CHARACTERIZATION AND ANALYSIS OF THE ANTIOXIDANT CAPACITY OF FUNCTIONAL PHENOLICS OXIDIZED BY *SCYTALIDIUM THERMOPHILUM* CATALASE PHENOL OXIDASE (CATPO)

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ABSTRACT

CHARACTERIZATION AND ANALYSIS OF THE ANTIOXIDANT CAPACITY OF FUNCTIONAL PHENOLICS OXIDIZED BY *SCYTALIDIUM THERMOPHILUM* CATALASE PHENOL OXIDASE (CATPO)

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Scytalidium thermophilum is a termophilic fungus that effectively produces the extracellular enzyme catalase phenol oxidase (CATPO). The enzyme is distinct among catalases with its bifunctionality of oxidising phenolic compounds in the absence of H_2O_2. CATPO is capable of oxidizing catechol, chlorogenic acid, caffeic acid and catechin which are *ortho* –diphenolic compounds. Diphenolic compounds are known as strong antioxidants. Catalase is one of the important antioxidant enzymes. Therefore, in this thesis the effect of CATPO on the final antioxidant capacity of the oxidized products was analysed. Antioxidant capacity measurements of oxidized and unreacted phenolic compounds were done using the two widely used methods TEAC and FRAP.

CATPO oxidized catechol showed 2.4 fold increase when compared to its non-oxidized form, which was highest among others. Catechol was followed by caffeic acid, chlorogenic acid, and catechin. This finding is new to the literature and may be of importance to the antioxidant mechanism of organisms. Results have also shown that the most well known phenol oxidases, laccase and tyrosinase, do not result in such high increases in antioxidant capacity upon oxidation of the substrates tested. Due to this finding, as a possible means of applying CATPO to increase the antioxidant capacity of products daily consumed, tea was selected.

Tea is the second most consumed beverage after water and it is known to possess high amounts of flavanols. Green tea is rich in catechins whereas black tea is a rich source of theaflavins and thearubigins. Fermentation is a critical process for production of good quality tea and is the key step differing between green and black tea production. During this process phenol oxidases catalyze the oxidation of polyphenolic compounds present in tea leaves to their corresponding *o*-quinones. Utilization of CATPO in tea samples resulted in an increase in antioxidant capacity and its effect was enhanced by an increase in brewing time. Interestingly, the addition of sugar decreased antioxidant capacity. Laccase and tyrosinase were ineffective in increasing the antioxidant capacity of tea samples.

Keywords; catalase, phenol oxidase, *Scytalidium thermophilum*, antioxidant capacity, tea phenolics

ÖZ

SCYTALİDİUM THERMOPHİLUM KATALAZ FENOL OKSİDAZI (KATFO) TARAFINDAN OKSİDE OLAN FONKSİYONEL FENOLİKLERİN KARAKTERİZASYONU VE ANTİOKSİDAN KAPASİTELERİNİN BELİRLENMESİ

Doktora, Gıda Mühendisliği Bölümü
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Scytalidium thermophilum etkin bir şekilde hücre dışı katalaz fenol oksidaz enzimini (KATFO) üreten termofilik bir mantardır. Enzim katalazlar arasında hidrojen peroksit yokluğunda fenolik bileşiklerin oksidasyonunu gerçekleştiriyor oluşuyla farklılık göstermektedir. KATFO katekol, klorojenik asit, kafeik asit ve kateşin gibi orto-difenolikleri oksitleme kapasitesine sahiptir. Difenolik bileşikler güçlü antioksidanlar olarak bilinirler. Katalaz ise önemli antioksidan enzimlerden biridir. Bu nedenle, bu tezde KATFO'nun oksitlenmiş ürünlerinin son antioksidan kapasiteleri üzerindeki etkisi incelenmiştir. Okside olmuş ve olmamış fenolik bileşiklerin antioksidan kapasite ölçümleri yaygın olarak kullanılan iki yöntemle (TEAC ve FRAP) yapıldı. KATFO tarafından okside edilen fenolikler içinde en yüksek artış katekolde tespit edildi.

Katekol, okside olmamış formu ile kıyaslandığında antioksidan kapasitede 2.4 kat artış gösterdi. Artış sıralamasında katekolü kafeik asit, klorojenik asit ve kateşin izledi. Bu bulgu, literatür için yeni ve organizmaların antioksidan mekanizması açısından çok önemli olabilir. Aynı zamanda sonuçlar iyi bilinen fenol oksidazlardan olan lakkaz ve tirozinaz için durumun bu şekilde olmadığını göstermektedir. Denenen fenolikler üzerinde lakkaz ve tirozinaz oksidasyonunun antioksidan kapasitede belirgin artışlara neden olmadığını göstermiştir. Bu bulguya bağlı olarak, günlük olarak tüketilen ürünlerin üretim aşamalarına KATFO eklenerek antioksidan kapasitesini artırmak için olası bir uygulama alanı olarak, çay üretimi seçilmiştir.

Sudan sonra en çok tüketilen ikinci içecek çay olup, çayın yüksek miktarda flavanol içerdiği bilinmektedir. Siyah çay teaflavin ve tearubigin açısından zengin bir kaynakken, yeşil çay içeriğinde yüksek oranda kateşinler bulunmaktadır. Fermantasyon kaliteli çay üretimi için kritik bir süreçtir ve hem yeşil hem de siyah çay üretimi arasındaki farklılığın oluşmasında önemli bir adımdır. Bu işlem sırasında fenol oksidazlar, çay yaprağında mevcut polifenolik bileşiklerin oksidasyonunu katalize ederler. Çay örneklerine KATFO eklenmesi yoluyla antioksidan kapasitede artış meydana gelmiştir ve bu etki demleme süresi ile doğru orantılıdır. İlginç bir şekilde, şeker ilavesi antioksidan kapasitede azalmaya neden olmuştur. Lakkaz ve tirozinaz uygulamaları çay örneklerinin antioksidan kapasitesini artırmamıştır.

Anahtar kelimeler; katalaz, fenol oksidaz, *Scytalidium thermophilum,* antioksidan kapasite, çay fenolikleri

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to my beloved family

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CHAPTER 1

INTRODUCTION

1.1 Plant Phenolics and Their Biosynthesis

Phenolic acids are plant metabolites widely spread throughout the plant kingdom. The term plant phenolics; refers to substances that possess an aromatic ring bearing one or more hydroxyl substituents. Phenolic compounds; in general terms, have a basic structural chemistry. There is only a limited number of metabolic pathways through which such compounds can be formed and so this enables the production of a wide range of compounds from the same origin. The majority of plant phenolics are formed via the shikimic acid and polyketide pathways as shown in Figure 1.1 and 1.2 (Geissman and Crout 1969).

Natural polyphenols can range from simple molecules such as caffeic acid to very large, highly poymerized compounds like tannins. The phenolic acids are known to be involved in the formation of lignin. They play a role in the structural stability of plant material. Their chemical structure allows them to form a variety of ester and ether cross-linkages. They are also responsible for the brightly colored pigments of many fruits and vegetables. The skins and seeds of fruits andthe leaves and stems of vegetables are rich sources of phenolic compounds. Tea plant (*Camellia sinensis)* is known to provide concentrated sources of these compounds.

The allocation of distinct phenolics in plants and dietary sources has not been well documented and this reflects the lack of current understanding of their potential role in human diet. There are not muchextensive food composition data available for the flavonoids or phenolic acids (Morton *et al.*, 2000). Polyphenols ubiquitously distributed in plant foods are listed in Table 1.1.

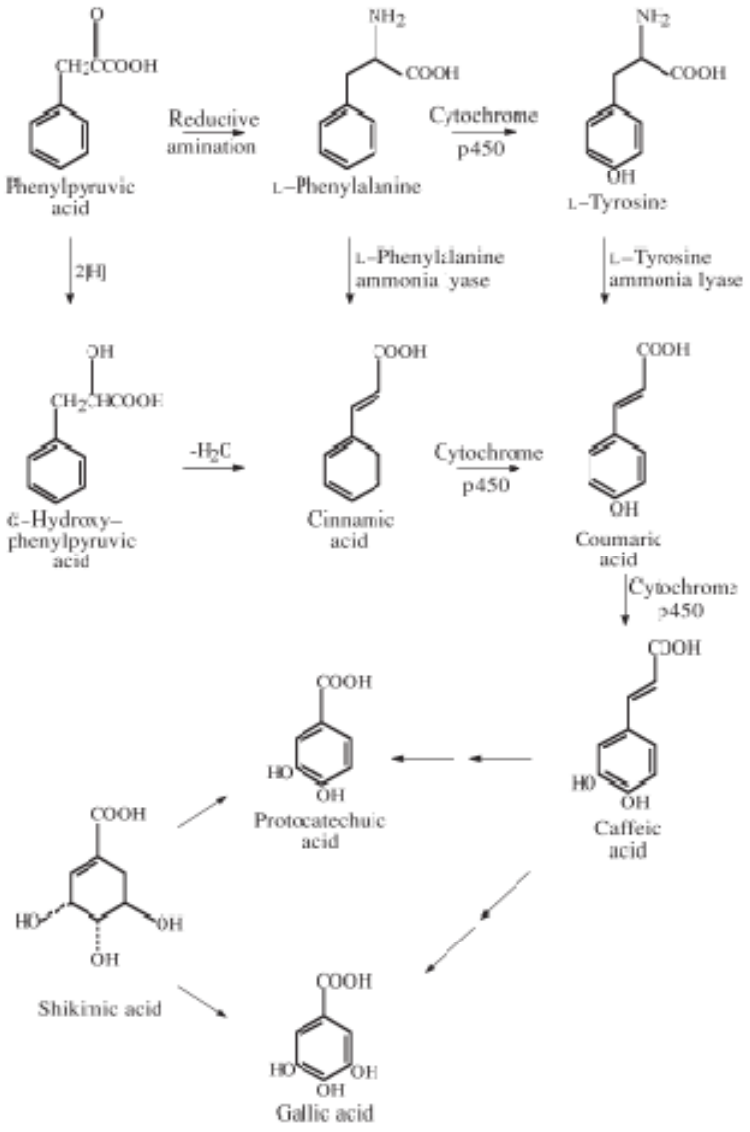


Figure 1. 1 Biosynthetic pathways for the phenolic acids in plants.

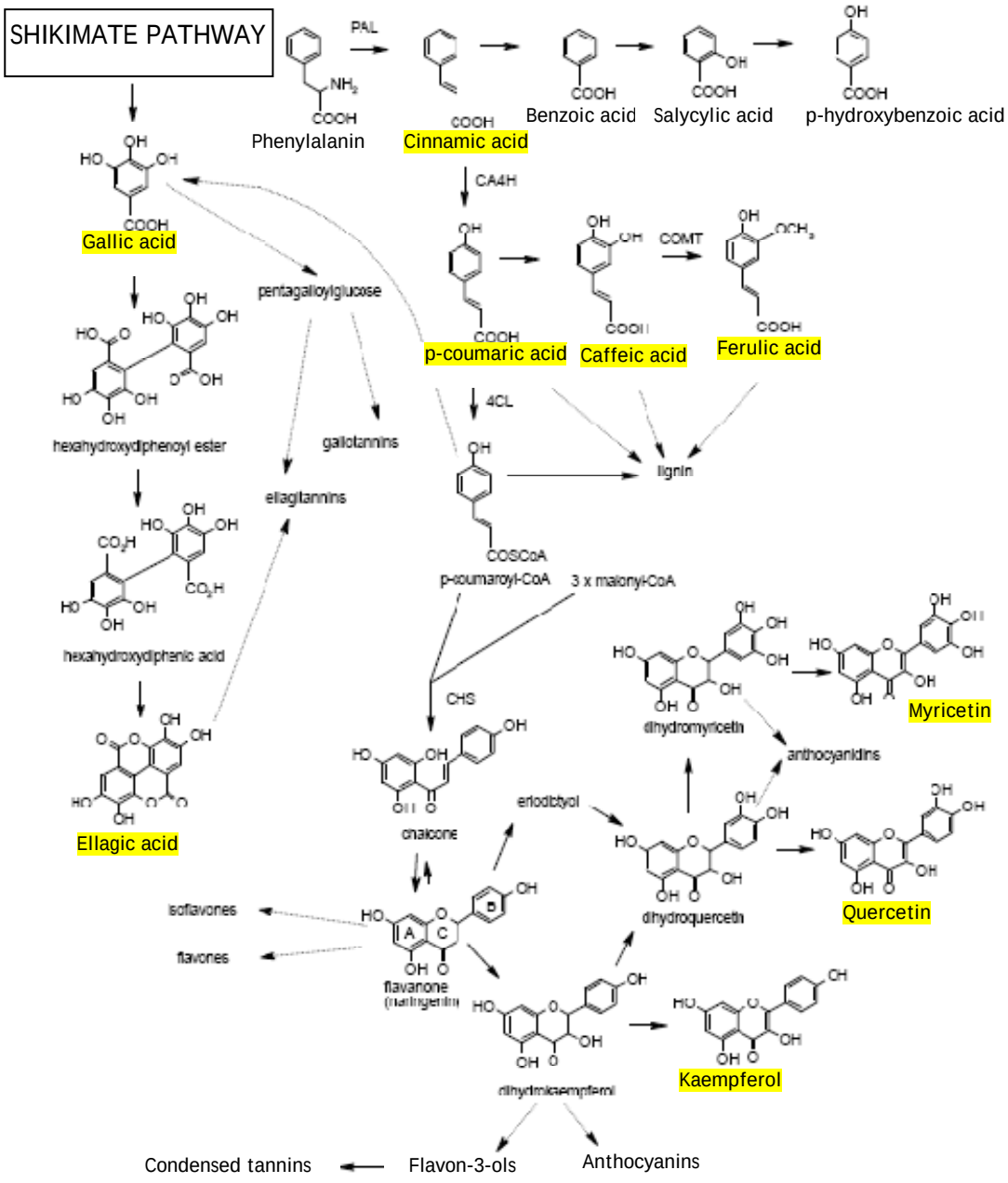


Figure 1. 2 Biosynthesis of hydroxycinnamic acids, hydroxybenzoic acids and flavonoids.

Table 1. 1 Dietary sources of phenolic compounds (abundant ones are listed).

Source	Compounds
Black Tea	Gallic and caffeic acids, theaflavin, thearubigin
Green Tea	Epigallocatechin gallate
Coffee	Hydroxycinnamic acids
Chocolate	Catechin
Cinnamon	Vanillic acid, procyanidin and epicatechin
Grapes	Cinnamic acid derivatives, anthocyanins, flavonols
Apricots	Chlorogenic acid, caffeic acid
Cherries	Caffeic and gallic acids
Apples and peaches	Caffeic, coumaric and ferulic acids
Raspberry	Ellagic, gallic and cinnamic acids
Citrus fruit	Coumaric and cinnamic acids
Berries in general	Kaempferol, quercetin and myricetin
Pomegranate	Ellagic acid
Olives	Dihydroxyphenylethanol, oleuropeine
Cauliflower	Coumaric acid
Broccoli	Caffeoylquinic acid
Artichoke	Benzoic and cinnamic acid derivatives
Parsley	Procatechuic, caffeic and p-coumaric acids
Soybean	Trans-cinnamic acid
Rosemary	Rosmarinic and carnosic acid
Thyme	Thymol and carvacrol

There has been influential scientific interest in the possibility that increased intake of dietary antioxidants may protect against some chronic diseases.

Plant phenolics are classified as potent antioxidants.Phenolic phytochemicals are important components of fruits and vegetables and are partly responsible for their beneficial health effects against oxidation linked diseases like cancer, diabetes and cardiovascular diseases.

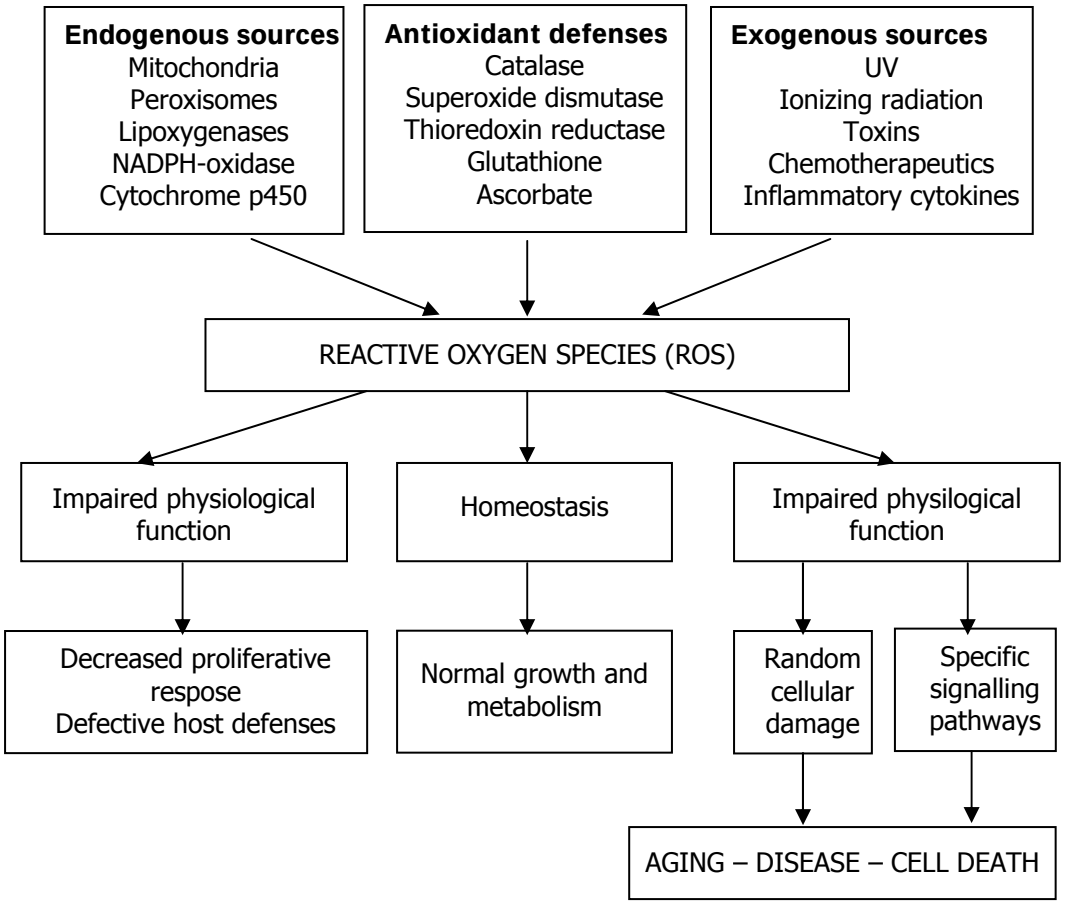


Figure 1. 3 Pathogenicitydriven by reactive oxygen species.

It is believed that phenolic compounds function either by countering the negative effects of oxidative stress by directly acting as an antioxidant or by activating cellular antioxidant enzyme systems (Vattem and Shetty 2005).

Flavonoids and phenolic acids can act as antioxidants by a number of pathways, but perhaps the most significant is by free radical scavenging in which the phenolic compound can break the free radical chain reaction. The resulting radical must be stable or it will result in a chain-propagating radical species (Morton *et al*., 2000).

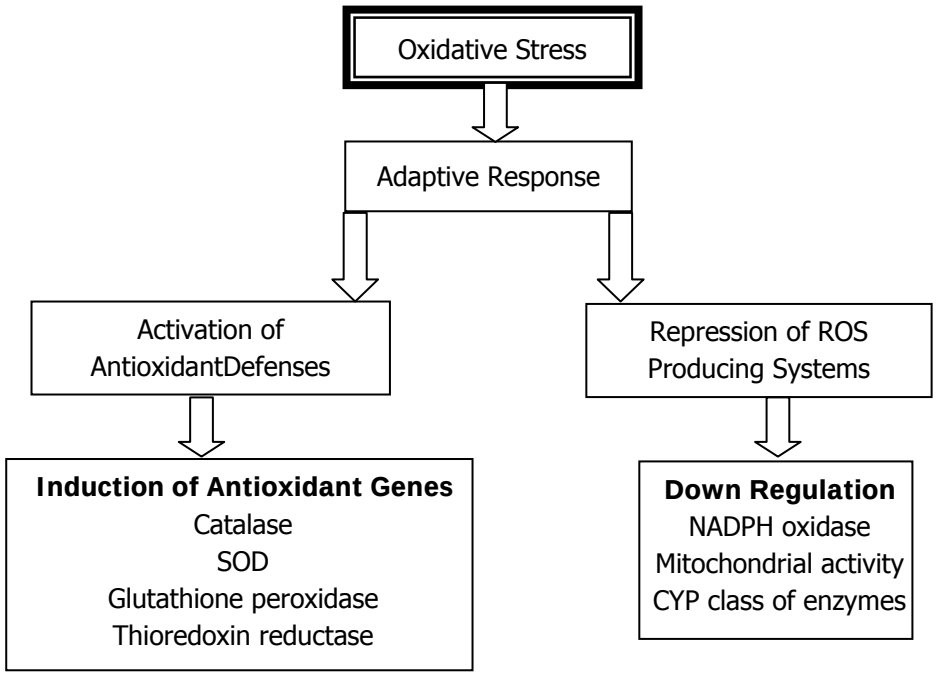


Figure 1. 4 Adaptive responses in living organisms to manage oxidative stress.

Reactive oxygen species are theoreticallyserved to be signaling molecules, and at low levels, they have been implicated in many cellular processes; especially intracellular signaling, which is responsible for proliferation or apoptosis, modulation of immune response and for mounting a defense response against pathogens (Sculley and Langley-Evans 2002) (Fig. 1.4).

Although the exact mechanism of the action of reactive oxygen species in effecting these physiological processes is not very well explained, there is evidence that reactive oxygen species, at some concentration, are capable of activating or repressing many biological effector molecules (Morel and Barouki 1999). It has been shown to activate or to repress transcription factors by directly activating them, by oxidizing the sulfhydryl groups present or by regulating a complex array of kinases which are important in signal transduction (Lusini *et al.* 2001).

1.2Biological Function of Phenolic Compounds

Phenolic compounds show a wide variety of biological effects and can be broadly divided into twomain categories. The first and the well understoodmode of action of these phytochemicals in fighting against oxidation stress related diseases is due to the direct involvement of the phenolics in quenching the free radicals from biological environments. It is known that free radicals cause oxidative damage to nucleic acids, as well as proteins and lipids. Oxidation of biological macromolecules, as a result of free radical damage, has now been strongly incorporated with the development of many physiological conditions which can alter into diseases(Droge 2002, Jakus 2000,Morel and Barouki 1999). Phenolic compounds, due to the hydroxyl substituents and phenolic ring possessed, can play a role as effective antioxidants because of their ability to scavenge free electrons. Phenolic antioxidants can therefore get rid of the harmful free radicals and eventually inhibit their oxidative reactions with key biological molecules (Rice-Evans *et al.*1997). Several studies have demonstrated the anticarcinogenic properties of phenolic phytochemicals such as gallic acid, caffeic acid, ferulic acid, catechin, quercetin and resveratrol (Yamada and Tomita 1996).

1.2.1 Sources, Structure and Chemistry of Selected Phenolics

A general overview and a preliminary literature review was given in the Section 1.1 with the help of Table 1.1 listing the very well known classes of edible plants and the abundant phenolic compounds in them. Here in this section a focus on four phenolic compounds that has an importance for this study is introduced.

a) Catechol

Catechol, or 1,2-dihydroxybenzene, is an organic compound with the molecular structure drawn in Figure 1.5. It is the orthoisomer of the three isomeric benzenediols, catechol, hydroquinone and resorcinol, in the order of ortho, para and meta.

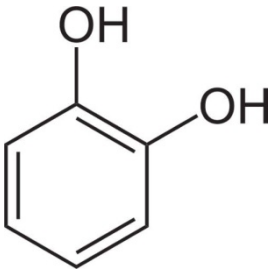


Figure 1. 5 Chemical structure of catechol.

Catechol occur naturally in fruits and vegetables in small amounts, like onions, potatos, eggplants and apples (Hollman and Arts, 2000). It is one of the main natural phenols in Argan oil (Charrouf and Guillaume, 2007).

b) Chlorogenic Acid

Chlorogenic acid is the ester formed between caffeic acid and L-quinic acid. The chemical structure of which is shown in Figure 1.6.

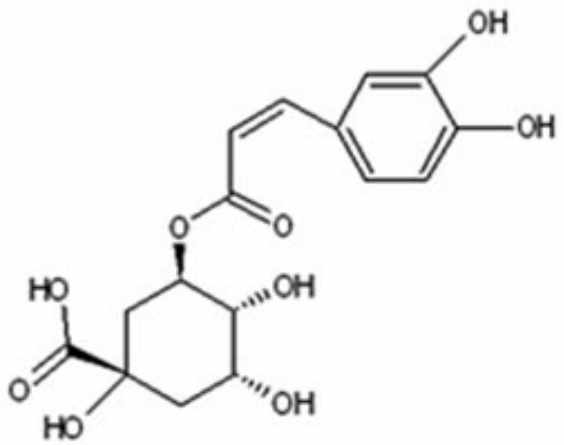


Figure 1. 6 Chemical structure of chlorogenic acid.

Chlorogenic acid is found in all higher plants and have a role in plant stress response (Dixon and Palma, 1995). The quantities present in most plants are very small compared to other phenolics, and thus they do not play an important role in human diet. However, a few plants accumulate chlorogenic acid, like green coffee beans, typically containing 6-7% of chlorogenic acid. Roasting results in a significant decline in the content of chlorogenic acif in green coffee beans and there is a rapid decrease in the content of chlorogenic acid with temperature increase throughout roasting.Prunes (Stacewicz-Sapuntzakis*etl. al.,* 2001) and peaches (Cheng and Crisosto, 1995) also contain a certain amount of chlorogenic acid .

c) Caffeic Acid

Caffeic acid is an organic compound consisting of bothphenolicandacrylic functional groups (Figure 1.7). It is a hydroxycinnamic acid and a key intermediate in thebiosynthesis of lignin.Caffeic acid can be found in oats, wheat, rice, berries, cherries, olive oil (Dimitrios, 2006), kiwi, coffee, tea, (Manach *et. al.,*2004), sunflower oil (Marinova *et. al.,*2001), and lots of other foods consumed.

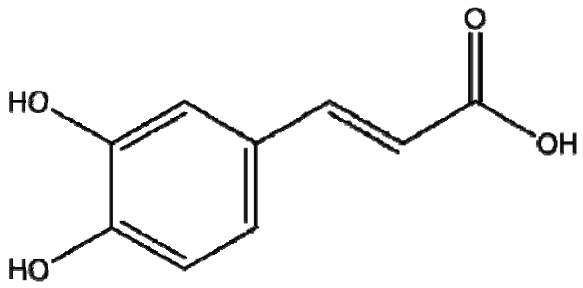


Figure 1. 7 Chemical structure of caffeic acid.

c) Catechin

Catechin is basicly a flavan-3-ol monomer (Figure 1.8). It is a plant secondary metabolite. The catechins are abundant in tea plant, *Camellia sinensis*, as well as in cocoas and chocolate(Manach *et. al.*, 2004). As a major constituent of gren tea 30 to 35 % of its total chemical components is catechin, whereas the number decreases to only 3 to 8 % because of the nature of black tea production process (Sharma and Rao, 2009).

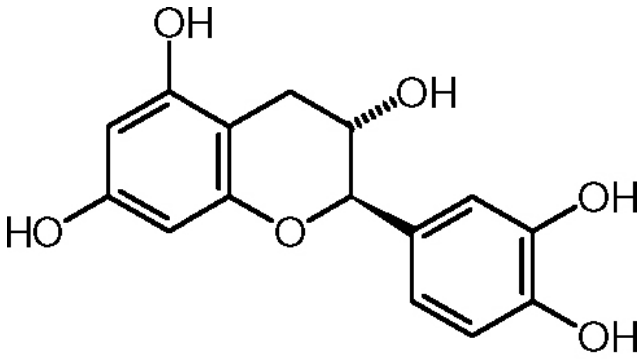


Figure 1. 8 Chemical structure of catechin.

1.2.2 Plant Phenolics as Antioxidants

One of the main roles of phenolic compounds is that they act as antioxidants. An antioxidant, by definition is a substance that when present in a reaction environment, significantly delays or totally inhibits the oxidation taking place. An effective antioxidant in one system is not neccessarily an effective one in another (Halliwell and Gutteridge, 1995). Phenolics act as antioxidants because of the hydroxyl group they posess. Many attempts at explaining the structure-activity relationships of some natural antioxidant compounds have been regarded in the literature. It has been reported that the antioxidant activity of phenolic compounds may result from the neutralization of free radicals initiating oxidation process.

The antioxidant acitivity of plant phenolics is either similar or in most cases even higher than that of Vitamin E (alfa-tocopherol) and Vitamin C (ascorbic acid).Extensively studied sources of natural antioxidants are fruits and vegetables, seeds, tea, cereals, berries, wine, olive oil and aromatic plants. A large number of studies are on tea and its antioxidant effects. The researches support the hyposthesis that tea is a major dietary supplement for the prevention of oxidative damage to DNA, development of cardiovascular diseases and cancer. Tea itself is a major source of flavanoids in the diet with levels approaching 200 mg per cup for a traditional brew of black tea (Lakenbrink *et. al.*, 2000).

1.3 Tea

By a simple definition, tea is an infusion drink made from the leaves of an evergreen shrub of Camellia family. Two main types of tea plant are cultivated worldwide in about 30 countries (Fig. 1.9); *Camellia sinensis* var. *sinensis* (Fig.1.10) is grown widely in China, Japan and Tibet, while *Camellia sinensis* var. *assamica* is cultivated mainly in India and Malaysia (FAO, 2004).

The plant grows mainly in tropical and subtropical climates. It takes about four to twelve years for a tea plant to bear seed, and about three years before a new plant is ready for harvesting (Magambo and Cannel, 1981). Tea is the most popular drink in the world other than water. The consumption of tea exceeds that of coffee, beer, wine and carbonated soft drink.

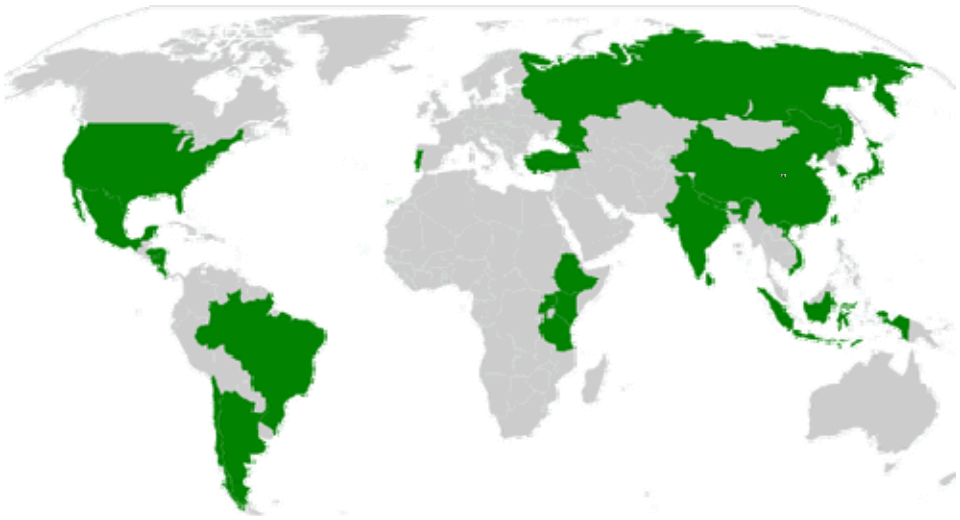


Figure 1. 9 Tea cultivated countries (green) on the world map.

Figure 1. 10a) The sketch of Camellia sinensis plant (Gleason, 2007)
b) A photograph captured from the fields (Rize, Turkey).

Many studies have demonstrated that both catechins and theaflavins, besides preventing free radical generation, have strong free radical scavenging abilities both in vitro and in vivo.

1.3.1 History of Tea

Tea cultivation began around 2000 BC in China and spread to Japan and to the near east first. Chinese mythology teaches that Emperor Shen Nung discovered tea while resting under a tree. Shen Nung, who always boiled his water saw a few leaves falling into his pot from a wild bush. When he drank the resulting brew, he found it delicious and refreshing, then he ordered the plant to be grown all over China (Gürsoy, 2005). The tea was first carried westwards during 5th centuryby Turkish Traders, who used it in barter trade for Chineseproduce. By the end of 6th century, tea had become sopopular that it was no longer considered a medicinal drinkbut a refreshing beverage.

Tea cultivation was first introduced in Batum(Republic of Georgia), neighboring Eastern Black searegion of Turkey by Russians in the last quarter of 19thcentury after importing seedlings from China. Since Russians hadsuccessfully introduced tea in Batum, it was felt that teacultivation must also be introduced in Turkey. Therefore,under the directions of the state, the Department of Agriculture selected Bursa in 1888 to evaluate the feasibility of teacultivation (Tekeli, 1976). Soon it was discovered that the teaplants needed very specific environmental conditions to be cultivated, Bursa soon jugded to be not feasible for the proper cultivation of *Camellia sinensis.* In 1917, after several locations visited and tested for ideal tea plantation Rize and its surroundings was chosen to be the place (Klasra *et. al.,*2007). In 1947 first commercial tea factory was established in Rize and Turkey prohibited importation of coffee and the national beverage turned to tea from coffee (www.caykur.gov.tr). At the start of tea cultivation in Turkey the goal was to meet the domestic demand only, but today Turkey is the 6th largest tea producer in the world (Özden, 2009).

1.3.2 Tea Production Processes

The important conventional teas are black and green tea. Among the numerous types of teas green, oolong and black are produced and consumed abundantly in different regions of the world. They are differing from each other because of the degrees of fermentation in the final product (Hara *et. al.*, 2001). Immediately after harvest the fresh leaves are brought to tea processing plants usually very close to the tea fields. It is the manufacturing process that determines the type of tea produced. Fermentation here in tea processing do not refer to an exogenous microbial activity as in wine or beer production, it is a natural browning caused by the endogenous enzymes of the plant itself (Harbowy and Balentine, 1997).

The green tea manufacturing process involves the rapid steaming or pan firing of the freshly harvested leaves to inactivate enzymes, preventing fermentation, producing a dry and stable product (Bokuchava and Skobeleva, 1980). Oolong tea, popular in Far East, is the partially fermented form of green tea. The tea leaves are wilted in sunlight, crushed and left to oxidize till the edges become dark in color, then they are heated and dried (Yamanishi *et. al.*, 1995). Black tea is fully fermented. Details of manufacturing black, green and oolong tea is summarized in the illustration given in Figure 1.11.

Fermentation is a critical process for production of good quality tea. During this process polyphenolases catalyze the oxidation of polyphenolic compounds present in tea leaves to their corresponding o-quinones. Oxidation of flavan-3-ols responsible for the formation of characteristic color and flavor of fermented tea is catalyzed by catechol oxidase. Therefore control of fermentation process has important effects on the flavor and color of tea and these depend on the degree of oxidation of tea phenolics. The rate and degree of polyphenolic oxidation depends on the composition, distribution, and content of flavanols in fresh tea shoot, the activity of oxidizing enzymes, the degree of tissue damage and cellular disruption, as well as temperature and oxygen content of the fermenting tea leaves.

Shoots in tea manufacture are composed of a bud andthree leaves on average. The distribution of thecatechins within a shoot varies with the age of leaves (Robertson, 1983). The content and distribution of catechins in fresh tealeaves may vary with the harvesting season and harvesting method (either by hand or mechanically). The conversion of catechins to theaflavins during tea manufacturing does not affect their radical scavenging potency.

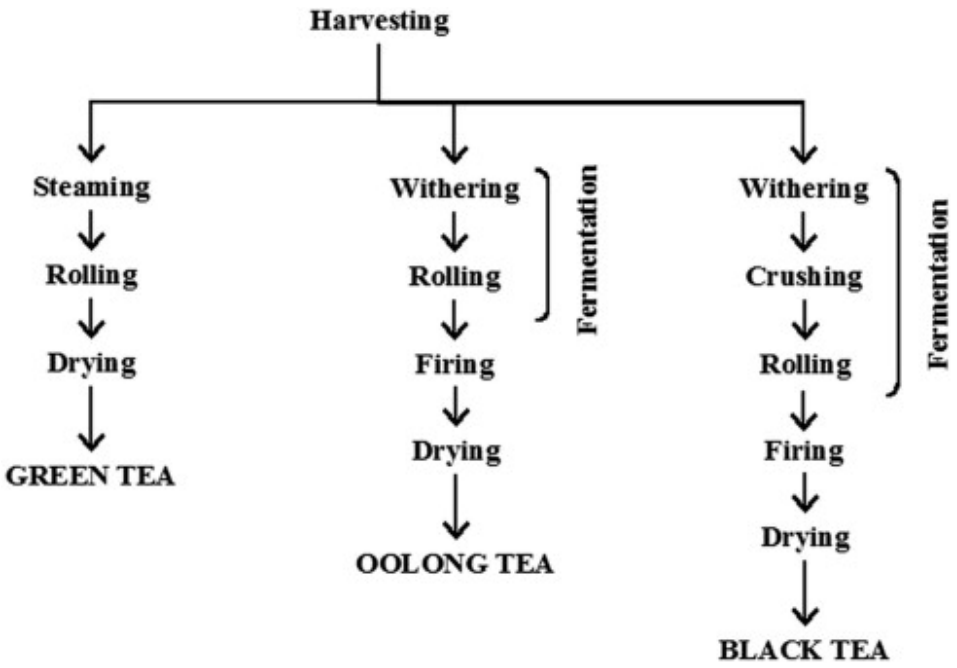


Figure 1. 11 Tea manufacturing process.

Manufacture process steps of black tea can be outlined as below;

1- *Withering;* Freshly plucked leaves are exposed to hot air for several hours (≈12-16) in order to reduce their moisture content by 50%.This step softens the leaves, preparing them to undergo subsequent operations without breaking.

2- *Rolling;* The leaves are rolled either by hand or mechanically allowing the essential oils to spread and to impregnate the buds. It is done mainly by two methods; Orthodox and CTC (Crush, Tear, Curl).

3- *Fermentation;* The chemical components present in tea leaves are allowed to undergo enzymatic oxidation in the presence of oxygen under controlled temperature and humidity. Enzymatic oxidation would lead to the formation of taste, aroma and coloring compounds.
4- *Drying;* After the required fermentation the leaves are dired to stop the reactions. The final moisture content in black tea is 3%. Exposure to at least 80°C is neccessary but temperature above 110°C would be harmful to tea.

1.4 Catalases

Catalases are antioxidant enzymes that catalyze the conversion of hydrogen peroxide (H_2O_2) to water and molecular oxygen, serving to protect cells from its toxic effects (Bai and Cederbaum, 2001). H_2O_2 is produced as a consequence of oxidative cellular metabolism and can be converted to the highly reactive hydroxyl radical via transition metals, this radical being able to damage a wide variety of molecules within a cell, leading to oxidative stress and cell death.

Catalases act to neutralize H_2O_2 toxicity, and are produced by all aerobic organisms ranging from bacteria to human. Most catalases are mono-functional, heme-containing enzymes, although there are also bifunctional heme-containing peroxidase/catalases that are closely related to plant peroxidases, and non-heme, manganese-containing catalases that are found in bacteria (Chelikani *et al.*, 2004). In addition to this, a new 4^{th} group of catalases, the catalase-phenol oxidases (CATPO), are introduced previously by our group (Sutay *et al.*, 2008). CATPOs are capable of H_2O_2 decomposition (catalase activity) and phenolic (o-diphenolic compound, especially catechol) oxidation in the absence of H_2O_2. There is only 1 study describing phenol oxidase activity of a mammalian catalase (Vetrano *et al*. 2005).

As a result of their striking ability to evolve molecular oxygen, catalases have the subject of observation and study for over 100 years. The overall reaction for the enzyme is very simple:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

A phlylogenetic analysis (Fig.1.12) of 113 monofunctional catalase sequences has revelaed a subdivision into 3 distinct groups (Sykes and Mauk, 2000). Clade I comtains majorly the plant enzymes with a little involvement from bacteria, Clade II contains only large subunit catalases with bacterial and fungal origin. Clade II includes all the rest with less similarities to others.

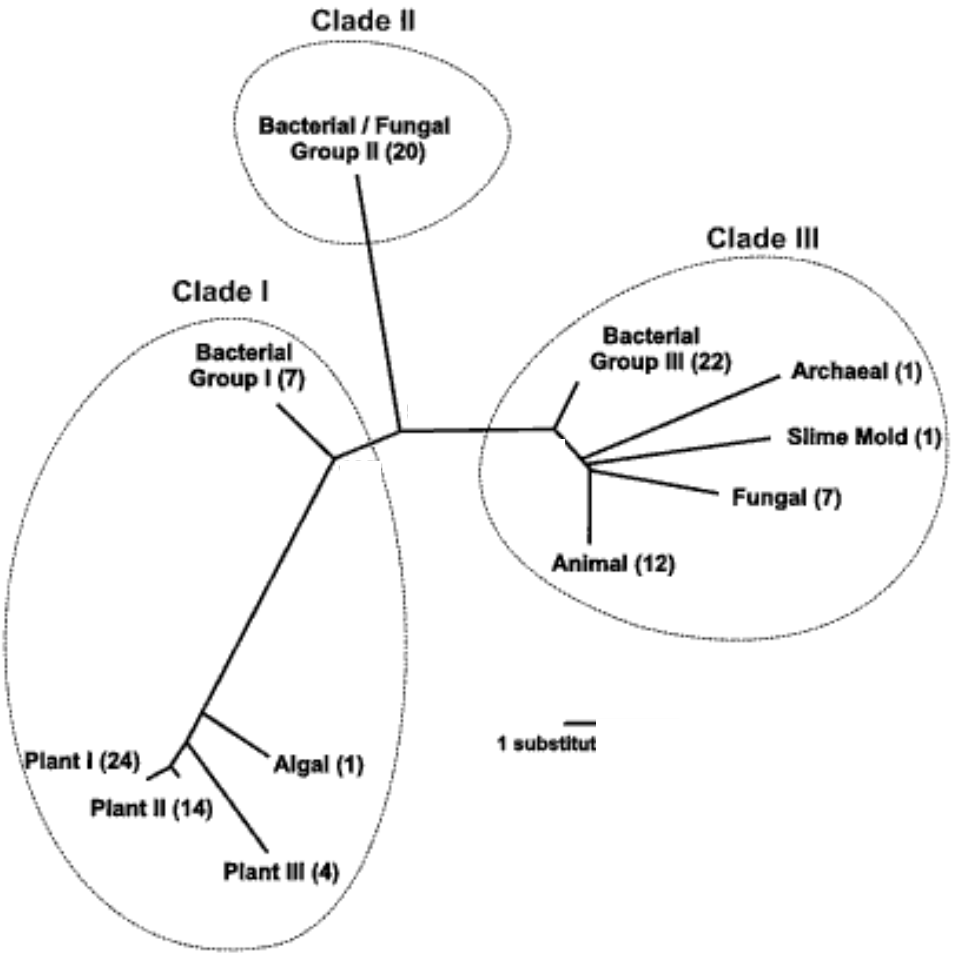


Figure 1. 12 Phylogenetic tree based on the amino acid sequences of 113 catalases.

1.5 Phenol oxidases

Phenoloxidases are oxidoreductases that catalyzeoxidation of phenolic compounds in the presence of molecular oxygen. There are different types of phenol oxidases present in nature and three major groups of these enzymes are laccases, catechol oxidases and tyrosinases.These enzymes catalyzes oxidation of phenols to highly active quinones. Quinones may contribute to the formation of brown pigments due to participation in polymerization and condensation reactions with proteins.

Laccases are widely distributed in plants and fungi. Their functions are related to sexual differentiation, pigmentation of fruiting bodies, lignolysis, detoxification and others. These enzymes act on *p*- and *o*-diphenols, showing more affinity for the first group (Sanchez-Amat and Solano, 1997).

Catechol oxidases are the key enzymes for melanin synthesis, acting on a variety of substituted *o*-diphenols to yield the corresponding *o*-quinones (Fig. 1.13). They have a pair of copper ions at the active site. These enzymes may also show cresolase activity, so that they are able to catalyze the hydroxylation of monophenols to *o*-diphenols. Thus, they catalyze the straightforward formation of *o*-quinones either from monophenols or *o*-diphenols. The molecular and structural differences between catechol oxidases and cresolases are not defined well enough since they are exclusively based on the substrates oxidized. There are enzymes from numerous sources that display both catalytic activities (Sanchez-Amat and Solano, 1997).

Tyrosinase catalyzes the hydroxylation of monophenols to *o*-diphenols (cresolase activity) as shown in Figure 1.13. Tyrosinases are a group of copper proteins that also catalyze the reaction of catechol oxidase, if only 1, 2-benzenediols (*o*-diphenols) are available as substrate. Tyrosinases have putative common ancestor with oxygen binding and oxygen transporting proteins.

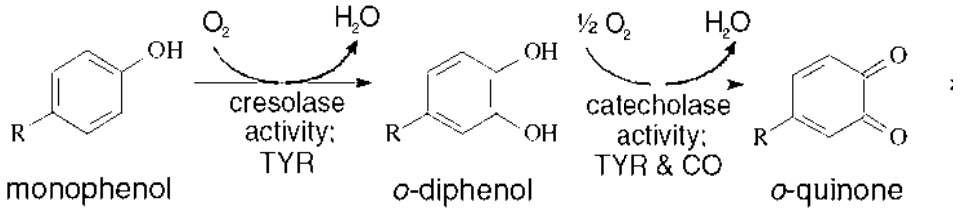


Figure 1. 13 Oxidation of phenolics by tyrosinase and catechol oxidase.

Phenol oxidases are very common in nature, they can be found in almost all plants, animals and microorganisms. Phenol oxidases have been implicated in a range of roles, one of which is in mycelial morphogenesis where the fungal mycelium undergoes changes in response to the presence of another fungus.

Laccase isoenzymes can vary between and within species, and can also have different functions within these species and under different environmental conditions. Tyrosinase is involved in the biosynthesis of melanins and other polyphenolic compounds in bacteria, fungi, plants and animals. All of these enzymes may have some role to play in the offence and defense of fungi during interactions (Score *et al.*, 1997).

Phenol oxidases have very wide substrate range and final oxidation products of these substrates are quinones, which are highly reactive molecules and polymerize into brown, red or black water-insoluble compounds (Durán and Esposito, 2000).

Phenols, substrates of phenol oxidases, are compounds having hydroxyl group attached to the benzene ring and are generally named as derivatives of the simplest member of the family, phenol. However, phenolics can be very large in size, such as condensed tannins (Tomás-Barberán and Espin, 2001). Phenolics are present in wastes from several industrial processes, as coal conversion, olive oil production, petroleum refining, paper and pulp production, production of organic chemicals.

Polyphenol oxidation induced browning in foods are usually referred with its detrimental influence. Some enzymatic browning reactions are nevertheless very beneficial to the overall acceptability of foods. Black, oolong and green tea are reliant on enzymatic browning for color and flavor development. Coffee, cocoa, dried prunes, dates and figs can also be added to the list.

1.6 The thermophilic fungus *Scytalidium thermophilum*

Thermophilic fungi from the *Torula-Humicola* complex are important in compost selectivity (Straatsma *et al*. 1989, Op den Camp *et al*. 1990), and are the dominant community inside the compost.

Table 1. 2 Taxonomy of the species fromwww.mycobank.org

Non abbreviated authority:	(Cooney and Emerson) Austwick
Bibliography:	Austwick, P.K.C., 1976, New Zealand Z. J. Agric. Res. 19:29.
Year of publication:	1976
Status of the name:	combination; legitimate
Classification:	1. Fungi(Anamorphic) 2. Ascomycota 3. Pezizomycotina 4. Leotiomycetes 5. Leotiomycetidae 6. Helotiales 7. *Scytalidium*
Basionym:	*Torula thermophila*(Cooney &Emerson 1964)
Morphic status:	Anamorph
Taxonomic synonym:	None

Scytalidium thermophilum is the climax species in mushroom compost and is the exclusive species at the end of the process. Cooney and Emerson (1964), reported that a divergence existed between isolates of the *Torula* and *Humicola* and their assignment into a species (Table1.2) since been accepted (Austwick 1976).

Scytalidium thermophilum(Fig. 1.14 and 1.15), also known as *Humicola insolens*, *Torula thermophila* and *Humicola grisea* var. *thermoida,* is a thermophilic fungus. It belongs to *Deuteromycetes* class having no sexual state in their life cycle. *S. thermophilum* plays an important role in determining selectivity of compost produced for growing *Agaricus bisporus*. Large numbers of isolates assigned to *Scytalidium thermophilum* are examined by Straatsma and Samson in 1993, which are *Torula thermophila*, *Humicola insolens* and *Humicola grisea var. thermoidea* as members of the Torula-Humicola complex (Emerson 1968, Ellis and Griffiths 1976).

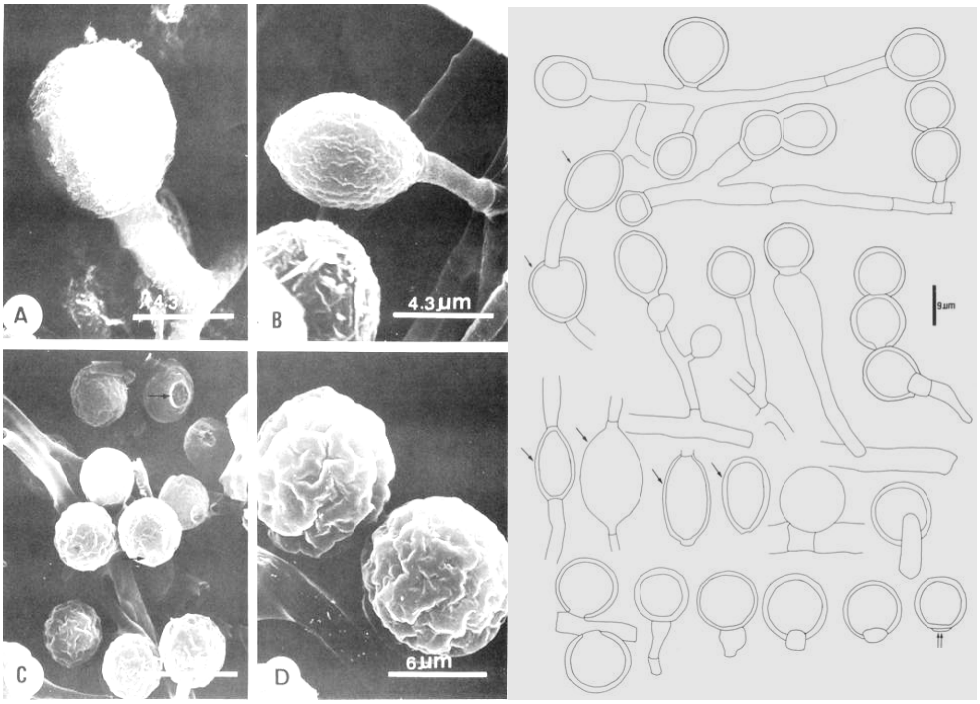


Figure 1. 14*Humicola insolens* (Cooney & Emerson) var. thermoidea (Cooney & Emerson) Ellis, Trans. Br. mycol. Soc. 78: 129-139. 1982.

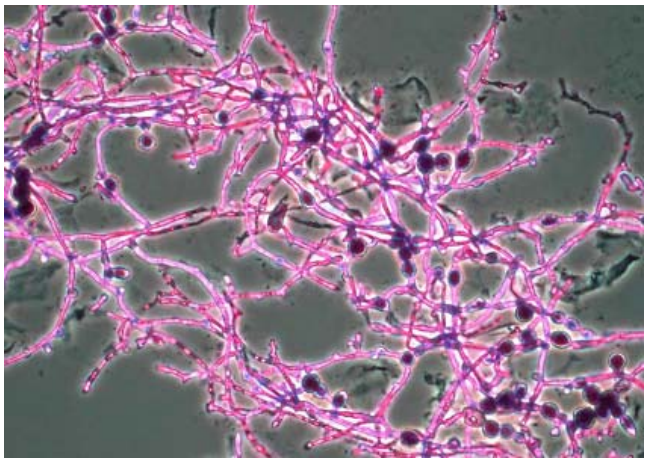


Figure 1. 15 Electron microscobe image of *S. thermophilum*hypae taken by, Corinne Darmond, Concordia University, Canada (March 2011).

Torula thermophila has single very dark spores borne on short lateral hyphal branches and were designated. The mature spores of *Humicola insolens* (Fig. 1.16) are smooth, colorless at first, turning light brown, variable in shape, generally globose (Cooney and Emerson, 1964).

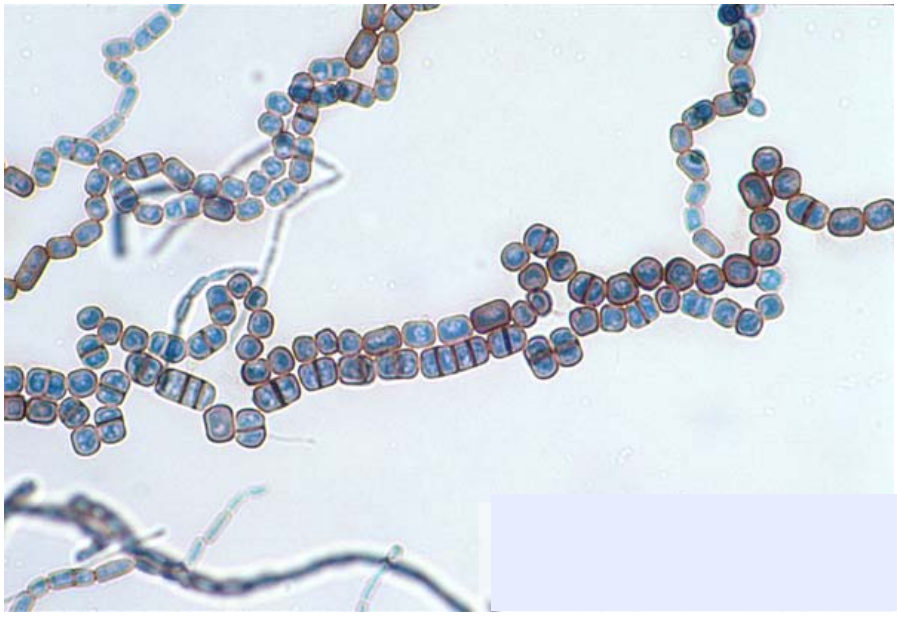


Figure 1. 16 Spore structures of *S. thermophilum*(www.drfungus.com).

1.7 *Scytalidium thermophilum* catalase phenol oxidase (CATPO)

In our laboratory group, *Scytalidium thermophilum* extracellular enzymes has been studied for more than a decade. The enzymes of this thermophilic fungus is valuable for the industrial use in two means, the ability of enzymes to work at high temperatures and the ability of fungi to grow on various lignocellulosic compounds (Ögel *et. al.,*2001, İfrij and Ögel, 2002). The phenol oxidase gained the scientific attraction because *S. thermophilum* produces immense amounts of melanin and melanin and other pigment production is mostly associated by phenol oxidases (Kaptan, 2004). Production and partial characterization of *S. thermophilum* phenol oxidase has been studied by Mete, S. (2003), a member of Ögel laboratory also. In the PhD study of Sutay (2007), the enzyme was characterized as a bifunctional enzyme possesing catalase activity together with phenol oxidase. The enzyme was renamed as catalase phenol oxidase from that point on. The study revealed that the enzyme was a tetramer of 320 kDa, with monomer molecular weight of 80 kDa. The isoelectric point was reported as 5.0 (Kocabas *et. al.,*2008). A latter study conducted by Avcı (2012) shed light to the oxidation products of this bifunctional enzyme. 14 phenolics were tested to be the putative substrates of *S. thermophilum* catalase phenol oxidase, and only four of them, namely, catechol, chlorogenic acid, caffeic acid and catechin were successfully analyzed using HPLC and LC-MS. It can be easily deduced from this study that the enzyme appears to have specificity over ortho diphenolics.

1.8 Aim of the study

This work represents an overview of enzymatic reactivity against CATPO over selected phenolic compounds and both black and green tea, and how oxidation affects their antioxidant capacities. The previous studies suggest that the enzyme may be playing a very important role in antioxidant defense mechanism of the fungus. It also promises the utilization of the valuable enzyme in certain areas of food industry, especially tea production. In this thesis, it was aimed to discover the effect of CATPO oxidation on selected phenolic compounds and the effect of CATPO oxidation on tea phenolics.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and enzymes

All phenolic compounds used as substrate were obtained from Sigma-Aldrich (Germany) or Merck (Germany). All organic solvents used were of analytical grade and obtained from Sigma, Fluka or AppliChem.

ABTS (2,2'-azino-bis(3-ethyl benzothiazoline-6-sulphonic acid)), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), sodium acetate trihydrate ($CH_3COONa.3H_2O$), ascorbic acid, ferric chloride hexahydrate ($FeCl_3.6H_2O$) and potassium persulfate ($K_2S_2O_8$) were obtained from Sigma or Merck.

All further chemicals, as well as the *Agaricus bisporus* tyrosinase and *Trametes versicolor* laccase were purchased from Sigma-Aldrich.

2.1.2 Fungal strain

The *Scytalidium thermophilum* (type culture *Humicola insolens*) industrial strain was provided by ORBA Inc., İstanbul (Simbiyotek Biyolojik Ürünler San. Tic. A.Ş.). *Scytalidium thermophilum* (type culture *Torula thermophila*) was isolated from mushroom compost (Ögel et al., 1998).

2.2 Methods

2.2.1 Storage, Maintenance and Growth of Microorganisms

Scytalidium thermophilum was cultivated on YpSs (Yeast-Starch) agar (constituents listed in Appendix A) plates and incubated at 45ºC for 4-5 days until complete sporulation was observed. Then, those cultures were stored at room temperature for maximum 2 months to utilize as stock cultures (Arifoğlu and Ögel, 2000).

For optimum enzyme production pre-cultivation and cultivation were carried out at 45°C according to the procedure described by Sutay (2007). At the end of the fifth day of incubation, 1 L fungal culture was harvested; filtered through filter paper (Whatman, Grade 1: 11 µm). The filtrate was centrifuged at 6000 rpm for 15 minutes to obtain the supernatant used as crude enzyme solution.

2.2.2 Enzyme assays

2.2.2.1 Phenol oxidase assay

Extracellular phenol oxidase activity measurements were determined using a SHIMADZU UV-1700 PharmaSpec temperature controlled spectrophotometer set to 60°C. Enzyme activity is followed by examining the increase in absorbance at 420 nm of the reaction mixture. The reaction mixture consisted of 0.5 mL 100 mM catechol solution in 100 mM phosphate buffer (pH 7.0) as substrate, 0.5 mL enzyme solution and 1 mL 100 mM phosphate buffer at pH 7.0. The substrate solution was prepared fresh in preincubated buffer and used directly to minimize the effects of rapid autooxidation of catechol with molecular oxygen in the air. Both buffer and enzyme solutions were preincubated for 5 minutes at 60°C. All activity measurements were contrasted to an enzyme blank sample, where 0.5 mL 100 mM phosphate buffer (pH 7.0) was added instead of enzyme solution.

Enzyme activity was determined by the initial rate of the reaction and the extinction coefficient (ε) as 3450 M^{-1} cm^{-1} for catechol (Ögel *et al*., 2006). One enzyme unit was defined as the amount of enzyme required for the formation of one nanomole of product/minute. Phenol oxidase activity was calculated by the following formula:

$$\text{Enzyme Activity } (\frac{\mathrm{U}}{\mathrm{L}}) = (\frac{\Delta \mathrm{OD}}{\Delta \mathrm{t}}) \,.\, (\frac{1}{\varepsilon})\, (10^6) \,.\, (4) \,.\, (2)$$

U: unit

ΔOD: change in optical density (in terms of absorbance)

Δt: change in time

ε: extinction coefficient

2.2.2.2 Catalase assay

Catalase activity was measured by the method of Beers and Sizer (1952) in a 50 mM potassium phosphate solution buffer. The substrate was prepared by adding 10,8 µL of a freshly prepared 30% H_2O_2 solution to 10 mL buffer followed by vortexing yielding a final concentration of 0.32 g H_2O_2 per liter solution. 1.5 mL of substrate solution was taken and its optical density was followed in an UV spectrophotometer (SHIMADZU UV-1700) at 240 nm wavelength. Catalase activitiy was expressed in units per mL of culture medium (U/mL) which is similar to 1 µmole of substrate oxidized in one minute by 1 mL of culture supernatant and calculated by the equation:

$$\text{Enzyme Activity } (\frac{\mathrm{U}}{\mathrm{mL}}) = (\frac{\Delta \mathrm{OD}}{\Delta \mathrm{t}}) \,.\, (\frac{1}{\varepsilon}) \,.\, (10^3)\, (\frac{Vk}{Vs}) \,.\, (\text{Dilution factor})$$

V_k: total volume of the solution in cuvette (mL)

V_s: volume of the crude enzyme in cuvette (mL)

ΔOD: change in optical density (in terms of absorbance)

Δt: change in time

The extinction coefficient (ε) of H_2O_2 is 39.4 M^{-1} cm^{-1}.

2.2.3 CATPO purification

Enzyme purification was performed with a Varian ProStar FPLC system, following the two step purification procedure described by Sutay (2007) with slight modifications for the run times and elutions. Crude CATPO solution was prepared according to the steps in section 2.2.1. The pH of the solution was adjusted to 8.0 by adding 1 M Tris-HCl buffer and filtrated through a 0.22 µm-pore-size Durapore membrane (Millipore). The crude enzyme solution was stored up to one month at -80°C until the purification procedure. Catalase and phenol oxidase acitivities were measured before purification and total protein concentration data was collected in each step.

2.2.3.1 Ion exchange chromatography

For anion exchange chromatography a 20 mL prepacked HiPrep 16/60 Q XL column (GE Life Sicences, USA) was used under 15 psi. The column was operated with a 50 mM pH 8.0 Tris-HCl buffer at 1.2 mL/min flow rate. Enzyme was eluted by collecting 3 mL fractions with a salt gradient in the range of 0-1 M NaCl, prepared in flow-through buffer. The wavelength was set to 280 nm and the analysis time was 165 minutes. All fractions were examined for both catalase and phenol oxidase activities. CATPO active fractions of anion exchange were kept for further use and stored at room temperature.

2.2.3.2 Gel filtration chromatography

The semi purified protein elutions from the previous step were first 5 to 10 times concentrated by Amicon Ultra-15 Centrifugal Filter Units (Millipore, USA). Gel filtration chromatography was conducted in a prepacked HiPrep 16/60 Sephacryl S-100 high resolution gel filtration column (GE Life Sicences). The column was equilibriated with 50 mM pH 8.0 Tris-HCl buffer and operated at a flow rate of 0.5 mL/min by collecting 5 mL fractions of a total 175 mL elution volume. Similar to the anion exchange procedure (sec. 2.2.3.1), each fraction was tested for both catalase and phenol oxidase activities. All solutions used for purification studies were filtered through 0.45 µm-pore-size membrane (Millipore, USA) before use.

2.2.3.3 Total protein determination by Bradford assay

Bradford reagent was prepared by dissolving 300 mg Coomassie Brilliant Blue in 150 mL 95% ethanol and adding 300 mL 85% (w/v) phosphoric acid to it (Bradford, 1976). Then 15 mL of this stock solution was diluted to 100 mL with water and filtered before use. The Bradford reagent was light brown in color. Bovine serum albumin (BSA) was used as the protein standard. 1 mg/mL stock BSA solution was prepared by dissolving 5 mg BSA in 5 mL of 100 mM pH 7.0 sodium phosphate buffer. To measure the protein content, 20 µL appropriately diluted BSA solution, 80 µL water, and 1 mL of Bradford reagent were mixed. Absorbance at 595 nm was measured after 10 minutes using a spectrophotometer to obtain a BSA standard curve.

2.2.4 Oxidation of phenolics

2.2.4.1 Reaction set-up with crude enzyme

Oxidation products of a number of phenolic substrates were examined by dissolving the substrates in ethanol and mixing with culture supernatants to the order of magnitude determined to be optimum after several trials.

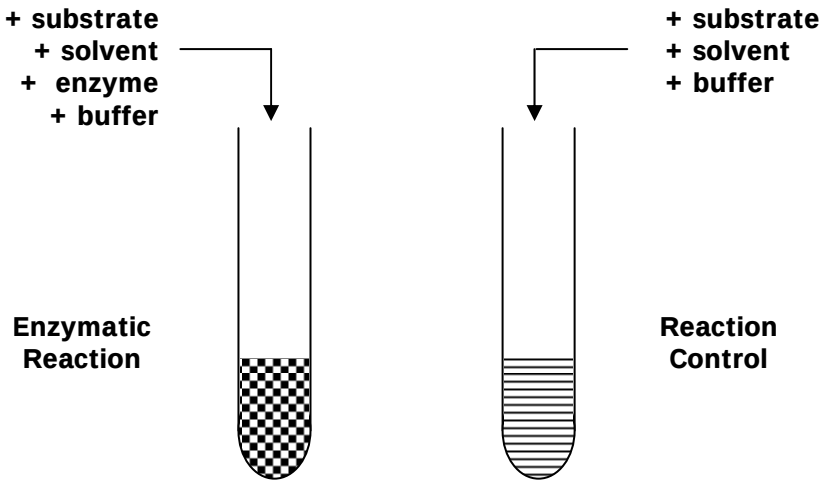


Figure 2. 1 Demonstration of reaction set-up for bioconversions.

A number of phenolic substrates (catechol, hyroquinone, resorcinol, caffeic acid, chlorogenic acid, L-dopa, quercetin, catechin, gallic acid and phenyllactic acid (chemical structures given in Appendix D)) with a concentration range between 0.5 mM to 100 mM for different conditions, 2 mL ethanol, 2 mL supernatant with an average phenol oxidase activity of 25 U/mL and 1 mL phosphate buffer pH 7.0; constituted the reaction mixture where in control tubes buffer was added instead of crude enzyme. All reactions took place at 60°C (Fig. 2.1).

Product formation was analyzed by thin layer chromatography (TLC) at 1 h, 4 h, 10 h, 24 h and 48 h after incubation. Preliminary color changes were scored visually and using an UV-VIS spectrophotometer. Either precipitation or extraction of the products from the reactions set-up was immediately carried out after terminating each reaction with liquid nitrogen to analyze the product by Fourier transform infrared spectroscopy (FTIR).

2.2.4.2 Reaction set-up with pure enzyme

Pure CATPO was obtained using the protocol described in section 2.2.3. and stored at -80°C until use. As activity measured after gel filtration step was 30 U/mL, 500 µL enzyme solution was used to get 15 unit enzyme for all reactions. 0.1mM, 1mM, 10mM phenolic compound concentrations were dissolved in 2 mL methanol, 2.5 mL phosphate buffer pH 7.0. Enzymatic reactions took place at 60°C and were terminated after 1 hour. Antioxidant capacity measurements were performed afterwards using these reactions.

2.2.4.3 Reaction set-up with laccase

Trametes versicolor laccase was used to set up oxidation reactions for the antioxidant activity assays. 10 unit enzyme was weighed and added to 2 mL methanol dissolved 0.1/1/10 mM phenolic substance solution. 3 mL 100 mM sodium acetate buffer (pH = 4.8) was added also. The reactions were carried out for an hour in 30°C water bath.

2.2.4.4 Reaction set-up with tyrosinase

10 unit *Agaricus bisporus* tyrosinase was utilized for the reactions set up during this stage. Similar to the reaction with laccase 2 mL methanol, 01./1/10 mM phenolic substance and 3 mL 100 mM pH 6.5 potassium citrate buffer were added into the reaction tube and waited for 60 minutes at 25°C.

2.2.5 Thin layer chromatography (TLC)

TLC silica gel 60 plates (Merck) were cut to 10 × 20 cm slices. Butanol : acetic acid : water in 12:3:5 (v/v) ratio was used as runner solvent. 5 µL aliquots of the oxidation reaction and their control were spotted on TLC plates at 1 cm above the bottom. The chromatograms were developed (Fig. 2.2) at room temperature for about an hour, air-dried and spots were detected under UV light.

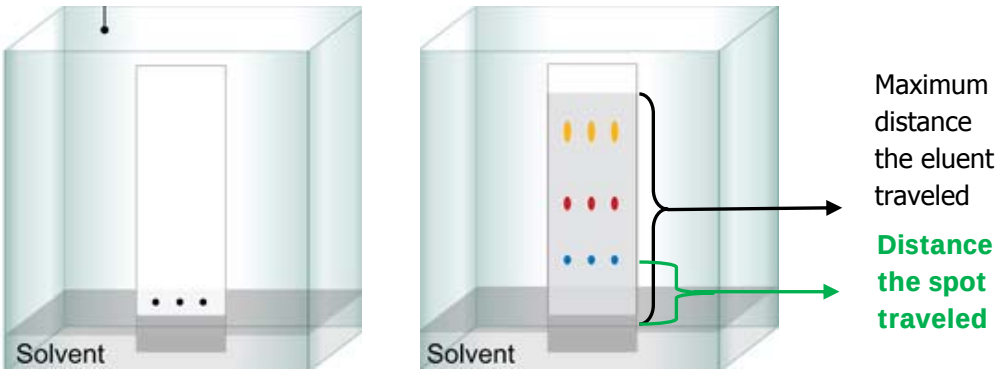


Figure 2 .2 Schematic description of the TLC method.

2.2.6 Fourier transform infrared spectroscopy (FTIR)

A) Sample Preparation

After oxidation reaction termination, the reaction and control tubes were stored overnight at 4°C. 15 mL falcon tubes containing the reactions were centrifuged at 6000 rpm for 20 minutes and the supernatant was separated from solid precipitate.

To remove residual nonpolymerized substrate the pellet was dissolved in 2 mL 50 : 50 distilled water : ethanol followed by centrifugation at 13 000 rpm for 15 minutes. The supernatant was discarded and the liquid remnants were evaporated in SpeedVac (Eppendorf, Germany). The precipitate was left drying at 45°C for 2 days. 0.2 g dried KBr was thoroughly mixed with 1 mg sample with a mortar and pestle (sample to KBr ratio; 1:200). The fine powder was then pressed with a hydraulic press (Shimadzu, Japan) up to 6 bar to obtain transparent pellets.

B) FTIR Data Analysis

Bruker Equinox 55 (Bruker Optik GmbH, Germany) operated at room temperature was used to get FTIR spectra as transmittance in between the 4000 – 400 cm^{-1} region (Fig. 2.3). OPUS software was used to analyze the spectra. IR spectrum of air and KBr transparent disk were recorded together as background and were substracted automatically. A total of 120 scans were taken for each interferogram at 4 cm^{-1} resolution for each sample.

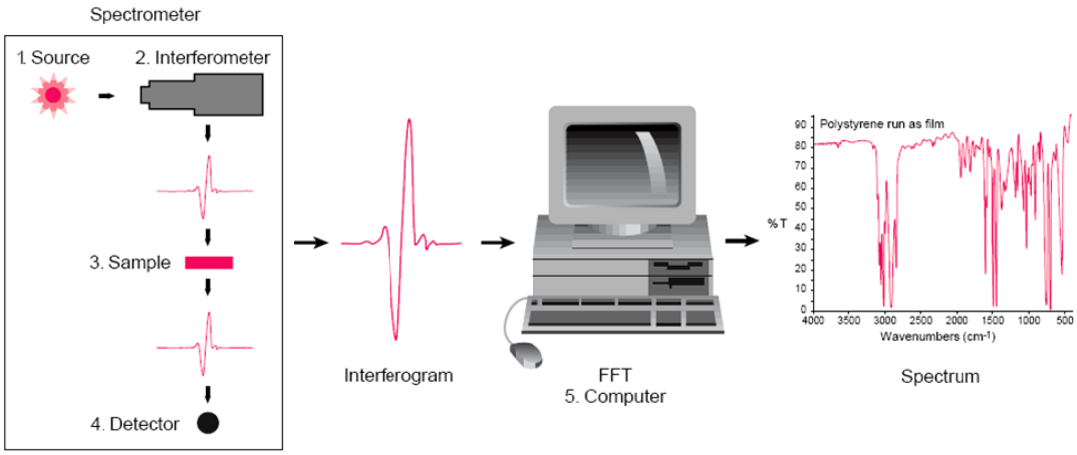


Figure 2. 3 The schematic drawing showing the basis of FTIR process.

2.2.7 Antioxidant capacity measurements

2.2.7.1 TEAC (Trolox equivalent antioxidant capacity assay)

The Trolox Equivalent Antioxidant Capacity (TEAC) assay is based on the ability of antioxidant molecules to quench the long-lived ABTS, a blue-green chromophore with characteristic absorption at 734 nm, compared with that of Trolox, a water soluble vitamin E analog (Fig. 2.4).

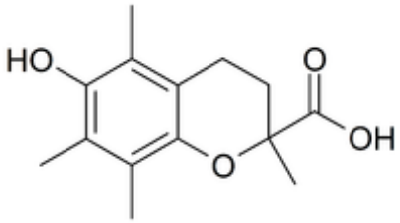


Figure 2. 4 Chemical structure of trolox

The addition of antioxidants to the preformed radical cation ($ABTS^{\cdot+}$) reduces it to ABTS, instigating a decolorization. In this study, the standard TEAC assay (Re et al., 1999) was been used with minor modifications for determination of the TEAC value. A stable stock solution of ABTS was made by reacting a 7 mmol/L aqueous solution of ABTS with 2.45 mmol/L potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The concentrated $ABTS^{\cdot+}$ solution was diluted with ethanol to a final absorbance of 0.70±0.02 at 734 nm at room temperature. A reaction mixture containing ABTS and Trolox (concentration range, 0–50 μmol/L) was incubated for 4 minutes, 6 minutes, or 10 minutes and subjected to spectrophotometric measurements. 10 μL of each sample prepared at different concentrations was mixed with were 1000 μL $ABTS^{\cdot+}$ solution and the absorbance was read at 734 nm. This was compared to a blank where 10 μL of the solvent was added to 1000 μL of the $ABTS^{\cdot+}$ solution. The reduction in absorbance 6 minutes after addition of the antioxidant was determined. The TEAC of the antioxidant was calculated by relating this decrease in absorbance to that of trolox calibration curve.

2.2.7.2 FRAP (Ferric reducing antioxidant power assay)

FRAP assay utilized antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue colour) was monitored by measuring the change in absorption at 593nm. The reaction was non specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, has driven the ferrous (Fe III —› Fe II) ion formation. The change in absorbance was therefore, directly related to the total reducing power of the electron donating antioxidants present in the reaction mixture. 3 mL working FRAP reagent [(a) Acetate buffer 300 mM pH 3.6 was prepared by weighing 3.1 g sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$) and adding 16 mL of glacial acetic acid to make the volume to 1 L with distilled water. b) TPTZ (2, 4, 6-tripyridyl-*s*- triazine) (MW 312.34) 10 mM in 40mM HCl (MW 36.46) c) $FeCl_3 \cdot 6H_2O$ (MW 270.30) 20 mM. The working FRAP reagent was prepared by mixing a, b and c in the ratio of 10 : 1 : 1 at the time of use)] was mixed with 100 µL sample and absorbance at 593 nm was measured at 0 minute after thorough vortexing. Thereafter, samples were placed at 37°C in water bath and absorption is again measured after 4 minutes. The standard curve was constructed using $FeSO_4$ solution, and the results were expressed as mmol Fe(II)/g dry weight . 1 mM $FeSO_4$ stock solution was prepared by weighing 0.139 g $FeSO_4 \cdot 7H_2O$ into 500 mL distilled water. While generating the calibration curve 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM dilutions of the $FeSO_4$ stock solutions were run through the spectrophotometer.

2.2.8 Preparation of Tea Samples

Fresh tea leaves and samples from each production stage were kindly provided by Doğuş Çay (Fig. 2.5). The tea leaves were harvested in mid June 2012 from Rize. All oxidation reactions were set up immediately after receiving the samples avoiding storage and the loss of primary properties of *Camelia sinensis*.

All tea samples were prepared using a ratio of 1 g per 100 mL boiling water and infused for a certain amount of time according to the nature of the study to ensure standardization. Infusions were prepared by adding 2 g of dry tea to 200 mL boiling deionised water (90 ± 5°C). The resulting infusion was stirred with a glass rod for about 30 s to ensure proper wetting, covered and steeped for 3, 5 and 10 minutes for different brewing times. The steeped infusion was then immediately filtered through Whatman fitler paper to remove contact with the tea leaves and allowed to cool to 60 °C. Thereafter, the cooled solution was analysed for its antioxidant capacity value using the procedure outlined above (Section 2.2.7.1).

Figure 2. 5 Tea samples delivered freshly from production area (a)harvested fresh tea leaves, b) after bruising, c) before fermentation, d) after fermentation, e) after drying, f) remaining waste).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Experimental Strategy

In this thesis; the oxidation products of *Scytalidium thermophilum* catalase phenol oxidase (CATPO) were analyzed (Figure 3.1). First crude enzyme was used in a quick screening method of tracking the color changes and results were compared with those of *Trametetes versicolor* laccase and *Agaricus bisporus* tyrosinase to find out the observational differences after oxidation for different substrates. The substrate list was limited to 12 different phenolic compounds that were chosen according to the abundance in food matrices and being a putative substrate for CATPO. These were catechol, hydroquinone, resorcinol, chlorogenic acid, coumaric acid, vanillic acid, caffeic acid, phenyllactic acid, gallic acid, L-dopa, quercetin and catechin. After revealing the putative phenolic compounds to be eligible to work with CATPO, the reactions were set up with the crude enzyme.

Next, Thin Layer Chromatograms were obtained using crude CATPO. TLC allowed more specific observation of putative products of the oxidation reactions. Following TLC; FTIR experiments were conducted to explore the bonds of the oligomers likely to be formed after the oxidation of certain phenolics by CATPO.

Following these initial screening studies, in our lab, (Avcı, 2011), the oxidation products of CATPO were studied by both HPLC and LC-MS analysis. CATPO resulted in distinct oxidation products only with catechol, chlorogenic acid, catechin and caffeic acid.

Of these four substrates laccase was unable to oxidize chlorogenic acid reaction; and tyrosinase oxidized only catechol and catechin. Based on those studies, these four phenolic compounds (catechol, chlorogenic acid, catechin and caffeic acid) were chosen to be the target substrates to be utilized in antioxidant capacity experiments. Two well known methods (TEAC and FRAP) were used for determining antioxidant capacities of both pure compounds and their oxidation products, and results were compared with laccase and tyrosinase CATPO gave the most significant increase for the oxidized products among the tested enzymes; catechol being the lead molecule with the 2.4 fold increase in its antioxidant potential after *S. thermophilum* catalase phenol oxidase catalysis.

Finally, the influence of CATPO on the antioxidant capacity of tea samples was investigated, to analyse its effect on phenols, polyphenols and flavonols in tea. Research was both conducted using tea in bags, as well as with samples obtained from each step of a tea production line. Tea samples were treated with the enzyme and the initial and final antioxidant capacity values were recorded. There were an increase for each type of tea sample differing for different steps of production line. The results will shed light on the possible usage of CATPO either immobilized, or direct addition before consumption of tea. The future studies may cover whether it is feasible to employ the extracellular enzyme of GRAS (generally recognized as safe) microorganism *Scytalidium thermophilum* (Kahl, FDA Report, 2006) in hot or cold served tea and tea products.

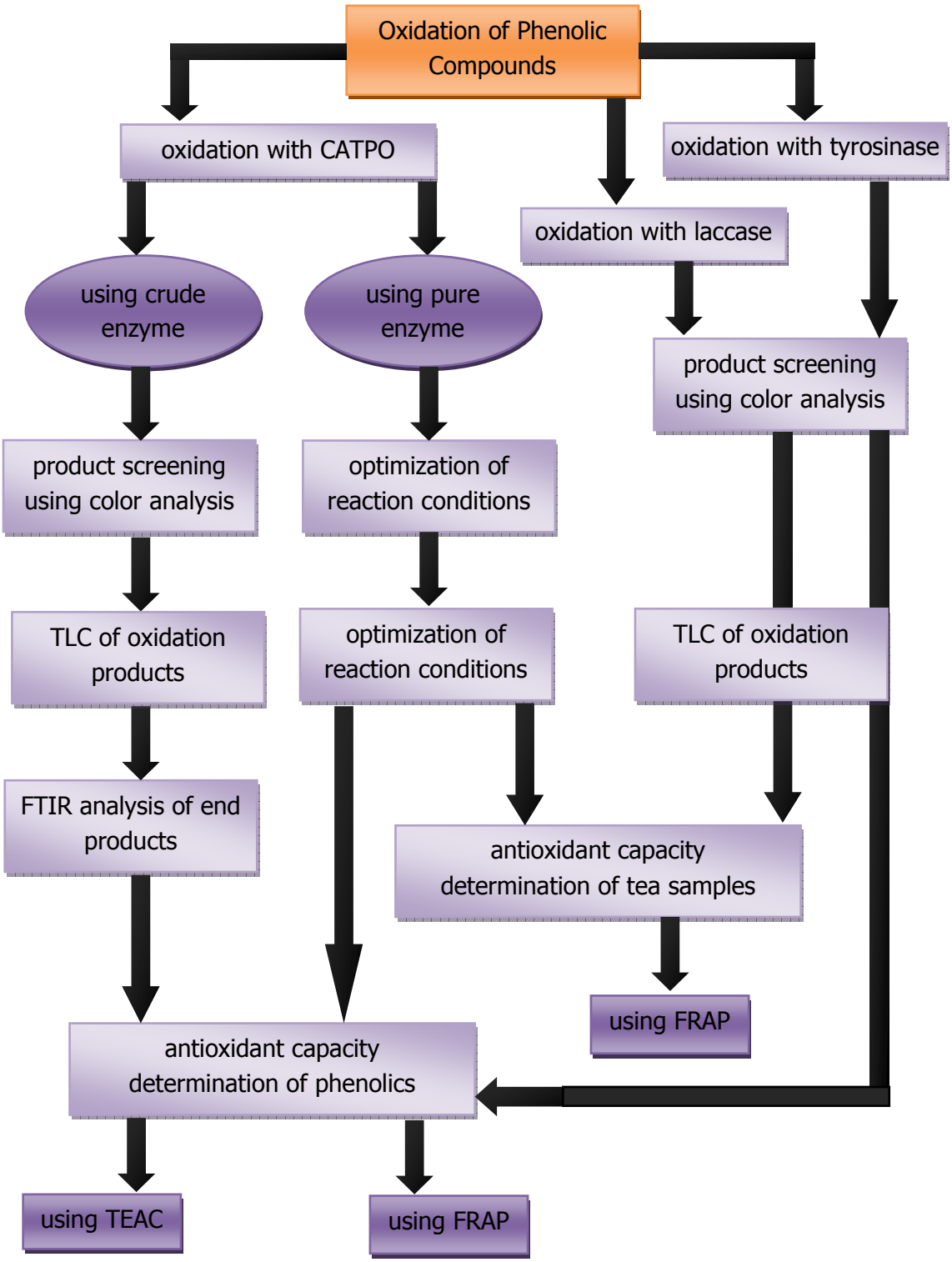


Figure 3. 1 A scheme describing the experimental strategy followed.

3.2 Preliminary Screening of Phenol Oxidation by Using Crude CATPO

3.2.1 Observations Based on Color Changes

Oxidation of phenolic compounds results first in the formation of reactive free radicals and quinones and this is followed by spontaneous chemical reactions, resulting in the formation of polymeric pigments, such as the formation of melanin (Land *et. al.*, 2004). Here, pigmentation upon oxidation, was used as a preliminary screening method for the analysis of phenol oxidation by crude CATPO. Crude enzyme was prepared as described in section 2.2.1, and the reaction was prepared by the method described in section 2.2.4.1. However, prior to colorimetric observations with crude CATPO, it was of interest to set up control experiments first with laccase from *Trametes versicolor* and tyrosinase from *Agaricus bisporus*. These are the two well-known phenol oxidases in the literature. Results were later used for the sake of comparison.

3.2.1.1 Observations Based on Color Changes with *Trametes versicolor* Laccase and *Agaricus bisporus* Tyrosinase

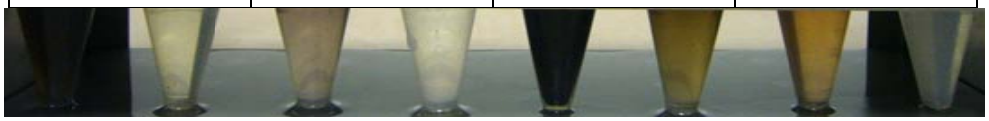
There are numerous studies that have reported the substrates of *Trametes versicolor* laccase (E.C. 1.10.3.2) and *Agaricus bisporus* tyrosinase (E.C. 1.14.18.1).

L-dopa and L-tyrosine are the natural substrates of mushroom tyrosinase (Xie *et. al.,* 2007, Takahashi and Miyazawa, 2010). Selinheimo (2007) and his group compared plant tyrosinases with the activity of commercially available *A. bisporus* tyrosinase. They have shown that *A. bisporus* tyrosinase is capable of effectively reacting with catechol, epicatechin, catechin, caffeic acid, L-dopa, and is unable to oxidize ferulic acid and coumaric acid. In a later study (2009) again Selinheimo and his friends conducted experiments to compare *Trichoderma reseii* tyrosinase substrates with *A. bisporus* tyrosinase subtrates.

T. versicolor laccase is an ezyme widely studied for its certain properties such as substrate specificities. In numerous articles (Rogalski *et. al.*, 1991, Sakurai and Kataoka, 2007, De Leonardis *et. al.*,2010,) different compounds were tested as possible substrates of this diphenol oxidase. Catechol, hydroquinone, resorcinol, caffeic acid, ferulic acid and vanillic acid were identified as being oxidized by *T. versicolor* laccase. On the other hand, a colleague from our laboratory has found that both enzymes oxidize catechol and catechin, but did not oxidize chlorogenic acid; whereas caffeic acid was only oxidized by laccase (Avcı, 2011).

The reactions were set up with 10 mM phenolic substance, 4 mL ethanol, 4 mL buffer and 2 mL either laccase or tyrosinase solution and for control, 2 ml buffer was added instead of enzyme solution. Color changes were observed at 1 hour, 4 hours, 12 hours and 48 hours. Results of 48 h reactions are shown in Figures 3.2, 3.3 and 3.4. Distinct color changes are marked as (+)ve.

+	-	+	+

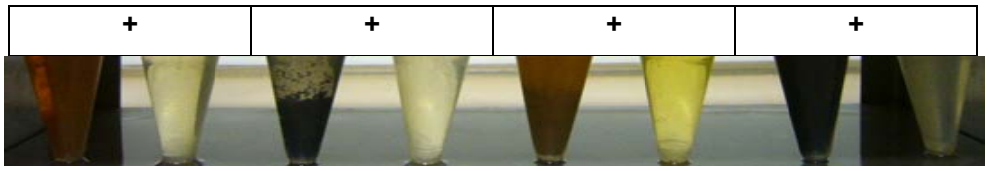


1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Cat	Cat contr	Hydroq	Hydroq contr	Cat	Cat contr	Hydroq	Hydroq contr
TYR		TYR		LAC		LAC	

Figure 3. 2 Oxidation of catechol (Cat) and hydroquinone (Hydroq) with tyrosinase (TYR) and laccase (LAC), 48 hr, 25°C, pH 6.5 for tyrosinase, 48 hr, 30°C, pH 4.8 for laccase

According to the results catechol was immediately oxidized, resulting in a distinct brown color even after 30 minutes of enzyme addition to the reaction mixture. This treat is common both for tyrosinase and laccase.

Mushroom tyrosinase and *A. bisporus* laccase can readily oxidize catechol, but only the latter one can oxidize hydroquinone. The two isomers catechol and hyroquinone gave different results for the preliminary screening (Figure 3.2). It was expected to come across with such a difference. Tyrosinase as the enzyme which catalyzes oxidation of monophenols to *o*-dihydroxyphenols and further to *o*-quinones, was found to oxidize catechol but not hydroquinone under different conditions for different purposes (a database approach and a comprehensive list is given under the heading *A. bisporus* tyrosinase www.brenda-enzymes.info). Laccases are welll known for their substrate diversity. In accord with what was published in the literature, the quick screening of color change brought out that ortho and para benzenediols are readily oxidizable by laccase.



1	2	3	4	1	2	3	4
Catech	Catech contr	L-dopa	L-dopa contr	Catech	Catech contr	L-dopa	L-dopa contr
TYR		TYR		LAC		LAC	

Figure 3. 3 Oxidation of catechin (catech) and L-dopa with tyrosinase (TYR) and laccase (LAC) 48 hr, 25°C, pH 6.5 for tyrosinase, 48 hr, 30°C, pH 4.8 for laccase.

As shown in Figure 3.3, tyrosinase oxidizes its natural substrate L-dopa and forms a distinct insoluble black precipitates after 48 hr reaction time. In fact, a distinct precipitate was observed both for catechin and L-dopa and for both enzymes. Autooxidation of phenolic substrates was not significant.

+	+	+	+

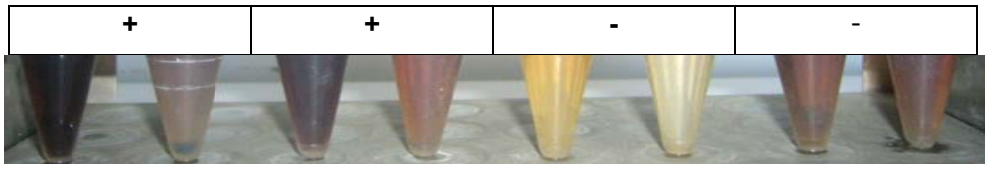
1	2	3	4	1	2	3	4
Caffeic	Caffeic contr	Gallic	Gallic contr	Caffeic	Caffeic contr	Gallic	Gallic contr
TYR		TYR		LAC		LAC	

Figure 3. 4 Oxidation of caffeic acid and gallic acid with tyrosinase (TYR) and laccase (LAC) 48 hr, 25°C, pH 6.5 for tyrosinase, 48 hr, 30°C, pH 4.8 for laccase.

Caffeic acid is phenolic compound with an *o*-diphenolic nature, and can be oxidized by tyrosinase (Deng *et. al.*, 2008, Gasparetti *et. al.*, 2012), in contrast to caffeic acid; gallic acid has been identified as a tyrosinase inhibitor and the mechanism has been well studied by Kubo *et al.* (2000). In the presence of gallic acid, L-dopa oxidation by tyrosinase was inhibited (Chang, 2009). However, gallic acid was oxidized and yielded the colored oxidation products, same as in our observations. Therefore, gallic acid itself, can also be referred to as as alternate substrate for tyrosinase under optimal conditions, like pH, temperature, oxygen amount and phenolic acid concentration with respect to the amount of enzyme provided.

3.2.1.2 Observations of Color Changes with Crude CATPO

The crude CATPO preparation was obtained by filtering the culture supernatant of *S. thermophilum* from the 5th day of incubation. Next, 10mM phenolic substance, 4 mL ethanol, 4 mL buffer was mixed with 2 mL of the crude enzyme. For control, 2 ml buffer was added instead of enzyme solution, and the reaction tubes were immersed in 60 °C water bath and remained there for 48 hr. The color change was recorded visually at the first hour, 4 hours, 12 hours and 48 hours. Results of 48 hours are presented in Figure 3.5, 3.6 and 3.8. Those resulting in oxidation due to crude enzyme are marked as (+)ve.



1	2	3	4	5	6	7	8
Cat	Cat contr	Hydroq	Hydroq contr	Res	Res contr	Chloro	Chloro contr

Figure 3. 5 Oxidation of catechol (Cat), hydroquinone (Hydroq), resorcinol (Res) and cholorogenic acid (Chloro) with crude CATPO, 48 hr, 60°C, pH 7.0.

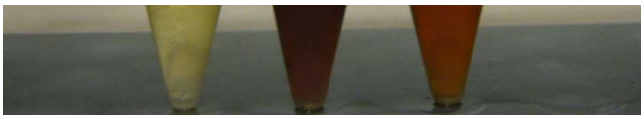
The substrates used for quick scrrening with crude CATPO were catechol, hydroquinone, resorsinol, chlorogenic acid, coumaric acid, vanillic acid, caffeic acid, phenyllactic acid, gallic acid, L-dopa, quercetin and catechin. The list is broader than the one used to screen the oxidation products of laccase and tyrosinase. It was because of the previous experiments conducted by colleagues that have studied catalase phenol oxidase of *S. thermophlium* (Mete, 2003, Kaptan, 2004, Yüzügüllü, 2010) utilized many phenolic compounds as being putative substrtaes of this bifunctional enzyme. CATPO appears to oxidize two of the three isomers of benzenediols, 1,1 and 1,3 catechol and hyroquinone respectively, but not 1,2 benzenediol (resorcinol). Hydroquinone showed color change only after laccase oxidation but not tyrosinase. During the studies of Avcı (2011), it was observed that the pure CATPO seemed to oxidize hydroquinone but it was not possible to run the product of the oxidation throught HPLC or LC-MS, because of the nature of the initial compound and probably the highly reactive *p*-quinone formed by CATPO. As a chromatogram is not present for that finding, it is not suggested to be oxidized by CATPO in the published article. Catechol oxidation is expected, since all the activity assays are based on the capability of CATPO oxidizing catechol. Autooxidation of both catechol and hydroquinone are intensive that the color change was close to the change in enzyme catalyzed reactions. Cholorogenic acid oxidation with crude CATPO gave the same color change both in reaction and control tubes, meaning that the initial screening gave negative for this compound.

-	-	+	-

1	2	3	4	5	6	7	8
Coum	Coum contr	Vanillic	Vanillic contr	Caffeic	Caffeic contr	PLA	PLA contr

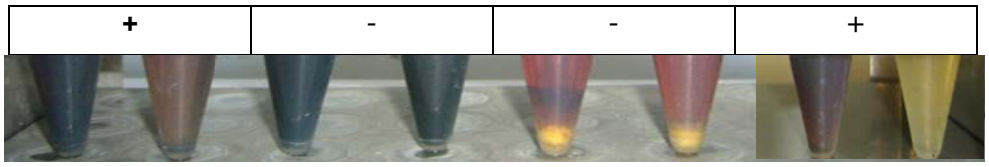
Figure 3. 6 Oxidation of coumaric acid (Coum), vanillic acid, caffeic acid and phenyllactic acid (PLA) with crude CATPO.

As can be seen from the Figure 3.6, coumaric acid, vanillic acid and phenyllactic acid was not oxidized by CATPO. Similar to the case of resorcinol (Fig. 3.5), the slight color change is generated by the presence of 2 mL supernatant in the mixture. The crude enzyme solution, i.e., centrifuged and filtered supernatant itself is a colored solution (Fig. 3.7) giving the difference between the control and reaction tubes. The minor color change is due to this fact. Caffeic acid on the other hand, oxidized readily and transformed to a molecule which then reacted with the compounds present inside the mixture to form insoluble polymers that precipitated.



1	2	3
Supernatant	Catech	Catech contr

Figure 3. 7 Comparision of supernatant exposed to same conditions as phenolics in reactions with crude CATPO oxidized catechin (Catech) and its control.



1	2	3	4	5	6	7	8
Gallic	Gallic contr	L-dopa	L-dopa contr	Querc	Querc contr	Catech	Catech contr

Figure 3. 8 Oxidation of gallic acid, L-dopa, quercetin (Querc) and catechin (Catech) with crude CATPO.

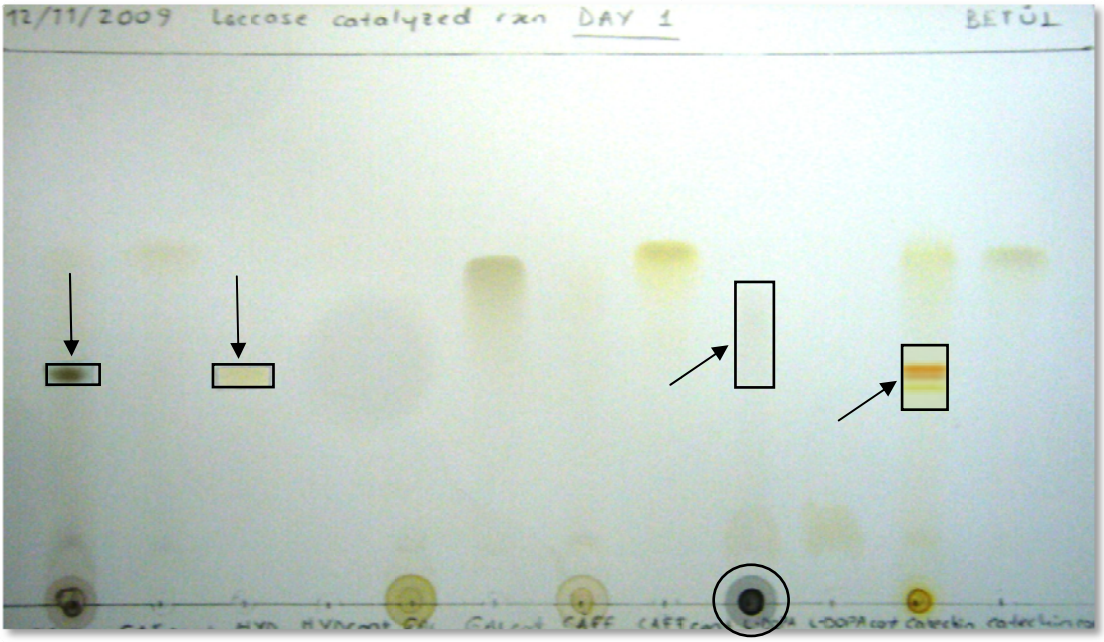
The last four substates tested for CATPO oxidation were gallic acid, L-dopa, quercetin and catechin. Among these; both for L-dopa and quercetin there was a gradual visible color change of the solution from light yellow to brown, even after 1 hour of reaction. Gallic acid can be said to be oxidized by CATPO and particularly for the substrate catechin, a prominent color change is present. But for the other two phenolic compounds L-dopa and quercetin; which are bulky molecules (App. B), it cannot be said that the have been oxidized only by the effect of enzyme. The autooxidation dominates for L-dopa and quercetin. They were not effectively dissolved in the reaction mixture (Fig. 3.8). Moreover, it is not possible to distinguish the color difference between reaction and control tubes.

The radicals formed by the CATPO catalysis tend to polymerize unspecifically with the surrounding compounds, including the products of reaction which are highly reactive radicals, proteins other than CATPO and polysaccharides still present in culture supernatant. This results in high molecular weight products which tend to precipitate. Defined reaction conditions like short reaction times, low molar concentrations of substrates and the utilization of pure CATPO will minimize the undesired polymerization.

3.2.2 Observations Based on Thin Layer Chromatography (TLC) Analysis of Phenol Oxidation Products of Crude CATPO

The aim of performing TLC over oxidation reaction products of CATPO was to investigate the differences in its final product mixture pattern. The method also allowed the determination of the most proper reaction conditions for phenol oxidations. Laccase and tyrosinase were again used for the sake of comparison. *T. versicolor* laccase and *A. bisporus* tyrosinase were utilized to explore their oxidation effect on catechol, hydroquinone, gallic acid, caffeic acid, L-dopa and catechin (Fig. 3.9 and Fig. 3.10). The reaction mixtures were spotted on 10x20 cm TLC plates. Buffer instead of enzyme solution was added to the control tubes. Ethanol was used as solvent.

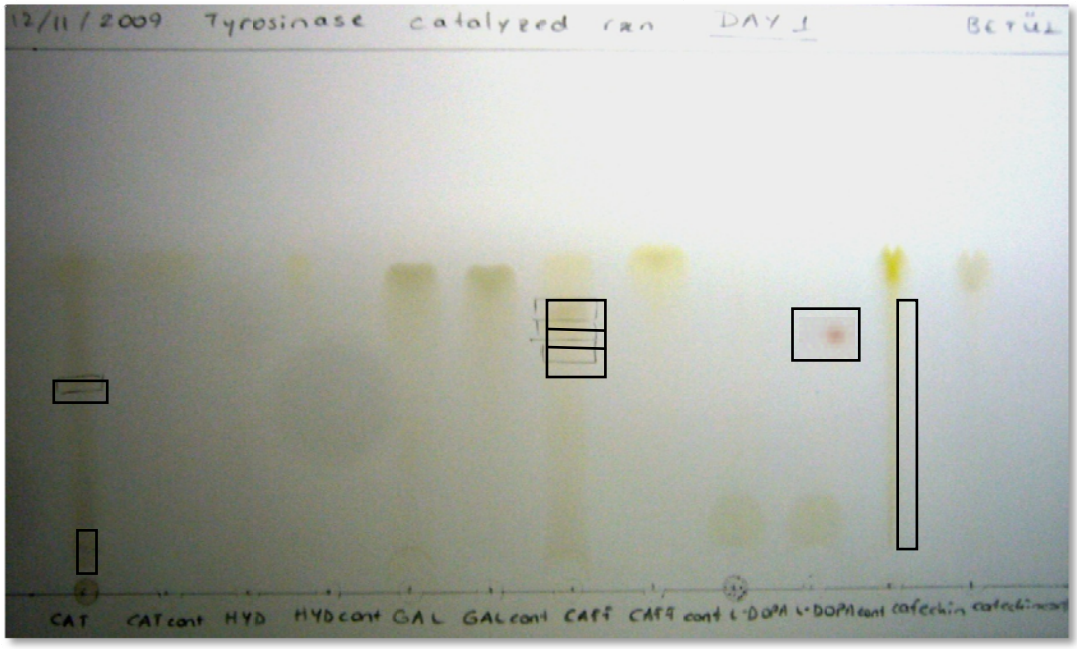
Crude CATPO was used to set up reactions with phenolics previously screened via color change observation and found to be either (+)ve or as a putative substrate. Results shown in Figure 3.11 indicate the oxidation products of catechol, hydroquinone, catechin, caffeic acid are detected as a single band. Three bands were observed for chlorogenic acid, while L-dopa, quercetin, coumaric acid and gallic acid did not yield distinct bands but rather smears.



1	2	3	4	5	6	7	8	9	10	11	12

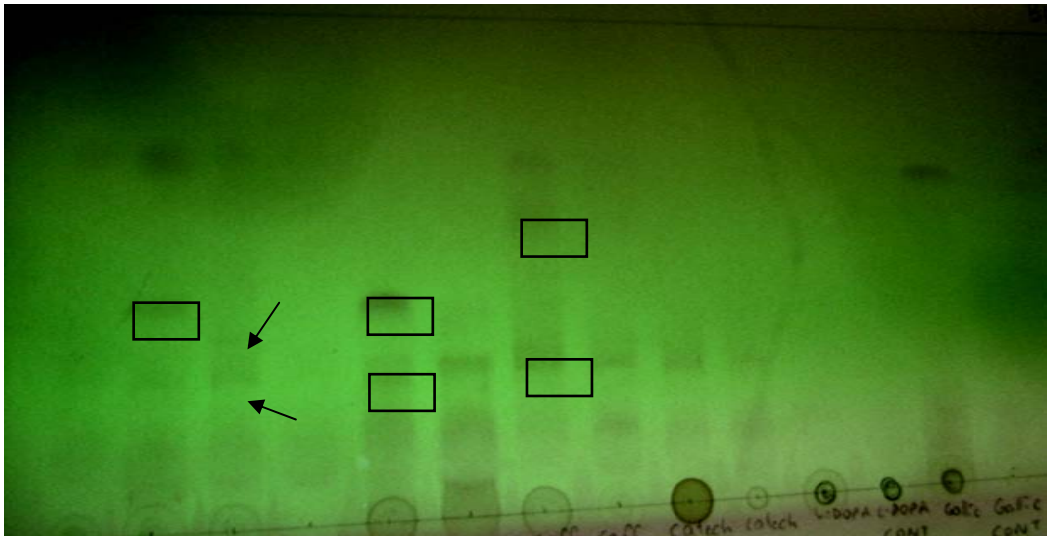
Figure 3. 9 TLC image for the oxidation of catechol (1, 2), hydroquinone (3, 4), gallic acid(5, 6), caffeic acid (7, 8), L-dopa (9, 10) and catechin (11, 12) with laccase after 24 h.

In further studies by Avcı (2011), it was shown using pure CATPO and by HPLC that indeed catechol, catechin, caffeic acid and chlorogenic acid are substrates of CATPO. It was not possible to perform HPLC analysis for hydroquinone. Based on TLC results, hydroquinone is very likely to be also one of the substrates of CATPO. This suggestion is also supported by the study of Yüzügüllü (2010). On the other hand, compounds resulting in smears were later identified by HPLC analysis not to be substrates of CATPO. Smear formation, therefore, is likely to be due to the use of crude enzyme solution, instead of pure enzyme. Results obtained by using crude enzyme are summarized in Table 3.1.



1	2	3	4	5	6	7	8	9	10	11	12

Figure 3. 10 TLC image for the oxidation of catechol (1, 2), hydroquinone (3, 4), gallic acid(5, 6), caffeic acid (7, 8), L-dopa (9, 10) and catechin (11, 12) with tyrosinase after 24 h.



1	2	3	4	5	6	7	8	9	10	11	12	13	14

Figure 3. 11 TLC image for the oxidation of catechol (1, 2), hydroquinone (3, 4), chlorogenic acid (5, 6), caffeic acid (7, 8), catechin (9, 10), L-dopa (11, 12) and gallic acid with crude CATPO after 24 h.

Table 3. 1 Summary of Preliminary Screening Results for laccase, tyrosinase and CATPO Oxidized Phenolic Substrates

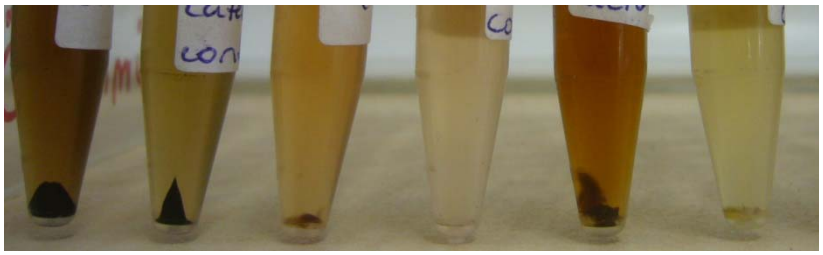
Enzyme/ Substrate	Laccase		Tyrosinase		CATPO	
	CC	TLC	CC	TLC	CC	TLC
Catechol	+	+	+	+	+	+
Hydroquinone	+	+	-	-	+	+
Resorcinol	nt	nt	nt	nt	-	nt
Chlorogenic Acid	nt	nt	nt	nt	-	+
Coumaric Acid	nt	nt	nt	nt	-	nt
Vanillic Acid	nt	nt	nt	nt	-	nt
Caffeic Acid	+	-	+	+	+	+
PLA	nt	nt	nt	nt	-	nt
Gallic Acid	+	-	+	-	+	-
L-dopa	+	+	+	+	-	-
Quercetin	nt	nt	nt	nt	-	nt
Catechin	+	+	+	+	-	-

*nt; not tested

In Table 3.1, the results in Sections 3.2.1 and 3.2.2 are summarized for the preliminary observation done with obtaining color changes and performing TLC over oxidation products of three enzymes, laccase, tyrosinase and CATPO.

3.2.3 FTIR Spectra of Oxidation Products

The CATPO oxidized phenolics were prepared (Section 2.2.6 a)) for the FTIR analysis by obtaining the polymerized pellet with the help of methods to remove the liquid part of the resulting product. The pellets formed are shown in Figure 3.12. Catechol, hydroquinone and chlorogenic acid were subjected to FTIR analysis to explore whether some data could be gathered for the bonds formed enzymatically using this method.



catechol and its control	hydroquinone and its control	chlorogenic acid and its control

Figure 3. 12 The image for the centrifuged tubes of pellets.

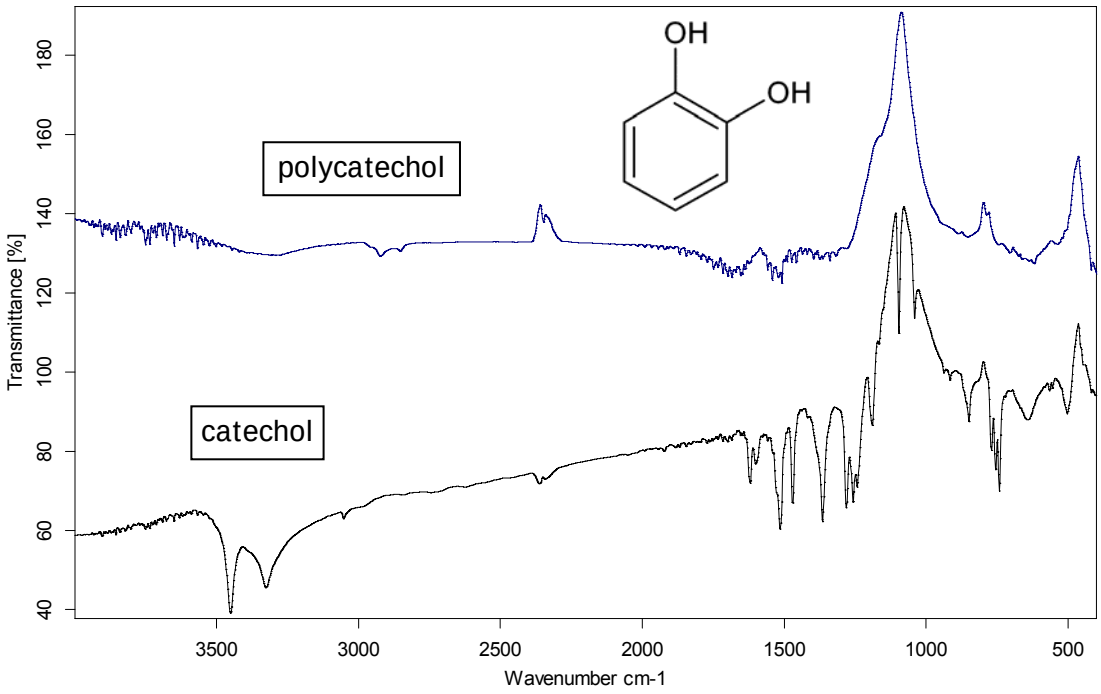


Figure 3. 13 The average FTIR spectra of catechol (black line) and polycatechol (blue line) in 400-4000 cm^{-1} region.

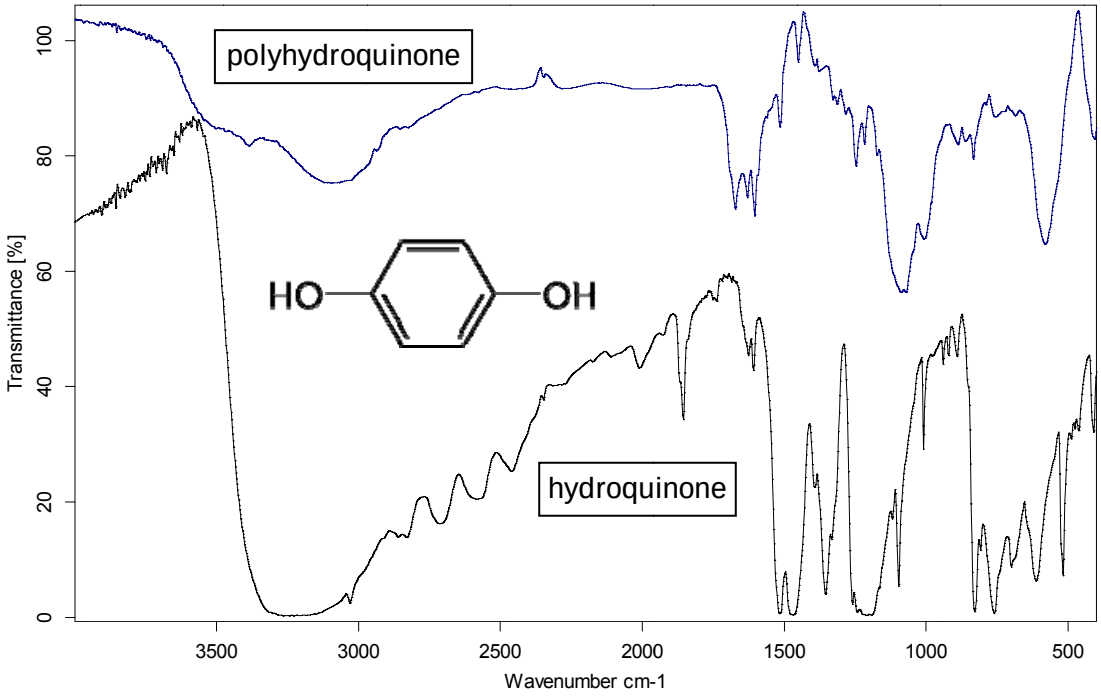


Figure 3. 14 The average FTIR spectra of hydroquinone (black line) and polyhydroquinone (blue line) in 400-4000 cm^{-1} region.

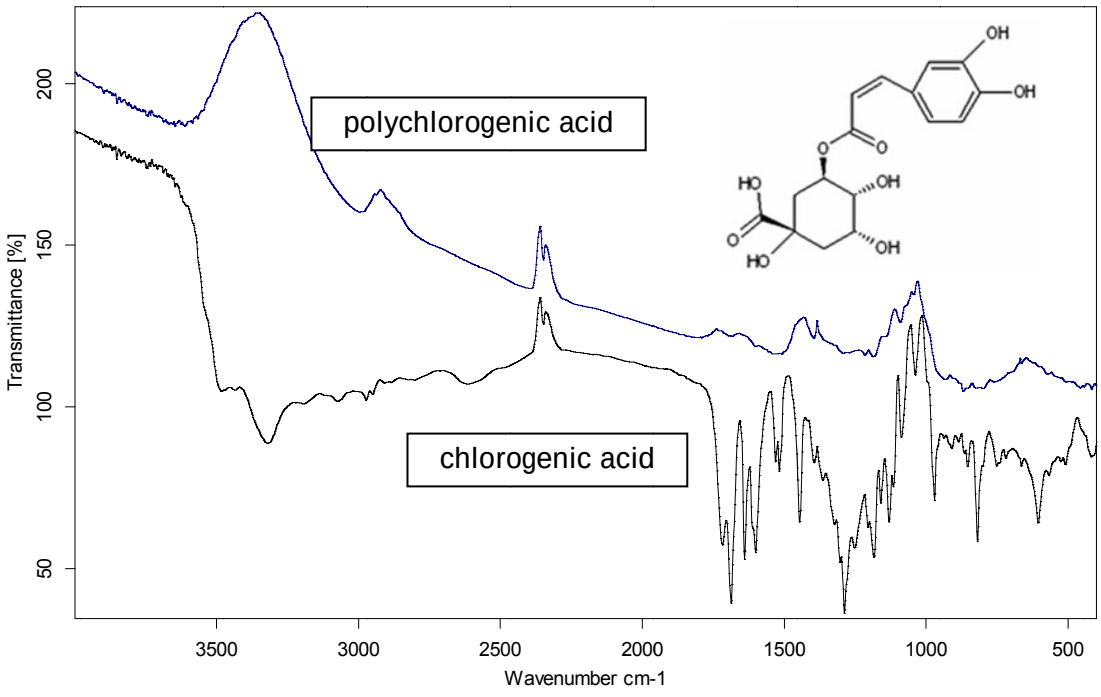


Figure 3. 15 The average FTIR spectra of chlorogenic acid (black line) and polychlorogenic acid (blue line) in 400-4000 cm^{-1} region.

In the overall picture FTIR results suggest that there is polymerization after the enzymatic reaction takes place. The peak broadening is apparent in every product spectra. For the catechol spectra, specifically the expected C-C and C-O-C bonds are present (NIST/EPA Infrared Database, http://webbook.nist.gov). Broad doublet peaks at 3451 cm^{-1} and 3326 cm^{-1} belong to characteristic hydrogen-bonded phenolic O–H vibration bands for catechol. Four absorption peaks between 1470 cm^{-1} and 1622 cm^{-1} are attributed to the aromatic ring C–C vibration bands; these are characteristic for the benzene aromatic ring. C–O vibration bands for catechol are at 1281 cm^{-1} and 1250 cm^{-1}. The other absorption bands at 850 cm^{-1} and 746 cm^{-1} are ascribed to out-of-plane bending of –C–H bonds of an aromatic ring.

For hydroquinone and chlorogenic acid, it is very difficult to make any suggestions. The main reason for the spectra not telling much is that the samples were taken from the reactions set up with supernatant. The supernatant is a complex media and the phenolics are open to precipitation with other molecules like proteins and polysaccharides. The spectra searched against known substance libraries the C-N bonds dominate and this points out that protein-phenolic complexes are formed.

3.3 Analysis of the Oxidation of Phenolics Using Pure CATPO

3.3.1 Purification of CATPO

The purification procedure was previously developed and optimized by Kocabaş *et. al.*, 2008. In that study, the bifunctionality of CATPO was introduced and a thorough research was conducted to find out this extracellular enzyme's molecular weight, isoelectric point and optimal pH for both activities. CATPO was purified 10 fold with 46% yield and was found to be 320 kDa overall with four 80 kDa subunits and an isoelectric point of 5.0. Catalase and phenol oxidase activities were both most stable at pH 7.0. An adapted method (Section 2.2.3) from this study was used in this thesis to obtain pure CATPO. A two-step purification was performed to purify the extracellular *S. thermophilum* CATPO. Ion exchage was followed by gel filtration under defined conditions.

For the first step, anion exchange chromatography; elution time correlated to absorbance data is represented in Figure 3.16, where UV absorbance values are plotted against elution volume. The crude enzyme solution was run through prepacked Hiprep 16/10 Q XL column at a flow rate of 1.2 mL/min at room temperature. The enzyme was separated from many other proteins having negative charge at pH 8.0, according to the previous findings that CATPO is bound to the column at this pH (Kocabaş *et. al.*, 2008). In total, analysis time was 165 minutes. CATPO activity was measured for all tubes collected between peak starting at 70 minutes and peak ending at 151 minutes, when added up the eluate in hand was 27 tubes that were each 3 mL in volume. The catalase and phenol oxidase active fractions were found to be in between 110 and 125 minutes in 5 tubes; as it can be followed through the graph (Fig. 3.15) and expected from the maxima of absorbance peaks.

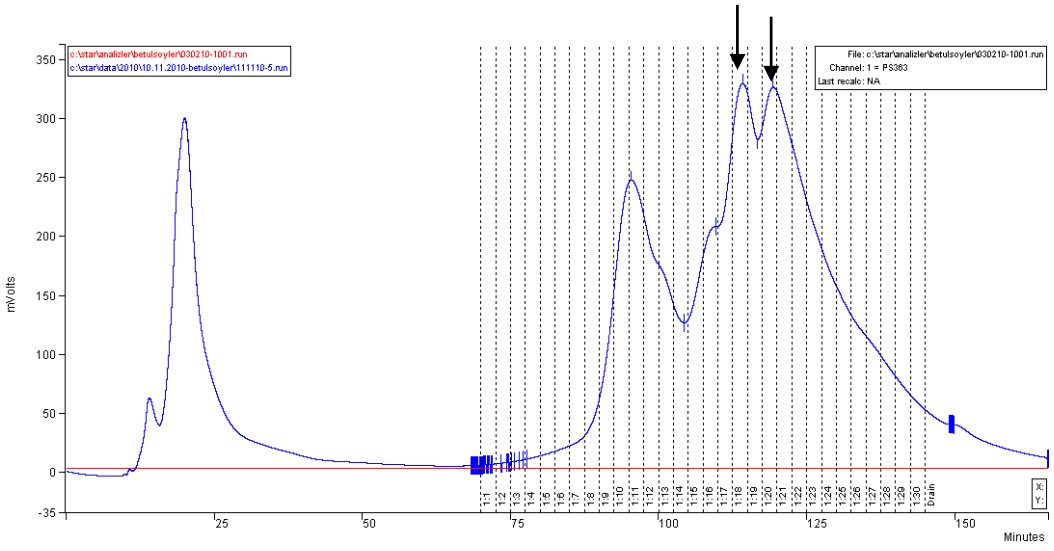


Figure 3. 16 Anion exchange chromatogram of *S. thermophilum* CATPO purification.

The output of anion exchange purification step was; CATPO active 5 tubes each being 3 mL volumetrically. They were combined in one falcon and then concentrated using Amicon ultra centrifugal filter unit.

Then, the 3 times concentrated protein mixture was loaded to Hiprep 16/60 Sephacryl S-100 gel filtration column (fractionation range (M_r) being 1000-100 000 Da) as 5 mL. The last step of CATPO purification was performed at room temperature at a flow rate of 0.5 mL/min, with the buffer Tris-HCl 50 Mm pH 8.0. Analysis was terminated at 350 minutes (Fig. 3.16). CATPO was eluted through the column between peak starting at 150 and peak ending at 240, to 5 mL collection tubes. 18 putative tubes were tested for catalase and phenol oxidase activity in a synchronized manner. The active fractions were caught over the period of 175 and 190 minutes. Therefore, it can be deduced that the first main peak in the chromatogram (Fig. 3.16) belongs to the CATPO.

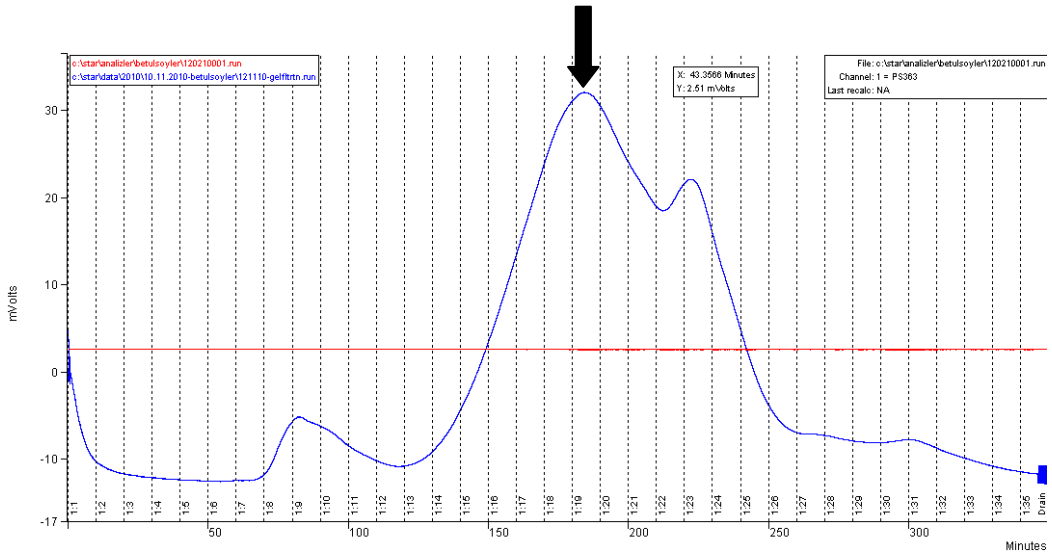


Figure 3. 17 Gel filtration chromatogram of *S. thermophilum* CATPO purification.

The course of purification procedure, purity and molecular weight of CATPO was checked by running portions from each step and different fractions on SDS-PAGE (Fig.3.17). The enzyme is known to be a tetrameric enzyme with four identical subunits, each subunit has a molecular weight of 80 kDa (Kocabaş *et. al.* 2008). The SDS-Page profile should reflect this fact. The image of the SDS-PAGE run after purification steps is shown in Figure 3.18.

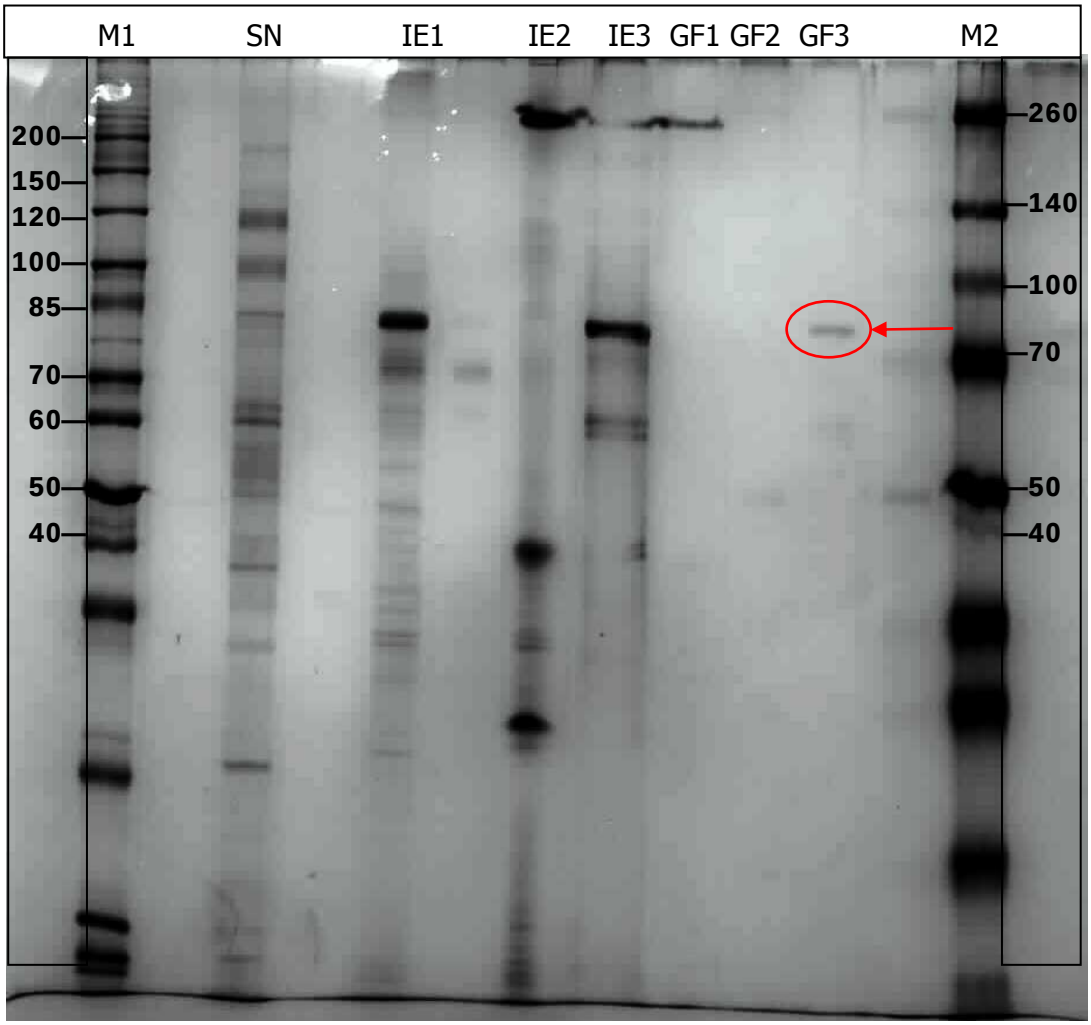


Figure 3. 18 SDS-PAGE of protein fractions from *S.thermophilum* CATPO purification steps.

M1: Marker (Fermentas PageRuler™ Unstained Protein Ladder)

SN: Crude enzyme solution (before Ion Exchange)

IE1: after Ion Exchange step catalase and phenol oxidase active fraction

IE2 and IE3: after Ion Exchange step catalase inactive fractions

GF1 and GF2: after Gel Filtration step catalase inactive fractions

GF3: after Gel Filtration step catalase and phenol oxidase active fraction

M2: Marker (Fermentas Spectra™ Multicolor Broad Range Protein Ladder)

The SDS-Page result clearly shows that the purification was successful and at the end of gel filtration pure CATPO was obtained. 80 kDa monomer is circled with red in lane GF3. The expected result was to see only CATPO fragment but not the other proteins after purification steps. The starting material was the supernatant obtained from 5th day incubation of *S. thermophilum* at 45 °C. The SN lane (Fig. 3.17) is the supernatant itself representing the variety of different extracellular proteins of *S. thermophilum* culture. After first attempt to purify CATPO, the abundancy of CATPO is apperant from lane IE1. There is still the complexity of proteins together with concentrated fraction of CATPO. Gel filtration step was there to finalize the purification process and get CATPO only.

The fractions that were loaded to the SDS-Page gel were also analyzed by Bradford Assay (Section 2.2.3.4) to determine the total protein content and compare with those reported by Kocabaş *et. al.* (2008). Ion exchange was resulted in 16 % activity recovery and 1.13 fold purification for CATPO activity and at the end of gel filtration 11 % activity recovery and 5.5 fold purification for CATPO activity was achieved (Table 3.2). Both the yield and purification fold was slightly lower than what was reported by Sutay (2007), however the ultimate activity value of 30 U / mL was ideal and adaquate for further studies.

Table 3. 2 Purification results for two-step isolation of *S. thermophilum* CATPO

	Volume (mL)	Activity (U/mL)	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification Fold
Supnt	50	28	1400	1.8	778	100	1
IE	10	22	220	0.25	880	16	1.13
GF	5	30	150	0.035	4285	11	5.5

Supnt; filtered supernatant (crude CATPO)
IE; after ion exchange purification (step one)
GF; after gel fitration purification (step two, final)

3.3.2 Effect of CATPO Oxidation on the Antioxidant Capacities of Phenolics

The aerobic metabolism of living organisms constantly produces reactive oxygen species (ROS), such as hydrogen peroxide, organic peroxide, superoxide anion and hydroxyl radical which are receiving particular attention because of their possible involvement in several diseases. Protection against ROS is provided by a range of different compounds and by enzymatic antioxidants, such as catalase. In the past decades, a large number of scientific reports have described the properties of phenolic compounds from numerous natural products. Phenolics can potentially interact with biological systems as efficient free radical scavengers, and play an important role in antimicrobial, antioxidant, anticarcinogenic, and anti-inflammatory activities. Different phenolic compounds may act as antioxidants at varying degrees depending on the polarity and molecular characteristics. Normally, one should expect oxidation of antioxidants to yield a lower antioxidant capacity. Oxidation itself may produce dimers, trimers and various other polymers, and also reactions between oxidized phenolic compounds may bring about formation of novel antioxidants, it would appear rather impossible to predict the antioxidant properties of these new compunds formed. The enzyme studied along this thesis is a catalase in nature and bears phenol oxidase activiy together with its catalase activity. At the core of this specific study, the investigation of the biological importance of phenol oxidase activity coupled with catalase activity, lies. It was aimed to explore the effect of oxidation on antioxidant capacities of selected phenolic compounds. The major biological function attributed to catalase is to take part in antioxidant defense mechanism of organisms. Catalase works closely with superoxide dismutase to prevent free radical damage. Hydrogen peroxide is a naturally occurring but destructive waste product of all oxygen-dependent organisms. It has been theorized that one of the primary reasons cells age is the damage to DNA caused by free radicals and oxidizing agents such as hydrogen peroxide. The phenol oxidation property of the extracellular catalase of *S. thermophilum* may contribute to the formation of more stable polymers or oligomers that possess an antioxidant activity different than the initial substrate.

14 phenolic compounds among different classes of plant phenolics were analyzed and 4 of them were found to be oxidized by CATPO (Avcı *et. al.*, 2012). Catechol, chlorogenic acid, caffeic acid and catechin were the phenolic substances having the common property of being an *ortho*-diphenolic compound and were detected to be oxidized by CATPO. The oxidation products were analyzed by HPLC, and subsequently by LC-MS. These four phenolic substances were subjected to an antioxidant activity measurement assay, together with the products oxidized by CATPO, as well as laccase and tyrosinase for the sake of comparison. Two methods were used; 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) – based one (TEAC) and Ferric Reducing Antioxidant Power (FRAP) among other possible assays. The analytical results are presented in tables where the antioxidant capacity was expressed in mM Trolox for TEAC assay and mmol Fe^{2+} equivalents per g of substance tested for FRAP assay.

3.3.2.1 TEAC Results of CATPO Oxidized Phenolics

Four antioxidant compounds known to be oxidized by CATPO, namely catechol, catechin, caffeic acid and chlorogenic acid, were analyzed by TEAC method to obtain the antioxidant capacity values in terms of ABTS oxidation. The products of CATPO oxidation reaction were subjected to antioxidant capacity measurements also, first by the most known method in phenolic substance studies; TEAC. The reaction parameters were at 60°C for 1 hr with pH 7.0. There were two different solvents used for setting up the reactions. All the measurements were triplicate and represented with the standard deviation. The TEAC literature value of initial substrate was also given in Table 3.3. The results obtained with both the ethanol and methanol as solvents did not differ much from each other, and the reactants were dissolved more easily in methanol than ethanol, for the rest of the study methanol was the choice of solvent.

The fold increase between the TEAC values of unreacted phenols and CATPO oxidized products were determined according to the average value obtained from triplicate measurements.

Table 3. 3 TEAC values of the phenolic substrates and CATPO oxidized products at 60°C, for 1 hr.

	TEAC VALUE (mM Trolox) methanol	*	TEAC VALUE mM Trolox ethanol	*	Literature value for the substrate
Catechol	1.08 ± 0.09		1.23 ± 0.11		1.42 (Arts *et. al* 2004)
Catechol product	2.59 ± 0.28	2.40	2.74 ± 0.25	2.23	
Chlorogenic acid	0.89 ± 0.04		0.97 ± 0.06		0.95 (Re *et. al.* 1999)
Cholorogenic acid product	1.36 ± 0.12	1.53	1.49 ± 0.08	1.54	
Caffeic acid	0.94 ± 0.05		0.98 ± 0.08		0.99 (Pellegrini *et. al* 2000)
Caffeic acid product	1.67 ± 0.16	1.78	1.85 ± 0.14	1.89	
Catechin	2.44 ± 0.22		2.69 ± 0.37		2.4 (Rice-Evans *et. al.* 1996*)*
Catechin product	3.41 ± 0.51	1.40	3.74 ± 0.34	1.39	

* Fold increase in TEAC values upon oxidation.

According to the results shown in Table 3.3, oxidation of phenolic compounds in the presence of CATPO caused an increase in antioxidant capacity. Among all the tested compounds catechol was the one with highest fold of increase in the antioxidant capacity after oxidation.

Thus the oligomeric and polymeric products (Avcı *et. al.*, 2012) appear to have higher antioxidant capacities as compared to their unreacted polymers.

3.3.2.2 FRAP Results of CATPO Oxidized Phenolics

In this present study FRAP was chosen to be the method for comparison of TEAC results. Although TEAC is a reliable, fast and reproducible assay, in antioxidant capacity studies it is a common practice to employ at least two methods to compare the results.

The phenolics dissolved in methanol were reacted with CATPO at 60 °C for an hour and then subjected to antioxidant capacity measurement analysis assays. The values in Table 3.4 are represented as mean of triplicate measurements ± standard deviation.

Table 3. 4 FRAP values of both the substrates and the CATPO oxidized products (expressed in mmoles Fe^{2+}/g).

	FRAP VALUE (mmol Fe^{II}/g)	*	Literature value for the substrate
Catechol	2.29 ± 0.47		2.28 (deGraft-Johnson *et. al* 2007)
Catechol product	4.53 ± 0.38	1.98	
Chlorogenic acid	1.38 ± 0.26		0.99 (Nilsson *et. al* 2005)
Cholorogenic acid product	1.79 ± 0.32	1.30	
Caffeic acid	1.45 ± 0.19		1.64 (Pulido *et. al.* 2000)
Caffeic acid product	1.96 ± 0.33	1.35	
Catechin	3.97 ± 0.29		2.47 (Luximon-Ramma *et. al.* 2005)
Catechin product	4.74 ± 0.41	1.19	

* Fold increase in FRAP values upon oxidation.

Even though the final scores for both methods show slight differences, the antioxidant potential in terms of FRAP value of CATPO oxidized phenolic compounds on a molar basis against radicals in order of effectiveness was not different than the results obtained by TEAC. Accordingly, again catechol oxidation showed the highest fold increase, which was followed by caffeic acid, chlorogenic acid and catechin.

3.3.2.3 TEAC and FRAP Results of Laccase Oxidized Phenolics

The effect of CATPO oxidation on the four ortho-diphenolic compounds was experimented to discover the functionality of phenol oxidase activity, in addition to the catalase activity. In order to suggest a function or come up with a hypothesis on top of known and studied mechanisms for the family of phenol oxidases in general, two other phenol oxidase namely laccase and tyrosinase were utilized for the comparison of the effect on oxidized products' antiradical power.

Table 3. 5 TEAC values of laccase oxidized products

	TEAC Value (mM Trolox)	*
Catechol	1.085 ± 0.09	
Catechol product	1.192 ± 0.08	1.09
Catechin	2.403 ± 0.22	
Catechin product	2.976 ± 0.31	1.24
Caffeic acid	0.941 ± 0.05	
Caffeic acid product	1.068 ± 0.11	1.13

* Fold increase in TEAC values upon oxidation.

The reaction of phenolic compounds with laccase and tyrosinase tested using HPLC and LC-MS (Avcı, 2011) yielded the result that laccase gave characterizable products only with catechol, caffeic acid and catechin. Therefore, the antioxidant capacity measurement studies were conducted only with these three phenolics.

The reaction was set up using methanol as the solvent, a sodium acetate buffer to sustain pH at 4.8 and a water bath adjusted to 30 °C. The average values of three measurements were represented in Table 3.5 for TEAC method and in Table 3.6 for FRAP method.

Table 3. 6 FRAP values of laccase oxidized products

	FRAP Value (mmol Fe^{2+}/g)	*
Catechol	2.29 ± 0.47	
Catechol product	2.68 ± 0.23	1.17
Catechin	3.97 ± 0.29	
Catechin product	5.54 ± 0.41	1.39
Caffeic acid	1.45 ± 0.19	
Caffeic acid product	1.58 ± 0.34	1.09

* Fold increase in FRAP values upon oxidation.

The products of catehol, catechin and caffeic acid oxidation by laccase showed only slightly higher antiradical efficacy. The result obtained with CATPO oxidized catechol was the most striking one with the fold increase value of 2.4 for TEAC value when compared to the starting material, pure catechol. Such a striking increase in the antioxidant capacities of the products was not apparent upon laccase oxidation.

3.3.2.4 TEAC and FRAP Results of Tyrosinase Oxidized Phenolics

Tyrosinase was found to oxidize catechol and catechin effectively and forming a dimer from catechol and a dimer as well as a trimer and a tetramer from catechin (Avcı, 2011). Therefore, the antioxidant capacity measurement studies were conducted only with these two phenolics.

The reaction was set up using methanol as the solvent, a potassium citrate buffer to sustain pH at 6.5 and a water bath adjusted to 25 °C. The average values of three measurements were represented in Table 3.7 for TEAC method and in Table 3.8 for FRAP method.

Table 3. 7 TEAC values of tyrosinase oxidized products

	TEAC Value (mM Trolox)	*
Catechol	1.09 ± 0.09	
Catechol product	1.24 ± 0.17	1.14
Catechin	2.40 ± 0.22	
Catechin product	2,58 ± 0.43	1.07

* Fold increase in TEAC values upon oxidation.

Table 3. 8 FRAP values of tyrosinase oxidized products

	FRAP Value (mmol Fe^{II}/g)	*
Catechol	2.29 ± 0.47	
Catechol product	2.53 ± 0.31	1.10
Catechin	3.97 ± 0.29	
Catechin product	4.09 ± 0.33	1.03

* Fold increase in FRAP values upon oxidation.

The oxygenated substrate showed almost the same antioxidant activity of catechol and catechin itself in this enzymatic reaction with tyrosinase. The results obtained for all the tested compounds were higher than that of the initial substrates in a significant amount with the oxidation of CATPO only, but not with laccase or tyrosinase. This highlights the unique property of CATPO when compared to the very well known phenol oxidases; laccase and tyrosinase.

These two enzymes have industrial importance such as in biotechnology, food processing, medicine, and the textile and paper industry, especially due to their ability to polymerize compounds. The polymerized products of their common substrates, however do not have an explicitly different antioxidant capacity when compared to the unreacted initial compounds.

3.3.3 Effect of CATPO Oxidation on the Antioxidant Capacities of Tea Samples

3.3.3.1 Antioxidant Capacity Values of CATPO Treated Green and Black Tea Preparations

Fresh tea leaves are very rich in catechins, which may constitute up to 30% of dry weight (Balentine *et. al.*, 1997). The type of processiong applied to the fresh tea leaves and amounts of flavonoids present in green, oolong and black teas. Polyphenol oxidases and catechins exist in separate kayers of the tea leaf. The Rolling disrupts the leaf; allowing catechins to enzymatically oxidize. In black tea production process, the freash leaves are withered and then rolled to provide the oxidation neccessary for the catehins. The oxidation of catechins resuults in the formation of dimers, trimers and polymers such as theaflavins and thearubigins. Thearubigins are a general name for a heteregenous group of compounds that may comprise up to 75 % of total flavonoids in black tea (Lakenbrink *et. al.*, 2000). Even though the conditions vary for different types of tea, in general fermentation step in black tea production is carried out at 24 – 27 °C for 3 – 4 hr. Green tea, on the other hand, is unfermented. The leaves are steamed or a hot air is blown over for 30 sec to inactivate the polyphenol oxidases. The reactions that would have polymerized the catechins present abundantly in leaves therefore are prevented this way. The total flavonoid majority is consequently composed of catechin monomers in green tea. 20 to 30 % of these flavonoids may also undergo a relatively restricted oxidation during withering of the leaves. The catechin polymers are are present in certain amounts also in green tea such as those found in black and oolong teas (Graham, 1992).

It was aimed to study the effect of CATPO addition to the tea leaves during the different stages of black tea production. The change in antioxidant capacity was monitored and recorded. The previous studies conducted with pure phenolic compounds gave promising results for the industrial usage of this powerful bifunctional enzyme in tea production. CATPO was added to the water extract of tea samples. Preliminary studies were done using the commercial teas in tea bags that can be obtained from a regular supermarket.

To prepare each infusion, standard 1% rule (1 g of tea per 100 mL water) was employed (Borse and Jagan Mohan Rao, 2012). Tea samples were brought to 60 °C and CATPO was added (15 U/ml) and left for an hour. Then the CATPO treated and untreated tea infusions were subjected to antioxidant capacity measurements using TEAC method. Each measurement was made in triplicates. Three different brewing times were experimented; 3, 5 and 10 minutes. These were selected according to standard percent infusion and brewing times of 4 to 5 minutes which are satisfactory for most purposes (Peterson *et. al.*, 2005).

Table 3. 9 Antioxidant capacities of commercial tea samples treated with CATPO

	TEAC value (mmol/L) 3 min brew	*	TEAC value (mmol/L) 5 min brew	*	TEAC value (mmol/L) 10 min brew	*
Green tea	2.21 ± 0.19		3.02 ± 0.24		5.59 ± 0.43	
CATPO treated green tea	2.34 ± 0.11	1.06	3.27 ± 0.35	1.08	6.72 ± 0.39	1.20
Black tea	1.58 ± 0.17		2.66 ± 0.28		4.78 ± 0.26	
CATPO treated black tea	1.73 ± 0.25	1.09	2.98 ± 0.31	1.12	6.02 ± 0.42	1.26

* Fold increase in TEAC values upon oxidation.

The TEAC values of CATPO added tea infusions are given in Table 3.9. According to the results, upto 5 minutes a significant increase in the antioxidant capacities of the samples was not observed. The maximum fold increase in the antioxidant capacity was 1.26, obtained for 10 minutes brewing of black tea. This result is expected because as the brewing time increases the phenolic compounds that are transferred to water increase. Thus an increase in the amount of phenolic compounds is likely to increase the effect of CATPO. The flavonoid content of a tea infusion depends on 2 factors; namely composition of tea, and brewing characteristics. The tea itself is influenced by tea type and processing method (blend, green versus black, etc.). The brewing characteristics are affected by the strength and type of infusion (tea particle size and weight), the flavonoid extraction rate and efficiency and brewing conditions such as tea to hot water ratio, time and temperature. Tea particles in teabags are smaller than in the regular conventional loose tea leaves used to brew tea in pots in Turkey. This particle size concept gains importance in tea water contact to assure the adequate transfer of phenolics to the brew. The great surface area to volume ratio of the leaves in tea bags exposes them to more hot water. This suggests that the antioxidant capacity of ready to use teas in tea bags would be higher than that of the loose leaves, but it was not the case (Table 3.10). The tea used in tea bags is commonly dust or low quality tea. The small space of the bag does not allow leaves to diffuse and steep properly. The obvious difference between

3.3.3.2 Effect of CATPO Oxidation on Antioxidant Capacity Values of Tea Leaves from Production Line

Tea samples were used as they were received from the produciton area, no further chopping were done. In the case of freash tea leaves, intact leaves were utilized for antioxidant capacity assays. Since most flavonoid compounds are extracted quite rapidly in preparation of tea brew, brewing times are not that important when infusion time is greater than 4 minutes (Arts *et. al.,* 2000). The brewing time of 5 minutes was chosen for the analysis of antioxidant capacity difference in CATPO oxidized tea leaves (2 g tea was infused in 200 mL boiling water).

Table 3. 10 Antioxidant capacity values of CATPO treated tea leaves from production line

	TEAC value	*
Fresh tea leaves	4.57 ± 0.36	
CATPO treated fresh tea leaves	5.16 ± 0.41	1.13
Tea leaves after rolling	4.40 ± 0.29	
CATPO treated tea leaves after rolling	4.98 ± 0.33	1.13
Tea leaves just before fermentation	4.25 ± 0.38	
CATPO treated tea leaves just before fermentation	4.87 ± 0.26	1.15
Tea leaves after fermentation	3.72 ± 0.22	
CATPO treated tea leaves after fermentation	4.21 ± 0.30	1.13
Tea leaves after drying	3.05 ± 0.21	
CATPO treated tea leaves after drying	3.89 ± 0.25	1.28

* Fold increase in TEAC values upon oxidation.

The tea brews were analyzed immediately after the preparation, no refrigerated or freeze dried storage was present to prevent the oxidation via ambient oxygen. The tea leaves brewed for 5 minutes were subjected to CATPO oxidation for an hour at 60°C. Antioxidant capacities of the leaves decrease by process steps. 4 % decrease after rolling, 7 % decrease just before fermentation, 8 % decrease after fermentation and finally 15 % decrease in total after drying was calculated from the initial value of 4.57 TEAC value of fresh tea leaves plucked from the plant from the cultivation area. More free gallic acids are formed during tea fermentation because more gallated catechins are transformed to non-gallated catechins by releasing free gallic acid before forming theaflavin. Theaflavin, one of the most prevalent phenolic compounds in black tea shows similar antioxidant capacity with EGCG, a major tea catechin in green tea (Lin, Chen, Ho, & Lin-Shiau, 2000), the concentration of tea catechins including EGCG was significantly higher than theaflavin and thearubigin. Gradual lowering of flavanol content is another consequence of tea leaves fermentation process (Sava *et al.*, 2001).

The effect of CATPO oxidation is most significant after drying of tea leaves. The polyphenol oxidase of its own is inactivated via drying and the phenolic substances are only subjected to the oxidation effect of CATPO after this point. For the processes before, the enzymes of tea plant itself has an activity on flavanols and phenolic acids present in the leaves.

3.3.3.3 Effect of Laccase Oxidation on Antioxidant Capacity Values of Tea Leaves from Production Line

The effect of *S. thermophlium* catalase phenol oxidase (CATPO) was aimed to be compared with the effect of *Trametes versicolor* laccase. 10 unit enzyme was added to 1 mL infusion prepared the same as in CATPO treatment experiments, and left for an hour at 30°C. 10 unit of laccase was used whereas CATPO treatment was made with 15 units of purified CATPO. Since the laccase was a commercially available enzyme with high purity a lower amount would be sufficient to conduct the analyses.

Table 3. 11 Antioxidant capacity values of laccase treated tea leaves from production line

	TEAC value	*
Fresh tea leaves	4.57 ± 0.36	
Laccase treated fresh tea leaves	5.44 ± 0.43	1.19
Tea leaves after rolling	4.40 ± 0.29	
Laccase treated tea leaves after rolling	5.15 ± 0.31	1.15
Tea leaves just before fermentation	4.25 ± 0.38	
Laccase treated tea leaves just before fermentation	5.12 ± 0.40	1.20
Tea leaves after fermentation	3.72 ± 0.22	
Laccase treated tea leaves after fermentation	4.53 ± 0.28	1.22
Tea leaves after drying	3.05 ± 0.21	
Laccase treated tea leaves after drying	3.14 ± 0.43	1.03

* Fold increase in TEAC values upon oxidation.

Laccase has common oxidation patterns with CATPO, on the contrary, the substrates that can be oxidized by CATPO are reported to be broader than that of laccase (Avcı, 2011). A fungal laccase derived from *Pleurotus* species was reported in a patent issued on 2001 by Unilever for the color enhancement of tea. During the experiments, it was also visible by eye that the laccase addition to tea infusions darkened the color of the solution. The antioxidant capacity increase for the dired tea leaves is not remarkable as in the case of CATPO. However, other process steps showed approximately 1.2 fold increase in antiradical power of tea leaves when laccase was added to the brew.

3.3.3.4 Effect of Tyrosinase Oxidation on Antioxidant Capacity Values of Tea Leaves from Production Line

Mushrrom tyrosinase was utilized as the third enzyme to reveal the effect of oxidation via its catalytic property on antioxidant capacity of tea phenols and polyphenols. The brewing time was 5 minutes again as in the previous two enzyme applications and for obtaining an infusion 2 g tea was added in 200 mL boiling water. 10 unit *Agaricus bisporus* tyrosinase was added to 1 mL infusion, and left for an hour at 25°C.

Throughout the study laccase and tyrosinase were used as the enzymes to discover the shared and differing properties of phenol oxidation in CATPO. CATPO is actually a catalase possessing an evident acitivity of phenol oxidation. Black tea production steps were chosen to be the food industry that may utilize CATPO in one of the process steps to enhance antioxidant capacity of tea leaves or the final product to be consumed.

Green tea itself, different extracts and isolated catechins were reported as tyrosinase inhibitors in a numerous researches (Kyung No *et. al.,* 1999, Sangsrichan and Ting 2010, Young-Hong *et. al.*, 2012, Parvez *et. al.*, 2007, Chang 2009, Kima and Uyama, 2005).

Table 3. 12 Antioxidant capacity values of tyrosinase treated tea leaves from production line

	TEAC value	*
Fresh tea leaves	4.57 ± 0.36	
Tyrosinase treated fresh tea leaves	5.03 ± 0.28	1.10
Tea leaves after rolling	4.40 ± 0.29	
Tyrosinase treated tea leaves after rolling	4.75 ± 0.32	1.08
Tea leaves just before fermentation	4.25 ± 0.38	
Tyrosinase treated tea leaves just before fermentation	4.68 ± 0.27	1.10
Tea leaves after fermentation	3.72 ± 0.22	
Tyrosinase treated tea leaves after fermentation	4.06 ± 0.39	1.09
Tea leaves after drying	3.05 ± 0.21	
Tyrosinase treated tea leaves after drying	3.19 ± 0.27	1.05

* Fold increase in TEAC values upon oxidation.

In this study it can be seen from Table 3.12 that tyrosinase addition to tea infusions do not significantly increase antioxidant capacity, and this can be explained by the conclusion from the studies gathered from literature. All agree on the fact that tea phenols inhibit the activity of tyrosinase. The tyrosinase added in tea infusions does not contribute to an increase that was found for CATPO (Table 3.10) and laccase (Table 3.11).

3.3.3.5 Effect of Sugar Addition on Antioxidant Capacity Values of Commercially Available Green and Black Tea

It was of interest also to see the effect of sugar addition on the antioxidant capacities of tea samples. The studies in literature on the addition of sugar, milk or honey to tea is limited to the bioavailability of tea catechins to human body (Dubeau *et. al.*, 2010).

In solutions catechins may interact with other compounds like polysaccharides and proteins to form complexes. This can result in either an increase or a decrease in antioxidant capacity of unreacted monomers. The effect of sugar addition, the conventional and common way to consume black tea in Turkey, was studied.

2 g commercial tea from tea bags was brewed for 5 and 10 minutes and table sugar (one cube ≈ 2.8 g) was added. The brew was left to cool down to 60°C and the antioxidant measurement assay TEAC was applied. The addition of sugar significantly decreased the antioxidant capacity of both green and black tea samples and under both brewing conditions.

Table 3. 13 TEAC values of sugar added tea samples

	TEAC value (mmol/L) 5 min brew	*	TEAC value (mmol/L) 10 min brew	*
Green Tea extract	3.78 ± 0.17		6.04 ± 0.08	
Green Tea with sugar	2.95 ± 0.09	- 1.28	3.92 ± 0.11	- 1.54
Black Tea extract	2.86 ± 0.14		3.02 ± 0.18	
Black Tea with sugar	2.04 ± 0.19	- 1.40	2.34 ± 0.12	- 1.29

* Fold increase in TEAC values upon oxidation.

CHAPTER 4

CONCLUSIONS

CATPO is a bifunctional enzyme possessing both activities, catalase and phenol oxidase. It is found to increase the antioxidant capacity of *ortho*-diphenolics, namey catechol, chlorogenic acid, caffeic acid and catechin. The antixidants measurements of oxidized and unreacted phenolic compounds were done using the two widely used methods TEAC and FRAP. The results obtained with the two methods were in accord with each other. CATPO oxidized catechol showed 2.4 fold increase when compared to its non-oxidized form, which was highest among others. Catechol was followed by caffeic acid, chlorogenic acid, and catechin. This finding is new to the literature and may be of importance to the antioxidant mechanism of organisms.

Effect of CATPO oxidation on antioxidant capacity of phenolics was compared with the effect of oxidation of laccase and tyrosinase. The results have shown that *T. versicolor* laccase and mushroom tyrosinase do not result in such high increases in antioxidant capacity upon oxidation of the substrates tested. Due to this finding, as a possible means of applying CATPO to increase the antioxidant capacity of products daily consumed, tea was selected.

Black tea is a rich source of theaflavins and thearubigins whereas green tea is rich in catechins. Besides whitering, rolling or drying; fermentation is a critical process for production of good quality tea and is the key step differing between green and black tea production. During this process phenol oxidases catalyze the oxidation of polyphenolic compounds present in tea leaves to their corresponding *o*-quinones.

Utilization of CATPO in tea samples resulted in an increase in antioxidant capacity and its effect was enhanced by an increase in brewing time. Interestingly, the addition of sugar decreased antioxidant capacity. Laccase and tyrosinase were ineffective in increasing the antioxidant capacity of tea samples.

CATPO can be added to a conventional black tea fermentation during the primary oxidation of the green tea or prefarbly it can be used as a supplement to produce an iced tea product having a higher antioxidant capacity.

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APPENDIX A

MEDIUM COMPOSITIONS

YpSs Agar Medium for Stock Cultures

Yeast extract	4.0 g/L
K_2HPO_4	1.0 g/L
$MgSO_4.7H_2O$	0.5 g/L
Soluble starch	15.0 g/L
Agar	20.0 g/L

Preculture Medium for 1 L (5 % v/v)

Yeast extract	0.2 g
K_2HPO_4	0.05 g
$MgSO_4.7H_2O$	0.025 g
Glucose	1.0 g

Mainculture Medium (modified for optimum CATPO production)

Yeast extract	4.0 g/L
K_2HPO_4	1.0 g/L
$MgSO_4.7H_2O$	0.5 g/L
$CuSO_4.5H_2O$	0.1 g/L
Glucose	15.0 g/L

(0.172 g/L Gallic acid - optional)

APPENDIX B

BUFFERS AND SOLUTIONS

Phosphate Buffer (100 mM, pH 7)

Dissolve 1.74 g K_2HPO_4 in 100 mL distilled water and adjust the pH.

Sodium acetate buffer (100 mM, pH 4.8)

Combine the following proportions of 0.1 N acetic acid and 0.1 N sodium acetate;

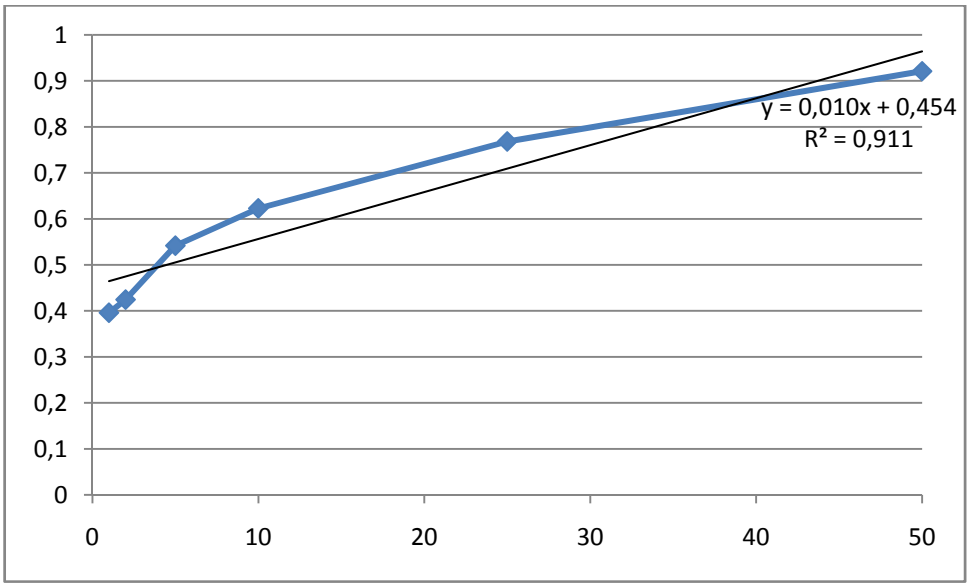
acetic acid	sodium acetate	pH
185	15	3.6
176	24	3.8
164	36	4.0
147	53	4.2
126	74	4.4
102	98	4.6
80	120	4.8
59	141	5.0
42	158	5.2
29	171	5.4
19	181	5.6

Potassium citrate buffer (100 mM, pH 6.5)

Dissolve 3.24 g potassium citrate in 100 mL distilled water and adjust the pH.

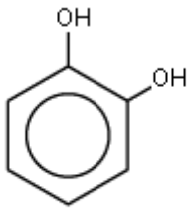
APPENDIX C

BOVINE SERUM ALBUMIN STANDARD CURVE FOR BRADFORD ASSAY

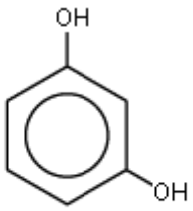


APPENDIX D

CHEMICAL STRUCTURES OF PHENOLIC COMPOUNDS

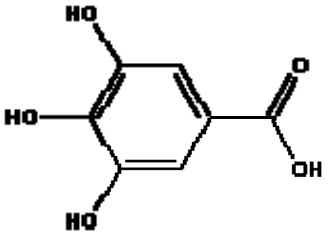


catechol

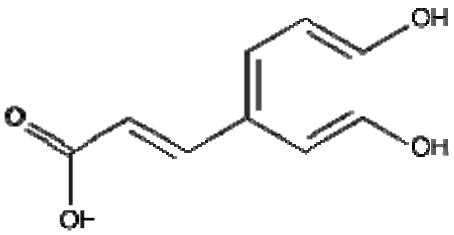


resorcinol

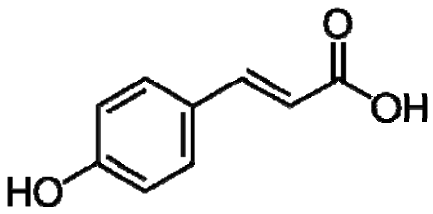
hydroquinone



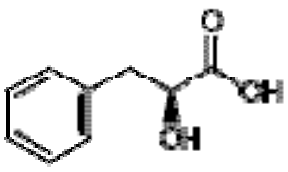
Gallic Acid



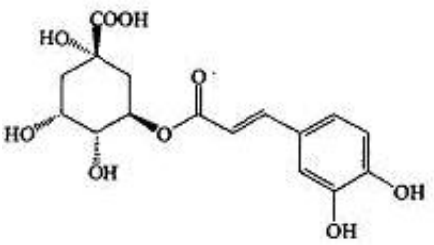
Caffeic acid



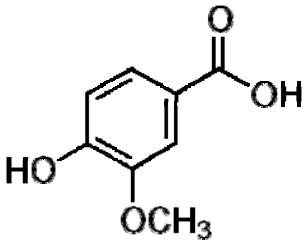
Coumaric acid



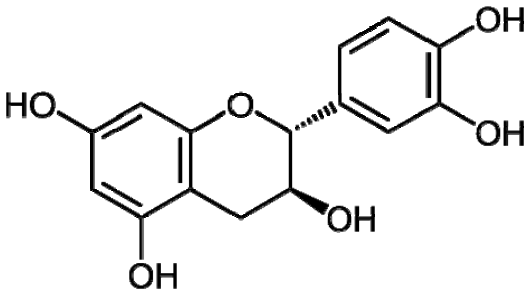
Phenyllactic acid



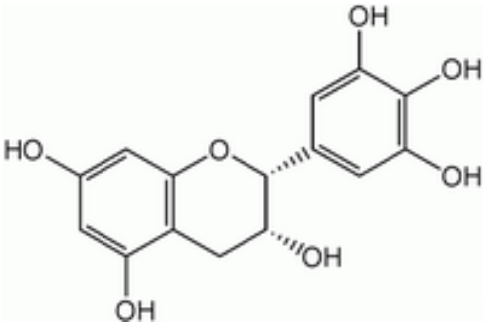
Chlorogenic acid



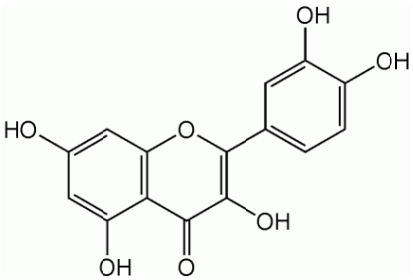
Vanillic acid



Catechin



Epicatechin



Quercetin

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FOREIGN LANGUAGES

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PUBLICATIONS

Gürkök S., **Söyler B.**, Biely P., Ögel Z.B., "Cloning and Heterologous Expression of the Extracellular α-Galactosidase from *Aspergillus fumigatus* in *Aspergillus sojae* Under the Control of *gpd*A Promoter". Journal of Molecular Catalysis B: Enzymatic, 2010, vol.64, pp. 146-149

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Gürkök, S., **Söyler, B.**, Ögel, Z.B., "*Aspergillus fumigatus* alpha-galactosidase heterologously expressed in *Aspergillus sojae* under gpdA promoter". 1-5 October 2008, Kuşadası, Turkey (Oral Presentation by Gürkök S.)

Gürkök, S., **Söyler, B.**, Ögel, Z.B., "Optimization of culture conditions for heterologous expression of alpha-galactosidase from *Aspergillus fumigatus* in *Aspergillus sojae*", June 28 - July 2, 2009, 3rd Congress of European Microbiologists (FEMS 2009) on the theme, "Microbes and Man - interdependence and future challenges", Gothenburg-Sweden (Poster presentation)

Gürkök S., **Söyler B.**, Ögel Z.B., "Heterologous expression of alpha-galactosidase from Aspergillus fumigatus in Aspergillus sojae, the influence of osmotic stress and optimization of culture conditions", August 5-9, 2008, XII. International Congress of Mycology, Istanbul-Turkey (Poster Presentation)

Gürkök, S., **Söyler, B.**, Ögel, Z.B., "Heterologous expression of *Aspergillus fumigatus* alpha-galactosidase in *Aspergillus sojae* and the influence of osmotic stress on the expression pattern". 21-23 May 2008, Erzurum, Turkey (Oral Presentation by Gürkök S.)

Gürkök S., **Söyler B.**, Ögel Z.B., "*Aspergillus fumigatus* alfa-galaktosidaz enziminin *Aspergillus sojae*'de heterolog ifadesi ve osmotik stresin etkisi", October 28- 31 2007, XV. Biotechnology Conference, Antalya- Turkey (Poster presentation)

Gürkök S., **Söyler B.**, Ögel Z.B., "Production of alpha-galactosidase from Aspergillus fumigatus in Aspergillus sojae under the control of the gpdA promoter and the influence of osmotic stress", June 13-16 2007, 3rd European Federation of Biotechnology Conference on Physiology of Yeasts and Filamentous Fungi (PYFF3), Helsinki- Finland (Poster presentation)

Söyler, B., Ögel, Z.B., "A life under stress; Growth, adaptation and enzyme production by Aspergillus in the presence of phenolics". April 12-14, 2007, International Symposium on Microbial Adaptation to Stress and Environment Marburg, Germany.

Söyler, B., Biely, P., Ögel, Z.B., "Heterologous expression of alpha-galactosidases from human pathogen *Aspergillus fumigatus*". Human Fungal Pathogens: Molecular Mechanisms of Host- Pathogen Interactions and Virulence", May 21-28, 2005, La Colle sur Loup, France

Söyler, B., Ögel, Z.B., Bakır, U. "Cloning and characterization of α-galactosidase genes from the human pathogen *Aspergillus fumigatus*", 9-11 September 2004, San Francisco, California, USA (Poster Presentation)

Söyler, B., Ögel, Z.B., "Cloning and characterization of *Aspergillus fumigatus* α-galactosidase genes". Bioinformatics II Summer School, 15-21 August 2004, Istanbul, Turkey (Poster Presentation).

Söyler, B., June 2004, "Cloning and Characterization of Industrially Important Alpha-Galactosidase Genes from the Human Pathogen *Aspergillus fumigatus*" (MSc. Thesis)

Söyler, B., Ögel, Z.B., Bakır, U., "Cloning studies for the heterologous production of *Aspergillus fumigatus* α-galactosidase", 13th Biotechnology Congress, 20-26 August 2003, Çanakkale, Turkey (Poster Presentation)

COURSES TAKEN

- "IRCA certified ISO 22 000 Lead Assesor Training", Ankara, February 20-25 2012
- "ISO 17043 and Proficiency Testing", Strengthening The Quality Infrastructure in Turkey Project, Ankara, 13-15 June 2011
- "Training for Measurement Uncertainty and ISO 13528 statistics", Strengthening The Quality Infrastructure in Turkey Project, Ankara, 16-18 June 2011
- "Real Time ile GDO Analizi (GMO Analysis using Real Time)", EuroFins-Ulusal Gıda Referans Laboratuvarı, Ankara, 7-8 December 2009
- GLP "Good Laboratory Practice" Applications, SWEDAC, Ankara, September 2009
- "Quality Management and Accreditation of PT/ILC Providers", EU MEDA Programme, TURKAK, Ankara, January 9-10 2007
- "Process Management and Improvement", KalDer, Ankara, September 18-19 2006
- GPBA 2006 Virtual Summer Session", Philadelphia, June 26-August 11 2006
- "Microarray Workshop", METU Central Laboratory Ankara, May 2006
- "Workshop on Accreditation with a Focus on Requirements Related to Quality and Technical Aspects for Inspection Bodies", BAM-ECA-TÜV Consortium, Ankara, June 28-30-July 1 2005
- "Quality Management System Basics", Türk Standardları Enstitüsü, Ankara, May 2-3 2005
- "Uncertainty Measurement and Traceability", TURKAK, Ankara, February 7 2005
- "TS EN ISO/IEC 17025 - General Requirements for the Competence of Calibration and Testing Laboratories", TURKAK, Ankara, February 8-9 2005

- "TS EN ISO/IEC 17025 – Documentation and Laboratory Quality Manual Preparation", TURKAK, Ankara, February 9 2005
- "Internal Auditing in Laboratories", TURKAK, Ankara, February 10-11 2005
- "Deney Laboratuvarlarında Kalite Güvence – TURKAK Denetçi Eğitim Prosedürü Modül G", TURKAK, Ankara, April 9-10 2003
- "Sampling Techniques - TURKAK Denetçi Eğitim Prosedürü Modül G", TURKAK, Ankara, April 7-8 2003
- "Training Course based on EA 3-05 and TURKAK Assesor Training Procedure Module F", TURKAK, Ankara, February 22 2003
- "Training Course based on EA 3-05 and DAR-3-EM-07: Framework Program to Train Assesors in Accreditation Procedures and TURKAK Assesor Training Procedure Module E2 – Assessment Under Stres Conditions", TURKAK, Ankara, November 13-14 2002
- "TURKAK Denetçi Eğitim Programı Modül G ve EA 3-05 Dokümanı", TURKAK, Ankara, October 5 2002
- ODTÜ PAL için düzenlenen "Ölçümlerin İzlenebilirliği ve Ölçüm Belisrziliği", TURKAK, ODTÜ Ankara, June 20 2005
- ODTÜ PAL için düzenlenen "TS ISO/IEC 17025 Standardı", TURKAK, Ankara, March 9-10 2002
- "Training Course based on EA 3-05 and DAR-EM 12: Framework Program to Train Assesors in Accreditation Procedures Part B and C", TURKAK, Ankara, February 4-6 2002