

THE EFFECTS OF HYDROGEN PEROXIDE, GALLIC ACID AND
RESVERATROL ON GROWTH AND CATALASE PRODUCTION OF
ASPERGILLUS FUMIGATUS

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**THE EFFECTS OF HYDROGEN PEROXIDE, GALLIC ACID AND
RESVERATROL ON GROWTH AND CATALASE PRODUCTION OF
*ASPERGILLUS FUMIGATUS***

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ABSTRACT

THE EFFECTS OF HYDROGEN PEROXIDE, GALLIC ACID AND RESVERATROL ON GROWTH AND CATALASE PRODUCTION OF ASPERGILLUS FUMIGATUS

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The aim of this study was to analyze the effect of hydrogen peroxide and selected phenolic compounds on growth and catalase production of *Aspergillus fumigatus*. As a result of growing *A. fumigatus* at different temperatures it was observed that, growth and catalase production of this species were highest at 37 °C. Catalase production was highest in the presence of 1 mM H₂O₂, yielding a significant 3 fold increase with respect to the control. Biomass was also increased by 1,44 fold with respect to the control sample. H₂O₂ increased catalase production possibly by inducing oxidative stress as biomass production significantly increased after the depletion of H₂O₂. Both gallic acid and trans-resveratrol significantly enhanced biomass generation of *A. fumigatus* (1,17 fold increase at 10 mM gallic acid and 1,45 fold increase at 3 mM resveratrol with respect to controls) and decreased extracellular catalase production (4,33 fold at 25 mM gallic acid and 16,7 fold decrease at 3 mM resveratrol with respect to controls) especially in the first 5 or 6 days of the cultivation where the anti-oxidant activity of the compounds were

possibly at their maximum. A sudden and significant rise was observed in extracellular catalase activity between 5th and 7th days of the cultivation in phenolic compound applied samples, possibly owing to the depletion of the antioxidant activity of gallic acid and resveratrol followed by fungal cells' response to a sudden increase of oxidative stress by boosting catalase production.

Keywords: Catalase, *Aspergillus fumigatus*, hydrogen peroxide, gallic acid, resveratrol, enzyme production.

ÖZ

HİDROJEN PEROKSİT, GALLİK ASİT VE RESVERATROLÜN ASPERGILLUS FUMIGATUS'UN BÜYÜMESİ VE KATALAZ ÜRETİMİ ÜZERİNDEKİ ETKİLERİ

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Bu çalışmada amaç, hidrojen peroksit ve seçilmiş fenolik maddelerin, *Aspergillus fumigatus*'un büyümesi ve catalaz üretimi üzerinde ki etkilerini analiz etmektir. *A. fumigatus*'un farklı sıcaklıklar altında büyütülmesi sonucunda, bu küf mantarı türünün, 37 °C'de büyüme ve katalaz üretimi açısından en yüksek değerlerine ulaştığı gözlenmiştir. 1 mM konsantrasyonunda uygulanan hidrojen peroksit, katalaz üretimini, kontrol grubuna göre anlamlı şekilde 3 kat arttırmıştır. Bunun yanında büyümede de, 1,44 katlık anlamlı bir artış gözlenmiştir. Hidrojen peroksitin katalaz üretimini arttırması, bu maddenin oksidatif strese yol açması olarak yorumlanmıştır. Hidrojen peroksit eşliğindeki büyüme artışının ise, bu maddenin ortamda tükenmesinden sonra anlamlı hale geldiği gözlenmiştir. Gallik asit ve trans-resveratrol maddelerinin ikisinde anlamlı biçimde *A. fumigatus*'un büyümesini arttırdığı (kontrol grubuna oranla, 10 mM'lık gallik asit örneğinde, 1,17 katlık, 3 mM'lık resveratrol örneğinde ise 1,45 katlık) ve hücre dışı katalaz üretimini, özellikle antioksidan aktivitenin muhtemelen en yüksek değerlerinde olduğu, ekimin ilk 5

veya 6 gününde düşürdüğü (kontrol grubuna oranla, 25 mM'lık gallik asit örneğinde 4,33 katlık, 3 mM'lık resveratrol örneğinde ise 16,7 katlık) gözlenmiştir. Fenolik madde eklenmiş örneklerde, ekimin beşinci ve yedinci günleri arasında, hücre dışı katalaz aktivitesinde ani ve anlamlı bir artış gözlenmiştir. Bu artışın muhtemel nedeni ise, gallik asit ve resveratrolün antioksidan aktivitelerinde tükenmenin gerçekleşmesi ve bunu takiben küf mantarı hücrelerinin, oksidatif stresdeki ani artışa bir tepki olarak katalaz üretimlerini arttırmaları olarak yorumlanmıştır.

Anahtar Sözcükler: Katalaz, *Aspergillus fumigatus*, hidrojen peroksit, gallik asit, resveratrol, enzim üretimi.

**DEDICATED
TO
MY BELOVED WIFE...**

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LIST OF ABBREVIATIONS

ROS : Reactive oxygen species

H₂O₂ : Hydrogen peroxide

FeSO₄ : Iron sulphate

OD : Optical density

Rpm: Revolutions per minute

U : Enzyme activity unit

YpSs : Yeast peptone, soluble starch

UV: Ultra violet

Cont. : Control

H₂O₂ : Hydrogen peroxide (H₂O₂)

mic.M : Micro molar

Gal. : Gallic acid

Resv. : Resveratrol

Max. : Maximum

Ext. : Extracellular

Int. : Intracellular

CHAPTER 1

INTRODUCTION

1.1 Definition and Mechanism of Oxidative Stress

Oxygen is a crucial element for all aerobic species. It can be used by an aerobic organism in the form of molecular oxygen which is called dioxygen. Oxygen is present in the atmosphere in this form. Dioxygen is produced from water by cyanobacteria, algae and plants during photosynthesis. Energy that is required to sustain life in aerobic organisms is provided by enzyme-mediated oxidation of sugars and carboxylic acids.

Oxidation is a chemical reaction that transfers electrons from an element or a molecule to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells.

1.1.1 Reactive Oxygen Species (ROS)

Free radicals are atomic or molecular species with unpaired electrons on an otherwise open shell configuration. These unpaired electrons are usually highly reactive that free radicals are very likely to take part in chemical reactions.

Reactive oxygen species (ROS) are free radicals associated with the reactions in the cells of aerobic species during the utilization of molecular oxygen called respiration (Harman, 1972). ROS are generally very small molecules which contain oxygen in

their structure. Some important molecules in the group of ROS are the superoxide anion, hydrogen peroxide, and hydroxyl radical (Valko *et al.*, 2007). ROS are highly reactive due to the presence of unpaired valence shell electrons. ROS are formed as natural byproducts of the utilization of oxygen and they also have important roles in cell signaling.

The superoxide anion is produced as a by-product of several steps in the electron transport chain (Lenaz, 2001). Particularly important is the reduction of coenzyme Q in complex III, since a highly reactive free radical is formed as an intermediate ($Q\cdot^-$). This unstable intermediate can lead to electron "leakage" when electrons jump directly to molecular oxygen and form the superoxide anion, instead of moving through the series of well-controlled reactions of the electron transport chain (Finkel and Holbrook, 2000). Moreover, free iron ions present in the cell occasionally convert hydrogen peroxide into hydroxyl radicals which are highly reactive.

Normally the level of ROS in the cell are kept at a steady level by readily neutralizing them by the enzymes that are produced by the cell's own defence system. Important proteins of this system are superoxide dismutase and catalase. When the balance between the formation and breakdown of the ROS in the cell is lost due to some internal or external factors such as disturbance of the cell's own defence system or addition of ROS to the cell from outside, a condition is formed in the cell called oxidative stress. As the concentration of ROS increases in the cell, they damage the cell by reacting with the natural components of the cell such as proteins, lipids or nucleic acids. Normally cell's own defence system repairs the damaged components of the cell but as the concentration of ROS in the cell increases, they give more and more damage and the defence system can no longer be effective.

The hydroxyl radical is particularly unstable among the free radicals and will react rapidly and non-specifically with most biological molecules. This species is produced from hydrogen peroxide in metal-catalyzed redox reactions (Stohs and

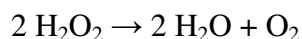
Bagchi, 1995). These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins (Sies, 1997). Damage to DNA can cause mutations and in turn sometimes bring about cancerous transformations, if not reversed by DNA repair mechanisms, (Nakabeppu *et al.*, 2006; Valko *et al.*, 2004) while damage to proteins causes enzyme inhibition, denaturation and protein degradation (Stadtman, 1992).

Oxidative stress is also thought to contribute to the development of a wide range of diseases including Alzheimer's disease, (Christen, 2000; Nunomura *et al.*, 2006) Parkinson's disease, (Wood-Kaczmar *et al.*, 2006) the pathologies caused by diabetes, (Davì *et al.*, 2005) (Giugliano *et al.*, 1996) rheumatoid arthritis, (Hitchon and El-Gabalawy, 2004) and neurodegeneration in motor neurone diseases (Cookson and Shaw, 1999). In many of these cases, it is unclear if oxidants trigger the disease, or if they are produced as a consequence of the disease and cause the symptoms (Valko *et al.*, 2007).

1.1.2 Hydrogen Peroxide

Hydrogen peroxide is a weak acid with strong oxidizing properties and is therefore a powerful bleaching agent. The oxidizing capacity of hydrogen peroxide is so strong that the chemical is considered a highly reactive oxygen species.

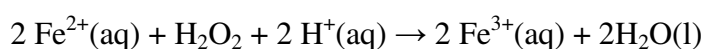
Hydrogen peroxide decomposes (disproportionates) exothermically into water and oxygen gas spontaneously:



This process is thermodynamically favorable; it has a ΔH of $-98.2 \text{ kJ}\cdot\text{mol}^{-1}$ and a ΔG of $-119.2 \text{ kJ}\cdot\text{mol}^{-1}$ and a ΔS of $70.5 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$. The rate of decomposition is dependent on the temperature and concentration of the peroxide, as well as the pH and the presence of impurities and stabilizers. Hydrogen peroxide is incompatible with many substances that catalyse its decomposition, including most of the

transition metals and their compounds. Common catalysts include manganese dioxide, and silver. The same reaction is catalysed by the enzyme catalase, whose main function in the body is the removal of toxic byproducts of metabolism and the reduction of oxidative stress. The decomposition occurs more rapidly in alkali, so acid is often added as a stabilizer.

In aqueous solution, hydrogen peroxide can oxidize or reduce a variety of inorganic ions. When it acts as a reducing agent, oxygen gas is also produced. In acid solution Fe^{2+} is oxidized to Fe^{3+} ,



and sulfite (SO_3^{2-}) is oxidized to sulfate (SO_4^{2-}). However, potassium permanganate is reduced to Mn^{2+} by acidic H_2O_2 . Under alkaline conditions, however, some of these reactions get reversed; for example, Mn^{2+} is oxidized to Mn^{4+} (as MnO_2).

In the industry, H_2O_2 is mostly used for bleaching paper, but has also found use as a disinfectant, as an oxidizer, and in rocketry (particularly in high concentrations as high-test peroxide (HTP) as a monopropellant, and in bipropellant systems.

Hydrogen peroxide is GRAS as an antimicrobial agent, an oxidizing agent and for other purposes by the US Food and Drug Administration (http://a257.g.akamaitech.net/7/257/2422/04nov20031500/edocket.access.gpo.gov/cfr_2001/aprqrtr/21cfr184.1366.htm) with a condition that, after the use of it, hydrogen peroxide should be removed from the medium with catalase application. Hydrogen peroxide has been used as an antiseptic and anti-bacterial agent for many years due to its oxidizing effect. While its use has decreased in recent years with the popularity of better-smelling and more readily available over the counter products, it is still used by many hospitals, doctors and dentists in sterilizing, cleaning and treating everything from floors to root canal procedures. Hydrogen peroxide and benzoyl

peroxide are sometimes used to treat acne (Milani *et al.*, 2003). The molecule is also used as an emetic in veterinary practice.

H₂O₂ is produced by the cells of organisms and associated with the mitochondrial respiratory chain, as well as various hydroxylation and oxygenation reactions. Inside the cells, superoxide dismutase (SOD) which is an antioxidant produced by cells, serves to convert the toxic superoxide radical (O₂⁻) into H₂O₂, as do the flavin-linked oxidases. After that, the heme-containing catalase (located in mitochondrial peroxisomes) decompose the H₂O₂ to oxygen in order to protect the cells from subsequent oxidative damage (<http://www.h2o2.com>).

Since H₂O₂ induces oxidative damage when present, production rate of enzymes such as catalases are increased as the concentration of H₂O₂ increases in the medium. Due to this reason, in order to increase the catalase production rate of cells, H₂O₂ can be added to the medium from outside. In this study, different concentrations of H₂O₂ are added to the growth medium of fungi and catalase production rates are measured. The enabled data provided values for catalase production rates to compare with the values enabled under the effect of phenolics and by this way, the effect of these phenolics on the fungi were then comprehended.

1.2 Anti-oxidants and Pro-oxidants

An antioxidant is a molecule capable of slowing down or totally preventing the oxidation of biological molecules. Antioxidants remove free radical intermediates by reacting with them and inhibit other oxidation reactions. Antioxidants are often reducing agents such as thiols or polyphenols.

Many organisms maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or

inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells.

As oxidative stress might be an important cause of many diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is not well known whether oxidative stress is the cause or the consequence of disease. Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease. Although some studies have suggested antioxidant supplements have health benefits, other large clinical trials did not detect any benefit for the formulations tested, moreover, excess supplementation may occasionally be harmful. In addition to these uses in medicine, antioxidants have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline.

Antioxidants are used as food additives to help guard against food deterioration. Exposure to oxygen and sunlight are the two main factors in the oxidation of food, so food is preserved by keeping in the dark and sealing it in containers or even coating it in wax, as with cucumbers. However, as oxygen is also important for plant respiration, storing plant materials in anaerobic conditions produces unpleasant flavors and unappealing colors (Kader *et al.*, 1989). Consequently, packaging of fresh fruits and vegetables contains an ~8% oxygen atmosphere. Antioxidants are an especially important class of preservatives as, unlike bacterial or fungal spoilage, oxidation reactions still occur relatively rapidly in frozen or refrigerated food (Zallen *et al.*, 1975). These preservatives include ascorbic acid (AA, E300), propyl gallate (PG, E310), tocopherols (E306), tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA, E320) and butylated hydroxytoluene (BHT, E321) (Iverson, 1995) (<http://www.ukfoodguide.net/enumeric.htm>).

Some antioxidants are added to industrial products. A common use is as stabilizers in fuels and lubricants to prevent oxidation, and in gasolines to prevent the

polymerization that leads to the formation of engine-fouling residues (Boozer *et al.*, 1955). They are also used to prevent the oxidative degradation of rubber, plastics and adhesives that causes a loss of strength and flexibility in these materials (<http://www.specialchem4adhesives.com/tc/antioxidants/index.aspx?id=>).

Antioxidant preservatives are also added to fat-based cosmetics such as lipstick and moisturizers to prevent rancidity.

Pro-oxidants are the chemicals which either create ROS or inhibit anti-oxidant systems of the cells and as a result, causing induction of the oxidative stress (Puglia and Powell, 1984).

1.3 Phenols and Polyphenols

Phenols or phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) attached to an aromatic hydrocarbon group. Phenols are similar to alcohols with respect to their chemical structure and some properties. Phenols are difunctional compounds. Hydroxyl group and the aromatic ring present in the phenols interact strongly and this effects reactivity of each other.

Polyphenols are characterized by the presence of more than one phenol group per molecule. Most polyphenols are naturally present in the structures of plants. These chemicals are accumulated in the body of the organism or microorganism that consume the plant. The effects of these chemicals to the health of the consumers are being searched for many years.

Phenols are known to effect the cells of the living organisms and microorganisms in various ways when they come into contact. Some of these effects are, pro-oxidant effects, anti-oxidant effects and toxic effects. Toxic effects are observed mostly when a very high amount of the chemical enters the cell and causes toxicity. Attention here is on the pro-oxidant and the anti-oxidant effects. Certain phenols can act as a part of the natural anti-oxidant defence system of the cells when taken in certain doses. They react with free radicals and reduce them. With this effect, they

protect the cell from oxidative damage and increase the cell's life span. On the other hand, some phenols act as pro-oxidants and damage the cells by creating more free radicals or by directly damaging anti-oxidant defence mechanism of the cell. Moreover, some phenols can act either as an antioxidant, or as a pro-oxidant, depending on the different kinds of conditions such as the concentration of the phenol in the cell. (Herbert, 1996) (Podmore *et al.*, 1998). It is reported in the literature that, some compounds with known anti-oxidant properties, can act as a pro-oxidant in certain concentrations and under certain conditions. It is claimed that, excessive antioxidant action can adversely affect key physiological processes (Herbert, 1996). Harms reported from pharmacological amounts of antioxidant vitamin supplements include promotion of heart disease, cancer and liver and kidney disease (Herbert, 1996). Also in a study by Podmore *et al.*, describe the potential pro-oxidant effects of daily supplementation with 500 mg of vitamin C on DNA base oxidation *in vivo*. The authors found that the oxidation of adenine (a purine) in DNA was significantly elevated after vitamin C supplementation (500 mg). The oxidation of nucleic acids is indicative of oxidative stress being placed on DNA with the concomitant exposure to reactive oxygen species, such as hydroxyl radical. The oxidation of adenine suggests that vitamin C (supplemented at 500 mg) participated as a pro-oxidant (Podmore *et al.*, 1998).

A detailed research in the subject of revealing these conditions and the exact mechanism of the phenomena is required in order to maximize the beneficial effects and to prevent the harmful effects of these chemicals.

1.3.1 Resveratrol

Resveratrol is a polyphenolic compound, naturally produced by plants under stress conditions. Anticarcinogenic, antiviral, antioxidative, antiaging and lifespan extending effects of resveratrol are discovered on yeast, animals and human (Sinclair, 2005) (Howitz *et al.*, 2003; Wood *et al.*, 2004; Baur *et al.*, 2006). Resveratrol is found at high amounts in the skin of red grapes and is a constituent of

red wine. Because of its drastic effects on cells and its availability among plants, this phenolic compound drew attention and is being studied by many scientists over the last 2 decades.

Resveratrol first came into question in 90's with the famous "french paradox" phenomena. Resveratrol in red wine is believed to be responsible for the low coronary heart disease rates of people in Southern France area, despite high dietary intake of saturated fats (Renaud and Ruf, 1994).

Resveratrol is under extensive investigation as a cancer chemopreventive agent (Saiko *et al.*, 2006; Delmas *et al.*, 2006). Indeed, there are studies showing that small doses of dietary resveratrol can reduce colon carcinogenesis in rats and mice (Sale *et al.*, 2005). One German study has already shown that under special conditions, resveratrol induces apoptosis in human fat cells. In addition, it inhibits production of cytokines which are involved in the development of obesity-related disorders (Fischer-Posovszky *et al.*, 2007).

A study of Dr. David Sinclair from Harvard Medical School, which is published in *Nature* in 2003 demonstrated that resveratrol significantly extends the lifespan of the yeast *Saccharomyces cerevisiae* (Howitz *et al.*, 2003). After these findings, studies began for the commercialization of resveratrol and related compounds as anti-aging drugs.

Later studies showed that resveratrol prolongs the lifespan of the worm *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* (Wood *et al.*, 2004). It also extended the maximum lifespan of a short-lived fish, *Nothobranchius furzeri*, by 59%, and extended its median lifespan by 56%. Also noted were an increase in swimming performance, an increase in cognitive performance (learning tasks), and a lack of neurofibrillary degeneration (found in a control group). The authors specify that "resveratrol's supplementation with food extends vertebrate lifespan and delays motor and cognitive age-related decline could be of high

relevance for the prevention of aging-related diseases in the human population. (Valenzano et al., 2006).

Later in 2006, another report in *Nature* from Sinclair's laboratory and the Laboratory of Experimental Gerontology at the National Institute on Aging showed that the compound improves health and survival of mice on a high-calorie diet. The study reveals that, resveratrol shifts the physiology of middle-aged mice on a high-calorie diet towards that of mice on a standard diet. The compound significantly increases survival by, increasing insulin sensitivity, increasing mitochondrial number, improving motor functions. This stands as a new approach for treating obesity-related disorders and diseases of ageing in mammals (Baur et al., 2006).

1.3.1.1 Structure of Resveratrol

Resveratrol (3,5,4'-Trihydroxystilbene) is a phytoalexin produced naturally by several plants as a part of the plant's defence mechanism against pathogens. Phytoalexins are antibiotics produced by plants that are under the attack of pathogens. Phytoalexins act as toxins to the pathogenic organism. They may damage the cell wall, delay maturation, disrupt metabolism or prevent reproduction of the pathogen. Phytoalexins are target specific, different phytoalexins are produced against different pathogens. Resveratrol is produced by several plants, apparently due to its antifungal properties. Resveratrol has a molecular formula as $C_{14}H_{12}O_3$ and it has a molar mass of 228,25 g/mole. It has a very low solubility in water but very soluble in DMSO and ethanol.

Resveratrol is a phenolic compound that belongs to stilbenoid group. Stilbene, or more specifically, (E)-stilbene, is an alkene, that contains an ethene with two phenyl groups on either carbon of the parent chain (Figure 1.1). Stilbenoids are the hydroxylated derivatives of stilbene (Figure 1.2). Resveratrol is produced in plants with the help of the enzyme stilbene synthase.

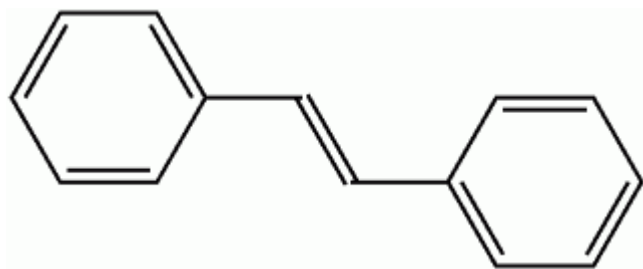


Figure 1.1 Structure of stilbene (<http://en.wikipedia.org/wiki/Image:Stilbene.png>).

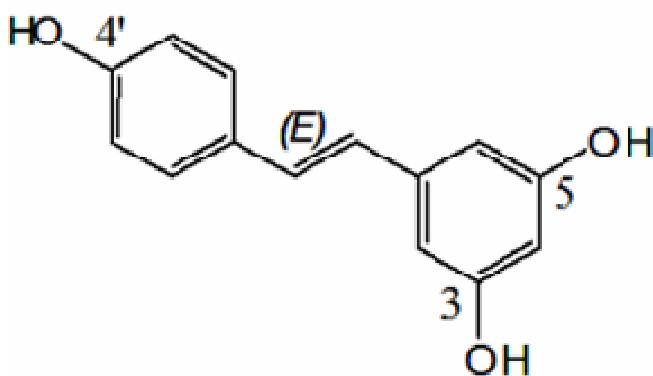


Figure 1.2 Structure of resveratrol (<http://en.wikipedia.org/wiki/Image:Resveratrol.svg>).

It exists as two structural isomers: *cis*- (*Z*) and *trans*- (*E*). The *trans*- form can undergo isomerisation to the *cis*- form when heated or exposed to ultraviolet irradiation (Lamuela-Raventos 1995). It has been stated by Dr. Sinclair of Harvard University that, it is hard to protect resveratrol from oxidation. It has been claimed that it is readily degraded by exposure to light, heat, and oxygen (Sinclair, 2005). However, studies find that *trans*-resveratrol undergoes negligible oxidation at room temperature (Bertelli, 1998). The four stilbenes *cis*- and *trans*-resveratrol, and *cis*- and *trans*-piceid are similar and related, and sometimes analyzed together as a group (LeBlanc, 2006). Piceid is a stilbenoid glucoside and is a major resveratrol derivative

in grape juices (Romero-Perez *et al.*, 1999; Wang *et al.*, 2002) (Figure 1.3). According to some of the previous studies on resveratrol, the fortifying effects of this compound on the cell structure can only be achieved with *trans*-resveratrol and *cis*-resveratrol has not shown any beneficial effects on cells.

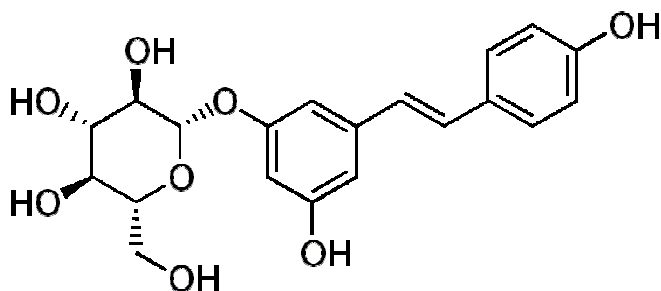


Figure 1.3 Structure of piceid (<http://en.wikipedia.org/wiki/Image:Piceid.png>)

1.3.1.2 Availability In Plants

It is found in widely varying amounts in grapes (primarily the skins), raspberries, mulberries, in plums, peanuts, berries of *Vaccinium* species, including blueberries, bilberries, and cranberries, some pines, such as Scots pine and eastern white pine, and the roots and stalks of giant knotweed and Japanese knotweed (Wang *et al.*, 2002).

Resveratrol concentration is relatively high in the skins of red grapes and in the red wines. Resveratrol amount is highest in *Vitis vinifera*, *labrusca*, and muscadine among grapes. Ordinary non-muscadine red wine contains between 0.2 and 5.8 mg/L (Gu *et al.*, 1999), depending on the grape variety. White wine has much less resveratrol, the reason of that is, red wine is fermented with the skins, allowing the wine to absorb the resveratrol, whereas white wine is fermented after the skin has

been removed. Wines produced from muscadine grapes, however, both red and white, may contain more than 40 mg/L (Ector *et al.*, 1996) (LeBlanc, 2006).

In grapes, resveratrol is found primarily in the skin and seeds, with fresh grape skin typically containing about 50 to 100 µg of resveratrol per gram (Tuma, 2007). This is particularly true for muscadine grapes, whose skin and seeds have about 100 times the concentration as the pulp (Ho *et al.*, 2004). The amount found in grape skins also varies with the grape cultivar, its geographic origin, and exposure to fungal infection. The amount of fermentation time a wine spends in contact with grape skins is an important determinant of its resveratrol content (Roy and Lundy, 2005).

1.3.1.3 Molecular Mechanisms of Resveratrol

Resveratrol interferes with all three stages of carcinogenesis - initiation, promotion and progression. Experiments in cell cultures of varied types and isolated subcellular systems *in vitro* imply many mechanisms in the pharmacological activity of resveratrol. These mechanisms include modulation of the transcription factor NF-κB (Leiro *et al.*, 2005) inhibition of the cytochrome P450 isoenzyme CYP1A1 (Chun *et al.*, 1999), alterations in androgenic (Benitez *et al.*, 2006) actions and expression and activity of cyclooxygenase (COX) enzymes.

In some lineages of cancer cell culture, resveratrol has been shown to induce apoptosis, which means it kills cells and may kill cancer cells (Benitez *et al.*, 2006; Faber and Chiles, 2006; Riles *et al.*, 2006; Sareen *et al.*, 2006; Tang *et al.*, 2006; Aziz *et al.*, 2006). Resveratrol has been shown to induce Fas/Fas ligand mediated apoptosis, p53 and cyclins A, B1 and cyclin-dependent kinases cdk 1 and 2. Resveratrol also possesses antioxidant and anti-angiogenic properties (Cao *et al.*, 2005; Hung *et al.*, 2000).

Resveratrol was reported effective against neuronal cell dysfunction and cell death, and in theory could help against diseases such as Huntington's disease and

Alzheimer's disease (Parker *et al.*, 2005; Marambaud *et al.*, 2005). Research at the Northeastern Ohio Universities College of Medicine and Ohio State University indicates that resveratrol has direct inhibitory action on cardiac fibroblasts and may inhibit the progression of cardiac fibrosis (Olson *et al.*, 2005).

The mechanisms of resveratrol's apparent effects on life extension are not fully understood, but they appear to mimic several of the biochemical effects of calorie restriction. This seems to function by means of lipase inhibition, reducing the absorption of fat through intestinal walls.

According to Sinclair *et al.* 2003, life-prolonging effects of resveratrol is based on that it activates sirtuins in the cell. Sirtuin is a class of enzyme, specifically NAD-dependent histone deacetylases (class 3), found in both prokaryotes and eukaryotes. They have been known to effect cellular metabolism through selective gene expression in eukaryotes. Sirtuins play an important role in the survival of damaged cells and delaying the cell death in all organisms from fungi to mammals. Sirtuins are a part of a feedback mechanism that provide the survival of the cell under stress conditions. Molecular effect of resveratrol on yeast cell is expressed as, providing a longer stability of DNA by activating Sir2 enzyme and as a result of this, prolonging cell's life span (Howitz *et al.*, 2003).

Sir2 is a member of a sirtuin family and among this family there is Sir2.1 which is an enzyme that controls life span in *C.elegans* and SIRT1 which is a deacetylase that controls p53 tumor repressor gene in human (Howitz *et al.*, 2003). It is indicated that resveratrol also activates SIRT1 and PGC-1 α and improve functioning of the mitochondria (Lagouge *et al.*, 2006). Only the "Trans" form of the molecule is capable of activating the mammalian SIRT1 gene in vitro; this is also the form predominantly found in red grape skins and red wine.

According to Auwerx *et al.*, mice fed resveratrol for 15 weeks had better treadmill endurance than controls (Lagouge *et al.*, 2006). The study supported Sinclair's

hypothesis that the effects of resveratrol are indeed due to the activation of SIRT1 (Howitz *et al.*, 2003).

Resveratrol also seems to increase the potency of some antiretroviral drugs against HIV in vitro (Heredia *et al.*, 2000). Infection by herpes simplex virus ordinarily activates the cell protein Nuclear Factor κ B (NF- κ B). A Northeastern Ohio Universities College of Medicine study undertaken in Vero cells found that resveratrol suppresses the activation of this transcription- and apoptosis-related protein. The study further found that multiple viral protein products were reduced or completely blocked, as well as a reduction in viral DNA production (Faith *et al.*, 2006).

A cell culture study found that resveratrol blocks the influenza virus from transporting viral proteins to the viral assembly site, hence restricting its ability to replicate. The effect was 90% when resveratrol was added six hours after infection and continued for 24 hours thereafter (Palamara *et al.*, 2005).

While the health benefits of resveratrol seem promising, one study has theorized that it may stimulate the growth of human breast cancer cells, possibly because of resveratrol's chemical structure, which is similar to a phytoestrogen (Gehm *et al.*, 1997; Bowers *et al.*, 2000). However, other studies have found that resveratrol actually fights breast cancer (Levi *et al.*, 2005; Garvina *et al.*, 2006). Because of this remaining dilemma, more studies should be done on this subject in order to get a full understanding of this molecule's effects and mechanism of effects.

1.3.2 Gallic Acid

Gallic acid is a polyphenolic compound produced by plants like resveratrol. Gallic acid is found in almost all plants. It is presented in concentrated amounts in gallnuts, sumac, witch hazel, tea leaves, oak bark, grapes and hops (<http://www.phytochemicals.info/>).

Gallic acid (3,4,5-Trihydroxybenzoic acid) is a colourless crystalline organic powder. Gallic acid occurs as a free molecule or as part of a tannin molecule (<http://www.phytochemicals.info/>). It has a molecular formula of $C_7H_6O_5$ and molar mass of 170.12 g/mol (Figure 1.4).

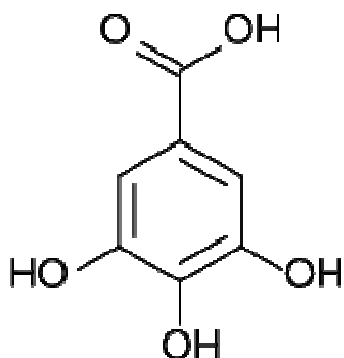


Figure 1.4 Structure of gallic acid (http://en.wikipedia.org/wiki/Image:Gallic_acid.svg).

Gallic acid is believed to have anti-fungal and anti-viral properties, but scientific data to prove this theory is not yet presented. It is commonly used in the pharmaceutical industry. Some gallic acid derivatives act as a antioxidant and helps to protect host cells against oxidative damage. Gallic acid was found to show cytotoxicity against cancer cells, without any effect on healthy cells (<http://www.phytochemicals.info/>). Further studies should be done in order to fully understand the effects of this molecule on cell structure and the mechanism of effect.

Gallic acid is obtained from plants or by the hydrolysis of tannic acid with sulfuric acid. Gallic acid has two functional groups in the same molecule, hydroxyl groups and a carboxylic acid group. They can yield numerous esters and salts including

digallic acid which is formed by the reaction of two moles of gallic acid with one another. When heated above 220°C, gallic acid loses a carbon dioxide to form pyrogallol, or 1,2,3-trihydroxybenzene, which is used in the production of azo dyes and photographic developers and in laboratories for absorbing oxygen. Salts and esters of gallic acid are termed as gallates. Some gallates are used as antioxidants in foods. Tannins (tannic acid or gallotannic acid) are esters of digallic acid (<http://chemicalland21.com/>). They are widely used in tanning leather, dyeing fabrics, manufacturing inks, and in various medical applications such as astringents and to treat some skin diseases and in cases of internal haemorrhage. This molecule also has usage in treating albuminuria and diabetes (<http://chemicalland21.com/>). Benzoic acid derivatives substituted by hydroxyl group or ether containing oxygen atom have bacteriostatic and fragrant properties. They are typically used in pharmaceutical and perfumery industry (<http://chemicalland21.com/>).

1.4 Catalase

Molecular oxygen which is used for energy production in cells can easily be converted to a ROS at the time the molecule is in contact with the cell. These ROS then readily attack natural components of cells such as DNA and mutate them. Cell's own defence mechanism produce enzymes that neutralize these free radicals. Two of these important enzymes are superoxide dismutase and catalase. Superoxide dismutase's function is that it converts superoxide radicals into hydrogen peroxide. Catalase's major function is the degradation of hydrogen peroxide to dioxygen and water (Goldberg and Hochman, 1989) (Figure 1.5).

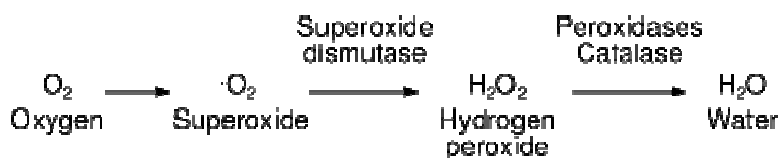
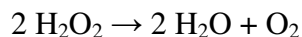


Figure 1.5 Enzymatic pathway for detoxification of reactive oxygen species (http://en.wikipedia.org/wiki/Image:Antioxidant_pathway.svg).

The decomposition of H₂O₂ is as follows:



Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long (Boon *et al.*, 2007). It contains four heme groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for catalase is approximately neutral (pH 7.0) (Glick, 1954) while the optimum temperature varies by species (Gibbs, 2005).

Catalase is an enzyme which belongs to oxidoreductases group. Oxidoreductases are enzymes that catalyze the transfer of electrons from one molecule which is the reductant, (also called the hydrogen acceptor or electron donor) to another molecule which is here the oxidant (also called the hydrogen donor or electron acceptor).

Catalase also belongs to a group of metalloenzymes. A metalloprotein is a generic term for a protein that contains a metal cofactor. The metal may be an isolated ion or may be coordinated with a nonprotein organic compound. Metalloenzymes constitutes a part of metalloproteins. These group of enzymes contain one or more metal atoms in their structures as functional groups. These metals are often involved in enzyme catalysis.

Catalase enzyme contains this metal atom as a heme group. A heme is a prosthetic group that consists of an iron atom contained in the center of a large heterocyclic organic ring. A substantial fraction of metalloproteins have heme as their prosthetic subunit; these are known as hemoproteins. Hemoproteins have diverse biological functions including the transportation of diatomic gases, chemical catalysis, diatomic gas detection, and electron transfer. The heme iron serves as a source or sink of electrons during electron transfer or redox chemistry. In the transportation or detection of diatomic gases, the gas atoms binds to the heme iron and this binding induces conformational changes in the surrounding protein. It has been hypotized that the original evolutionary function of hemoproteins was electron transfer in

primitive sulfur-based photosynthesis pathways in ancestral cyanobacteria before the appearance of molecular oxygen (Hardison, 1999).

There are many types of catalases differing in their functions and structures in different organisms. Even in one organism, there can be more than one type of catalase. Some catalases are monofunctional and some are bifunctional, for example showing both catalase and phenol oxidase activities. Catalases are some of the most efficient enzymes found in cells. Each catalase molecule can decompose millions of hydrogen peroxide molecules in one second. Most of the catalases use an iron ion to assist in this reaction. The enzyme is composed of four identical subunits, each with its own active site buried deep inside (Fita *et al.*, 1985). The iron ion, is gripped at the center of a disk-shaped heme group. Catalases are incredibly stable enzymes and this feature provides the protection from the damage that these highly reactive molecules could cause.

All members of the animal and plant kingdoms contain catalase in their structures. Animals contain catalase in every organ, with particularly high concentrations occurring in the liver. Some species of fungi have been found to produce the enzyme when growing in stress conditions such as in a low pH (Isobe *et al.*, 2006). Some of these fungi are reported as effective producers of catalases (Isobe *et al.*, 2006).

1.4.1 Molecular Mechanisms of Catalase

Catalases catalyse the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor (Zámocký, 1999; Chelikani, 2004). This protein is localized to peroxisomes in most eukaryotic cell (Del Río, 1992). Catalase is an unusual enzyme since, although hydrogen peroxide (Figure 1.6) is its only substrate, it follows a ping-pong mechanism. Here, its cofactor is oxidised by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate (Hiner, 2002).

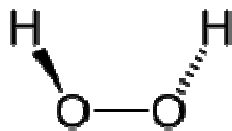
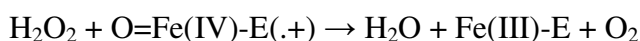
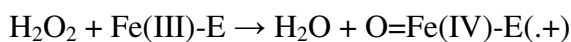


Figure 1.6 Structure of hydrogen peroxide (<http://en.wikipedia.org/wiki/Image:Hydrogen-peroxide-2D.png>).

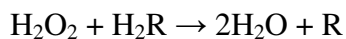
The exact mechanism of catalase is not currently known, the reaction is hypotized to occur in two stages (Boon *et al.*, 2007):



Fe(-)E represents the iron center of the heme group attached to the enzyme. Fe(IV)-E(.+) is a mesomeric form of Fe(V)-E, meaning that iron is not completely oxidized to +V but receives some "supporting electron" from the heme ligand. This heme has to be drawn then als radical cation (.+).

As hydrogen peroxide enters the active site, it interacts with the amino acids Asn147 (asparagine at position 147) and His74, causing a proton (hydrogen ion) to transfer between the oxygen atoms. The free oxygen atom coordinates, freeing the newly-formed water molecule and Fe(IV)=O. Fe(IV)=O reacts with a second hydrogen peroxide molecule to reform Fe(III)-E and produce water and oxygen (Boon *et al.*, 2007). The reactivity of the iron center may be improved by the presence of the phenolate ligand of Tyr357 in the fifth iron ligand, which can assist in the oxidation of the Fe(III) to Fe(IV). The efficiency of the reaction may also be improved by the interactions of His74 and Asn147 with reaction intermediates (Boon *et al.*, 2007). In general, the rate of the reaction can be determined by the Michaelis-Menten equation (Loew, 1900).

Catalase can also oxidize different toxins, such as formaldehyde, formic acid, and alcohols. For that, catalase uses hydrogen peroxide according to the following reaction:



Again, the mechanism of this reaction is not exactly known.

Any heavy metal ion (such as copper cations in copper(II) sulfate) acts as a noncompetitive inhibitor on catalase. Also, cyanide is a competitive inhibitor of catalase, strongly binding to the heme of catalase and stopping the enzyme's action.

1.4.2 Applications of Catalases

Catalase is used in the food industry for removing hydrogen peroxide from milk prior to cheese production (Schroeder *et al.*, 1969). Another use is in food wrappers, where it prevents food from oxidizing (Murthy *et al.*, 1981). Catalase is also used in the textile industry, removing hydrogen peroxide from fabrics to make sure the material is peroxide-free. A minor use is in contact lens hygiene - a few lens-cleaning products disinfect the lens using a hydrogen peroxide solution; a solution containing catalase is then used to decompose the hydrogen peroxide before the lens is used again (Cook *et al.*, 1994). Recently, catalase has also begun to be used in the aesthetics industry. Several mask treatments combine the enzyme with hydrogen peroxide on the face with the intent of increasing cellular oxygenation in the upper layers of the epidermis. In microbiology, the *catalase test* is used to differentiate between bacterial species in samples. H_2O_2 is added to the samples and bubble formation is observed in order to see if the microorganism can produce catalase or not (Boon *et al.*, 2007).

1.4.3 Catalases of *A. fumigatus*

There are 3 different catalases produced in *A. fumigatus* discovered so far. One of these catalases is a conidial catalase which is only produced by conidia. Named CatAp, this spore specific catalase is a monofunctional catalase that is resistant to heat, metal ions and detergent. This catalase is believed, not to be a virulence factor. Other 2 catalases are both mycelial catalases namely, Cat1p (or CAT1) and Cat2p (or CAT2). Cat1p is found to be an extracellular catalase since, its detection in the cell wall, membrane fractions, culture supernatant and also possession of a signal peptide are observed. Also, this enzyme is believed to be a virulence factor of *A. fumigatus* (Calera *et al.*, 1997). Cat2p is defined as a bi-functional enzyme with catalase-peroxidase activities and also sensitive to heat, metal ions and detergent. This catalase is portrayed as an important virulence factor for *A. fumigatus* infections since it has both catalase and peroxidase activities (Paris *et al.*, 2003).

1.5 Aim of The Study

Aim of this study is to analyze biomass generation and catalase production of *A. fumigatus* in the presence of H₂O₂, gallic acid and resveratrol. As catalase is a vital enzyme for fungal life which is produced in order to deal with hazardous cellular oxidation, the observation of catalase production may give ideas about the mechanisms related to oxidative stress. To understand these mechanisms, H₂O₂, will be added to the growth medium as an inducer of catalase, and phenolics, which are reported to have anti-oxidant effect on cells. Analyzing catalase production may reveal the effects of these phenolics on the cell by altering oxidative stress condition. Also some anti-oxidant compounds are claimed to act as pro-oxidant under certain circumstances. The application of gallic acid and resveratrol to the growth medium of *A. fumigatus* may enable the observation of a possible anti-oxidant to pro-oxidant effect and the determination of the conditions of this transition in relation to growth and catalase production.

CHAPTER 2

MATERIAL AND METHODS

2.1. Materials

2.1.1 Fungal Strains

Aspergillus fumigatus IMI 385708 was kindly provided by Prof. Dr. Peter Biely from the Slovak Academy of Sciences, Institute of Chemistry.

Aspergillus sojae (ATCC11906) was kindly supplied by Dr. Peter Punt from TNO Nutrition and Food Research, Department of Microbiology Holland.

Aspergillus niger, *Aspergillus nidulans*, *Aspergillus flavus* and *Aspergillus oryzae* were kindly provided by Dr. Ceyda Pembeci from Food Science and Technology Research Institute, Marmara Research Center, TUBITAK, from the TUBITAK MRC Culture Collection.

2.1.2 Chemicals and Enzymes

The list of chemicals used and their suppliers are given in Appendix A.

2.1.3 Growth Media, Buffers and Solutions

The preparation of the growth media, buffers and solutions used are given in Appendix B.

2.2 Methods

2.2.1 Maintenance and Cultivation of the Strains

Stock cultures of *Aspergillus fumigatus* were inoculated onto YpSs agar plates (Appendix B) and incubated at 37 °C for 4-5 days until sporulation, followed by storage at 4 °C for a maximum of 2 months. Every 2 months, stock cultures were periodically renewed.

Stock cultures of *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus flavus* and *Aspergillus oryzae* were inoculated onto YpSs agar plates (Appendix B) and incubated at 30 °C for 4-5 days until sporulation, followed by storage at 4 °C for a maximum of 2 months. Every 2 months, stock cultures were periodically renewed.

To prepare spore suspensions, spores of the fungal strains were collected by pouring 1 ml of Saline Tween Solution (Appendix B) on to the surface of the agar plate and by gentle mixing. Saline Tween Solution (Appendix B) with spores, was pipetted from the surface and stored.

Enumeration of the spores in the spore suspension was done by using a haemocytometer (Chang Bioscience Inc.).

For the comparison of the growth of *Aspergillus* species, 100 spore/ml of medium was used. Spores were directly inoculated into medium containing 100 ml YpSs broth (Appendix B) with glucose instead of starch, in 250 ml erlen mayers. Totally, six erlen mayers are used for each experiment.

For the analysis of growth and catalase production of *A. fumigatus*, maximization of the enzyme production was crucial to clearly see the differences in enzyme productivities between the cultivations. For this purpose, 10000 spores/ml of medium was selected as the optimum. Spores were directly inoculated into medium containing 100 ml YpSs broth (Appendix B) with glucose instead of starch in 250 ml erlen mayers. Biomass is measured every 48 hours. Totally, eight erlen mayers are used for each experiment.

The analysis of growth and catalase production in the presence of phenolics and other compounds are done by applying 0,0085% (wt/vol) FeSO₄, H₂O₂ (100 µM, 500 µM, 1 mM, 2 mM), trans-resveratrol (100 µM, 500 µM, 3 mM) and gallic acid (500 µM, 3 mM, 10 mM, 25 mM) (as final concentrations).

2.2.2 Analysis of Biomass Generation

Growth was analysed every 24 hours after inoculation. 10 ml's of samples were taken from each 100 ml flask. A flask was only used twice for sampling and then discarded. Samples were filtered through 3 to 4 layers of pre-weighed aluminium foil with 4-5 holes that has a size sufficient to prevent passage of the mycelium. Filter paper caused errors and was, therefore, not preferred. Samples were dried in 60 °C oven overnight and weighed.

2.2.3 Catalase Enzyme Activity Analysis

Catalase activities in culture supernatants (extracellular catalase activity) was determined every 24 hours of growth. Catalase activity assay was done according to (Beer and Sizer, 1952). 50 mM potassium phosphate solution was used as the buffer. To prepare the substrate, 10,8 µl from the 30% H₂O₂ solution was pipeted and added to 10 ml buffer and vortexed giving a final concentration of 0,32 g H₂O₂ per liter solution. 1,5 ml of substrate solution was taken and it's optical density was measured by a UV spectrophotometer (SHIMADZU UV-1700) at 240 nm wavelength and

recorded. Blanks contained only the buffer solution. Assay was done at 25 °C. 1,45 ml substrate solution was placed into cuvettes. 50 µl of culture supernatant was added to the cuvette to complete the volume to 1,5 ml and mixed. The assay was done by recording absorbance values of each sample every 30 seconds for a total of 10 minutes. Assay should be started immediately after the addition of the enzyme to the substrate because the reaction rate is very high. After the assay was done, curves were plotted with the absorbance values versus time. Initial rate of the reaction expressed the activity. Slopes were used to calculate the activities. Catalase activity was expressed in units per ml of the culture medium (U/ml) which is, 1 µmole of substrate oxidised in one minute by 1 ml of culture supernatant and calculated by the formula given below:

$$\text{Enzyme Activity (U/ml)} = (\Delta\text{OD}/\Delta t) (1/\epsilon) (1000) (V_k/V_s) (\text{Dilution factor})$$

ΔOD : Change in the optical density

Δt : Change in time (min)

ϵ : Extinction coefficient of the substrate (39,4 for hydrogen peroxide) ($\text{M}^{-1}\text{cm}^{-1}$)

V_k : Total volume of the solution in the cuvette (ml)

V_s : Volume of the supernatant in the cuvette (ml)

The coefficient “1000” in the formula is used to adjust the unit of the extinction coefficient from 1/(mole/L) to 1/(µmole/ml).

2.2.3.1 Intracellular Catalase Enzyme Activity Analysis

10 ml of samples were taken from each culture medium and filtered through filter paper. The sample was washed with distilled water several times. Biomass was then wrapped in a piece of aluminum foil and put in a container filled with liquid nitrogen. In order to lyse the cells, a micro dismembrator was used. Its working principle was based on mixing at very high speeds (1500 – 2000 rpm) in the presence of metal spheres. Thus cells were crushed and lysed. Before dismembration started, Complete Mini (EDTA free) protease inhibitor (Appendix A) was added to the container (one

tablet for a total of 10 ml cell extract) in order to prevent the activity of proteases. This compound contains different types of protease inhibitors for the proteases of fungi and it was important for the protease inhibitor to be EDTA free since our enzyme was a metallo enzyme and the metal chelating agent EDTA could react with and decompose our enzyme besides metallo proteases. After lysis, 400 μ l of 50 mM potassium phosphate buffer was added on the cell lysate and mixed. The mixture was then placed in a 1,5 ml eppendorf tube. After centrifugation at 12000 rpm for 8 minutes, the pellet formed was discarded and the clear lysate was used in activity analysis. The same formula presented above in the section 2.3.3 is used for intracellular catalase assay. In this procedure, “ml” value which is the denominator of activity unit (U/ml) does not represent ml of the culture supernatant instead, it represents, ml of the solution that was prepared by adding 400 μ l of 50 mM potassium phosphate buffer on dismembrated cell fragments.

To compare the extracellular activities with intracellular ones, both were translated into U/g biomass units. This is done for extracellular activity by multiplying U/ml activity values with 100 (since there was totally 100 ml of supernatant in the flask) and dividing by the total biomass (g/100 ml) in the flask. For intracellular activity, the solution prepared by adding 400 μ l of 50 mM potassium phosphate buffer on dismembrated cell fragments contain 10% of the total catalase enzyme present in the flask (since 10 ml of sample was taken and dismembrated out of 100 ml culture medium). Intracellular Activity (U/ml) value was divided by 2,5 to get the enzyme activity in 400 μ l solution. This value was then multiplied with 10 to get the total activity in the flask and divided by the total biomass (g/100 ml) in the flask.

2.2.4 Statistical Analysis of the Experimental Data

Statistical analysis of the experimental data was carried out by using SPSS software v15.0 Copyright (c) SPSS Inc. Data were subjected to ANOVA and Multiple Comparisons (Scheffe model) tests in order to test the significance of the changes in the results obtained by the application of various compounds to the growth medium

of the fungus with respect to the controls. Outputs are presented in Appendix C. The samples were subjected to statistical tests in four different subjects as, maximum biomass generation, maximum growth rate, maximum extracellular catalase production and maximum intracellular catalase production. ANOVA test determines if there is a significant difference in results between the groups (significance level was set to: $p < 0,01$ for all tests). A value, lower than 0,01 in the column named “Sig.” shows that there is significance between the groups and to observe which samples’ results were significantly different from the control, Multiple Comparisons (Scheffe model) test is applied. Again a value lower than 0,01 in the “Sig.” column shows that sample’s result is significantly different than the control.

CHAPTER 3

RESULTS AND DISCUSSIONS

It was aimed in this study to analyze growth and catalase production of *A. fumigatus* in the presence of H₂O₂ and some functional phenolic compounds. Biomass generation and extracellular and intracellular catalase productions of *A. fumigatus* were measured every 24 hours for 8 days in the presence of iron sulphate (0.3 mM), H₂O₂ (0,1; 0,5; 1 and 2 mM), gallic acid (0,5; 3; 10 and 25 mM) and resveratrol (0,1; 0,5 and 3 mM). Results were statistically analyzed with ANOVA and Multiple Comparison (Scheffe model) tests (significance level was set to: $p < 0,01$) (Appendix C).

3.1 Comparison of Growth of *Aspergillus* Species at Different Temperatures

First, it was of interest to compare the growth rate of *A. fumigatus* with those of some other *Aspergillus* species namely *A. niger*, *A. flavus*, *A. oryzae* and *A. sojae* at both 30 and 37 °C in order to have some idea about the general fitness of the *Aspergillus* strains present in our collection. Initial pH values of the media were measured as 7,1.

Accordingly, *A. niger* showed the best growth rate at 30 °C (Figure 3.1), while at 37 °C, *A. fumigatus* showed the highest growth rate (Figure 3.2).

It can be observed from Figures 3.1 and 3.2, *A. fumigatus* cell fitness was higher at 37 °C than at 30 °C yielding a 1,2 fold higher biomass at 37 °C than at 30 °C. Also

the growth rate ($\text{gml}^{-1}\text{day}^{-1}$) of the fungus at 37 °C was 1,6 fold higher than value at 30 °C.

Due to the high growth rate of *A. fumigatus* especially at 37 °C, which is close to body temperature, and due to the significance of *A. fumigatus* as an opportunistic human pathogen, it was decided to continue studies on catalase production using *A. fumigatus*. This would also contribute to the literature by providing more data on the catalase production of this fungus, which is known to be a part of the defence system of *A. fumigatus* (Shibuya *et al.*, 2006).

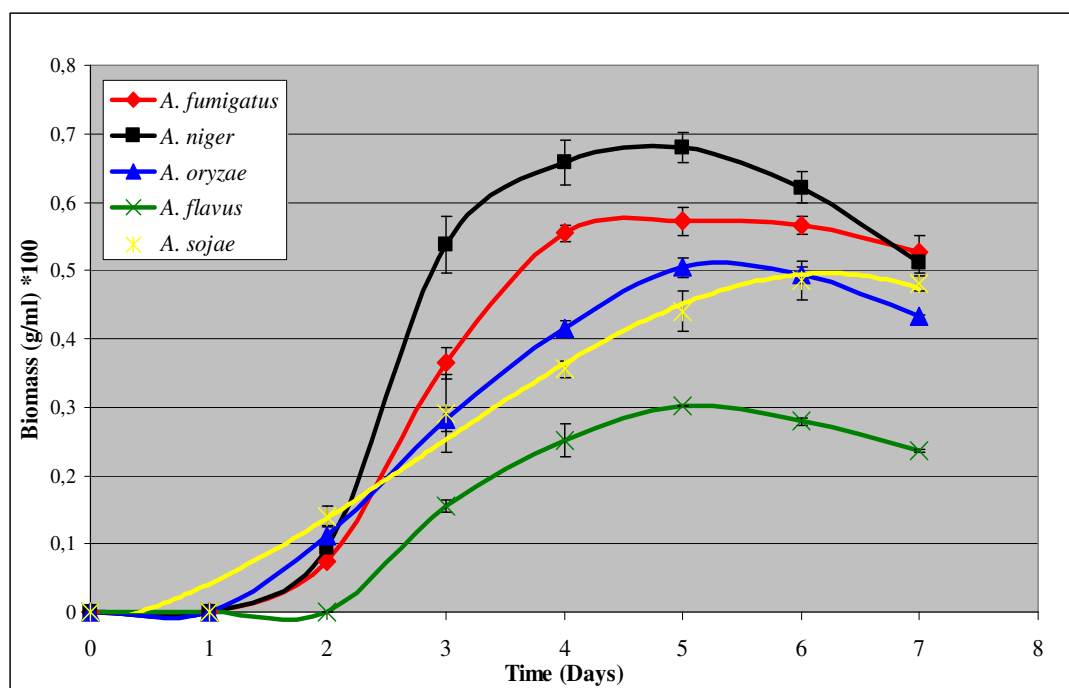


Figure 3.1 Growth of *Aspergillus* species at 30 °C and 160 rpm in 100 ml YpSS medium with glucose as the sole carbon source. Spore inoculum was 100 spores/ml of the medium.

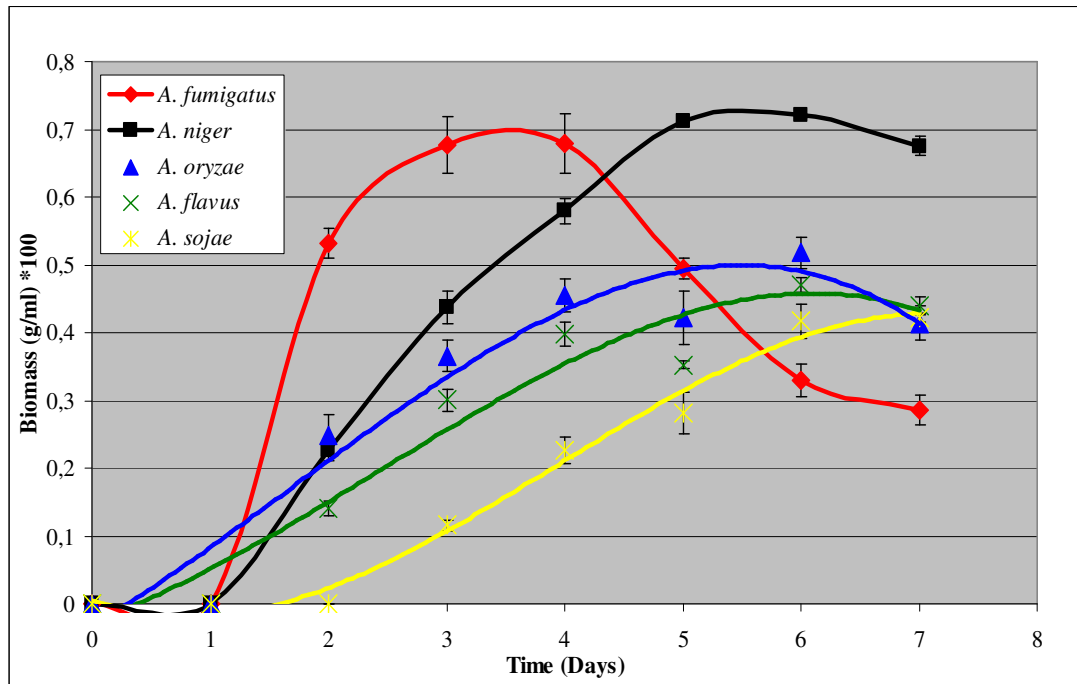


Figure 3.2 Growth of *Aspergillus* Species at 37 °C and 160 rpm in 100 ml YpSS medium with glucose as the sole carbon source. Spore inoculum was 100 spores/ml of the medium.

3.2 Effect of Temperature on the Growth of *A. fumigatus*

A. fumigatus is a thermotolerant fungus and the strain IMI 385708 is known to show optimum growth at 45 °C. However this was not analyzed before. Therefore, in this study, the optimum temperature for growth of *A. fumigatus* was analyzed.

In the literature, optimum growth temperature for *A. fumigatus* strains are between 37 to 45 °C. Clinical isolate strains' optimum growth temperature are close to 37 °C. Although IMI 385708 was not a clinical isolate, according to the results, best growth was at 37 °C, rather than 45 °C (Figure 3.3). Furthermore, at 37 °C, growth rate was significantly higher than at other temperatures (nearly two fold higher than the other

samples) (Figure 3.4). Similar results were obtained when the inoculum concentration was increased from 100 spores per ml to 10000 spores per ml (Figure 3.5). With respect to its optimum growth temperature, IMI 385708 showed similarities with human pathogenic *A. fumigatus* strains.

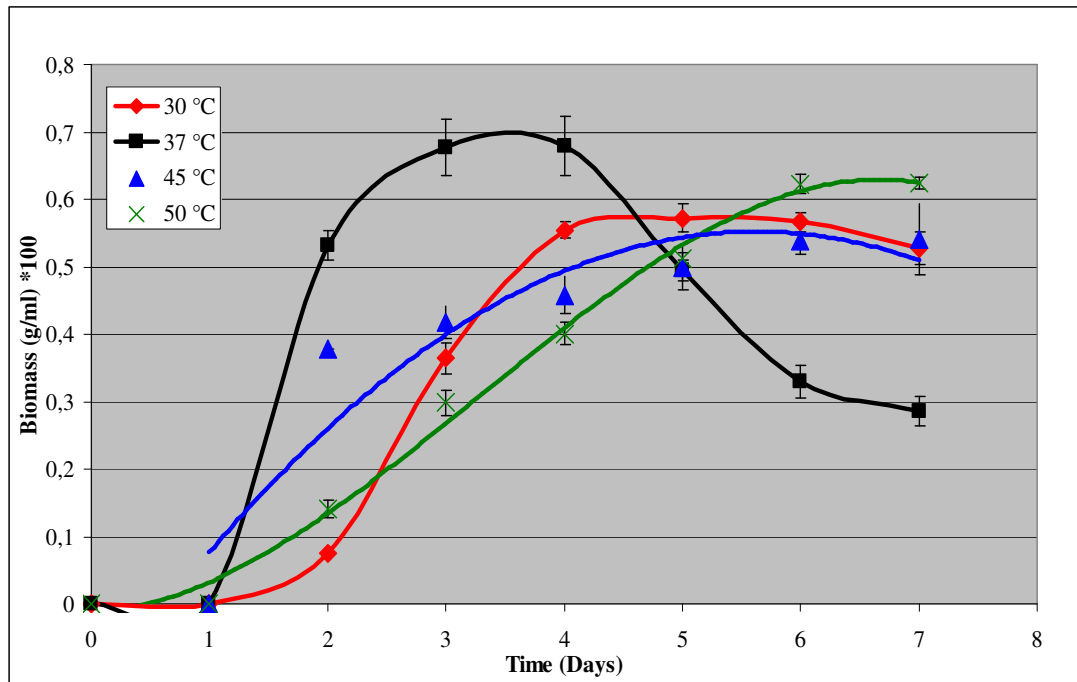


Figure 3.3 Effect of temperature on the growth of *A. fumigatus* IMI 385708, 160 rpm in 100 ml YpSS medium with glucose as the sole carbon source. Spore inoculum was 100 spores/ml of the medium.

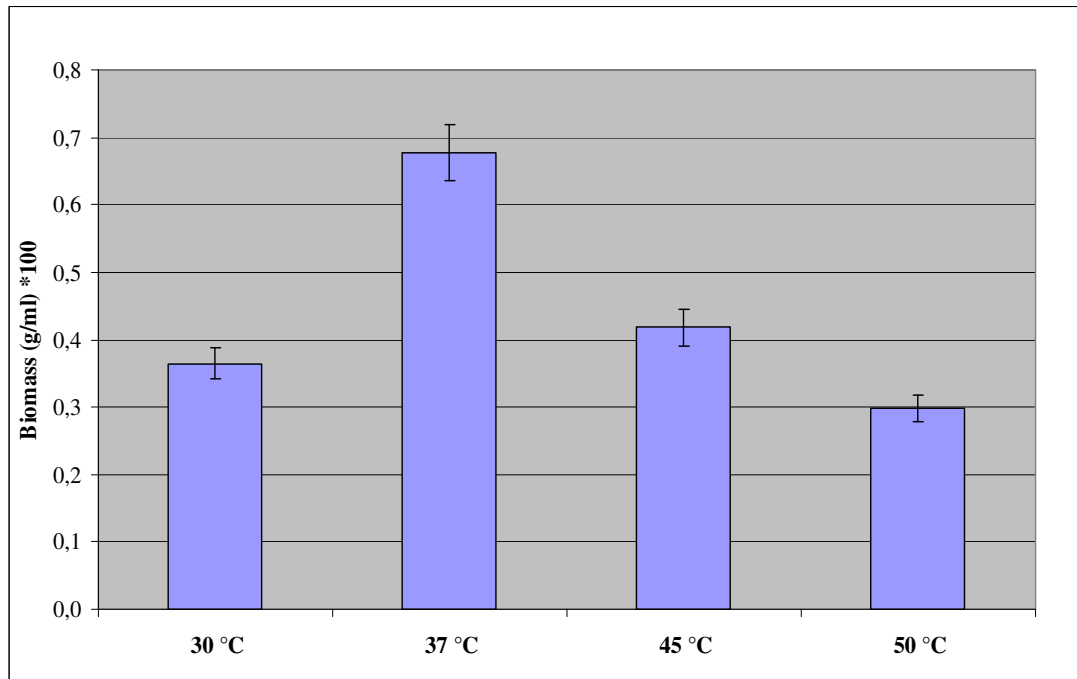


Figure 3.4 Comparison of biomass generation of *A. fumigatus* IMI 385708 at the third day of the cultivation at 30, 37, 45 and 50 °C and 160 rpm in 100 ml YpSS medium with glucose as the sole carbon source. Spore inoculum was 100 spores/ml of the medium.

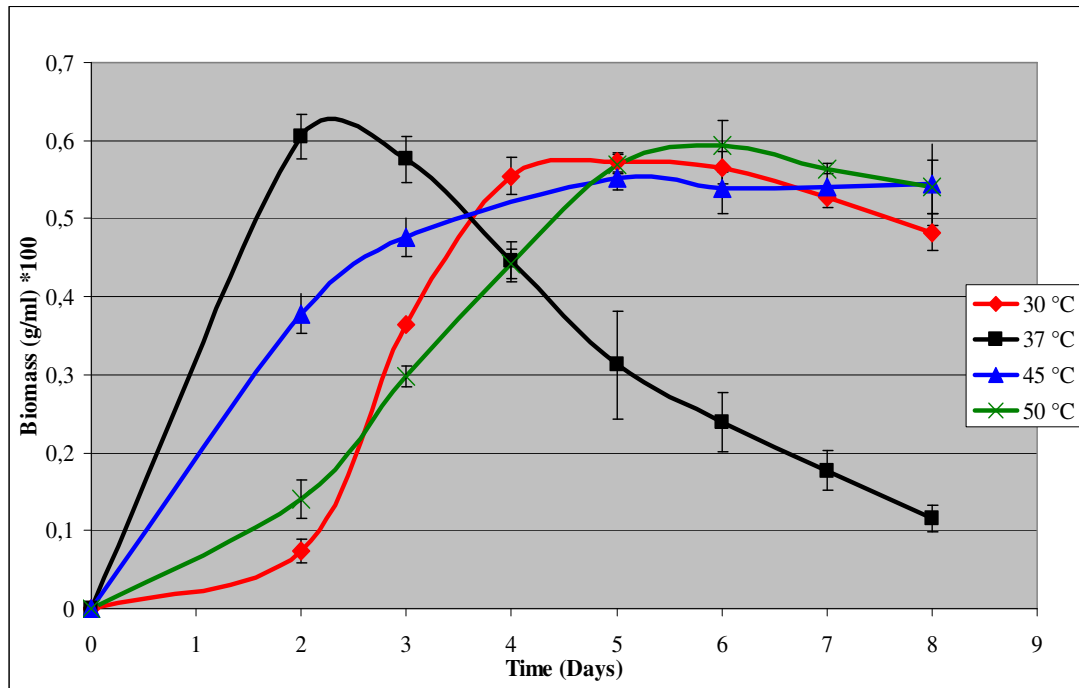


Figure 3.5 Growth of *A. fumigatus* IMI 385708 at different temperatures and 160 rpm in 100 ml YpSS medium with glucose as the sole carbon source. Spore inoculum was 10000 spores/ml of the medium.

3.3 Effect of Temperature on Extracellular Catalase Production by *A. fumigatus*

As shown in Figure 3.6, *A. fumigatus* produces extracellular catalase (U/ml) highest at 37 °C. Activity was 4 times the maximum activity at 30 and 45°C.

As indicated before in the introduction, *A. fumigatus* is an opportunistic pathogen causing Aspergillosis in immunosuppressed patients with AIDS, cancer or organ transplants. Thus the pathogenicity of *A. fumigatus* might be, at least partly, related to it's efficient growth at 37 °C, which is close to human body temperature. Catalase is crucial for the fungus in order to survive in the host body. Phagocytes which are a part of the defence mechanism ingest foreign matters present in the body. As a part

of the phagocytosis, phagocytes release H_2O_2 to the medium in order to induce oxidative stress on the pathogen. In this case, *A. fumigatus*' response to this attack is the production of catalases, which neutralizes H_2O_2 in the medium (Shibuya *et al.*, 2006). In this study it was shown that catalase production occurs in the absence of H_2O_2 , either as part of the secondary metabolism or as a result of hyphal degradation and cell lysis in aged cultures. In the latter case the catalase produced should be intracellular catalase that leaks out of lysed cells.

For the remaining experiments, 37 °C was selected due to the observed high catalase production. As shown in Figure 3.7 catalase production was observed in the late stationary and the death phases of the growth.

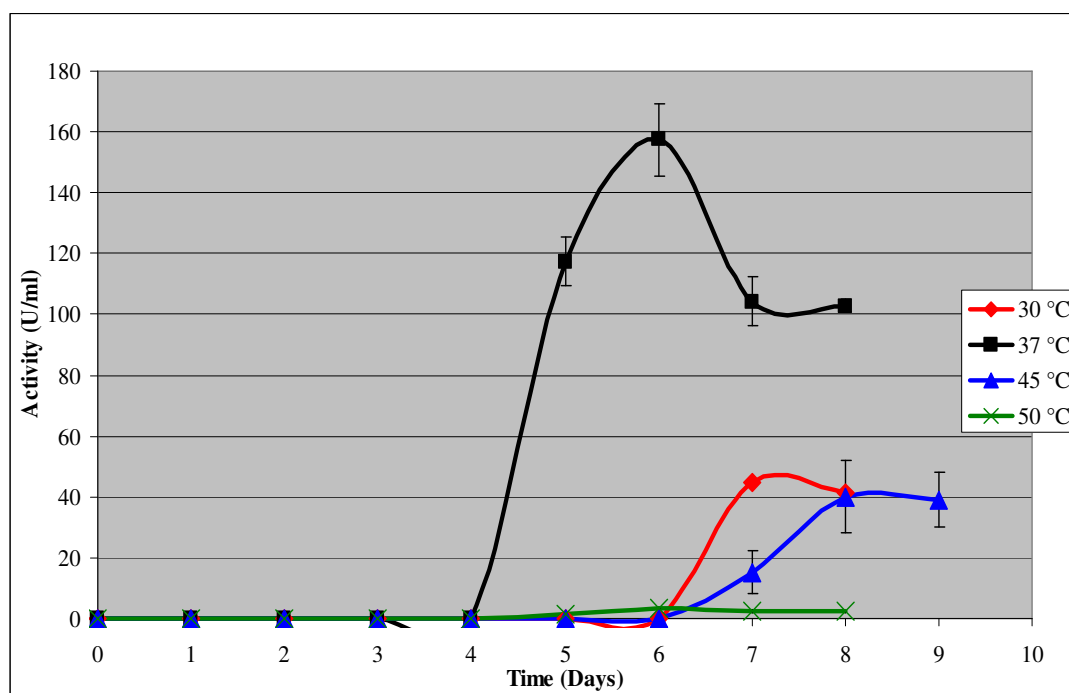


Figure 3.6 Extracellular catalase production by *A. fumigatus* IMI 385708 at different temperatures .

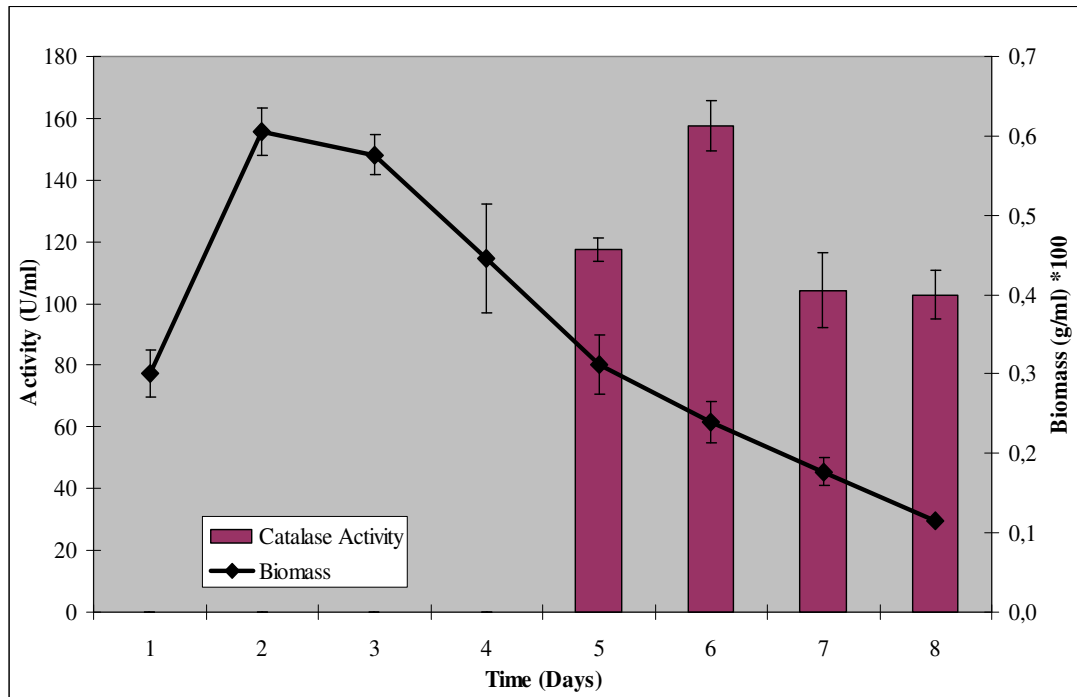


Figure 3.7 Extracellular catalase production by *A. fumigatus* IMI 385708 with respect to biomass generation at 37 °C.

3.4 Effect of Temperature on Intracellular Catalase Production by *A. fumigatus*

Both intracellular and extracellular catalase activities of *A. fumigatus* were determined at 37 °C (Figure 3.8). According to the results, time course of catalase production was not altered and the production values for intra and extracellular catalases were nearly four fold different. It was concluded at this point that, the intracellular and extracellular catalase activities observed are not related to each other. Thus, *A. fumigatus* appears to produce catalase as a part of senescence and not as a result of the leakage of intracellular enzyme. However, this result was valid in the absence of a compound such as H₂O₂, inducing oxidative stress at an earlier stage of growth.

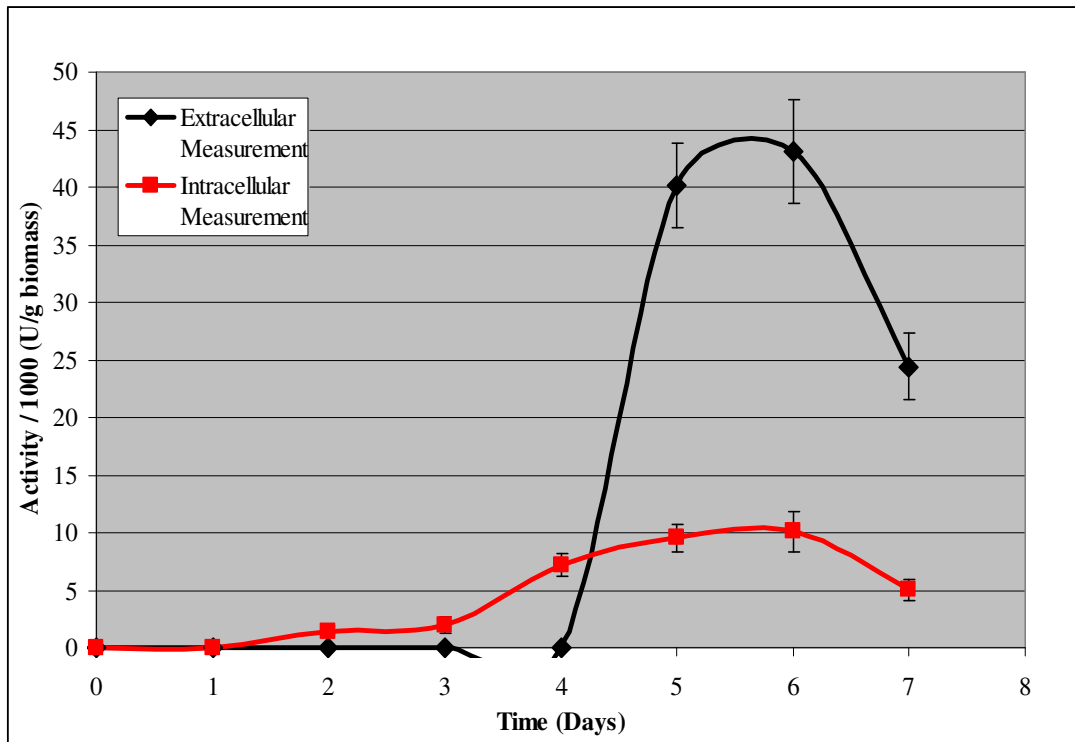


Figure 3.8 Time course of extracellular and intracellular catalase production by *A. fumigatus* IMI 385708 per gram biomass at 37 °C.

A possible question that can be raised here is, why this extracellular catalase was produced from fungal cells so late in their growth phase? A possible explanation to this question might be that fungi respond to oxidative damage and aging. Cell death is followed by cell lysis and secreting the interior components of the cells to the medium including the molecules that cause oxidative stress such as ROS. This is expected to increase the ROS concentration in the medium resulting in catalase production.

3.5 Effect of Iron on Growth and Catalase Production by *A. fumigatus*

Metal ions in the medium are reported to increase the production of ROS and as a result, increasing the oxidative stress. Moreover, it was suggested that metal-induced oxidative stress in cells can be partially responsible for the toxic effects of heavy metals (Ercal *et al.*, 2001). Therefore it was of interest to see the effect of iron supplementation of the medium on catalase production by *A. fumigatus*.

It can be observed from Figure 3.9 that at both 37 and 45 °C, biomass generation was higher when FeSO₄ was added to the medium both at 37 °C and 45 °C. Biomass generation increase at 37 °C was insignificant as shown in Appendix C (Figure C.1). Extracellular catalase activity was also enhanced in the presence of FeSO₄ at both temperatures (Figure 3.10) and this change was significant with respect to the control as shown in Appendix C (Figure C.2). Extracellular catalase activity increase at 37 °C was 1,35 fold whereas, it was 2 fold at 45 °C with respect to the control.

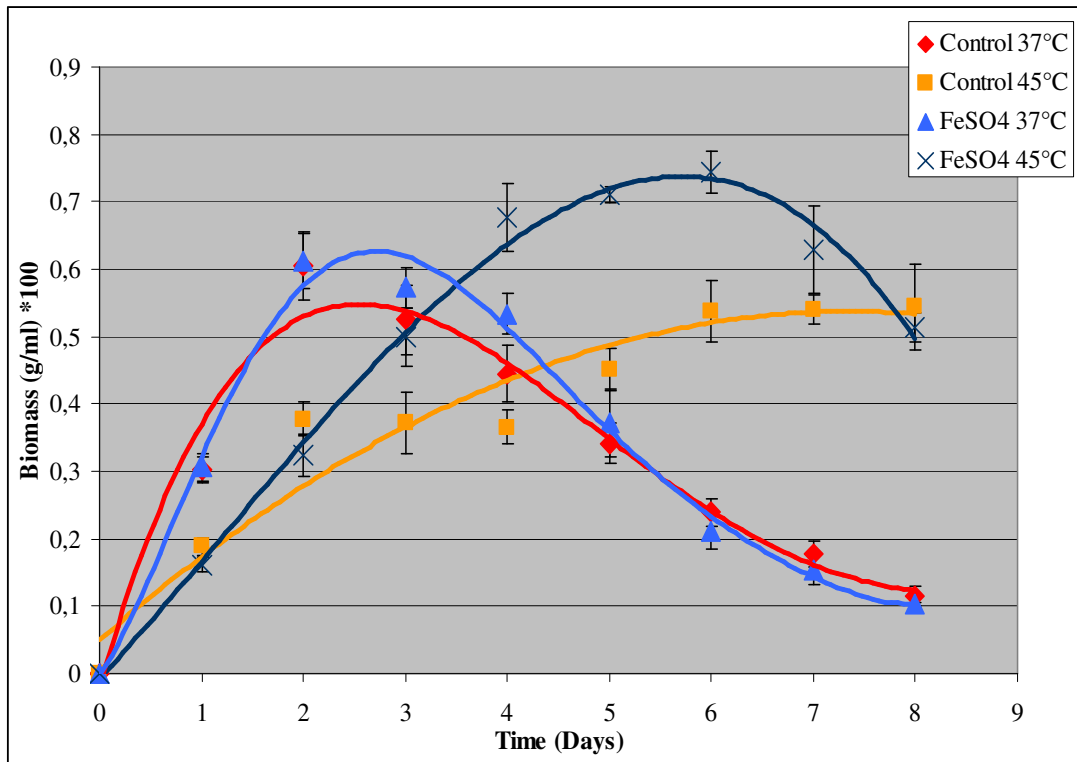


Figure 3.9 Growth of *A. fumigatus* in the presence of 0,0085% (wt/vol) FeSO₄ at 37 °C and 45 °C.

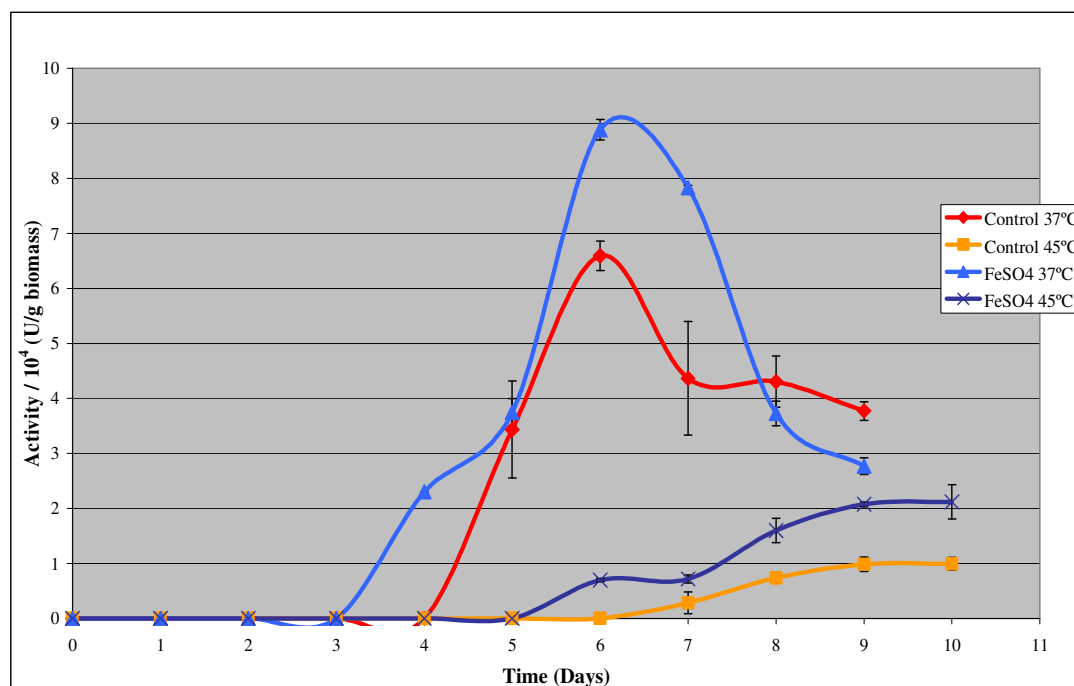


Figure 3.10 Extracellular catalase production by *A. fumigatus* per gram biomass in the presence of 0,0085% (wt/vol) FeSO₄ at 37 °C and 45 °C.

3.6 Effect of H₂O₂ on Growth and Extracellular Catalase Production by *A. fumigatus*

In order to compare native – uninduced conditions with induced conditions for catalase production, H₂O₂ was used. H₂O₂ is an oxidative stress agent widely used in catalase enzyme studies. Since H₂O₂ is also one of the natural products of aerobic respiration inside cells, the cells' response to this situation is the production of higher amounts of catalase.

To observe the effect of the H₂O₂ on biomass generation and catalase production by *A. fumigatus*, four different concentrations of H₂O₂ (100 μM, 500 μM, 1 mM, 2 mM) were added into the growth medium of the fungus at 37 °C. Very limited growth with no enzyme activity was observed at 5 mM (data not shown). In addition to the

analysis of growth and catalase production, to get a better understanding of the mechanism, remaining H_2O_2 concentration was monitored every 12 hours starting from the beginning of the experiment (Figure 3.13). Also initial pH values of the media with different H_2O_2 concentrations were measured (found as 7,1) and it was observed that, the application of H_2O_2 at these concentrations does not change the pH of the medium.

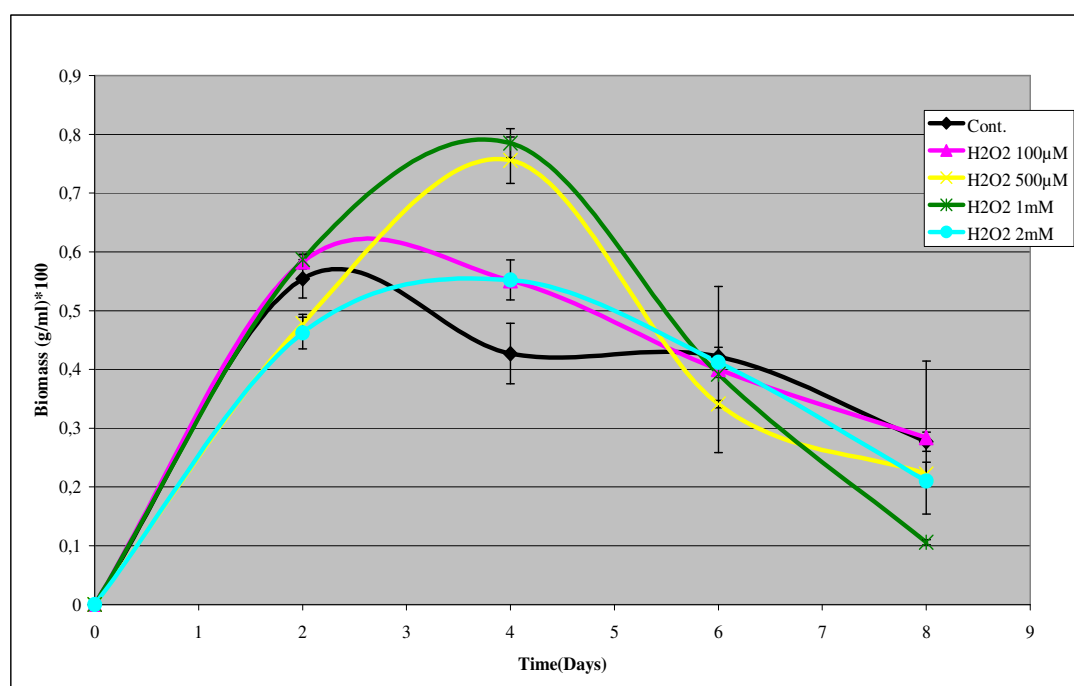


Figure 3.11 Growth of *A. fumigatus* in the presence of H_2O_2 at 37 °C.

Interestingly it was observed that, upto a certain concentration, H_2O_2 increases the growth of *A. fumigatus* at 37 °C (Figure 3.11). As H_2O_2 increases, biomass production increases until an optimum value at 1 mM, followed by a decline in the biomass production (Figure 3.12). 1 mM H_2O_2 increase the maximum biomass generation by 1,4 fold with respect to the control. 500 µM and 1 mM H_2O_2 addition

has effected the maximum biomass generation significantly as shown in Appendix C (Figure C.1).

These results are unexpected because H_2O_2 increases the oxidative stress applied on the cells and should have a negative effect on growth. Thus, biomass generation was expected to decrease. This may be due to more efficient catalase production resulting in an overall higher fitness of the fungus in coping with oxidative stress that naturally occurs, in addition to H_2O_2 .

The reason of 2 mM H_2O_2 sample's showing a biomass production performance not higher than the control may be, due to the fact that this high concentration of H_2O_2 may be more than the fungal cells could endure, and this may damage even the strongest cells with highest catalase production potential. Supporting this idea is the growth pattern of 2 mM H_2O_2 in Figure 3.11, showing decreased growth rate.

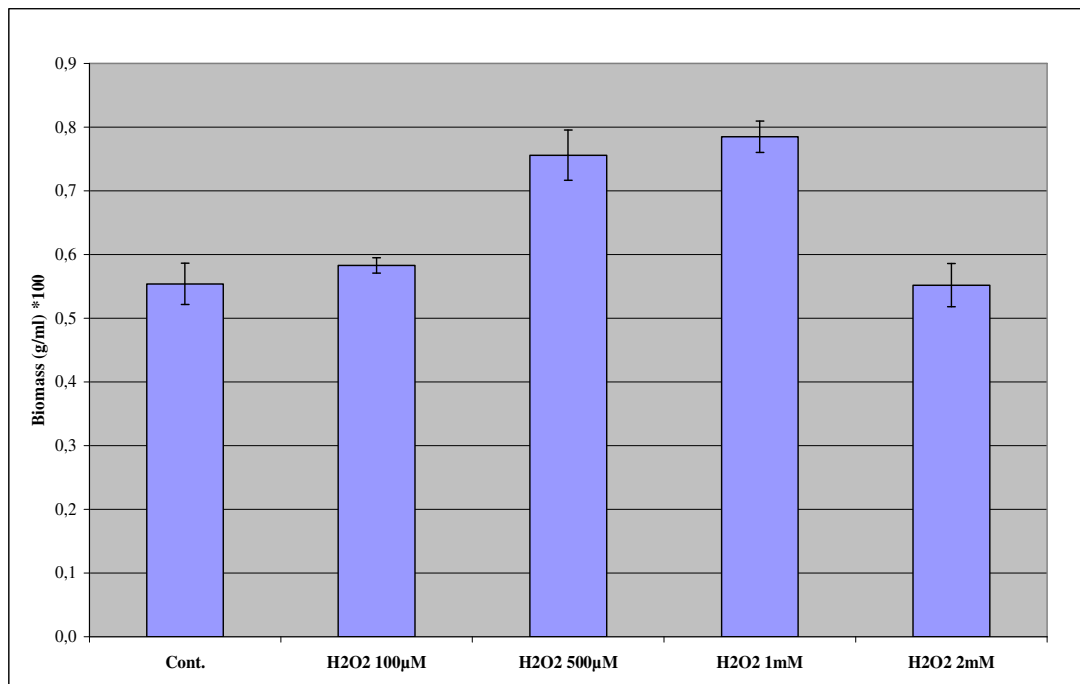


Figure 3.12 Maximum biomass generation of *A. fumigatus* in the presence of H_2O_2 at 37 °C.

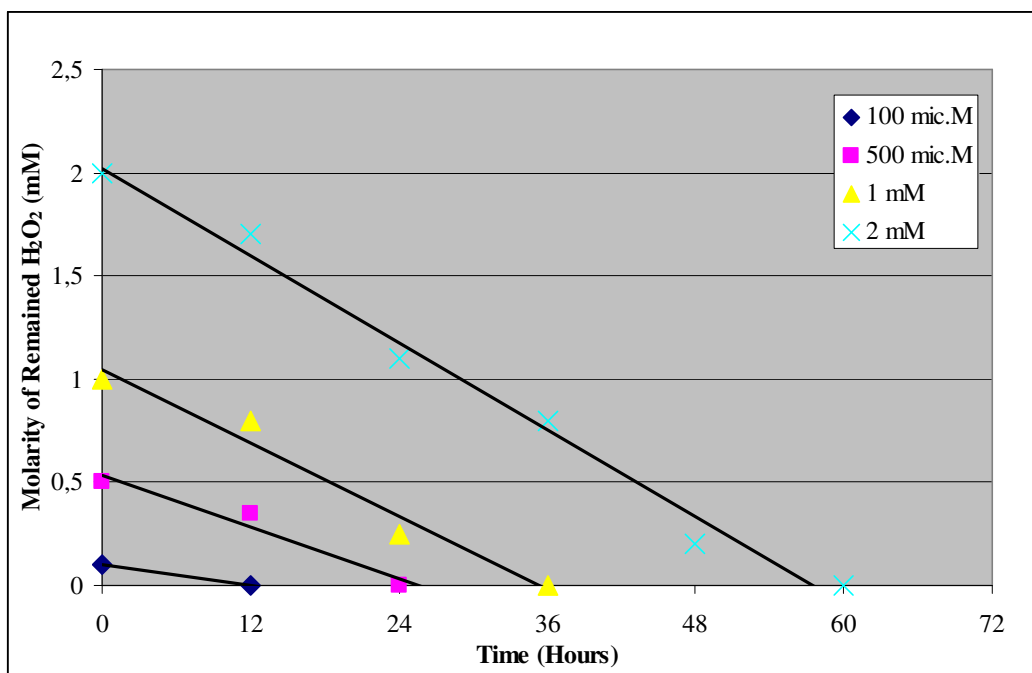


Figure 3.13 Determination of H_2O_2 concentration in the medium during the growth of *A. fumigatus* at 37 °C.

Considering catalase production shown in Figures 3.14 and 3.15, H_2O_2 effects catalase activity significantly at all concentrations except 100 μM as shown in Appendix C (Figure C.2). Especially by looking at activity per g biomass values in Figure 3.15, it was observed that, 1mM sample has the highest activity by far while the other activities were similar. There was nearly a 3 fold increase in catalase production for 1 mM sample with respect to the control in 7th and 8th days of the cultivation. This elevated catalase production at 1 mM is in parallel to an increase in the growth of *A. fumigatus*. At this concentration there seems to be a sensitive balance between oxidative stress upon the cells and their survival. The time course of the extracellular catalase production was not changed with the addition of H_2O_2 , with respect to the control. The activity was observed in the death phase of the growth after the fourth day of the cultivation in all samples (Figures 3.14 and 3.15).

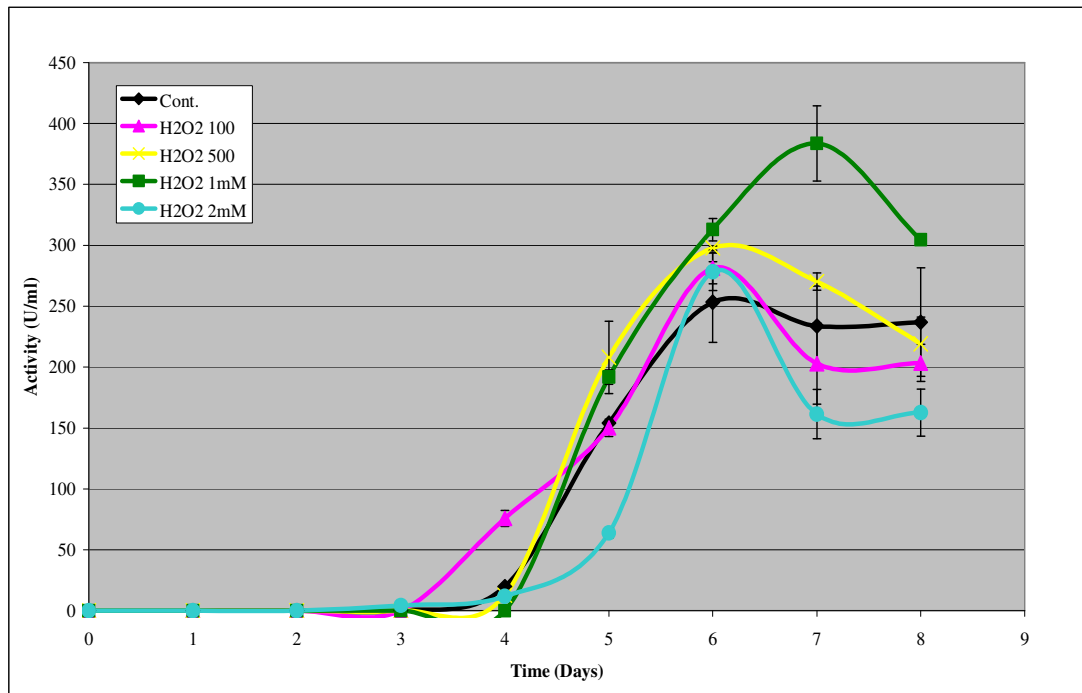


Figure 3.14 Extracellular volumetric catalase production by *A. fumigatus* in the presence of H₂O₂ at 37 °C.

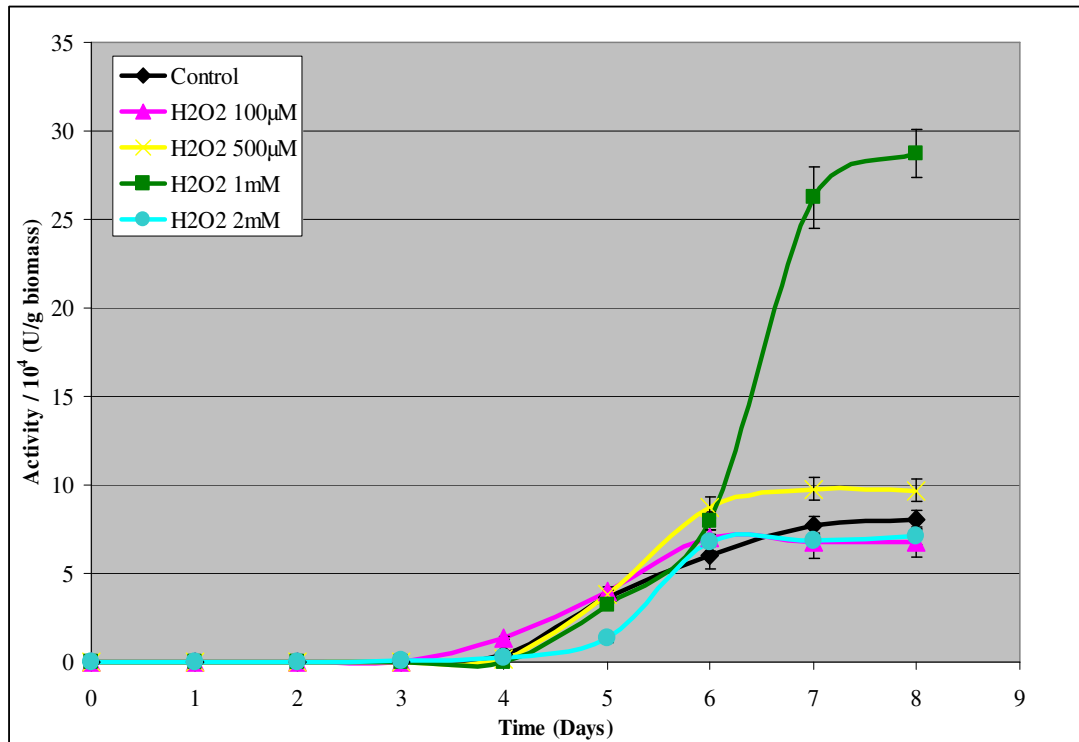


Figure 3.15 Extracellular catalase production by *A. fumigatus* per gram biomass in the presence of H₂O₂ at 37 °C.

3.7 Effect of H₂O₂ on Intracellular Catalase Production by *A. fumigatus*

As presented in Figure 3.16, H₂O₂ also increases intracellular catalase production. The increases were significant for all samples except 100 µM sample as shown in Appendix C (Figure C.3). Increasing the concentration of H₂O₂ results in higher intracellular activities except for the 2 mM sample, which has a slightly lower activity pattern than the control sample. 1mM sample has the highest intracellular production values similar to extracellular production. 1 mM samples' intracellular catalase production was nearly 1,3 fold higher than 500 µM sample and 2,3 fold higher than the control after fourth day of cultivation. 100 µM sample showed a similar pattern with the control sample since this concentration was very low and

H₂O₂ was depleted completely within 12 hours (Figure 3.13). 500 μM sample displayed a catalase production pattern that was significantly higher than control, 100 μM and 2 mM samples (Figure 3.16) unlike its pattern for the extracellular activity where 500 μM sample's catalase activity was very close to the samples except 1 mM (Figure 3.15). Intracellular catalase activity of 500 μM sample was nearly 1,9 fold higher than the other samples except 1 mM sample after the fourth day of cultivation. For the time course of the catalase production, intracellular activity was started to be observed after the first day of the cultivation for all samples but after the third day, 500 μM and 1 mM samples' intracellular catalase production rate increases significantly and reached their maximum values one day before than control. This may be due to the fungal response to high H₂O₂ concentration in the medium. At 500 μM and 1 mM samples, intracellular catalase activity was observed close to the beginning of the death phase.

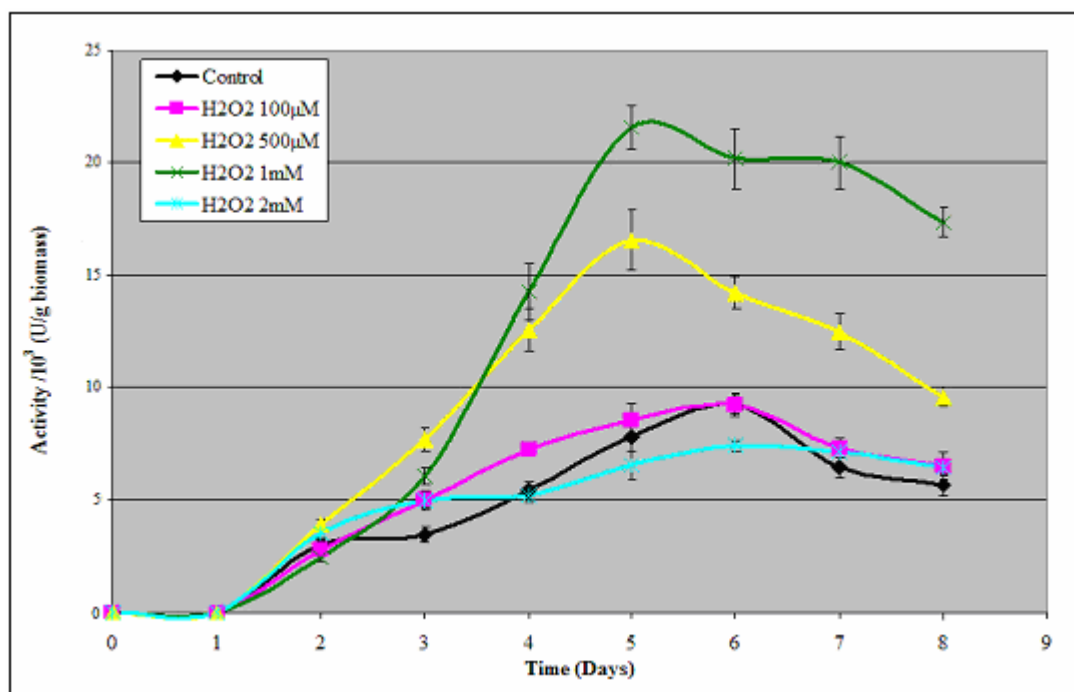


Figure 3.16 Intracellular catalase production by *A. fumigatus* per gram biomass in the presence of H₂O₂ at 37 °C.

3.8 Effect of Gallic Acid on Growth and Extracellular Catalase Production by *A. fumigatus*

Gallic acid is a natural plant originated polyphenolic compound and it is found almost in all plants. Gallic acid derivatives are reported to act as antioxidants and protect host cells against oxidative damage. Gallic acid was found to show cytotoxicity against cancer cells, without any effect on healthy cells (<http://www.phytochemicals.info/>). The abundance of this compound in plants makes it a potential antioxidant supplement or as an additive in functional foods. Scientific studies including gallic acid and the effects of this molecule on cells are limited and further studies should be done to understand its effects on cell structure and the mechanism of this effect.

To observe the effect of gallic acid on growth and catalase production, four different final concentrations of gallic acid 500 μ M, 3 mM, 10 mM and 25 mM were added into culture medium. Although it was a very high concentration, gallic acid at 50 mM was also applied but at this concentration, growth was significantly impaired and no catalase activity was observed throughout the cultivation (data not shown). Also initial pH values of the media with different gallic acid concentrations were measured and it was observed that, the application of gallic acid at 0,5 mM and 3 mM does not change the pH of the medium significantly (observed around 7) whereas, at 10 mM, pH was measured as 4,6 and at 25 mM, pH was observed as 3,9.

It can be observed from Figures 3.17 and 3.18 that, the addition of gallic acid into the growth medium slightly enhances the growth upto 10 mM gallic acid, followed by a slight decrease at 25 mM. Significant changes in maximum biomass generation was only at 3 mM and 10 mM samples with respect to the control as shown in Appendix C (Figure C.1).

Gallic acid's effect on biomass generation can be explained by the anti-oxidant potential of this compound. Gallic acid might neutralize ROS that are naturally

produced during aerobic respiration of the fungus thereby, decreasing oxidative stress. At 25 mM gallic acid, however, the compound might act as a pro-oxidant instead of an anti-oxidant due to the towering concentration of the phenolic as explained in the introduction part section 1.3. This may be the reason of the observation of the slight decrease in the growth at 25 mM sample.

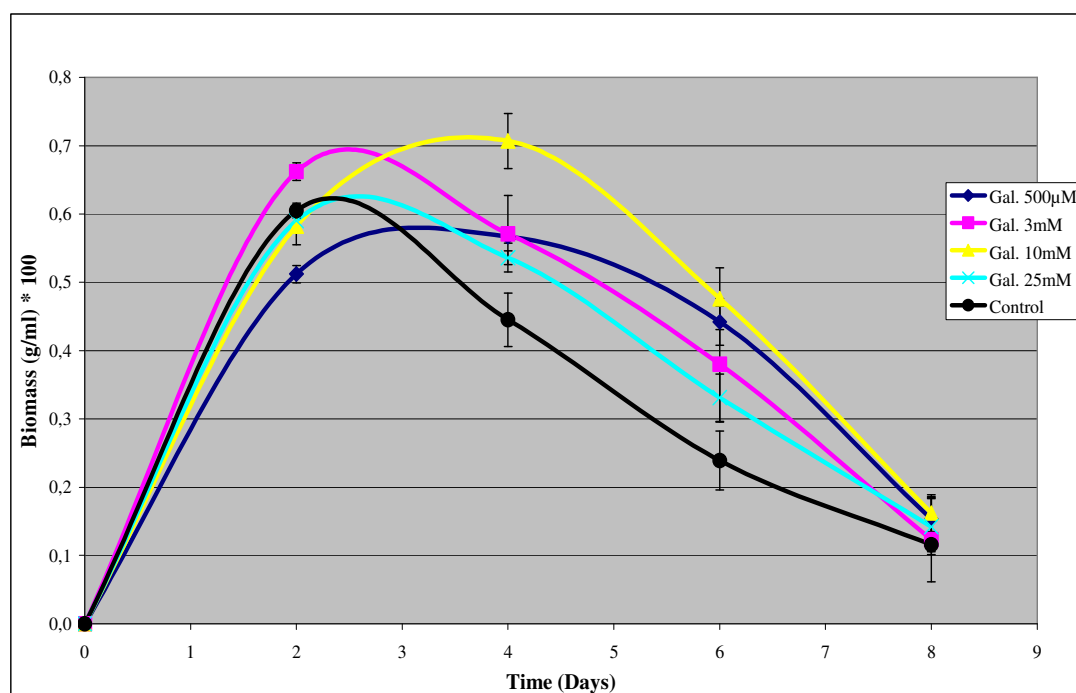


Figure 3.17 Growth of *A. fumigatus* in the presence of gallic acid at 37 °C.

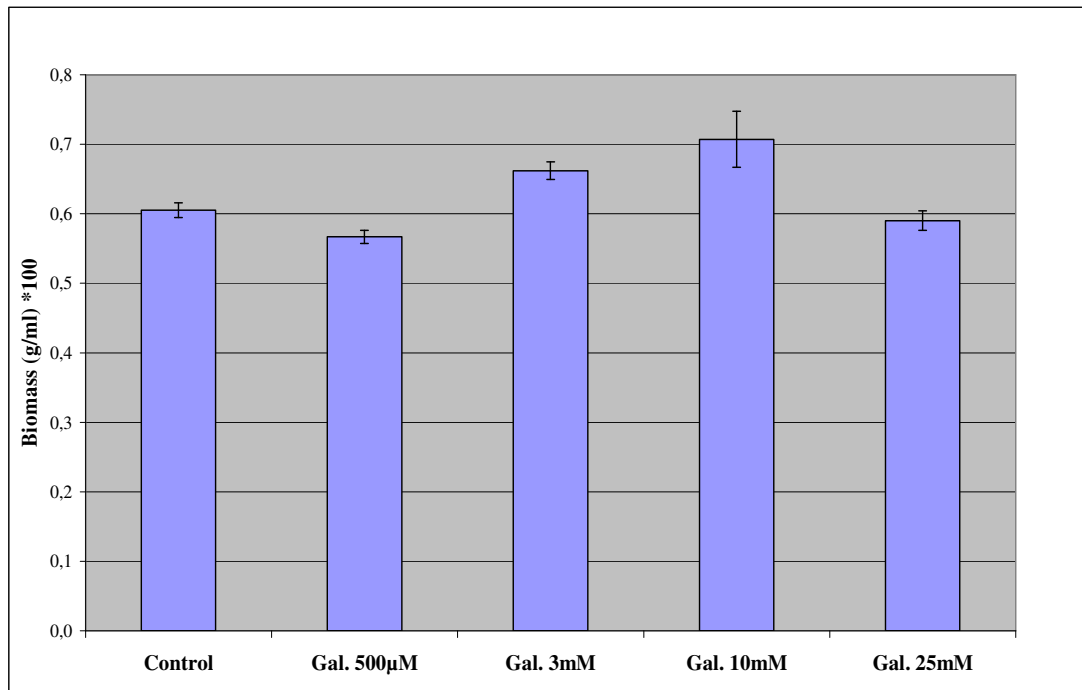


Figure 3.18 Maximum biomass generation of *A. fumigatus* in the presence of gallic acid at 37 °C.

As shown in Figure 3.19, gallic acid caused an increase in catalase production by *A. fumigatus* at 37 °C except for 25 mM sample. Maximum activity (U/ml) was at 500 µM gallic acid and after this concentration, activity slightly decreased and remained nearly the same at 3 mM and 10 mM gallic acid. At 25 mM, activity was lowered significantly and has a maximum value lower than the control sample. Figure 3.20 presents U per gram biomass values, in this figure, again there is a similar trend as in Figure 3.19. Both the increase and the decrease in the maximum extracellular catalase production in all gallic acid applied samples were significant as shown in Appendix C (Figure C.2). In gallic acid applied samples, catalase production occurs at a later stage of growth as compared to the control sample. At the fifth day, control sample had an activity of nearly 120 U/ml while gallic acid applied samples were nearly the same and about 50 U/ml, which is more than a 2 fold difference. This may

be explained by the antioxidant potential of gallic acid. It is possible that, as gallic acid presented in the medium, it may decrease oxidative stress and thus, reduce catalase necessity.

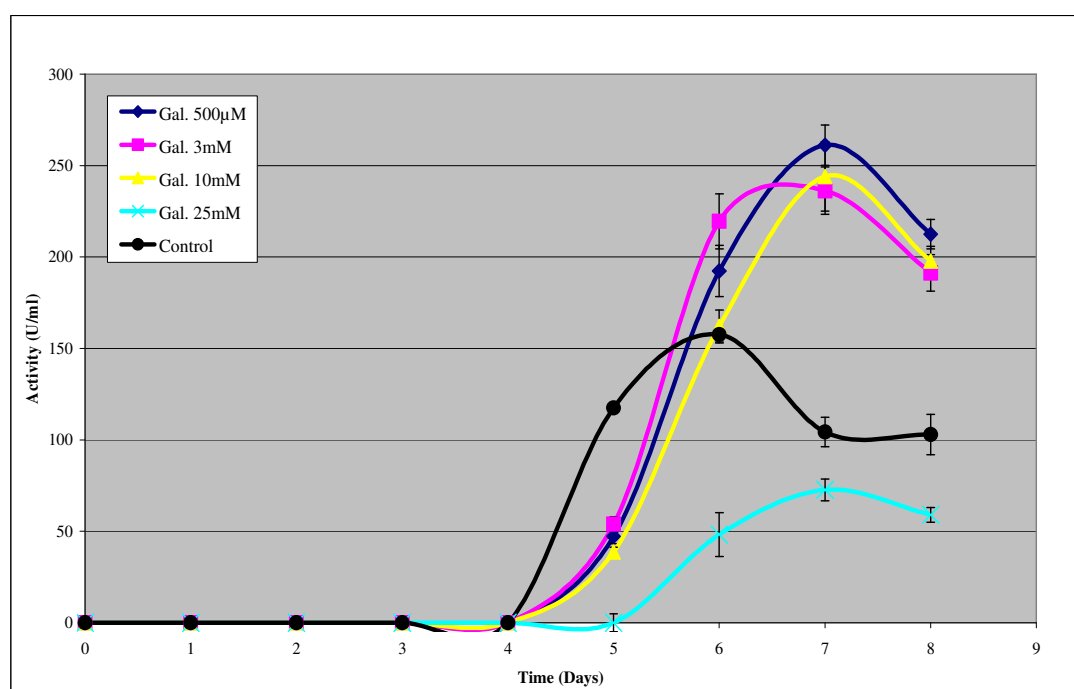


Figure 3.19 Extracellular volumetric catalase production by *A. fumigatus* in the presence of gallic acid at 37 °C.

On the other hand, at the 7th day, gallic acid samples except 25 mM, have similar activities around 240 U/ml which is 2,4 fold higher than the activity of control sample on that day. Also these activity values of gallic acid samples on the 7th day, were nearly 5 fold higher than the values at 5th day, indicating a sudden rise in extracellular catalase production between 5th and 7th days.

This might be explained by the depletion of the antioxidant activity of gallic acid followed by fungal cells' response to this sudden increase of oxidative stress by boosting catalase production.

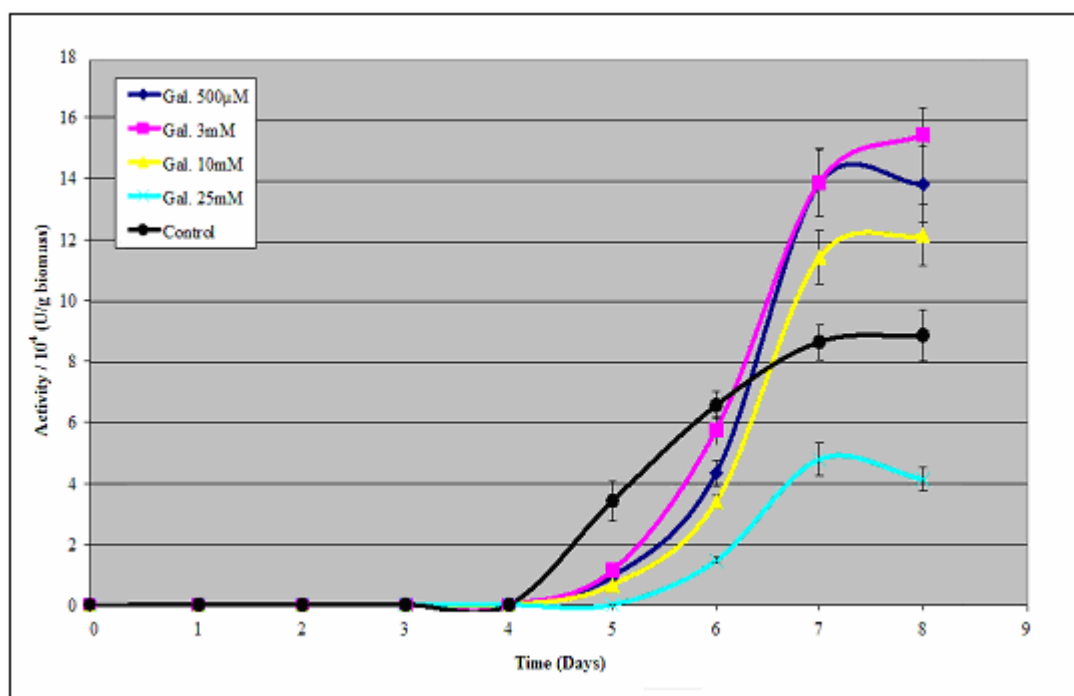


Figure 3.20 Extracellular catalase production by *A. fumigatus* per gram biomass in the presence of gallic acid at 37 °C.

3.9 Effect of Gallic Acid on Intracellular Catalase Production by *A. fumigatus*

Figure 3.21 shows intracellular catalase activity per g biomass in the presence of gallic acid in the growth medium. Similar intracellular activities were observed except the 25 mM sample. The changes in the maximum intracellular catalase production in all gallic acid samples were significant as shown in Appendix C

(Figure C.3). Gallic acid samples' activity remained lower than the control samples until the fifth day and after the sixth day, there is a distinct difference between gallic acid samples and control sample as the control sample's activity decreases but those with gallic acid show a sharp increase followed by a decrease after the seventh day. This difference may be attributed to the same boosting effect on catalase production explained above for the extracellular catalase activity. The higher activity of the control sample maybe due to the antioxidant effect of gallic acid which may lower the need for catalase at the earlier stages of growth. Control sample's activity decreases after the sixth day possibly because most of the cells have died already. The adverse effect of 25 mM gallic acid was again apparent, in consistence with extracellular activity.

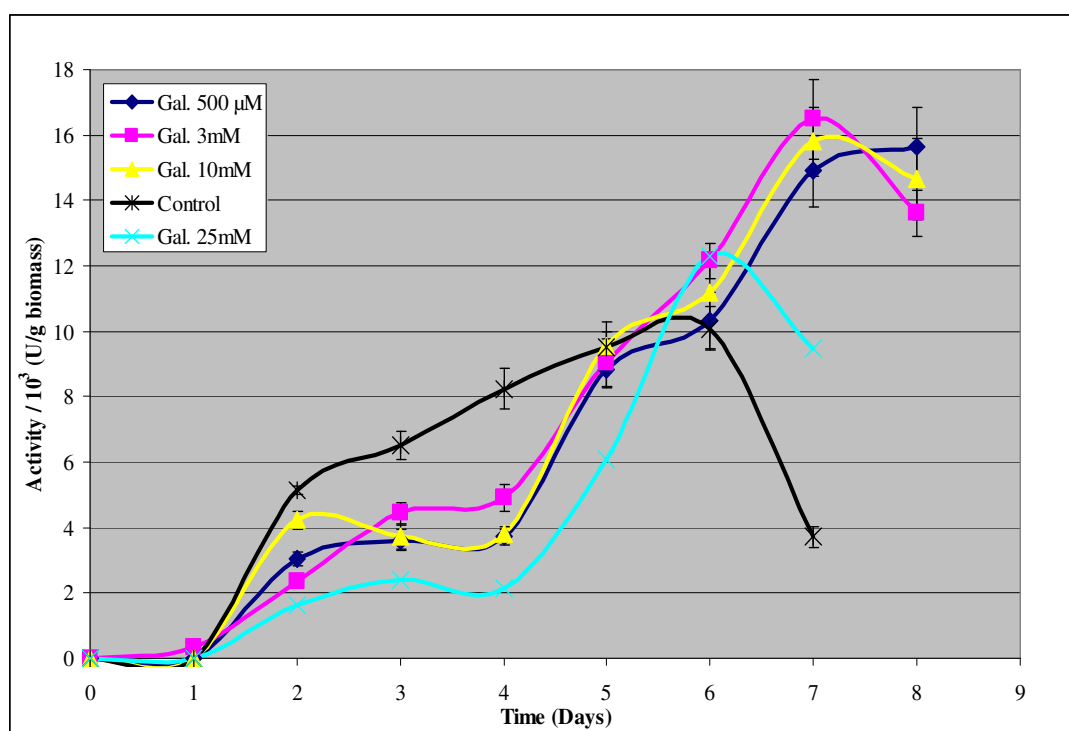


Figure 3.21 Intracellular catalase production by *A. fumigatus* per gram biomass in the presence of gallic acid at 37 °C.

3.10 Effect of Resveratrol on Growth and Extracellular Catalase Production by *A. fumigatus*

To observe the effect of resveratrol on growth and catalase production by *A. fumigatus*, three different concentrations of trans-resveratrol (100 μ M, 500 μ M and 3 mM) were added into the growth medium of the fungus at 37 °C. Biomass generation, intracellular and extracellular catalase activities were then determined. Also initial pH values of the mediums with different resveratrol concentrations were measured (found as 7,1) and it was observed that, the application of resveratrol at these concentrations does not change the pH of the medium.

As can be seen from Figure 3.22, trans-resveratrol significantly enhanced the growth of *A. fumigatus* at all concentrations as shown in Appendix C (Figure C.1). As the concentration of resveratrol increases, biomass generation increases upto 3mM reaching 1,5 fold the biomass generated in the control sample (Figure 3.22). This was even higher than the effect of gallic acid which yielded an increase of 1,16 fold at 10 mM (Figure 3.18).

For control, 100 μ M & 500 μ M samples, decrease in the biomass was clear with the death & lysis of the cells, but for 3 mM sample, reached biomass remains nearly the same for the last four days of the cultivation. It was observed that cell lysis do not take place at this sample and it is also possible that, cell death may not be occurred too. These results support the data about life increasing effects of resveratrol in the literature (Howitz *et al.*, 2003). Resveratrol might prevent the hyphal degradation by increasing DNA stability with the activation of sirtuins.

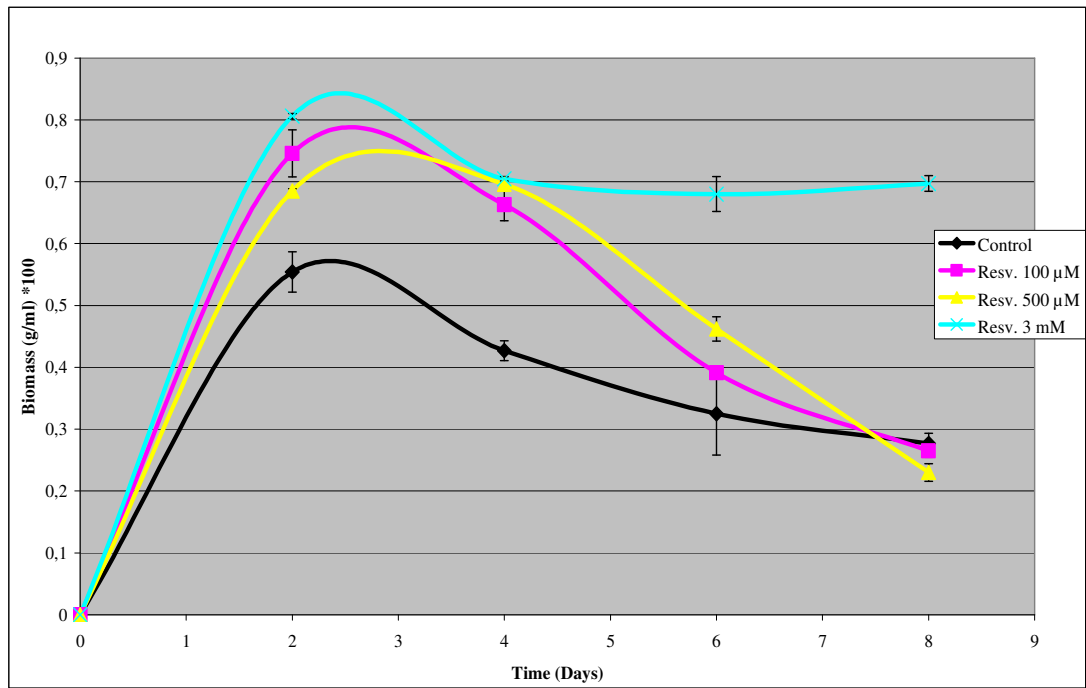


Figure 3.22 Growth of *A. fumigatus* in the presence of trans-resveratrol at 37 °C.

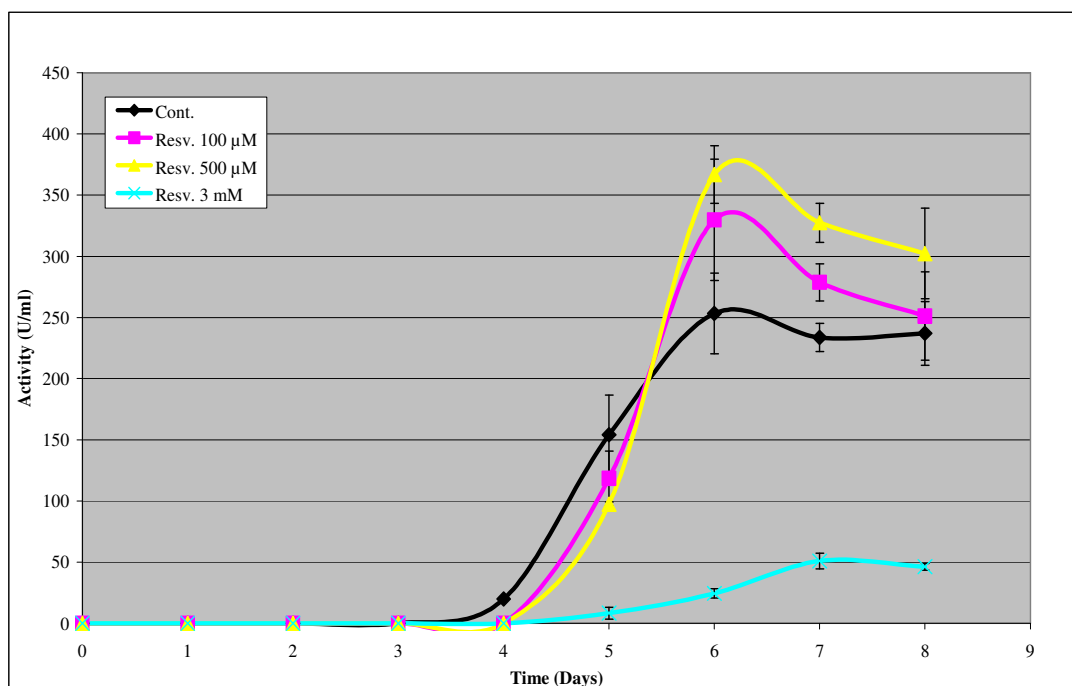


Figure 3.23 Extracellular volumetric catalase production by *A. fumigatus* in the presence of trans-resveratrol at 37 °C.

As for catalase production, results similar to those of gallic acid were obtained. Changes in maximum extracellular catalase production at all concentrations were significant as shown in Appendix C (Figure C.2). Namely at lower concentrations of resveratrol, an increase in activity was observed at a later stage of growth. However at higher resveratrol concentration (at 3 mM) catalase activity was much lower (Figures 3.23 and 3.24). The negative effect of gallic acid on growth at 25 mM, however, was not observed at 3 mM resveratrol, while 25 mM gallic acid resulted in a 2 fold decrease in activity with respect to the control, 3 mM resveratrol caused a 8 fold decrease in the maximum activity (Figures 3.20 and 3.24). These results may be related to the more effective antioxidant activity of resveratrol, as well as its influence of the lifespan of cells by effecting the activity of sirtuins (Howitz *et al.*, 2003).

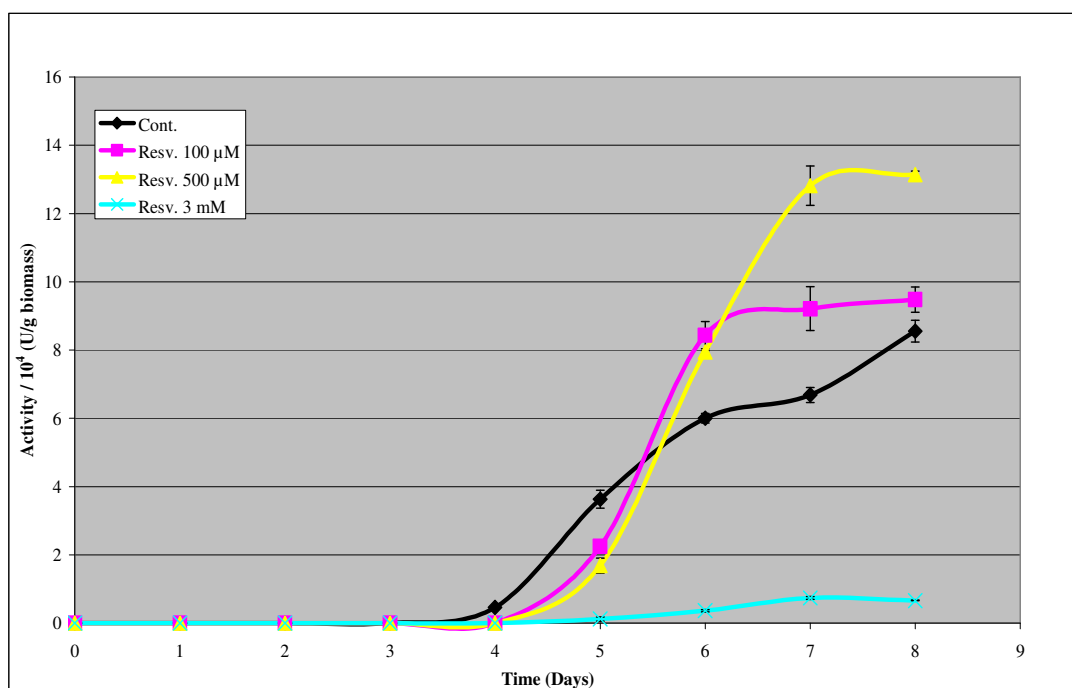


Figure 3.24 Extracellular catalase production by *A. fumigatus* per gram biomass in the presence of trans-resveratrol at 37 °C.

3.11 Effect of Resveratrol on Intracellular Catalase Production by *A. fumigatus*

Intracellular catalase activities of *A. fumigatus* in the presence of trans-resveratrol at 37 °C are presented in Figure 3.25. As seen from this figure, a distinctive difference in intracellular catalase activities was not observed, as compared to the control, except the 3 mM sample which was somewhat lower. Significance was observed for the changes at the maximum intracellular catalase production for 100 µM and 500 µM samples, however, for 3 mM sample, the change was insignificant with respect to the control as shown in Appendix C (Figure C.3).

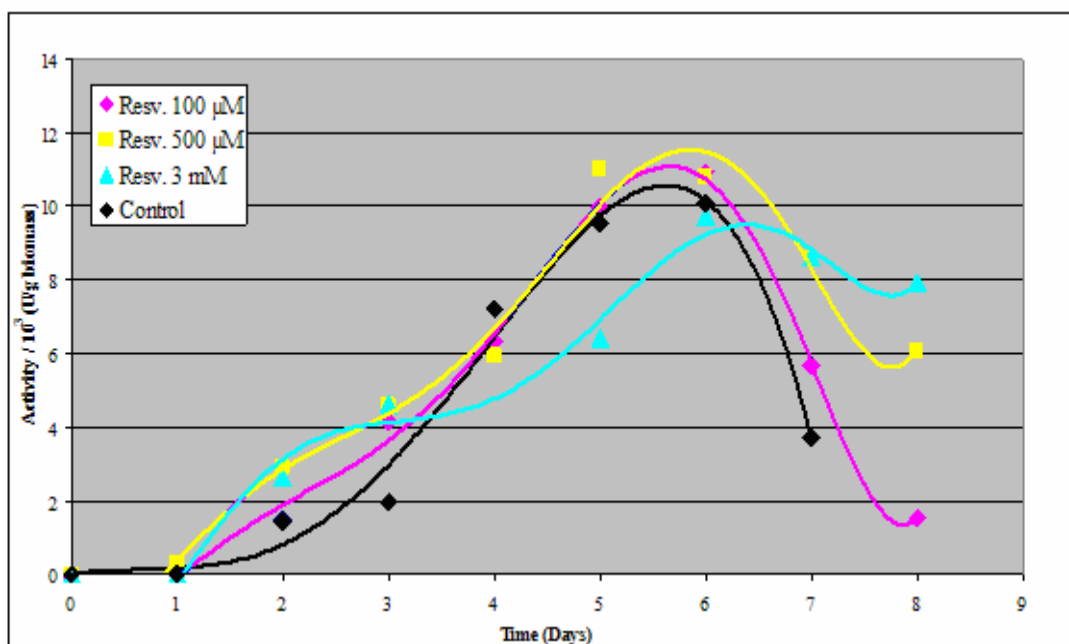


Figure 3.25 Intracellular catalase activity of *A. fumigatus* per gram biomass in the presence of trans-resveratrol at 37 °C.

3.12 Relative Comparison of Growth and Catalase Production by *A. fumigatus* at Different Treatments

As presented in Table 3.1 % relative maximum biomass generation column, application of resveratrol at 3 mM increases the biomass generation with 45%, 1 mM H₂O₂ with 42%, 0,5 mM gallic acid with 17% and 0,3 mM iron sulphate with an insignificant change of 1%. It is observed from % relative maximum extracellular catalase activity (U/g biomass) column that, application of all compounds increase maximum catalase production, by %235 for 1 mM H₂O₂, 83% for 3 mM gallic acid, 54% for 0,5 mM resveratrol and 35% for iron sulphate. 1 mM H₂O₂ increases the maximum intracellular catalase production by 128%, 3 mM gallic acid by 65% and 0,5 mM resveratrol by 10%. Last column in Table 3.1 presents % relative maximum

intracellular catalase activities and 1 mM H₂O₂, 3 mM gallic acid and 0,5 mM resveratrol samples all produced significant differences from control sample.

Table 3.1 Comparison of growth, extracellular and intracellular catalase productions of *A. fumigatus* in the presence of different compounds relative to control sample.

Treatment	% Relative Maximum Biomass Generation	% Relative Maximum Extracellular Catalase Activity **	% Relative Maximum Extracellular Catalase Activity ***	% Relative Maximum Intracellular Catalase Activity **	% Relative Maximum Intracellular Catalase Activity ***
None	100	100	100		100
FeSO₄ (0,3 mM)	101	120 *	135 *	-	-
H₂O₂ (0,1; 0,5; 1 & 2 mM)	142 * (1 mM)	151 * (1 mM)	335 * (1 mM)	325 * (1 mM)	228 * (1 mM)
Gallic Acid (0,5; 3; 10 & 25 mM)	117 * (0,5 mM)	165 * (0,5 mM)	183 * (3 mM)	164 * (0,5 mM)	165 * (3 mM)
Resveratrol (0,1; 0,5 & 3 mM)	145 * (3 mM)	144 * (0,5 mM)	154 * (0,5 mM)	156 * (0,5 mM)	110 * (0,5 mM)

* The mean difference is significant (with respect to control sample named as “none” in the table above) at the .001 level according to Multiple Comparisons test (Scheffe model) (Appendix C).

** Results are based on U/ml

*** Results are based on U/g biomass

CHAPTER 5

CONCLUSIONS

A. fumigatus is an opportunistic pathogenic fungus. In this study, the aim was to investigate the effect of phenolic compounds and H₂O₂ on the growth and catalase production of *A. fumigatus*.

According to the results the following conclusions are made:

- Among 5 different species of *Aspergillus*, best growth was observed with *A. niger* at 30 °C and *A. fumigatus* at 37 °C.
- *A. fumigatus* showed the highest catalase production rate at 37 °C which may be linked to protection against host defence in the human body.
- Production of extracellular catalase in *A. fumigatus* was observed in the death phase of the growth. Extracellular activity was observed after the fourth day of the cultivation and reach its maximum value of 160 U/ml at the sixth day. Intracellular catalase however was produced during exponential, stationary and death phases starting from the second day of the cultivation with the maximum of 37 U/ml at the fifth day.
- Application of H₂O₂ to the growth medium increased catalase production (U/g biomass) (by 3 folds) possibly by inducing oxidative stress. Upon the depletion of H₂O₂, biomass production significantly increased (by 1,44 fold)

with respect to controls. This may be due to more efficient catalase production resulting in an overall higher fitness of the fungus in coping with oxidative stress that naturally occurs, in addition to H₂O₂.

- Application of gallic acid to the growth medium at the time of inoculation increased the biomass generation of *A. fumigatus* (by 1,17 fold in 10 mM sample) possibly by reducing oxidative stress and oxidative damage upon cells. This is supported by the observation of an initial decrease in catalase production (by 4,33 fold in 25 mM sample), followed by an increase at the later stages of growth.
- Application of resveratrol to the growth medium produced results similar to those with gallic acid application, but at the same concentrations, the effect of resveratrol was more pronounced (on biomass generation, 1,45 fold increase in 3 mM sample and on extracellular catalase production, 16,7 fold decrease in again 3 mM sample in the first six days of the growth).
- Applications of phenolics and H₂O₂ effect the intracellular catalase activity in a similar way as they effect the extracellular one, but with less significant changes in activity values than that of extracellular activities (even less significant in phenolic compound applied samples with respect to H₂O₂ applied ones).

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APPENDIX A

CHEMICALS AND THEIR SUPPLIERS

Table A.1 Chemicals that were used in the experiments and their suppliers.

Chemical	Supplier
Agar	Oxoid
Complete Mini (EDTA free)	Roche
Ethanol	Gurup Deltalar
FeSO ₄	Sigma
Gallic Acid	Merck
Glucose	Merck
H ₂ O ₂	Merck
K ₂ HPO ₄	Merck
KH ₂ PO ₄	Merck
KOH	Merck
MgSO ₄ .7H ₂ O	Merck
NaCl	Merck
Resveratrol	Sigma
Starch	Merck
Tween 20	Sigma
Yeast Extract	Merck

APPENDIX B

PREPARATIONS OF GROWTH MEDIA, BUFFERS AND SOLUTIONS

1. Glucose (40% w/v, 50 ml)

20 g of glucose is dissolved in 50 ml dH₂O and sterilized by filtration with filters that has a pore diameter of 0,2μ.

2. YpSs Agar (per Liter)

4 g yeast extract

1 g K₂HPO₄

0.5 g MgSO₄·7H₂O

15 g soluble starch

20 g agar

The final volume is adjusted to 1 liter with deionized water, and autoclaved.

3. YpSs Broth (per liter)

4 g yeast extract

1 g K₂HPO₄

0.5 g MgSO₄·7H₂O

The final volume is adjusted to 950 ml with deionized water, and autoclaved.

After sterilization, 50 ml sterile glucose solution is added to the YpSs solution and stirred.

4. Saline tween solution (per liter)

1% Tween solution is prepared by adding 1ml of Tween80 into 99ml of deionized water in a 100ml beaker and stirring.

800 ml of distilled water is added to a 1L beaker, 5 g of sodium chloride is added to the beaker and stirred until dissolved. 2ml of 1% Tween solution is added and stirred well. The final volume is brought up to 1 liter by deionized water, autoclaved and stored at 4 °C.

5. Potassium phosphate buffer (50mM, pH:7,0) (per liter)

6.80 g Monobasic potassium phosphate (KH_2PO_4)

1.90 g potassium hydroxide (KOH)

The final volume is adjusted to 1 liter with deionized water.

The pH should be around 7.00. Any adjustments in pH that are required are made with:

- (i) Adding 50 mM potassium phosphate solution (6.80 g KH_2PO_4 is added to an empty beaker and final volume is adjusted to 1 liter by adding dH_2O) dropwise, to lower the pH, or
- (ii) Adding 0.1 M potassium hydroxide (5,61 g KOH is added to an empty beaker and final volume is adjusted to 1 liter by adding dH_2O) dropwise, to raise the pH.

APPENDIX C

RESULTS OF ANOVA AND MULTIPLE COMPARISONS (SCHEFFE MODEL) TESTS

Table C.1 Explanation of the sample numbers that were used in statistical tests.

Sample No.	Treatment	Concentration
0	Control	-
1	FeSO ₄	0,3 mM
2	H ₂ O ₂	0,1 mM
3	H ₂ O ₂	0,5 mM
4	H ₂ O ₂	1 mM
5	H ₂ O ₂	2 mM
6	Gallic Acid	0,5 mM
7	Gallic Acid	3 mM
8	Gallic Acid	10 mM
9	Gallic Acid	25 mM
10	Resveratrol	0,1 mM
11	Resveratrol	0,5 mM
12	Resveratrol	3 mM

1. Statistical Analysis of Maximum Biomass Generation

ANOVA

Growth

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8358,615	12	696,551	1393,103	,000
Within Groups	6,500	13	,500		
Total	8365,115	25			

Multiple Comparisons

Dependent Variable: Growth

Scheffe

(I) SampleNo	(J) SampleNo	Mean Difference (I-J)	Std. Error	Sig.	99,9% Confidence Interval	
					Upper Bound	Lower Bound
0	1	-1,00000	,70711	,998	-7,2542	5,2542
	2	-5,00000	,70711	,012	-11,2542	1,2542
	3	-37,00000*	,70711	,000	-43,2542	-30,7458
	4	-43,00000*	,70711	,000	-49,2542	-36,7458
	5	,00000	,70711	1,000	-6,2542	6,2542
	6	5,50000	,70711	,011	-,7542	11,7542
	7	-9,50000*	,70711	,000	-15,7542	-3,2458
	8	-17,00000*	,70711	,000	-23,2542	-10,7458
	9	2,00000	,70711	,755	-4,2542	8,2542
	10	-34,50000*	,70711	,000	-40,7542	-28,2458
	11	-26,50000*	,70711	,000	-32,7542	-20,2458
	12	-45,50000*	,70711	,000	-51,7542	-39,2458

* The mean difference is significant at the .01 level

Figure C.1 ANOVA and Multiple comparisons (Scheffe model) test results of the samples presented in Table C.1. for their maximum biomass generation.

2. Statistical Analysis of Maximum Extracellular Catalase Production (U/g biomass)

ANOVA

ExtracellularCatalaseProduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	143010,462	12	11917,538	19366,000	,000
Within Groups	8,000	13	,615		
Total	143018,462	25			

Multiple Comparisons

Dependent Variable: ExtracellularCatalaseProduction

Scheffe

(I) SampleNo	(J) SampleNo	Mean Difference (I-J)	Std. Error	Sig.	99,9% Confidence Interval	
					Upper Bound	Lower Bound
0	1	-35,50000*	,78446	,000	-42,4384	-28,5616
	2	6,50000	,78446	,011	-,4384	13,4384
	3	-15,50000*	,78446	,000	-22,4384	-8,5616
	4	-236,00000*	,78446	,000	-242,9384	-229,0616
	5	17,50000*	,78446	,000	10,5616	24,4384
	6	-58,50000*	,78446	,000	-65,4384	-51,5616
	7	-84,00000*	,78446	,000	-90,9384	-77,0616
	8	-43,50000*	,78446	,000	-50,4384	-36,5616
	9	39,50000*	,78446	,000	32,5616	46,4384
	10	-11,00000*	,78446	,000	-17,9384	-4,0616
	11	-54,50000*	,78446	,000	-61,4384	-47,5616
	12	91,00000*	,78446	,000	84,0616	97,9384

* The mean difference is significant at the .01 level.

Figure C.2 ANOVA and Multiple comparisons (Scheffe model) test results of the samples presented in Table C.1. for their maximum extracellular catalase production.

3. Statistical Analysis of Maximum Intracellular Catalase Production (U/g biomass)

ANOVA

IntracellularCatalaseProduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	41041,833	11	3731,076	7462,152	,000
Within Groups	6,000	12	,500		
Total	41047,833	23			

Multiple Comparisons

Dependent Variable: ExtracellularCatalaseProduction
Scheffe

(I) SampleNo	(J) SampleNo	Mean Difference (I-J)	Std. Error	Sig.	99,9% Confidence Interval	
					Upper Bound	Lower Bound
0	2	-,50000	,70711	1,000	-6,7649	5,7649
	3	-73,50000*	,70711	,000	-79,7649	-67,2351
	4	-128,50000*	,70711	,000	-134,7649	-122,2351
	5	21,50000*	,70711	,000	15,2351	27,7649
	6	-54,50000*	,70711	,000	-60,7649	-48,2351
	7	-65,00000*	,70711	,000	-71,2649	-58,7351
	8	-57,50000*	,70711	,000	-63,7649	-51,2351
	9	-21,00000*	,70711	,000	-27,2649	-14,7351
	10	-9,00000*	,70711	,000	-15,2649	-2,7351
	11	-10,50000*	,70711	,000	-16,7649	-4,2351
	12	3,50000	,70711	,092	-2,7649	9,7649

* The mean difference is significant at the .01 level.

Figure C.3 ANOVA and Multiple comparisons (Scheffe model) test results of the samples presented in Table C.1. for their maximum intracellular catalase production.