weights range between 120-340 kDa, they have been generally homodimers and homotetramers have not been observed. Their most distinctive characteristic feature is the fact that they show bio functional catalytic behavior. Maximum turnover number of catalase-peroxidases is two or three times lower than mono functional catalases.

All known catalase peroxidases have different hem spectra. Contrary to typical hem catalases they have approximately 6.5 sharp pH optimum. However catalase-peroxidases are more sensitive to the parameters like high temperature and pH and H_2O_2 concentration as compared with other typical catalases.

Manganese catalases; there are three manganese catalases, one from lactic acid bacterium (Beyer and Fridovich, 1985) and two from thermophilic organisms (Barynin and Grebenko, 1986). These enzymes are called as pseudocatalase since they use manganese ions. Contrary to the hem including types, catalases are not inhibited by CN^{-} and N_3^{-} ions. Their weights vary between 170 and 210 kDa and form different oligometric structures (hemopentamers or homohexamers).

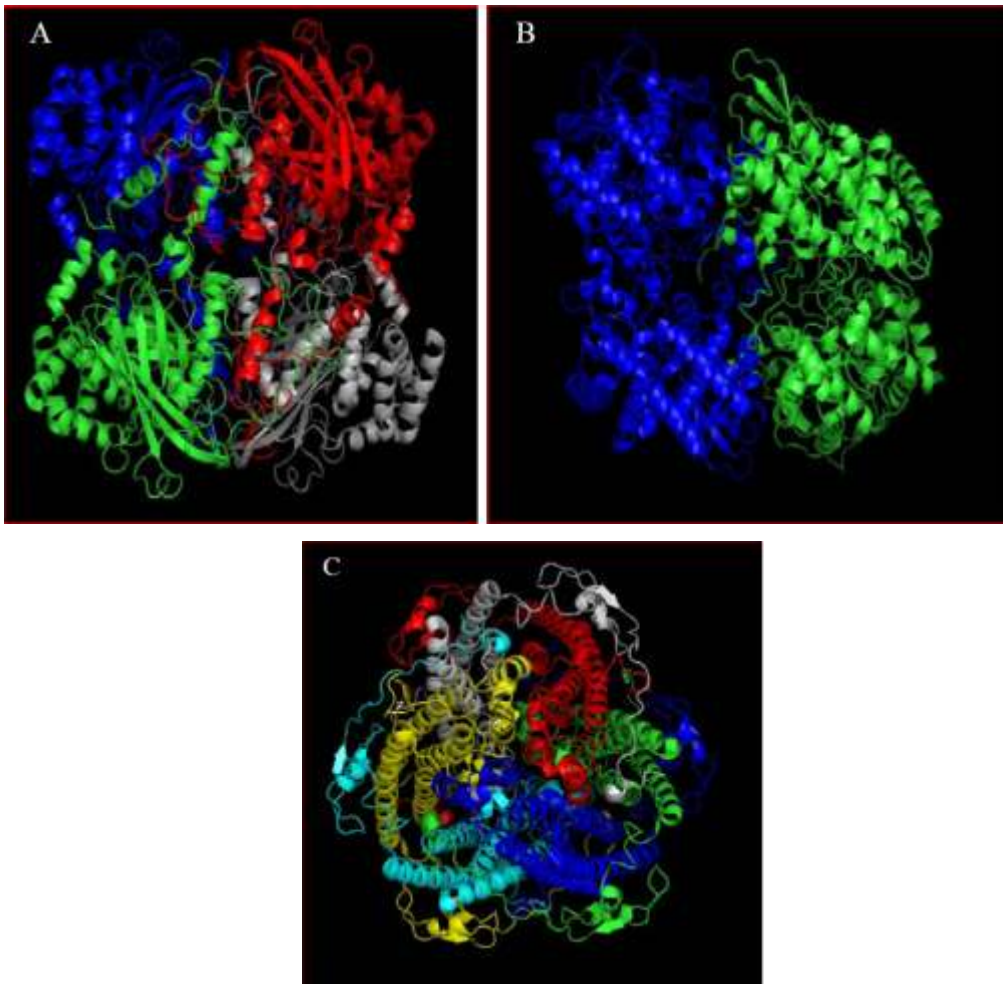


Figure 1.1 (A) Mono-functional catalase; (B)Catalase-peroxidase; (C) Manganese-catalase

1.1.3.4 Literature Summary

Kang et all determined that photosynthetic bacterium Rhodospirillum rubrum S1 produces three different catalases when grown under anaerobic conditions and purified and characterized the heaviest one from these catalase types. At the beginning total specific catalase activity in raw cell extract was 88 U/mg. After the calculation of last purification, enzyme was purified 14.27 times and specific activity value reached 1256 U/mg. Procedures used in purification procedures were respectively 30-70% ammonium sulfate precipitation, DAEA-cellulose anion change chromatography, Sephadex G-200 gel filtration colon and 7,5% non-denature polyacrylamide gel electrophoresis. Pure enzyme is made up of 4 equal subunits of

79 kDa, its total molecular weight has been determined approximately as 318 kDa. K_m and V_{max} values of pure enzyme against hydrogen peroxide have been found respectively 30.4 mM and 2564 U. Pure enzyme preparation has a wide pH range (5.0~9.0) and protects its stability in a wide temperature range (20°C~60°C). Sodium cyanide, sodium aside and hydroxylamine known as hem including catalase inhibitors have inhibition effect on Rhodospirillum rubrum S1 catalase. According to findings purified catalase has been defined as mono-functional catalase (Kang, Lee, Yoon ve Oh, 2005).

Nakamura et all purified catalase enzyme from dog erythrocytes and compared the characteristics of this enzyme with characteristics of human erythrocyte catalase. In the event glycerol was not used as stabilizing agent, purification procedure couldn't be carried out. Dog erythrocyte hemolysate was dialyzed to 1 mM EDTA and 1.5 mM phosphate tampon (pH 6.8) including 10% glycerol during all night and centrifuged at 9000 g during 10 minutes. Supernatant was mixed with Fractogel EMD DEAE gel suspension and mixture was waited at 4 ^{0}C during 1 hour. Gel was washed with the tampon used in dialysis in order to take the hemoglobins away and filled to colon (5 x 6 cm). Catalase was eluted with 30 mM phosphate tampon (pH 6,8) including 10% glycerol. DEAE fractions indicating catalase activity have been collected. DEAE fraction's pH is adjusted to pH 5.4 with 200 mM acetic acid and applied to Fractogel EMD COO column (1.5 x 3 cm) balanced with 30 mM acetate tampon (pH 5.4) including 10% glycerol. After column was washed with same buffer, catalase elution was carried out with the linear increase of NaCl concentration in buffer from 0 to 300 mM. Active COO fractions were collected in terms of Catalase, they were dialyzed against 20 mM Tris (pH 8.3) including 10% glycerol and applied to polybuffer exchanger PBE 94 gel column (1 x 25 cm). After column was washed with same buffer, catalase was eluted with pure water including 10% glycerol and mixture (pH 5.0) of polybuffer 74 and polybuffer 96 (9: 0.7: 0.3). Fractions where catalase activity is observed were regulated to 20% ammonium sulfate saturation and then balanced with 1.5 mM phosphate buffer (pH 7.4) including 1mM EDTA, 10% glycerol and 20% ammonium sulfate and applied to butyl-toyopearl gel column. Column was washed with balance buffer, elution of

catalase was carried out with 1.5 mM phosphate buffer (pH 7.4) including 1mM EDTA and 50% ethylene glycol. After the purification steps catalase enzymes were purified 4823 times (Nakamura, Watanabe, Sasaki and Ikeda, 2000).

Yanık and Donaldson benefited from ion change chromatography (DEAE-Sepharose), gel filtration (Sephacryl-300 HR) and hydroxylapatite chromatography in their studies where they investigate the relationship between catalase protein and other proteins in the glioksizoms of sprouted castor bean (Yanik and Donaldson, 2005).

Ebara and Shigemori purified alkali-tolerant catalase from thermophlic bacterium Metallosphaera hakonensis. Isolation and purification procedure can be summarized as follows. 10 g cell is washed with 50 mM Tris-HCI buffer (pH 7.6) and suspended in 50 mL of same buffer. After cells are degraded with sonication, centrifuge has been made at 48000 g during 30 minutes in order to take away the cell debris. Obtained supernatant was heated at 68 ^{0}C during 10 minutes. Contaminated proteins denatured as a result of heat application are taken away with centrifuge at 4800 g during 30 minutes. Catalase remained at supernatant. Enzyme solution was dialyzed against 50 mM Tris-HCl buffer including 1 $MnCl_{2.}$ After enzyme solution is regulated to 1.0 M ammonium sulfate saturation and it is applied to hydrophobic interaction chromatography column(Butyl-Toyopearl 650-M, TOSO).Washing was made with 50mM potassium phosphate buffer including 1.0 M ammonium sulfate and elution was made with 50 mM potassium phosphate buffer including 1.0-0 M decreasing ammonium sulfate gradient. After fractions performing catalase activity are collected and dialyzed, fraction desalted is applied to DEAE-cellulose anion exchanger column.DEAE fractions including catalase activity were collected and applied to Sephacryl S-200 HR gel filtration as final step after concentrated (Ebara and Shigemori, 2008).

Zemocky et all purified hem including catalase-1 which is the form found mostly in NH3 catalases from soil bacterium Comamonas terrigena N3H. Cell of which wet weight was approximately 26 g was harmonized with glass beads with 0.2 mm

diameter at 450 mL of 20 mM Tris-HCl (pH 7,5) buffer. Catales was separated with 0% →45% (w/v) ammonium sulfate fraction from other intracellular proteins. Obtained pellet was degraded at 20 mM Tris-HCl (pH 7.5) buffer including 0.85 M ammonium sulfate at the minimum volume as much as possible. Supernatant obtained after centrifuge was applied to hydrophobic spheron 100.000 column (2.6 x 6 cm). Balance operation was made with 20 mM Tris-HCl (pH 7.5) buffer including 1.7 M ammonium sulfate. 1.7 M → 0.0 M ammonium sulfate linear gradient was used in elution step. Fractions showing highest catalase activity were gathered and applied to Superose 12 column (Pharmacia, "Superose P12," 2,5 x 40 cm) as more advanced chromatographic step. Balance operation was made with same buffer also. Active fractions were applied to Q Sepharose Fast Flow column of which channel volume is 10 cm^3. In last chromatographic step Mono Q HR 5/5 (Pharmacia) column was used. Specific activity of purified catalase example was determined as 55900 U/mg (Zamocky et al, 2004).

Shi et al. used ammonium sulfate fraction, anion change chromatography and Macro-prep Ceramic hydroxyapatite as purification steps in their studies where they targeted high level expression and purification of recombinant catases at pichia pastoris (Shi et al., 2007).

Wang et all purified thermo stabile catalase from culture liquid of Thermoascus aurantiacus grown in ethanol environment as electrophoretic and isoelectrophoretic. Culture liquid was distilled from filter paper and previously cooled ethanol with equal volume was mixed to obtained filtrate. Solution was centrifuged during fifteen minutes at 10000g. Obtained precipitate was degraded at 20 mM Tris-HCl buffer (pH 8.0) and dialyzed against same buffer during 24 hours. Dialyzate was applied to DEAE cellulose DE-52 (Whatman) column (1.6 x 40 cm). Tris-HCl buffer was used as balance buffer, 1 L 0 →500 mM NaCl gradient was used in elution. Flow rate 20 mL/h. Fractions having highest catalase activity in10 mL fractions (60 mL) were combined and concentrated to 8 mL. Concentrated sample was applied to Sepharose CL-6B column (1.6 x 100 cm). Fractions were collected at 3mL volume and 10 mL/h

flow rate. 18 mL active fraction was concentrated to 3 mL and obtained preparation was used as pure enzyme extract (Wang et al., 1998).

Aydemir and Kuru purified the catalase from chicken erythrocyte 136 times by applying acetone precipitation, ethanol-chloroform treatment, CM-cellulose and Sephadex G-200 chromatography (Aydemir and Kuru, 2003).

Bangyukova et all investigated in vivo inhibition effect of 3-amino 1,2,4- triazole (AMT) in the brain of Carassius auratus on catalase enzyme. It was determined that injection of AMT in 0.1 mg/g concentration decreases increasingly brain catalase activity during 72 hours. When AMT concentration was increased to the level of 0.5-1.0 mg/g, catalase activity was decreased at two thirds rate within 5-10 hours. Antioxidant enzyme activity of AMT and its effects on oxidative stress markers were examined by treating fish in 0.5 mg/g AMT concentration during 24-168 hours. After 24 hours of injection, levels of thiobarbituric acid reagents increased 6.5 times and then decreased after 168 hours. Content of carbonylprotein (CP) increased twice within 24 hours. CP levels perform inverse correlation and it was suggested that catalase enzyme protects proteins against oxidative modification (Bagnyukova, Vasylkiv, Storey and Lushchak, 2005).

Altınordulu and Eraslan investigated the effects of ernofloxacin , ciprofloxacin and norfloxacin antibiotics on plasma malondialdehid (MDA) and erythrocyte catalase activity at therapeutic and higher doses. For this purpose three-day 160 female chickens were used within the scope of study. When applied at therapeutic levels, it was determined that no one of the examined three type antibiotics causes radical production. (Altınordulu and Eraslan, 2009).

Aksoy et all investigated the inhibition kinetics of human erythrocyte catalase in the presence of azide. It was seen that azide has non-competitive inhibition effect on catalase activity. K_m for human erythrocyte catalase and K_i for azidewere found respectively 10.97 and 1.107 mM (Aksoy, Balk, Öğüş and Özer, 2004).

Wolfe et all determined the concentration required for cyanide bacterial catalase to bind to or inhibit 50% of hem group (i.e I_{50}) in the presence and absence of hydrogen peroxide (Wolfe, Beers and Sizer, 2004).

Mitchell et all purified Trichoplusia ni. larval catalase by using the techniques of ethanol-chloroform fractionation and standard column chromatography. Specific activity of purified catalase was determined as 22 x 10^5 U/mg. Molecular weight of purified natural catalasewas 247000–259000 Da and molecular weight of each subunit 63000 Da and determined to have tetra metric structure. K_m value of purified catalase is 54.2 mM against hydrogen peroxide. 3-amino-1,2,4-triazole was determined to be an effective inhibitor in the presence and absence of hydrogen and I_{50} value was determined to be 100 mM (Mitchell, Ahmad and Pardini, 2002).

1.2 Saprophytic Funguses

Saprophytic funguses are known especially to be able to degrade the sclerous structures like hemicelluloses, cellulose and lignin. In accordance with their affect styles on their substrates are divided into three sub-class; brown saprophytic funguses, soft saprophytic funguses and white saprophytic funguses.

Brown saprophytic fungus group is generally made up of Basidiomycetes like Armillariella mellea, Laccaria laccate and Lentinus. They can break cellulose and hemicellulose into pieces effectively and lignin partially. Brown saprophytic funguses weak the resistibility of sclerous structure (Kirk and Adler, 1970; Kirk, 1975).

Soft saprophytic funguses attack humid sclerous structures and their effects are just to soften sclerous structure surface (Blanchette, 1991). Soft saprophytic funguses are generally in soil (Carlie and Watkinson, 1994).

White saprophytic funguses degrade cellulose, hemicelluloses and lignin at the same time. Sclerous structure turns to fibrous, white and unstable with the effect of white saprophytic fungus. Most effective white saprophytic fungi are most effective ligninolytic microorganisms. This fungus group is used commonly in the processes like microbial delignification, microbial bioremediation and microbial whitening of pulp.

1.2.1 White Saprophytic FungusesPhanerochaete chrysosporium

Phanerochaete chrysosporium (P. *chrysosporium*) is a white saprophytic fungus and especially known to be able to degrade the aromatic polymer lignin (Kenealy and Dietrich, 2004). P. *chrysosporium*, secretes extracellular enzymes enabling three dimensional complex structure to be degraded into components to be used by metabolism.

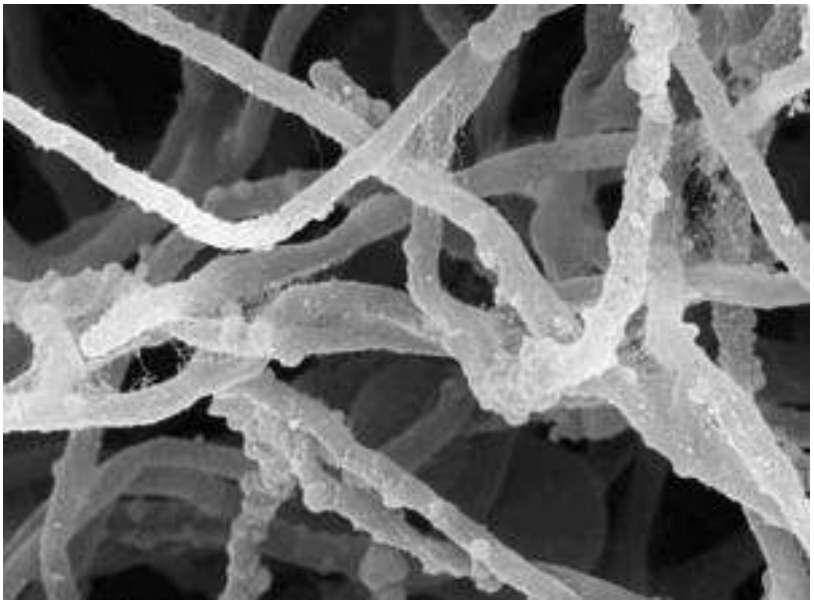


Figure 1.3 View of P. *chrysosporium* fungus in electron microscope

Because P. *chrysosporium* produces ligninase, laccase and Mn-peroxidase, they have become the subject of many studies.

1.2.1.1 P. chrysosporium Industrial Applications

P. *chrysosporium* which is one of the most studied white saprophytic funguses produces raw proteins glycosylated extracellulary and a model organism used in the biodegradation studies of xenobiotics (Zollinger, 1991; Vaidya and Datye, 1982; Chung and Stevens, 1992). P. *chrysosporium* is able to transform many various organic components thanks to the their extracellular, non-specific ligninolitic enzymes like lignin peroxidase, LiP (EC 1.11.1.14), manganase peroxidase, MnP (EC 1.11.1.13) and laccase (EC 1.10.3.2) as well as oxidases producing hydrogen (Cripps, Bumpus and Aust, 1990; Bakshi, Gupta and Sharma, 1999; Chivukula, Spadaro and Renganathan, 1995; Bumpus and Brock, 1988). P. *chrysosporium* can degrade not only the derivations of natural and industrial lignin but also human made aromatic compounds like various environmental pollutants (Bonnarme and Jeffries, 1990). Thus the fact that white saprophytic fungus P. *chrysosporium* is used in biodegradation of industrial textile paints fall with the most remarkable study subjects. Industrial applications of P. *chrysosporium* are available in the removal of various heavy metals, ammunition wastes, pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, blanchers, synthetic polymers and wood protectors (Pointing, 2001) as well as synthetic paints. Summaries of the studies made for the matter concerned are as follows.

Pazarlıoğlu et al studied on in vitro and in vivo biodecolorization of various commercial textile paints principally Direct Blue 15 with P. *chrysosporium* cells and batch systems immobilized on pumice (Pazarlıoğlu, Ürek and Ergün, 2004).

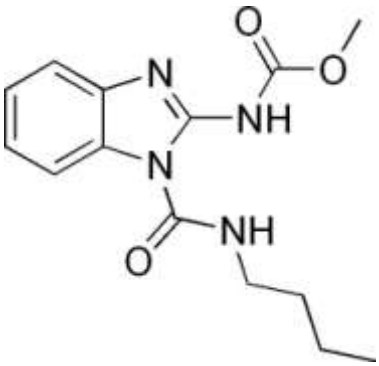
Faraco et all investigated bioremidation effects of P. *chrysosporium*and Pleurotus ostreatus cells and ligninolitic enzymes on various model textile paints like Direct Blu 71, Direct Red 80, Direct Yellow 106, Reactive Blue 222, Reactive Red 195, Reactive Yellow 145, Reactive Black 5, Acid Blue 62, Acid Yellow 49 and Acid Red 266 (Faraco et al., 2008).

Say et all studied on biosorption of some heavy metals like cadmium, lead and copper from artificial waste waters with P. *chrysosporium*fungi. Maximum absorption of different metal ions were obtained at pH 6.0 and biosorption balance was reached after approximately 6 hours (Say, Denizli and Arıca, 2001).

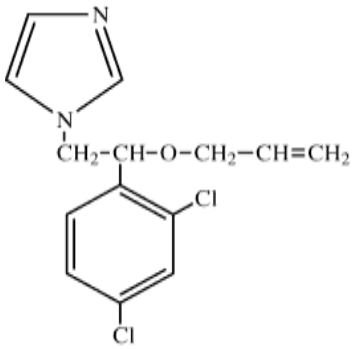
1.3 Fungicides

Fungicides are chemical compounds or biological organisms used in order to kill or inhibit fungi or fungal spore. Fungi cause agricultural damages causing productivity, quality and profit loss. Fungicides are used against fungal infections in animals as well as agriculture(Hogan, 2011).

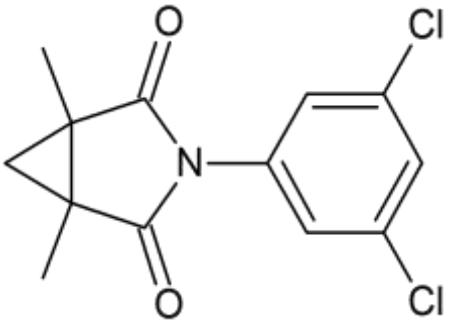
Inhibition effects of benomyl, imazalyl and procymidon fungicides are researched within the scope of thesis.



Benomyl, $C_{14}H_{18}N_4O_3$



Imazalyl, $C_{14}H_{14}Cl_2N_2O$



Procymidon, $C_{13}H_{11}Cl_2NO_2$

The aim of the study is the purification and characterization of some kinetic parameters of catalase enzyme which is of great importance in terms of industrial applications.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

All chemicals used in this work were analytically pure and purchased from Sigma and Merck companies.

Two different strains of white rot fungus *Phanerochaete chrysosporium* (DSM-1547 ve DSM-6909) used as catalase enzyme source were purchased from German Collection of Microorganisms and Cell Culture (DSMZ).

The experiments were performed using hydrogen peroxide, coomassie brilant blue G-250 and R-250, sodium dodecyl sulfate (SDS), bromo phenol blue, amoniumpersulphate (APS), phenylmethylsulfonyl floride (PMSF), N,N,N',N'-tetramethylethylenediamine (TEMED), glucose, malt extract, soybean peptone, yeast extract, agar, thiamin-HCl, potato dextrose agar, asparagine, tween-80, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulphate, calcium chloride, ammonium chloride, sodium tartrate, ammonium sulphate, citric acid, sodium citrate, ferrous sulfate hepathydrate, zinc sulphate hepathydrate, manganase sulfate dihydrate, sodium chloride, cobalt (II) chloride hexahydrate, aluminum potassium sulfate dodecahydrate, copper sulphate, boric acid, sodium molybdate dihydrate, nitrilotriacetic acid, ethanol, methanol, acetone, polyethylene glicol (PEG)- 400; 1000; 2000; 3350; 6000; 8000, DEAE cellulose, sephacryl s-200, catalase, L-glutamate dehydrogenase, bovine serum albümin, alcohol dehydrogenase, apoferritin, benomyl, procymidon and imazolyl.

Optical measurements were made up by a spectrophotometer with thermocouple system. In addition, thermostat, cryostat, homogenizator, pH-meter, peristaltic pump, electrophoresis, refrigerated centrifuge, vortex were used at the experimental studies

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2.2 Mediums Components and Conditions of P. *chrysosporium* Strains

2.2.1 The Solid Mediums Components and Conditions of P. chrysosporium Strains

The spore cultures of two different P. *chrysosporium* strains obtained from DSMZ (DSM-1547 and DSM-6909) were prepared by inoculating five different solid mediums. The components and symbolic codes of the used solid mediums (K) were indicated in the Table 2.1. After pH of the mediums were adjusted to 4.5 for K1 and 5.6 for all of the other solid mediums, the sterilization was carried out by auto-claving at 121 ^{0}C and for 20 minutes. Approximately 50 mL of the mediums were taken to the petri plates and inoculation was doneafter freezing. The inoculation was carried out for 7 days andat 28 ^{0}C.

2.2.2 The Liquid Mediums Components and Conditions of P. chrysosporium Strains

The spore suspensions were prepared in the liquid mediums (pH 4.5) given in the table 2.2 from 5 different agar mediums referred to in Table 2.1 for both P. *chrysosporium* strains (DSM-1547 and DSM-6909).

Prior to inoculation, liquid mediums were sterilized by auto-claving at 121 ^{0}C and for 20 minutes. Inoculation was carried out for 12 days at 28 ^{0}C and with 150 rpm agitation in the 250 mL erlenmayer flask containing 90 mL liquid medium and 10 mL spore suspension.

In the course of incubation period, samples were taken at 6th, 9th and 12th days. The harvested cells were washed several times with 20 mM potassium phosphate buffer at +4 ^{0}C and stored at -20 ^{0}C until used.

Table 2.1 The Solid Mediums Components of P. *chrysosporium* Strains

No	Code	Litterateur	The Components of the Mediums	
			Component	**Amount (g/L)**
1	**K1**	Pridham and et al., 1956 (modified)	Glucose	10.0
			Malt extract	10.0
			Soybean Peptone	2.0
			Yeast extract	2.0
			Agar	20.0
			Magnesium sulphate	1.0
			Potassium dihydrogen phosphate	2.0
			Thiamin-HCl	1.10^{-3}
2	**K2**	Galloway and Burgers, 1952	Malt extract	30.0
			Soybean Peptone	3.0
			Agar	15.0
3	**K3**	Beever and Bollard, 1970	Potato dextrose agar	39.0
4	**K4**	Wickerham, 1951	Yeast extract	3.0
			Malt extract	3.0
			Soybean Peptone	5.0
			Agar	15.0
			Glucose	10.0
5	**K5**	Pridham and et al., 1956 (modified)	Glucose	10.0
			Malt extract	10.0
			Soybean Peptone	2.0
			Yeast extract	2.0
			Agar	20.0
			Magnesium sulphate	1.0
			Potassium dihydrogen phosphate	2.0
			Asparagine	1.0
			Thiamin-HCl	1.10^{-3}

Tablo 2.2 The Liquid Mediums Components of P. *chrysosporium* Strains

No	Code	Litterateur	The Components of the Mediums	
			Component	**Amount (g/L)**
1	**S1**	Tien and Kirk, 1988 (modified)	Potassium dihydrogen phosphate	2.0
			Calcium chloride	0.114
			Magnesium sulphate	0.7
			Ammonium chloride	0.12
			Glucose	2.0
			Thiamin-HCl	1.10^{-3}
			Tween-80	0.05
			Trace elements*	
2	**S2**	Tien and Kirk, 1988 (modified)	Potassium dihydrogen phosphate	2.0
			Calcium chloride	0.1
			Magnesium sulphate	0.5
			Glucose	10
			Thiamin-HCl	1.10^{-3}
			Sodium tartrate	0.417
			Ammoniumsulphate	0.284
			Acetate buffer **	
			Trace elements***	

*$FeSO_4.7H_2O$, 70 µg; $ZnSO_4.7H_2O$, 46 µg; $MnSO_4.2H_2O$, 35 µg; $CoCl_2.6H_2O$, 7 µg

**20 mM acetate buffer, pH 4.5

***$MgSO_4$, 0.3 g; $MnSO_4$, 0.05 g; NaCl, 0.1 g; $FeSO_4.7H_2O$, 0.01 g; $CoCl_2$, 0.01 g; $ZnSO_4.7H_2O$, 0.01 g; $CuSO_4$, 0.01 g; $AlK(SO_4).12H_2O$, 0.001 g; H_3BO_3, 0.001 g; $Na_2MoO_4.2H_2O$, 0.001 g; Nitrilotriacetic acid, 0.15 g.

2.3 Preparation of Crude Extract from *P. chrysosporium*

The cells harvested with centrifugation were resuspended in 20 mM potassium phosphate buffer, pH 7.4, in a volume equal to 3.0 times its weight. The homogenization procedure was performed for 3 min at 9000 rpm with 30 seconds time intervals. Cell debris in the homogenate was removed by centrifuge at 15000 rpm and 15 min at +4 ^{0}C. The purification of the catalase enzyme from the crude extract was the aim of the study.

2.4 Catalase Activity Assay Conditions

The catalase enzyme activity was spectrophotometrically determined with Aebi method (Aebi, 1974), depending the absorbance decrease at 240 nm of H_2O_2 by the hydrolsis to H_2O and O_2.

The catalase activity was measured by adding 10 µl enzyme extract to H_2O_2 solution (≈ 40 mM), initial absorbance 0,450-0,470 at 240 nm, prepared in 50 mM potassium phosphate buffer, pH 7.0, and against blank containing same buffer and enzyme extract. The spectrophotometric assay was carried out at 1 mL cuvette.The activity value was determined by measuring the time (second) required for 0.050 units decrease in the initial absorbance of H_2O_2. The extinction coefficient (E) for H_2O_2at 240 nm is 43.6 $M^{-1}cm^{-1}$.

The specific activity of catalase (U/mg protein) was described as the enzyme amount necessary for the decrease of the H_2O_2absorbance from 0.450 to 0.400 in 20 s at 240 nm and in 50 mM potassium phosphate buffer, pH 7.0

2.5 Total Protein Assays

UV (A_{280}) and Bradford (A_{595}) methods were used for the quantification of protein amounts in the samples.

In the direct determination of protein amounts in the ultraviolet region, the absorbance values of the samples were read at 260 and 280 nm against air (A_2). The same procedure was repeated against blank (A_1). The total protein amounts in the unknown samples were calculated according to equation given in the below.

$$\Delta A = 1.55 \times (A_2\text{-}A_1)_{280} - 0.76 \times (A_2\text{-}A_1)_{260}$$

$$p = 15 \times \Delta A \text{ g/l}$$

Under certain conditions, the acidic and basic groups of the proteins can interact with the dissociated groups of organic dyes. Due to the this nature of the proteins, organic dyes can be used as a reactive in the protein amount assays. However, many organic dyes are insensitive(Orange G) to the method or have some disadvantage. This approach only became popular with the development by Bradford of a method using Coomassie Brillant Blue G-250. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 nm (red form) to 595 nm (blue form). The dye is prepared as a stock solution in either phosphoric or perchloric acid.

The application of the procedure is so simple. The reagent is added to the sample and the absorbance is measured at 595 nm. The procedure is very sensitive, working in the range 0.2 – 1.4 mg protein mL^{-1} for the standard assay and 5 – 100 μg protein mL^{-1} for the microassay procedure.

A diffuculty observed in performing the assay is the tendency of protein-dye complex to bind to glass surfaces. Disposable cuvettes can, therefore, be used to advantage.

The method exhibits a significant dependence on protein amino acid composition and this has recently been shown to be consequence of the dye binding primarily to basic and aromatic amino acid residues.

The procedure shows interference with the chemicals such as potassium chloride, sodium chloride, magnesium chloride, Tris, EDTA, ammonium sulfate, glycerol, ethanol, acetone.

The preparation of required reagents and the procedure were discussed in the below.

The preparation of dye reagent; use vigorous homogenization or agitation to dissolve 100 mg of Coomassie Brillant Blue G250 in 50 mL of 95% ethanol. This

solution is mixed with 100 mL of 85% w/v phosphoric acid, diluted with water to 1 liter and filtered.

The preparation of protein standard; BSA was used as a protein standard. 1000 ppm stock BSA solution was diluted to 10, 25, 50, 75 ve 100 ppm in the 20 mM potassium phosphate buffer (pH 7.4).

Protein amount assay;900 µl reagent is added to 100 µl sample and shaked gently. Absorbance value was read in the end of 2 minutes against blank.

The protein amounts were determined by using calibration curve drawn between known concentration of BSA standards and their absorbance values at 595 nm (Figure 2.1).

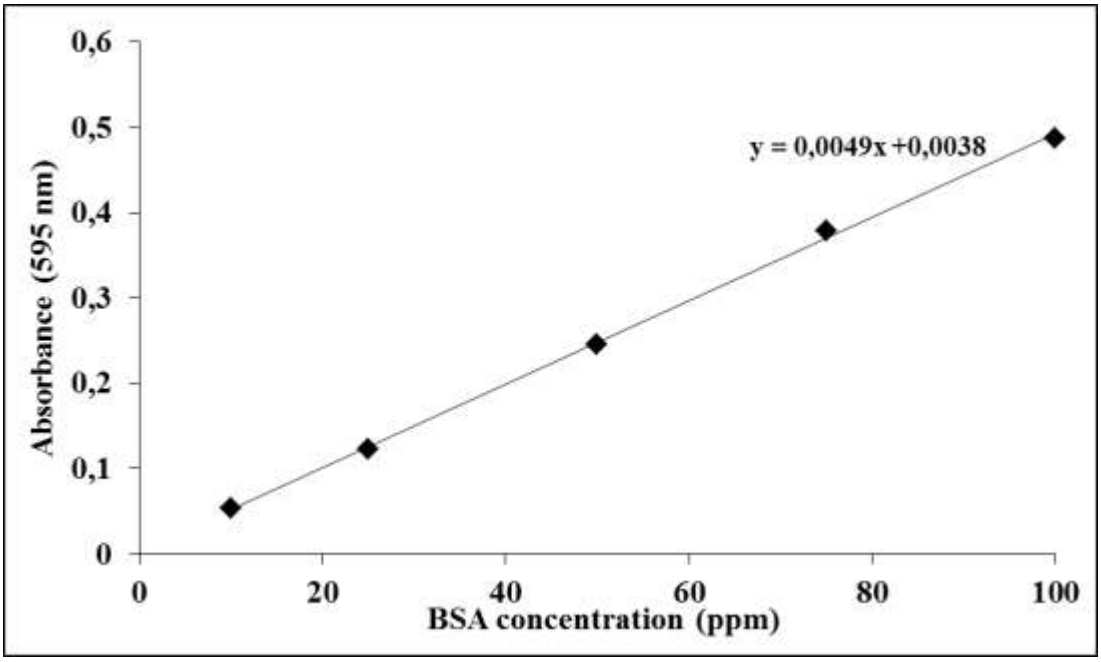


Figure 2.1 The calibration curve and equation for the Bradford method

2.6 The Purification Steps of Catalase

2.6.1 Ammonium Sulfate Precipitation

Precipitation by addition of neutral salts is probably the most commonly used method for fractionating proteins by precipitation. The precipitated proteins is usually not denatured and activity is recovered upon redissolving the pellet. In addition, these salts can stabilize proteins against denaturation, proteolysis and bacterial contamination.

The appropriate amounts of ammonium sulfate were added to the catalase enzyme extract to give 40, 45, 50, 55, 60, 65, 70, 75 and 80% ammonium sulfate concentration. Solid ammonium sulfate was gradually added by mixing and prior to any other addition, it was ensured that there was no unsolved salt in the solution. The catalase enzyme extracts with certain amounts of ammonium sulfate were allowed to stand for 3 h. by mixing in certain time intervals. Pellet and supernatant were seperated from each other by centrifuge at 15000 rpm for 15 min and at +4 ^{0}C. Pellet was solved in the least possible volume of 20 mM potassium phosphate buffer (pH 7.4). Both phases were ultrafiltered by using 10 kDa ultrafiltrate for preventing any interference effect of the salt in the protein amount assays.

To calculate the number of grams of ammonium sulfate (g) to add one liter at 20 ^{0}C to give a desired concentration use the following equation:

$$g\ (NH_4)_2SO_4 = 533\ (S2\text{-}S1) / (100 - 0.3.\ S2)$$

S_1, Starting ammonium sulfate concentration.

S_2, Final ammonium sulfate concentration.

2.6.2 Precipitation by Organic Solvent

Many proteins can be precipitated by addition of water-miscible organic solvents such as acetone and ethanol. The factors which influence the precipitation behaviour of a protein are similar to those involved in isoelectric precipitation and different from those involved in salting out; thus, this method can be used as an alternative to isoelectric precipitation in a purification steps, perhaps in conjunction with salting-out.

The fractionation of the proteins in the extract was carried out by using organic solvents acetone, methanol and ethanol and in the concentration of 10, 20, 30, 40, 50, 60, 70 and 80%. Before the precipitation process, organic solvents were freezed at -20 ^{0}C.

As the ammonium sulfate precipitation process, organic solvents were gradually added by mixing and prior to any other organic solvent addition, it was ensured that the solution was completely homogenous. The process was carried out at +4 ^{0}C. The catalase enzyme extracts with certain amounts of organic solvents were allowed to stand for 1 h. by mixing gently in certain time intervals. Pellet and supernatant were seperated from each other by centrifuge at 15000 rpm for 15 min and at +4 ^{0}C. Pellet was solved in the least possible volume of 20 mM potassium phosphate buffer (pH 7.4). Both phases were ultrafiltered by using 10 kDa ultrafiltrate for preventing any interference effects of the organic solvents in the protein amount and activity assays.

The following equation is used to calculate the volume, in mL, of organic solvent to add to one liter:

$$\text{mL organic solvent} = 1000\,(P2 - P1) / (100 - P2)$$

P_1, Starting percentage of organic solvent.

P_2, Desired percentage of organic solvent.

2.6.3 Precipitation by Organic Polymers

PEG is the most commonly used organic polymer. The mechanism of precipitation is similar to that of precipitation by organic solvents, however, lower concentrations are required.

The fractionation of the proteins in the crude extract was carried out PEG with different molecular weights and in the concentrations of 10 and 20%.All process was carried out at +4 ^{0}C and next PEG addition was done after it was ensured that there was no unsolved PEG.The catalase enzyme extracts with certain amounts of PEG were allowed to stand for 3 h. by mixing in certain time intervals. Pellet and supernatant were seperated from each other by centrifuge at 15000 rpm for 15 min and at +4 ^{0}C. Pellet was solved in the least possible volume of 20 mM potassium phosphate buffer (pH 7.4). Both phases were ultrafiltered by using 10 kDa ultrafiltrate for preventing any interference effect of the polymers in the protein amount assays.

To calculate the number of grams of PEG (g) to add one liter at 20 ^{0}C to give a desired concentration use the following equation:

$$g\ PEG = 533\ (S2-S1) / (100 - 0.3.\ S2)$$

S_1, Starting PEG concentration.
S_2, FinalPEG concentration.

2.7 Chromatographic Applications

2.7.1 DEAE Cellulose Ion Exchange Chromatography

The sample which the best purification fold was obtained was concentrated to 1 mg protein/mL by ultrafiltration (10 kDa) and then applied to the DEAE cellulose anion exchange column. Column dimensions were 10 x 1 cm and flow rate was 0.75

mL/min. 10 mM potassium phosphate buffer (pH 7.4) was used as an equilibration buffer. Elution of proteins was carried out with 0 →1.0 M NaCl gradient prepared in the same buffer. Volume of the each fraction was 2 mL. All of the collected fractions were analyzed at 280 nm and 240 nm in terms of protein amounts and catalase activities, respectively. The fractions with catalase activity were analyzed Bradford method instead of other direct UV method for the correction of protein amounts more sensitively.

2.7.2 Sephacryl S-200 Gel Filtration Chromatography

The sample which the best purification fold from anion exchange column was obtainedwas concentrated by ultrafiltration (10 kDa) and then applied to the sephacryl s-200 gel filtration column. Column dimensions were 75 x 1.5 cm and flow rate was 0.2 mL/min. 10 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl was used as equilibration and elution buffer and 3 mL fractions were collected. All of the collected fractions were analyzed at 280 nm and 240 nm in terms of protein amounts and catalase activities, respectively. The fractions with catalase activity were analyzed Bradford method instead of other direct UV method for the correction of protein amounts more sensitively. Active fractions were combined and used for characterization of the purified catalase.

In order to determine the molecular weight of purified catalase, standard protein mixture instead of sample was applied to the gel filtration column under same conditions. 2.0 mL standard protein mixture was prepared with 1 mg BSA, 1 mg alcohol dehydrogenase, 1 mg catalase, 1 mg L-glutamate dehydrogenase and 1 mg apoferritin. The calibration curve was given in figure 2.2.

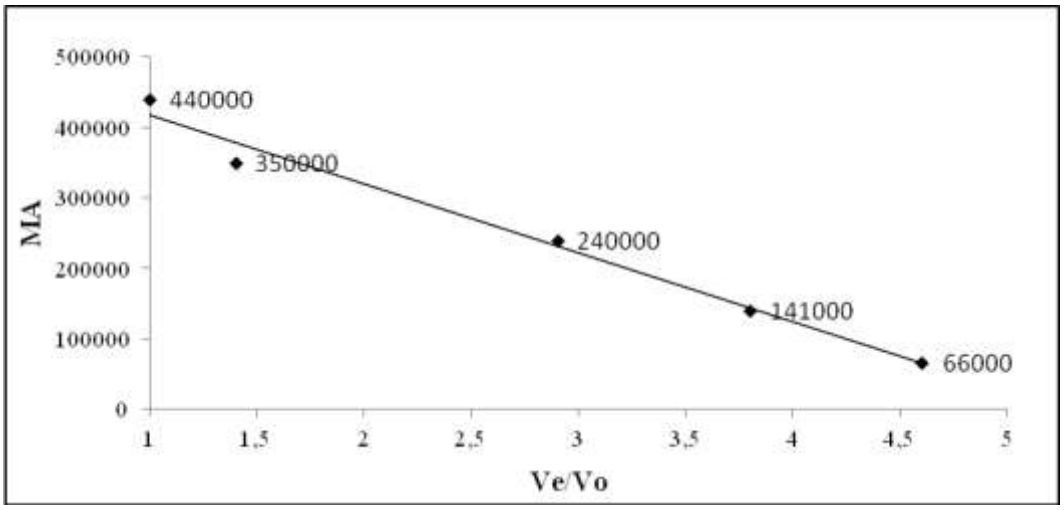


Figure 2.2 Sephacryl S-200 Gel Filtration Chromatography Molecular Weight Calibration Curve. Apoferritin (440000 Da), L-glutamate dehydrogenase (350000 Da), Catalase (240000 Da), Alcohol dehydrogenase (141000 Da), Bovine serum albümine (66000 Da).

2.8 Electrophoretic Procedures

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (Laemmli, 1970) using a vertical slab gel apparatus.

2.8.1 Stock Reagent Preparation

1. Acrylamide/Bis: 146.0 g acrylamideand 4.0 g N,N'-Methylene-bis Acrylamide were diluted with distilled water to 500 mL and then filtered and stored +4 ^{0}C in the dark.

2. 1,5 M Tris-HCl (pH 8.8): 54.45 g Tris-base was dissolved in 150 mL distilled water and adjusted to pH 8.8 and then fulled with distilled water to 300 mL and stored +4 ^{0}C.

3. 0,5 M Tris-HCl(pH 6.8): 6 g Tris-base was dissolved in 60 mL distilled water and adjusted to pH 6.8 and then fulled with gently stirring to 100 mL and stored +4 ^{0}C.

4. %10 (w/v) SDS: 10 g SDS was dissolved in 60 mL water with gentle stirring and fulled with distilled water to 100 mL.

5. %10 (w/v) Ammonium Persulfate: 100 mg ammonium persulfate was dissolved in 1 mL distilled water.

6. SDS Sample Buffer (4X Laemmli buffer): 2.4 mL 1 M Tris-HCl, pH 6.8; 0.8 g SDS stock; 4 mL %100 glycerol; 0.01% bromophenolblue; 1 mL β-mercaptoethanol.

7. 5xElectrode (Running) Buffer: 45.0 g Tris-base, 216.0 g glycineand 15.0 g SDS were dissolved in 3 L distilled water and stored at +4 ^{0}C. 300 mL of this 5x buffer was diluted water for one electrophoretic run.

8. Gel Preparation: The gels used in SDS polyacrylamidegel electrophoresis usually contain acrylamide, bisacrylamide, SDS and a buffer with known pH. To prevent the formation of air bubbles during polimerization degas should be made under vacuum. The initiators such as ammoniumpersulfate and TEMED are used.

	%12	%4
Acrylamide/bis (%30T, %2.67C Stok)	40.0 mL	1.3 mL
Distilledwater	33.5 mL	6.1 mL
1.5 M Tris-HCl, pH 8.8	25.0 mL	-
0.5 M Tris-HCl, pH 6.8	-	2.5 mL
%10 SDS	1.0 mL	100 µL
%10 ammoniumpersulfate (fresh)	500 µL	50 µL
TEMED	50 µL	10 µL
Total Monomer	100 mL	10 mL

The following proteins were used as SDS electrophoresis molecular weight standarts: carbonic anhydrase (29000 Da), ovalbumin (45000 Da), bovine serum

albumine (66000 Da),phosphorylaseb (97400), β-galactosidase (116000) and myosin (205000). Molecular weight markers and purified catalase were dissolved with sample buffer and denaturated by incubation in a boiling water-bath for 4 min.

Electrophoresis was performed according to discontinious methods with %12 separating and %4 stacking polyacrylamide gels at room temperature with a 150 volts for approximately 5 h. Gel was stained with Coomassie Brillant Blue R-250 dye reagent overnight prior to destaining. Molecular weight of purified catalase per subunit was determined.

2.9 Characterization of P. *chrysosporium* Catalase

2.9.1 Catalase Activity and Stability Changes Depending pH

pH-dependent catalase activity variations were investigated at 25 ^{0}C and with 40 mM H_2O_2 using 20 mM potassium phosphate buffer in pH 5.0-8.0 and 20 mM Tris-HClin pH 8.0-9.0 ranges. Optimum pH value of the purified catalase was determined from the graph drawn between pH and relative activity percentage.

Purified P. *chrysosporium*catalase was incubated in 20 mM potassium phosphate buffer, pH 6.0; 7.0 and 8.5 for 10 h. The remaining activity values were determined every two hours during incubation under the standard activity assay conditions.

2.9.2 Catalase Activity and Stability Changes Depending Temperature

Temperature-dependent catalase activity variations were investigated with 40 mM H_2O_2 using 20 mM potassium phosphate buffer, pH 7.0, at 10, 20, 30, 40, 50 and 60 ^{0}C. Optimum temperature of the purified catalase was determined from the graph drawn between temperature and relative activity percentage.

Purified P. *chrysosporium* catalase was incubated in 20 mM potassium phosphate buffer, pH 7.0, at 10 – 80 ^{0}C for 2 h. The remaining activity values were determined after incubation under the standard activity assay conditions.

2.10 Kinetic Characterization of P. *chrysosporium* Catalase

2.10.1 K_m ve V_{max}

Purified P. *chrysosporium* catalase activity was measured in the different substrat concentrations (5 – 40 mM H_2O_2) and under standard conditions. Lineweaver-Burke graph was drawn and K_m – V_{max} values were determined.

2.10.2 Determination of Inhibition Effects of Some Fungucits

For the determination of the inhibition effects of benomyl, imazolyl and procymidon fungucits on purified catalase, activity was measured with variable substrat (5 – 40 mM) and fungucit concentrations (0,9 and 2 ppm for benomyl; 5, 10 and 20 ppm for imazolyl and procymidon) and then by drawing Lineweaver-Burke graph, inhibiton type was decided.

2.11 Evaluation of the Data

All of the presented data is the average of at least three independent experiment. Analysis of the data was done by using SPSS 11.0 and Graph Pad Prism 5.04 programmes.

CHAPTER THREE

RESULT AND DISCUSSION

3.1 Alteration of Catalase Activity of P. *chrysosporium*Strains (DSM-1547 and DSM-6909) Depending Solid and Liquid Mediums

Two different P.*chrysosporum* strains (DSM-1547 and DSM-6909) were inoculated to 5 different solid medium defined as K1-K5.After inoculation, spores covered overall surface in all of the mediums except K4for 5-7 days during incubation process at 28 ^{0}C. Suspensions ($OD_{650} \approx 0.8$)which were prepeared with the spores obtained from solid medium were inoculated to S1 and S2 separately at 28 ^{0}C and incubated by shaking at 150 rpm.In the crude extracts, prepared by taking time-dependent samples in the incubation period of liquid mediums for 12 days, the activity alterations of the target catalase enzyme were determined. As seen from Table 3.1, spesific catalase activity values of P. *chrysosporium* (DSM-1547) determined as a result of combination solid and liquid mediums were higher than P. *chrysosporium* (DSM-6909) for all of the medium combinations. The highest catalase activities forP. *chrysosporium* (DSM-1547) were observed in the K5, K1, K3 and their combinations with nitrogen and carbon limited medium S2 at 12th day with the values of 260.61; 254.98 and 238.56 U/mg, respectively.

According to the obtained results,there was no statistically significant difference when compared this three findings with each other.

When the mediums were evaluated in terms of produced biomass, economy or price and the simplicity of preparing the mediums, potato dextrose agar (PDA) defined as K3 was prefered. At all the other stage of the research, the production of biomass of P. *chrysosporium* was carried out in K3-S2 medium.

Table 3.1 Specific catalase activities depending mediums and incubation times

Solid Medium	Liquid Medium	Incubation time (day)	DSM-1547	DSM-6909
			U/mg	U/mg
K1	S1	6	155.27	10.23
		9	179.92	12.56
		12	183.43	15.67
	S2	6	201.19	56.54
		9	212.55	64.34
		12	**254.98**	**68.65**
K2	S1	6	135.03	5.43
		9	165.70	6.87
		12	173.46	12.43
	S2	6	133.66	34.12
		9	151.78	43.89
		12	181.86	45.67
K3	S1	6	111.77	2.34
		9	137.95	2.45
		12	156.85	3.56
	S2	6	183.02	14.78
		9	192.65	19.88
		12	**238.56**	**22.34**
K4	S1	6	85.09	16.45
		9	94.90	18.56
		12	115.16	20.54
	S2	6	105.16	24.67
		9	132.37	30.12
		12	145.10	38.45
K5	S1	6	155.28	12.32
		9	176.15	14.65
		12	191.79	17.43
	S2	6	211.11	58.54
		9	227.80	66.43
		12	**260.61**	**72.88**

3.2 Purification Steps of Catalase

In the initial purification steps which were carried out with ammonium sulphate, organic solvents andpolyethyleneglycol in order to fractionation of proteins by precipitation,the crude catalaseextract of P. *chrysosporium* which was concentratedwith 10 kDa ultrafiltrat was used.Initial volume activity proteinamount and spesific activity of the concentrated extract was 1279.88 U/ml, 5.370 mg/mLand 238.34 U/mg, respectively.

3.2.1 Ammonium Sulphate Precipitation

The fractionation of proteins by using neutral salts depending on the their solubility differences is a widespread method for the first step of purification. One of the neutral salts used for this purpose is ammonium sulphate. With this method, many of proteins are not denatured and activity is recovered with dissolving pellet in a suitable buffer.

In the research, P. *chrysosporium*crude catalase extract samples were saturated to 40, 45, 50, 55, 60, 65, 70, 75 ve 80% ammonium sulphate, supernatant and pellet phases were seperated with centrifuge and specific activity alterations were determined.

Table 3.2 Catalase activity and protein amount distributionsin the supernatant and pellet phases depending $(NH_4)_2SO_4$concentrations

% $(NH_4)_2SO_4$	**Supernatant (top phase)**				**Pellet (bottom phase)**			
	U/mL	**mg/mL**	**U/mg**	**PF**	**U/mL**	**mg/mL**	**U/mg**	**PF**
40	1099.58	4.89	224.58	0.9422	26.04	0.26	100.15	0.42
45	781.4	4.68	166.96	0.7005	72.42	0.38	190.58	0.79
50	409.56	4.68	87.51	0.3671	304.54	0.38	801.42	3.36
55	75.86	3.85	19.69	0.0826	672.3	0.84	800.36	3.35
60	**2.94**	**3.96**	**0.74**	**0.0031**	**950.18**	**0.78**	**1218.18**	**5.11**
65	2.44	3.78	0.64	0.0027	706.98	0.88	803.39	3.37
70	11.08	3.78	2.93	0.0122	927.92	1.06	875.39	3.68
75	6.08	3.45	1.75	0.0073	824.8	0.84	981.9	4.11
80	20.62	3.85	5.35	0.0224	742.34	0.86	863.18	3.62

As seen from Table 3.2 and Figure 3.1, the highest purification fold is determinedas 5.11at%60$(NH_4)_2SO_4$concentrationinthe bottomphase.

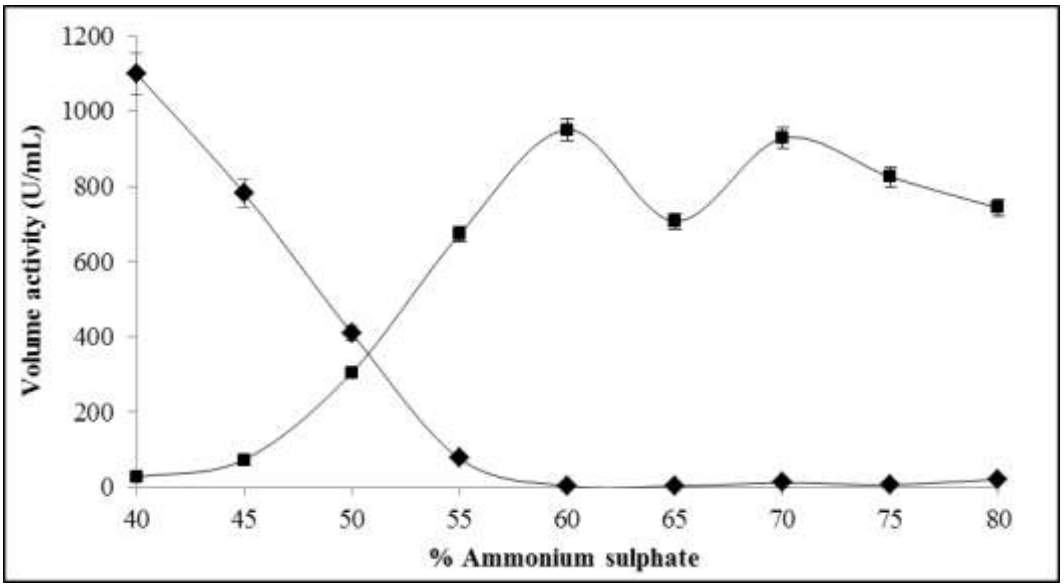


Figure 3.1 Catalase volume activity distributions in the top (--♦--) and bottom (--■--) phases depending on ammonium sulphate concentrations

3.2.2 Precipitation with Organic Solvents

The proteins in the crude catalase extract of P. *chrysosporium*were precipitated by fractionation with organic solvents. Acetone, methanol and ethanol were selected as organic solvents and used in the concentrations of 10, 20, 30, 40, 50, 60, 70 and 80%. Catalase activity alterations in the supernatant and pellet phases were determinedin a way similar to ammonium sulphate precipitation.

Table 3.3 Catalase activity and protein amount distributionsin the supernatant and pellet phases depending acetoneconcentrations

Acetone (%)	**Supernatant U/mL**	**Pellet U/mL**	**Pellet mg/mL**	**Pellet U/mg**	**PF**
10	1444.4	----	----	----	----
20	1042.6	----	----	----	----
30	426.62	9.06	----	----	----
40	180.64	860.68	----	----	----
50	20.72	1223.9	1.11	1096.68	4.60
60	**17.12**	**1522.72**	**1.33**	**1143.18**	**4.79**
70	11.26	1041.86	1.26	826.87	3.46
80	4.72	957.84	1.00	950.23	3.98

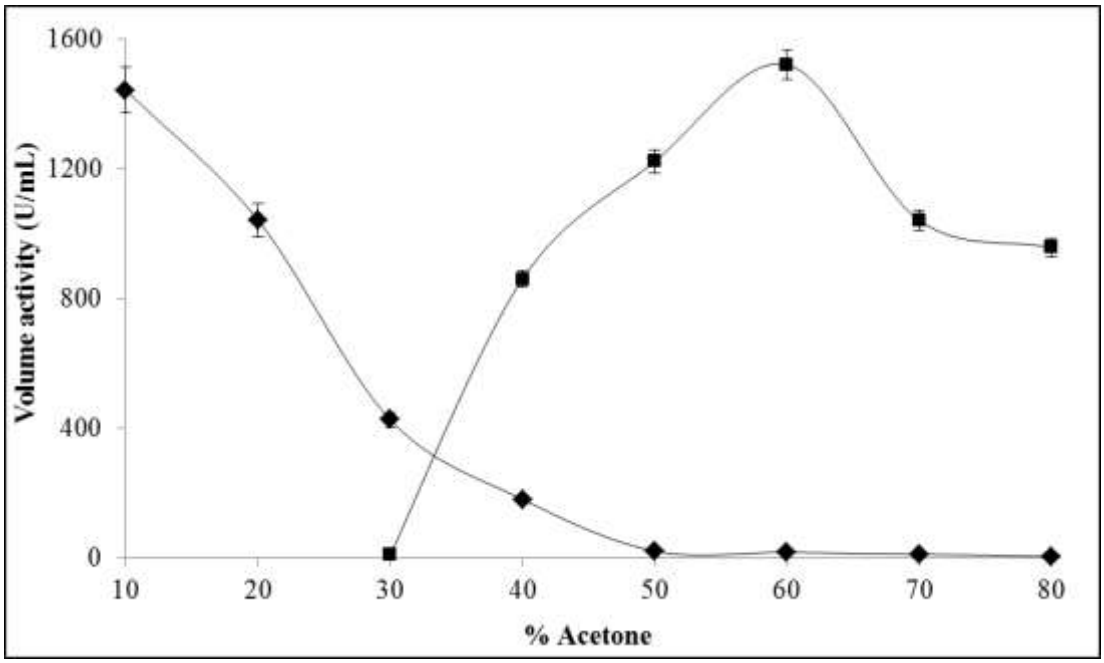


Figure 3.2 Catalase volume activity distributions in the top (--♦--) and bottom (--■--) phases depending on acetone concentrations

As seen Table 3.3 and Figure 3.2, the best purification fold was determined as 4.79 at 60% acetone concentration in the pellet phase.

Precipitation process in the methanol medium were carried out under same conditions and the highest purification fold, 5.05, was observedin 70% methanol concentration from the pellet phase (Table 3.4 and Figure 3.3).

Table 3.4 Catalase activity and protein amount distributions in the supernatant and pellet phases depending methanol concentrations

Methanol (%)	**Supernatant U/mL**	**Pellet U/mL**	**Pellet mg/mL**	**Pellet U/mg**	**PF**
10	1462.7	15.3	----	----	----
20	1374.68	16.3	----	----	----
30	1268.92	17.88	----	----	----
40	1159.9	129.1	0.324	398.45	1.67
50	957.84	424.18	0.396	1071.16	4.49
60	200.72	1211.96	1.188	1020.16	4.28
70	**104.62**	**824.8**	**0.684**	**1205.84**	**5.05**
80	20.46	530.24	0.72	736.44	3.08

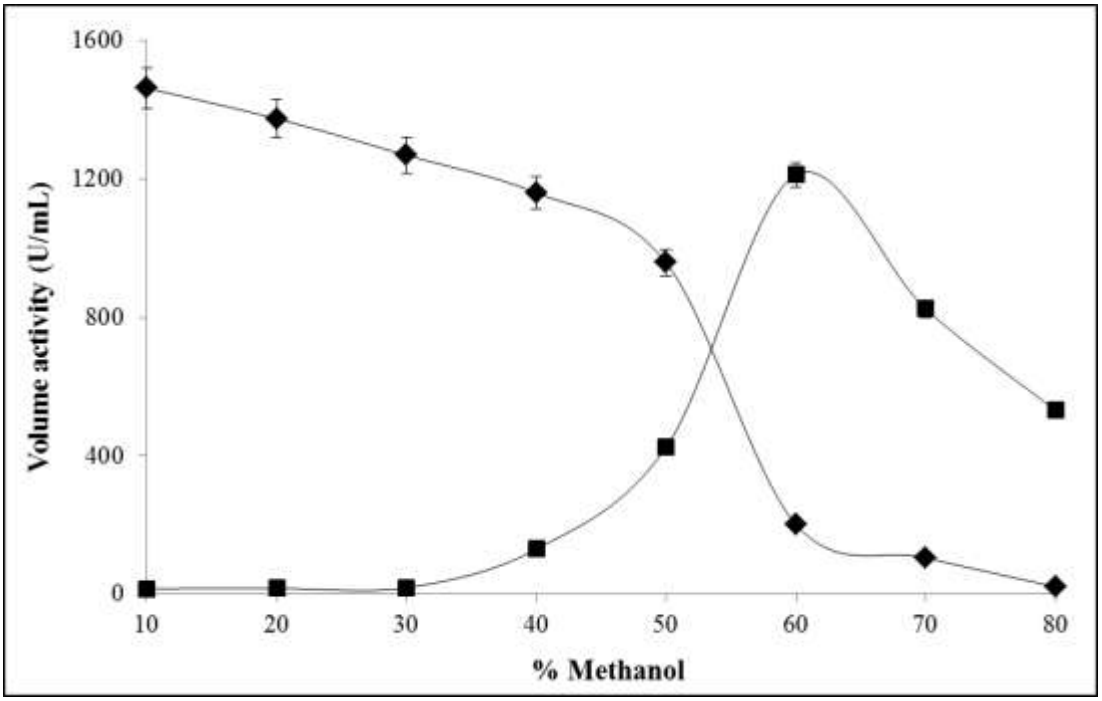


Figure 3.3 Catalase volume activity distributions in the top (--♦--) and bottom (--■--) phases depending on methanol concentrations

As seen from Table 3.5 and Figure 3.4,as the result of ethanol precipitation of proteins in the P. *chrysosporium* crude catalase extract, the best purification fold was found as 7.33 at 60% concentration and in the pellet phase. This result was found as the best value among precipitations with organic solvent.

Table 3.5 Catalase activity and protein amount distributions in the supernatant and pellet phases depending ethanol concentrations

Ethanol (%)	**Supernatant U/mL**	**Pellet U/mL**	**Pellet mg/mL**	**Pellet U/mg**	**PF**
10	1631.48	----	----	----	----
20	1596.4	----	----	----	----
30	1268.92	20.18	----	----	----
40	1070.66	195.34	----	----	----
50	315.88	1413.96	1.116	1266.98	5.31
60	**25.36**	**1448.44**	**0.828**	**1749.32**	**7.33**
70	23.26	998.1	0.792	1260.22	5.28
80	17.12	----	----	----	----

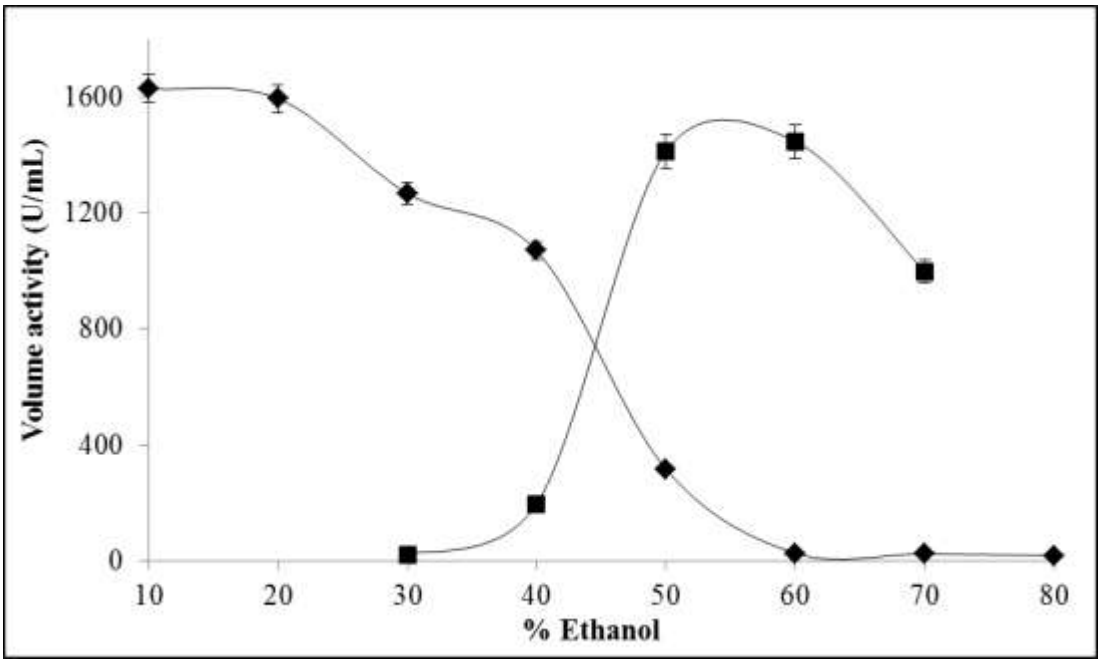


Figure 3.4 Catalase volume activity distributions in the top (--♦--) and bottom (--■--) phases depending on ethanol concentrations

3.2.3 Precipitation with Organic Polymers

Polyethylene glycols which had different moleculer weights (PEG 400, 1000, 2000, 3350, 6000, 8000) were usedas organic polymer. It was determined that viscosity increased when worked with higher concentration than 20% and there was no pellet formation when worked under 10% concentration of PEG.

Purification fold found as a result of precipitation with PEGs were so low when compared to ones obtained from precipitation with ammonium sulphate and organic solvent, so it was decided that precipitation with PEG is not suitablemethodfor catalase fractionation from extract of P.*chrysosporium* as pre-purification step(Table 3.6).

Table 3.6 Catalase activity and protein amount distributions in the supernatant phase depending concentration and molecular weight of PEG

PEG MW	**% 20 PEG**			
	U/mL	**mg/mL**	**U/mg**	**PF**
400	1035.8	4.33	238.97	1.00
1000	1023.9	4.39	232.93	0.97
2000	988	4.34	227.56	0.95
3350	817.24	4.26	191.40	0.80
6000	802.52	4.19	191.18	0.80
8000	788.32	4.19	187.96	0.78

3.2.4 Two Stage Precipitations with Ammonium Sulphate and Organic Solvents

For the purpose of fractionation of catalase with the best purification foldfrom crude extract, organic solvent precipitations after ammonium sulphate precipitation as a two stage precipitations were carried out instead of using ammonium sulphate, organic solvent and organic polymer precipitation separately and results were evaluated.

Because of getting the best result at 60% concentration, at the first step crude extract was brought to 60% $(NH_4)_2SO_4$ concentration, obtained pellet was dissolved in minimum amounts of appropriate buffer and in the second steppprecipitation was done with organic solvents.

As seen from Table 3.7 and Figure 3.5, when supernatant and pellet phases were analyzed separately in terms of catalase activity and total protein amounts, best result

with approximately 14 fold was gained in the precipitation with 60% $(NH_4)_2SO_4$ - 60%ethanol in the pellet phases.

Table 3.7 Two stages precipitations with ammonium sulphate and then organic solvents

1. 60% Ammonium sulphate 2. Organicsolvent	**Pellet**			
	U/mL	**mg/mL**	**U/mg**	**PF**
Acetone (60%)	1258.18	1.2096	1040.16	4.36
Methanol (60%)	742.34	0.5148	1441.99	6.05
Ethanol (60%)	1903.42	0.5706	3335.82	13.99

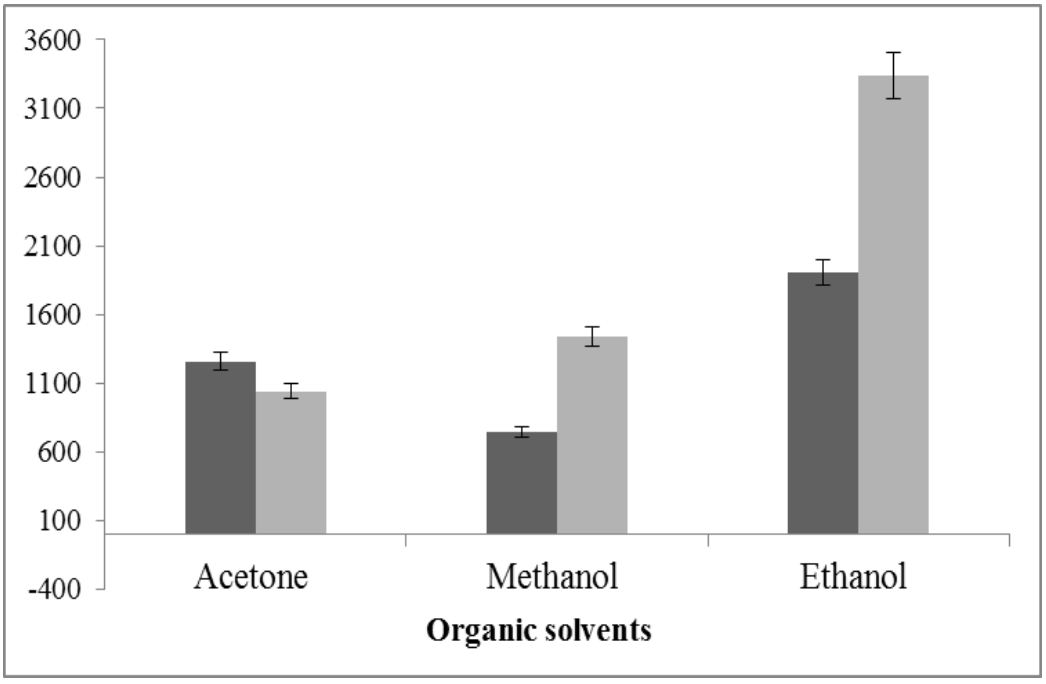


Figure 3.5 Two stage precipitation. 60% ammonium sulphate and then 60%organic solvents; volume aktivity (-- ■ --), specific activity (-- ■ --)

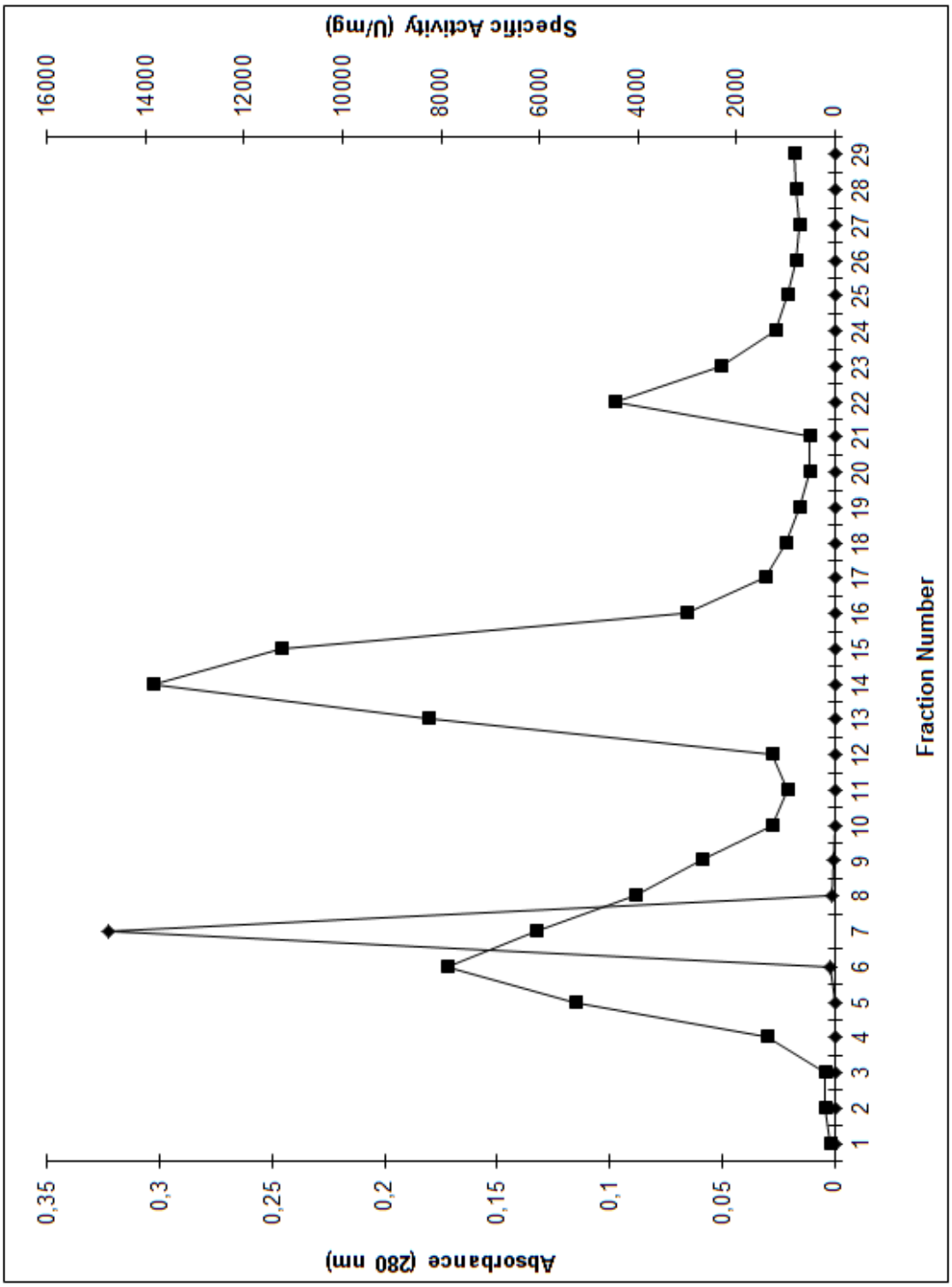


Figure 3.6 DEAE Cellulose anion exchangechromatography; distribution of total protein amount (--■--) andcatalase specific activity (--♦--)

3.3 Chromatographic Applications for the Purification of Catalase

3.3.1 DEAE Cellulose Ion Exchange Chromatography

As a second purification step, the fraction of 60% $(NH_4)_2SO_4$- 60% ethanol which approximately 14 fold purification was obtained, was applied to the DEAE cellulose ion exchange chromatographycolumn based on the charge differentations of proteins depending on medium pH.

Catalase activities were observed in the 6, 7, 8 and 9 numbered fractions with the values of 100.36; 14777.08; 54.32 ve 10.25 U/mg, respectively. Because activity values of the fractions were low by comparison with 7^{th} fraction, they were neglected and 62-fold purification was obtained with the 7^{th} fraction (Figure 3.6).

3.3.2 Sephacryl S-200 Gel FiltrationChromatography

The active catalase fractionobtained from DEAE cellulose ion exchange chromotography was applied to sephacryl s-200 gel filtrationcolumn. The protein amounts and catalase activity distributions obtained from gel filtration applicationwas shown in figure 3.7. The highest activity with 30770.31 U/mg was found in the 23^{th} fraction. Negligible level of activities in 22^{nd} and 24^{th}fractions were observed. As the result of sephacryl s-200 gel filtration chromotography, 129.10-fold purification was obtained. All of the purification steps of catalase enzyme from P.*chrysosporium* were presented in table 3.8.

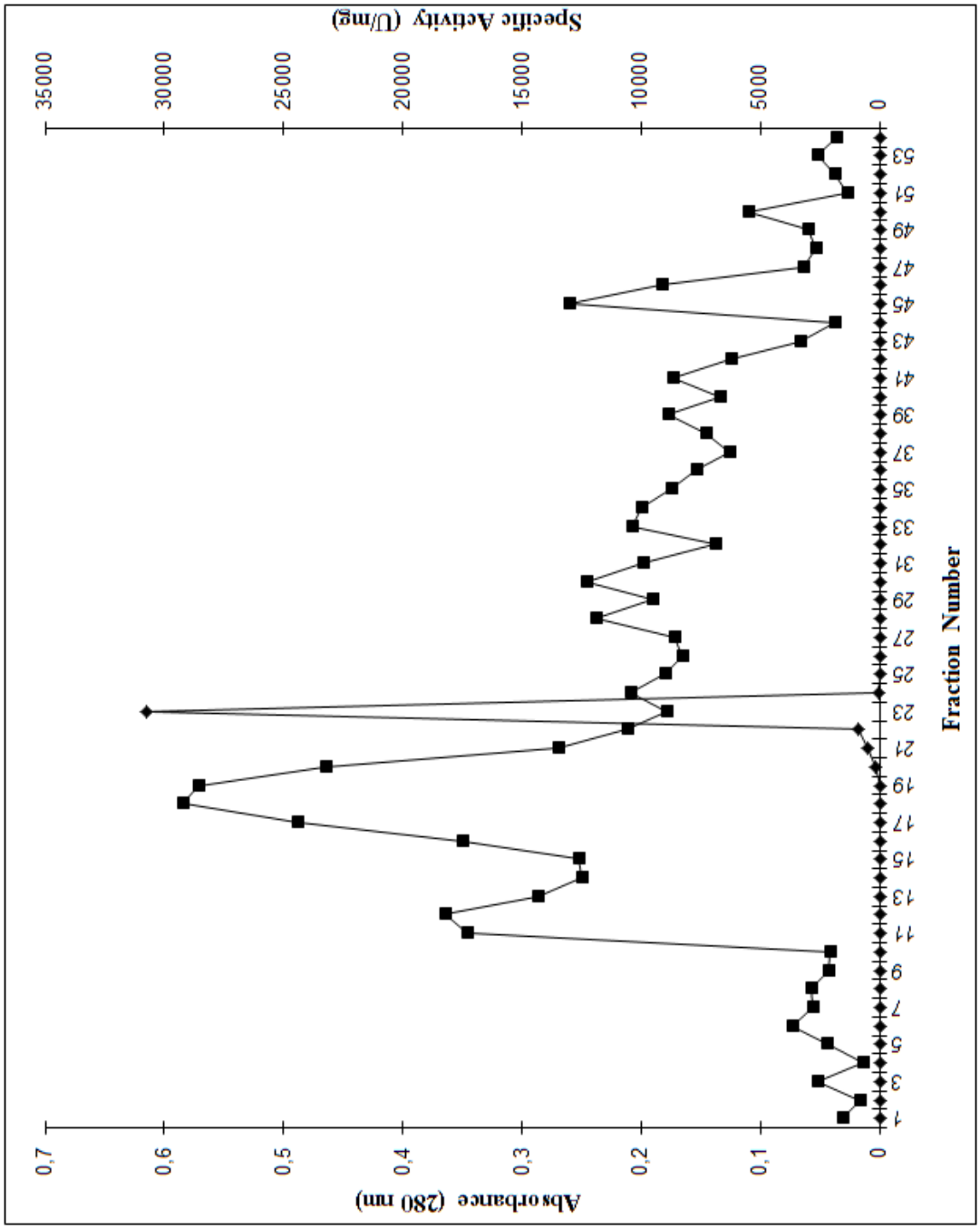


Figure 3.7 Sephacryl s-200 gel filtration chromatography; distribution of protein amount (--■--) andcatalase specific activity (--♦--)

Table 3.8 The purification steps of *Phanerochaete chrysosporium* catalase

Purification step	**Protein amount (mg/mL)**	**Aktivity (U/mL)**	**Specific activity (U/mg)**	**PF**
Crude extract	5.370	1279.88	238.34	----
%60 $(NH_4)_2SO_4$ %60 Ethanol	0.361	1204.57	3336.76	13.99
DEAE Cellulose	0.102	988.94	14777.08	62.0
Sephacryl-s-200	0.021	660.28	30770.31	129.10

In terms of determining the molecular weight of purified catalase by using sephacryl-s-200 gel filtration chromatography, standard protein mixture being molecular weight range with 66000 - 440000 Da was applied to the gel filtration column under same conditions.

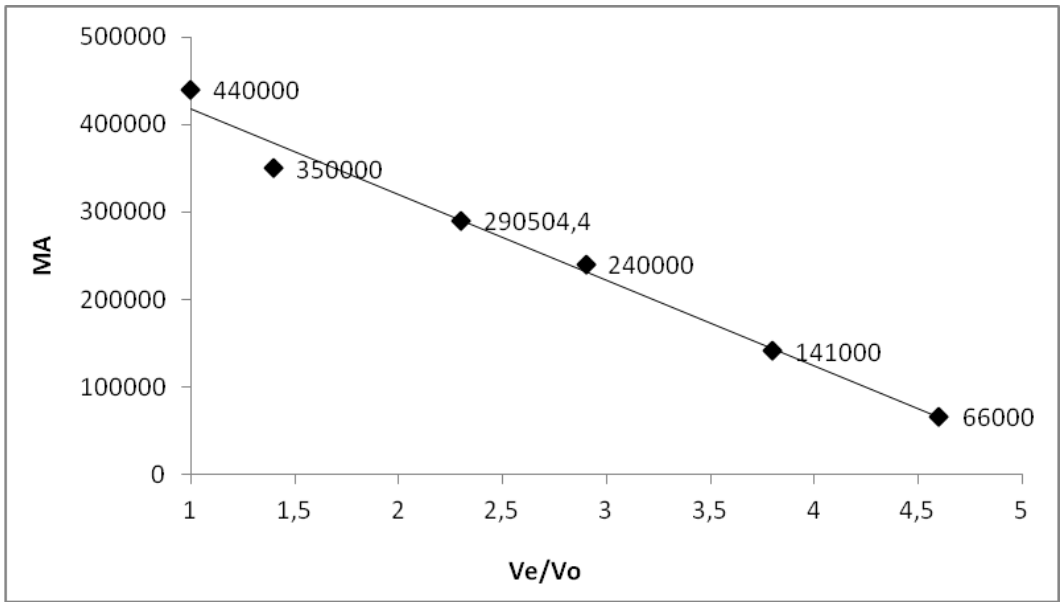


Figure3.8 Sephacryl s-200 gel filtration chromatography molecular weight calibration curve and molecular weight of purified catalase. Apoferritin (440000 Da), L-glutamate dehydrogenase (350000 Da), Catalase (240000 Da), Alcohol dehydrogenase (141000 Da), Bovine serum albümine (66000 Da).

As seen from figure 3.8, molecular weight of purified catalase was found 290504 Da.

3.3.3 SDS-PAGE Gel Electrophoresis

The Rf values of standard protein markers (carbonic anhydrase, 29000 Da; ovalbumin, 45000 Da; bovine serum albumine, 66000 Da; phosphorylaseb, 97400; β-galactosidase, 116000 and myosin, 205000 Da) and purified catalase were compared and the molecular weight of purified enzyme was found 70 kDa.

3.4 Enzymatic Characterization of Purified Catalase

3.4.1 pH Dependent Activity Changes

pH dependent activityalterations of purified catalase from P. *chrysosporium* was studied at 25 ^{0}C, between pH 5.0-8.0 in 20mM potassium phosphate buffer and pH 8.0-9.0 in 20 mM Tris-HCl buffer.

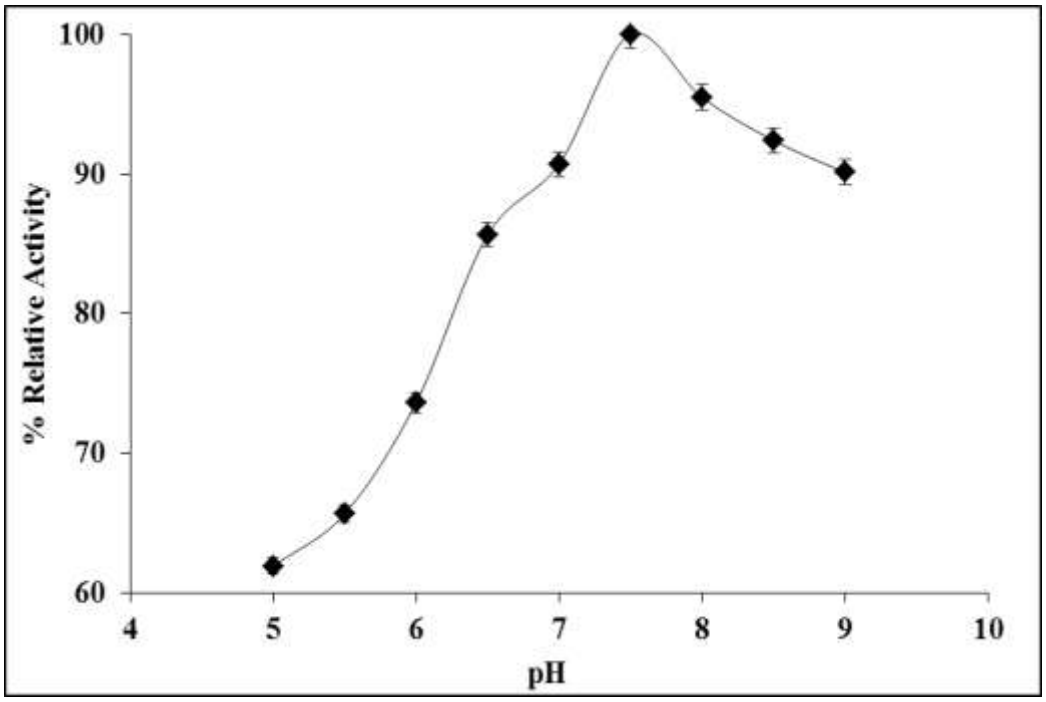


Figure 3.9 pH dependent activity changes at 25 ^{0}C

As seen from figure 3.9, pH activity values showed almost linear increase up to pH 7.5. On the other hand,it was observed that there was little declinein higher pH values. Results showed that the optimum pH of the P. *chrysosporium* catalase was 7.5.

3.4.2 Temperature Dependent Activity Changes

Purified catalase activity alterations depend on temperature was determined in 50 mM potassium phosphate buffer (pH 7.5) at 10 - 80°C.

As seen from figure 3.10, optimum temperature for the purified catalase was 30 ^{0}C and the activity of the enzyme showed faster decreases above so-called temperature. Purified enzyme did not have activity at 70 and 80 ^{0}C in practice.

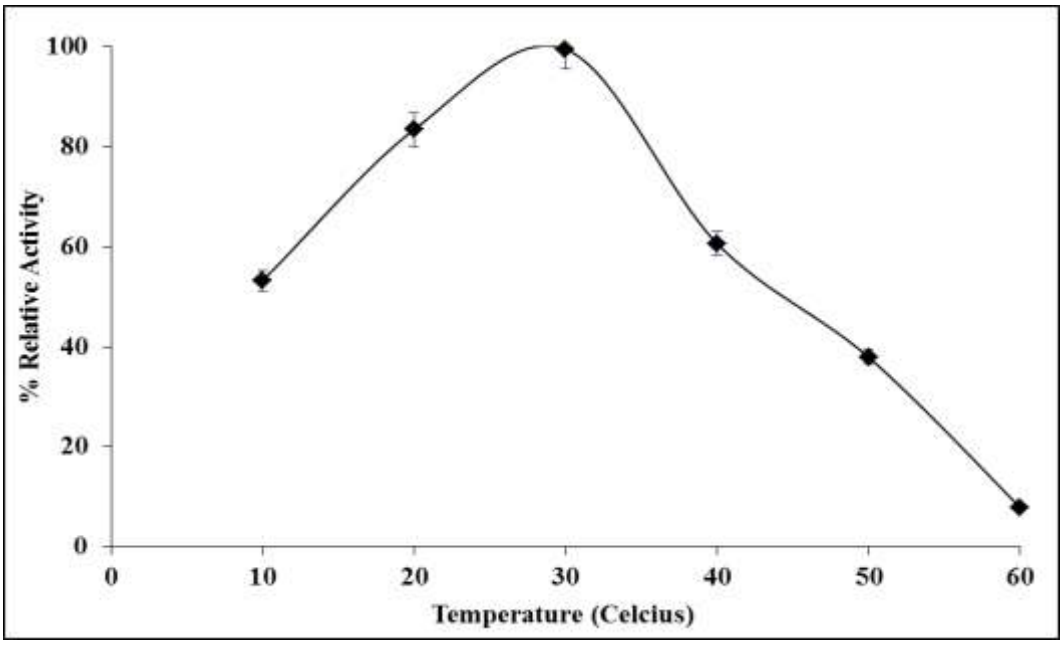


Figure 3.10 Temperature dependent activity changes in 50 mM potassium phosphate buffer, pH 7.5

3.4.3 pH Stability Changes

The activity stability depend on pH of the purified catalase from P. *chrysosporium*was determined under the standard activity assay conditions every two hours during 10 hours incubation period in 20 mM potassium phosphate buffer, pH 6.0; 7.0 ve 8.5.

As seen from figure 3.11, stability of the purified catalase was the highest at pH 7.0.After incubation periodat 25 ^{0}C for 10 hours in pH 7.0 buffer, the activity

decreased in the ratio of 17 percentage. Results showed that purified catalase was more stable in the basic region.

3.4.4 Thermal Stability Changes

Purified catalase from P. *chrysosporium* incubated in 20 mM potassium phosphate buffer at different temperatures for 2 hours and the remained activity was determined at the end of two hours under standart conditions(Figure 3.12).

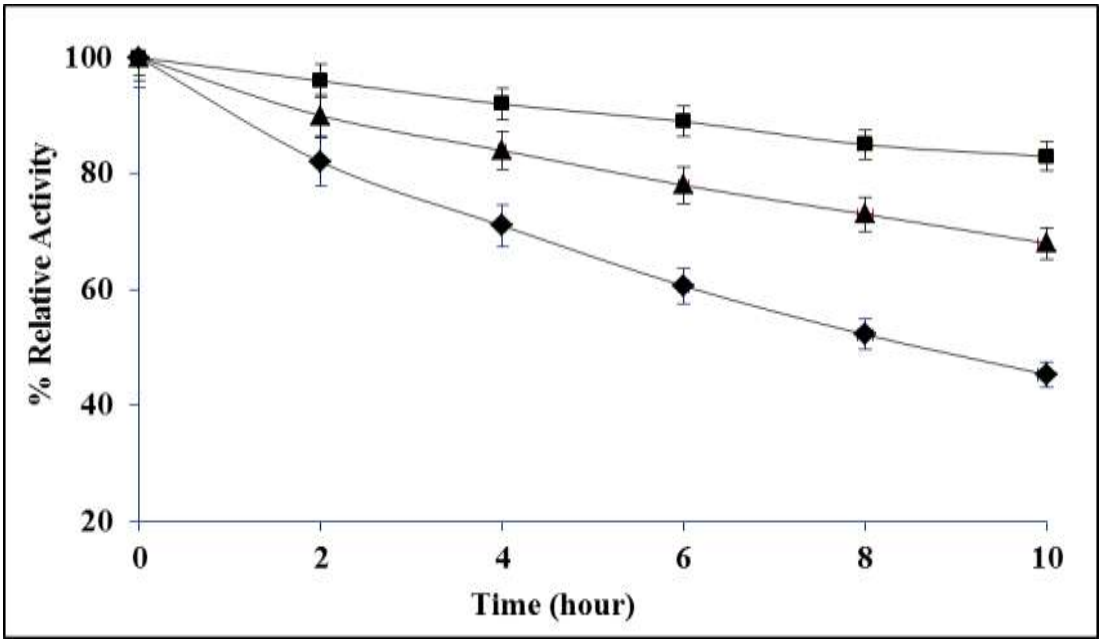
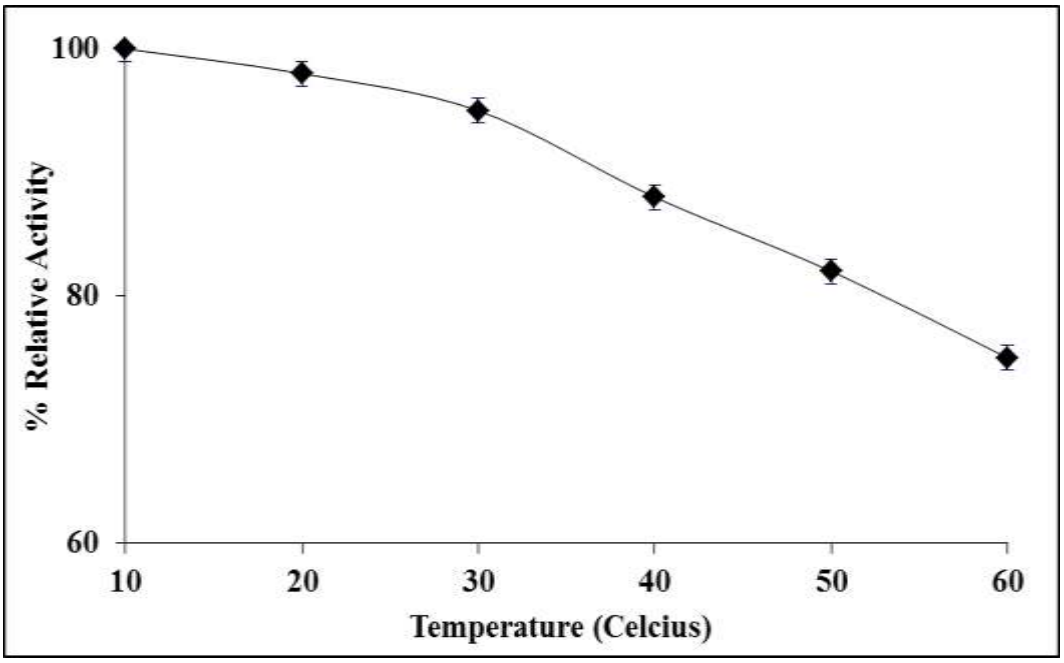


Figure 3.11 pH - % relative activity graphic; pH 6.0 (--♦--); pH 7.0 (--■--) ve pH 8.5 (--▲--)



Figures 3.12 Temperature - % relative activity graphic

3.5 Kinetic Parameters of Purified Catalase

3.5.1 K_m ve V_{max}

Activity changes of purified catalase from P. *chrysosporium* were investigated under standard conditions in H_2O_2 concentrations of 5-40 mM. $K_m$and V_{max} values were found by Lineweaver-Burke graphic (Figure 3.13).

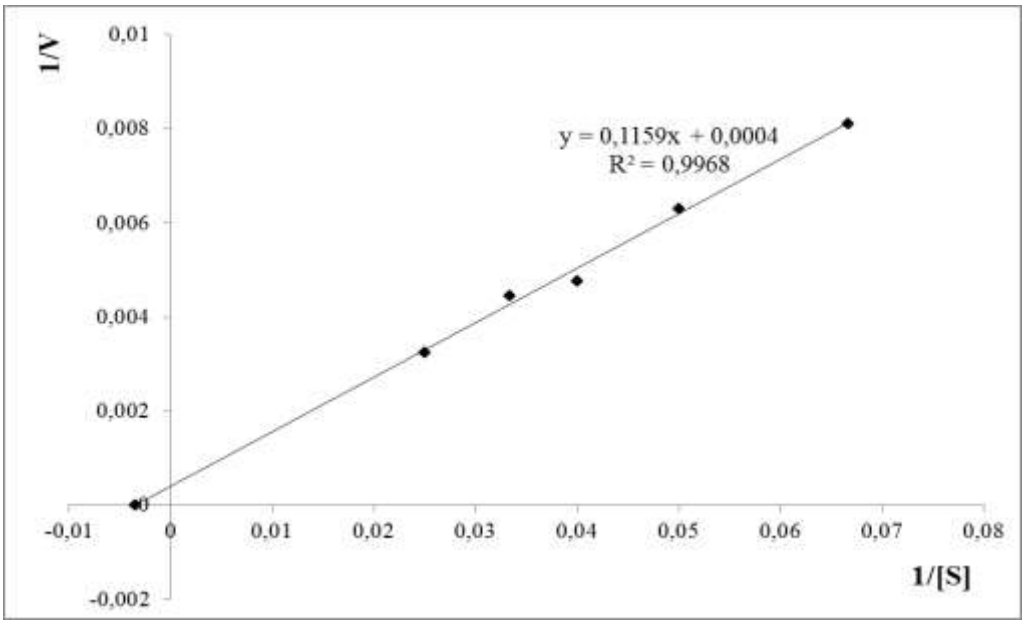


Figure 3.13 Lineweaver Burkegraphic

According to Lineweaver-Burk graphic, K_m and V_{max} values were found as 290.69 mM and 250000 U/mg for H_2O_2 and at 25^0C, respectively.

3.5.2 Inhibition Kinetics of Benomyl, Imazolyl ve Procymidon Fungucits

For the purpose of specifing the inhibition effects of benomyl, imazolyl and procymidon fungucitsupon the activity of catalase enzyme purified from *P. chrysosporium,* activity changes were investigated under standard assay conditionsby altering fungucit and substrate concentrations.

It was determined that imazolyl and procymidon had no inhibition effects on purified catalase activity up to 20 ppm. On the other hand, other fungucit benomyl had an inhibition effect in the concentrations of 0.9 and 2.0 ppm.

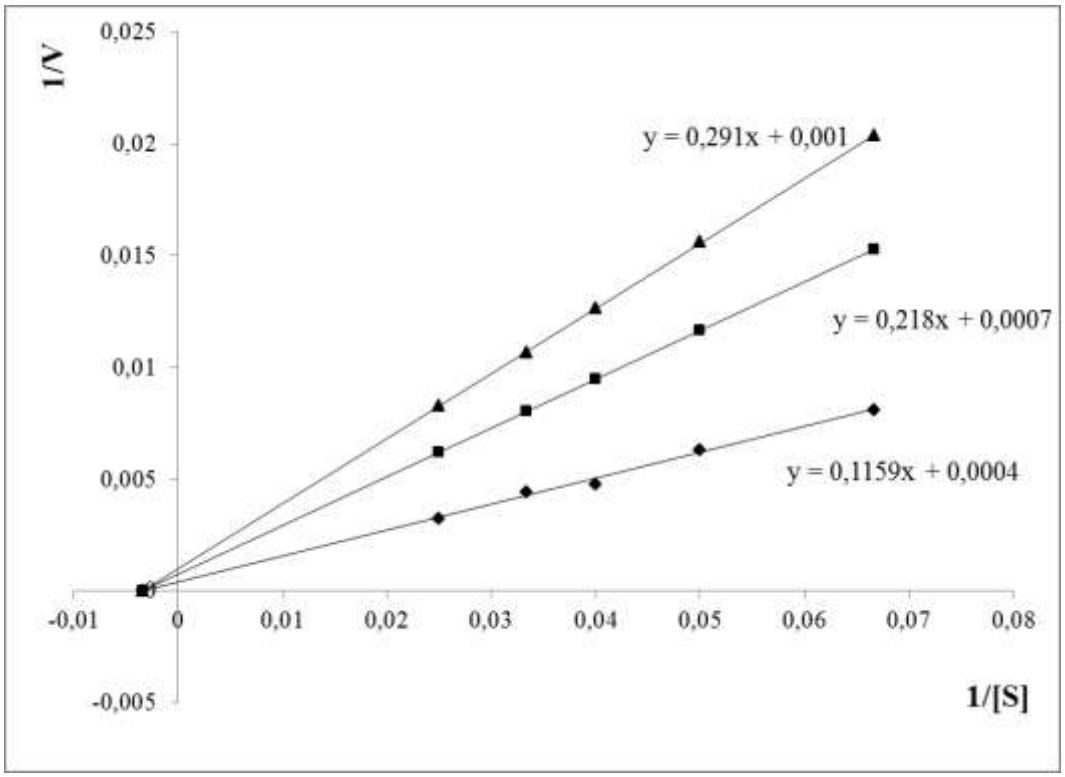


Figure 3.14 1/[S] – 1/V graph; 0ppm (--♦--), 0,9ppm (--■--) and 2ppm (--▲--) ppmof benomyl

As seen from figure 3.14, in the presence of benomyl, non-competitive inhibition was occured and K_i value was found 1.158 mM.

CHAPTER FOUR

CONCLUSIONS

The purification of enzymes, catalysts in the biologic systems, is of vital importance in terms of their usage in many industrial fields such as clinic, analytic and food. For the purpose of providing economy, there are comprehensive researches about purification from an economic source, immobilization and then long-time usage of enzymes (Tukel & Alptekin, 2004; Yanik & Donaldson, 2005; Shi et al., 2007). Catalase which is one of the target enzymes for this purpose, is usedespecially in food industry. In food industry, foods are sterilized by H_2O_2 and after sterilization remaining H_2O_2 may cause toxic effects for living being. To prevent these toxic effects of H_2O_2, catalase has widespread use. In the presented thesis,P.*chrysosporium* which can be easily produced was used as the source of catalase. Purification and characterization of catalase from P.*chrysosporium*was aimed in the scope of this thesis.

In the isolation of an enzyme from a microbial source, the solid and liquid mediums used for the production of the so-called microorganism is effective factors on target enzyme levels. Therefore, nitrogen and carbon limited K3-S2 medium with the specific activity of 238.56 U/mg was chosen for the production of catalase. When this specific activity value compared with other specific activities obtained from other researches, it can be said that an efficient source was produced within the scope of study (Kuusk, Björklund & Rydströrm, 2000; Nakamura et al., 1998; Monti, Baldaro & Riva, 2003). In addition, the high yield in biomass production with the mediums determined as a result of research as a part of thesis can be thought as a contribution to the economy of the process.

The precipitation process used as an initial purification steps were carried out with neutral salt, organic solvents and organic polymer as oneor two-step. The precipitationwith 60% ammonium sulphate and then 60% ethanol had the highest purification fold with the 14-fold increase in specific activity. Many of the proteins

do not undergo denaturation by precipitation with neutral salts and when the obtained pellets dissolve a suitable buffer, the activity is recovered. Therefore especially ammonium sulphate salt is prefered at initial purification process to fractionate the proteins because of having high solubility(Reilly et al., 2001; Wang et al., 1998).

Processing time for precipitation with organic solvent was short when compared with salt and PEG precipitation. This situation has an advantage in terms of denaturation factor which is depending on process.

When PEGs with different molecular weight were used, there was no precipitation at lower concentration than 10% concentration. On the other hand at 20% PEG concentration and over, viscosity and solubility problems were risen. According to these results, when PEG chain was elongated, although there was no significant differences in protein distribution decreasing catalase enzyme activity at the supernatant could be arised from dissociation affect of long chain hydrofobic structure to catalase subunits. Results showed that precipitation with PEG is not suitable as an initial purification step to fractionate the catalase from P. *chrysosporium*.

In order to obtain further purification fold, the fraction of 60% ammonium sulphate and 60% ethanol was applied to DEAE cellulose anion exchange chromotography column and 60-fold purification was obtained. Elution was carried out by using NaCl gradient and catalase activity was observed in the early fractions. It can be supposed that there were negatively charged proteins and they interacted with column matrix stronger than catalase. Obtained catalase fractions from DEAE cellulose anion exchange chromotography was applied gel filtration chromotography and 129.10 fold purification was achieved. Based on the results, it can be said that when compared with the other researches used similar purification steps, high purification fold was obtained in this research (Kang, Lee, Yoon & Oh, 2005; Yörük, Demir, Ekici & Savran, 2005).

According to the results of gel filtration chromotography, the molecular weight was found 290504 kDa on the other hand with SDS-PAGE electrophoresisthe molecular weight per subunit was found 70 kDa.It means that catalase has four identical subunits. Molecular weight of catalase is about 240 kDa in general and our result is higher than this value but this result is in accordance with other studies which used fungus at their worked. Torii et al(1982) found the molecular weight of purified catalase from Aspergillus niger as 385 kDa and Jacob et al.(1979) studied with Neurospora crassa and molecular weight of isolated catalase was found 320 kDa.

The characterization results of purified enzyme from P. *chrysooporium* showed that optimum pH was 7.5 and temperature was 30 ^{0}C. (Akertek and Tarhan, 1995; Alptekin et al, 2009; Lee et al, 2007; Claiborne and Fridovich, 1979; Tarhan, 1991). Because pH-dependent activity of purified catalase in basic region was changed more slightly than acidic region, it is thougt that purified catalase activity is not effected significantly in the basic region when compared with the acidic one.This situation is in accordance with the result that stability at pH 8.5 is higher than the stability at pH 6.0. K_m and V_{max}values were found as 290.69mM and 250000U/mg for H_2O_2, respectively. It is indicated in the literature thatdepending on the sources of purified enzyme and the growing conditions of sources, K_m and V_{max} values show diversity(Switala & Loewen, 2001).Imazolyl and procymidon fungucids did not shown inhibition effect on purified catalase P. *chrysosporium*, so it can be said that antioxidant defense system protects an indication of the resistance. However, another studied fungucid benomyl had an non-competitive inhibition effect on purified catalase activity. Çatalgöl had also determined that benolmyl reduced the activity of catalase from rats(Çatalgöl, 2008).

As a result of the research, catalase enzyme from P. *chrysosporium* fungus was purified with 130 fold as a consequence of four step purification.

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