

Thesis:

FETOUCHE Abdelmadjid 2008

Title:

Effect of small antioxidants molecules on the viability of oxidative stress defective yeast.

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List of Abreviation

DMSO: Dimethyl sulfoxide

CA: Caffeic acid

AA: Aascorbic acid

Quer:Quercetine

BC: Beta-carotene

CHP: Cumene hydroperoxide

EDTA:Eethylene diamid tetra acetic acid

GSH:Glutathione

GSSG:Oxidized glutathione

ROS:Reactive oxygen species

PCD:Programmed cell death

HIV:Human immuno deficiency virus

GFP:Green fluorescence protein

OYE:Old yellow enzyme

PCR: Polymerase chain reaction

YPD: Yeast peptone dextrose

Glu/CM:Glucose complet medium

ABSTRACT

Oxidative stresses is produced in cells by oxygen derived species resulting from cellular metabolism and from interaction of cells with exogenous sources such as carcinogenic compounds, red ox-cycling drugs and ionizing radiations; H₂O₂ and CHP are compounds that can generate free radicals.

In this work we studied the capacity of seven (7) small antioxidants molecules (Ascorbic Acid, Caffeic Acid, Catechine, Beta-carotene, Quercetine, Tocotrienol (β and γ) and Hesperidin) to protect 3 strains of yeast from oxidative stress in the presence of defined concentration of one of the two pro-oxidant H₂O₂ or CHP

Two of the strains used have different mutations in their antioxidant machinery Δ sod1 a strain that is deficient in super oxide dismutase enzyme and a double knockout strain Δ oye2glr1 deficient in the same time in Old yellow enzyme and glutathione reductase, characterized by a high concentration of oxidized glutathione (GSSG), the third strain used as control was the wild type BY4741.

Regarding the results we found that AA showed a big protective effect for all strains, even for the Δ gsh1 strain which has lacking in endogenous glutathione and need an exogenous concentration of glutathione to grow.

Against that, a high concentration of quercetine and beta-carotene showed an inhibitory effect on all strains which have been confirmed by measuring the level of ROS inside the cells.

When quercetine has the strongest inhibitory effect, we measured the level of GSH/GSSG in the cells to see the effect of quercetine in this level and we have seen that quercetine not only increase the concentration of GSH and GSSG inside the cell but also could not help the cells to maintain the quantity of GSH when it is exposed to H₂O₂.

Old yellow enzyme OYE2 and OYE3 has a major role in oxidative stress and the modulation of programmed cell death processes. For this we used cells that have an endogenous carboxy-terminal fusion of the green fluorescent protein (GFP) to the OYE2 or OYE3 gene and we tried the induction of genes with small 5 μ M and high concentration 50 μ M of BC. (GFP fluorescence was quantified by fluorimetry), BC gave the induction of OYE2-GFP in both concentration and Quercetine lead to the super induction of OYE2-GFP

The formation of oye2p-oye3p heterodimer has previously been associated with sensitization of cells to H₂O₂ iduced programmed cell death.

GFP-YAP1 fusion strain was generated in order to examine the change of yap1 expression to quercetine supplementation by fluomitery.

The results showed no change in fluorescence intensity compared to the different doses of quercetine supplemented.

1. Free radical

The appearance of oxygen in the atmosphere made possible respiratory metabolism and efficient energy generation systems, but led also to the formation in cells of reactive oxygen species (Temple *et al.*, 2005).

Free radicals are a chemical species that possess an unpaired electron in the outer shell of the molecule. The fact that they are highly reactive means that they have low chemical specificity, they can react with most molecules in its vicinity, and this includes proteins, lipids, carbohydrates and DNA. It also means that in trying to gain stability by capturing the needed electron they don't survive in their original state for very long time and quickly react with their surroundings. When the "attacked" molecule loses its electron, it becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, finally resulting in the disruption of a living cell

2. Free radical generation

Free radicals can be generated both in-vivo and in-vitro by one of the following mechanisms:

1. Homolytic cleavage of a covalent bond, in which a normal molecule fragments in two, each fragment retaining one of the paired electrons. Homolytic cleavage occurs less commonly in biological systems, as it requires high-energy input from ultra-violet light, heat or ionising radiation.
2. Loss of a single electron from a normal molecule.
3. Addition of an electron to a normal molecule.

In cells ROS are generated by a number of pathways.

- A consequence of normal aerobic metabolism: approximately 90% of the oxygen utilized by the cell is consumed by the mitochondrial electron transport system.
- Oxidative burst from phagocytes (white blood cells) as part of the mechanism by which bacteria and viruses are killed, and by which foreign proteins (antigens) are denatured (Percival *et al.*, 1998).
- Xenobiotic metabolism, detoxification of toxic substances.

All organisms suffer some exposure to $\text{OH}\cdot$ because it is generated during the splitting of water, driven by environmental radiation.

Cells have evolved a highly sophisticated and complex system to be protected against the reactive species. (Percival *et al.*, 1998)

.Antioxidants are the first line to defense against free radical damage and are critical to maintain optimum health and well being.

3. Antioxidant:

Antioxidants are molecules capable of slowing or preventing the oxidation of other molecules.

Antioxidant acts using this mode of action: using four routes:

1. Chain breaking reactions, e.g. alpha-tocopherol which acts in lipid phase to trap "ROD" radical.

2. Reducing the concentration of reactive oxygen species e.g. glutathione.

3. Scavenging initiating radicals e.g. superoxide dismutase which acts in aqueous phase to trap superoxide free radicals.

4. Chelating the transition metal catalysts: A group of compounds serves an antioxidant function by sequestration of transition metals that are well-established pro-oxidants. In this way, transferrin, lactoferrin, and ferritin function to keep iron induced oxidant stress in check and ceruloplasmin and albumin as copper sequestrants.

Both endogenous and exogenous in origin the antioxidants function interactively and synergistically to neutralize free radicals.

Table 1. Enzymic antioxidants (Young and Woodside 2001)

Enzymatic antioxidants	Presence	Properties
Superoxide dismutase	Mitochondria and cytosol	Dimutase superoxide radicals
Glutathione peroxidase	Mitochondria and cytosol	Remove hydrogen peroxide and organic hydroperoxide
Catalase	Mitochondria and cytosol	Remove hydrogen peroxide

Table 2. Nonenzymic antioxidants (Young and Woodside 2001)

Nonenzymatic antioxidants	Presence	Properties
Bilirubin	Product of heme metabolism in blood	Extracellular antioxidant
Uric acid	Product of purine metabolism	Scavenger of OH radicals
A lipoic acid	Endogenous thiol	Effective in recycling vitamin C. It may also be effective glutathione substitute
Ubiquinone (coenzyme Q10)	Mitochondria	In the reduced form it is an efficient antioxidant

3.1 Dietary and phytonutrients antioxidants

3.1.1 Established antioxidants

Vitamins C and E are the principal nutrients which possess radical quenching properties. Both are powerful antioxidants, and the most important difference between these two compounds stems from their different solubility in biological fluids. Vitamin C is water soluble and is therefore especially found in the aqueous fractions of the cell and in body fluids whereas vitamin E is highly lipophilic and is found in membranes and lipoproteins

3.1.1.1 Vitamin E:

The term vitamin E refers to a family of at least eight related fat-soluble antioxidants compounds

The tocopherol which is considered to be the true vitamin E and has this structure:

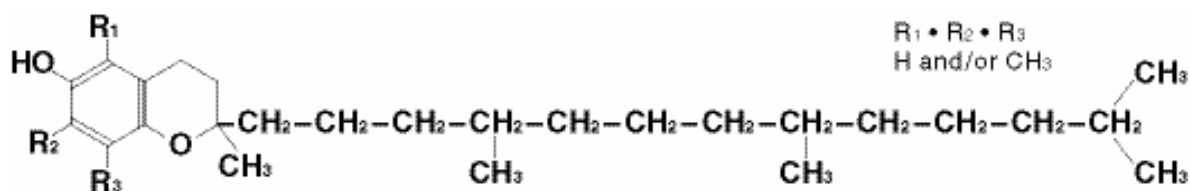


Figure 1.Chemical structure of Tocopherol

Natural TOCOPHEROL exists as a mixture of 4 homologues, Alpha, Beta, Gamma, and Delta - available as a mixed product known as MIXED TOCOPHEROL when the different isomers: The tocotrienol which is named by analogy to tocopherol including a chemical difference, we can concenter tocotrienol as tri-enes with tocopherol which mean that they share an identical structure except for the addition of three double bonds to their side chains.

Homologues	R1	R2	R3
Alpha- TOCOPHEROL	Me	Me	Me
Beta- TOCOPHEROL	Me	H	Me
Gamma- TOCOPHEROL	H	Me	Me
Delta- TOCOPHEROL	H	H	Me

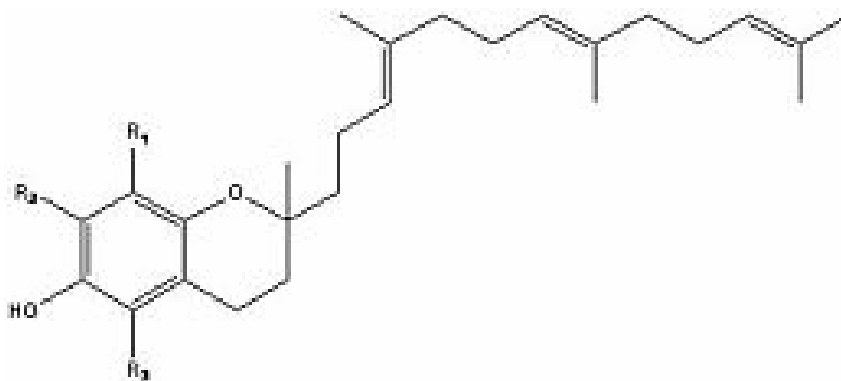


Figure 2.Chemical structure of Tocotrienol

The different types of Tocotrienol are four and we can differentiate between them in this table:

Type	R ₁	R ₂	R ₃
Alpha Tocotrienol	Me	Me	Me
Beta Tocotrienol	Me	H	Me
Gamma Tocotrienol	Me	Me	H
Delta Tocotrienol	Me	H	H

1) The two groups can be divided on the basis of the degree of saturation of their side chains. Tocopherols have saturated side chains whereas tocotrienols have unsaturated side chains.

2). Tocopherols are found in polyunsaturated vegetable oils and in the germ of cereal seeds, whereas tocotrienols are found in the aleurone and subaleurone layers of cereal seeds and in palm oil.

The synthesis of the tocotrienols is distinguished by the addition of a prenylPP (geranylgeranyl-PP), rather than phytyl-PP, to homogentistic acid. It is possible that the prenyl side chain might alter the metabolism or subcellular distribution of tocotrienols when compared with tocopherols. (Michael *et al.*, 1991)

3.1.1.2 Health benefits and antioxidant activity of vitamin E

A better reason for using natural vitamin E is that an excess of alpha-tocopherol can actually displace gamma-tocopherol from the membranes of cells, gamma-tocopherol protects the membranes of cells including brain cells from peroxynitrite damage for this the balance between alpha and gamma tocopherol is needed.

-Tocotrienol has beneficial effects in cardiovascular diseases by inhibiting the oxidation of LDL cholesterol and down regulates 3-hydroxyl-3-methylglutaryl-coenzyme A (HMG CoA) reductase a key enzyme in the synthesis of cholesterol (Qureshi *et al.*, 1996).

-Tocopherol inhibits the inflammatory pathways, for instance, those involving 5-lipoxygenase and phospholipase A2 in the same time it is believed that alpha-tocopherol inhibits cell proliferation, platelet aggregation and monocyte adhesion.

- Tocotrienols have a better distribution in the fatty layers of the cell membrane than Tocopherols which explain their superior antioxidant activity.

-Tocotrienol and tocopherol, inhibit both the proliferation and tube formation of bovine aortic endothelial cells, with δ -tocotrienol appearing the highest activity. Also, δ -tocotrienol reduces the vascular endothelial growth factor-stimulated tube formation by human umbilical vein endothelial cells (Hitoshi *et al.*, 2003).

-It is believed that the transformation of vitamin E to CEHC is mostly a detoxification mechanism, useful to maintain the malignant properties of prostate cancer cells.

3.1.1.3 Vitamin C

Vitamin C or Ascorbic acid is the enolic form of 3-oxo-L-gulofuranolactone. It can be prepared by synthesis from glucose, or extracted from plant sources such as rose hips, blackcurrants or citrus fruits. It is easily oxidised in air.

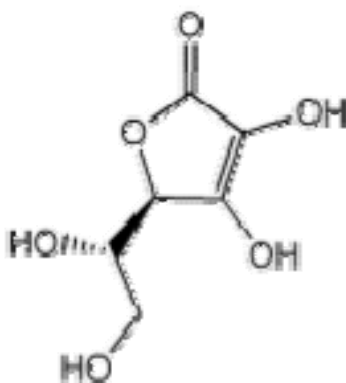


Figure 3.Chemical structure of ascorbic acid

Chemically, ascorbic acid is a simple carbohydrate material, related to glucose, of rather unique properties. The presence of the ene-diol group in the molecule confers electron lability, which makes it a member of an oxidation-reduction system having electron donating and electron accepting properties.

The body does not produce vitamin C, so it must be obtained through the diet and/or in the form of supplements. All fruits and vegetables contain some amount of vitamin C. Foods that tend to be the highest sources of vitamin C include green peppers,

citrus fruits and juices, strawberries, tomatoes, broccoli, turnip greens and other leafy greens, sweet and white potatoes, and cantaloupe.

Vegetables such as broccoli, sweet green and red peppers, potatoes (with skin), tomatoes, and Brussels sprouts are good sources.

Ascorbic acid is a relatively fragile molecule and it may be lost from foods during preparation, cooking, and/or storage. Ascorbic acid is easily destroyed by oxidation, particularly in the presence of heat and alkalinity, and because it is highly soluble in water, it is often discarded in cooking water. Although the vitamin occurs in small amounts in animal tissues, it is usually destroyed either by exposure to air or by processing before it reaches the table.

3.1.1.4 Vitamin C functions, uses, and health benefits

Vitamin C has multiple functions as either a coenzyme or cofactor it is responsible for helping to build and maintain our tissues and strengthening our immune system. It is essential for the oxidation of phenylalanine and tyrosine, the conversion of folacin to tetrahydrofolic acid and may modulate prostaglandin synthesis to favor the production of eicosanoids with antithrombotic and vasodilatory activity.

Vitamin C is required for synthesis of dopamine, noradrenaline and adrenaline in the nervous system or in the adrenal glands. Vitamin C is also needed to synthesise carnitine, important in the transfer of energy to the cell mitochondria. Ascorbic acid is required for collagen synthesis and has a structural role in bone, cartilage and teeth

*Vitamin C's antioxidant activity may be helpful in the prevention of some cancers and cardiovascular disease. The antioxidant properties of vitamin C are thought to protect smokers, as well as people exposed to secondhand smoke, from the harmful effects of free radicals. Vitamin C strengthens the collagen structure of arteries, lowers total cholesterol, and blood pressure, and inhibits platelet aggregation.

*Vitamin C may against heart disease by reducing the stiffness of arteries and the tendency of platelets to clump together. Vitamin C reverses endothelial vasomotor dysfunction in patients with coronary artery disease. Under most circumstances, dietary

vitamin C is adequate for protecting against the development of or consequences from cardiovascular disease.

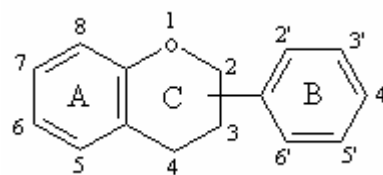
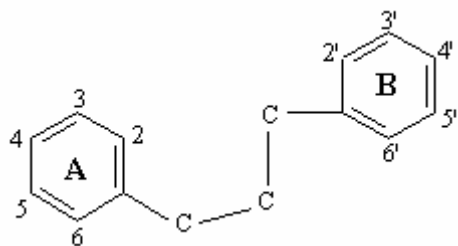
*Vitamin C may have cancer-preventive activity, at least for certain types of cancer. As a powerful antioxidant, vitamin C may help to fight cancer by protecting healthy cells from free-radical damage and inhibiting the proliferation of cancerous cells.

*As a participant in hydroxylation, vitamin C is needed for the production of collagen in the connective tissue. These fibers are ubiquitous throughout the body; providing firm but flexible structure. Vitamin C is involved in the hydroxylation of proline to form hydroxyproline in the synthesis of collagen, a protein substance on which the integrity of cellular structure in all fibrous tissues depends. Collagen is the "glue" that strengthens many parts of the body, such as muscles and blood vessels. Collagen is a protein needed to develop and maintain healthy teeth, bones, gums, cartilage, vertebrae discs.

*Vitamin C has been reported to reduce activity of the enzyme, aldose reductase, in people. Aldose reductase is the enzyme responsible for accumulation of sorbitol in eyes, nerves, and kidneys of people with diabetes. Vitamin C levels in the eye decrease with age and that supplementing with vitamin C prevents this decrease, possibly leading to a lower risk of developing cataracts. Vitamin C may be helpful in protecting against some of the lipid oxidation caused by smoking. Vitamin C may be helpful in chronic diseases characterized by oxidative damage to biological molecules. People with recurrent boils (furunculosis) may have defects in white blood cell function that are correctable with vitamin C supplementation

3.1.1.5 Flavonoids as antioxidants

Flavonoids are natural substances with variable polyphenolic structures (Kelly et al., 2005), compounds possessing 15 carbon atoms; two benzene rings joined by a linear three carbon chain.

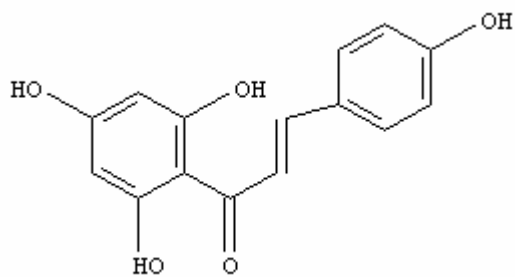
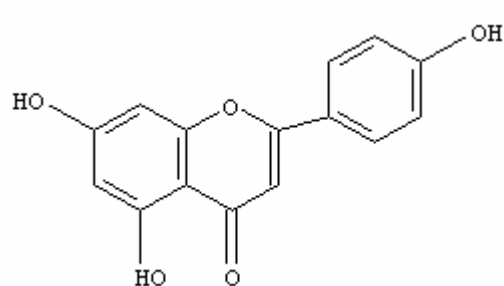
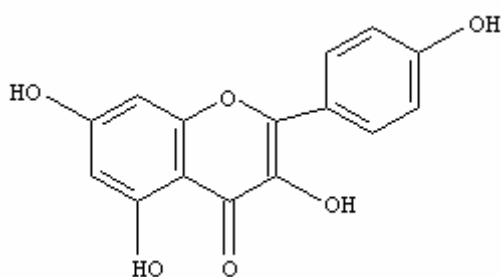
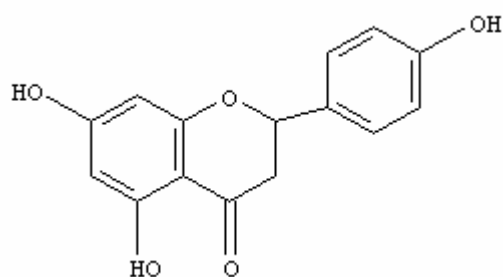
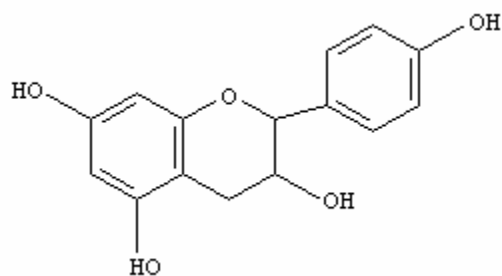
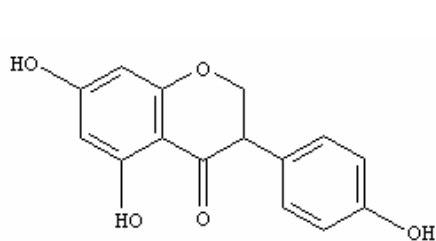
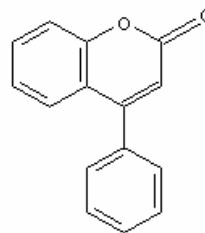


The skeleton of Flavonoids

C₁₅ skeleton with a CHROMANE ring

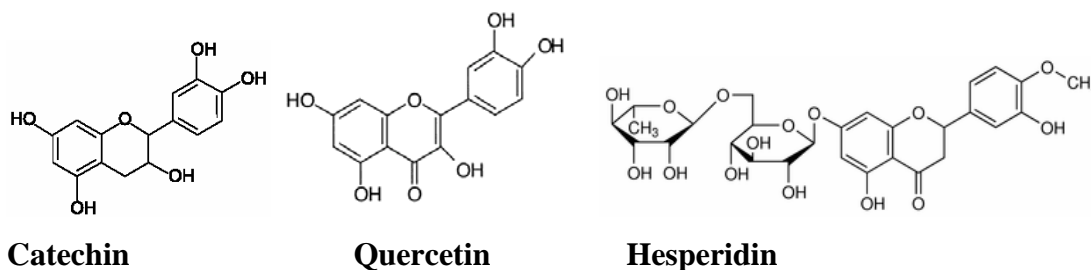
The flavonoids skeletons is characterized by its (C₆-C₃-C₆) to be differentiated according to the saturation level and saturation level and opening of central pyran ring into 6 different subgroups (Yao *et al.*,2004).

Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification.

**Chalcone****Flavone****Flavonol****Flavonone****Anthocyanin****Isoflavonoid****Neoflavonoid**

The Isoflavonoids and the Neoflavonoids can be regarded as ABNORMAL FLAVONOIDS.

In this work we used 3 different flavonoids from different subgroups that we can show its characteristics in this table:



Flavonoid	Subgroup	Substitution
Catechin	Flavanol	3,5,7,3',4'-OH
Hesperidin	Flavanone	3',5,7-trihydroxy-4'methoxy flavanone
Quercetin	Flavanol	3,5,7,3',4'-OH

3.1.1.6 Flavonoids food sources:

Tea: Green, white or black tea are a rich source of flavonoids, especially flavonols (catechin, epicatechin, epigallocatechin, and epicatechin gallate). Tea is a good source of quercetin.

Onions: The major flavonoid in onions is quercetin. Other flavonoids in onion are kaempferol and myricetin.

Honey: Depending on the flower type the bees feed on, honey contains myricetin, and quercetin.

Other dietary flavonoid sources are beans, spinach, buckwheat, strawberry, blueberry, rooibos plant. The concentration and composition of flavonoids in plants may vary depending on the growing condition, maturity, plant part, and variety.

3.1.1.7 Health benefits and antioxidant activity

Beneficial effects of flavonoids on human health are partly explained by their antioxidant activity.

A prolonged oxidative stress produced by chronic consumption leads to increased formation of lipofuscin in hippocampal and cerebellar neurons which is the end result of lipid peroxidation, flavonols prevented the accumulation of neuronal lipofuscin submitted to ethanol feeding so the flavonols can reduce the effects of oxidative activity brought about by alcohol consumption, indicating that these compounds might display neuronal beneficial effects under oxidative stress (De fretas *et al.*, 2004).

Because of its bioactive potential, flavonoids have an anticarcinogenic effect because they can interact with the different steps of cancer development.

Flavonoids intake is inversely correlated with mortality from cardiovascular diseases which can be explained by its beneficial effect on atherosclerosis, including lipoprotein oxidation, blood platelet aggregation and vascular reactivity. (Pleuso.,2006).

Flavonoids can inhibit the cyclic nucleotide phosphodiesterase (PDE), an elevation in cAMP level and activation of protein kinase A.

Tea flavonoids including catechin inhibit LDL lipid peroxidation in vitro, and do same work in vivo but with smaller effect. (Rimersma *et al.*, 2001)

Dietary flavonoids with a catechol structure increase α -tocopherol in rats and protect the vitamin from oxidation (Jan Frank *et al.*, 2006)

It was found that the flavonoids have a big positive effect on the immune system especially in the anti-inflammatory reaction (Ielpo *et al.*, 2000)

Quercetin has demonstrated significant anti-inflammatory activity because of direct inhibition of several initial processes of inflammation.

It inhibits both the manufacture and release of histamine and other allergic/inflammatory mediators. And it exerts potent antioxidant activity and vitamin C-sparing action. Quercetin's proven anti-inflammatory properties help the body counter allergic reactions to pollen. Also seems to reduce inflammation in the lungs and other air passages, making breathing easier.

Flavonoids have prooxidant and cytotoxicity effects which give them the ability to scavenge the ROS and generate more oxidative stress (Loo *et al.*, 20003) which is responsible for its cytotoxic and proapoptotic effects.

3.1.1.8 Phenolic acid as anti oxidants

Phenylpropanoid acids are compounds widely present in plants, including many edible vegetable staples. Their biological properties, particularly the antioxidant activity, are well known and depend on the structural characteristics of these compounds. Because of their relative polar properties, important efforts have been made in order to increase their hydrophobicity and therefore produce amphiphilic molecules of industrial value. Thus, esters of hydro-p-coumaric (Lee *et al.*, 1995), hydroferulic (Hegazi *et al.*, 2002) and hydrocaffeic acids, as well as alkyl coumarates and ferulates, have been widely reported as antioxidants in food, cosmetic and pharmaceutical formulations. Interestingly, hydrocinnamic esters have been used in the synthesis of HIV-1 protease inhibitors or as precursors for the synthesis of 1, 3, 4, 9-tetrahydropyrano indole-1-acetic acid, which is used as an analgesic, inflammation inhibitor and antipyretic.

In spite of the remarkable industrial potential of phenylpropanoid acid esters as food antioxidants, their use in oil-based food processing has been limited, because of their high water solubility.

In this work we used caffeic acid as a member of phenylpropanoid family

Caffeic acid, $C_9H_8O_4$ is a naturally occurring phenolic compound, (formerly called a carboic acid), which is found in many fruits, vegetables, and herbs, including coffee, although varying in amounts depending on the plant

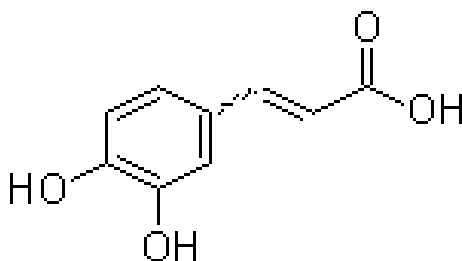


Figure 4. Caffeic acid structure

Chlorogenic acid, an ester of caffeic acid and quinic acid, is a major phenolic compound in coffee; daily intake in coffee drinkers is 0.5–1 g. Chlorogenic acid and caffeic acid are antioxidants in vitro and might therefore contribute to the prevention of cardiovascular disease.

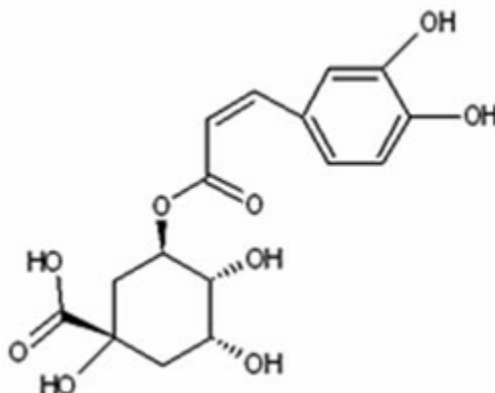


Figure 5.Chlorogenic acid

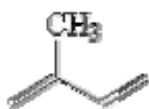
3.1.1.9 Carotenoids

Occurring in chromoplasts of plants and some other photosynthetic organisms (some types of fungus and some bacteria) Carotenoids are organic pigments that absorb blue light. Carotenoids serve two key roles in plants and algae: they absorb light energy for use in photosynthesis, and they protect chlorophyll from photodamage.

There are over 600 known carotenoids; they are split into two classes, xanthophylls and carotenes.

Carotenoids are found in colored fruits and vegetables. Apricots, cantaloupe, carrots, pumpkin and sweet potato are sources of α -carotene and β -carotene; pink grapefruit, tomatoes and watermelon are sources of lycopene, α -carotene, β -carotene, phytofluene and phytoene. Mango, papaya, peaches, prunes, squash and oranges are sources of lutein, zeaxanthin, and β -cryptoxanthin, α -, β - and α -carotene, phytofluene and phytoene, whereas green fruits and vegetables such as green beans, broccoli, brussel sprouts, cabbage, kale, kiwi, lettuce, peas and spinach are sources of lutein, zeaxanthin, α - and β -carotene. Carotenoid concentrations in fruits and vegetables vary with plant variety, degree of ripeness, time of harvest, and growing and storage conditions.

Bioavailability of carotenoids (bioavailability, as used here, relates to the intestinal uptake and passage of a compound into the systemic circulation) is influenced by several factors such as characteristics of the food source, interactions with other dietary factors and various subject characteristics (Sergio *et al.*, 1999). In this work we used the β -carotene which is the molecule that gives carrots their orange colour. It is part of a family of chemicals called the carotenoids, which are found in many fruit and vegetables, as well as some animal products such as egg yolks. Carotenoids were first isolated in the early 19th century, and have been synthesised for use as food colourings since the 1950. Biologically, beta-carotene is most important as the precursor of vitamin A. It also has anti-oxidant properties and may help in preventing cancer and other diseases. Structure Beta-carotene is a member of a family of molecules known as the carotenoids. These have a basic structure made up of isoprene units.



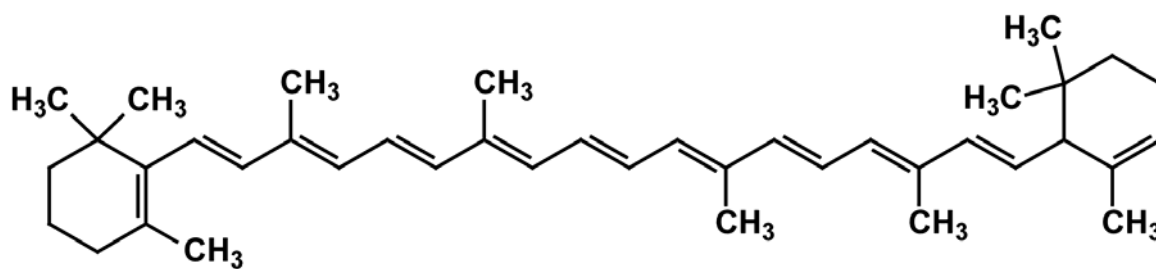
Isoprene

These are joined end-to-end to give a conjugated chain which is common to all carotenoids. Notice that the two centre isoprene units are joined differently to the others ("head-to-head" rather than "head-to-tail") so that the chain has a centre of symmetry.

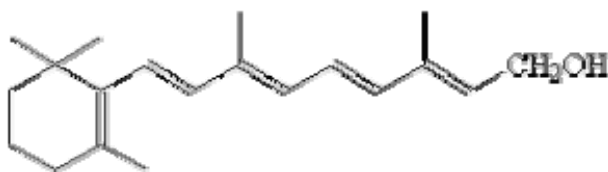


The central part of a carotenoid

Beta-carotene is made up of eight isoprene units, which are cyclised at each end.



Beta-carotene is also known as provitamin A, because it is one of the most important precursors of vitamin A in the human diet. If you compare the two molecules, it is clear that vitamin A (retinol) is very closely related to half of the beta-carotene molecule.



Vitamin A

There are two ways in which beta-carotene can be converted to vitamin A: either by cleavage at the centre or by breaking the molecule down from one end (see here for a diagram illustrating these processes).

The second of these is thought to be the most important biologically. The breakdown of beta-carotene occurs in the walls of the small intestine (intestinal mucosa) and is catalysed by the enzyme beta-carotene dioxygenase.

The retinol formed is stored in the liver as retinyl esters. This is why cod liver oil used to be taken as a vitamin A supplement.

All carotenoids help to prevent heart disease and heart attack by inhibiting the formation of harmful LDL cholesterol. Because they are very soluble in fat and very insoluble in water, carotenoids circulate in lipoproteins along with cholesterol and other fats. Carotenoids are able to absorb light in the visible range of the spectrum. By filtering out the sun's harmful ultraviolet (UV) rays and by keeping free radicals from damaging the retina, carotenoid may help to protect against macular degeneration. Reducing the amount of blue light that reaches the structures of the eye that are critical to vision may protect them from light-induced oxidative damage. It is commonly perceived that vitamin

E, beta-carotene, and other carotenoids act similarly to antioxidants, their unique biologic activities distinguish them from each other.

For example, individual antioxidants and carotenoids act differently in different components of the immune system in addition to contributing to cell-to-cell communication, cellular differentiation, and regulation of cell growth or induction of apoptosis (Maureen *et al.*, 2003).

The antioxidant actions of carotenoids are based on their singlet oxygen quenching properties and their ability to trap peroxy radicals. The best documented antioxidant action of carotenoids is their ability to quench singlet oxygen. This results in an excited carotenoid, which has the ability to dissipate newly acquired energy through a series of rotational and vibrational interactions with the solvent.

Under no physiological circumstances, carotenoids may act as prooxidant. These results can be modified by altering the oxidant stress, the cellular or subcellular system, the type of animal and environmental conditions.

4 .Yeast

Yeast are unicellular organisms. The precise classification is a field that uses the characteristics of the cell, ascospore and colony. Physiological characteristics are also used to identify species. One of the more well known characteristics is the ability to ferment sugars for the production of ethanol.

Yeasts are characterized by a wide dispersion of natural habitats. Common on plant leaves and flowers, soil and salt water. Also found on the skin surfaces and in the intestinal tracts of warm-blooded animals, where they may live symbiotically or as parasites.

4.1 Cellular Growth of cell

4.1.1 Vegetative Reproduction in Yeast

Vegetative reproduction takes place in yeasts by the following methods:

4.1.1.1 Budding: It is the most common method of reproduction under favorable conditions. The protoplast of the yeast cell bulges out in the form of a bud which ultimately develops into a daughter cell. Meanwhile, the nucleus divides into two daughter nuclei and of these one migrates into the enlarging bud. The bud grows and eventually gets separated from the mother cell. Quite often, the daughter cell also starts producing buds before getting separated from the mother cell and the process may be repeated giving rise to chains or groups of yeast cells. Such chains may be branched or unbranched and give the appearance of a false mycelium called **pseudo mycelium**. The cells of pseudo mycelium are loosely attached and sooner or later they detach.



Figure 6. Representation of the budding in yeast: *Saccharomyces cerevisiae* or budding Baker's yeast is Latin for "Sugar Fungus Beer". It converts a carbon source such as sugar and water into carbon dioxide and ethanol. Budding yeast can also feed on acetate, glycerol and other carbon sources. Note all images are hot-linked to their respective sources (L. Van Warren, 2005)

4.1.1.2 Fission:

It is the simple splitting of a cell into two daughter cells by the constriction and formation of a transverse partition wall. During this process, the parent cell elongates and its nucleus divides into two daughter nuclei by intranuclear mitosis. Then, a constriction appears somewhere near the middle of the mother cell, followed by the formation of a transverse septum and two uninucleate daughter cells are formed. The two daughter cells finally separate.

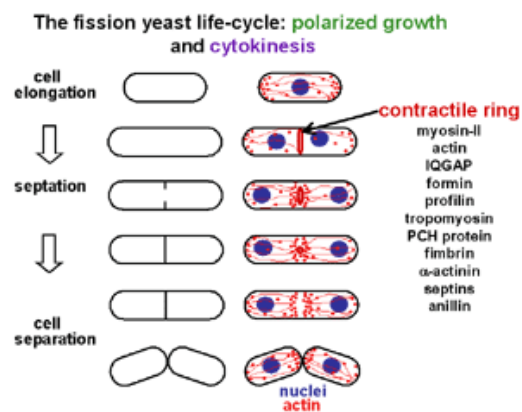


Figure 7. The fission yeast life cycle exhibits two dynamic changes in morphology: cell elongation (polarized growth) and cell separation (cytokinesis). The left scheme depicts the cell wall outline; right scheme illustrates the position of nuclei and the localization pattern of the actin cytoskeleton (university of vermont, Dept. of Molecular Physiology & Biophysics)

4.1.2 Sexual reproduction in yeast:

Yeasts are categorized into two groups, based on their methods of sexual reproduction: the ascomycetous (Division Ascomycotina) and basidiomycetous (Division Basidiomycotina) yeasts.

The sexual spores of the ascomycetous yeasts are termed ascospores, which are formed in simple structures, often a vegetative cell. Such asci are called naked asci because of the absence of an ascocarp, which is a more complex fruiting body found in the higher Ascomycetes. If the vegetative cells are diploid, a cell may transform directly into an ascus after the $2n$ nucleus undergoes a reduction or meiotic division. See also Ascomycota.

Certain yeasts have been shown to be heterothallic; that is, sporulation occurs when strains of opposite mating type (usually indicated by “a” and α) are mixed on sporulation media. However, some strains may be homothallic (self-fertile), and reduction division and karyogamy (fusion of two haploid nuclei) take place during formation of the sexual spore. Yeasts that produce sporogenous cells represent the teleomorphic form of the life cycle. In cases, in which sexual cycles are unknown, the yeast represents the asexual or anamorphic form. A species of yeast may be originally discovered in the anamorphic form and named accordingly; subsequently, the sexual state may be found and a name applied to represent the teleomorph. Consequently, the anamorphic and teleomorphic names will differ.

Basidiospores and teliospores are the sexual spores that are produced in the three classes of basidiomycetous yeasts: Urediniomycetes, Hymenomycetes, and Ustilaginomycetes. Sexual reproduction and life cycle in these yeasts is typical of other basidiomycetes in that it can include both unifactorial (bipolar) and bifactorial (tetrapolar) mating systems. See also Basidiomycota.

4.2 Using yeast as a model to study oxidative stress

Saccharomyces cerevisiae is probably the best studied eukaryotic organism. This is because yeast is easily grown in laboratory and is extremely favorable organism for studies of genetic phenomena (Lushchak., 2006). *Saccharomyces cerevisiae* is a well studied unicellular eukaryotic organism when its genome has been completely sequenced.

All aerobic organisms possess special systems which defend them against reactive oxygen species, the involvement of so-called antioxidant enzymes, particularly superoxide dismutase and catalase, in the protection of proteins (Lushchak *et al*, 2006) .

4.3 Effect of Hydrogen Peroxide in Cells.

Reactive oxygen species include superoxide anion (O_2^-), peroxides of hydrogen (H_2O_2) and other compounds ($ROOH$), hydroxyl radical ($\bullet OH$), etc. The group of RNS consists of nitric oxide ($\bullet NO$) and its derivatives, for example, peroxynitrite ($OONO^-$). Thiyl radical ($RS\bullet$) and its derivatives are examples of reactive sulfur species are very active and can cause big modifications in the level of cells from which we can mention the cell and DNA lyses when it is generally thought that is the major cause of mutations and the ROS in this case are generated as normal part of oxygen metabolism but also produced by ionizing radiations, by attacking almost all cell components ROS can include many types of DNA damage including DNA breaks , base and sugar modifications (Sandrine *et al.*,2007),on the other hand due to its high activity ROS trigger several proximal and distal signaling pathways ,affect the activities of transcriptional factors ,and lead to expression of specific genes (Saso *et al.*,2002) .

By the interaction of ROS with complex proteins like acotinase, succinate, isocitrate and malate dehydrogenase which are enzymes for Krebs cycle a chemical modification of polypeptide chains and nonprotein components should be distinguished on the other hand the abstraction of hydrogen by hydroxyl radical from the α -carbon atom of any amino acid residue begins a sequence of reactions resulting in the formation of alkyl radical and water which will give the cleavage of the peptide bond (Lushchak *et al*, 2006) by the way we can mention that all amino acid residues of peptides are susceptible to oxidation by hydroxyl radicals which can give a highly toxic and active molecules and that partial oxidation of proteins, modifying surface charge or hydrophobic, is one of the mechanisms determining their spatial distribution in the cell.

In the level of organism's state the oxidation of proteins may be reliable marker of the intensity of oxidative stress process which is very well seen in the case of the Ischemia/reperfusion, the neurodegenerative disorder and the diabetes mellitus.

4.4 ROS and apoptosis

Apoptosis is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area. Apoptosis plays a crucial role in developing and maintaining health by eliminating old cells.

Under stress conditions, ROS are believed to cause damage to cell membranes and DNA fragmentation in somatic cells (Hyun *et al.*, 1998) yeast cells undergo a form of apoptosis in which there is nuclear fragmentation, accumulation of DNA-strands breaks and inversion of the plasma membrane that all depend on the activity of a years caspase-like protein.(Mark *et al.*,2005)

This occurs in cells that are exposed to stresses such as H₂O₂, acetic acid and other ROS generators.

It is commonly perceived that vitamin E, beta-carotene, and other carotenoids act similarly to antioxidants; their unique biologic activities distinguish them from each other.

For example, individual antioxidants and carotenoids act differently in different components of the immune system in addition to contributing to cell-to-cell communication, cellular differentiation, and regulation of cell growth or induction of apoptosis.

4.5 Enzyme used to protect cells under oxidative stress

4.5.1 Antioxidant enzymes:

Microorganisms such as yeasts have evolved to survive stressful changes in their environment. Sudden challenge can result in disturbance of cellular functions and even growth arrest (Bayliak *et al.*, 2006), The yeast *Saccharomyces cerevisiae* possesses two different but overlapping stimulons responsible for cell defense against hydrogen peroxide and superoxide anion (Jamieson *et al.*, 2008) The adaptive response to H₂O₂ in *S. cerevisiae* is under control of Yap1 and Skn7 proteins, and it involves a change in the expression of at least 167 proteins. Cytosolic catalase T, and Mn₂₊ superoxide dismutases

(SOD), glutathione reductase (GR), and glucose_6_phosphate dehydrogenase (G6PDH) are among them.

4.5.2 Super oxide dismutase (SOD)

During electron transport an inappropriate donation of electrons to molecular oxygen can be done which results to the formation of superoxide anion O_2^- . An enzyme that catalyzes the conversion of superoxide into hydrogen peroxide and oxygen; "oxygen free radicals are normally removed in our bodies by the superoxide dismutase enzymes".

Two forms of SOD are found in complex organisms, the first contains copper and zinc (Cu/ZnSOD) which represented by SOD1 which is found outside the mitochondrial matrix and SOD3 and the second form is SOD2 which is localized inside the mitochondrial matrix the main site of superoxide production .

SOD1 contains zinc ion and disulphide bonds which are important for the stability of the enzyme, and a cooper ion essential for dismutase activity (James *et al.*, 2004)

The copper ion is directly inserted into active site by CCS, which binds copper tightly and transfers the ion specifically to SOD1, a process that probably involves the formation of a heterodimeric complex of SOD1 and CCS in the intermembrane space (Rae *et al.*, 1999).

SOD1 transcription and translation can be induced by various conditions, including exposure to high oxygen concentration, cooper ions and oxidative stress, the posttranslational activation of SOD1 need the presence of CCS and the oxygen (or superoxide).

One of the mediators in the transcriptional activation of yeast SOD1 is the yAP-1, which acts as sensor of the redox state cell, under oxidative stress it moves from the cytoplasm to the nucleus and induces the expression of a number of target genes encoding protective enzymes including SOD1.

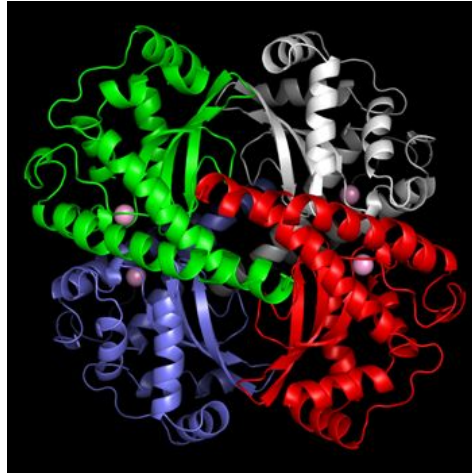


Figure 8. Crystal structure determination of Superoxide Dismutase from *C. elegans* (Emma *et al.*, 2007)

Although H_2O_2 is relatively innocuous, it is unstable and can give rise to the destructive hydroxyl ($\cdot\text{OH}$). And to avoid the formation of $\cdot\text{OH}$ radicals, other antioxidant defense, including catalase (CAT) and the glutathione system, have evolved to convert H_2O_2 to water and oxygen.

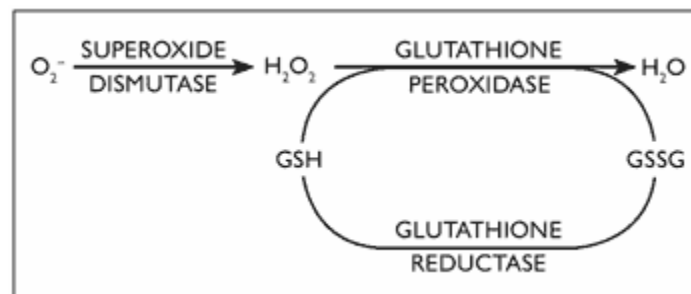


Figure 9. Glutathione peroxidase (GPX) role in oxidative stress and its interaction with superoxide dismutase (SOD) (Monica Pinto *et al.*, 2002)

4.5.3 Catalase (CAT)

Catalases are produced by aerobic organisms ranging from bacteria to man. Catalases (EC 1.11.1.6) are haem-containing proteins that catalyse the conversion of hydrogen peroxide (H_2O_2) to water and molecular oxygen, thereby protecting cells from the toxic effects of hydrogen peroxide:

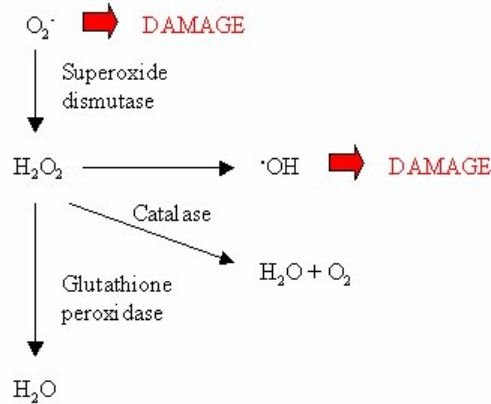


Figure 10. Catalase role in oxidative stress (advanced enzyme)(www.enzymeindia.com)

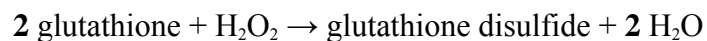
Some haem-containing catalases are bifunctional, acting as a catalase and a peroxidase (EC 1.11.1.7). In these bifunctional catalase-peroxidases, a variety of organic substances can be used as a hydrogen donor, for example alcohol, which can be oxidised in the liver. These bifunctional catalases are closely related to plant peroxidases. There are also non-haem manganese-containing catalases, which occur in bacteria. This review concentrates on the mono-functional, haem-containing catalases (EC 1.11.1.6).

4.5.4 Glutathione reductase

Is an enzyme (EC 1.8.1.7) that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant.

For every mole of GSSG one mole of NADPH is required.

In cells exposed to high levels of oxidative stress, like red blood cells, up to 10% of the glucose consumption may be used for production of the NADPH needed for this reaction.



4.5.6 Old Yellow Enzyme (OYE)

OYE or NADPH oxidoreductase is a single domain protein of around 45 KDa with an α/β barrel fold (Fox *et al.*, 1994) was isolated from brewer's bottom yeast by Warburg and Christian (1932) and they identified that it permitted the system to form a complete respiratory chain reacting with molecule oxygen .

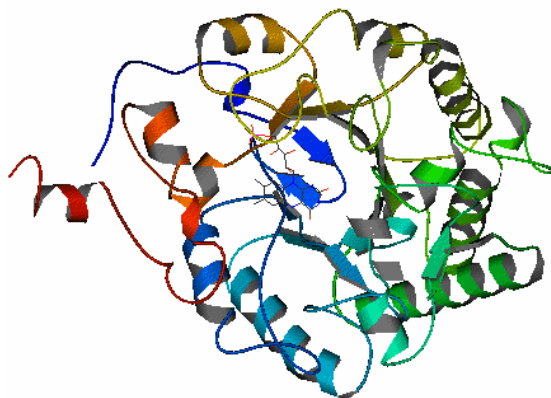
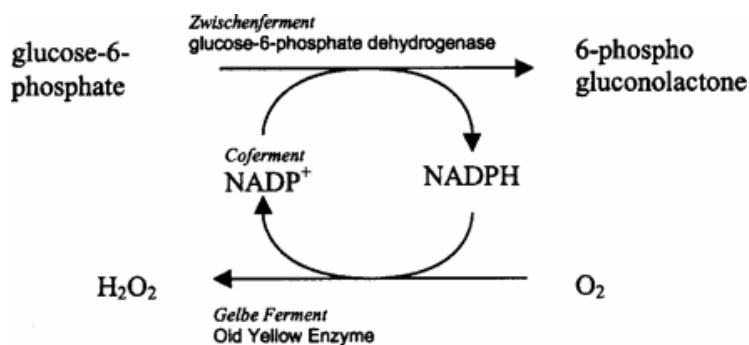


Figure 11. Crystal structure of the Old yellow enzyme. (www.enzymeindia.com)

The enzyme was termed old yellow enzyme because it comprise a yellow dye essential for the enzyme activity ,the yellow dye was similar in nature to vitamin B2 (riboflavin) when the yellow cofactor was in fact riboflavin 5'-phosphate (FMN) Flavin Mono Nucleotide (Williams *et al.*, 2002).



The OYE catalyses the oxidation of β -NADH, β -NADPH and α -NADPH which are assumed to be physiological reductants ,the OYE can be reoxidized by methylene blue, Fe^{3+} ,quinine , cytochrome, in the same time the reoxidation can be affected by molecular oxygen to yield hydrogen peroxide and superoxide .

It has been found that *Sacharomyces carbergensis* (Saito *et al.*, 1991) and *sacharomyces cervisiae* contains pair of closely related genes and the application of the standard strategy knockout mutagenesis to the OYE gene function was initially frustrated by the presence of two paralogous genes in *sacharomyces cervisiae* . Δ oye2 yeast strains are

sensitive to prooxidant-induced PCD .Over expression and knock-out analysis indicate these OYE2 anti-oxidant activities are proposed by OYE3 (Osama *et al.*,2007). .

OYE family members catalyze other reactions from which we can mention, reductive denitration of penta erythritol tetranitrate by PETN reductase (Binks *et al.*, 1995), reduction of morphine (R=H)/cadinone (R=CH₃) to form hydro morphine and hydrocodone (R=CH₃) by morphinone reductase (Hailes *et al.*, 1993).

1. Recovery assays of different strains of yeast

Three strains of yeast were chosen (BY4741, Δ Sod1, Δ Oye2glr1.). These three strains were chosen for their effect on different components of the antioxidant machinery as representing physiologically relevant conditions in higher organisms encountering various types of oxidative stress.

1.1 *Saccharomyces cerevisiae* strains

1.1.1 BY4741 Mat a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0: This strain is derived from strain S288C in which commonly used selectable marker genes are deleted by design, based on the yeast genome sequence which has been constructed and analyzed. These strains minimize or eliminate the homology to the corresponding marker genes in commonly used vectors without significantly affecting adjacent gene expression.

1.1.2 Δ sod1 Mutant derivatives of *S. cerevisiae* that lack either of the SODs exhibit phenotypic deficiencies which are particularly extreme in the Δ sod1 mutants. Even though they are viable, Δ sod1 strains grow poorly in air, are extremely sensitive to redoxcycling drugs, die quickly in the stationary phase, and display aerobic lysine and methionine auxotrophies. It is generally believed that the loss of superoxide-scavenging activity, which is due to the absence of *SOD1* gene product, leads to a serious burden of oxidative stress and causes havoc in cellular metabolism and growth processes (Jia-Perng and al., 2001)

Δ oye2glr1 was previously described by (odat *et al.*,2007).

1.2 Media used:

1.2.1 YPD medium

YPD Agar Medium is a blend of peptone, yeast extract, and dextrose in optimal proportions for growth of most strains of *Saccharomyces cerevisiae*.

YPD mixture was prepared as follows:

20 g/L Difco peptone

10 g/L Yeast extract

20 g/L Agar (for plates only)

Add H₂O to 950 ml. Adjust pH to 6.5, and autoclave.

Allow medium to cool to ~ 55°C and then add dextrose (glucose) to 2% (50 ml of a Sterile 40% stock solution).

Note: If you add the sugar solution before autoclaving, autoclave at 121°C for 15 min. Autoclaving at a higher temperature, for a longer period of time, or repeatedly may cause the sugar solution to darken and will decrease the performance of the medium.

1.2.2 Glu/CM

The glucose completes (Glu/CM):

The medium was prepared by mixing 20 gr of glucose, 6.7 gr of yeast nitrogen base and 1.33 gr of complete amino acid. 1L of deionised water was added and well mixed after an autoclave was necessary for the media.

Autoclaving is necessary before using of the media, the flasks, and micropipettes tips.

1.3 Overnight culture

An overnight culture was prepared for the strain. This overnight culture was prepared in 100 ml flasks adding around 50 ml of media and the inoculum was added.

The flasks were placed in an incubator, at 30°C for around 18 hours. The inoculums were taken from YPD plates, which were stored at 4°C

1.4 Experiments

The adjustment of the absorbance to 0.1 was done after mixing the inoculums, and the antioxidant.

The measurement of the optical density (at 600 nm) of the mixture was taken every two hours, and the values were multiplied by 30 to give the approximate number of cells in **millions/ml** and those values were plotted in function of the time (hours).

1 ml was taken from the prepared Glu/CM medium before adding the culture was placed in a small 10 mm x10 mm plastic cuvette.

This cuvette was closed with Para film to protect it from any contamination. The cuvette was used before each reading in the spectrophotometer for the calibration. So each flask had a different blank. Different blanks were used because of the difference in the color of the antioxidants.

2. Determination of the viability of cells treated with pro-oxidants

The strains were grown in 5ml Glu/CM overnight. The next day cells were diluted at OD₆₀₀= 0.1 in 40 ml Glu/CM in 200 ml flasks and the cultures were kept shaking at 30°C until they reached OD₆₀₀ 0.5-0.7. For each strain, 50ml FALCON tubes containing 20ml media (Glu/CM) were prepared supplemented with 50 µM of the tested antioxidant and 200 µl of the culture were inoculated into the set of prepared tubes containing media with antioxidant, after mixing 100 µl sample was taken from each tube and **10⁻¹, 10⁻²** dilutions was prepared and spread on YPD plates. Prooxidant (H₂O₂) was added to each tube to a final concentration of **1.5mM** final. The treated cultures were incubated shaking in 30°C shaker for 2 hours. After a two-hour incubation period, samples were taken as before (100µl sample from each tube was taken, and **10⁻¹, 10⁻²** dilutions were made on YPD plates and the plates were incubated in 30°C incubator until the appearance of the colonies).

3. ROS measurement using a fluorimetry

3.1 Phosphate buffer saline (PBS)

NaCl 140 mM

KCl 2.7 mM

Na₂HPO₄ 110 mM

KH₂PO₄ 1.8 mM

The solids were dissolved in 800 ml ddH₂O and the pH was adjusted to 7.4, then the volume of the solution was completed to 1 litre, and sterilized by autoclaving (20 min 151 lb/sq.in on liquid cycle).

3.2 MitoTracker Red CMX ROS

95µl of DMSO was added to a vial containing 50µg of the stain, which gave a final concentration of 1 mM stock solution, working solution: 1:2000 or 0.5 µl of stock in 1 ml solution

3.3 Experiment

The following stocks of pro-oxidants and antioxidants were prepared:

H₂O₂ 100 mM, cumene hydroperoxide (CHP) 100 mM

Overnight cultures of cells in Glu/CM were adjusted until the OD₆₀₀= 0.5 was reached.

1 ml aliquots from each culture were split in labeled eppendorf tubes (2ml). The cells were washed once with sterile PBS, and resuspended in PBS. The pro-oxidants and antioxidants were added to their respective tubes at the following final concentrations: 1.25mM H₂O₂, 80µM CHP (60µM for Δsod1), 5µM and 50µM of each antioxidant tested, and one tube was kept untreated (control).

All vials were incubated for 30 minutes shaking at 30°C. At the end of incubation the cells were washed one time with PBS, were resuspended in 1 ml PBS, and the cells were stained with 1:2000 dilution of 1mM mitrotracker CMXROS stock solution (0.5 µl of stain stock to 1 ml of cells), the stained cells were incubated in the dark for 15 minutes with occasional shaking.

At the end of incubation cells were washed one time with PBS, and they were resuspended in 2 ml PBS.

Fluorescence was measured using a Fluorimeter (PERKIN ELMER LS55 luminescence spectrometer), at 540nm excitation wavelength and 560 to 750nm emission wavelength, scan speed used was 500nm/minute, 5nm excitation slit, and 5nm emission slit.

Cells harbouring endogenous OYE2-GFP or OYE3-GFP fusions were treated with small antioxidant molecules in PBS and were observed for induction of GFP 130 fluorescence under X1000 magnification in a fluorescent microscope.

4. Glutathione measurements

4.1 Solutions:

1% Oxalic acid (or 0.5% formic acid)

2-vinyl pyridine

Stock buffer: 125 Mm Na-phosphate, 6,3 Mm NA-EDTA .Adjust pH to 7.5

5Mm Triethanolamine :Make a 1 M stock by mixing 132 μ l Triethanolamine into a total volume of 1 ml H₂O, To prepare a 5 Mm stock add 50 μ l from the 1 M stock into 10 mls volume of H₂O.

Prepare 3 working solutions made in stock buffer

0.3 Mm NADPH (24 mgs in 10 ml stock buffer)

6 Mm DTNB (0.115 gr in 50 ml stock buffer)

50 Units of Glutathione reductase

4.2 Experiment

Grow 5 ml cultures of the yeast strains in Glu/CM overnight Next day resuspend cells into 50 ml medium in Erlenmeyer flasks at OD₆₀₀=0.15 and grow cells till saturation for about 10 hours ,Label 50 ml FALCON tubes and weight them empty,

Centrifuge cells in the FALCONS (3800 rpm for 8 min) ,Wash 2X in sterile H₂O, At the end of the second centrifugation make sure you dry with a paper towel the walls of the tubes to remove any excess water. Weight the tubes + pellets and calculate the wet pellet weight. resuspend cells in equal concentrations in 1% Oxalic acid .To do so ,resuspend the cells with the lowest weight in 5 ml ,and all other cells in proportional to their weight volume of 1% oxalic acid (make sure you write down the weight and the volume),Sonicate cells suspension to break cells (1 min should be enough ,check turbidity) ,Centrifuge the samples and collect the supernatants(13000 ,2min),Label 2 sets of eppendorf tubes : set A for total glutathione (GSH) and set B for oxidized glutathione (GSSG),Transfer 200 µl of supernatant to each respective tube

To all samples add 1.8 µl of 5Mm stock Triethanolamine 45 µ M final concentration

To set B (GSSG) Add 4 µl of 2-vinyl pyridine and incubate for 30 min at RT.

Prepare the following mastermix in accordance to the total number of reactions

700 µl	Sol 1 (0.3 Mm NADPH)=210 µ M NADPH Final
100 µl	Sol 2 (6 Mm DTNB) 0.6 Mm DTNB Final

Add 800 µl of mix per reaction, equilibrate the reaction at 30 c for 5 min. Transfer contents to 1 ml cuvette , add 1 µl enzyme (0.5 units of enzyme Glutathione reductase) and monitor absorbance at 412 nm,Control reactions containing known quantities of GSH and GSSG should be prepared (250 µ M, 500 µ M, 1m M, 2 mM ,5 mM, 10 mM) in 1 % oxalic acid.

GSH Stock: Prepare a 250 mM stock of GSH by measuring 0.15 gr and dissolving it in 2 mls of st .d H₂O .Prepare the following dilutions.

500 µM	2 µl IN 1ml of 1% oxalic acid
1 mM	4 µl in 1 ml of 1% oxalic acid
2 mM	8 µl in 1ml of 1% oxalic acid
5 mM	20 µl in 980 of 1% oxalic acid
10 mM	40 µl in 960 of 1% oxalic acid

Make additional 1:10 dilutions from the mM stock to cover the 5 mM the μ M region,
Add 10 μ l Triethanolamine
GSSG stock: 100 mM stock GSSG .Dissolve 0.12 grams in 2 mls d H₂O.
Make dilutions as above.

5. PCR (polymerase chain reaction)

5.1 Materials

Primers 100 pmol/ μ l. :with a 5'-ends(70 nucleotides) that correspond to the desired gene sequence:YAP1-F2,5'-TTG TCA TCA ATG CAG AAG ACG TTC AAT TAG CTT TGA ATA AGC ATA TGA ACC GGA TCC CCG GGT TAA TTA A-3',and the 3'-ends that anneal and allow amplification of the selectable marker (HA tag,GFP.,or MYC tag) YAP-R1,5'-TTG TAA CAT TAT AGA AAA AGT TCT TTC GGT TAC CCA GTT TTC CAT AAA GTTT CCC GAA TTC GAG CTC GTT TAA AC-3'.

DNA Template (Usually the amount of template DNA is in the range of 0.01-1ng for plasmid (PFA-6a #30 plasmid) DNA and 0.1-1 μ g for genomic DNA, for a total reaction mixture of 50 μ l).

Taq DNA polymerase,

Platinum Taq buffer – MgCl₂ 50mM,

10mM, dNTPs

PCR Water.

5.2 Method

The final concentration of primers was 100pmol/ μ l. A master mix was prepared, consisting of 5 μ l Taq buffer (10x), 1 μ l dNTPs (10mM) and multiplied by the number of reaction tubes needed. After adding the master mix to the PCR tubes, the other components were added: 2 μ l DNA template (diluted 1:10); 1 μ l of each primer, and then water was added to reach 49 μ l volume. Reactions were incubated allowed to cycle in Gene Amp PCR 2400 system (Perkim-Elmer) under the conditions needed depending on

the reaction, 1µl of Taq polymerase was added to the reaction mixture when the temperature reached 94°C, to have final volume of 50µl .

6. Purification of the PCR product

6.1 Purification of the PCR product by ethanol chloroform precipitation

Phenol is naturally water soluble; the phenol used as a water saturated solution with Tris buffer and gives a fuzzy interface that is sharpened by the presence of chloroform. Ethanol precipitation is a method used to concentrate DNA. DNA is polar soluble in water, which is also polar. Based on the principle of (like dissolves like), it is in soluble in the relatively less polar ethanol. The DNA precipitation in this step is due to the ethanol interacting with the water so that fewer water molecules are available to dissolve the DNA molecules.

6.2 Materials:

Tris-buffered 50% phenol

Tris-buffered 50% chloroform

100% ethanol

70% ethanol

6.3 Method

To 200µl of DNA sample to a 1.5 ml micro centrifuge tube and one volume of phenol: chloroform is added which will give a sharp interface , vortexes vigorously one minute to mix the phases. Centrifugation is carried at 13000rpm for 3min to separate the phases, and the aqueous phase is removed to a new tube. The aqueous phase is usually the upper

The loss of DNA into the organic phase can be reduced by adding a second volume of water, mixing, centrifuging, and removing again. The extraction step with 1V of phenol : chloroform is repeated once , then followed with extraction with 1V of chloroform , which will ensure that no phenol what's over remains .DNA is initially ethanol-precipitated by adding 2V of ice cold 100% ethanol and 1/10 V of 3M sodium Acetate Ph 5.8 incubate the samples 30 min at -80°C or 2 hours at -20°C .This will precipitate DNA , as well as the salts that form ionic bonds with it .The suspension was centrifuged for 20 min in a micro centrifuge tube at 4 °C and the supernatant was removed , leaving a pellet of the crude DNA . IN the next step ,70% ethanol was added to the precipitated pellet , without vortexing or disturbing the pellets (fast) ,to re-suspend the DNA .This allows 20-30% water to access the salts present in the pellet . This pellet is air-dried giving the purified DNA and the pellets were resuspended with the 10-20 µl of 1 x TE or elution buffer.

7. Agarose gel electrophoresis

7.1 Materials

Agarose (Invitrogen)

Running Gel Buffer, TAE Buffer 50X stock

24.2% (w/v) Tris base,

3.72% (w/v) EDTA,

The pH was adjusted to 8.0 with ~57 ml Glacial acetic acid and brought to 1 liter with distilled water. The working solution was 1x TAE prepared by dilution of the 50x buffer (40ml 50x TAE and the volume completed to 2 liters with distilled water).

STEB (6X Loading buffer) for DNA

100 mM Tris HCl, pH 7.5,

20% Glycerol (w/v),

1 mM EDTA,

Few grains of Bromophenol blue.

Staining solution: 50 µl of 1 mg/ml Ethidium bromide stock added to 500 ml of distilled deionized water.

Destaining solution: clean deionized distilled H₂O

7.2 Method

Agarose gel electrophoresis was used to separate, identify and purify DNA fragments.

1% agarose gel was prepared by adding 100 ml of 1x TAE to 1g of agarose, mixed and then heated for 2-3 min in a microwave oven until the solution became clear. The tray was assembled with the comb into the gel apparatus. After being cooled down to 55°C, the gel was poured into the tray and left until solidified. The tank was filled with 1xTAE solution covering the top of the gel. The DNA samples were mixed with the corresponding volume of 6x loading buffer (to a final concentration of 1x) and the samples were applied to the wells.

The gel was run at 150V for at least half an hour to ensure an optimal separation of the Fragments, then stained with Ethidium Bromide for 15-20 min. The gel was transferred to the distaining solution for 5 minutes to remove the excess ethidium bromide before visualizing it under UV light.

8. Yeast transformation

8.1 Reagents

For LiOAc yeast transformation

10X Lithium acetate solution

Lithium acetate FW: 102.02 g/mol

The lithium acetate solution is prepared as a 1M stock in distilled water and filter sterilized.

The final pH should be between 8.4-8.9 (pH 7.5).

10X TE buffer (pH 7.5)

Tris HCL

0.5 M EDTA stock

The buffer is a mixture of 100 mM Tris HCL (pH 8.0), and 10 mM EDTA, pH is then adjusted to 7.5 and sterilized by standard autoclaving for 20 minutes. Polyethylene glycol (PEG 50% w/v) the polyethylene glycol (PEG), MW 3350 (Sigma) is made up to 50% (w/v) with distilled water and then filter sterilized. For optimal transformation efficiencies, care must be taken to ensure that the PEG solution is at the proper concentration. In addition, it is important to store the PEG in a tightly capped container to prevent evaporation of water and a subsequent increase in PEG concentration. 50 g of polyethylene glycol is dissolved in 35 ml of water in a 150 ml glass beaker by stirring for at least 30 minutes. The liquid is then transferred to a 100 ml graduated cylinder and the volume is then adjusted to 100 ml by adding distilled water. The solution is then filter sterilized and stored in a securely capped bottle.

Sonicated single stranded carrier DNA (sssDNA) sssDNA was used as a carrier which enhances the transformation efficiency, 2mg of high-molecular weight DNA (deoxyribonucleic acid sodium salt, type 3 from salmon testes, Sigma) per 1ml of sterile TE buffer was sonicated, aliquoted and stored at -20°C. Prior to use, it should be boiled for 10 minutes and quick-cooled on ice.

Solution 1: 1M Lithium Acetate 10x TE Buffer dd H2O 1:1:8 ratios respectively.
Solution 2: 1M Lithium Acetate 10x TE Buffer 50% PEG 1:1:8 ratios respectively.

8.2 The medium (Glu/CM-His):

The medium was prepared by mixing 20 gr of glucose, 6.7 gr of yeast nitrogen base and 1.33 gr of complete amino-acid-histidine. 1L of deionised water was added and well mixed after an autoclave was necessary for the media. We add 20 g/L Agar (for plates only) and the ph is adjusted (5.8-6.2) .

8.3 Method

5ml of an overnight culture were diluted at OD600 to ~ 0.1 in 50ml fresh medium and grown till OD600 ~0.5. Cells were pelleted by centrifugation at 4000 rpm for 5 minutes, and then washed twice with ddH₂O water and resuspended with 300µl of solution 1. 50µl of these cells were added to new eppendorfs containing 3µl of plasmid DNA mixed with 3µl of the carrier sssDNA. 300µl of Solution 2 was added to each tube, and then incubated at 30°C for 30-45 minutes. Cells were then heat shocked at 42°C for 10 minutes. Cells were then plated on the desired plate at a volume depending on the expected transformation efficiency

9. Yeast Genomic DNA Isolation

9.1 Materials

YPD growth medium (1% yeast extract, 2% peptone, 2% glucose),

Lysis buffer: 2% Triton-X100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH8.0, 1mM EDTA

pH 8.0.

100% Ethanol

Chloroform

TE buffer: (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0)

RNase A

9.2 Method:

5 ml of liquid culture grown for 20-24h (saturation) at 30°C was transferred into a microcentrifuge tube, and cells were pelleted by centrifugation at 13,000 rpm for 3 minutes. The pellet was resuspended in 0.5µl of water and then centrifuged for 3 min. Supernatant was poured and briefly vortex to resuspend cells in residual liquid.

0.2 ml of Buffer A (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0), 200 µl glass beads, and 0.2 ml phenol:chloroform:isoamyl alcohol

(25:24:1) was added and Vortexed for 3 minutes. After vortexing add 0.2 ml TE . centrifuge for 5 minutes. The aqueous was transferred to a new tube. Chloroform extraction two times.(supernatant was transferred to a new ependorff 1 ml 100% EtOH and 10 µl of 4M ammonium acetate was added (cold EtOH will create a large, dirty pellet), and tube was inverted to mix before a spinning of 2 min, and then 1 ml of 70% EtOH was added. Spin for 2 min and dry the pellet and resuspend in 50 µl TE.

10.chemicals

Caffeic acid, sigma (Germany)

Catechin ,98 % sigma (Germany)

Quercetin,sigma (USA)Ascorbic acid,serva (USA)

B-carotene ,95% sigma (Germany)

Methano and Acetone ,Fluka (Germany)

Hydrogen peroxide 30% v/v Merck (Germany)

All amino acids used are from Gibco .BRL(UK)

D(+)-Glucose-monohydrate,Merck (Germany)

Chlorogenic acid ,95% sigma (Germany)

Tocotrienol generously donated by the professor Couladouros.

11. Apparatuses:

UV-Visible diode array spectrophotometer 8452 A,Hewlett-Packard.

Balance ,AT 261 Deltarange ,METTLER

Balance METTLER PM2000

pH meter,LAMOTTE,USA

Ultrasound ,Bandelin,Sonorex TK 52H

Leica DM LB Microscope equipped with a 100-mercury lamp linked to a leica DC 300F digital camera,Microscope slides and cover slips

Perkin elmer LS luminescence spectrometer.

RESULTS AND DESCUSSION

1. Recovery assays of different strains of yeast.

Three mutated yeast strains were chosen: The wild type BY4741, $\Delta sod1$ with a deletion in super oxide dismutase and $\Delta oye2 glr1$ with the double knockout in Old yellow enzyme and glutathione reductase.

The strain $\Delta gsh1$ carries a deletion of the GSH1 gene which encodes the enzyme γ -glutamyl synthetase catalyzes the first step for glutathione (GSH) biosynthesis.

The cells are unable to synthesise GSH and although they are viable, they exhibit a dramatic reduction in growth rate and sensitivity to cold and heat.

1.1 Effect of antioxidant supplementation in H_2O_2 induced yeast programmed cell death (PCD)

We used 7 different small molecules antioxidants (**Ascorbic Acid, β -carotene, Caffeic Acid, Catechine, Hesperidin, Quercetine, and Tocopherol**) at high concentration (50 μ M) and low concentration (5 μ M), the concentration of H_2O_2 used was 1.25 mM.

The first strain used was the control wild type BY4741 which showed the fastest recovery comparing to the other two strains.

Among the compounds used AA showed a beneficial effect at both low and high concentration. The wild type cells showed a 12 % improvement in growth with the supplementation of 5 μ M and 15 % improvement with 50 μ M of AA.

In $\Delta sod1$ the AA showed a 10% and 47 % improvement using 5 μ M and 50 μ M respectively then for the double knockout strain $\Delta Oye2glr1$ the improvement was 18% improvement at 5 μ M AA and 47% at 50 μ M AA.

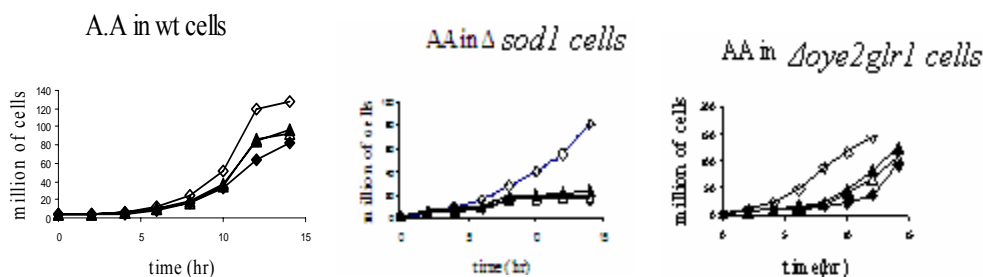


Figure 12. Effect of A.A in H_2O_2 treated cells. Cells were treated with 1.25 mM H_2O_2 (◆) or untreated (◇). Treated cells were additionally supplemented with ascorbic acid (AA) at 5 μ M (△) and 50 μ M (▲). Growth was monitored by measuring OD_{600} every 2 hours.

The treatment of the wild type cells with both 5 μM and 50 μM of β -carotene gave an improvement of 12% and 4 % respectively in cell growth which is not the case in the two other strains where we can see a marked improvement of 30 % at 5 μM and a reduction of 18 % at 50 μM in Δsod1 cells, Finally in $\Delta\text{Oye2glr1}$ cells there was an improvement by 18 % and 46 % at 5 μM and 50 μM respectively.

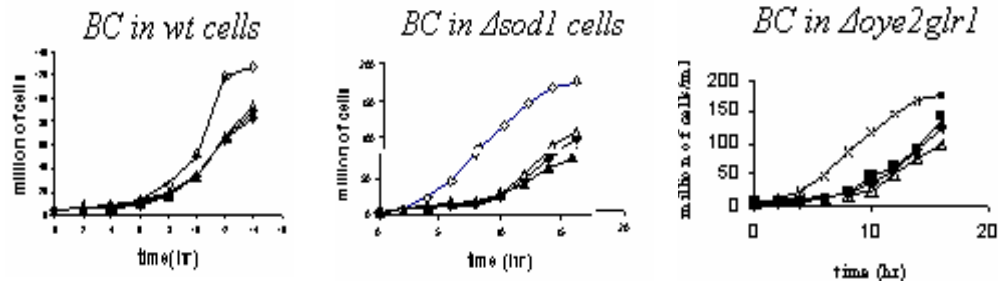


Fig 13 . Effect of BC in H_2O_2 treated cells. Cells were treated with 1.25 mM H_2O_2 (-♦-) or untreated (-◇-). Treated cells were additionally supplemented with ascorbic acid (BC) at 5 μM (-Δ-) and 50 μM (-▲-). Growth was monitored by measuring OD_{600} every 2 hours

When we treated the BY4741 cells with CA there was no effect at all at 5 μM but at 50 μM slight improvement of 4% was seen. On Δsod1 cells the addition of 5 μM of CA gave 43% and a 25% improvement for 50 μM .

In $\Delta\text{oye2 glr1}$ cells a 22% and 27% improvement was seen with 5 μM and 50 μM respectively.

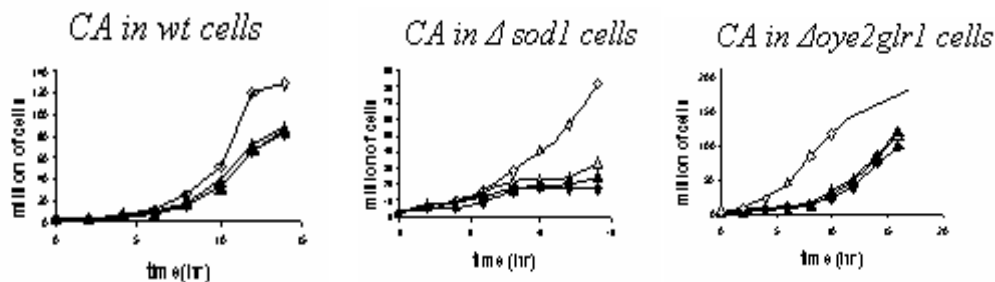


Figure 14. Effect of CA in H_2O_2 treated cells. Cells were treated with 1.25 mM H_2O_2 (-♦-) or untreated (-◇-). Treated cells were additionally supplemented with ascorbic acid (CA) at 5 μM (-Δ-) and 50 μM (-▲-). Growth was monitored by measuring OD_{600} every 2 hours

The treatment of the wt cells with the fourth compounds which is the Catechine did not show any effect that we can mention (slight inhibition of 8 % with 5 μM supplementation) but the both concentrations gave a very slight growth inhibition of Δsod1 strain. And the best activity of catechine was seen on $\Delta\text{oye2glr1}$ strain when we saw a growth improvement of 27% using the two concentrations 5 μM and 50 μM .

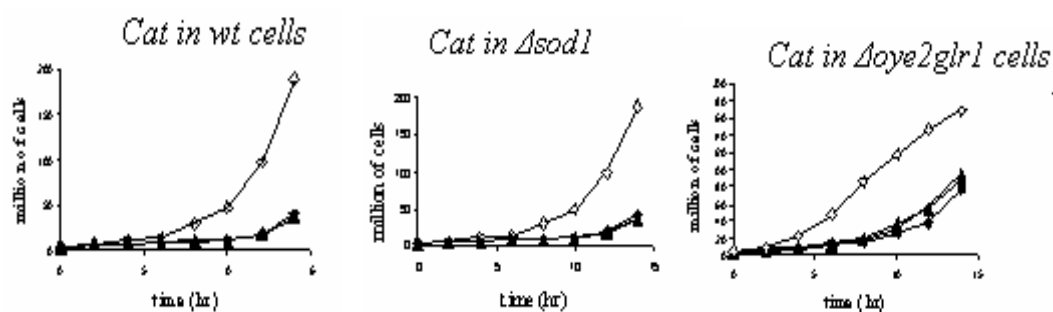


Figure 15 Effect of Cat in H_2O_2 treated cells. Cells were treated with 1.25 mM H_2O_2 (-◆-) or untreated (-◇-). Treated cells were additionally supplemented with ascorbic acid (Cat) at 5 μM (-△-) and 50 μM (-▲-). Growth was monitored by measuring OD_{600} every 2 hours

Hesperidin has different effects on the wild type cells, with 5 μM the growth curve show a 23 % of improvement but with 50 μM it is totally the inverse and it gives an inhibition of 31 % of growth. Supplementation with 5 μM and 50 μM hesperidin was also growth inhibitory in a similar manner to catechin on Δsod1 , in $\Delta\text{oye2glr1}$ strain there was no effect for the 5 μM supplementation but with 50 μM Hesperidin show an inhibition of 21%.

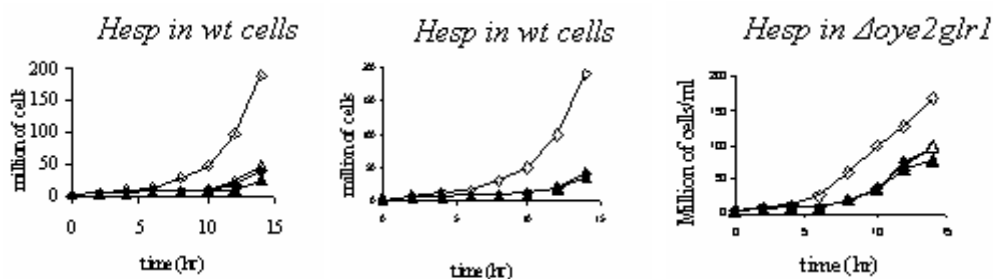


Figure 16 Effect of Hesp in H_2O_2 treated cells. Cells were treated with 1.25 mM H_2O_2 (-◆-) or untreated (-◇-). Treated cells were additionally supplemented with ascorbic acid (Hesp) at 5 μM (-△-) and 50 μM (-▲-). Growth was monitored by measuring OD_{600} every 2 hours.

In wild type cells at 5 μ M Quercetine no protection was seen, whereas at 50 μ M Quer totally inhibited recovery, in $\Delta sod1$ a significant improvement by 28% 5 μ M Quer and almost no protection at 50 μ M Quer and in $\Delta oye2glr1$ cells a 20% improvement at 5 μ M QC and none at 50 μ M QC.

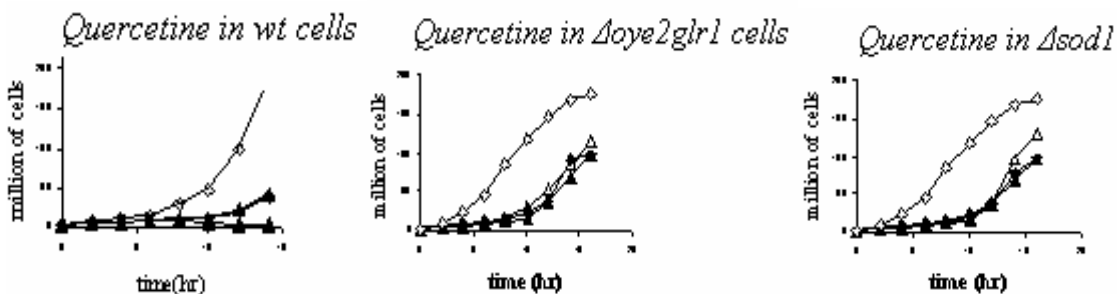


Figure 17 Effect of Quer in H_2O_2 treated cells. Cells were treated with 1.25 mM H_2O_2 (- \blacklozenge -) or untreated (- \diamond -). Treated cells were additionally supplemented with ascorbic acid (Quer) at 5 μ M (- Δ -) and 50 μ M (- \blacktriangle -). Growth was monitored by measuring OD_{600} every 2 hours.

The last compound used was the Tocopherol; in this test we used three different concentrations for both β -tocopherol and γ -tocopherol.

The two isomers show an identical activity and 100% similarity in there effects.

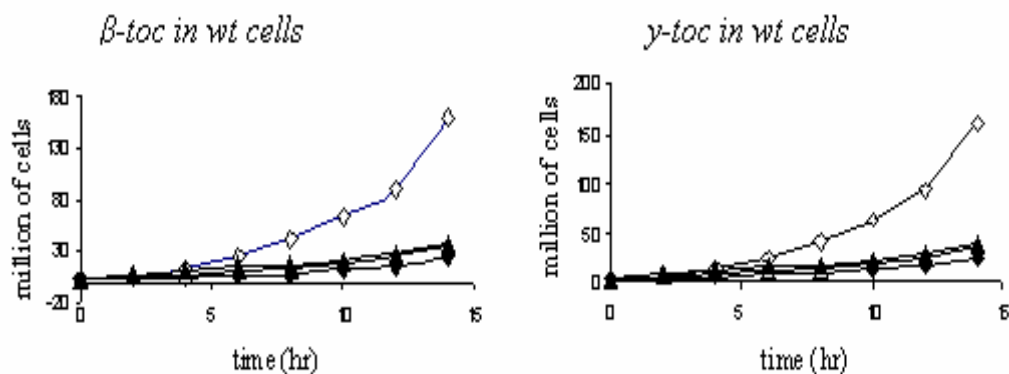


Figure 18. The similarity of effect between β -tocotrienol and γ -tocotrienol.in wt cells

On wt cells we used two different concentrations (5 μ M, 50 μ M) which improve the growth of cells by 46% and 59 % respectively. On β -toc $\Delta sod1$ strain there was an improvement of 8 % t with 5 μ M supplementation and 59 % improvement when we add 50 μ M of tocotrienol, in $\Delta oye2glr1$ strain the improvement was 0% at 5 μ M whereas at 50 μ M the improvement was 14 %.

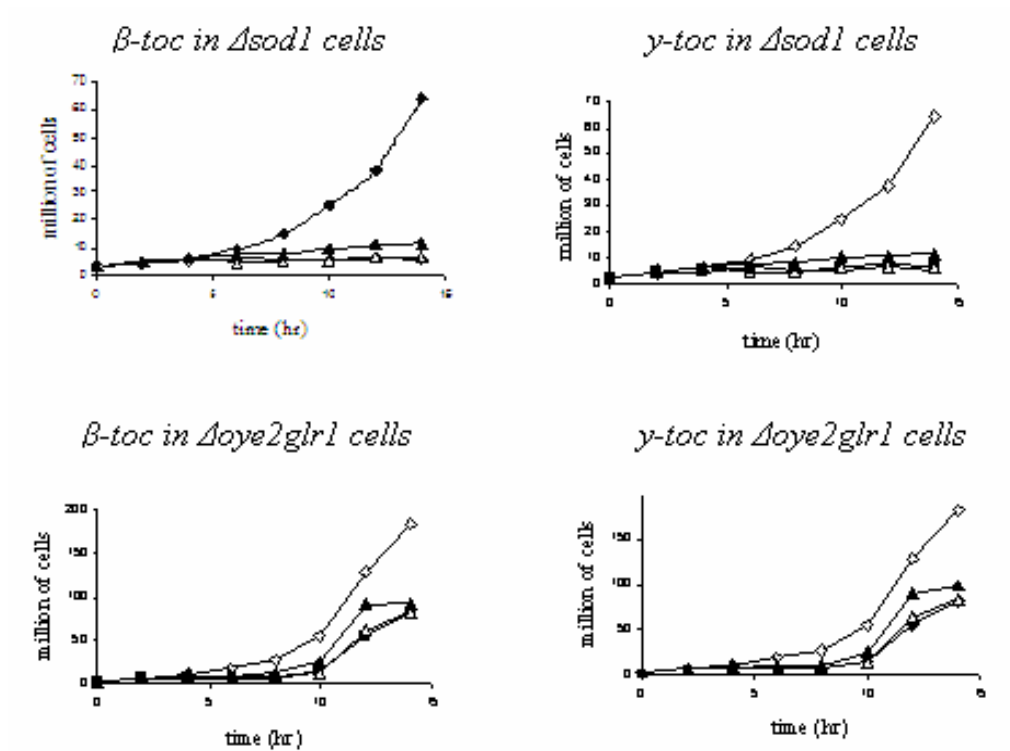


Figure 19. Effect of Toc in H₂O₂ treated cells.

1.2 Effect of antioxidant supplementation in cumene hydroperoxide (CHP) induced cell death

In this test we used the same protocol like H₂O₂ but the concentrations used were 80 mM CHP for the wild type and the $\Delta oye2glr1$ strain and 60 mM for $\Delta sod1$ strain due to its sensitivity.

In wt cells the AA has a small improvement of growth at 5 μ M but 50 μ M improved the growth by 54%, in $\Delta sod1$ protection was significant for both concentrations 22% and

28% for low and high regiment respectively, and in *Δoye2glr1* there was 9% improvement at 5μM and 24% improvement at 50μM.

Beta-carotene supplementation of CHP treated cells exhibited protection only in *Δsod1* cells and was inhibitory under several treatments. In wild type cells there was a reduction of recovery by 28% at 5μM BC and an improvement by 8% at 50μM BC, in *Δsod1* cells a 22% Improvement at 5μM BC and 5% for 50μM BC and in *Δoye2glr1* cells it was totally Inhibitory at 5μM BC and showed an 8% reduction of recovery at 50μM BC.

In the same manner as with H₂O₂-induced PCD, caffeic acid supplementation upon CHP treatment was highly beneficial in most cases. In wild type cells there was a 23% Improvement at 5μM CA that reached 102% at 50μM CA, in *Δsod1* cells a 22% improvement at 5μM CA and 38% improvement at 50μM CA were seen, and in *Δoye2glr1* there was a 12% and 21% improvement for low and high doses of CA respectively.

Quercetin supplementation had beneficial effects only in wild type cells at low doses showing a 28% at 5μM QC. In all other cases there no change or a mild inhibition. It is noteworthy though that no drastic inhibition was observed as was the case for H₂O₂ treated QC.

On wt cells, Hesperidin did not show any effect at small concentration but at 50 μM there was an 8% improvement of cell growth, in *Δoye2glr1* cells Hesperidin improved cell growth with with 13% in the both concentrations for *Δsod1* at 5mM concentration Hespertine inhibit cell growth with 20% and 11% for 50μM .

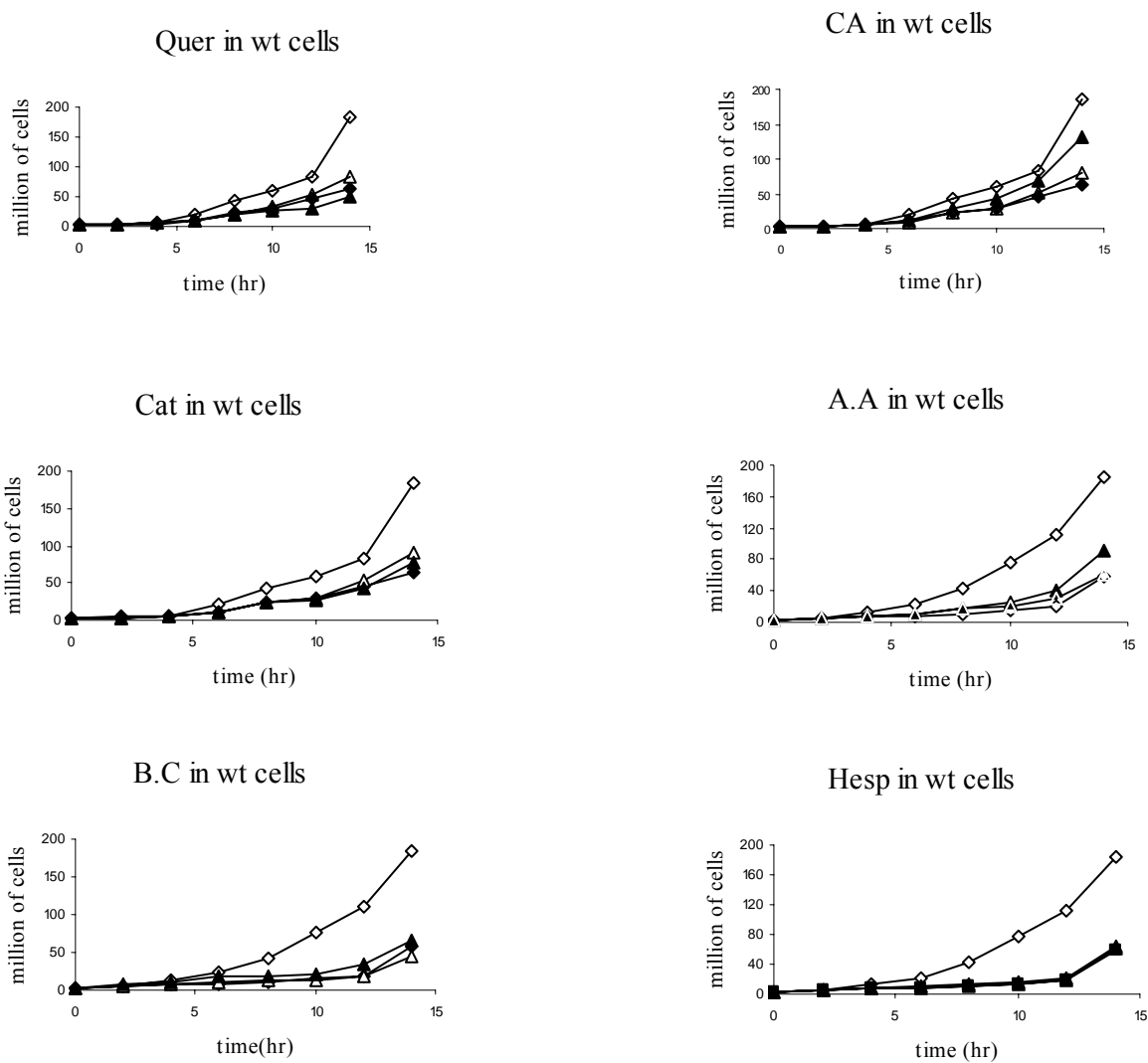


Figure 20. Effect of small antioxidants molecules in CHP treated wt cells. Cells were treated with 80 mM CHP (-◆-) or untreated (-◇-). Treated cells were additionally supplemented with ascorbic acid (Toc) at 5 μM (-△-) and 50 Mm (-▲-). Growth was monitored by measuring OD₆₀₀ every 2 hours

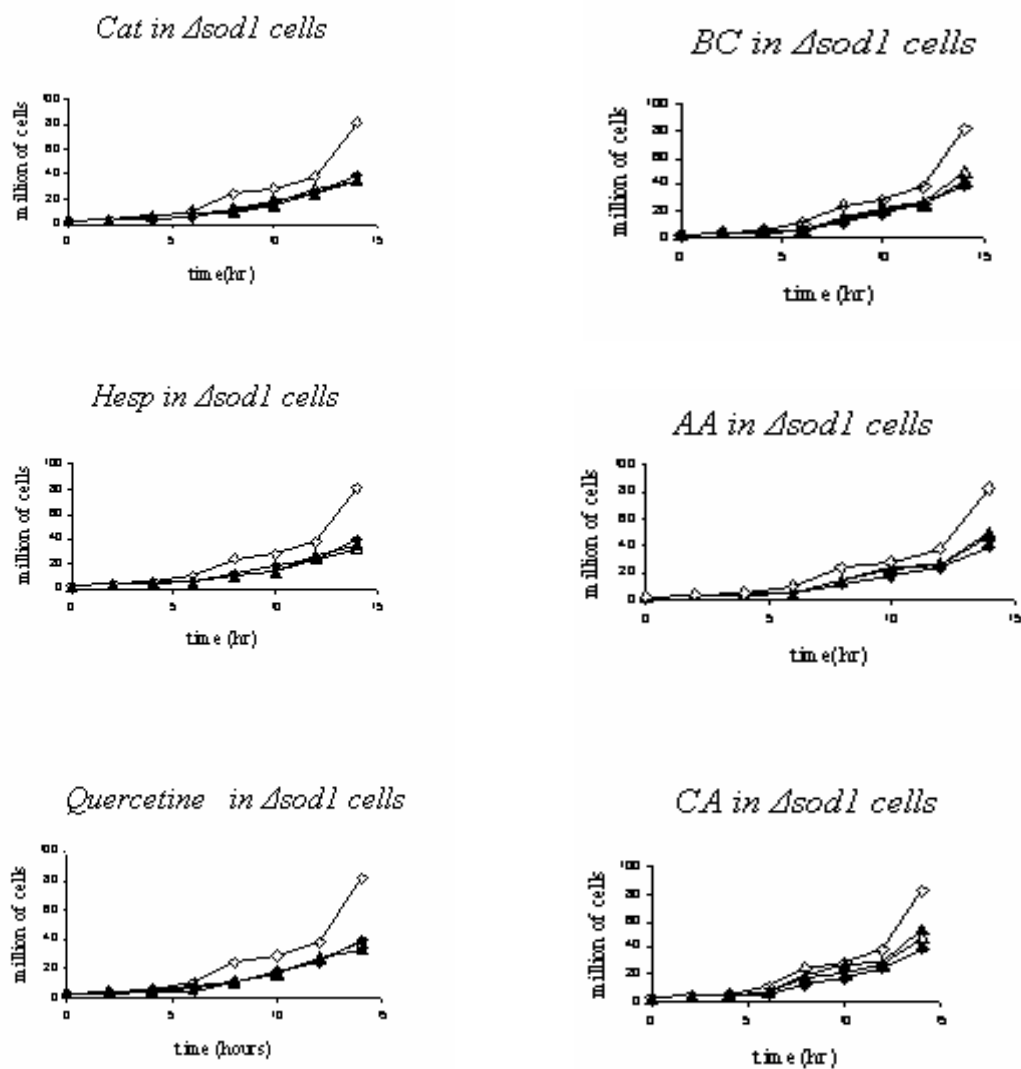


Figure 21 Effect of small antioxidants molecules in CHP treated $\Delta sod1$ cells. Cells were treated with 60 mM CHP (\blacklozenge) or untreated (\diamond). Treated cells were additionally supplemented with ascorbic acid (Toc) at 5 μ M (\triangle) and 50 μ M (\blacktriangle). Growth was monitored by measuring OD₆₀₀ every 2 hours

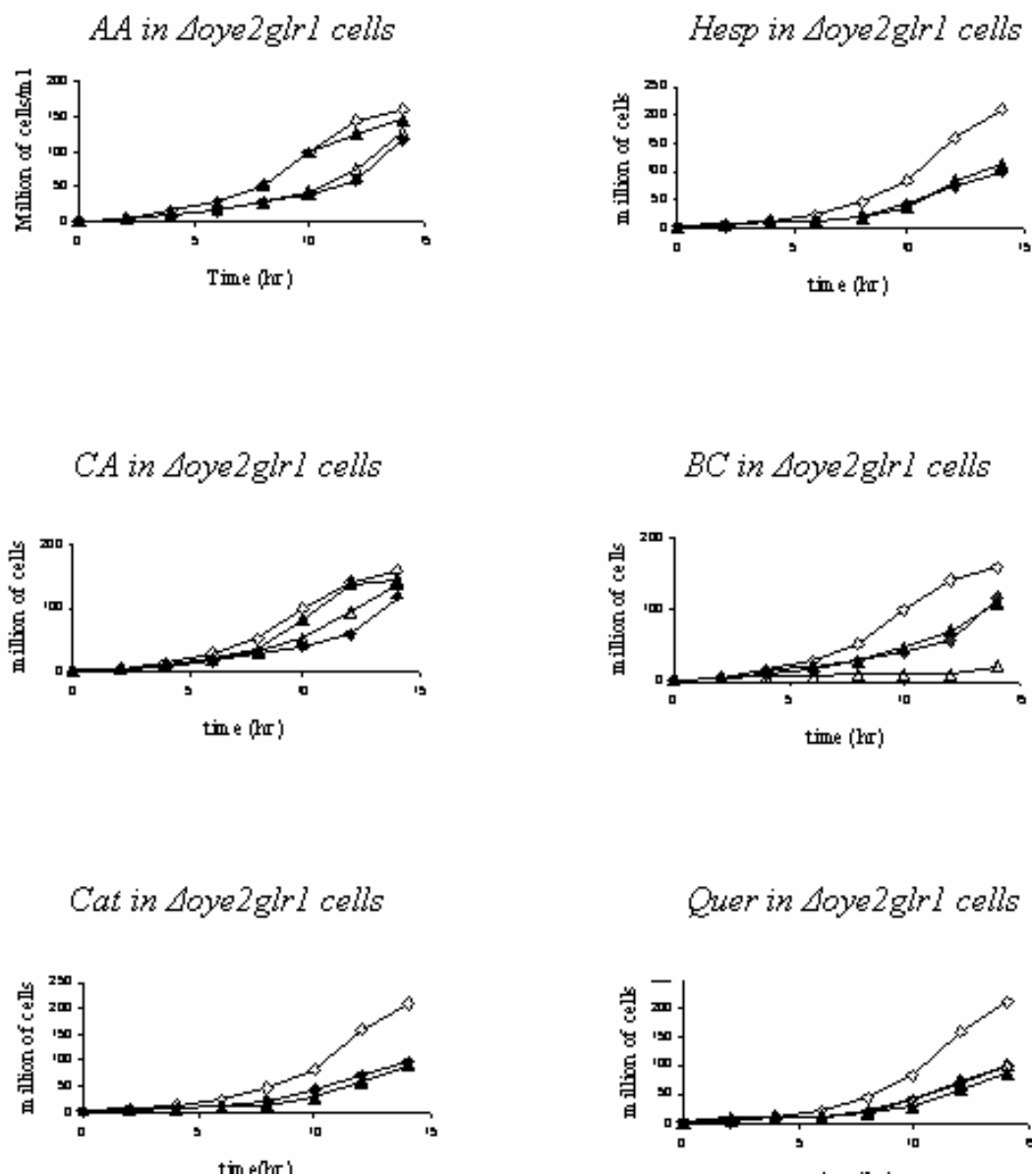


Figure 22. Effect of small antioxidants molecules in CHP treated $\Delta oye2glr1$ cells. Cells were treated with 80 mM CHP (\blacklozenge) or untreated (\diamond). Treated cells were additionally supplemented with ascorbic acid (Toc) at 5 μ M (\triangle) and 50 μ M (\blacktriangle). Growth was monitored by measuring OD_{600} every 2 hours

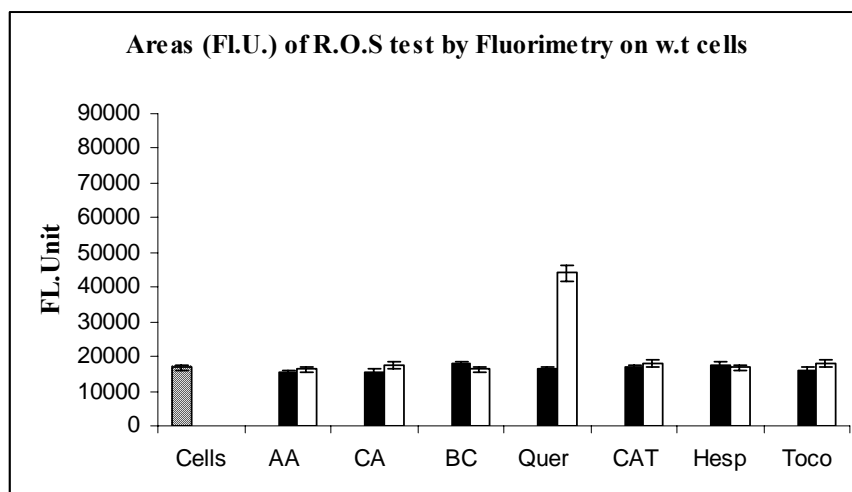
For $\Delta gsh1$ strains deficient in GSH1 (gene responsible for the synthesis of gamma glutamylcysteine synthetase catalyzing the first step in glutathione biosynthesis) can not grow in the Glu/CM which must be supplemented with a concentration of GSH.

No compound could improve the growth of $\Delta gsh1$, only AA has improved the growth with 5% and 16 % using 5 μ M and 50 μ M respectively.

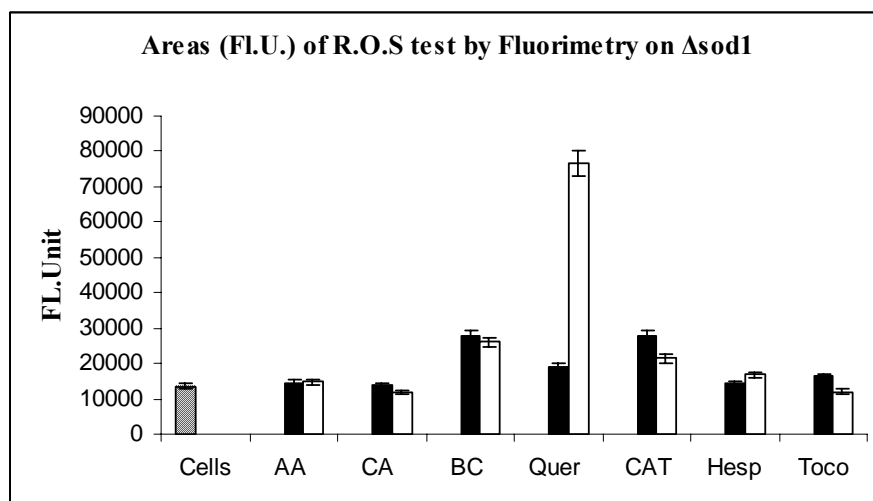
2. Reactive oxygen species and glutathione levels in cells treated with antioxidant small molecules

We performed these experiments to know the effect of each antioxidant on the level of endogenous reactive oxygen species (ROS) inside the cells which were resuspended in PBS at mid- log phase and supplemented with 5 μ M and 50 μ M of each antioxidant for 30 min and stained at the end of incubation with mitotracker CMXRos and fluorescent emission was monitored by fluorimetry.

A)



B)



C)

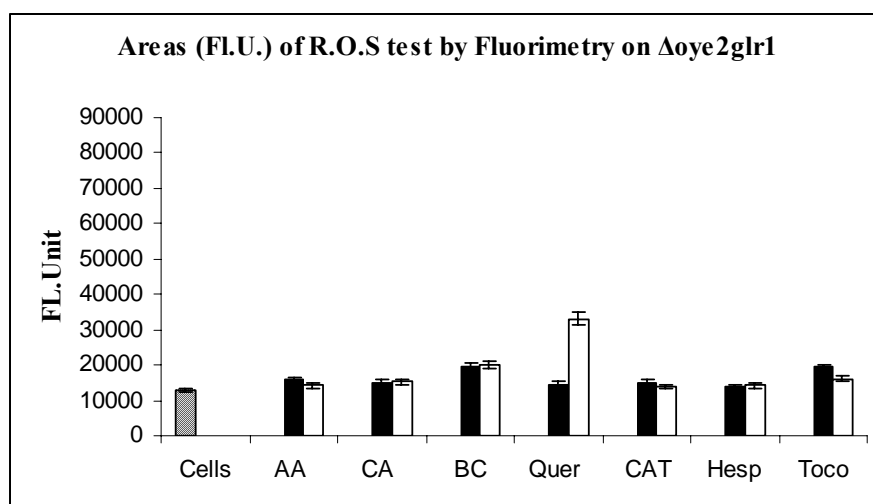


Figure 23. Intracellular ROS levels of cells treated with Antioxidants **A)** in wt cells, **B)** in Δ sod1 cells and **C)** in Δ oye2glr1 cells.

In the three different strains AA does not have any effect on the level of ROS inside the cells and CA has a small effect on $\Delta sod1$, when we used it with high concentration a decrease of ROS level in $\Delta sod1$ was seen.

Against that Cat has an inverse effect by increasing the level of ROS in $\Delta sod1$ when we used it in both small and high concentration.

BC increases the level of ROS in both $\Delta sod1$ and $\Delta oye2glr1$ but not in wild type, the big effect was seen on $\Delta sod1$.

Quercetin and especially with its high concentration 50 μM has a dramatic effect by increasing the level of ROS in the three different strains used.

Glutathione (GSH) is the most abundant thiol (SH) compound in animal tissues, plant tissues, bacteria and yeast. GSH plays many different roles such as protection against reactive oxygen species and maintenance of protein SH groups. During these reactions, GSH is converted into glutathione disulfide (GSSG: oxidized form of GSH). Since GSSG is enzymatically reduced by glutathione reductase, GSH is the dominant form in organisms.

We assayed the effect of Quercetine on the Glutathione level inside the cells

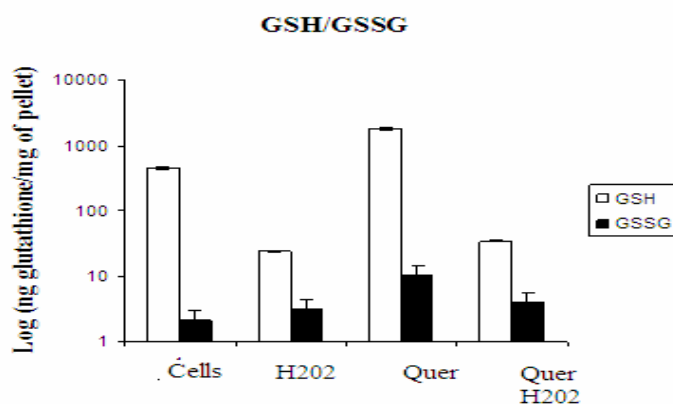


Figure 24. Glutathione levels of cells treated with antioxidants

Quercetin supplemented cells exhibited a significant increase in GSH and GSSG levels compared to wild type cells. However, upon addition of H₂O₂, quercetin failed to protect cells from loss of GSH.

3. Induction of the stress responsive machinery by beta-carotene and Quercetin

As presented in the chapter one, old yellow enzyme OYE2 and OYE3 plays a major role in oxidative stress and the modulation of programmed cell death processes.

For this we used a cells that have an endogenous carboxy-terminal fusion of the green fluorescent protein (GFP) to the OYE2 or OYE3 gene and we tried the induction of genes with small 5 μM and high concentration 50μM of BC.

GFP fluorescence was quantified by fluorimetry

BC caused the induction of OYE2-GFP at i both concentrations and for Quercetin even if did not give any effect with 5 μM ,it lead to the superinduction of OYE2-GFP.

The formation of oye2p-oye3p heterodimer has previously been associated with sensitization of cells to H₂O₂-induced PCD.

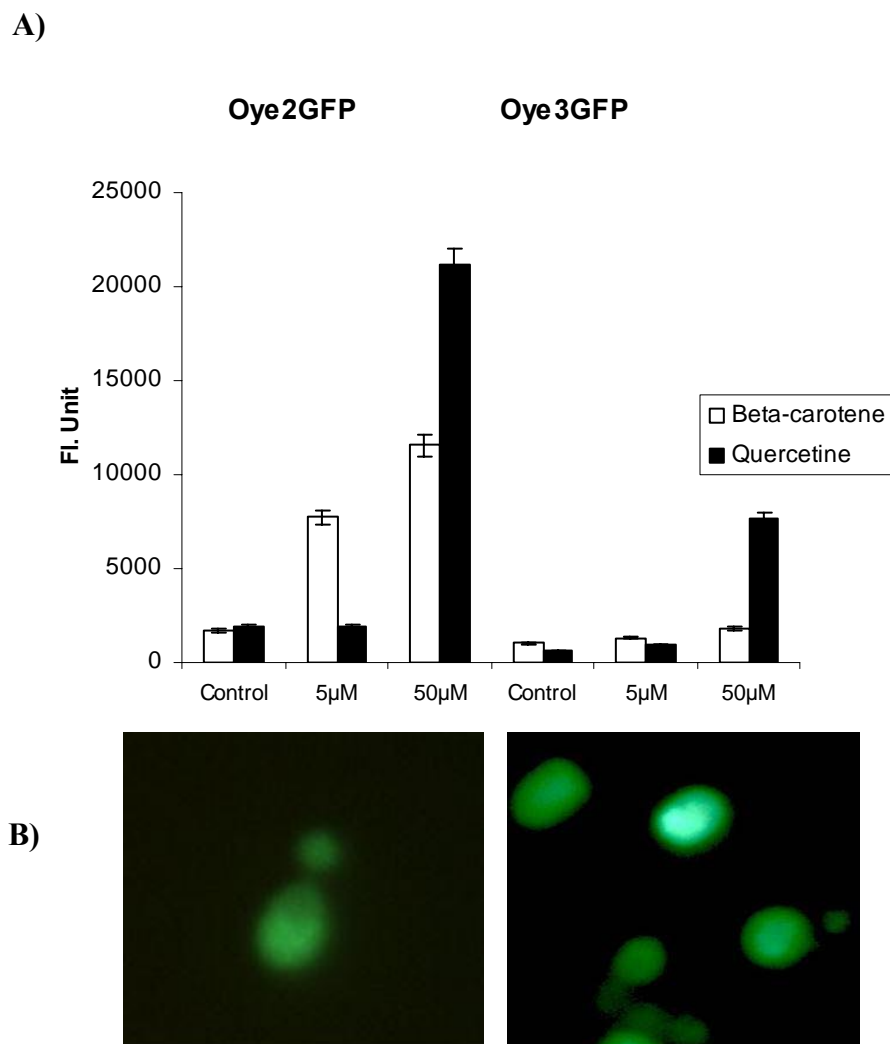
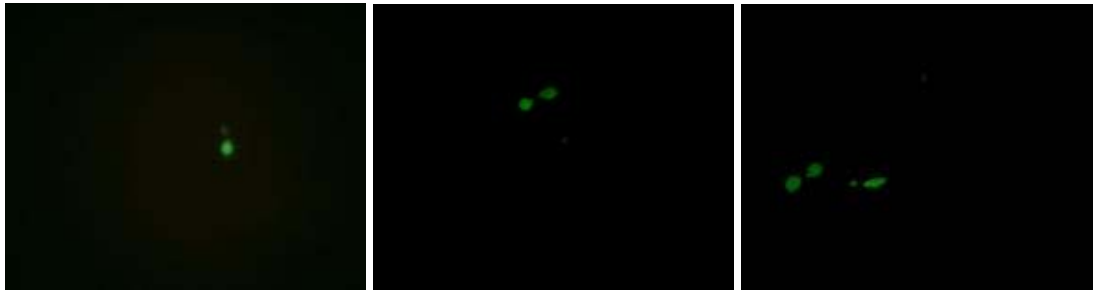


Figure 25. Response of the yeast antioxidant machinery in cells treated with antioxidant small molecules. **(A)** Yeast cells engineered to express a chromosomally encoded C-terminal fusion of GFP to the OYE2 or OYE3 gene from their native promoters, were supplemented with beta-carotene (white bars), or quercetin (black bars) at 5µM and 50µM concentration. GFP fluorescence revealing the extent of OYE Protein induction was monitored by fluorimetry. **(B)** OYE2-GFP cells treated with beta-carotene (left) and quercetin (right) were visualized for GFP localization microscopically.

A)



Control cells

5μM BC

5μM BC

B)



Control cells

5μM BC

50μM BC

Figure 26. FLUO MICROSCOPY (A) OYE3-GFP cells treated with beta-carotene, (B) OYE2-GFP cells treated with beta-carotene

4.Determination of the viability of cells treated with pro-oxidants

The strains were grown in 5ml Glu/CM overnight. The next day cells were diluted at $OD_{600}= 0.1$ in 40 ml Glu/CM in 200 ml flasks and the cultures were kept shaking at 30°C until they reached $OD_{600} 0.5-0.7$.

When the AA, CA, BC, and QC, cells where treated with 1 mM of H_2O_2 and 50 μM of one the antioxidants cited.100 μl of each culture was plated in YPD plates .

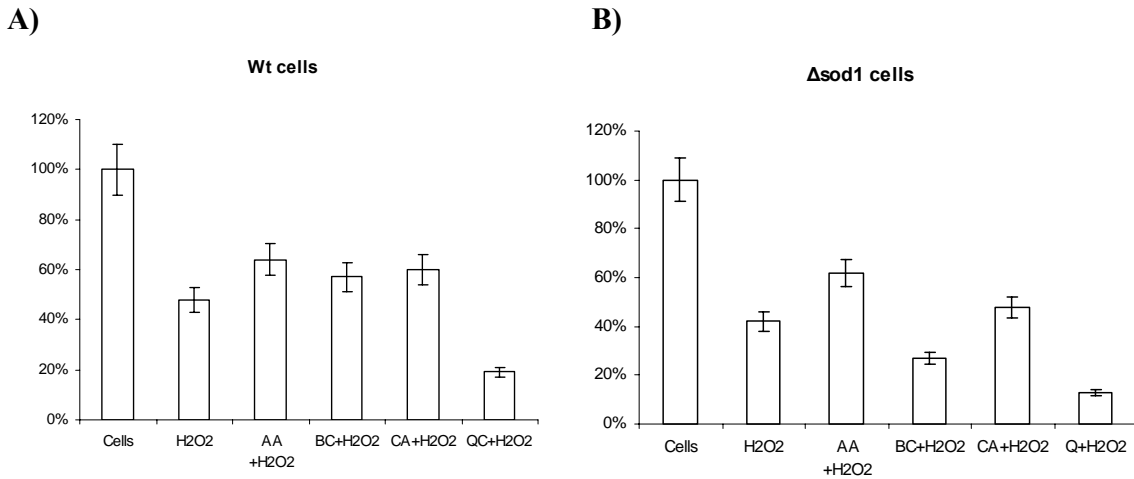


Figure 27. Viability test for 2 different strains A) wild type cells and B) Δ sod1 cells.

Comparing the growth of cells without any treatment and their growth with different treatment we can say that the AA has a big antioxidant effect whereas the quercetin showed a dramatic effect which improves our first results with growth assay.

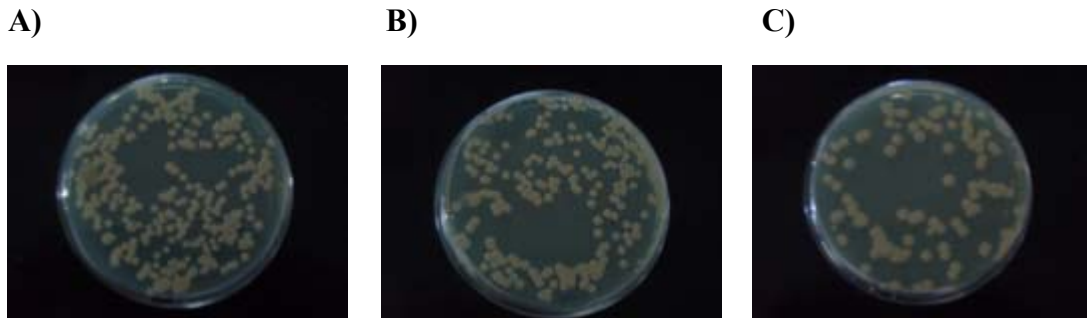


Figure 28: Viability test for Δ sod1 cells **A)** AA+H₂O₂, **B)** CA+ H₂O₂, **C)** Q+ H₂O₂.

Creating GFP-YAP1 fusion strain

PCR reaction (see material and methods) was run, and then the 5 μ l of each reaction were run on gel to quantify the PCR method

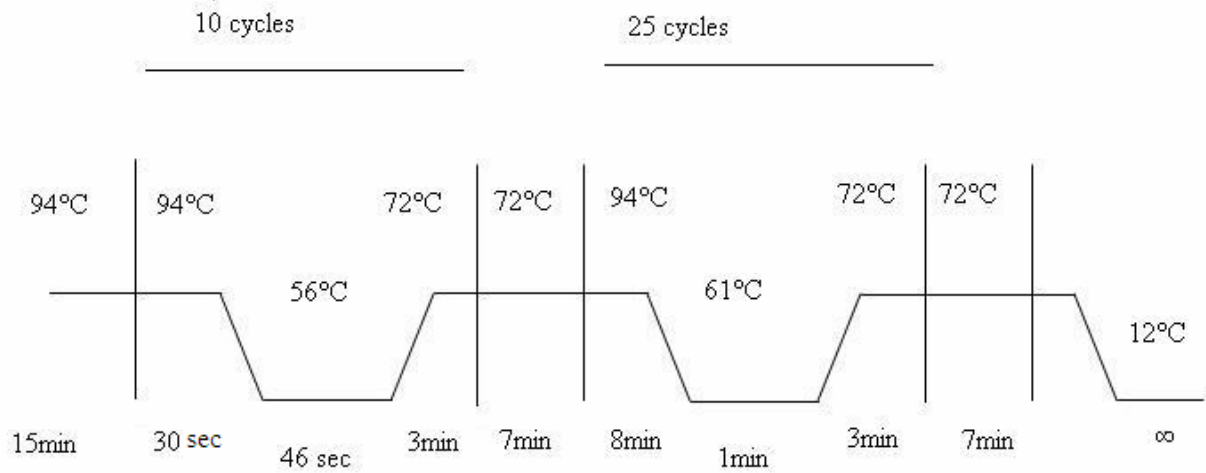


Figure 29: PCR program used for amplification of 5' yap1-pfa6a#30 cassette-yap1 3'

The PCR products were collected in the same ependorff and purified by phenol / chloroform protocol (see phenol/chloroform purification in material and methods), the concentrated linear DNA fragments were transformed directly to BY4742 cells, and then the transformants were selected on Glu/ his plates. 8 different colonies from the colonies that grow on the selective medium were randomly chosen

Genomic DNA was isolated and was used a template in a PCR reaction to confirm the insertion of the cassettes.

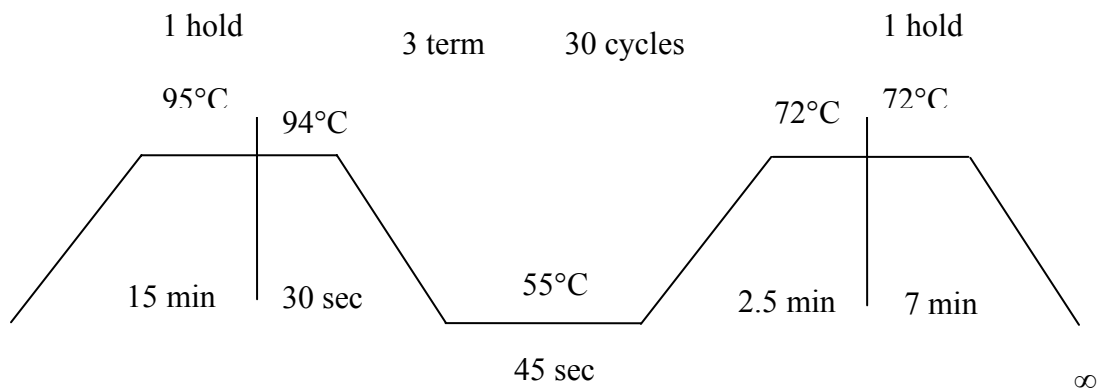


Figure 30: PCR confirmation program of GFP-yap1 fusion

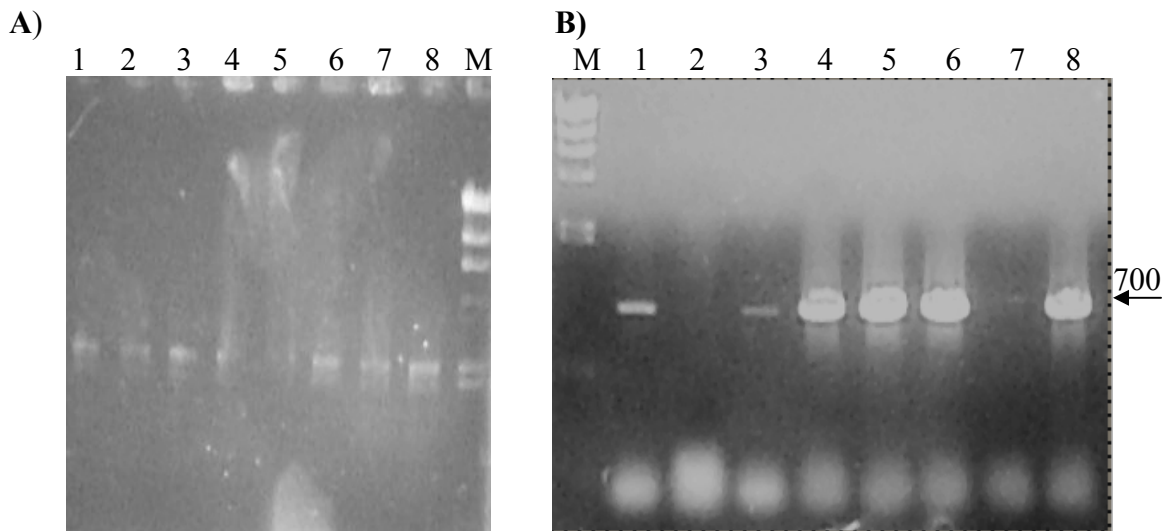


Figure 31. (1A) 1-8 PCR amplifications of *pfa6a#30* cassette flanked by 50b *yap1* on both sides were generated by preparing 8 parallel reactions. (1B) Verification of GFP-*yap1* fusion M: Gene ruler, λ DNA/*HindIII* marker, 1: PCR product using the primers *yap* confirmation and *pfa6a* (conf) the negative result is expected as BY4742 genomic DNA used as template for negative control. 2-5: PCR product using the primers *yap* confirmation and *pfa6a*(conf), clear band appeared ~ 700 bp which confirm the fusion of GFP to *yap*.

Cells BY4742 with *yap1-gfp* were grown for over night growth and exposed to different treatments in PBS for 2 hours:

- A) Control cells (only cells)
- B) Cells treated with H_2O_2
- C) Cells treated with 100 μ M of Quercetine

And were observed for induction of GFP fluorescence, under X1000 magnification in a fluorescent microscope.

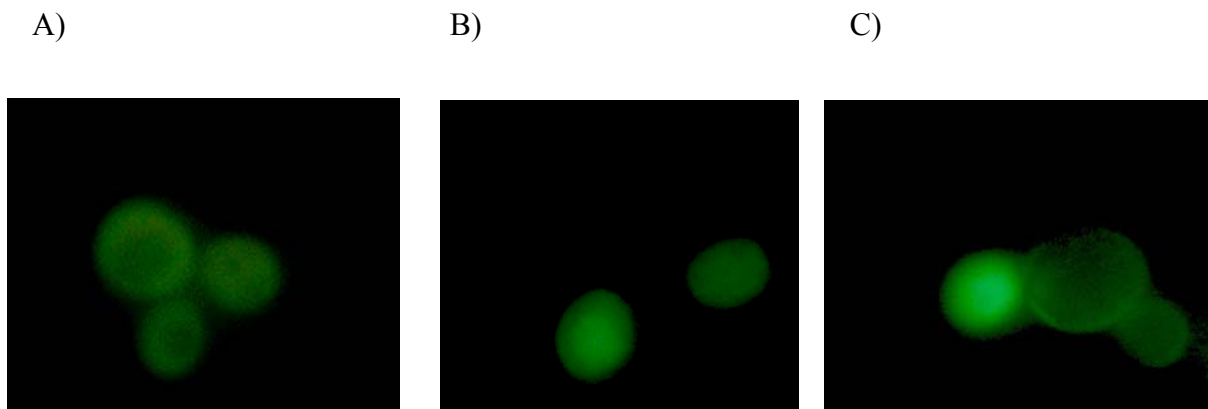


Figure 32: Response of the BY4741 with YAP1-GFP fusion antioxidant machinery in cells treated with antioxidant small molecules A) control cells B) cells treated with 1 mM of H₂O₂ C) cells treated with 100 mM of Quer.

100 μ M of Quercetine shows an over expression of YAP1-GFP gene which explain the pro-oxidant role of quartertone in high concentration comparing to H₂O₂ alone.

General discussion

We have chosen in this work 3 different strains with different mutations in their oxidative stress machinery which protects cells in oxidized environment represented in this work by the addition of H₂O₂ hydrogen peroxide and CHP which is an organic pro-oxidant (Cumene hydroperoxide) used to mimic oxidative stress induced by lipid hydroperoxide, which is mediated by the formation of hydroxyl radicals (OH[•]) (Rietjens. *et al.*, 2006).

The first strain Δ sod1 has a deletion in sod gene, gene responsible for the synthesis of super oxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (Young *et al.*, 2001), the second was the double knockout strain Δ oye2glr1 with double deletion in Old yellow enzyme and glutathione reductase genes that remove hydrogen peroxide (Young *et al.*, 2001), the third strain used was Δ gsh1 which can not synthesize the glutathione and it can not grow in medium that do not contain exogenous GSH.

As control we used the wild type BY4741.

Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or glutathione (GSH) peroxidase. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. Additionally, oxidative stress and ROS have been implicated in disease states, such as Alzheimer's disease, Parkinson's disease, cancer, and aging (Fiers *et al.*, 1999).

Among the 7 antioxidants molecules studied in this work, AA showed the best antioxidant activity not only in high concentration but also in low concentration, and the only compound that improved the growth of Δ gsh1 with a minimal addition of exogenous glutathione.

Ascorbic acid is a small, water-soluble anti-oxidant molecule which acts as a primary substrate in the cyclic pathway for enzymatic detoxification of hydrogen peroxide. In addition, it acts directly to neutralize superoxide radicals, single oxygen or superoxide

and as a secondary anti-oxidant during reductive recycling of the oxidized form of α -tocopherol, another lipophilic anti-oxidant molecule (Abed Shalata., 2001).

Caffeic showed an antioxidant activity not so important as the case of ascorbic acid but it was clearly observed.

Caffeic acid is usually related with its antioxidant and anti cancer activity, it has been widely reported as antioxidants in food, cosmetic and pharmaceutical formulations (Pridya *et al.*, 2003). Caffeic acid is among the major hydroxycinnamic acids present in wine - scavenging superoxide anion radical, total reducing power and metal chelating on ferrous ions activities (Gülçin *et al.*,2005).

Against that two compounds showed a pro-oxidant activity which cause a dramatic effect on yeast growth.

β -carotene acts as an antioxidant, as resonant stable carbon-centered, carotenoid radical is formed. However, in the presence of high oxygen tension, oxygenation of this carotenoid radical is possible, which would result in pro-oxidant activity (which explain its pro-oxidant behavior when used in high concentration of 50 μ M). In this case, the carotenoid radical undergoes autooxidation to form eccentrically cleaved β -carotene, which could be indirectly responsible for the higher incidence of lung cancer (Russell *et al.*).

The most dramatic effect was seen with quercetine and especially in its high concentration. Quercetine can be considered as an antioxidant and pro-oxidant in the same time. The hydroxyl group in position 3 should be blocked to prevent its auto-oxidation (Kessler *et al.*, 2003) which led to its high pro-oxidant activity shown in our study.

We can say that Quercetine acts as xenobiotics and stimulates the stress responsive machinery which explains the high concentration of intracellular ROS and give no equilibrium in GSH synthesis and increasing of GSSG level.

We created a new strain BY4742 with a fusion of GFP-YAP1 (Green fluorescence protein fused with YAP1) and we treated this strain with different concentrations of Quercetin.

One of the mediators in the transcriptional activation of yeast SOD1 is the YAP-1, which acts as a sensor of the redox state of the cell, under oxidative stress it moves from the cytoplasm to the nucleus and induces the expression of a number of target genes encoding protective enzymes including SOD1, which explain the high GFP induction seen by microscopy when we treated cells with a high concentration of Quercetin.

The combination of the results of the recovery assays which have been confirmed by colony assays, and the results of measuring GSH levels, can explain easily the high antioxidant activity of ascorbic acid and the pro-oxidant activity of quercetin which gave a dramatic effect on strains.

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