DOKUZ EYLUL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE ANTIOXIDANT RESPONSE MECHNASIMS OF *PHANEROCHAETE CHRYSOSPORIUM* DEPENDING MENADIONE STRESS FACTOR

by
Burcu TONGUL

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İZMİR

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We have read the thesis entitled **"THE ANTIOXIDANT RESPONSE MECHNASIMS OF *PHANEROCHAETE CHRYSOSPORIUM* DEPENDING MENADIONE STRESS FACTOR"** completed by **BURCU TONGUL** under supervision of **PROF. DR. LEMAN TARHAN** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

In order to identify the antioxidant response mechanism of *Phanerochaete Chrysosporium* against menadione, cells from stationary phase were treated with multiple concentration of menadione for different incubation times. Then the menadione-treated cells were investigated in terms of intracellular superoxide anion radical, hydroxyl radical and hydrogen peroxide levels, variations of SOD, CAT, G6PDH, NADH, NADPH oxidase enzyme activities, variations of energy metabolism and cell membrane peroxidation and protein oxidation levels and the results were compared with control.

The highest values of hydrogen peroxide and hydroxyl radical were observed two point two-fold and one point seven-fold higher than control for menadione treated samples, respectively. The investigated antioxidant enzymes, SOD, CAT and G6PDH, gave considerable response to menadione-induced oxidative stress. The highest activities were obtained five point four-fold, five point one-fold and two point five-fold higher than control for SOD, CAT and G6PDH enzymes, respectively.

In addition, NADH and NADPH oxidase enzymes that play important roles in formation of ROS were induced by menadione treatment and when compared with control the activities of these enzymes were observed four point nine-fold and five-fold higher than control.

In order to decide adequacy of antioxidant system against menadione-induced oxidative stress, damages of protein and membrane lipids were investigated. The result shows that although menadione induces the formation of ROS and oxidative stress, antioxidant system is able to resist against menadione-induced oxidative stress up to relatively high concentrations of menadione.

Keywords: Menadione, *Phanerochaete chrysosporium*, superoxide anion radical, hydrogen peroxide, hydroxyl radical, SOD, CAT, G6PDH, NADH oxidase, NADPH oxidase, ATP, ADP,AMP, LPO, Protein carbonyl content

PHANEROCHAETE CHRYSOSPORİUM UN MENADİON STRES FAKTÖRÜNE BAĞIMLI ANTİOKSİDAN CEVAP MEKANİZMALARI

ÖZ

Phanerochaete Chrysosporium un menadiona karşı geliştirdiği antioksidan cevap mekanizmasını belirlemek amacıyla, stasyoner fazda bulunan hücreler çeşitli konsantrasyonlardaki menadiona farklı inkübasyon saatlerinde maruz bırakılmıştır. Ardından menadiona maruz bırakılan hücreler; süperoksit anyon radikali, hidrojen peroksit ve hidroksil düzeyleri; SOD, CAT, G6PDH, NADH ve NADPH oksidaz enzimlerinin aktivite değişimleri, enerji metabolizmasındaki değişimler, Phanerochaete *Chrysosporium* da menadiona bağımlı lipit peroksidasyonu ve protein oksidasyonu açısından incelenerek kontrol ile kıyaslanmıştır.

En yüksek hidrojen peroksit ve hidroksil radikal düzeyleri kontrole kıyasla, sırası ile iki onda iki ve bir onda yedi kat bulunmuştur. İncelenen antioksidan enzimler, SOD, CAT ve G6PDH, menadion indüklü oksidatif strese karşı belirgin cevaplar vermişlerdir. En yüksek enzim aktiviteleri SOD, CAT ve G6PDH için kontrole kıyasla, sırası ile beş onda dört, beş onda bir ve iki onda beş kat daha yüksek çıkmıştır.

ROS üretiminde önemli rol oynayan NADH ve NADPH oksidaz enzim aktiviteleri de menadion muamelesinden etkilenmiştir. en yüksek aktiviteleri, kontrolden dört onda dokuz ve beş kat daha yüksek oldukları bulunmuştur.

Antioksidan sistemin, menadion indüklü oksidatif strese karşı yeterli olup olmadığına karar vermek amacıyla lipit peroksidasyonu ve protein oksidasyonu seviyeleri incelenmiştir. Sonuçlar, menadionun ROS oluşumunu ve oksidatif stresi indüklemesine rağmen *Phanerochaete Chrysosporium* un menadion-indüklü oksidatif strese karşı verdiği cevabın, onu menadion-indüklü oksidatif strese karşı koruyabildiğini göstermektedir.

Anahtar sözcükler: Menadion, *Phanerochaete chrysosporium*, süperoksit anyon radikali, hidrojen peroksit, hidroksil radikali, SOD, CAT, G6PDH, NADH oksidaz, NADPH oksidaz, ATP, ADP, AMP, LPO, Protein karbonil içeriği

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CHAPTER ONE
INTRODUCTION

1.1 Oxidative Stress

In physiological conditions there is a balance between reactive oxygen species (ROS) and antioxidant system. When organism expose to ROS antioxidant systems act as scavenger and protector in order to remove the disturbing effects of ROS.

When imbalance between antioxidant system and reactive oxygen species occurs at the side of ROS generation, deterioration of the structures and functions of the major component in cells as lipids, proteins and DNA occurs and this imbalance is defined as oxidative stress. If this deterioration is compensated by some metabolic regulations cells can perform their functional tasks but if damages aren't compensated the functions of cells will be lost and result in aging, cancer and other degenerative diseases.

1.1.1 ROS and ROS Generation

Radicals have high reactivity and relatively short half-life due to their unpaired electrons in the orbitals so they can react with the other molecules especially vital biomolecules as lipid, proteins and DNA in cells (Halliwell, 1994).

The most important class of radicals is oxygen-derived species in living systems (Miller, Buettner, & Aust, 1990). Oxygen-derived species can be defined as "reactive oxygen species" (ROS). ROS include either the radicals superoxide anion radical ($O_2^{\cdot}$), hydroxyl ($^{-}OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), and nitric oxide ($NO^{\cdot}$) or the non-radical hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), peroxynitride ($ONOO^{-}$), ozone (O_3), and H_2O_2 molecules (Aruoma, 1998).

Molecular oxygen (O_2) is a radical itself and the addition of one electron to molecular oxygen forms the superoxide anion radical ($O_2^{\bullet -}$) (Valko, Morris &

Cronin, 2005). The generation of $O_2^{\bullet-}$ mainly occurs in mitochondria that is responsible for ATP synthesis in cells (Cadenas & Sies, 1998). There is small electron leakage in the mitochondrial electron transport chain while energy transduction occurs (Kovacic, Pozos, Somanathan, Shangari & O'Brien, 2005; Valko, Izakovic, Mazur, Rhodes & Telser, 2004). The leak electrons transfer to oxygen and this transfer results in generation of $O_2^{\bullet-}$. Superoxide anion radical joins dismutation reaction in order to oxidize $O_2^{\bullet-}$ O_2 and reduce to H_2O_2 (Halliwell, & Gutteridge, 1999).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Dismutation is most rapid at the acidic pH values needed to protonate $O_2^{\bullet-}$ and will become slower at more alkaline pH values.

$O_2^{\bullet-}$ in aqueous solution can act as a reducing agent. For example, it reduces the haem protein cytochrome c:

$$\text{cyt c (Fe(III))} + O_2^{\bullet-} \longrightarrow O_2 + \text{cyt c (Fe(II))}$$

and the chloroplast copper-containing protein plastocyanin:

$$\text{plastocyanin (Cu(II))} + O_2^{\bullet-} \longrightarrow O_2 + \text{plastocyanin (Cu(I))}$$

Superoxide can also act as an oxidizing agent, it can oxidize ascorbate:

$$AH_2 + O_2^{\bullet-} \longrightarrow A^{\bullet-} + H_2O_2$$

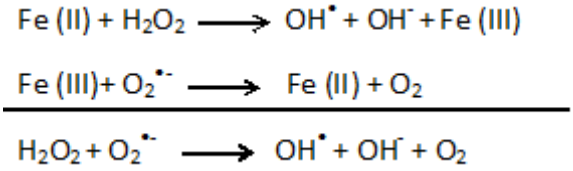
Superoxide does not oxidize NADH or NADPH at measurable rates. However, it can interact with NADH bound to the active site of the enzyme lactate dehydrogenase to form an $NAD^{\bullet}$ radical:

$$\text{enzyme-NADH} + O_2^{\bullet-} + H^+ \longrightarrow \text{enzyme-NAD}^{\bullet} + H_2O_2$$

In general $O_2^{\bullet-}$ in aqueous solution at pH 7.4 is not highly reactive. The direct biological damage that can be caused by $O_2^{\bullet-}$ is highly selective and often includes the reaction with other radicals as $NO^{\bullet}$ or iron ions in iron-sulphur proteins. The interaction between $O_2^{\bullet-}$ and iron ions is notably important for organisms. $O_2^{\bullet-}$ can reduce Fe(III) and also oxidize Fe(II). The former reaction may proceed through intermediate species as perferryl:

The values of the rate constants are much affected by binding ligands to the iron. For example Fe (III) bound to the chelating agent EDTA is still reduced by $O_2^{\bullet -}$ whereas Fe(III) attached to the chelators transferrin, lactoferrin or desferrioxamine is reduced much more slowly. Reduction of Fe(III) chelates of citrate, ADP by $O_2^{\bullet -}$ is also possible but the rate constants appear fairly low.

Reduction of Fe(III) by $O_2^{\bullet -}$ can accelerate the Fenton reaction, giving a superoxide-assisted Fenton reaction:



Due to presence of high SOD activity in mitochondria determination of occurrence of superoxide anion radical is considerable difficult but generation of superoxide radical is proved three decades ago (Loschen, & Flohe, 1971).

Hydroxyl radical ($OH^{\bullet}$) has fairly short half-life, approximately 10^{-9}, and it makes $OH^{\bullet}$ notably dangerous that can react with all molecules where exist in the environment the radical is produced (Pastor, Weinstein, Jamison, & Brenowitz, 2000).

Hydroxyl radical can be generated in biologically relevant systems by multiple reactions. One is Fenton reaction which is catalyzed by transition metals. In fact there is not any free iron in organism but under stress conditions, with the effect of superoxide; free irons are released from iron-containing molecules (Valko, Leibfritz, Moncola, Cronin, Mazura, & Telser, 2007). The released Fe^{+2} can participate in Fenton reaction which generates highly reactive hydroxyl radical;

$$Fe(II) + H_2O_2 \longrightarrow OH^{\bullet} + OH^{-} + Fe(III)$$

At the same time superoxide radical participates in the Haber–Weiss reaction

$$Fe(III) + O_2^{\bullet -} \longrightarrow Fe(II) + O_2$$

and in a result of this reaction hydroxyl radical is generated.

UV-induced hemolytic fission of the O-O bond in H_2O_2 also generates $OH^{\bullet}$ and this could conceivably happen to H_2O_2 generated in sunlight-exposed skin. In addition ·OH can be generated from ozone and during ethanol metabolism and peroxynitrous acid decomposition. Other sources of $OH^{\bullet}$ include ionizing radiation, the reaction between hypochlorous acid and superoxide anion radical, ultrasound, lithotripsy and freeze-drying ((Halliwell, & Gutteridge, 1999).

An important, biological relevant example of H-abstraction by hydroxyl is its ability to initiate lipid peroxidation. The reaction of hydroxyl radical with aromatic compounds often proceeds by addition (Kalpana, Srinivasan, Venugopal, & Menon, 2008).

Peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radical are another oxygen-derived radicals. Alkoxyl radical formed in biological systems often undergo rapid molecular rearrangement to other radical species.

The simplest peroxyl radical hydroperoxyl (HOO•) is the form of protonated superoxide. These radicals are generated as by product when lipid peroxidation chain reactions occur. $ROO^{\bullet}$ radicals oxidize ascorbate and NADH, the latter leading to $O_2^{\bullet -}$ formation in the presence of O_2:

$$ROO^{\bullet} + NADH \longrightarrow RO_2H + NAD^{\bullet}$$

$$NAD^{\bullet} + O_2 \longrightarrow NAD^{+} + O_2^{\bullet -}$$

$ROO^{\bullet}$ and $RO^{\bullet}$ radicals can abstract $H^{\bullet}$ from other molecules, a reaction important in lipid peroxidation. Some $ROO^{\bullet}$ breakdown to liberate $O_2^{\bullet -}$. For example when

glucose react with $OH^{\bullet}$, six different $ROO^{\bullet}$ radicals are formed, since $H^{\bullet}$ abstraction by $OH^{\bullet}$ can occur at any of the $OH^{\bullet}$ groups. Aromatic alkoxyl and peroxyl radicals tend to be less reactive, since electrons can be delocalized into the benzene ring.

Nitric oxide ($NO^{\bullet}$) include one unpaired electron. NO• can diffuse easily within cells. One-electron reduction would give nitroxyl anion, NO^{-}. nitroxyl is a reactive, short-lived species, it can react with $NO^{\bullet}$ to give nitrous oxide, N_2O and possibly hydroxyl radical

$$NO^{\bullet} + NO^{\bullet} \longrightarrow ONNO^{\bullet-}$$

$$ONNO^{\bullet-} + NO^{\bullet} \longrightarrow N_2O + NO_2^{\bullet}$$

$$ONNO^{\bullet-} + H^{+} \longrightarrow N_2O + OH^{\bullet}$$

Specific nitric oxide synthases (NOSs) metabolize arginine to citrulline with the formation of $NO^{\bullet}$ (Ghafourifar, & Cadenas, 2005).

During inflammatory processes, cells which are responsible for immun system produce superoxide anion radical and nitric oxide. Under these conditions nitric oxide and super oxide may react together in order to generate peroxynitrite (ONOO−), which has relatively high reactivity and can cause DNA fragmentation and lipid peroxidation (Carr, McCall, & Frei, 2000).

$NO^{\bullet}$ synthesized by the vascular endothelial cells that line the interior of blood vessels presumably diffuses in all directions, but some of it will reach the underlying smooth muscle, bind to guanylate cyclase and activate it. As a result more cyclic GMP is made, which lower intracellular free Ca^{+2} and relaxes the muscle, dilating the vessel and lowering blood pressure. Much of the $NO^{\bullet}$ generated in vivo I eventually lost by interaction with haem groups of haemoglobin.

Hydrogen peroxide is not a radical but can easily pass through the cell and oxidize the cellular compounds including energy-producing system at high concentrations. Several enzyme found in vivo can generate H_2O_2, including xanthin, urate and D-amino acid oxidases (Allegra, Reiter, Tan, Gentile1, Tesoriere1, & Livrea, 2003). In

addition many biological system that generates $O_2^{\bullet-}$ will also produce H_2O_2 by $O_2^{\bullet-}$ dismutation. H_2O_2 is only a weak oxidizing and reducing agent. H_2O_2 appears capable of inactivating a few enzymes directly, usually by oxidation of labile essential thiol groups in active site. As an example chloroplast fructose biphosphatase is inactivated in cells treated with H_2O_2.

Hypochlourous acid is not free radical too. When inflammation occurs hypochlorous acid is produced by myeloperoxidase (Weiss, 1989). Although its importance in bacterial killing by phagocytes is uncertain, HOCl has the ability of damaging biomolecules, both directly and by decomposing to form chlorine. HOCl addition can oxidize thiols, ascorbate, NAD(P)H and lead to chlorinatin of DNA bases, especially pyrimidines and tyrosine residues in proteins.

ROS can be generated by endogenous and exogenous sources. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation (Inoue, et al, 2003).

NADH and NADPH oxidase are responsible for ROS production as an endogenous source. The enzyme NAD(P)H oxidase plays a major role as the most important source of superoxide anion, especially for vascular and endothelial cells. NAD(P)H oxidase transfer electrons from NAD(P)H across the membrane and when this electrons encounter with molecular oxygen, generates superoxide anion radical.

Other endogenous sources of cellular reactive oxygen species are neutrophils, eosinophil and macrophages. Generation of reactive oxygen species including superoxide anion, nitric oxide and hydrogen peroxide is accreted due to increased oxygen uptake for activated macrophage (Stohs, &Bagchi, 1995). Neutrophils and macrophages can produce superoxide anion by means of NADPH oxidase which reduces oxygen to superoxide (El-Benna, Dang, Gougerot-Pocidalo, & Elbim, 2005). Superoxide is dismutated by superoxide dismutase to hydrogen peroxide, following the formation; hydrogen peroxide may be converted to hypochlorous acid by myeloperoxidase (Kettle, & Winterbourn, 2001; Winterbourn, Hampton, Livesey, &

Kettle, 2006). This hypochlorous acid can react with superoxide anion radical and generate hydrogen peroxide. These radicals provide killing bacteria effectively that is why they are important for immune system.

There can be disruption or uncoupling in the P450 catalytic cycle and due to the result of this situation superoxide anion and hydrogen peroxide can be produced (Gupta, Dobashi, Greene, Orak, & Singh, 1997).

Although hydrogen peroxide is weak agent besides other ROS when DNA, lipid and proteins incubated H_2O_2, oxidation may not appear even at millimolar level but H_2O_2 can diffuse easily into membranes and produce hydroxyl by reacting with copper and iron ions in the cells so this generated hydroxyl can damage the biomolecules instead of H_2O_2 (Spencer, Jenner, Chimel, Aruoma, Cross, Wu, & Halliwell, 1995).

1.1.2 Antioxidant Defence Systems

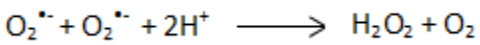
Only aerobic organisms survive in the presence of oxygen because these organisms have evolved a series of defence mechanism to protect themselves against toxicity of oxygen, ROS and other reactive metabolites (Cadenas, 1997). Antioxidant defence system includes enzymes as superoxide dismutase, catalase, peroxidases which catalytically remove free radicals and other reactive species; and includes non-enzymatic proteins as transferrins, haptoglobins, haemopexin and metallothionein which interact with pro-oxidants such as iron ions, heat shock proteins that protect biomolecules against damages especially oxidative damages; ROS and RNS scavengers having low-molecular mass as glutathione, α-tocopherol, ascorbic acid (Halliwell, & Gutteridge, 1999).

1.1.2.1 Enzymatic Antioxidant System

Enzymatic antioxidant system includes various enzymes as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR).

These enzymes have the ability of catalytically removing free radicals and other reactive species.

1.1.2.1.1 Superoxide dismutase (SOD) EC1.15.1.1. Superoxide dismutase is a metallo enzyme and catalyses the dismutation of superoxide to oxygen and H_2O_2 that is less reactive than superoxide anion radical.



SOD has several isoforms. Cu/ZnSOD is present in virtually all eukaryotic cells. Cytosol, lysosomes, peroxisomes, nucleus and mitochondrial membranes have been reported to contain some Cu/ZnSOD (Halliwell, & Gutteridge, 1999).

Cyanide is a powerful inhibitor of Cu/ZnSOD. These enzymes are also inactivated on incubation with diethyldithiocarbamate a compound that binds to the copper and removes it from active sites.

Manganese SOD is not inhibited by cyanide or diethyldithiocarmate but it is inactivated by chloroform plus ethanol. Despite these differences, MnSOD catalyse same reaction as Cu/ZnSOD. MnSOD are widespread in bacteria, plants and animals. In most animal tissue and yeast, MnSOD is largely located in the mitochondria (Halliwell, & Gutteridge, 1999). The relative activity of MnSOD and Cu/ZnSOD depend on the tissue and on the species; one obvious variable is the number of mitochondria present. Mammalian erythrocytes, with no mitochondria contain no MnSOD; MnSOD is about 10% of total SOD activity in rat liver.

Iron containing SOD (FeSOD) usually contains two protein subunits although some tetrameric enzymes have been described. The dimeric enzymes usually contain one or two ions of iron per molecule of enzyme. FeSOD show decreased activity at high pH values when compared to pH 7.0 and are not inhibited by CN^-. The rate constant for reaction with superoxide anion radical is lower for FeSOD than for the other types of SOD. Some bacteria contain both FeSOD and MnSOD, whereas others contain only one. No animal tissue has been found to contain FeSOD, but some

higher plant tissues have. Mitochondria from mustard leave apparently contain Cu/ZnSOD in the intermembrane space, and MnSOD in matrix, but the FeSOD appears to be located in the chloroplast.

In human there are three isoforms of SOD; cytosolic Cu/Zn SOD, Zn-SOD, mitochondrial MnSOD, and extracellular SOD (EC-SOD).

1.1.2.1.2 Catalase (CAT) EC 1.11.1.6. Catalase is a water-soluble enzyme and catalyses the reaction of hydrogen peroxide to water and molecular oxygen Qi, Hauswirth, & Guy, 2007). Most aerobic cells contain catalase activity (Halliwell, & Gutteridge, 1999).although a few do not, such as *Bacillus popilliae*, *mycoplasma pneumonia*, *Euglena*. A few anaerobic bacteria contain catalase but most do not. Catalase is present in blood, bone marrow, liver, kidney, and mucous membrane in high quantity. Catalase in erythrocytes may help protect them against H_2O_2 generated by dismutation of superoxide anion radical generated by haemoglobin autoxidation. The brain, heart and skeletal muscle contain lower levels of catalase than other tissues (Halliwell, & Gutteridge, 1999). Mitochondria, chloroplast and endoplasmic reticulum contain little catalase. In cases where the low rate of formation of H_2O_2, catalase reduces H_2O_2 to H_2O with peroxidative reaction. In the high rate of formation of H_2O_2, catalase reduces H_2O_2 to H_2O and oxidizes other H_2O_2 to O_2 catalytically.

$$H_2O_2 + AH_2 \longrightarrow 2H_2O + A$$

$$H_2O_2 + H_2O_2 \longrightarrow 2\,H_2O + O_2$$

1.1.2.1.3 Glutathione peroxidase family (GPX) EC 1.11.1.19. Glutathione peroxidases (GPX) remove H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione, GSH (Halliwell, & Gutteridge, 1999).

$$H_2O_2 + 2GSH \longrightarrow GSSG + H_2O$$

Glutathione peroxidases are not generally present in higher plants or bacteria, although they have been reported in a few algae and fungi. GSH, their substrate, is a

low molecular mass thiol-containing tripeptide. It present in animals, plants and many aerobic bacteria at intracellular concentration that are often in millimolar range, but rarely is it present in anaerobic bacteria. In all cases the peroxide group is reduced to alcohol. Glutathione peroxidases cannot act upon fatty acid peroxides esterified to lipid molecules in lipoproteins or membranes: they have to be first released by the action of lipase enzymes.

The ratio of reduced to oxidized glutathione (GSH/GSSG) in normal cells are high so there must be a mechanism for regeneration to GSH. Glutathione reductase enzymes are responsible for this process:

$$GSSG + NADPH + H^{+} \longrightarrow 2\,GSH + NADP^{+}$$

1.1.2.1.4 The Glutathione S-Transferase Superfamily (GSTs) EC 2.5.1.18. Glutathione is also involved in the metabolism of herbicides, pesticides and xenobiotics generally in both animal and plant tissues ((Halliwell, & Gutteridge, 1999). Many xenobiotics supplied to living organisms are metabolized by conjugation with GSH, catalysed by glutathione S-transferase (GST) enzymes:

$$RX + GSH \longrightarrow RSG + HX$$

Liver is especially rich in these enzymes and resulting glutathione conjugates are often excreted into bile using ATP-dependent glutathione S-conjugate " efflux pump"; the same pumps are involved in the export of GSSG when liver is subjected to oxidative stress (Halliwell, & Gutteridge, 1999). Compounds metabolized by GST in animals include chloroform, organic nitrates, bromobenzene, aflatoxin, DDT, naphthalene and paracetamol. The presence of large amounts of such xenobiotics can decree hepatic GSH concentrations, thereby impairing the antioxidant defence capacity of the liver. Some glutathione transferases can metabolize aldehydes produced during lipid peroxidation, as 4-hydroxynonal.

Some GSTs show a glutathione-peroxidas-like activity, with organic hydroperoxides, which was formerly called non-selenium glutathione peroxidase. They catalyse reaction of organic peroxides with GSH to form GSSG and alcohols.

All eukaryotes have multiple cytosolic and membrane-bond GST isoenzymes, each with distinct substrate specificities and other properties (Halliwell, & Gutteridge, 1999). As well as their catalytic functions, many GSTs appear to serve as intracellular carrier proteins for haem, bilirubin, bile pigments and steroids which bind non-enzymatically to the proteins.

1.1.2.1.5 Glucose-6-phosphate dehydrogenase (G6PD) E.C 1.1.1.49. Glucose-6-phosphate dehydrogenase is the rate-limiting enzyme of the pentose phosphate pathway (PPP) (Fujita, Hirao, & Takahashi, 2007). G6PD is responsible for generating NADPH which is mainly used to regenerate GSH.

Recent results have showed that G6PD has the protective role in the eukaryotic cells which have alternative routes for the production of NADPH. The study has showed that the mutant in G6PD gene of Saccharomyces cerevisiae is more sensitive than non-mutant Saccharomyces cerevisiae because of depletion in the intracellular pool of GSH (Nogae, & Johnston, 1990). In the study conducted on mouse ES cells similar results have shown (Pandolfi, Sonati, Rivi, Mason, Grosveld, & Luzzatto, 1995). Oxidative stress resulting in increasing superoxide anion radical or decreasing the amount of GSH causes the gene expression of G6PD. Because of depending on G6PD to provide the equilibrium of GSH, G6PD is known as an antioxidant enzyme.

1.1.2.2 Non-enzymatic Antioxidant System

1.1.2.2.1 Glutathione (GSH). Glutathione is main thiol antioxidant which has several functions in antioxidant system (Masella, Benedetto, Vari, Filesi, & Giovannini, 2005). Apart from its role as a cofactor for the glutathione peroxidase family, GSH is involved in many other metabolic processes, including ascorbic acid metabolism, maintaining communication between cells, and in generally preventing protein –SH groups from oxidizing and crosslinking (Halliwell, & Gutteridge, 1999). It also seems involved in intracellular copper transport (Halliwell, & Gutteridge, 1999). GSH can chelate copper ions and diminish their ability to generate free radicals, or at least to release radicals into solution (Halliwell, & Gutteridge, 1999).

GSH is a radio protective agent and a cofactor for several enzymes in different metabolic pathways, including glyoxylases (Halliwell, & Gutteridge, 1999) and enzymes involved in leukotriene synthesis.

Glutathione also play a role in protein folding and degradation of proteins with disulphide bonds, such as insulin.

In vitro, GSH can react with $OH^{\bullet}$, HOCl, peroxynitrite,$RO^{\bullet}$, $RO_2^{\bullet}$, carbon-centred radicals. Its reaction with free radicals will generate thiyl ($GS^{\bullet}$) radicals. $GS^{\bullet}$ radicals can generate superoxide anion radical by the reaction:

$$GS^{\bullet} \xrightarrow{GS^-} GSSG^{\bullet-} \xrightarrow{O_2} GSSG + O_2^{\bullet-}$$

Protein sulphydrls which are involved in DNA repair and expression system are preserved in nucleus by GSH through stabilization of redox state of these proteins (Ji, Akerboom, Sies, & Thomas, 1999).

The reaction of glutathione with the radical R• can be represented as (Karoui, Hogg, Frejaville, Tordo, & Kalyanaraman, 1996):

$$GSH + R^{\bullet} \longrightarrow GS^{\bullet} + RH$$

For the formation of non-radical product, thiyl radicals ($GS^{\bullet}$) can dimerise and generate oxidized glutathione (GSSG):

$$GS^{\bullet} + GS^{\bullet} \longrightarrow GSSG$$

Oxidized glutathione GSSG is gathered the cell and the ratio of GSH/GSSG is important for being criteria of oxidative stress (Hwang, Sinskey, & Lodish, 1992).

GSSG can react with protein sulphydryl groups and as a result protein–glutathione disulphides may occur so many enzymes can be damaged by too high concentration of oxidised glutathione (GSSG)

$$GSSG + \text{protein-SH} \rightleftharpoons \text{protein-SSG} + GSH$$

1.2.2.2.2 The Thioredoxin System. The Thioredoxin System contains two adjacent –SH groups in its reduced form that are converted to a disulphide unit in oxidized thioredoxin which is undergoing redox reactions with multiple proteins (Nakamura, & Yodoi, 1997) :

$$\text{Thioredoxin-(SH)}_2 + \text{Protein-S}_2 \longrightarrow \text{Thioredoxin-S}_2 + \text{Protein-(SH)}_2$$

The regeneration of the disulphide to the dithiol form is catalysed by thioredoxin reductase (TR), the source of electrons is NADPH:

$$\text{TR-S}_2 + \text{NADPH} + \text{H}^+ \longrightarrow \text{TR-(SH)}_2 + \text{NADP}^+$$
$$\text{Thioredoxin-S}_2 + \text{TR-(SH)}_2 \longrightarrow \text{Thioredoxin-(SH)}_2 + \text{TR-S}_2$$

Thioredoxin also responsible for controlling some transcription factors with a redox regulation mechanism which affect cell proliferation and death.

1.2.2.2.1 Vitamin C .Vitamin C is a very important and powerful antioxidant. Because of being water-soluble antioxidant it operates in aqueous environment of cells. Besides fulfil its duty with antioxidant enzymes it operates co-ordinately with Vitamin E and the carotenoids. Vitamin C regenerates α-tocopherol (Vitamin E) which exposed to radicals in membranes and lipoproteins (Kojo, 2004; Carr, & Frei, 1999).

It has been reported that vitamin C is decreased the risk of being stomach cancer (Knekt, et al, 1991). It is thought that the positive effect of vitamin C may be depending on the inhibitory effects of vitamin C in the formation of N-nitroso compounds. It is also reported that Vitamin C protects the organism against lung and colorectal cancer.

Although in vivo studies show contrary tendency, in vitro studies show that Vitamin C does not affect the lipid peroxidation depend on transition metals neither effect nor inhibits transition metals- dependent lipid peroxidation. By contrast with this situation under unphysiological conditions Vitamin C can lead the ion-dependent formation of hydroxyl radical (Smith, Harris, Sayre, Beckman, & Perry, 1997).

1.2.2.2.2 Vitamin E. Vitamin E is a fat-soluble enzyme and has eight isoform. α-tocopherol is notably active form of Vitamin E and known as a major membran-bound antioxidant (Burton, & Ingold, 1989).

It mainly protects cells from lipid peroxidation (Pryor, 2000). α-tocopherol and ascorbate work together in a cyclic-type of process. While α-tocopherol acts as an antioxidant α-tocopherol is transformed to α-tocopherol radical by labile hydrogen from lipid or lipid peroxyl radicals. The mission of vitamin C in this process is regenerating of α-tocopherol (Kojo, 2004).

1.2.2.2.3 Carotenoids. Carotenoids are a group of coloured pigment that are widespread in plant tissue. They are also found in some animals and certain bacteria. Carotenoids from the diet can be found in the tissues of humans and some other mammals, but many other animals do not absorb them. In humans the largest amounts of carotenoids are found in adipose tissue and liver.

In plants, carotenoids play a key antioxidant role, helping to prevent the formation of ROS, especially singlet O_2 formed during photosynthesis. Indeed, β-carotene administration is protective against light-induced skin damage in patient with porphyria

In vitro studies have shown that β-carotene inhibits peroxidation of simple lipid systems at low O_2 concentration, but not at high O_2 concentration. However, studies with LDL show that β-carotene does not protect them against peroxidation whatever the O_2 concentration (Halliwell, & Gutteridge, 1999).

Although carotenoids are powerful quenchers/scavengers of singlet O_2, how important this would be to healthy animals is uncertain. Lycopene, one of the carotenoids appears to be the best singlet oxygen quencher in vitro. Exposure the sunlight can decrease carotenoids level in plasma and skin, and scavenging by carotenoids could be important in the eye.

In vitro studies also show the potential of carotenoids to act as free-radical scavengers (Halliwell, & Gutteridge, 1999). There have been suggestion that vitamin A can scavenge some free radicals in vitro.

Oxidizing radicals can react with carotenoids by electron transfer, for example nitrogen dioxide react with β-carotene so a radical cation is produced

$$NO_2^{\bullet} + Car \longrightarrow Car^{\bullet+} + NO_2^{-}$$

Possible fates of $Car^{\bullet+}$ include dismutation

$$2Car^{\bullet+} \longrightarrow Car + Car^{+2}$$

and if $Car^{\bullet+}$ is present at a membrane surface to interact with hydrophilic ascorbate the reaction with ascorbate can occur

$$Car^{\bullet+} + Asc \longrightarrow Car + Asc^{\bullet} + H^{+}$$

Carotenoids can also react with peroxyl radical, hydroxyl radical and thiyl radicals.

1.2.2.2.4 Heat Shock Proteins. When cells expose to oxidative stress however lots of protein level may be decreased the concentration of several proteins may be increased (Kristal, et all., 1997). This enhancement is independent from type of stress (Diller, 2006). Heat shock proteins act as molecular chaperones which regulate the functions of other protein through binding them, adjusting their function, transport and folding state (Lenaerts, et al., 2007)

1.2.2.2.5 Transferrins. Transferrins bind free metal ions in case of not stimulating free radical reactions (Gutteridge, Quinlan, & Evans, 1994). Transition metals are essential for numerous biological processes as DNA, RNA or protein synthesis, being cofactors of many enzymes. When amount of these metals is decreased the metabolic processes will be disturbed (Jime´nez Del Rı´o & Ve´lez-Pardo, 2004) But if cellular proteins obviate to bind transition metals because of collection of excessive amount of these metals in tissue it can be cytotoxic (Khan, Dobson, & Exley, 2006; Lo´pez, et al., 2006; Sayre, et al, 2005; Yu, Yang, & Wang, 2006). At this point the antioxidant properties of transferrins come into prominence.

1.1.3 The Meaning of Oxidative Stress?

The imbalance between oxidants and antioxidants occurs at the side of oxidant causes oxidative stress. Due to insufficiency of prevention and repair mechanisms or production of excessive amount of ROS, ROS can damage the most important constituent of cells such as lipids, proteins and DNA.

1.1.3.1 Oxidative Damage to Lipids: Lipid Peroxidation

Lipid peroxidation has been defined as "the oxidative deterioration of polyunsaturated lipids" by A. L. Tappel. Polyunsaturated fatty acids (PUFAs) contain two or more double bounds between Carbon-Carbon elements (Halliwell, & Gutteridge, 1999).

Some proteins are loosely attached to the surface of membranes but most are tightly attached, being partially embedded in the membrane, located in the membrane interior or sometimes traversing membrane. Because of this location of proteins in membranes lipid peroxidation can damage to membrane proteins (Halliwell, & Gutteridge, 1999).

Lipid peroxidation has three stages as all chain reactions: initiation, propagation, and termination. Initiation of lipid peroxidation occurs through abstraction a hydrogen atom from a methylene group. Fatty acids which have one or no double bounds are more resistant to such attacks than PUFAs (Halliwell, & Gutteridge, 1999). Because the presence of double bound weakens the C-H bonds on the adjacent carbon atom to the double bond (Gutteridge, 1995). This situation cause the sensitivity of polyunsaturated fatty-acid side chains of membrane lipids to peroxidation.

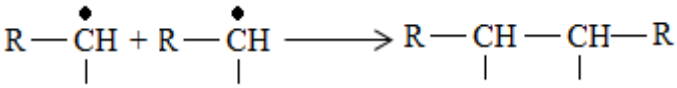
Carbon-centred radical occurs with the abstraction of a hydrogen atom from fatty acid and molecular rearrangement then this radical combines with oxygen and generate peroxyl radical which has the ability of abstracting hydrogen atom from

other fatty acids by itself in this case the chain reaction starts. If chain breaking antioxidants do not collide with fatty acid chain reaction adducts peroxidation can continue until substrates finish.

Hydroxyl radical can initiate peroxidation and generate C-centred radical. The hydroxyl which exists outside a membrane can also attack extrinsic proteins or “head groups of phospholipids”. Therefore lipid peroxidation can be prevented by scavengers of hydroxyl radicals as mannitol and formate (Halliwell, & Gutteridge, 1999). On the other hand, superoxide is insufficiently reactive to abstract H from lipid, its charge prevent it from entering the hydrophobic interior of membranes. The protonated form of superoxide, hydroperoxyl, is more reactive and being uncharged give it the ability of hydrogen atom from some isolated fatty acid as linoleic, linolenic and arachidonic acids.

In addition some researches have reported hydroperoxyl-dependent peroxidation of liposomes and lipoproteins (Halliwell, & Gutteridge, 1999). As well as RO•, RO_2•, OH• and HO_2•, several iron-oxygen complexes have been suggested to be capable of abstracting H and initiating peroxidation (Halliwell, & Gutteridge, 1999).

After abstraction of hydrogen atom from –CH2– groups an unpaired electron remains on the carbon. The carbon radical is usually stabilized by a molecular rearrangement to form conjugated diene. Carbon radicals can undergo various reactions for example; if two of them collided within membrane they may cross-link the fatty acid side-chains:



However, the most likely fate of carbon radicals under aerobic conditions is to combine with O_2. Reaction with O_2 results in the formation of peroxyl radical. Peroxyl radicals are able abstract H from another lipid molecule too; this is the propagation stage of lipid peroxidation. The peroxyl radical combines with the hydrogen atom in order to abstract it and lipid hydroperoxide (LOOH) is formed.

Indeed an alternative fate of peroxyl radical is to form cyclic peroxides. On the other hand lipid hydroperoxyl may react with Fe^{+2} and generate alkoxy radicals (RO•) alkoxyl radical can abstract H from PUFAs in order to propagation of lipid peroxidation by generating an alkyl radical.

In the termination stage the chain reaction is terminated by reaction of peroxyl with another radical or an antioxidant as a result of scavenging of peroxyl.

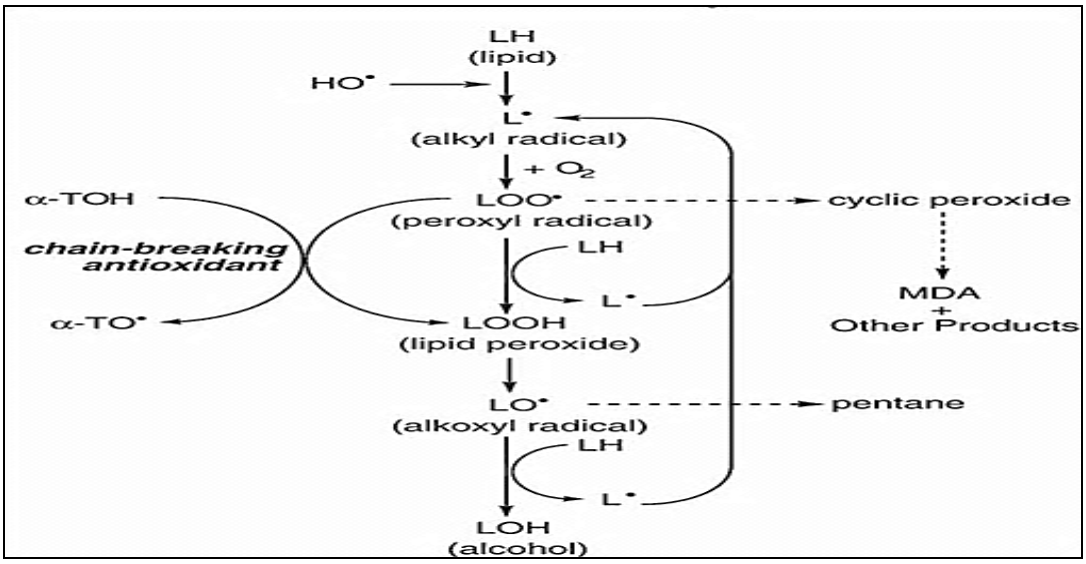


Figure 1.1 Lipid peroxidation chain reaction.

1.1.3.2 Oxidative Damage to Protein

ROS causes the oxidation of amino acid residue side chain, formation of cross linkages between two amino acids either of the same or of two different proteins and protein fragmentation with the result of oxidation of the protein backbone. In a result of this effect the structure of proteins can be differentiated, and the function of proteins can be disrupted and the catalytic activity of enzymes can be reduced or vanished (Stadtman, 1993; Naskalski, & Bartosz, 2000).

All amino acid residues of proteins are exposed to oxidation by hydroxyl as well as sulfur-containing amino acid residues are notably sensitive to oxidation by all forms of ROS. In order to redisintegrate these residues to their unmodified form, disulfide reductase and MeSOX reductase exist in most biological systems besides

the ROS scavenger system which prevent the formation of irreversible oxidation products. Aromatic amino acid residues are also target of ROS attacks.

Direct oxidation of lysine, arginine, proline and threonine residues may generate carbonyl derivatives besides α-amidation pathway and oxidation of glutamil side chains. Carbonyl groups are also generated by reacting with aldehydes which are produced during lipid peroxidation (Schuenstein, & Esterbauer, 1979). If aliphatic side chains of amino acid residues are removed via β-scission, carbonyl groups bound to proteins can be stabile as the residual structures. Therefore protein carbonyls are suitable as valid biomarkers for determination of protein oxidation

Oxidation of protein backbone is initiated with the abstraction of α- hydrogen atom from an amino acid residue by hydroxyl radical and this abstraction results in formation of C-centred radical. The formed C-centred radical rapidly reacts with O_2 to form an alkylperoxyl radical which cause the formation of alkoxyl radical and alkoxyl radical may be converted to hydroxyl protein derivatives. In the absence of oxygen, alkylperoxyl radical cannot be formed and the carbon-centred radical may react with other carbon-centred radical in order to form a protein-protein cross-linked derivatives. The generation of alkoxyl radical can cause the cleavage of peptide bond through both diamide and α-amidation pathways.

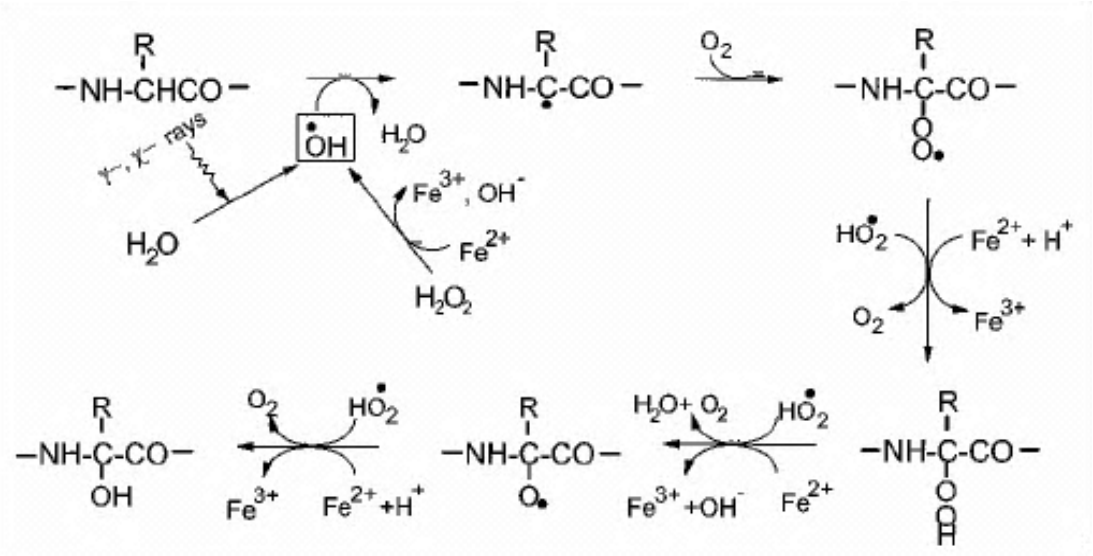


Figure 1.2 General aspect of protein oxidation.

1.1.3.3 Oxidative damage to DNA

Free radicals, especially hydroxyl radical, react with DNA through addition or abstraction. Hydroxyl radical can be added to double bonds of heterocyclic DNA bases and abstracts an H atom from the methyl group of thymine and also from each of the C–H bonds of 2′-deoxyribose (Sonntag, 1987). Further reactions of thus-formed C- or N-centred radicals of DNA bases and C-centred radicals of the sugar moiety result in a variety of final products (Evans, Dizdaroglu, & Cooke, 2004).

1.1.4 Effects of Oxidative Stress on Health

The endogenous and exogenous factors of ROS generation may be major factor of several degenerative diseases (Halliwell, 1994; Weisburger, 2001) due to the accumulation of damage on biomolecules as lipids, protein and DNA.

Lipid peroxidation is seen as the main reason of cancer, heart disease and aging and plays an important role in the pathogenesis of many diseases such as inflammation, atherosclerosis, alcoholic liver disease and trauma. In the result of intracellular protein oxidation, modulator functions of cellular metabolism can be changed (Kweon, Park, Sung, & Mukhtar, 2006) and in the result of oxidative modification of DNA bases causes mutation and alters the function of gene so cancer may occur (Morimura, et al., 2004; Nakabeppu, et al., 2004). Oxidative damage and mutations in mitochondrial DNA cause to mitochondrial dysfunction that result in a variety of disorders. Herewith these alterations, ROS influence cell cycle mechanism and ultimately lead to carcinogenesis (Horton, & Fairhurst, 1987).

The accumulation of oxidized protein and their by products such as protein aggregates and protein crosslinks is known as major factor for some disease as atherosclerosis, diabetes, Alzheimer's disease, Parkinson's disease, hepatitis, and rheumatic arthritis. Although protein oxidation can result in a loss function this loss of function does not affect whole cells in a deleterious consequence. Key enzymes of glycolytic pathway are inactivated under oxidative stress on the other hand

antioxidant responses are induced by NADPH from pentose phosphate pathway (Cabiscol, & Ros 2006). There are endogenous degradation or repair systems in order to annihilate the oxidative protein modifications in metabolism but in some cases this systems fall behind the formation of oxidative protein modifications so the oxidative protein modifications cause diseases as the above-mentioned.

Biochemical changes that occur during hypoxia due to the decrease in oxygen pressure can cause oxidative damage to the cell. It is seen that the undamaged cells during hypoxia are exposed to serious damage during reperfusion (Murray, Granner, Mayes, & Rodwell, 1991)

There are three basic mechanisms to explain the damage after ischemia reperfusion; the increase of enzymes and substrates that produce ROS, the increase of mitochondrial ROS production and the increase of ROS depends on the activated neutrophils.

It is seen in rats that the reactive oxygen species that are produced in case of the acute or short term hypoxia cause lipid peroxidation. the Studies on rats show that because of hypoxia lipid peroxide level are increased at brain, liver, aorta and serum although liver protect itself from the effect of hypoxia.

Rheumatoid arthritis is also known as free radical-induced disease. At the RA patients, ROS which are produced by the active neutrophils that presents at the rheumatoid anastomosis cause the breaking of hyaluronic acid polymers so this breaking results in increasing the viscosity of synovial fluids and damage to collagen tissues.

Reactive oxygen species have a great importance for carcinogenesis because of causing the changes in DNA sequence and gene expression. Superoxide anion radical and hydrogen peroxide play a role in triggering the formation of cancer through causing breakage the DNA chain and activating oncogenes (Hall, Holmin, & Barton, 1996; Hiraku, & Kawasaki, 1996).Superoxide and organic peroxides are reported to

be effective in tumour development phase. One of the mechanisms of the formation of cancer is ionizing radiation that causes the production of radicals in tissues. The released Ca^{+2} ions in a result of disruption of membrane can activate the Ca^{+2}-dependent proteases and nucleases.

One of the DNA repair enzymes poly (ADP ribose) synthetase is stimulated by DNA breakage and NAD^{+} serves as substrate for this enzyme increases. With enzyme activation and the increase in NAD^{+}, the ribolisation of Poly ADP increases in chromosome so gene expression is modulated. But if the poly ribose synthetase enzyme insufficiency occurs, cancer formation is triggered by ROS. Inflammation and phagocytes also play a role in the pathogenesis of cancer. The activation of leukocytes that activating the carcinogen during the respiratory burst is appeared as another factor for increasing incidence of cancer (Duthie, Colhns, Ross, & Ma, 1996).

1.1.5 Biomarkers of Oxidative Stress

A number of biomarkers of oxidized lipids, proteins and DNA have been found in order to exhibit the effects of oxidative stress on health. The successful determination of oxidation of biomolecules depends on the quality of sampling process.

As a result of lipid peroxidation a number of products are generated containing carbonyl derivatives (Benedetti, Casini, Ferrali, & Comporti, 1979). The unstable hyperoxides of fatty acids which are generated by PUFA peroxidation converts to more stable carbonyl groups. The most important carbonyls which can be detected in biological tissues are hexanal, 4-hydroxy-2, 3-transnonenal, propanol, 4-hydroxy-2, 3-transhexanal (Esterbauer, Cheeseman, Dianzani, Poli, & Slater, 1982). Because of being stabile aldehydes and their metabolites, they are convenient for detection of lipid peroxidation. Lipid peroxidation end up with the reactions between lipid hydroperoxide and aldehyde or other carbonyl compounds and some end products are generated in a result of this stop reaction (Thomas, & Aust, 1986). One of the end products that can be easily identified and used in the measurement of oxidative stress

is malondialdehyde (MDA) molecule. MDA which can be react with two amine groups in order to form schiff base is a bifunctional aldehyde (Parantainen, Vapaatalo, & Hokkanen, 1986).

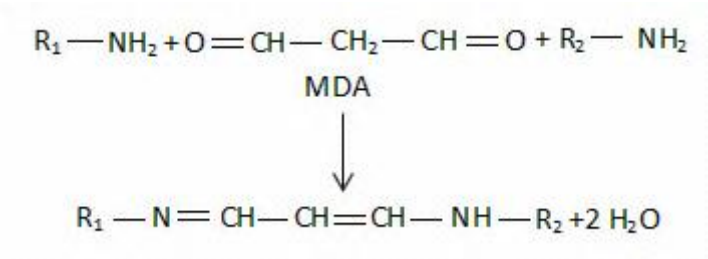


Figure 1.3 The Formation of Schiff Base from MDA.

With the formation of MDA, ion transport system can be damaged and enzyme activities may decrease. Due to having the ability of diffusing easily in membranes MDA can react with DNA bases and disrupt the structure and function of DNA (Draper, Mcgirr, & Handley, 1986).

Malondialdehyde, emerged as the product of in-vivo enzymatic LPO also occur as a product of prostaglandin metabolism, as the product of cyclo-oxygenase reaction.

The conditions as vitamin E deficiency, exposure of iron or carbontetrachloride and richness in PUFA enhance the level of MDA (Halliwell, & Gutteridge, 1988).

MDA levels which can be detected with Tiobarbutiric acid (TBA) correlates with the prevalence of lipid peroxidation (Cochrane, 1991). Because of having the ability of TBA to react with other substances as bilirubin lipid peroxidation level is expressed as TBARs (Knight, Pieper, & Clellan, 1988). This method is the most common used to measure the levels of lipid peroxide by spectrophotometric method. MDA reacts with TBA to form a pink-colored complex and this complex is measured at 532 nm by spectrophotometer.

Hydrolysis of proteins is required to liberate nitro tyrosine for the latter assay. Nitrotyrosine metabolize are excreted in human urine (Halliwell, & Gutteridge, 1999) although the possible confounding effect of dietary nitrotyrosine and of dietary nitrate/nitrite requires evaluation.

Addition of HOCl to proteins leads to multiple changes, including oxidation of thiol groups and methionine residues and chlorination of –NH2 groups. In addition, the aromatic ring of tyrosine can be chlorinated. Chlorination can also be caused by NO_2Cl. It is possible that chlorotyrosines may be marker of attack upon proteins by reactive chlorine species.

ROS produce a multiplicity of changes in proteins (Halliwell, & Gutteridge, 1999), including oxidation of –SH groups, hydroxylation of tyrosine and phenylalanine, conversion of methionine to its sulphoxide and generation of protein peroxides. Several assays for damage to specific amino acid residues in protein developed. The levels of anyone of these products in proteins could in principle be used to assess steady-state levels of oxidative protein damage in vivo. *Ortho*-tyrosine and dityrosine have measured in hair from *Homo tirolensis (*Halliwell, & Gutteridge, 1999*)* although whether they were formed during life or after death as a result of exposure of the body to sunlight or transition metals is unknown.

The carbonyl assay is a general assay of oxidative protein damage (Halliwell, & Gutteridge, 1999). It is based on the fact that several ROS attack amino acid residues in proteins to produce product with carbonyl group, which can be measured with suitable probes.

Classically, protein carbonyls are measured by the reaction between carbonyl groups and 2,4, dinitrophenylhydrazine (DNPH). However its applicability to biological sample is limited by the low inherent sensitivity of a direct spectrophotometric (Cao, Cutler, 1995). Thus, more sensitive Enzyme-Linked Immunosorbent assays (ELISA) have been developed (Alamdari, et al., 2005; Buss, Sluis, Domigan, & Winterbourn, 1997) for the measurement of protein carbonyls. But in this method, different results appear according to the kits. To resolve this dilemma, an alternative direct method having adequate sensitivity for biological systems was sought. Recent reports (Chaudhuri, et al., 2006; Fujita, Hirao, & Takahashi, 2007) have used a highly fluorescent compound, fluorescein 5-

thiosemicarbazide (FTC) that specifically reacts with carbonyl groups in oxidized proteins and not in oxidized lipids.

8-OH-G and 8-OH-dG are the products most frequently measured as indicators of oxidative DNA damage. This is sensible, as these products arise when several different ROS attack DNA. It should be noted, however that addition of HOCl or $ONOO^-$ to DNA can destroy 8-OHdg so its levels are not a quantitative estimate of oxidative DNA damage. Analysis of 8-OHdG using HPLC coupled to electrochemical detection is highly sensitive technique that is frequently used (Halliwell, & Gutteridge, 1999). Gas chromatography-mass spectrometry (GC-MS) with selected ion monitoring (SIM) has also been used to characterize oxidative DNA base damage by identification of a spectrum of products, including 8-OHdG. On the other hand comet assay (Halliwell, & Gutteridge, 1999) has been modified to allow an assessment of oxidative base damage.

1.1.6 Properties of Menadion that is Used as a Stressor Agent

Menadione (2-methyl-1,4-naphthoquinone, vitamin K3) was widely used in the prophylaxis of haemorrhagic disease of the new-born (Gasser,1959). On the back of, it was investigated that menadione is an artificial electron carrier in the treatment of certain mitochondrial myopathies (Eleff, et al,1984; Wijburg, et al., 1989) and adjunct to other drugs in cancer chemotherapy (Margolin, et al., 1995; Taper, & Roberfroid, 1992; Tetef, et al., 1995; Gold,1986; Chlebowski, Akman, & Block, 1985; Chlebowski, et al., 1983). On the other hand it is known that menadione induces oxidative stress in cells and tissue and damages them through two mechanisms. First, it increases oxidation of NADH and NADPH so induces the formation of ROS through redox cycling (Gutierrez, 2000). Secondly, menadione can conjugate with glutathione result in reducing this radical scavenger (Zadzinski, Fortuniak, Bilinski, Grey, & Bartosz, 1998).

Menadione is a quinon-containing compund which can be used as an agent for studies of oxidative damage (Chiou, & Tzeng, 2000). Menadione can be reduced by

either one or two electron intakes. Semiquinone radical is generated with one-electron reduction of quinone then because of being unstable semiquinone reacts with oxygen and re-forms the quinone with formation of ROS (Nutter, Ngo, Fisher, & Gutierrez, 1992). One electron reduction of quinones occurs by the agency of flavoenzymes (Powis, Svingen, & Appel, 1981; Chesis, Levin, Smith, Ernster, & Ames, 1984; Iyanagi, 1987) or by interaction with oxyhemoglobin (Munday, Fowke, Smith, & Munday, 1994; Goldberg, & Stern, 1976) and this reduction is known as a toxification reaction and responsible for the in vitro cytotoxicity of menadione (Chesis, Levin, Smith, Ernster, & Ames, 1984; Thor, et al., 1982; O'Brien, 1991).

It has been reported that the cytotoxicities of menadione include induction of macromolecular damage, disruption of calcium homeostasis, depletion of cellular thiols (Nutter, Ngo, Fisher, & Gutierrez, 1992; Tzeng, Chiou, Huang, & Chen, 1992; Tzeng, Chiou, Wang, Lee, & Chen, 1994; Tzeng, Lee, & Chiou, 1995; Chiou, Chou, & Tzeng, 1998). Two-electron reduction of a quinone generates the hydroquinone. Hydroquinones are less reactive form of quinone than semiquinones but hydroquinones are also cause autoxidation and again with production of ROS (Munday, 1997). Hydroquinones can also conjugate with glucuronide or sulphate in order to eliminate from body with using the properties of this conjugates as being hydrophilic and not being come into redox cycle (Cadenas, 1995; Losito, Owen, & Flock, 1967). Menadione is reduced to hydroquinone,menadiol, by DT-diaphorase NADPH:[quinone acceptor] oxidoreductase, E. C. 1.6.99.2) (Ernster, Danielson, & Ljunggren, 1962; Ernster, 1987) and menadiol is comparatively stable at neutral pH in the presence of diaphorase (Munday, 1997).

1.1.7 Properties of Phanerochaete Chrysosporium Used as Model Microorganism

Phanerochaete chrysosporium is an important white rot fungus because of having the ability of degrading the aromatic polymer lignin. *P. chrysosporium* generates extracellular enzymes that uses non-specific oxidizing agents in order to

fragment the three dimensional structure of lignin into components that can be utilized by its metabolism.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

All chemicals that used in this thesis have analytical purity and purchased from Sigma and Merck companies.

2.2 Microorganism and Culture Conditions

The strain of *Phanerochaete chrysosporium*, DSM-1547 obtained from German Collection of Microorganisms and Cell Culture (DSMZ) was used as model microorganism for investigating the antioxidant response to menadione-induced oxidative stress.

Spore suspension was generated in PDA medium (pH 5.6) as described by Beever and Bollard, (1970). Sterilization of medium was carried out by autoclave at 121°C for 20 minutes. Inoculation was carried out at 28 °C for 7 days in petri dishes.

P.chrysosporium was cultured in three different Tien and Kirk modified liquid medium shown in Table 2.1. The liquid mediums were sterilized by autoclave at 121 °C for 20 minutes. Incubation was carried out at 28 °C for 12 days and with 150 rpm agitation in the 250 mL erlenmeyer flask containing 90 mL liquid medium and 10 mL spore suspension (OD_{650}; 0.800).

Cells from stationary phase were treated with menadione at the concentrations of 0.1 mM; 0.2 mM; 0.3 mM; 0.5 mM; 0.75mM for 1 h; 2 h; 3 h; 4 h; 5 h; 6 h; 7 h; 8 h then the menadione-treated cells were harvested and washed several time with 20mM potassium phosphate buffer (pH 7.4) at 4 °C and stored at -20 °C.

Table 2.1 Liquid media components of *P. chrysosporium*

Code	Litterateur	The components of medium	
S1	Tien and Kirk, 1988 (Modified)	Component	Amount (g/L)
		Potassium dihydrogen phosphate	2.0
		Calcium chloride	0,114
		Magnesium sulphate	0,7
		Ammonium chloride	0,12
		D-Glucose	2.0
		Thiamin-HCl	1.10^{-3}
		Tween 80	0,05
		Trace Elements*	
S2	Tien and Kirk, 1988 (Modified)	Potassium dihydrogen phosphate	2.0
		Calcium chloride	0.1
		Magnesium sulphate	0.5
		D-Glucose	10
		Thiamin-HCl	1.10^{-3}
		Sodium tartrate	0.417
		Ammonium sulphate	0.284
		Trace elements**	
S3	Tien and Kirk, 1988 (Modified)	Potassium dihydrogen phosphate	2.0
		Calcium chloride	0.1
		Magnesium sulphate	0.5
		D-glucose	10.0
		Thiamin- HCl,	0.001
		Ammonium chloride	0.1
		Trace element***	

*$FeSO_4.7H_2O$, 70 µg; $ZnSO_4.7H_2O$, 46 µg; $MnSO_4.2H_2O$, 35 µg; $CoCl_2.6H_2O$, 7 µg
**$MgSO_4$, 0.3 g; $MnSO_4$, 0.05 g; NaCl, 0.1 g; $FeSO_4.7H_2O$, 0.01 g; $CoCl_2$, 0.01 g; $ZnSO_4.7H_2O$, 0.01 g; $CuSO_4$,
*** Nitrilotriacetate, 0,015 g; NaCl, 0,01 g; $MnSO_4.H_2O$,0,005g; $FeSO_4.7H_2O$, 0.001g; $ZnSO_4$, 0.001g; $CaSO_4$, 100 µg ; $CuSO_4.5H_2O$, 100 µg; $NaMoO_4.2H_2O$, 100µg.

2.2.1. Preparation of Crude Extracts

The harvested cells were resuspended in 20 mM potassium phosphate buffer at pH 7.4 in a volume equal to 3.0 times its weight. The homogenization procedure was performed for 3 min at 9000 rpm with 30 seconds time intervals. Cell debris in the homogenate was removed by centrifuge at 15000 rpm and 15 min at +4 °C. The crude extract was used with no-refreezing.

2.3 Determination of ROS Levels

2.3.1 Measurement of Superoxide Anion Radical Level

Superoxide anion radical level was determined with luminometer (Skatchkov, et al, 1999). Lucigenin was used as a probe for luminometrical measurement.

2.3.2 Measurement of Hydroxyl Radical Level

Hydroxyl radical level was determined fluorometrically (Neungnapa, Bao, Hetong, Feng, &Yueming, 2009). For the measurement of level of hydroxyl radical TBARS products of 2-deoxy-D-ribose was quantified. The fluorescent intensity was measured at the excitation wavelength of 532 nm and the emission wavelength of 553 nm against the reagent blank solution.

The amount of hydroxyl radical was calculated by equation of the calibration curve that was plotted with MDA as standard.

2.3.3 Measurement of Hydrogen Peroxide Level

H_2O_2 levels were measured by using a bit modification of the method described by Barja (1999). Fluoresence was determined at 312 nm excition and 420 nm emission wavelenghts.

The H_2O_2 levels were calculated with using a standard curve of H_2O_2 and expressed as nmol/gww.

2.4 Enzyme Activity Assays

2.4.1 Catalase Activity Assay:

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

The extinction coefficient for H_2O_2 at 240 nm is 43.6 $M^{-1}cm^{-1}$. The specific activity of catalase (U/mg protein) was described as the enzyme amount necessary for the decrease of the H_2O_2 absorbance from 0.450 to 0.400 in 20 s at 240 nm.

2.4.2 Superoxide Dismutase (SOD) Activity Assay

The SOD enzyme activity was determined by spectrophotometry at 490 nm. The procedure of SOD activity assay was based on the measurement of autoxidation of 6-hydroxydopamine (6-OHDA) of which SOD has the inhibitory effects on autoxidation (Crost, Serviden, Bayer, & Serra, 1987).

One unit is the amount of SOD required to inhibit the initial rate of 6-OHDA autoxidation by 50%.

2.4.3 NADH Oxidase Activity Assay

NADH oxidase activity was determined by spectrophotometry. The procedure was based on the disappearance of NADH at 340 nm (Anders, Hogg, & Jago, 1970). The decreases in A_{340} were recorded of two 5 min intervals.

A millimolar extinction coefficient of 6.22 was used to calculate the NADH disappearance.

2.4.4 NADPH oxidase Activity Assay

NADPH oxidase activity was determined by spectrophotometry. The procedure was based on the disappearance of NADPH at 340 nm (Anders, Hogg, & Jago, 1970).

A millimolar extinction coefficient of 6.22 was used to calculate the volume activity of the enzyme

2.4.5 Glucose-6 Phosphate Dehydrogenase Activity Assay

The conversion of $NADP^+$ to NADPH is catalysed by two dehydrogenase enzymes in pentose phosphate pathway (PPP), glucose 6-phosphate dehydrogenase (G6PD) and 6- phosphogluconate dehydrogenase (PGD) (Tian, Pignatare, & Stanton, 1994).

The activity of these two dehydrogenase enzyme was measured by the increase of absorbance at 340 nm, monitoring the conversion of $NADP^+$ to NADPH. Therefore either PGD activity alone or total dehydrogenase activities had to be measured to determine accurate enzyme activities for G6PD and PGD so G6PD activity was calculated by subtracting the activity of PGD from total enzyme activity.

In order to measure the total dehydrogenase activity both substrates for two dehydrogenase enzyme were added to the cuvette whereas to measure the activity of PGD only the substrate for PGD was added to the cuvette.

A millimolar extinction coefficient of 6.22 was used to calculate the volume activity of the enzyme

2.5 Determination of ATP, ADP and AMP Levels

2.5.1 Sample Preparation

The samples were prepared by the procedure of Ganzera et al. (2006) (Ganzera, Vrabl, Wörle, Burgstaller & Stuppner, 2006). The cell pellet was extracted in boiling water with shaking for 15 minutes and cooled immediately on ice and then centrifuged at 12000 rpm for 15 min. The supernatant was quickly frozen and lyophilised. The lyophilyzate was resolved in 200 µL of ultra-pure water

2.5.2 HPLC Conditions

The HP 1100 HPLC system used was equipped with a photodiode detector. 50 mM aqueous triethylamin (TEA) buffer (A) and acetonitrile (B). Gradient elution was performed from 99 A/1B in 10 min to 95A/5B and changed in another 10 min to 92.5A/ 7.5B. Each run was followed by a 5-min wash with 70B/30 parts 0.1% phosphoric acid Detection wavelength, flow rate, column temperature were set to 254 nm, 1 ml/min, 20 ^{o}C (Ganzera, Vrabl, Wörle, Burgstaller & Stuppner, 2006).

2.6 Determination of Damage Levels

2.6.1 Determination of Lipid Peroxidation

Lipid peroxidation was measured by the formation of MDA using the thiobarbutiric acid reaction (Schemedes, 1989). Briefly proteins were precipitated by

TCA. Then, supernatant was react with TBA in boiling water bath for 30 minutes. The mixture was cooled immediately and the absorbance was measured at 532 nm.

Lipid peroxidation was calculated using a molar extinction coefficient for MDA of $1.56x10^5$ L/mol per cm.

2.6.2 Determination of Protein Carbonyl Content

Protein carbonyl content was measured fluorometrically with using flourescein thiosemi carbazide.

The fluorescent intensity was measured at the excitation wavelength of 485 nm and the emission wavelength of 535 nm.

2.7 Total Protein Assay

Bradford method (A_{595}) was used for measurement of total protein amounts in the samples.

In the total protein assay, 900 µl reagent was added to cuvette then 100 µl sample is added and shaked gently. Absorbance value was read in the end of 2 minutes against blank (100 µl buffer instead of sample).

BSA was used as a protein standard. 1000 ppm stock BSA solution was diluted to 10, 25, 50, 75 and 100 ppm in the 20 mM potassium phosphate buffer (pH 7.4). The protein amounts were determined by using calibration curve drawn between known concentration of BSA standards and their absorbance values at 595 nm.

CHAPTER THREE
RESULT AND DISCUSSION

Within the scope of this thesis, *Phanerochaete Chrysosporium* treated with menadione at different concentrations and incubation periods. In order to investigate the antioxidant response system of *Phanerochaete Chrysosporium* against menadione-induced oxidative stress, intracellular superoxide anion radical, hydroxyl radical and hydrogen peroxide levels, variations of SOD, CAT, G6PDH, NADH, NADPH oxidase enzyme activities and energy metabolism, cell membrane peroxidation and protein oxidation levels were examined and the results were compared with control.

3.1 Determination of Culture Medium Depend on The Growth Curves

As can be seen in figure 3.1 *Phanerochaete Chrysosporium reached* stationary phase in S1, S2 and S3 respectively on the 8^{th}, 8^{th} and 4^{th} days.

S3 culture medium was poor in terms of biomass when compared with the other culture media. On the other hand, although S2 medium was more suitable than S1 medium in terms of biomass production, *P. chrysosporium grown* in S1 medium had higher antioxidant enzyme activities than grown in S2. The antioxidant response system of *P.chrysosporium*, was investigated in S1 culture medium.

Menadione, in the range from 0.1 mM to 0.75 mM, was added to culture medium of *P.chrysosporium* on the 10^{th} day and incubated between 1-8 hours. The samples were taken hourly. The results were compared with menadione-free control.

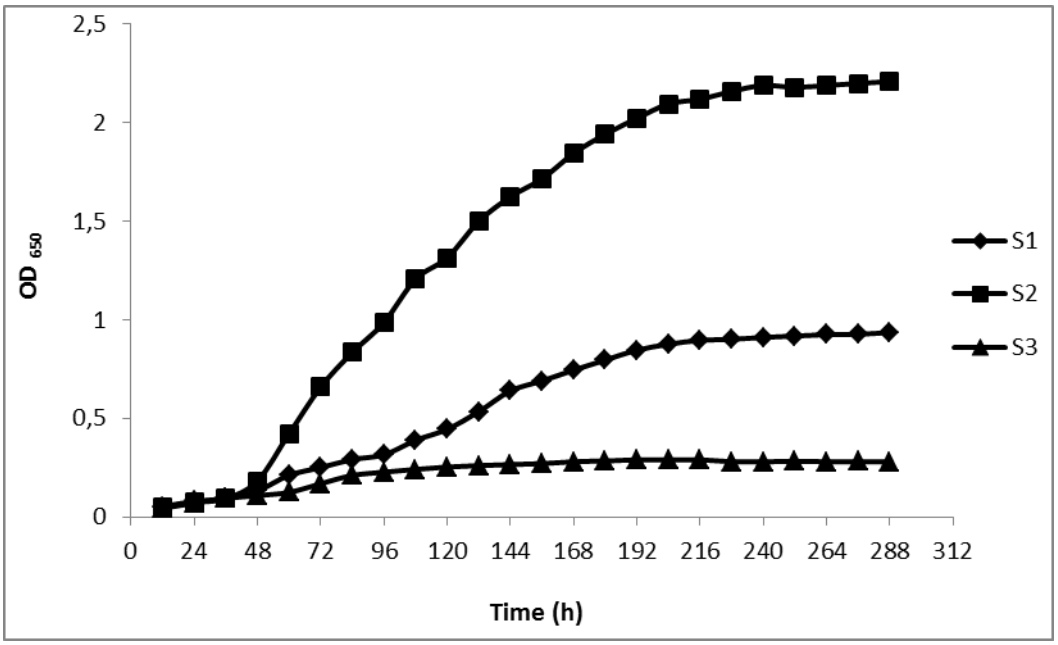


Figure 3.1 The growth curves of *P.chrysosporium* depend on the culture media (S1, S2 and S3 culture media are described in Table 2.1)

3.2 Investigation of Some Oxidative Stress and Antioxidant Parameters in *P.chrysosporium* at Menadione Treated Conditions

3.2.1 Investigation of Intracellular Reactive Species Levels

3.2.1.1 The Variation of Intracellular Superoxide Anion Radical Level

In menadion-free conditions superoxide anion radical levels decreased significantly after 5th hour ($p<0.01$).

In menadione treated conditions, for 0.1 mM sample, while the level was 54.67±1.24 RLU at 1st hour, it showed a rapid decrease at 2nd hour, then it showed no significant changes during following incubation period after 3rd hour ($p>0.01$) but it stayed at slightly significant upper level when compared with control ($p<0.05$). Similar situations were observed after 3rd hour for 0.2 mM after and 4th hour for 0.3mM. At 0.5 mM and 0.75 mM menadione threated samples decreases were slower and almost similar values with control were obtained. As a different situation,

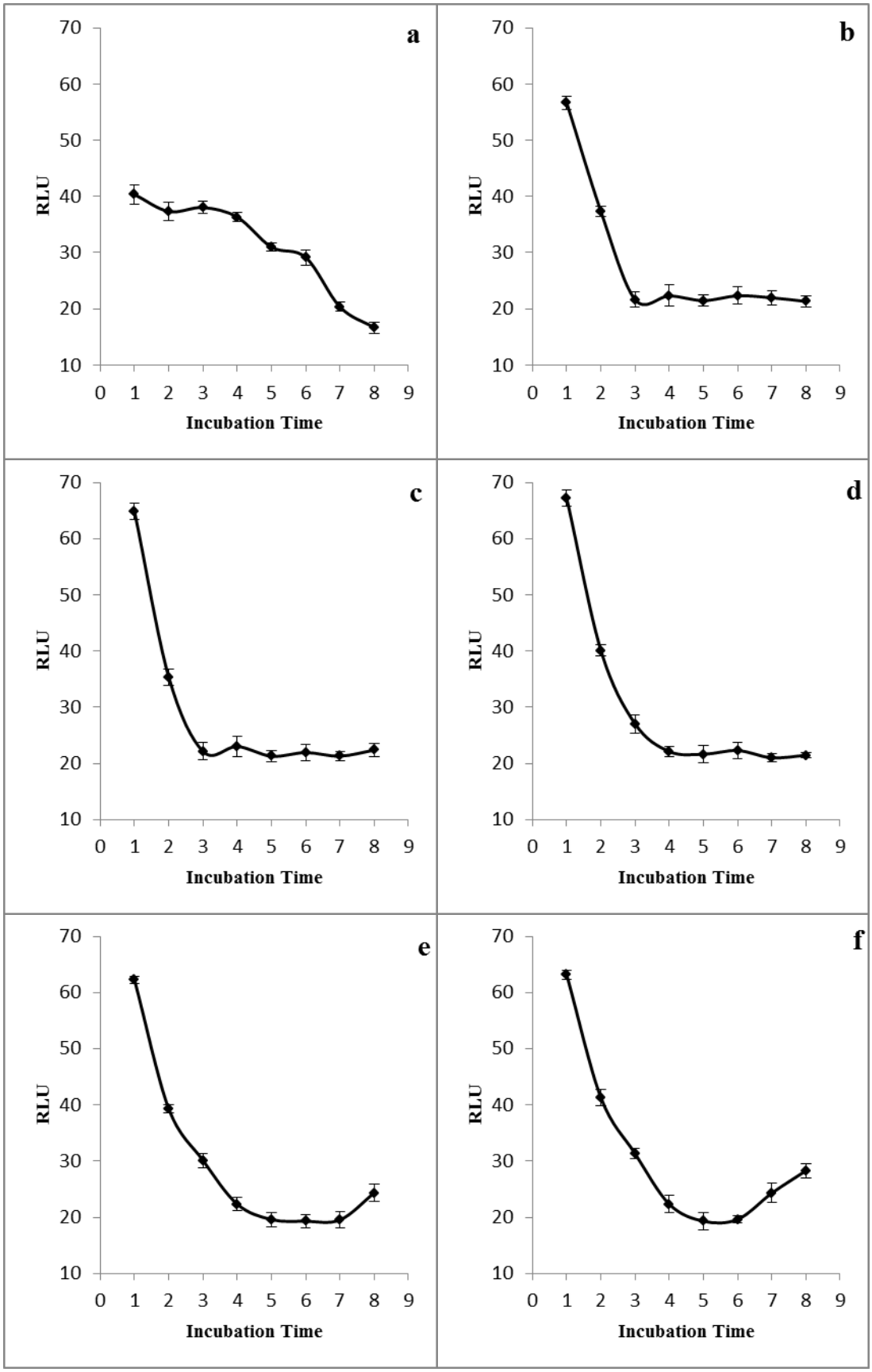


Figure 3.2 Variation of O_2^{-}• levels depend on the incubation period; control (a), 0.1 mM menadione (b), 0.2 mM menadione (c), 0.3 mM menadione (d), 0.5 mM menadione (e), 0.75 mM menadione (f).

significant increases were observed at 8th hour for 0.5 mM sample and at 7th hour for 0.75 mM sample ($p<0.01$).

For all menadione treated samples, the maximum values of superoxide anion radical were obtained at the 1st hour and then decreasing tendency was occurred. In this case, superoxide anion radical increased rapidly in an hour. At the following incubation periods, generation of this radical triggered antioxidant defence system that decreased the level of superoxide anion radical but the radical level was nevertheless higher than control values. The increases of superoxide anion radical at 0.5 and 0.75 mM treated samples is due to the decreases the activity of enzymes that responsible for scavenging superoxide anion radical at the end of the incubation periods depend on menadione-induced oxidative stress.

For all menadione treated samples, superoxide anion radical level were significantly higher at the 1st hour ($p<0.01$). Increases of superoxide anion radical level for 0.75 mM menadione treated sample after 6th hour may be caused by negative effects of high menadione concentration on antioxidant system or some intracellular metabolic balance.

3.2.1.2 The Variation of Intracellular Hydrogen Peroxide Level

In menadione-free culture medium, while intracellular hydrogen peroxide value of 1st hour sample was 5.8±0.15 nmol/ mg gww, at following incubation period this value reached to with rapid and significant decreases ($p <0.01$) after 5th hour.

In menadione treated samples, when compared with control, significant changes were not observed ($p>0.01$) at the initial incubation period and increases of H_2O_2 levels were obtained significantly after 3rd for 0.1mM menadione treatment and after 2nd hour for 0.2 mM menadione treatment ($p<0.01$). when compared with control, H202 levels of 0.1, 0.2 and 0.3 mM menadione treated samples showed positive correlations with increased menadione treatment ($p<0.01$; r= 0.731, r=0.877, r= 0.886, respectively). For 0.1 and 0.2 mM menadione treatments, the maximum H_2O_2

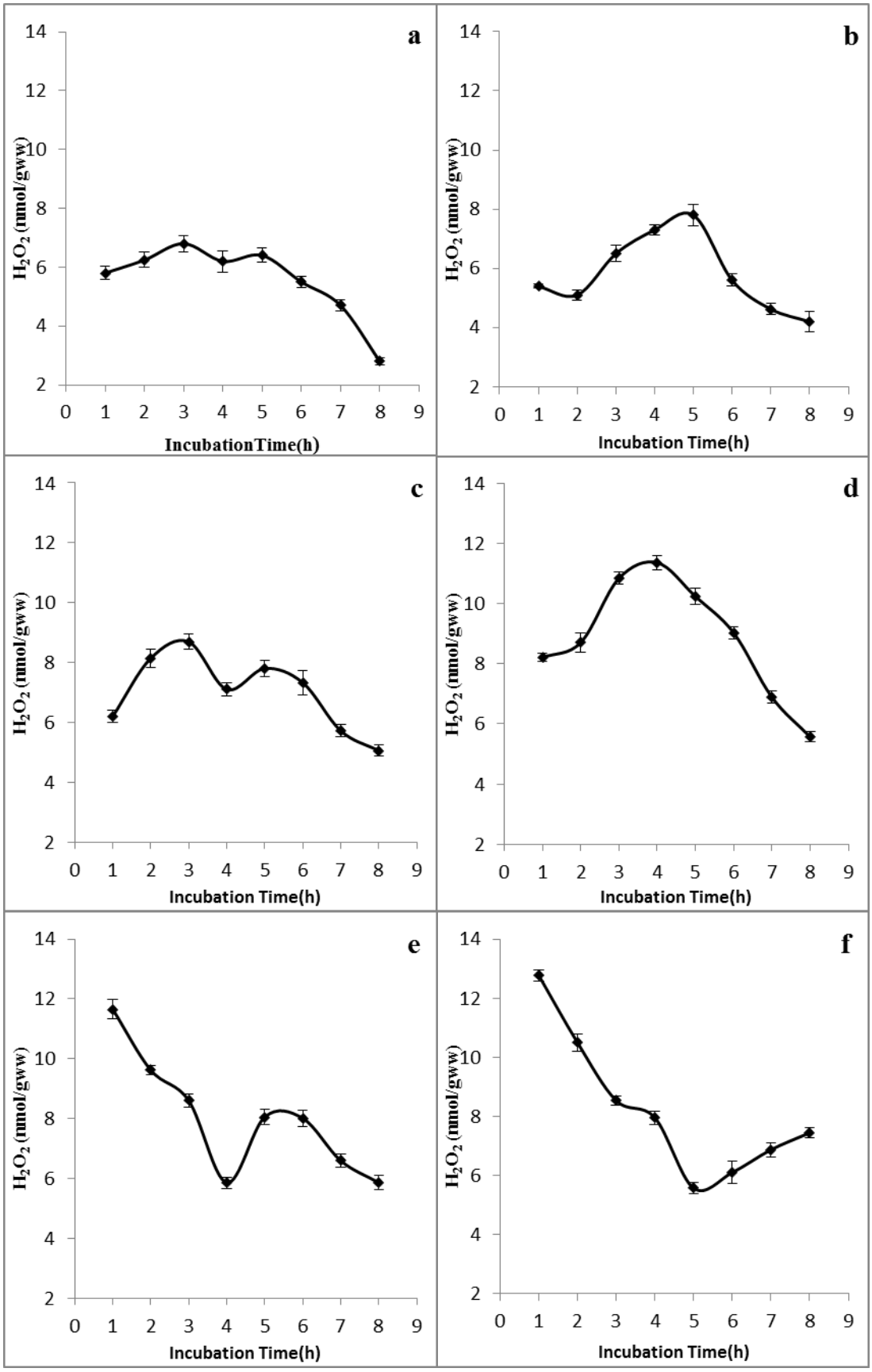


Figure 3.3 Variation of H_2O_2 levels depend on the incubation period; control (a), 0.1 mM menadione (b), 0.2 mM menadione (c), 0.3 mM menadione (d), 0.5 mM menadione (e), 0.75 mM menadione (f).

values were reached 7.8±0.19 nmol/gww and 8.68±0.26 nmol/g gww at the 5th and 3rd hours, respectively. For 0.3, 0.5 and 0.75 mM menadione treatment, the levels of H_2O_2 increased significantly with increasing menadione concentrations at the 1st hour ($p<0.01$). While the maximum value, 11.357±0.22 nmol/gww was obtained at 4th hour for the 0.3 mM menadione treatment, 0.5 mM menadione treated samples had tendency to decrease except for 4th hour fluctuation. In 0.75 mM menadione treated samples, H_2O_2 levels increased again significantly and continuously after 5th hour ($p<0,01$). H_2O_2 levels of all the menadione treated samples were generally over the controls during all incubation periods.

The highest value of H_2O_2 level observed for 0.75 mM menadione treatment at the 1st hour was 12.770±0.19 nmol/gww and it was 2.2-fold more than control.

According to another research, with 20 μM menadione treatment, the H_2O_2 level of isolated atria cells increased approximately 1.34-fold when compared with control (Floreani, Napoli, & Palatini, 2002). Salicylic acid, another agent for oxidative stress were investigated by researchers in *Arabidopsis fhalian* (Rao, Paliyath, Ormrod, Murr, &Watkins, 1997). 5mM salicylic acid treatment for 8 hour caused the 1.44-fold increase of H_2O_2 level when compared to control but CAT activity and SOD activity increased only 1.36-fold and 1.04-fold, respectively. When compared to our study, although concentration of salicylic acid was higher than concentration of menadione it could not induce antioxidant system as well as menadione. Another study compared the effect of 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) and menadione in terms of low and high concentration effects in A549-S cells (Watanabe, & Forman, 2003). The results showed that at the lower concentrations (25-50 μM) DMNQ caused a 3-4 fold higher H_2O_2 levels than menadione but at the higher concentrations (100 μM) the rate of H_2O_2 accumulation was similar for both quinone.

Generally obtained higher values of H_2O_2 levels for menadione treated samples than the values of controls may be a proof of intracellular oxidative stress due to menadione-induced H_2O_2 production. The decreasing of H_2O_2 levels for control

samples after the 5th hour may be caused by increasing activities of enzymes that use the H_2O_2 as a substrate and effects of forming the new equilibrium on the metabolism.

The decreasing of H_2O_2 levels after reaching the maximum values for 0.1-0.3 menadione treated samples may be caused by time-dependent menadion induced oxidative stress for *P.chrysosporium* and probable degradation reactions of menadione itself besides increasing activities of enzymes that use the H_2O_2 as a substrate.

At 0.5 and 0.75 mM menadione treatments, maximum H_2O_2 levels were obtained at the end of the 1st hour when compared with other incubation periods and decreases of following incubation periods can be explained by similar factors.

Increases of H_2O_2 levels for 0.75 mM menadione treated sample after 5th hour may be caused by negative effects of high menadione concentration on antioxidant system or some intracellular metabolic balance.

3.2.1.2 The Variation of Intracellular Hydroxyl Radical Level

In menadione-free culture medium, significant decreases of intracellular hydroxyl radical level were observed after the 5th hour ($p<0.01$). For all menadione treated samples, hydroxyl radical levels were increased significantly related with increasing menadione concentration at the 1st hour ($p<0.01$). When compared with control, hydroxyl radical levels of 0.1, 0.2, 0.3 and 0.5 mM menadione treated samples showed positive correlation with increased menadione concentrations ($r= 0.776$, $r= 0.649$, $r= 0.718$, $r=0.911$, respectively). The maximum hydroxyl radical levels were obtained at the 3rd hour for 0.1 mM, at the 2nd hour for 0.2 mM, at the 3rd hour for 0.3 mM, at the 4th hour for 0.5 mM and at the 2nd hour for 0.75 mM menadione treatments and the values were 14384±35.57; 1880.944±34.1; 1800.494±42.98; 1900.861±46.48; 1800.856±50.78 nmol/gww, respectively. During following incubation period continuous decreases were observed. For the 8th hour, hydroxyl

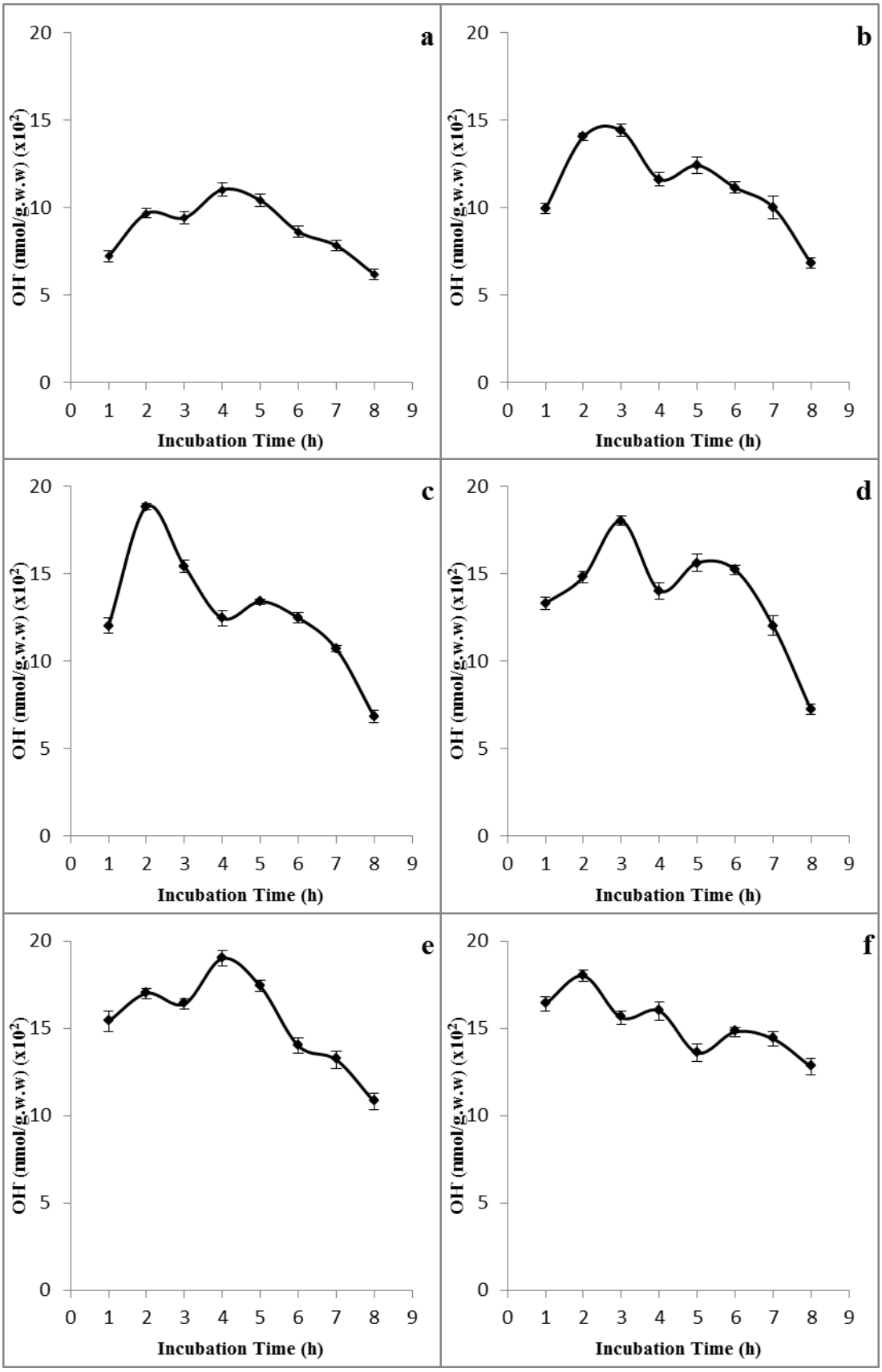


Figure 3.4 Variation of $OH^{\cdot}$ levels depend on the incubation period; control (a), 0.1 mM menadione (b), 0.2 mM menadione (c), 0.3 mM menadione (d), 0.5 mM menadione (e), 0.75 mM menadione (f).

radical levels which were similar ($p>0.01$) until the 0.3 mM menadione treatments, showed significant increases ($p<0.01$). The highest value of hydroxyl radical was 1.73-fold higher than control.

In general hydroxyl radical levels increased depending on incubation time and increasing level of menadione treatment to *P.chrysosporium*

It has been suggested that menadione-induced oxidative stress causes the generation of superoxide anion radical and formation of hydroxyl radical can be caused by generation of superoxide anion radical with Fenton reactions in metabolism. In addition hydroxyl radical can be generated under the condition that if Fe^{+2} ions or peroxidase catalyse the further reduction of H_2O_2 to $OH^{\cdot}$ utilizing $O_2^{\bullet -}$ as an electron donor. (Halliwell & Gutteridge, 1989; Chen & Schopfer,1999).

Increasing levels of hydroxyl radical related to menadione concentration may proof that menadione causes oxidative stress on *P.chrysosporium*. Notably reactive hydroxyl radical has effective role in intracellular damages. In general, decreasing of intracellular hydroxyl levels after a maximum value may be caused by time-dependent menadion induced oxidative stress, increasing activities of antioxidant enzymes, and probable degradation reactions of menadione itself.

3.2.2 Investigation of Some Oxidative Stress and Antioxidant Parameters in P.chrysosporium at Menadione Treated Conditions

3.2.2.1 Investigation of Superoxide Dismutase Activity Variations in Menadione Treated P.chrysosporium

In menadion-free medium, while the SOD activity did not show any significant changes until 6^{th} hour, during following incubation period SOD activity increased significantly and the activity reached 83,1672±4.12 U/mg at 8^{th} hour ($p<0.01$). For menadione treated samples SOD activities significantly increased based on the increased menadione concentration until 0.5 mM samples at the 1^{st} hour ($p<0.01$). As

can be seen Figure 3.4 the maximum enzyme activities were obtained at 3rd hour for 0.1 mM, at 2nd hour for 0.2 mM, at 3rd hour 0.3 mM, at 4th hour for 0.5 mM and for at 2nd hour 0.75 mM menadione treatment and the values were respectively, 200.2153±5.1; 225.235±4.92; 246.45±4.24; 370.2155±5.91; 357.248±7.72 U/mg. The highest SOD enzyme activity, observed in 0.5 mM-menadione treated samples at 5th hour was 5.4-fold higher than control. According to a research, SOD activity of 30μM treated *Bacillus sp. F26* was obtained 2-fold higher than SOD activity of non-treated *Bacillus sp. F26* (Yan, Hua, Du, & Chen, 2006). The results of another studies showed that SOD activity of *P. chrysogenum* was induced by 0.25 mM menadione treatment at 1.3-fold when compared to control (Emri, Pócsi, & Szentirmai, 1999). When compared to these studies, the obtained SOD activities that induced by menadione-treatment quiet higher. This means that *P.chrysosporium* gives response to menadione treatment and activates its antioxidant response system effectively. The 8th hour SOD activity were 1.5-fold higher than control but in the range of 0.1-0.3 mM menadione treatment the activity did not show significant differences when compared with each other ($p>0.01$). For 0.5mM and 0.75 mM menadione treated samples, 8th hour SOD activity values were 145.7854±4.12; 220.236±9.65 U/mg respectively.

When compared the superoxide anion levels and SOD activities, there were negative correlations for all menadione treated samples. The highest negative correlation was shown at 0.75 mM menadione treated samples ($r= -0.709$). This means that superoxide anion radical triggered SOD activity and the higher activities of SOD rapidly dismutated superoxide anion radical and decreased the level of superoxide anion radical but at the end of incubation periods, menadion-induced formation of superoxide radical affect SOD activity negatively in case enzyme activity decreased while superoxide anion radical increased.

It was shown by another group that the level of intracellular oxidation products for the 30 mM menadione treated sod 1 mutant *Saccharomyces cerevisiae was higher 4.3*–fold than *non*-mutant *Saccharomyces cerevisiae*. It shows us the

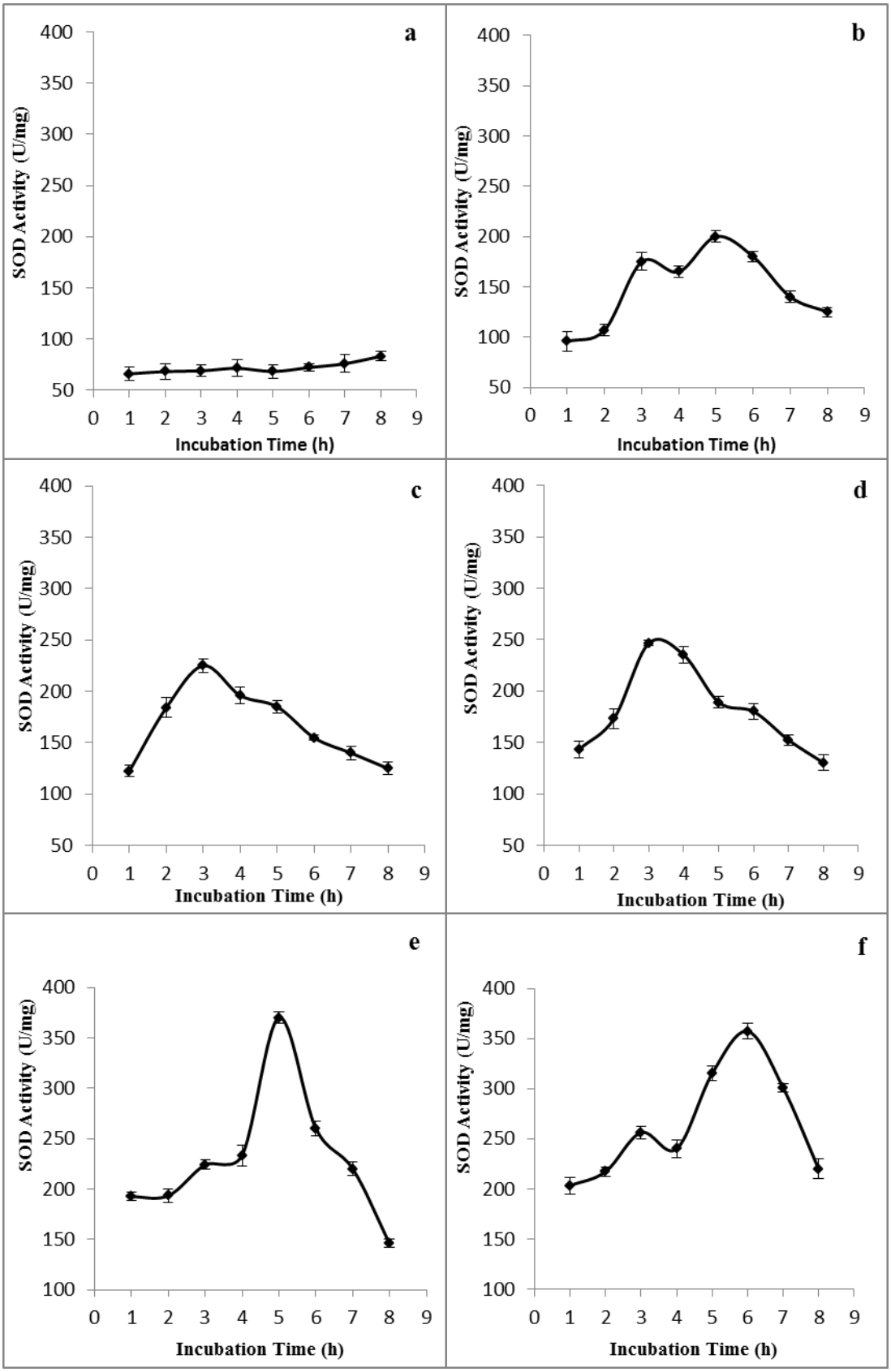


Figure 3.5 Variation of SOD activity depend on the incubation period and concentration of menadione; control (a), and menadione concentrations 0.1 mM (b), 0.2 mM (c), 0.3 mM (d), 0.5 mM (e), 0.75 mM (f).

important role of SOD in antioxidant system against menadione-induced oxidative stress (Pereira, Herdeiro, Fernandes, Eleutherio, & Panek, 2003).

In all investigated concentrations and incubation periods, significant increases in SOD activity compared with the controls may show us that the menadione-induced oxidative stress has a trigger effect on production of SOD enzyme of *P.chrysosporium*

Besides being higher than control values, observed decreases in SOD activity values after a maximum level may be related with superoxide radical level, degradation mechanism of menadione or inhibition of SOD enzyme activity by excessive superoxide radical.

3.2.2.2 Investigation of Catalase Activity Variations in Menadione Treated P.chrysosporium

In menadion-free medium, the CAT activity of *P.chrysosporium* increased after the 6^{th} hour significantly $p<0.01$. While the CAT value of 1^{st} hour sample activity was 2928.36±289 U/mg, CAT value of the 8^{th} hour sample increased significantly and reached 4300.235±363 IU/ mg ($p<0.01$). Although the CAT activity of 0.1-0.3 mM menadione treated 1^{st} hour samples were higher than control, the activity did not show significant differences when compared with each other ($p>0.01$). In general aspect, CAT activity showed instant increases at the 2^{nd} hour. As can be seen figure 3.6 the maximum enzyme activity values were obtained at 6^{th} for 0.1 mM hour, at 3^{rd} hour for 0.2 mM, at 4^{th} hour for 0.3 mM, for 0.5 and 0,75 mM menadione treatment at 6^{th} hour and the values were 10100,256±345; 15333,477±306; 17588,982±395; 18137,822±347; 18698,36±399 U/ mg, respectively. The highest value of CAT activity was determined in 0.75 mM menadione treated sample. CAT activity was higher 1.5-fold at the 1^{st} hour and 5.1-fold at the 6^{th} hour when compared to control. Although the 8^{th} hour CAT activity values were higher than control, concentration-dependent activities did not show significant differences when compared each other except for 0.75 mM concentration ($p>0.01$).

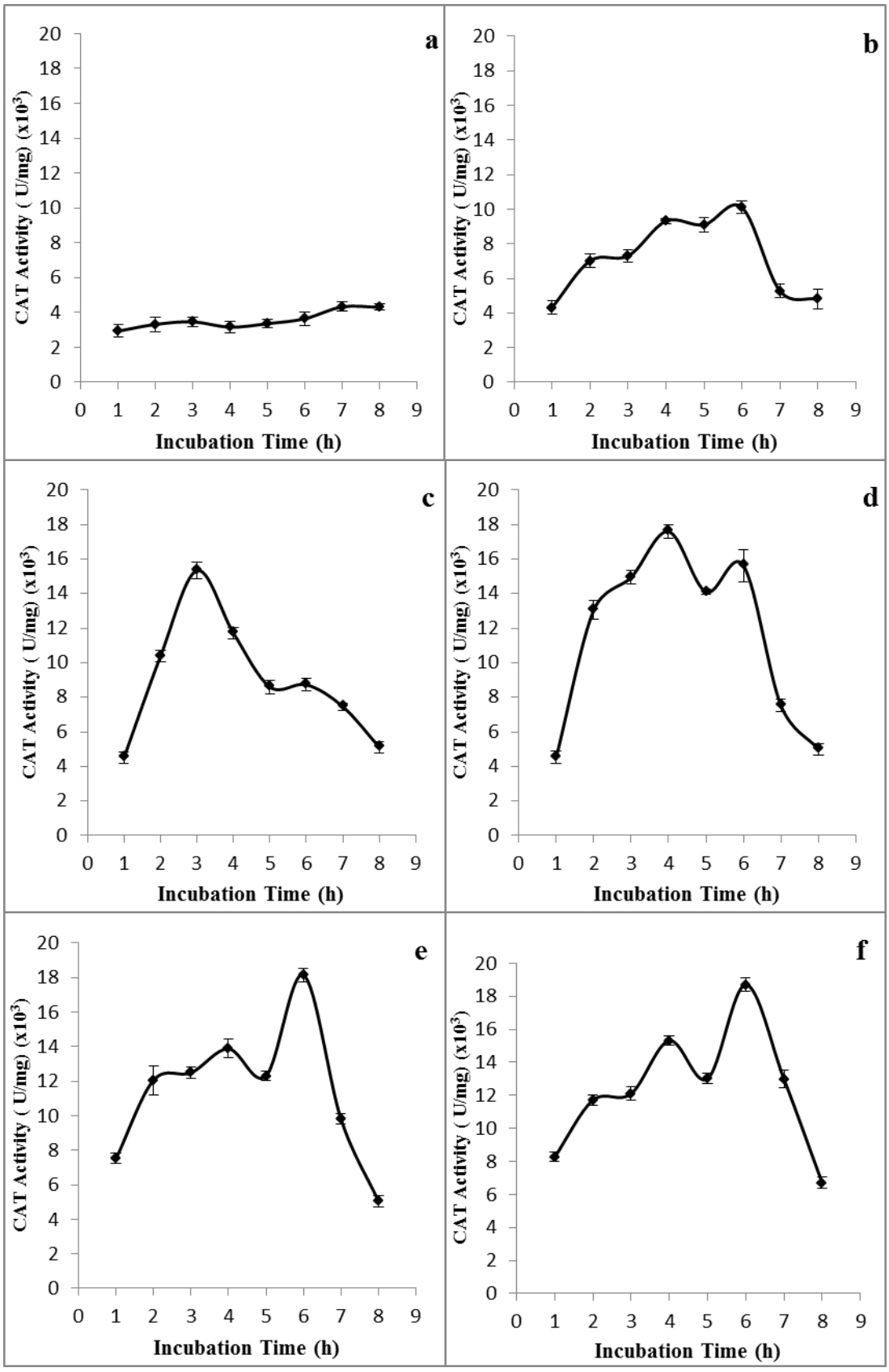


Figure 3.6 Variation of CAT activity depend on the incubation period and concentration of menadione; control (a), and menadione concentrations 0.1 mM (b), 0.2 mM (c), 0.3 mM (d), 0.5 mM (e), 0.75 mM (f).

According to a research, CAT activity of 0.2 mM menadione treated *Schizosaccharomyces pombe* increased 2-fold when compared with control (Lee, Dawes, & Roe, 1995). But at the other researchers did not observed any alteration of CAT activity of 0.25 mM menadione treated *P. chrysogenum* and in our study we observed 4.4-fold increases of 0.2 mM menadione treated *P.chrysosporium*. This show us that although the agent of oxidative stress is same and the treatment concentrations are similar, the agent may effects antioxidant system differently according to species or the antioxidant response against to oxidative stress may vary prominently depending on species.

Significant CAT activity increases observed after the 6th hour of control sample complies with significant decreases at H_2O_2 levels.

For 0.1-0.3 mM menadione treated samples, the 1st hour intracellular CAT activity levels are very close to each other just like H_2O_2 levels. In this concentration range, for the 1st hour of *P.chrysosporium*, while significant increases observed at CAT activity compared with control, observation of similar H_2O_2 levels with control sample shows us one of antioxidant enzymes CAT activity is induced by H_2O_2. During following incubation period, the ratio of maximum CAT activity to control is higher than the ones observed in H_2O_2 levels. This shows that, in fact, formation of H_2O_2 is induced considerably by menadione treatment but suppressed notably by increasing CAT activity. Either H_2O_2 levels or CAT activities are getting closed to control values with decreases during incubation periods after reaching the maximum values. This means, in menadione-induced oxidative stress on *P.chrysosporium*, CAT gives positive response to H_2O_2.

For 0,5- 0,75 mM menadione treated samples, the 1st hour intracellular CAT activities increased considerably when compared with other menadione concentrations. This situation complies with H_2O_2 level and CAT activity relation for the 1st hour. This shows relationship between CAT in antioxidant system and increasing menadione-induced oxidative stress. The increased enzyme activities depend on the following incubation time provides steadily decreases of H_2O_2 levels

for both concentration of menadione. Although at 0.1, 0.2 and 0.3 mM menadione treatment positive correlation occurred with H_2O_2 level and CAT activity (r= 0.802, r=0.816, r=0.85, respectively), at 0.75 mM menadione treatment negative correlation occurred with H_2O_2 level and CAT activity (r= -0.518). Menadione treatment triggered all CAT activities but the highest levels of H_2O_2 were obtained at higher menadione treatments. This means that although catalase activity was triggered by H_2O_2 formation, at higher menadione treatment CAT did not sufficient to hydrolyse H_2O_2 or higher H_2O_2 or other ROS formations affect CAT activity negatively.

Although similarity of decreases of CAT activities between these two concentrations after the 6th hour, increases at H_2O_2 levels for 0,75mM after 5th hour may be result of either other factors in antioxidant system rather than CAT or an effect from high menadione concentrated source.

3.2.2.3 Investigation of Glucose 6-Phosphate Dehydrogenase Activity Variations

In menadione-free medium, G6PDH activity significantly increased at the 8th hour when compared to the 1st hour sample ($p<0.01$). In 0.1 and 0.2 mM menadione treated samples, G6PDH showed similar activities with controls in first 3 hours ($p>0.01$). The maximum enzyme activity values were obtained for 0.1 mM at the 4th hour, for 0.2 mM at the 5th hour, and for the others at the 3rd hour and the values were 4.7523±0.13; 5.8864±0.12; 6.9494±0.15; 6.25±0.11 and 5.98±0.11 U/mg, respectively and although they were over the controls, after reaching the maximum values, activity showed decreasing tendency. The highest G6PDH value, 6.9494± 0.15 U/mg was obtained at 0.3 mM menadione treatment and this value was higher 2.54- fold than control.

G6PDH produces NADPH which is required for many detoxification reactions (Salvemini, Franzé, Iervolino, Filosa, Salzano, & Ursini, 1999). Increase of G6PDH activity of L-buthionine-(*S,R*)-sulfoximine (BSO), cause GSH depletion, treated Hep3B cells are investigated by reasearchers and G6PDH activity was observed 2-

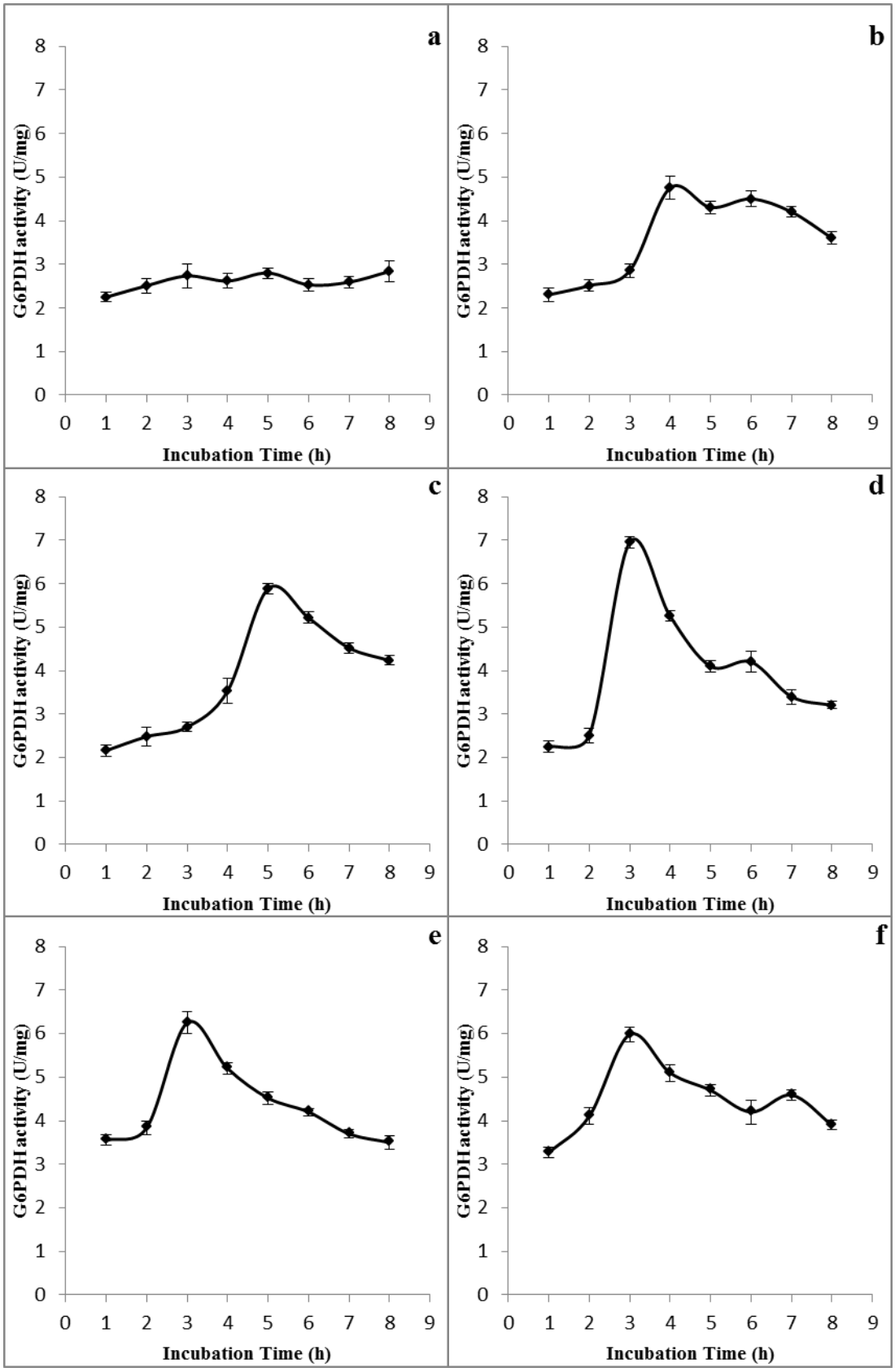


Figure 3.7 Variation of G6PDH activity depend on the incubation period and concentration of menadione; control (a), and menadione concentrations 0.1 mM (b), 0.2 mM (c), 0.3 mM (d), 0.5 mM (e), 0.75 mM (f).

fold higher than control samples. On the other hand, although menadione causes GSH depletion too, there was not any significiant changes in the actvity of G6PDH of 0.25 mM menadione treated *Penicillium chrysogenum*(Emri, Pócsi, & Szentirmai, 1999).

Observation of important activity increases after maximum shows that necessary equilibrium for stimulation of G6PDH activity settles about 3rd hour in cell. In menadione-induced oxidative stress, especially negative alterations occurred in Glutathione systems, may induce G6PDH activity.

Generally, slow and steady decreases of G6PDH activities that are higher than initial and control activities after maximum levels may reflect its support to balance against menadione-induced oxidative stress.

3.2.3 Investigation of NADH Oxidase and NADPH Oxidase Activity Variations

In menadione-free culture the activity of NADH oxidase increased significantly after the 5th hour ($p<0.01$). For all menadione treated samples, activity of NADH oxidase increased significantly with increased menadione concentration in all incubation periods ($p<0.01$). NADH activity of *P.chrysosporium* reached the maximum values at 3th hour for 0.2 and 0.3 mM menadione treatment and at 5th hour for all the other concentration ranges. The highest NADH oxidase activity was observed in 0.75 mM menadione treated sample at 5th hour and the value of activity was 4274.72 ±97.73 U/mg, 4.86-fold higher than control.

In menadione-free culture, the NADPH enzyme activity was 1678±117.8 IU/mg on the 1st hour and the activity did not show significant changes except for 7th hour ($p>0.01$). For the menadione treated samples, the time-dependent variation of NADPH oxidase showed increases with the increased concentration of menadione for all incubation periods ($p<0.01$). The NADPH activity of *P.chrysosporium* reached the maximum values at 5th hour in 0.1 mM menadione treated sample and at 6th hour in all the other concentration ranges. The highest NADPH oxidase activity

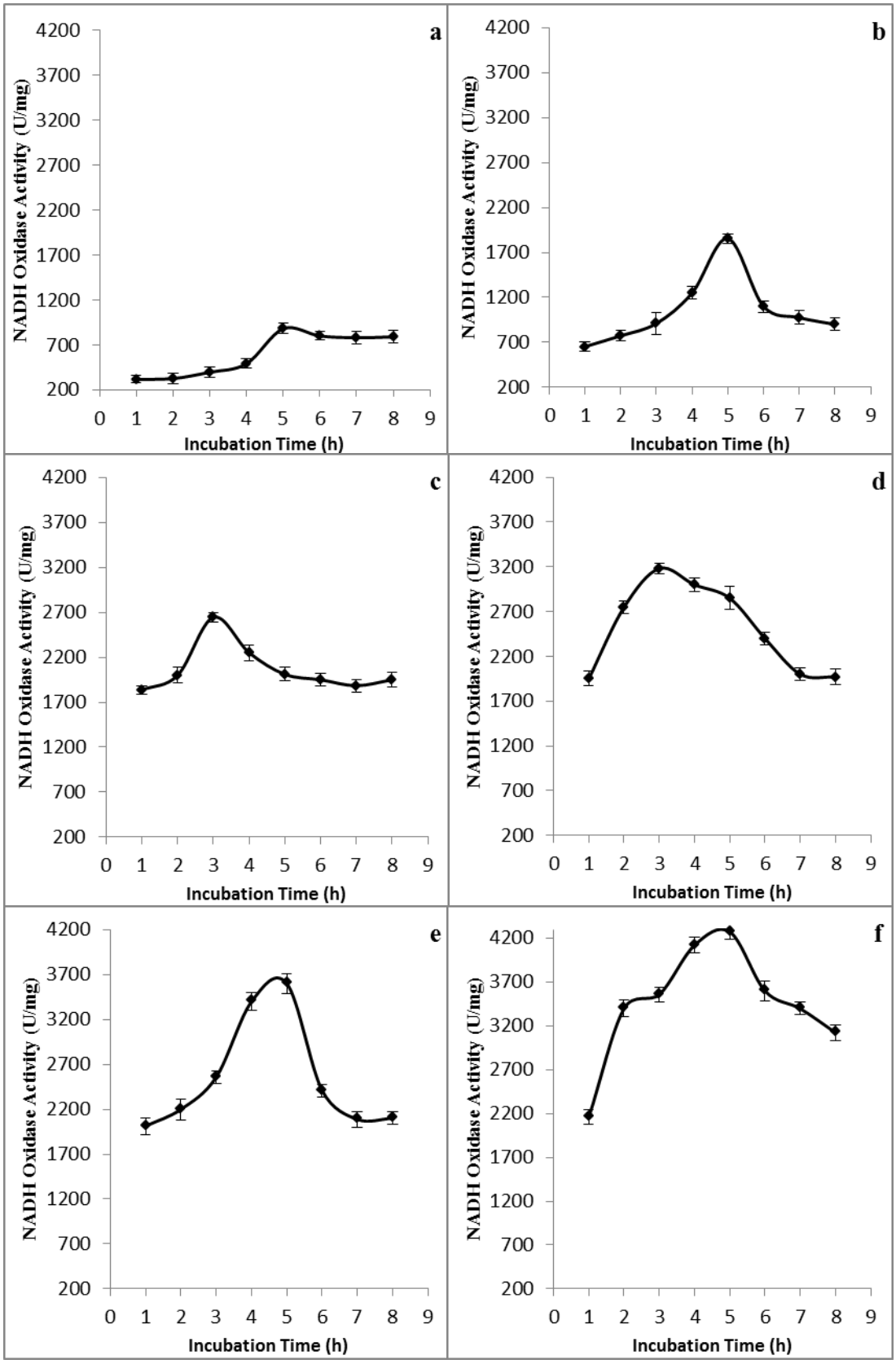


Figure 3.8 Variation of NADH Oxidase activity depend on the incubation period and concentration of menadione; control (a), and menadione concentrations 0.1 mM (b), 0.2 mM (c), 0.3 mM (d), 0.5 mM (e), 0.75 mM (f).

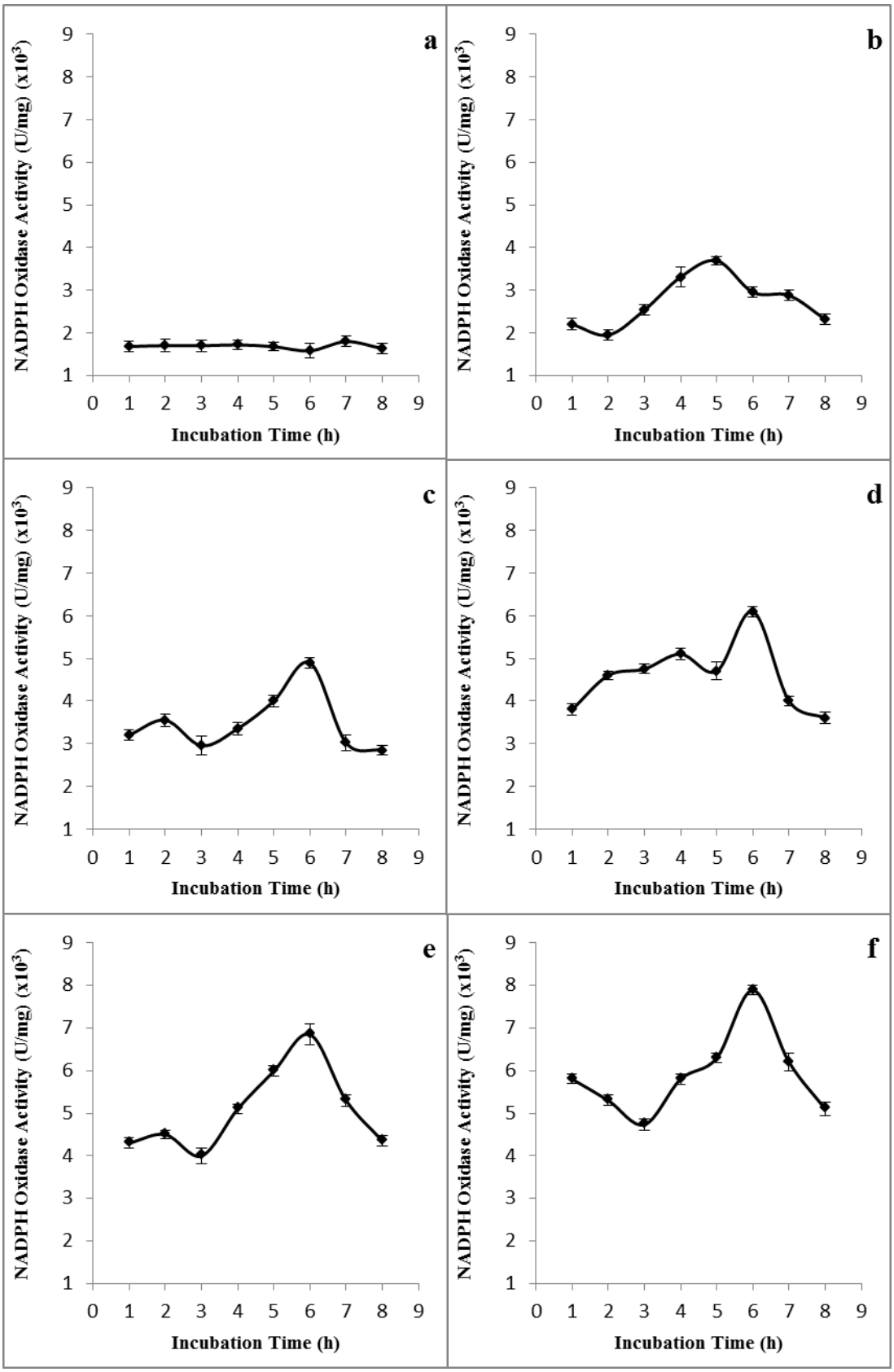


Figure 3.9 Variation of NADPH Oxidase activity depend on the incubation period and concentration of menadione; control (a), and menadione concentrations 0.1 mM (b), 0.2 mM (c), 0.3 mM (d), 0.5 mM (e), 0.75 mM (f).

was observed in 0.75 mM menadione treated sample and the value of activity was 7891.33±119 IU/mg, 5-fold higher than control

The higher values of all menadione treated conditions than control values reflect the important contributions of NADH oxidase to redox reaction in the presence of menadione. Increasing of NADPH oxidase activities similar to NADH oxidase in menadione treated conditions may be seen as a response to changes in antioxidant defence system and menadione-induced oxidative stress.

NAD(P)H oxidase is the significant enzyme that generate ROS, especially superoxide anion radical. It was shown by researchers that intracellular superoxide anion radical level increased a 2.7-fold for the angiotensin II-treated vascular smooth muscle cells. the group suggested that this superoxide production was related to stimulating NADH, NADPH oxidase activity. At this study, NADPH oxidase activity increased 3.65-fold and NADH oxidase activity increased 2.8-fold by stimulation with angiotensin II- treatment (Griendling, Minieri, Ollerenshaw, & Alexander,1994).

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3.2.4 Investigation of Variation of Energy Metabolism of P.chrysosporium Depend on Menadion Treatment

At menadione treated samples, ATP levels showed negative correlation with increasing menadione concentration. After 0.3 mM menadione treatment, ATP levels were significantly lower when compared with control ($p<0.01$) but not significant alterations when compared with each other ($p>0.01$).

At the 0.1 mM menadione treatment, ADP levels that approximately 1.2 fold higher than ATP in untreated *P.chrysosporium* samples, were observed significantly higher when compared with control ($p<0.01$) but there were not significant alteration depend on the incubation time when compared with themselves ($p>0.01$). ADP levels show significant increases until 5th and 3rd hours at 0.2 and 0.3 mM menadione treatment, respectively ($p<0.01$) and with the following incubation periods significant alteration of ADP were not observed and became closer the control ($p>0.01$). ADP levels in 0.5 and 0.75 mM menadione treated samples did not show any significant alterations when compared with control ($p>0.01$).

AMP levels were obtained higher than control in 0.1, 0.2 and 0.3 mM menadione treated samples until 5^{th}, 3^{rd} and 2^{nd} hours, respectively. With following incubation period the levels decreased to similar values with control. AMP levels in 0.5 and 0.75 mM treated samples were obtained significantly lower than control for all incubation periods.

Amount of total nucleotide was decreased after 4^{th} hour and 3^{rd} hour for 0.2 mM and 0.3 mM treatment respectively. At 0.5 and 0.75 mM menadione treatment amount of total nucleotide was decreased at the beginning of incubation period. This may be due to the damages of enzymes that involved in synthesis of nucleotide or adenine nucleotides may involve in the repair mechanisms of menadione-induced DNA damage.

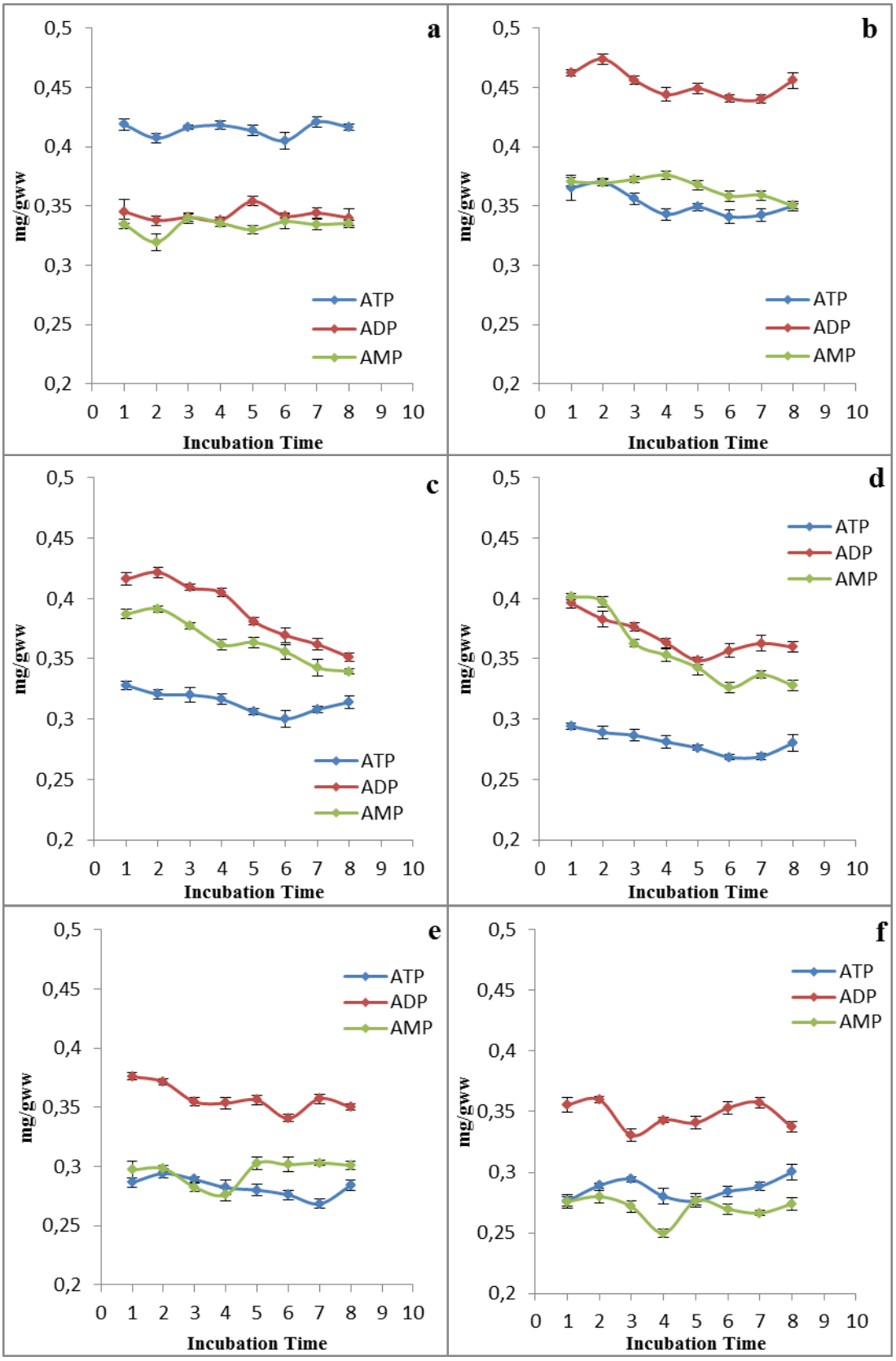

Figure 3.10 Variations of ATP, ADP, AMP levels depend on the incubation period; control (a), 0.1 mM menadione (b), 0.2 mM menadione (c), 0.3 mM menadione (d), 0.5 mM menadione (e), 0.75 mM menadione (f).

3.2.5 Investigation of Intracellular Damages of P.chrysosporium Depend on Menadion Treatment

3.2.5.1 Investigation of Protein Oxidation

The protein carbonyl content level was investigated in 0.2; 0.5 and 0.75 mM Menadione treated *P.chrysosporium*. Protein carbonyl levels did not show changes at the first two hours ($p>0.01$). As can be seen in figure 3.1, at the following incubation period, except for the 4-6th hour incubation period, increases were observed properly for all the menadione concentrations. At the 8th hour, protein carbonyl contents of *P.chrysosporium* increased 2-fold in 0.2 mM; 2.7-fold in 0.5mM and 3.0fold in 0.75 mM menadione treated samples.

According to a research, paraquat and H_2O_2 treatment to various fungi result in protein oxidation at the ratio between 2.5-4.0 when compared with control (Angelova, Pashova, Spasova, Vassilev, Slokoska, 2005). At another study, after exposure to 40 and 70 mg/ml Cu^{2+} , protein damages of the *Humicola lutea* were observed 2.4-fold higher than control (Krumova, Pashova, Dolashka, Stefanova, Angelova, 2009). On the 2'-3'-dideoxycytidine (ddC) treated cardiac muscles, protein carbonyl content was higher 1.94-fold higher than control (Skuta, et all., 1999).

No significant differences between control and menadione-treated samples in all values at the first two hours prove that antioxidant defence system has a protective role against menadione-induced oxidative stress. In menadione treated conditions the rapid increases of protein carbonyl levels, especially after the 6th hour is related with decreases of antioxidant defence enzyme activities.

3.2.5.2 Investigation of Lipid Peroxidation

P.chrysosporium membrane LPO level variations depend on the incubation period were significantly higher when compared to control at all the menadione treatment. At the range of 0.1-0.5 mM menadione treatment, did not change significantly when

compared to each other. LPO levels in the presence of 0.75 mM menadione reached the highest value, 5.32±0.13 on the 4th hour. It was 2-fold higher than control.

Intracellular membrane LPO level increases, which are indicator of oxidative stress damages, were not explosive when compared with general literature expectations, except of 0.75 mM menadione treated samples. Membrane LPO level showed its maximum value, by 2-fold, at 4th hour in 0,75 mM sample. This situation shows us important protective role of enzymatic and non-enzymatic antioxidant system.

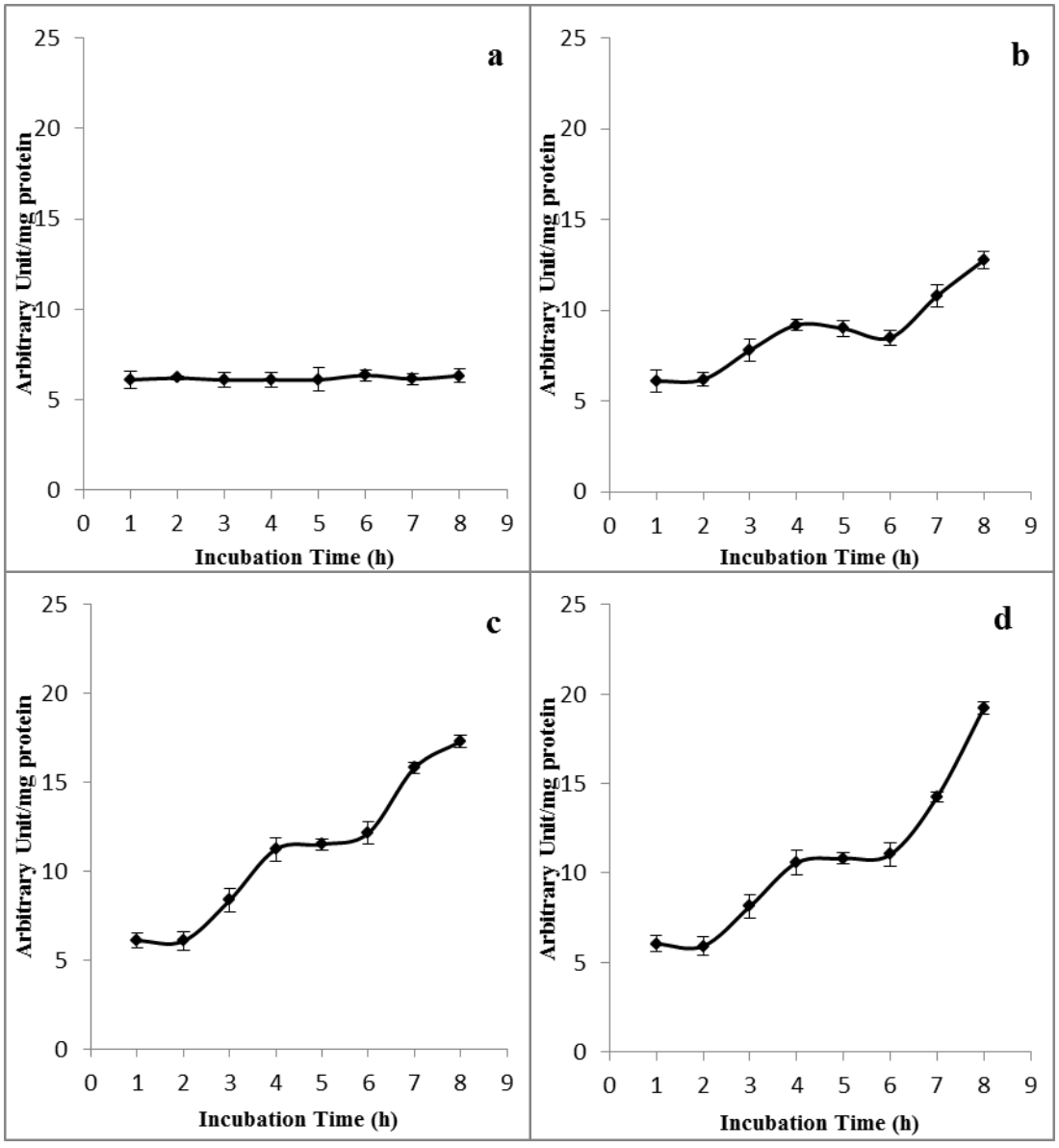

Figure 3.11 Variation protein carbonyl content levels depend on the incubation period and concentration of menadione; control (a), and menadione concentrations 0.2 mM (b), 0.5 mM (c), 0.75 mM (d)

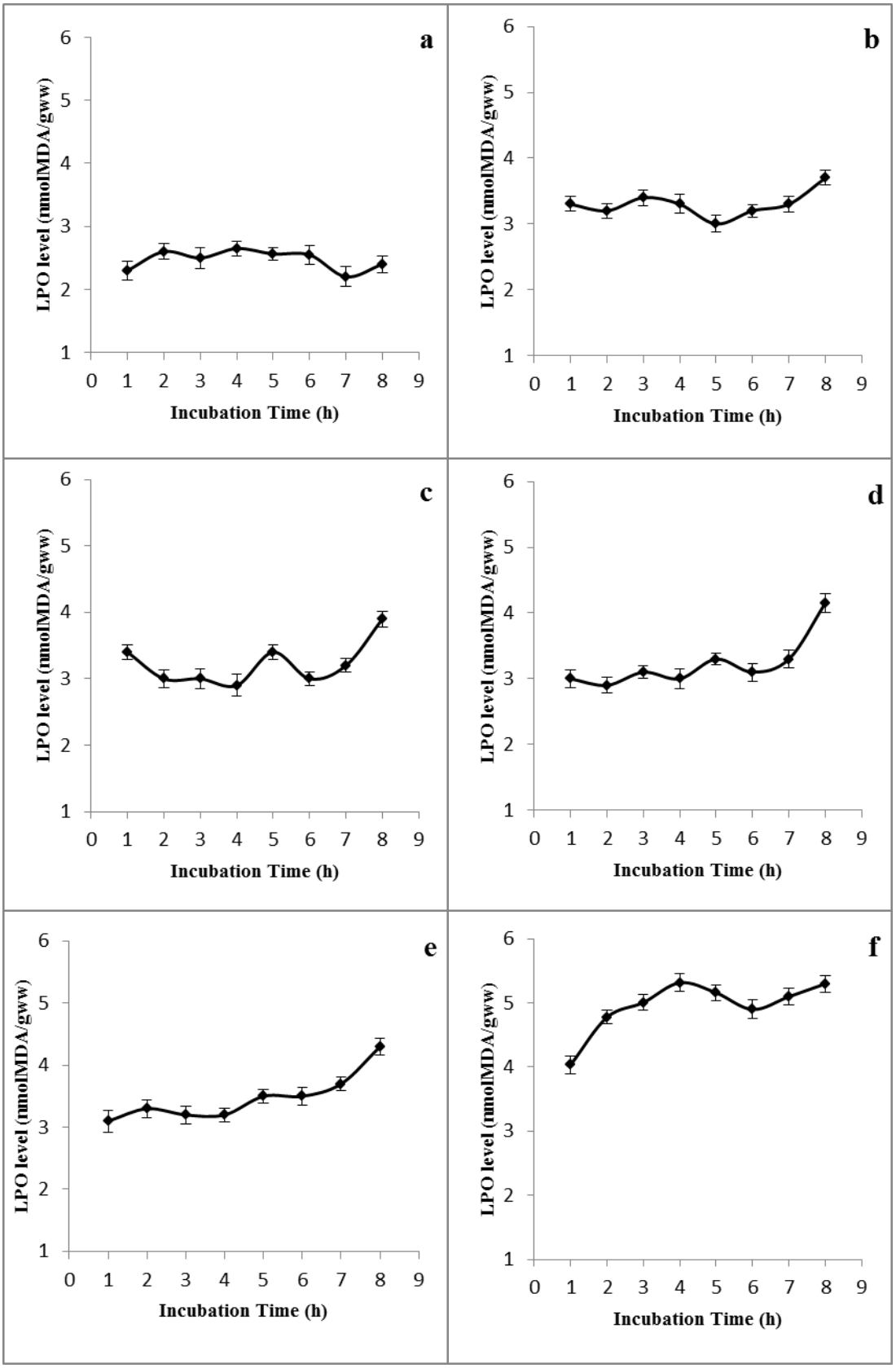

Figure 3.12 Variation of LPO level depend on the incubation period and concentration of menadione; control (a), and menadione concentrations 0.1 mM (b), 0.2 mM (c), 0.3 mM (d), 0.5 mM (e), 0.75 mM (f).

CHAPTER FOUR
CONCLUSION

Oxygen has a vital importance for aerobic organism but it can also cause damage to cells. The most important difference between aerobic and anaerobic organisms to survive in oxygenated environment is the antioxidant defence system that protects aerobics from toxicity of O_2. In physiological conditions there is a balance between reactive oxygen species (ROS) and antioxidant system. If this balance is not maintained, oxidative stress occurs and excessively generated ROS can damage DNA, lipids and proteins. Oxidative stress can be induced by a broad range of environmental factors including; chemotherapeutic, UV stress, pathogen invasion, and oxygen shortage. The other cause of oxidative stress can be endogenous sources including mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation.

In our study, we investigated the antioxidant response of *P.chrysosporium* against menadione-induced oxidative stress. For menadione supplementation, menadione, in the range from 0,1 mM to 0.75 mM, was added to culture medium of *P.chrysosporium* on the 10^{th} day and incubated between 1-8 hours and the samples were taken hourly.

Intracellular hydroxyl radical and hydrogen peroxide levels, variations of SOD, CAT, G6PDH, NADH, NADPH oxidase enzyme activities and cell membrane peroxidation and protein oxidation levels were examined and the results were compared with control within the scope of thesis.

Intracellular level of H_2O_2 did not remain stable in control samples. The decreasing of H_2O_2 levels for control samples after the 5^{th} hour may be caused by increasing activities of enzymes that use the H_2O_2 as a substrate and effects of forming the new equilibrium on the metabolism. The highest value of H_2O_2 level observed for 0.75 mM menadione treatment at the 1^{st} hour and it was 2.2-fold more than control. This increase would be considerably toxic for cells if the antioxidant

system and repair mechanisms worked properly. After decreasing of H_2O_2 level until 5^{th} hour, a tendency of increasing occurred. This downtrend can be caused by the effect of high concentration of menadione to antioxidant system or the H_2O_2 production mechanisms. Hydroxyl radical is one of the important ROS and has a quite damaging effect for cells. The highest value of hydroxyl radical was 1.73-fold higher than control. In general hydroxyl radical levels increased depending on incubation time and increasing level of menadione treatment to *P.chrysosporium*. It has been suggested that menadione-induced oxidative stress causes the generation of superoxide anion radical and formation of hydroxyl radical can be caused by generation of superoxide anion radical with Fenton reactions in metabolism. Increasing levels of hydroxyl radical related to menadione concentration is a proof of menadione caused oxidative stress on *P.chrysosporium*.

The investigated antioxidant enzymes, SOD, CAT and G6PDH, give considerable response to menadione-induced oxidative stress. The highest activities were obtained 5.4-fold, 5.1-fold and 2.5-fold higher than control. It is clear that antioxidant enzyme activities were induced by menadione treatment in order to protect *P.chrysosporium* against oxidatively occurred damages.

In order to decide adequacy of antioxidant system against menadione-induced oxidative stress, damages of protein and membrane lipids were investigated. The result shows that although menadione induces the formation of ROS, antioxidant system is able to resist against menadione-induced oxidative stress up to relatively high concentrations of menadione.

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