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Article

Effect of dietary antioxidants on the cytostatic effect of acrylamide during copper-deficiency *in Saccharomyces cerevisiae*

Upendra Nadh Kommuguri[§], Poorna Venkata Satyaprasad Pallem[§], Sreedhar Bodiga[#], and Vijaya Lakshmi Bodiga[§*]

[§]Department of Biotechnology, Krishna University, Machilipatnam, Andhra Pradesh, India.
[#]Department of Biochemistry, Kakatiya University, Warangal, Andhra Pradesh, India.

***Address for correspondence**:
Dr. Vijaya Lakshmi Bodiga
Assistant Professor,
Department of Biotechnology
Krishna University
Machilipatnam
Krishna Dist.
Andhra Pradesh
India. 521001
Fax: 08672 – 225962
Email: bodigavijayasri@gmail.com

Running head: Acrylamide and antioxidants

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Abstract

Acrylamide exposure increases oxidative stress and causes cytotoxicity. In order to understand the role of oxidative stress in acrylamide toxicity, we utilized *Saccharomyces cerevisiae* as a model organism grown in Yeast Peptone Dextrose (YPD) or Copper-Deficient Medium (CDM). Although the growth curves of yeast were comparable in these media, acrylamide treatment resulted in significant growth inhibition and colony formation only in CDM. Copper-deficiency induced decrease in intracellular metallothionein levels, along with reduced Cu, Zn-SOD activity appeared to increase the sensitivity of yeast to cytostatic effect of acrylamide. Increased DCF fluorescence, enhanced formation of PBN-hydroxyethyl adducts and lowered GSH content was observed under copper-deficient conditions, when challenged with acrylamide. The cytostatic effects and intracellular redox changes in response to acrylamide were ameliorated by antioxidant molecules viz. a viz. curcumin, β-carotene, vanillin and caffeic acid, which effectively decreased the oxidative stress and improved the growth recovery.

Keywords: Acrylamide; Antioxidants; Copper-deficient medium; Cytotoxicity; Electron Spin Resonance; Oxidative stress; Yeast

Abbreviations: YPD-Yeast extract peptone dextrose; BCS-Bathocuproiene disulfonate; CDM-Copper deficient medium; CUP1-metallothinein; ACT1-actin; SOD-superoxide dismutase activity; DCF-Dichlorofluorescein; TBARS-thiobarbituric acid reactive substances; GSH-glutathione (reduced); GSSG- glutathione (oxidized); PBN- para phenyl tertiary butyl nitrone; ESR- electron spin resonance

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1. Introduction

Acrylamide is a synthetic, water-soluble monomeric compound widely used in textile, paper industry, soil coagulation, but is also found in heat processed carbohydrate-rich foods[1]. It is thought to be formed by the Maillard reaction between asparagine residues and reducing sugars present within the foodstuffs at high temperatures[2, 3]. Diet, therefore appears to be one of the main sources of environmental acrylamide exposure in humans[4-6]. Current average exposure to acrylamide for the general population is estimated at 0.85 µg/kg body weight/day[7, 8]. Numerous studies, both *in vitro* and *in vivo*, conducted to evaluate the potential absorption of acrylamide indicate that it is readily absorbed[9, 10] and appears to be rapidly distributed in muscle, liver and gastrointestinal tissues [9, 11]. Once absorbed, acrylamide is metabolized *via* the cytochrome P450 enzyme system or conjugated by glutathione-S-transferase (GST) to glycidamide or N-acetyl-S-(3-amino-3-oxopropyl) cysteine[9, 12]. Acrylamide contains an α,β-unsaturated amide bond that can react with nucleophilic sulphydryl groups on various proteins *via* a Michael addition[13-16]. In addition to the toxicokinetic studies in various animal models, a recent study in Caco-2 cell line demonstrated that acrylamide transport in the intestine is primarily a passive process, combined with a modest energy- and pH-dependent active secretory component[17]. Intracellularly, acrylamide has been shown to increase oxidative stress due to depletion of GSH and also result in DNA damage[18-20]. With increasing attention towards understanding the mechanisms of acrylamide toxicity and to identify potential dietary/therapeutic compounds that can prevent or inhibit acrylamide toxicity, there has been an urgent need for a simple eukaryotic cellular model system for studying acrylamide toxicity. Very recently, yeast deficient in Cu, Zn-superoxide dismutase

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(Sod1) was shown to be hypersensitive to growth inhibitory effect of acrylamide and this effect was attributed to the generation of reactive oxygen species (ROS) and rescued by antioxidants like N-acetylcysteine, glutathione and dithiothreitol[21]. Although the role of ROS in the mechanism of cytostatic action when treated with acrylamide is yet to be clearly established, the accumulation of ROS can trigger a protective response such as metallothionein synthesis which can efficiently scavenge ROS [22-24] and thereby prevent the cytostatic effects. In the present study, we have used yeast grown on a copper deficient medium to study acrylamide toxicity, obviating the need for genetic manipulation and accompanying compensatory effects. Yeast grown on low copper medium is known to exhibit comparable growth as wild type, but decreased Cu, Zn-SOD activity and a small, but appreciable compensated increase in Mn-SOD activity[25, 26]. Further, copper deficiency also decreases CUP1p (metallothionein) expression, a polypeptide with copper, zinc buffering capacity and a potential scavenger of free radicals due to high content of sulfhydryls[26-29]. Thus, we hypothesized that copper deficient yeast model system would be hypersensitive to acrylamide toxicity, due to decreased endogenous antioxidant defenses and hence more helpful in evaluating potential exogenous antioxidants that can prevent cytostatic and cytotoxic effects of acrylamide.

2. Materials and Methods

2.1. Materials

Yeast extract, peptone, glucose were obtained from HiMedia (Mumbai, India), unless otherwise mentioned. Bacto agar was obtained from Difco, BD Biosciences, NJ, USA. Bathocuproiene disulfonate (BCS), Fluorescent probe 2',7'-dichlorofluorescein diacetate

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(DCF-DA), acrylamide, curcumin, β-carotene, vanillin, caffeic acid, para-phenyl tertiary butyl nitrone (PBN) were procured from Sigma, USA. All other chemicals were of analytical grade and obtained from local sources.

2.2. Growth conditions of BY4741

The *Saccharomyces cerevisiae* BY4741 (*MAT*α *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) strain used in this study was a generous gift from Dr. Anand Bachchawat, IMTECH, Chandigarh, India. Synthetic dextrose medium without copper (2% glucose, 0.17% yeast nitrogen base without Cu and Fe, 0.5% ammonium sulfate, amino acid, 1 µM $FeCl_3$), but containing 100 µM bathocuproine disulfonic acid disodium salt (BCS) was used as copper deficient medium (CDM). Yeast cultures were inoculated in standard yeast extract/peptone/dextrose (YPD) (1% yeast extract, 2% peptone and 2% glucose) or CDM overnight at 30°C and were seeded from single colonies grown on respective solid media. Growth of experimental cultures was initiated at $A_{600\ nm} = 0.05$; the cultures were allowed to grow to $A_{600\ nm} = 1.0$ (log phase, 1.5×10^{7} cells/mL) before use.

2.3. Effect of acrylamide on cell growth

Yeast cells (5×10^{4} cells/mL) were cultured in YPD or CDM containing various concentrations of acrylamide (0.1-10.0 mg/mL) for 24 h. The preliminary dosing study used these concentrations based on the estimated daily intake of acrylamide to be 0.85 µg/kg body weight, in the general population[7, 8]. A previous study in yeast lacking Cu, Zn- SOD has used acrylamide concentrations of 10 mM (0.7 mg/mL); 20 mM (1.4 mg/mL) and 40 mM (2.8 mg/mL) for cytotoxicity studies [21](TIV 2011). Further, a chronic exposure study in mice to study DNA damage in male germ cells reported 0.001 – 10 µg/mL for up to 1 year, which is equivalent to 0.0001-2 mg/kg body weight/day[30].

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Growth inhibition was assessed by measuring the time-dependent changes in the absorbance of the culture at 600 nm. The effective concentration of acrylamide at which the cell growth (OD 600) was reduced by 50% of the control was found to be 0.5 mg/mL.

Sensitivity to acrylamide toxicity was assessed using a colony formation assay. Cultures (1 ml) containing a total of 6×10^6 cells were exposed for 4 h to desired concentration of acrylamide (0.5-3.0 mg/mL) with or without antioxidants (curcumin, β-carotene, vanillin, caffeic acid), washed once in PBS, resuspended in YPD or CDM, diluted 1:5000, and plated onto 100-mm agar plates. After 2 days of growth at 30°C, the number of colonies was counted manually. Stock solutions of acrylamide and antioxidants were prepared fresh each day and maintained in the dark to eliminate photochemical ROS generation and degradation of phytochemicals.

2.4. Northern blot analysis of CUP1 mRNA levels

Total RNA was extracted from mid-exponential phase cultures of *S. cerevisiae* strain (1×10^7 cells/mL) by the SDS-hot phenol method as detailed elsewhere[26]. For Northern blot hybridization studies, 10 µg of *S. cerevisiae* total RNA were fractionated by electrophoresis in a formaldehyde-agarose gel (1.2% w/v) and blotted onto Hybond-membranes. Blots were prehybridized at 42°C for 4 h in a high stringency solution containing 50% formamide, 1% sodium dodecyl sulfate (SDS), 5× SSPE (Ambion Inc., Austin, TX), 5× Denhardt's solution and 100 µg/mL denaturated sheared non-homologous DNA. Northern hybridization was carried out at 42°C for 12–14 h in the same solution containing 1×10^7 cpm/mL of [α-^{32}P] dCTP-labelled cDNA probe prepared with the direct 5'-TCAATCATCACATAAAATGTTC-3' and reverse 5'-GTTTCATTTCCCAGAGCAG-3' oligonucleotides for *CUP 1* and direct 5'-

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ACACGGTATTGTCACCAACTGGG-3’ and reverse 5’-AGGACAAAACGGCTTGGATGG-3’ for *ACT1*. After hybridization, blots were washed in a 2× SSPE 1% SDS solution for 20 min at 42 ◦C and then washed again in a 0.1× SSPE 1% SDS solution for 10 min at 42°C. Blots were exposed on scientific autoradiographic imaging film (Kodak) at −80°C for 24 h.

2.5. GSH and GSSG levels

Samples were suspended in the 10% TCA and vortexed for 10 min to extract glutathione. After centrifugation at 10,000*g* for 30 min, reduced glutathione (GSH) and total glutathione was estimated in supernatant, exactly as mentioned before[26]. Levels of oxidized glutathione (GSSG) were calculated from the difference between the values of total glutathione and reduced glutathione (GSH) and the results were expressed as µg/mg protein

2.6. Evaluation of lipid peroxidation

Lipid peroxidation was quantified as thiobarbituric acid (TBA)-reactive substances (TBARS), according to the method described by us before[26]. The concentrations of TBARS in samples were calculated by using molar extinction coefficient of MDA–thiobarbituric chromophore 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol of TBARS/mg protein.

2.7. Quantification of protein bound carbonyls

For assessment of carbonyls, the reaction with dinitrophenylhydrazine was employed. Carbonyl content was calculated using the molar absorption coefficient of aliphatic hydrazones of 22,000 M^{-1} cm^{-1} and expressed as nmol carbonyl/mg of protein[26].

2.8. Measurement of intracellular oxidation level

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Intracellular ROS was detected by the oxidant-sensitive probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma). According to this procedure 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was added from a fresh 5 mM stock (prepared in ethanol) to a final concentration of 10 mM in 1 ml of yeast cell culture (10^7 cells) then incubated at 28 °C for 20 min. Finally, cells were cooled on ice, harvested by centrifugation and washed twice with distilled water. The pellet was resuspended in 500 µL of water and 1.5 g of glass beads was added. Cells were lysed by three cycles of 1 min agitation on a vortex mixer followed by 1 min on ice. The supernatant was obtained after centrifugation at 25,000*g* for 5 min. and after appropriate dilution with water the fluorescence was measured using a Shimadzu fluorimeter (RF-5301PC) with excitation at 502 nm and emission at 521 nm.

2.9. Cu, Zn-SOD and Mn-SOD activity

The SOD activity gel assay was conducted as previously described[26]. Briefly, cells were homogenized by glass bead agitation. Protein extracts (50 µg) were subjected to non-denaturing PAGE on 12% gels, and subsequently stained for SOD activity using Nitro Blue Tetrazolium (Sigma).

2.10. ESR spin trapping studies

200 µL of the overnight yeast cell culture was mixed with stock solutions of PBN (α-(pyridyl-4-N-oxide)-N-*tert*-butylnitrone) and buffer to give 250µL of a suspension that was 92.3 mM in PBN and 23.5 mM $NaHCO_3$, pH 7.4. It was incubated further at 30°C, with shaking at 220 rpm for 2 h, prior to ESR measurements. When the experiments required addition of acrylamide and antioxidant, these were added at the beginning of the 2 h incubation period. ESR spectra were measured using Joel JES-FE2A with JM-FE-2A

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electromagnet (Japan) at room temperature. The following settings were used: frequency 9.63 GHz; microwave power 20.7 mW, modulation frequency 100 kHz, modulation amplitude 1G, time constant 10 ms, receiver gain 2 x 10^6, sweep width 20 G with 1024 point resolution. Individual scans were averaged and integrated to obtain a single spectrum from 3 different experiments. These data were interpreted by computer simulation techniques using Winsim package according to Duling[23, 31].

2.11. Statistical analysis

Statistical analysis was performed using Sigma Stat 3.5 (Systat Software Inc., CA, USA). Significance of the results was tested with the unpaired t-test or one-way ANOVA with Tukey test. For all graphs, bars with different symbols are significantly different from each other at $p < 0.05$.

3.0. Results

3.1. Evaluation of low copper medium for induction of copper-insufficiency

The total average concentration of copper in standard YPD medium was 0.3 µM, while that in CDM was less than 0.02 µM. Exposure of cells to copper chelator, BCS in synthetic dextrose medium without copper (CDM) did not alter growth curve (Fig. 1, inset), but, showed lower intracellular copper content (< 50 nM) compared to those grown in YPD (> 200 nM), confirming copper deprivation.

3.2. Effect of acrylamide on cell growth and toxicity

Two independent methods, namely, growth inhibition and colony formation assays, were used to find out whether copper depletion causes any change in acrylamide toxicity. The effect of varying concentrations of acrylamide (0 .1 – 10.0 mg/mL) on growth of BY4741 in YPD and CDM was examined over 24 h and is shown in Fig. 1. The dose-ranging

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inhibition curve confirmed that the cells growing in CDM were distinctly more susceptible to acrylamide. There was a dose-dependent suppressive effect of acrylamide on cell growth starting at 0.2 mg/mL and drastic inhibition of growth with > 1.5 mg/mL in yeast cells grown on CDM. Cell growth in YPD over 24 h did not show any significant difference in the presence of acrylamide up to 10 mg/mL.

Colony-formation data with different concentrations of acrylamide in Fig. 2 revealed that cells growing in CDM showed significantly less colony formation and therefore increased sensitivity to acrylamide compared to those growing under copper-sufficient conditions (YPD) which exhibited more colony formation. Using 1.0 mg/mL acrylamide, we performed growth kinetics of BY4741 in YPD and CDM, for 12 hours. The growth curves as shown in Fig. 3 were not affected by acrylamide in YPD, but showed drastic inhibition in the presence of CDM. Thus, the copper-deficient cells were more sensitive to acrylamide. These results confirm further that the copper-deprived cells are more sensitive to acrylamide toxicity than copper-sufficient cells.

3.3. Effect of acrylamide on *CUP1* mRNA levels and SOD activity

To validate the copper-dependent changes in the expression, we monitored the levels of metallothionein *CUP1* levels that are known to be directly influenced by intracellular copper status. As depicted in Fig. 4a, levels of *CUP1* mRNA did not differ significantly between CDM and YPD. In the presence of acrylamide, the levels of *CUP1* mRNA increased significantly in YPD, but not in CDM. Expression levels of the internal control gene, *ACT1*, which encodes actin, were equivalent across all groups. These results demonstrate that up-regulation of *CUP1* in YPD, but not in CDM in response to acrylamide may be responsible for the observed effects.

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To investigate the role of Cu, Zn-SOD (*SOD1*) in increased toxicity of acrylamide, we examined the enzymatic activity of the well characterized copper dependent enzyme, *SOD1*, which is lowered in copper deficiency. As shown in Fig. 4b, the activity of *SOD1* was significantly reduced in CDM compared with that in YPD. Cu, Zn-SOD activity was significantly elevated by acrylamide in YPD, but was significantly lowered in CDM. However, the analysis of Mn-SOD showed that its activity was similar under YPD and CDM, but reduced after acrylamide exposure in CDM alone. Thus, it seems likely that copper directly affects Cu, Zn-SOD activity and Mn-SOD activity is influenced only after substantial exposure to acrylamide.

3.4. Effect of acrylamide on GSH levels

As can be seen in Fig. 5, copper deficiency did not alter GSH and GSSG levels. Further, GSH levels did not change significantly with acrylamide treatment in YPD, but lowered by almost 2-fold in CDM. GSSG levels increased significantly with acrylamide treatment in CDM, suggesting the oxidation of GSH.

To understand if the altered copper status affected intracellular oxidation differentially in YPD and CDM, we assessed DCF oxidation as a measure of intracellular oxidation. Cells grown in YPD showed no differences in DCF oxidation compared to those grown in CDM. Interestingly, 1.0 mg/mL acrylamide challenge in CDM showed a dramatic increase in DCF oxidation compared to those grown in YPD, indicating copper deprivation does indeed promote the intracellular oxidation levels when challenged with acrylamide due to lack of antioxidant defense (Fig. 6).

3.5. Effect of acrylamide on lipid peroxidation and protein oxidation

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Increased intracellular oxidation is associated with higher levels of oxidative stress markers, such as protein carbonylation and lipid peroxidation. As lipid peroxidation is a major consequence and useful marker of ROS-mediated oxidative damage, we tested whether lipid peroxidation in acrylamide-treated S. cerevisiae was enhanced. There was negligible difference between the basal lipid-hydroperoxide content of the BY4741 in YPD and CDM, prior to acrylamide exposure as shown in Fig. 7a, implying that copper deficiency did not play a major role in lipid peroxidation under non-stressed conditions. A significant increase in lipid peroxide accumulation was observed with acrylamide treatment in CDM, but not in YPD. The results show that protein carbonylation (Fig. 7b) was no different between YPD and CDM. Further, acrylamide was unable to induce protein carbonylation in YPD, but increased protein carbonyls by at least 2-fold in CDM. Similarly, lipoperoxidation measured as TBARS did not show any increase with acrylamide in YPD, but showed 2- fold increase in CDM.

3.6. Effect of antioxidants on acrylamide-induced cytostatic effect

To evaluate the capacity of the four small dietary antioxidant molecules, β-carotene, curcumin, vanillin and caffeic acid, to protect yeast cells grown in copper deficient medium and stressed with acrylamide, we used growth recovery and cell survival assays, in addition to determining their effect on the relative levels of DCF fluorescence. Treatment of the yeast cells with acrylamide (1.0 mg/mL) exhibited a pro-oxidant insult, resulting in growth suppression. Thus, the recovery of the cells in copper deficient medium when supplemented with low (5 μM), medium (25 μM) or high (50 μM) concentrations of antioxidants was monitored for a period of 12 h. All growth recovery assays were performed independently in triplicate. Detectable statistically significant

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differences could be seen starting at 4 -6 h for the antioxidants tested in this study. Overall, these differences reflected at the end of the assay period. Yeast cells exposed to acrylamide and supplemented with curcumin exhibited growth recovery at 5 and 25 μM, whereas 50 μM curcumin was inhibitory to cell recovery (Fig. 8a). This was evident in colony formation assay as well (Fig. 8b). Supplementation of cells with low (5 μM) and intermediate (25 μM) concentrations of β-carotene showed increased growth rates, whereas 50 μM β-carotene showed no beneficial effect (Fig. 9). Vanillin showed beneficial growth promoting effects at all concentrations tested that tended to increase in a concentration-dependent manner. Similar observations were made with colony formation assays using increasing concentrations of vanillin (Fig. 10). Caffeic acid also showed promising beneficial effects in promoting growth of yeast in the presence of acrylamide as shown in Fig. 11.

3.7. Electron spin resonance (ESR) studies

The ESR signals observed for WT BY4741 strain in YPD and CDM had spectral parameters characteristic of PBN-radical adducts; each spectrum shows a triplet of doublets, with characteristic isotropic spectra parameters: g_{iso} = 2.007(0.001), aN=15.1(0.1) G, and aH=2.7(0.2)G. However, these parameters did not correspond to the PBN-hydroxyl radical adducts, indicating that hydroxyl radical is not formed. The spectra obtained were identical to hydroxyethyl adducts of PBN [32]. These hydroxyethyl adducts were found to be higher in CDM compared to those in YPD as shown in Fig. 12. The ability of curcumin to attenuate the formation of these adducts was evident.

4.0. Discussion

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Previous studies have documented that *Saccharomyces cerevisiae* adapts to a nonlethal dose of exogenous compounds that generate intracellular reactive oxygen species by undergoing a temporary cell cycle arrest[33, 34]. This is believed to be a physiological protective mechanism to protect itself from pro-oxidant insult, when cellular antioxidant defense is not adequate and compromised. Reactive oxygen species are generated during normal cellular respiratory metabolism, but their damaging effects are generally suppressed by endogenous antioxidant defenses, that include superoxide dismutases, catalase and glutathione peroxidase. *S. cerevisiae* mutants lacking the principal cellular SOD (Cu, Zn SOD; encoded by the *SOD1*) display certain aerobic growth defects, e.g., reduced growth rate, and methionine and lysine auxotrophies [35]. The balance between the antioxidant defenses and the rate of production of reactive oxygen species (ROS) is believed to be a critical factor that determines the extent of injury in various experimental model systems. The antioxidant defenses, which consist of a network of enzymes, proteins and low molecular weight scavengers, function to protect cellular components against the damaging effects of ROS. Moreover, sod1Δ mutants are hypersensitive to several types of stress, including oxidative stress[36], metal toxicity [37], prolonged stationary incubation [38], and freeze-thaw stress [39]. Such evidence has underscored the central role of ROS in mediating various stresses. However, there is presently no evidence to suggest that antioxidant defenses play a role in the insensitivity of eukaryotes, such as *S. cerevisiae*, to the action of acrylamide. This work focused on the functional role of the copper deficiency-induced down regulation of two components, CUP1 and Cu, Zn-SOD activity in sensitizing acrylamide toxicity in yeast model. Further, we proposed that

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exposure to various antioxidants would markedly improve cell survival in response to acrylamide.

The aim of the present study was to determine the effect of Cu deficiency on acrylamide sensitivity and cytotoxicity in yeast cells. The data show that a significant decrease in intracellular Cu levels can be observed by employing low copper medium, along with BCS. We have earlier shown that the decrease in intracellular copper is reflected in a decrement of copper-dependent metallothionein expression (*CUP1*) and Cu, Zn-SOD activity[26]. The severity of copper deprivation had no effect on growth curve and cell density, without acrylamide challenge.

Compensatory to a drop in Cu, Zn-SOD activity, there was an increase in Mn-SOD activity. Cytochrome c oxidase (CCO) is the Cu-dependent, terminal respiratory complex of the electron transport chain. Inhibition of CCO due to copper deficiency can promote oxidative stress by increasing mitochondrial production of reactive oxygen species. Compensatory increase of Mn-SOD activity attempts to level off the ROS in the mitochondrial fraction in copper-insufficient conditions. Thus, the copper-deficient yeast cells behave very similar to WT BY4741 cells grown in YPD, in terms of growth rate, basal levels of oxidative stress. This is also supported by DCF fluorescence, TBARS formation and protein carbonylation data in the present study, where none of these parameters showed any differences between YPD and CDM.

Initial growth curves clearly demonstrated that acrylamide caused growth arrest in the BY4741 yeast strain only when the copper deficient medium was used. This was also confirmed with the colony formation assay, where increasing concentrations of acrylamide exposure resulted in decreased ability to form colonies. The cause of the

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growth arrest with acrylamide can be attributed to higher levels of oxidative stress, seen as increase in DCF fluorescence, enhanced formation/accumulation of TBARS and protein carbonyls. It is known that wild-type BY4741 grown in YPD medium possesses robust antioxidant defense and hence resists cytostatic/cytotoxic actions[26]. Since the action of acrylamide appears to be cytostatic, we tested whether the inhibition of the BY4741 growth in CDM by acrylamide can be reversed by the addition of antioxidant molecules. We used four different small molecule antioxidants, each at three different concentrations including 5 (low), 25 (intermediate) and 50 (high) μM. Vanillin, curcumin, caffeic acid and β-carotene were chosen because of their dietary origin. In a previous study with yeast mutants lacking antioxidant machinery for testing the antioxidant potential of small molecules, β-carotene and caffeic acid were used at concentrations of 5 (low) and 50 μM (high) [40]. 5, 25 and 50 μM concentrations of β-carotene used in the present study correspond to 0.027, 0.134 and 0.268 g/10 mL of the media, respectively. Mean daily intake of β-carotene is equivalent to 2 mg/d which is equivalent to approximately 3300 IU of vitamin A [41]. Caffeic acid is a major representative of hydroxycinnamic acids, which occurs in foods mainly as an ester with quinic acid called chlorogenic acid. Daily intake of caffeic acid is 0.5-1.0 g in coffee drinkers [42]. The concentrations of caffeic acid (5, 25 and 50 μM) used in the present study would be equivalent to 0.009, 0.045 and 0.09 g/10 mL of the medium. Vanillin is found in asparagus, beer, maple syrup, popcorn, rum, tomatoes, and wine. Confectioneries and frostings contain vanilla at concentrations up to 768 ppm while milk products contain as high as 1400 ppm. Survey data compiled by the National Academy of Sciences indicates that the average daily intake of vanillin in foods may be as high as 38.0 mg per person.

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The estimated daily intake is 11 mg/capita and possible average daily intake of vanillin from foods is 38.9 mg/capita [43]. 5, 25 and 50 μM vanillin corresponds to 0.0076 g, 0.038 g, and 0.076 g/10 mL. 5, 25 and 50 μM curcumin concentration used in the present work is equivalent to 0.0184 g, 0.092g and 0.184 g/10 mL of the incubation medium. The maximum dietary consumption of curcumin @1.5 g per person per day is observed in certain South East Asian communities, whereas smaller quantities of turmeric (comprising 2-8% of curcumin) tend to be used for medicinal purposes [44, 45]. Pretreatment of the cells with these antioxidants resulted in a partial or total reversal of cellular growth inhibition, in response to acrylamide exposure. Upon treatment with a compound, an increase in yeast growth above the acrylamide-induced arrest, which serves as a baseline, indicates positive antioxidant activity in this model system.

It therefore appears that these small antioxidant molecules of dietary origin are capable of promoting growth in the presence of acrylamide, but higher concentrations of these antioxidants inhibited growth rate, increased lag time. These results indicate that concentrations of these antioxidants can have a significant effect in inhibition of acrylamide-induced growth arrest.

Depletion of copper in our cell model did not alter growth curve, intracellular DCF oxidation, protein carbonylation and lipid peroxidation, unless challenged with acrylamide. However, it did affect the *CUP1* mRNA levels and Cu, Zn-SOD activity significantly. A significant decrease in both these antioxidant factors can be attributed to enhanced cytotoxicity gain for acrylamide in copper depleted cells, as observed from the reduced cytotoxicity in copper-sufficient cells with higher metallothionein levels and Cu, Zn- SOD activity. Yeast cells lacking SOD1 are highly sensitive to oxygen and to agents

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that lead to oxidative stress, such as paraquat and menadione [46]. Our observation that lowered SOD1 activity resulted in higher sensitivity to acrylamide suggests and confirms that acrylamide leads to oxidative stress and that copper-sufficient cells are relatively protected against this stress.

The progressive accumulation of oxidatively damaged molecules may result from decline in the levels of antioxidant defenses. The results clearly suggest that copper deficiency exacerbated the intracellular oxidation upon acrylamide challenge. There is now abundant evidence for a link between copper status and metallothionein levels. Metallothioneins are a group of cysteine-rich proteins with antioxidant properties and have the capacity to buffer intracellular Cu. Yeast metallothioneins are encoded by *CUP1* and *CRS5* genes and have been shown to play a role in protecting yeast cells against oxidants, as the oxidant-sensitive phenotype of strains lacking Cu, Zn-SOD can be complemented by the over-expression of metallothionein[46]. These data were consistent with unaltered levels of GSH and GSSG in YPD and CDM, but a moderate to dramatic decrease in GSH levels with acrylamide in YPD and CDM, respectively. These data point out that a mere decrease in copper pool inside the cells is not sufficient to pose cytotoxicity, but can predispose cells to be more susceptible to acrylamide due to impaired antioxidant defense. While a few studies have shown that acrylamide can lead to the production of free radicals, it is still not clear as to the precise mode of generation of these ROS. The increase in GSSG and decrease in GSH levels induced by acrylamide suggests that oxidative stress is involved in cytotoxicity. This is corroborated by the PBN-adducts formed during acrylamide exposure in CDM. It is understandable that the acrylamide challenge in copper-deprived cells causes an increase in steady state levels of

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lipoperoxides and carbonyls that exceeds the metabolic capacity of endogenous antioxidant systems, as they are compromised by copper-deficiency and lack of metallothionein and SOD activity.

The overall results suggest that copper-deficient cells exhibit a lower antioxidant defense and increased susceptibility to acrylamide, resulting in accumulation of oxidized proteins and lipids, exceeding the levels associated with YPD, because of a higher intracellular oxidation.

The growth recovery assay upon oxidative insult with acrylamide is a comparable assay to colony viability measurements. It was chosen as the assay of choice as populations of cells maintained in chemically defined copper deficient medium are supplemented with the small antioxidant molecules for the whole assay period, which resembles more closely a physiological setting. This enables testing concentrations of antioxidants at physiologically relevant concentrations.

Curcumin is the main phenolic pigment in turmeric and is a commonly used spice, food preservative, and flavoring and coloring agent. Extensive research over the last few decades strongly suggests that curcumin possesses potent antioxidant[47, 48], anti-inflammatory[49], anti-tumor[50], antimicrobial properties[51]. It also inhibits lipid peroxidation and scavenges superoxide anion, singlet oxygen, nitric oxide and hydroxyl radicals [52]. In the current study, curcumin showed excellent antioxidant capacity against acrylamide-induced oxidative stress and improved the growth recovery. These data reconfirm the ability of curcumin to scavenge acrylamide-induced ROS and attenuate cytotoxicity in HepG2 cells[53]. On the contrary, curcumin also appears to show pro-oxidant effect at higher concentrations (50 μM) during acrylamide challenge. Such a pro-oxidant activity

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was observed to be due to the generation of phenoxyl radical of curcumin by peroxidase-H_2O_2 system, which co-oxidizes cellular GSH or NADH, accompanied by oxygen uptake to form ROS [54]. Our results are in agreement with previous studies of dose-dependent inhibition of hemolysis in RBCs and cytotoxicity of macrophages at low concentrations of curcumin, but enhanced cytotoxicity at high concentrations [55, 56].

Vanillin (4-hydroxy-3-methoxbenzaldehyde), a major constituent of vanilla pods, has generally regarded as safe (GRAS) status and is one of the world's principal flavouring compounds. Currently it is added to foods such as ice cream, liquors, soft drinks, and confectionary in concentrations ranging from 1 to 26 mM depending on the nature of the product[57]. Vanillin has been shown to exhibit antioxidant properties[58], antimutagenic properties[59]. Vanillin exhibited dose-dependent protective effect against iron-dependent lipid peroxidation in brain tissue [60].

β-carotene is the most common carotenoid in fruits and vegetables. Beta-carotene along with other carotenoids has been studied for their antioxidant properties and their ability to prevent chronic diseases. The antioxidant actions of carotenoids are based on their singlet oxygen quenching properties and their ability to trap peroxyl radicals[61]. 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, thus regenerating the original unexcited carotenoid, which can be reused for further cycles of singlet oxygen quenching. The quenching activity of a carotenoid mainly depends on the number of conjugated double bonds of the molecule and is influenced to a lesser extent by carotenoid end groups (cyclic or acyclic) or the nature of

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substituents in carotenoids containing cyclic end groups. Lycopene (eleven conjugated and two non-conjugated double bonds) is among the most efficient singlet oxygen quenchers of the natural carotenoids[62]. The prevention of lipid peroxidation by carotenoids has been suggested to be mainly via singlet oxygen quenching[61]. β-carotene has been best characterized with regard to its singlet oxidant quenching and antioxidant capabilities. This ROS scavenging property of β-carotene appears to protect against the acrylamide-induced growth arrest of yeast cells at 5 and 25 μM, but not at 50 μM. β-carotene and others carotenoids have antioxidant properties, but the antioxidant capability is variable depending on the *in vitro* system used. The antioxidant activity of these compounds can shift into a pro-oxidant effect, depending on such factors as oxygen tension or carotenoid concentration. At 50 μM concentration, β-carotene showed no beneficial effect compared to acrylamide treatment. In a study using Δsod1 mutants, β-carotene was found to improve the growth recovery by 30% at low concentrations, but reduce the recovery by 18% at high concentrations of 50 μM[40]. Thus, carotenoids and β-carotene seem to be health promoting when taken at physiologic levels, but may take on circumstantially adverse properties when given in high dose and in the presence of highly oxidative conditions. There remains a great interest in the possible health promoting effects of carotenoids, but the mechanism by which these compounds are acting (antioxidant or other properties) is in need of further study.

Caffeic acid-(3,4-dihydroxycinnamic acid) is among the major hydroxycinnamic acids present in wine; According to recent research, caffeic acid was a superior antioxidant compared with *p*-coumaric and ferulic acids, in inhibiting LDL oxidation[63], but also quenching radicals[64] and singlet oxygen[65]. It is interesting to note that caffeic

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acid behaved as a potent antioxidant at all concentrations tested. However, 50 μM caffeic acid showed less antioxidant efficacy compared to 25 μM.

We conclude that excessive production of ROS after acrylamide exposure causes cytotoxicity and supplementation of antioxidants such as curcumin, β-carotene, vanillin and caffeic acid may reduce acrylamide mediate cytotoxicity due to their direct effects on ROS scavenging activity. Based on the growth recovery data and colony formation assays, the order of antioxidant efficacy and functional recovery with the four antioxidants was vanillin > caffeic acid > curcumin > β-carotene. These antioxidant molecules could exert their beneficial effects by abstracting reactive free electrons from free radical intermediates postulated to be formed in the Maillard reaction[66].

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Figure legends:

Fig. 1 Effect of acrylamide on *BY4741* cell growth. Determination of dose-ranging inhibition by acrylamide at concentrations varying from 0.1 to 10.0 mg/mL in YPD or CDM. Growth of cells was evaluated by reading the OD at 600 nm. The inset shows the growth curve of *BY4741* cells as a linear plot of OD 600 as a function of time (in hours) in YPD or CDM.

Fig. 2 Effect of acrylamide on BY4741 survival. Survival of *BY4741* cells in YPD and CDM as a function of acrylamide concentration as determined using colony-formation assay. Logarithmic-phase cells were exposed for 4 h to different concentrations of acrylamide (0.5 – 3.0 mg/mL) and incubated on YPD or CDM plates at 30°C for 2 days to allow formation of colonies. Colony formation data shown here is a representative image from three independent experiments, each performed with duplicate cultures for each concentration.

Fig. 3 Copper deprivation results in increased sensitivity of *BY4741* cells towards acrylamide. Growth of cells was evaluated by reading the *A*600 as a function of time in YPD or CDM with and without 1.0 mg/mL acrylamide concentration. Each data point represents the mean of three experiments, each performed with duplicate cultures.

Fig. 4a Effect of acrylamide on *CUP1* mRNA accumulation. A, BY4741 cultures were grown in YPD or CDM for 12 h. Aliquots were subsequently incubated in the presence of acrylamide (1.0 mg/mL) at 30°C for 12 h. Fifteen µg of total RNA were prepared and analyzed by Northern hybridization using the radiolabeled *CUP1* and *ACT1* oligonucleotide probes.

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Fig. 4b Effect of acrylamide on SOD activity. Cells grown in YPD or CDM were treated with 1.0 mg/mL acrylamide for 2 h and total soluble extracts were electrophoresed for SOD gel activity. 50 μg of crude extract from each treatment were analyzed on a 10% native acrylamide gel. The location of Mn SOD and Cu, Zn-SOD are indicated.

Fig. 5 Levels of glutathione. GSH (reduced form), GSSG (oxidized form) of cells grown in YPD and CDM, in the presence or absence of 1.0 mg/mL acrylamide. The data represent the mean ± standard deviation of at least three independent experiments. * $p < 0.05$ vs. YPD or CDM.

Fig. 6 Intracellular peroxide levels produced under acrylamide treatment. YPD or CDM grown cells loaded with 10 μM H_2DCFDA were treated with 1.0 mg/mL acrylamide for 30 min, and intracellular peroxides were measured as induction of fluorescent intensity with respect to control values in YPD. Results given are mean of four independent experiments. * indicates $p < 0.05$ vs. YPD.

Fig. 7 Lipid and protein oxidation. (a) TBARS values are given in nmols/mg protein was determined after 4 h of treatment with 1.0 mg/mL acrylamide. The values summarized here are mean values for three independent experiments. (b) Protein carbonyl content, given in nmol of carbonyl/mg of protein, was determined after 4 h of treatment with 1.0 mg/mL acrylamide. The values summarized here are mean values for three independent experiments.

Fig. 8 Curcumin improves the growth recovery of acrylamide-treated yeast. (a) Wild type BY4741 cells were treated with 1.0 mg/mL of acrylamide in CDM and curcumin was added to the cultures at 5, 25 and 50 μM. Growth was monitored by measuring optical density of the cultures at 600 nm. Inset shows the cell viability of acrylamide-treated cells

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with increasing concentrations of curcumin. (b) Changes in DCF fluorescence of yeast cells upon treatment with acrylamide and different concentrations of curcumin. * indicates $p<0.05$ relative to CDM+Acrylamide.

Fig. 9 β-Carotene improves the growth recovery of acrylamide-treated yeast. (a) Wild type BY4741 cells were treated with 1.0 mg/mL of acrylamide in CDM and β-Carotene was added to the cultures at 5, 25 and 50 μM. Growth was monitored by measuring optical density of the cultures at 600 nm. Inset shows the cell viability of acrylamide-treated cells with increasing concentrations of β-Carotene. (b) Changes in DCF fluorescence of yeast cells upon treatment with acrylamide and different concentrations of β-Carotene. * indicates $p<0.05$ relative to CDM+Acrylamide.

Fig. 10 Vanillin improves the growth recovery of acrylamide-treated yeast. Wild type BY4741 cells were treated with 1.0 mg/mL of acrylamide in CDM and vanillin was added to the cultures at 5, 25 and 50 μM. Growth was monitored by measuring optical density of the cultures at 600 nm. Inset shows the cell viability of acrylamide-treated cells with increasing concentrations of vanillin. (b) Changes in DCF fluorescence of yeast cells upon treatment with acrylamide and different concentrations of vanillin. * indicates $p<0.05$ relative to CDM+Acrylamide. ** indicates $p<0.05$ relative to vanillin.

Fig. 11 Caffeic acid improves the growth recovery of acrylamide-treated yeast. Wild type BY4741 cells were treated with 1.0 mg/mL of acrylamide in CDM and caffeic acid was added to the cultures at 5, 25 and 50 μM. Growth was monitored by measuring optical density of the cultures at 600 nm. Inset shows the cell viability of acrylamide-treated cells with increasing concentrations of caffeic acid. (b) Changes in DCF fluorescence of yeast cells upon treatment with acrylamide and different concentrations of caffeic acid. *

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indicates $p<0.05$ relative to CDM+Acrylamide. ** indicates $p<0.05$ relative to caffeic acid.

Fig. 12 EPR spectra of yeast in YPD or CDM, with or without acrylamide treatment. Yeast cells were grown in YPD or CDM with or without acrylamide and prepared for EPR analysis as described under “Materials and Methods”.

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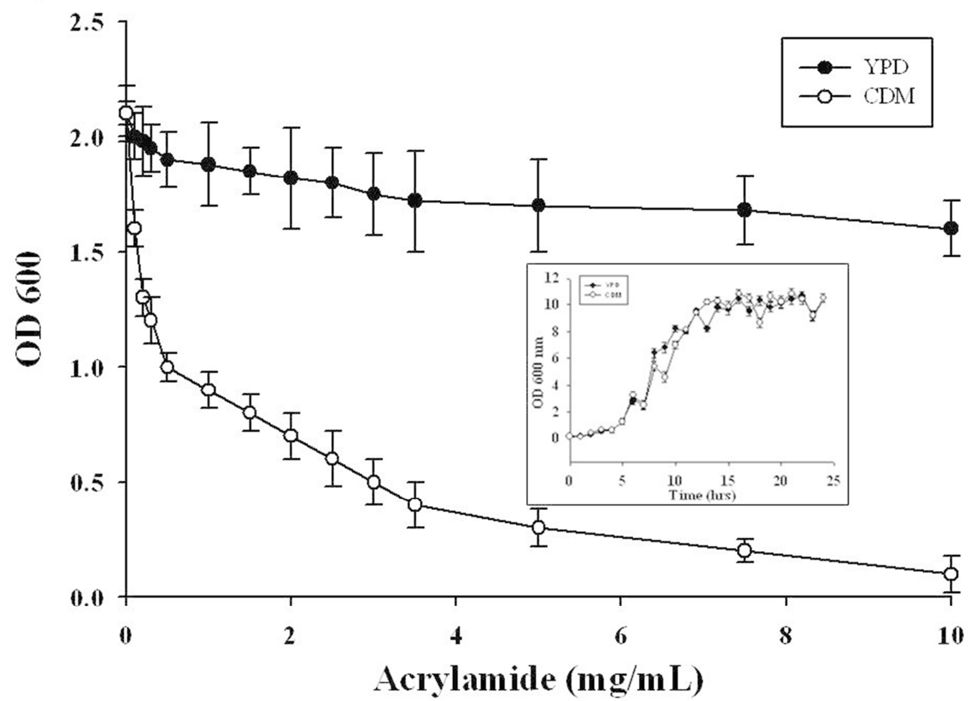
Figure 1.



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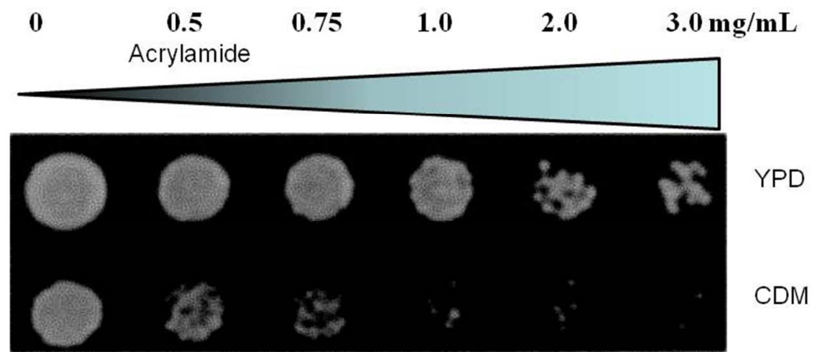

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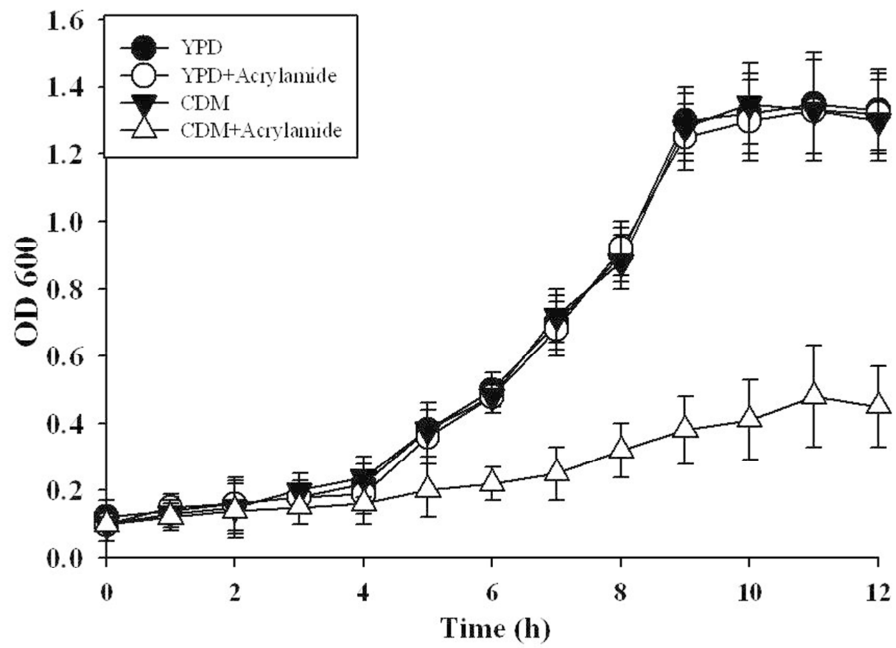

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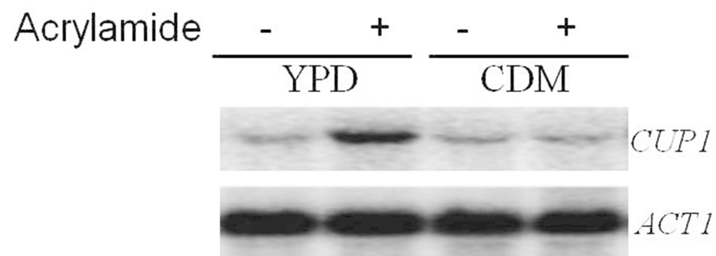

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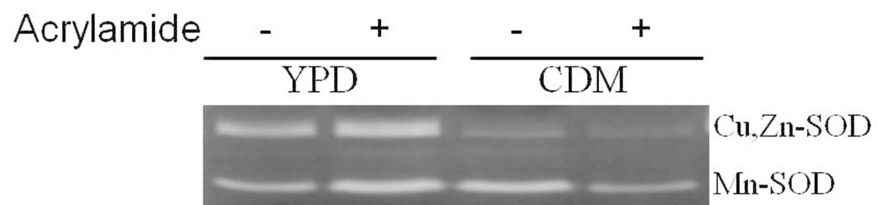

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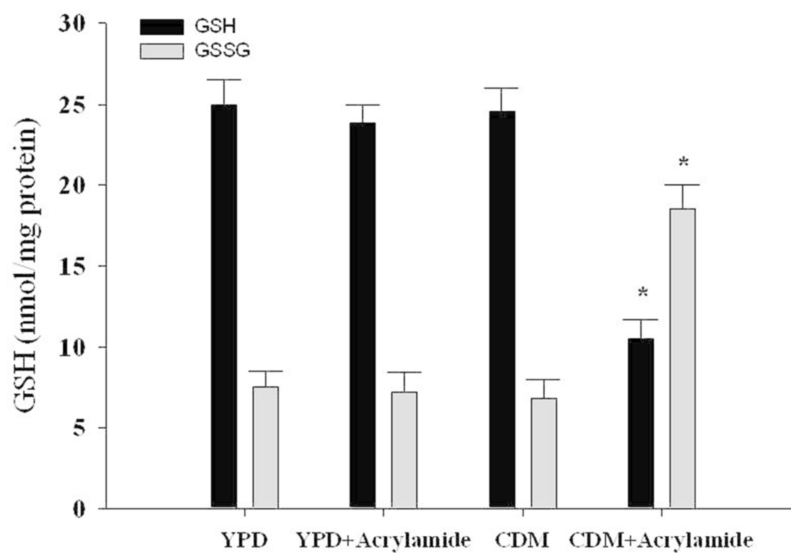

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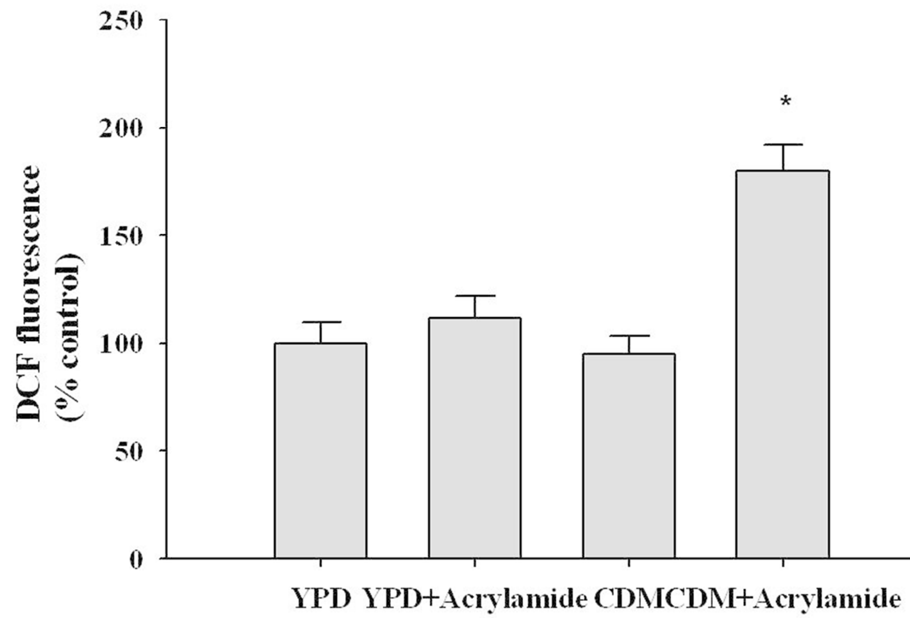

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Figure 7a.

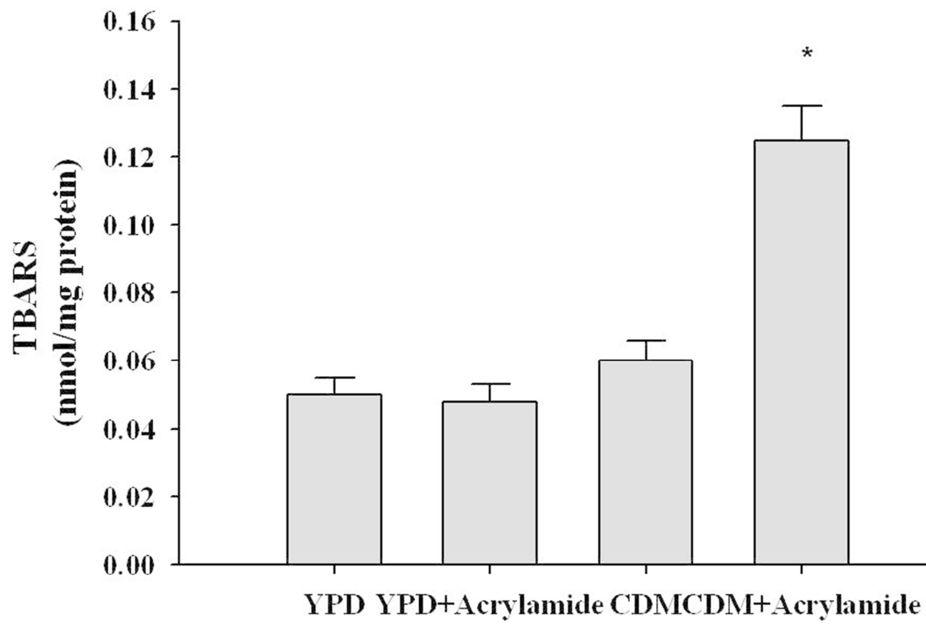

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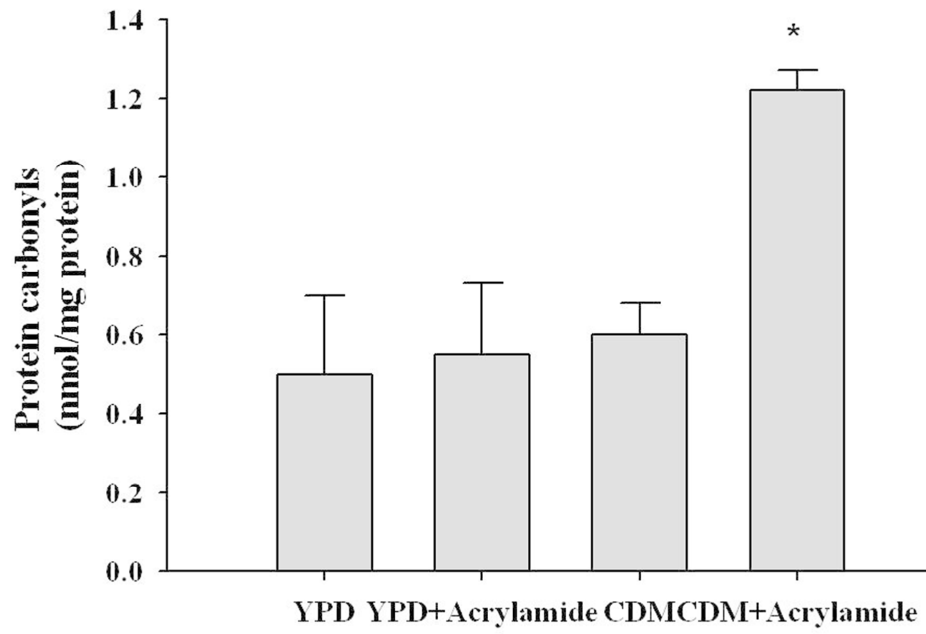
Figure 7b.



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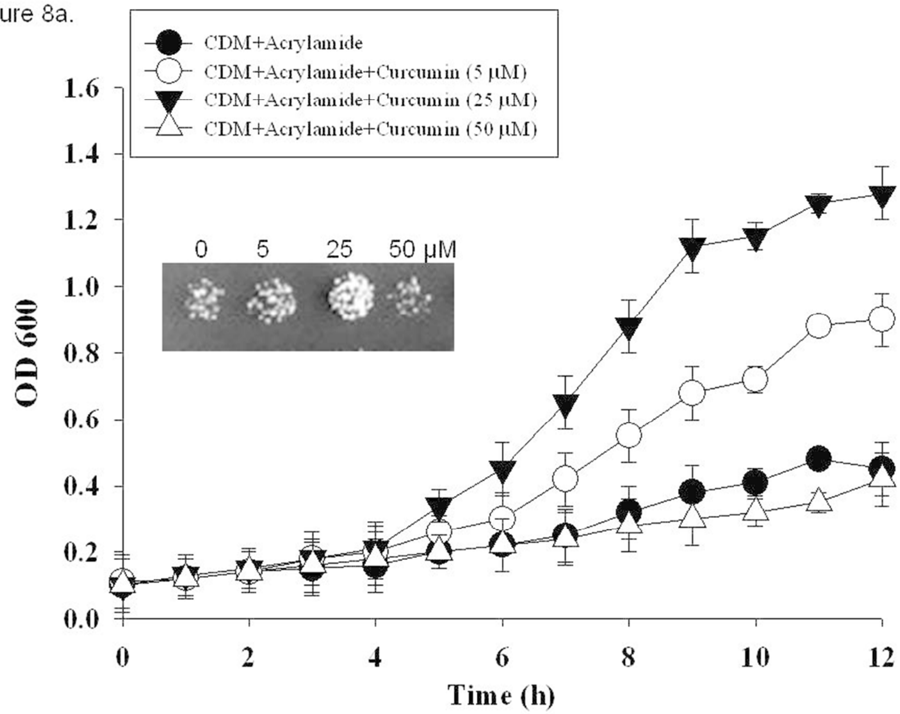

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Figure 8b.

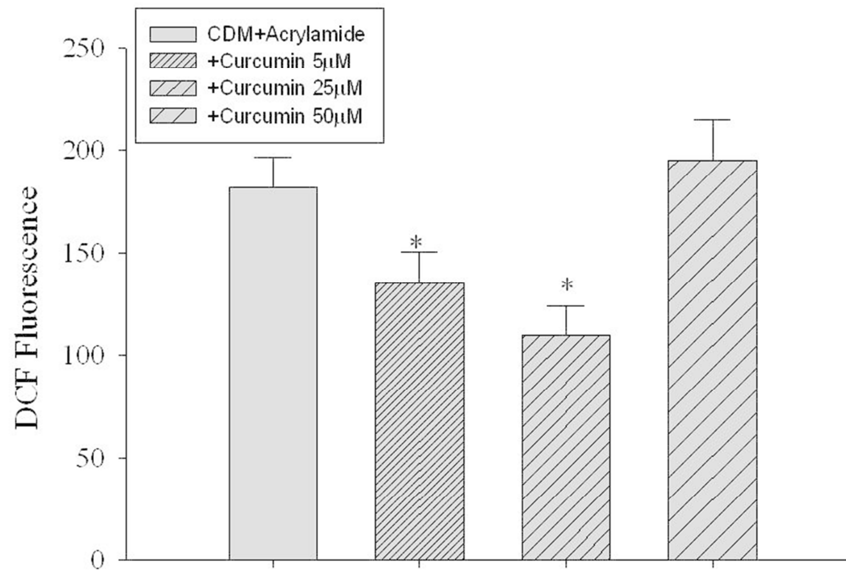

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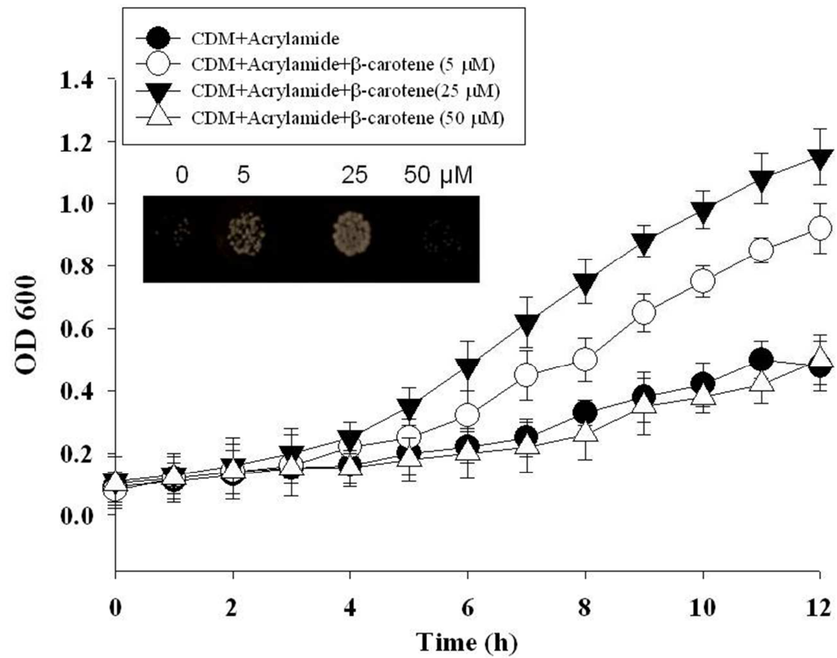
Figure 9a.



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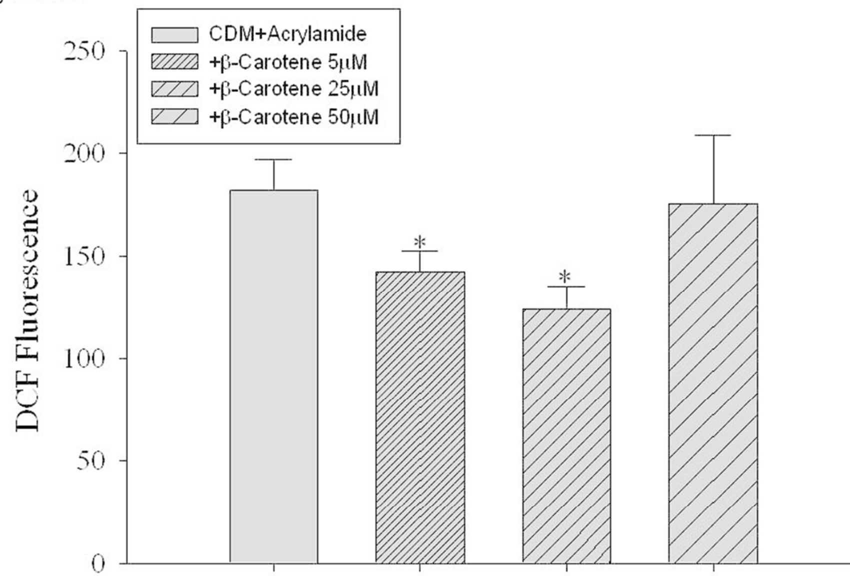

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Figure 10 a.

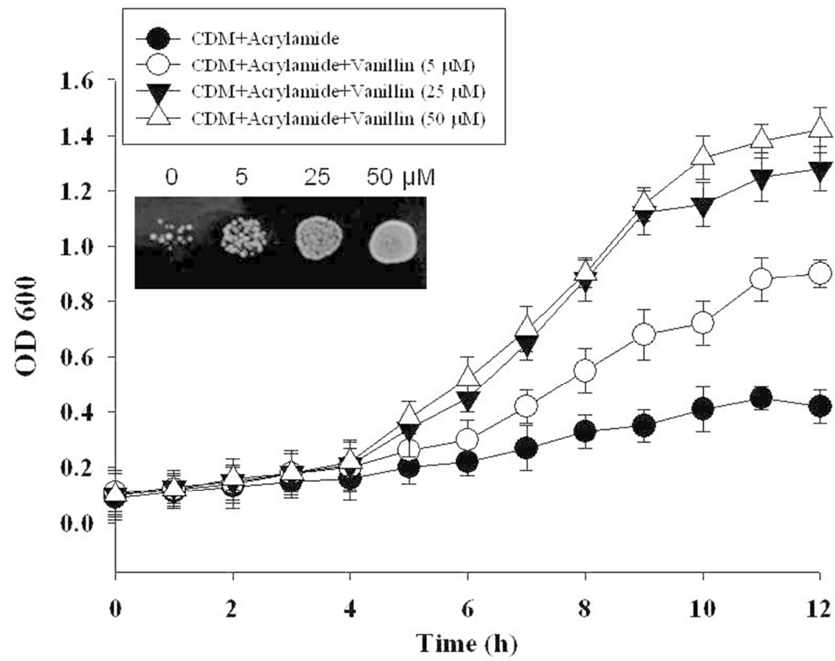

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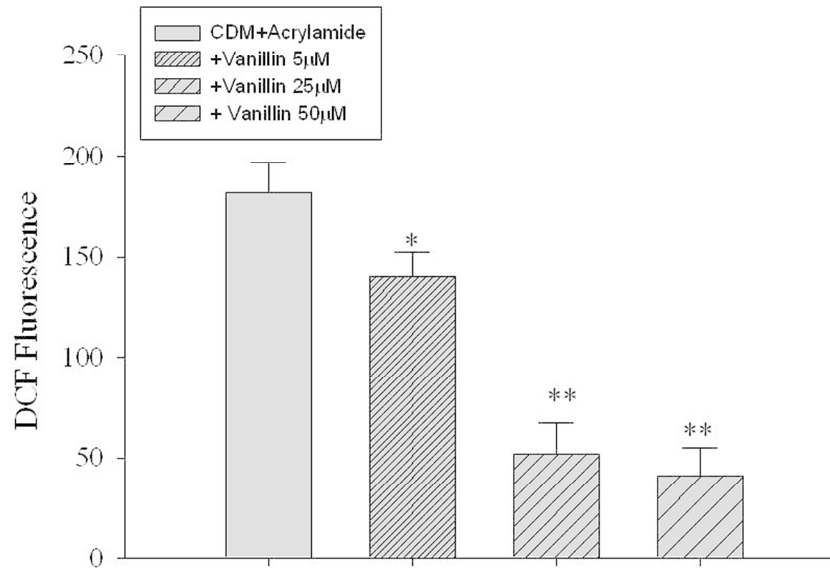
Figure 10 b



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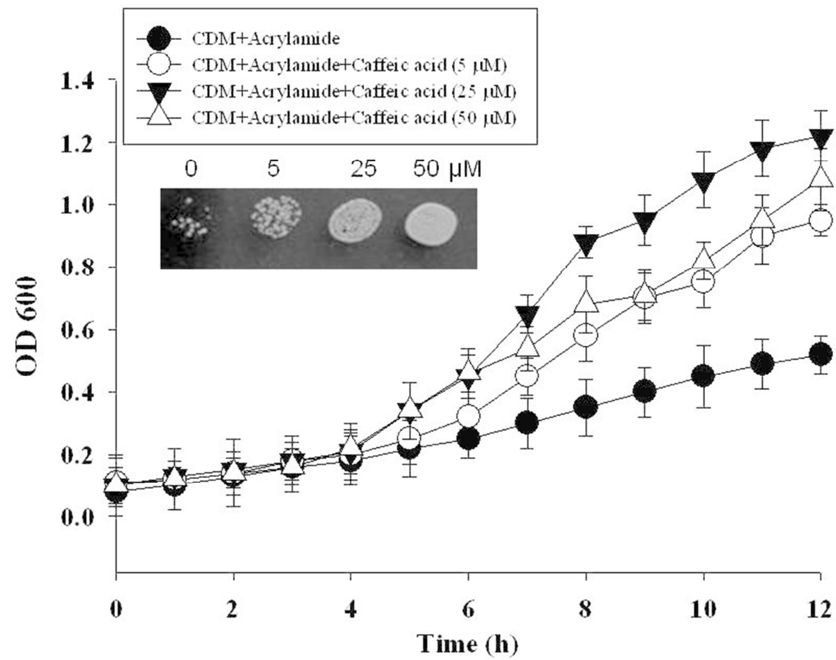

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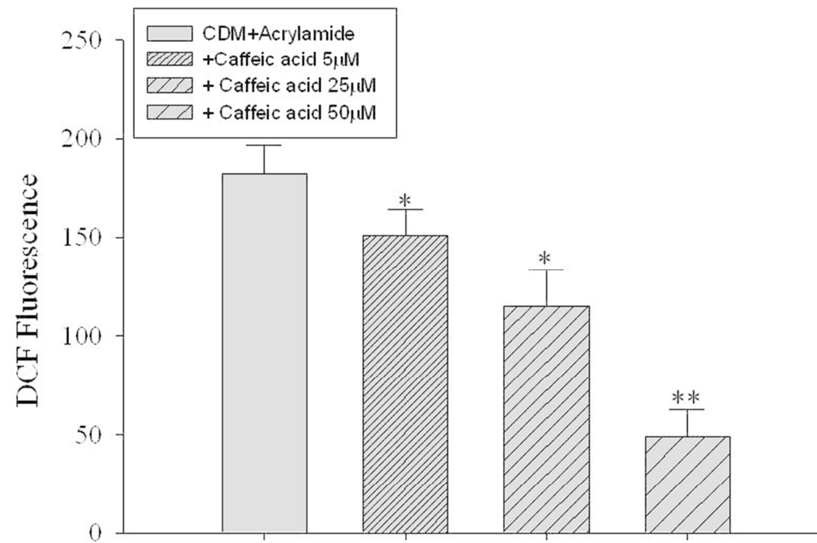

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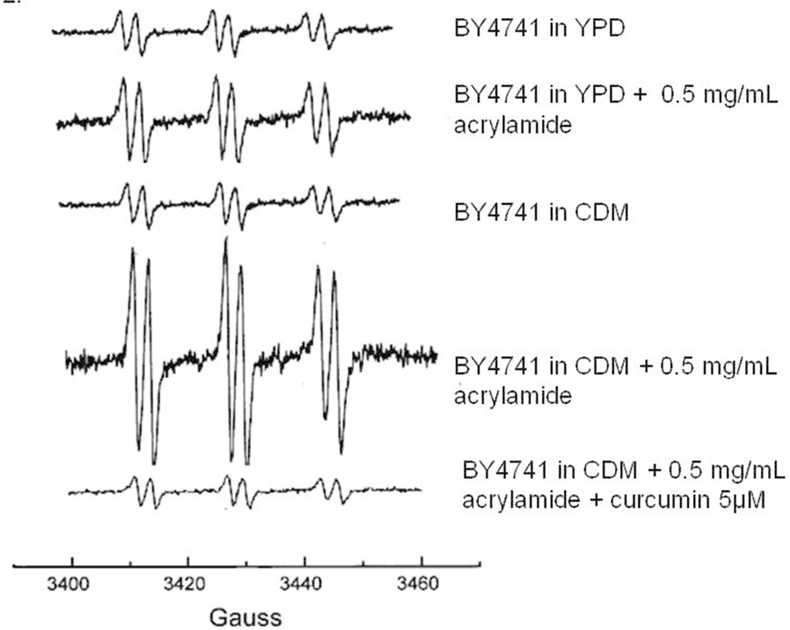

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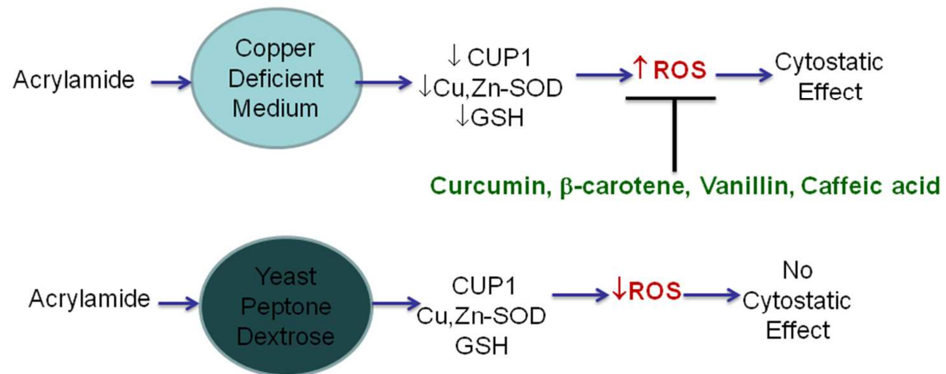
TOC Graphic



254x190mm (96 x 96 DPI)

Food & Function Accepted Manuscript