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The Effect of Cultural Conditions on the Variations of SOD, CAT and GSH-Px Activities and LPO Levels in the Filamentous Fungus *Fusarium equiseti*

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The changes in the activity of cellular detoxifying defence enzymes such as SOD, CAT and GSH-Px against superoxide anion radical and hydrogen peroxide, and in LPO levels in the filamentous fungus, *Fusarium equiseti* were measured under growth condition. Experiments were performed comparing changes in glycerol and saccharose (5-25 g/L) as carbon sources, and in glycine, peptone (5-35 g/L) as nitrogen sources in an AFM medium. While SOD activities correlate well with respect to the increase in the glycerol concentration ($r = 0.437$, $p < 0.001$), CAT activities showed negative correlation ($r = -0.663$, $p < 0.001$). The increase in SOD and CAT activities of *F. equiseti* correlated with increases in saccharose concentration. When glycerol and saccharose were used as carbon sources, the maximum SOD, CAT and GSH-Px enzymes activities and the minimum LPO level were determined in the medium containing 15 g/L of saccharose on the 12^{th} day at 66.61, 182.79, 1.045 IU/mg and 1.41 nmol MDA/ gr wet weight, respectively. The effects of peptone and glycine as nitrogen sources were also investigated: 53.4% and 48.03% decreases were observed in the SOD and CAT activities of *F. equiseti* in the presence of 10 g/L of peptone in the culture medium. The presence of 15 g/L of glycine caused 5.30% and 69.90% decreases in the SOD and CAT activities in comparison to the presence of 15 g/L of saccharose, respectively. On the other hand, LPO levels increased in proportion to the decrease in antioxidant enzyme activities in glycine and peptone supplemented media.

Key Words: Fusarium, glycerol, saccharose, glycine, peptone, SOD, CAT, GSH-Px, LPO (MDA).

Introduction

In aerobic organisms, reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radical are produced as by-products of a normal metabolism. They are also generated when cells are exposed to environmental insults including oxidative agents and radiation which are potentially toxic to cells. Oxygen radicals are implicated in damage to membrane lipids, proteins, and DNA[1,2], and toxicity

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results when the degree of oxidative stress exceeds the capacity of the cell defence systems. Microbial cell defences against the damaging effects of oxidative stress involve both enzymatic and non-enzymatic components[3,4]. The enzymatic components may directly scavenge active oxygen species or may act by producing a non-enzymatic antioxidant. A variety of non-enzymatic antioxidants, such as ascorbic acid, glutathione and thioredoxin, may play an important role in the cellular response to oxidative stress by reducing certain reactive oxygen intermediates[5]. Among the antioxidant enzymes, superoxide dismutase (SOD) catalyses dismutation of the superoxide anion (O_2^-) into hydrogen peroxide, catalase (CAT) detoxifies H_2O_2 and glutathione peroxidase (GSH-Px) both detoxifies H_2O_2 and converts lipid hydroperoxides into non-toxic alcohols. These defences provide protection from highly reactive free radical products which cause lipid peroxidation (LPO) and the destruction of biological molecules in the cell once the radicals are generated[6–8].

Most of the findings which have led to a greater understanding of the biological role of antioxidant enzymes were obtained in studies performed using bacteria, mainly *Escherichia coli*, yeast and complex eukaryotes[9–11]. From an evolutionary point of view it seems important to examine antioxidant enzyme biosynthesis by a wide range of organisms[12]. However, only a few reports have been concerned with lower multicellular eukaryotes, such as filamentous fungi[13]. On the other hand, filamentous fungi are suitable for SOD, CAT and GSH-Px investigation because of their potential advantages, abundant mycelium, intensive respiration and high level of cyanide-resistant respiration[14,15] which are prerequisites for the generation of reactive oxygen species[16].

The aim of this study was to determine the variations of SOD, CAT and GSH-Px enzyme activities and LPO levels in *F. equiseti* depending on the carbon and nitrogen sources.

Materials and Methods

Media and growth conditions

Cultures of *F. equiseti* were maintained on supplemented potato dextrose agar. Spore production in the slants usually requires 5 to 7 days of growth at 28 °C. Spore concentration was determined by measuring with a haemocytometer (5.10^7 conidia/mL).

Cultures were grown in Armstrong Fusarium Medium (AFM) a synthetic medium containing: 1.1 g of KH_2PO_4, 0.4 g of $MgSO_4.7H_2O$, 1.6 g of KCl, 7.27 g of KNO_3, 2 ppm of $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.2H_2O$, 1.0 mg of thiamine-HCI and 0.05 mL Tween 80 in 1 L of distilled H_2O[17], and the pH of the medium was adjusted to 4.5. Then 100 mL cultures were incubated with agitation at 100 rpm at 28 °C in 250 mL shaking flasks for 18 days. In the first step of the study, glycerol or saccharose were used as carbon sources in 5-25 g/L concentrations. In the second step, glycine and peptone in 5-35 g/L concentrations were used as nitrogen sources in the presence of 15 g/L of saccharose. All concentrations of the components in the culture medium were at initial values and any supporting was done during the incubation period. After the cultivation process, the cells were collected by centrifugation followed by washing twice with distilled water, and were kept at -20 °C.

Preparation of cell-free extracts

Wet *F. equiseti* cells frozen at -20 °C were thawed overnight at 4 °C and then suspended in a 20 mM phosphate buffer, pH 7.4, containing polypropylene glycol-1200 in a volume equal to 1.5 times their weight. A 600 μL cell suspension was ground in 1.5 mL plastic vials with 0.8 g of glass beads (0.5 mm ϕ) for 10 min. Cell debris was removed by centrifugation at 15,000 rpm for 15 min.

Assay methods in cell-free extracts

SOD activity was measured in cell free extracts by the method described by Crosti based on the inhibitory effect of SOD on the spontaneous autoxidation of 6-OHDA at 490 nm[18]. 1 IU is defined as the amount of SOD required to inhibit the initial rate of 6-OHDA autoxidation by 50%.

Catalase activity was determined in cell free extracts by the method Aebi. The decomposition of H_2O_2 was followed directly by a decrease in extinction at 240 nm and 37 °C [19].

GSH-Px activity was determined with Ransel kits using consecutive glutathione reductase reaction. The reaction was monitored by oxidation of NADPH at 340 nm and 37 °C. This assay, based on the method Paglia and Valentine, requires cumene hydroperoxide as a substrate[20].

Lipid peroxidation was estimated based on thiobarbituric acid (TBA) reactivity. Samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBA. The extinction coefficient of 153,000 M^{-1} cm^{-1} for the chromophore at 532 nm was used to calculate the MDA-like TBA produced[21].

The protein content was determined by the method described by Lowry et al. using bovine serum albumin as standard[22].

Statistical analysis

The Tukey test, for multiple comparison was used for statistical significance analyses. The values are the means of 3 separate experiments. Comparisons were also made with Pearson's correlation for each substrate and/or enzyme between the different carbon and nitrogen sources at each given incubation time.

Results

SOD, CAT and GSH-Px activities and LPO level variations depending on the carbon sources

The effects of glycerol and saccharose as carbon sources on SOD, CAT and GSH-Px enzyme activities and membrane LPO levels were investigated using *F. equiseti* in the AFM medium. Antioxidant enzymes and lipid peroxidation levels did not change significantly during the first 9 days of cultivation.

Figure 1a shows that the increase of SOD activity in *F. equiseti* correlates well with the increase in the glycerol concentration up to 20 g/L ($r = 0.437$, $p < 0.001$) and the maximum activity observed at this concentration was 80.4 4 $\pm$ 1.01 IU/mg on the 12^{th} day of incubation (Figure 1a). However, higher concentrations than 20 g/L had an inhibitory effect on SOD activity ($p < 0.001$).

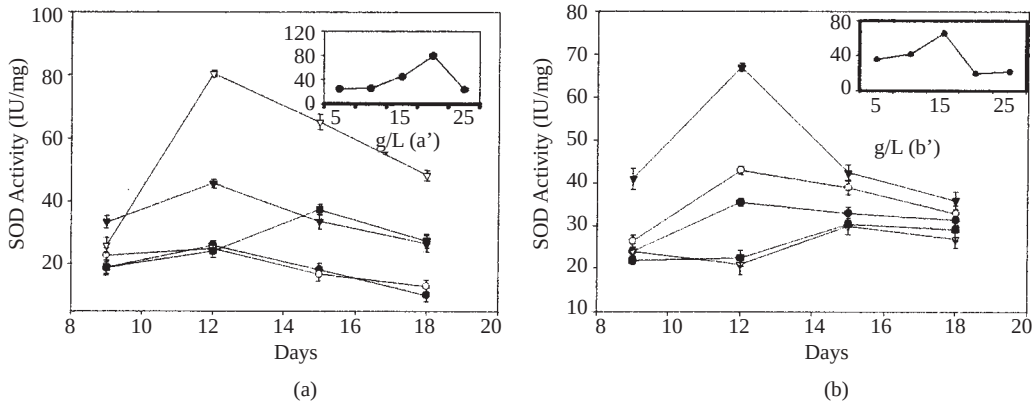

Figure 1. Variations in SOD activity in *F.equiseti* depending on the incubation period in media containing; 5g/L (–●–), 10g/L (–o–), 15 g/L (–▼–), 20 g/L (–∇–) and 25g/L (–■–) of glycerol (a) and saccharose (b) and variations in maximum SOD activity depending on the concentrations of glycerol (a') and saccharose (b').

The activity of the enzyme tended to increase in the saccharose containing medium up to 15 g/L and the maximum value was 67.00 ± 0.91 IU/mg on the 12^{th} day (Figure 1b). The maximum SOD activities observed for 20 and 25 g/L of saccharose shifted to 15 days and were lower than that seen with 15 g/L of saccharose ($p < 0.001$).

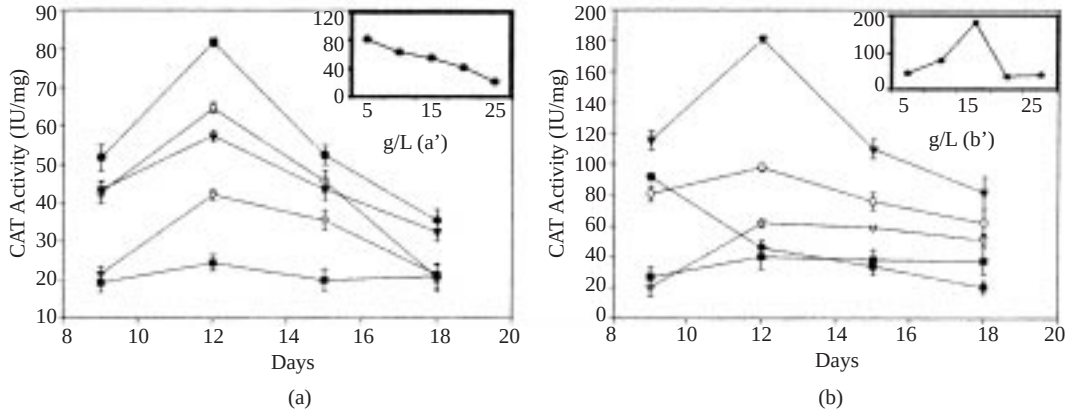

Figure 2. Variations in CAT activity in *F.equiseti* depending on the incubation period in media containing; 5g/L (–●–), 10g/L (–o–), 15 g/L (–▼–), 20 g/L (–∇–) and 25g/L (–■–) of glycerol (a) and saccharose (b) and variations in maximum CAT activity depending on the concentrations of glycerol (a') and saccharose (b').

As shown in Figure 2a, CAT activity in the filamentous fungus *F. equiseti* increased for all tested glycerol concentrations on the 12^{th} day, after which CAT activity began to decline. In contrast to SOD, CAT activity showed a negative correlation with glycerol concentration during the incubation period ($r = -0.663$, $p < 0.001$), and the maximum activity was 81.57 ± 1.1 IU/mg at 5 g/L of glycerol. CAT activity variations did not show the same trend when saccharose was used instead of glycerol as a carbon source (Figure 2b). CAT activity increased from 46.05 ± 4.71 IU/mg to 181.21 ± 1.80 IU/mg with increasing saccharose concentrations from 5 to 15 g/L on the 12^{th} day ($p < 0.001$). While CAT activity decreased continuously in 5 g/L of saccharose during the incubation period, the maximum values were reached on the

12^{th} day in the range 10-25 g/L of saccharose-containing medium and further incubation led to a decrease in enzyme activity ($p < 0.001$) (Figure 2b). While these decreases in CAT activity were observed with increasing saccharose concentrations in the range 5-15 g/L ($p < 0.001$), they did not change significantly at 20 and 25 g/L of saccharose after 12 days of incubation.

GSH-Px activities for the medium containing 20 g/L of glycerol and 15 g/L of saccharose, had the highest SOD and CAT activities compared to the other concentrations of the carbon sources ($p > 0.001$), at 1.88 ± 0.02 IU/mg and 1.045 ± 0.01 IU/mg, respectively.

The LPO level, which is an indicative marker of oxidative cell damage in *F. equiseti* reached a minimum in the range 5-20 g/L of glycerol concentrations on the 12^{th} day and then increased at higher concentrations ($p < 0.001$), (Figure 3a). However, LPO levels rose continuously in the medium containing 25 g/L of glycerol with respect to the incubation period ($p < 0.001$).

Figure 3b shows that the levels of LPO increased continuously in the media containing 5 and 10 g/L of saccharose during the incubation period ($p < 0.001$). While the lowest LPO levels were observed on the 12^{th} day at 15 g/L of saccharose, which had the highest SOD and CAT activities, the minimum shifted to 16 days for 20 and 25 g/L of saccharose.

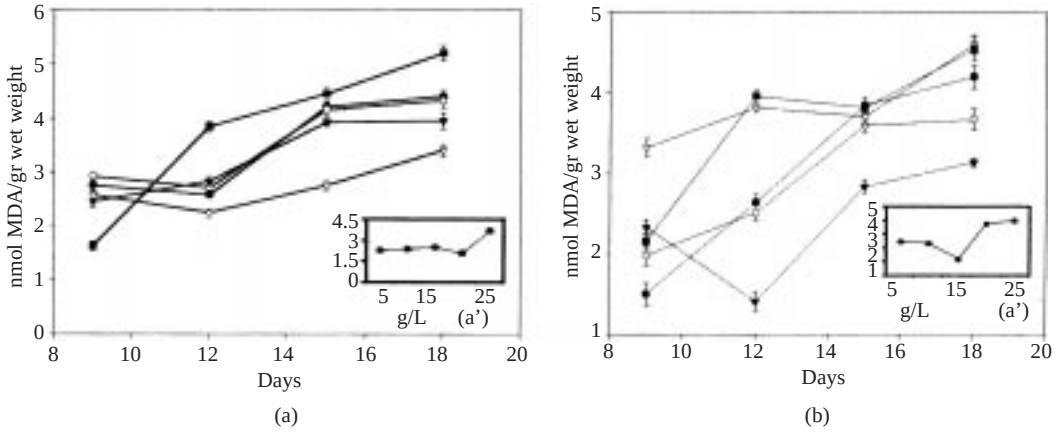

Figure 3. Variations in LPO levels in *F.equiseti* depending on the incubation period in media containing; 5g/L (–●–), 10g/L (–o–), 15 g/L (–▼–), 20 g/L (–∇–) and 25g/L (–■–) of glycerol (a) and saccharose (b) and variations in minimum LPO levels depending on the concentrations of glycerol (à) and saccharose (b̀).

The best correlation between the increase in antioxidant enzyme activities and LPO levels in the 5-25 g/L concentrations among the applied carbon sources was at 15 g/L of saccharose. Therefore, this was used in the following experimental stages.

SOD, CAT and GSH-Px activities and LPO level variations depending on the ritrogen sources

The variations of SOD, CAT and GSH-Px enzyme activity and the LPO level in *F. equiseti* were investigated with respect to glycine and peptone as nitrogen sources in the range 5-35 g/L in the AFM medium.

Figure 4a shows that SOD activity peaked on the 12^{th} day for 10 and 15 g/L of glycine, but on the 16^{th} day for 20 and 25 g/L after which it decreased towards the end of the incubation period. SOD activity

decreased continuously in the medium containing 5 g/L of glycine with respect to the incubation period. The highest SOD activity was 62.25 ± 0.78 IU/mg at 15 g/L of glycine ($p < 0.001$).

Figure 4b shows that SOD activity in *F. equiseti* increased markedly in the whole range of peptone concentrations for the first 12-day period ($p < 0.001$), and began to decrease thereafter ($p < 0.001$). The maximum obtained SOD activity values were similar for 10 and 15 g/L of peptone at 42.7 ± 0.98 IU/mg and 40.04 ± 0.95 IU/mg on the 12^{th} day ($p > 0.001$). On the other hand, SOD activity decreased approximately 2-fold by raising the peptone concentration from 15 to 25 g/L at 12 days ($p < 0.001$).

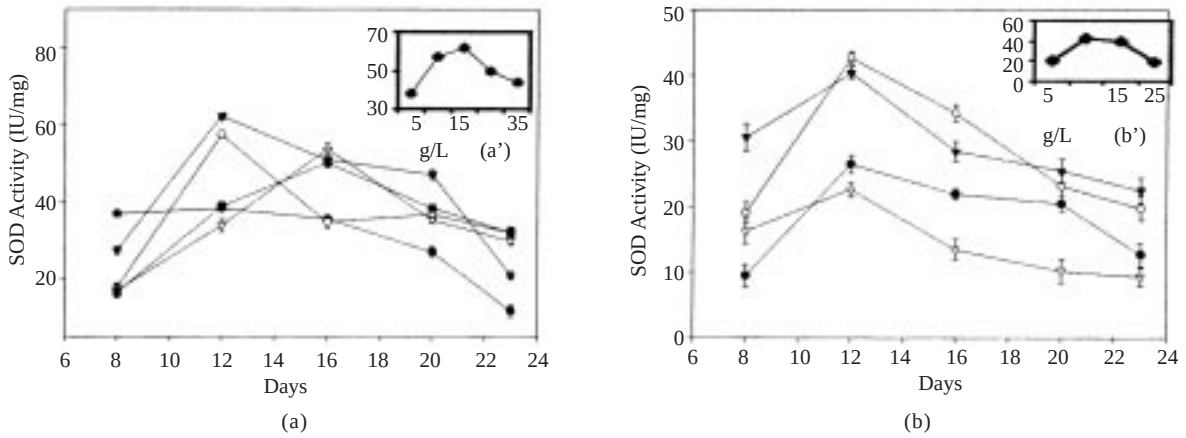

Figure 4. Variations in SOD activity in *F.equiseti* depending on the incubation period in media containing; 5g/L (–●–), 10g/L (–o–), 15g/L (–▼–), 25g/L (–▽–) and 35g/L (–■–) of glycine (a) and peptone (b) and variations in maximum SOD activity depending on the concentrations of glycine (a') and peptone (b').

As shown in Figure 5a, CAT activity increased significantly from 23.76 ± 1.10 IU/mg to 54.67 ± 0.75 IU/mg when the glycine concentration was increased from 5 to 15 g/L on the 12^{th}day ($p < 0.001$). The maximum CAT activity was observed on the 12^{th} day for all glycine concentrations used except for the medium containing 35 g/L of glycine.

Following supplementation with another nitrogen source, the maximum CAT activity in *F. equiseti* depending on the peptone concentrations was on the 12^{th} day, at 94.12 ± 2.91 IU/mg in the medium containing 10 g/L of peptone ($p < 0.001$). However, the maximum SOD and CAT activity values in peptone decreased relative to the values observed in glycine ($p < 0.001$). CAT activities decreased significantly in media containing peptone at concentrations higher than 10 g/L (Figure 5b).

GSH-Px activities were 1.07 and 0.74 IU/mg for the media containing 15 g/L of glycine and 10g/L of peptone, which were observed to have the highest SOD and CAT activities.

LPO level variations showed a negative correlation with the SOD and CAT activities in mediums containing glycine ($r = -0.446, -0.302$, $p < 0.001$) and peptone ($r = -758, -0.716$; $p < 0.001$). The minimum LPO levels determined for 15 g/L of glycine and 10 g/L of peptone were 1.65 ± 0.91 and 2.21 ± 0.07 nmol MDA/g wet weight respectively (Figures 6a,b). On the other hand, the highest LPO level on the 12^{th} day was 5.4 ± 0.15 nmol MDA/g wet weight at 5 g/L of peptone, which had the lowest SOD and CAT activities.

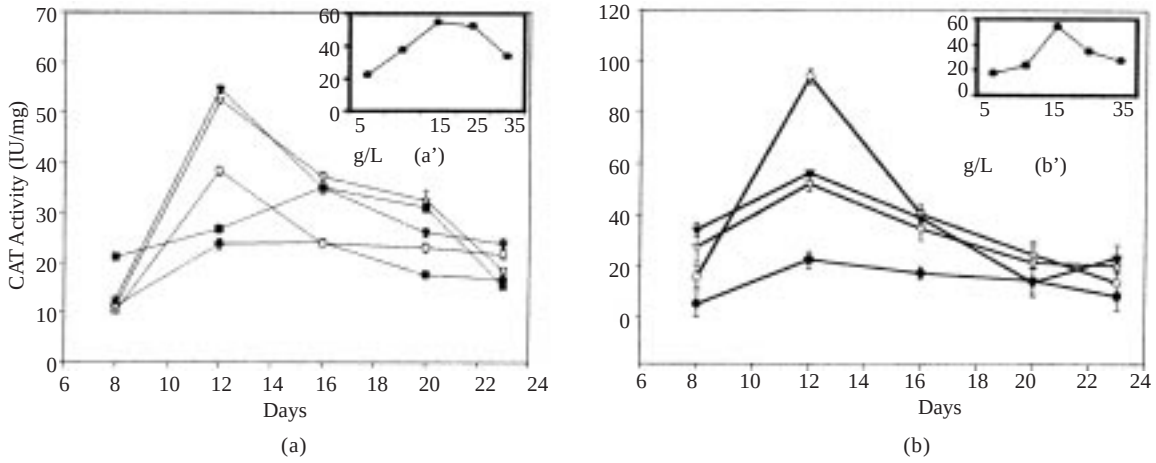

Figure 5. Variations in CAT activity in *F.equiseti* depending on the incubation period in media containing; 5g/L (–●–), 10g/L (–o–), 15g/L (–▼–), 25g/L (–▽–) and 35g/L (–■–) of glycine (a) and peptone (b) and variations in maximum CAT activity depending on the concentrations of glycine (a') and peptone (b').

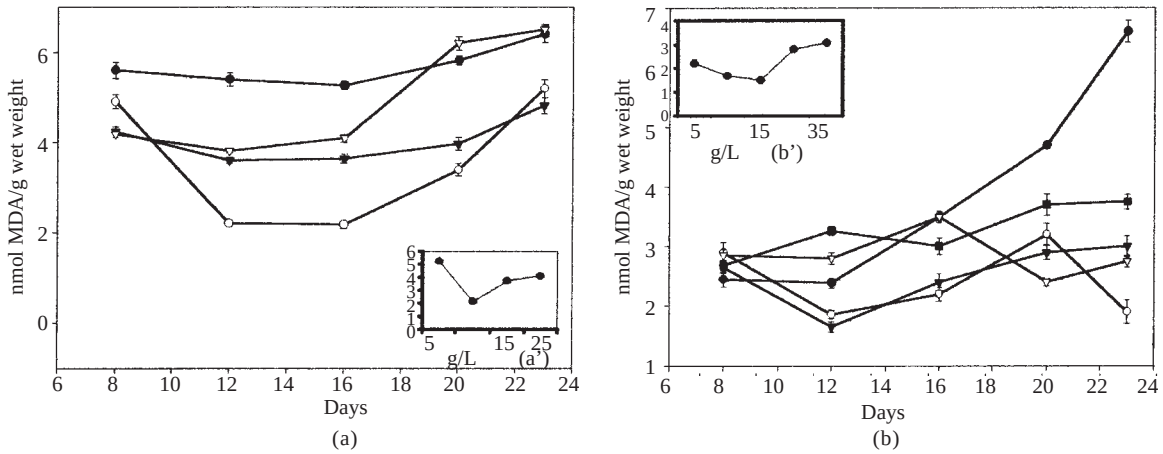

Figure 6. Variations in LPO levels in *F.equiseti* depending on the incubation period in media containing; 5g/L (–●–), 10g/L (–o–), 15g/L (–▼–), 25g/L (–▽–) and 35g/L (–■–) of glycine (a) and peptone (b) and variations in minimum LPO levels depending on the concentrations of glycine (a') and peptone (b').

Discussion

Under normal circumstances, the generation rate of ROS by various biochemical systems in all living cells is rather low and does little damage, simply because it is efficiently removed by the SOD, CAT and GSH-Px systems. However, circumstances can arise for a variety of reasons where high rates of ROS production do occur and therefore variations of antioxidant enzymes and LPO levels may be necessary.

Our results showed that the increase in SOD and CAT activities by 88.24% and 293.48% at 15 g/L of saccharose compared with 5 g/L of saccharose caused a decrease in LPO level of 96.41% on the 12^{th} day. Following supplementation with glycerol as an alternative carbon source, SOD activity increased by 210% although CAT activity decreased by 48.29% in medium containing 20 g/L of glycerol compared with 5 g/L of

glycerol and LPO levels thus decreased by 13.18%. In the light of the results for the carbon sources, despite lower SOD and GSH-Px activity values there were higher CAT activity and lower LPO levels in the medium containing 15 g/L of saccharose compared with 20 g/L glycerol. This indicated that CAT activity was an effective antioxidant enzyme which prevents excess H_2O_2 accumulation in the *F. equiseti* cells. According to our previous study, increases in membrane LPO levels in *F. acuminatum* with respect to decreases in CAT activity also showed the capacity of the CAT enzyme to dismutate of reactive oxygen species. The elevated levels of GSH-Px activity in glycerol medium in comparison to the saccharose concentrations, which determined maximum SOD and CAT activities, were also an important factor in the reduction of LPO[23,24].

The current investigation also shows that micro-organisms characteristically change their metabolic behaviour depending on the nature of the carbon source[25]. It has been reported that saccharose is transported into the cell by a saccharose inducible H^+-symport and is further hydrolysed by intracellular α-glucosidase in *C. albicans*[26]. The uptake of glycerol may be by passive diffusion, although active transport systems have been described in some yeasts, acting mainly in conditions of osmotic stress[27,28]. Changes in transport and concentrations of carbon sources can a effect the energy metabolism, specifically the electron transport in the inner mitochondrial membrane, in the process of which most of the cellular ROS are generated and may cause the variation in antioxidant enzyme activities. According to our results, the maximum antioxidant enzymes were determined on the 12^{th} day of cultivation. These results are supported by other studies which reported that after the 12^{th} day, Fusarium species enter a stationary phase which produces secondary metabolites like antioxidant enzymes[15].

Nitrogen sources and their concentrations are also important parameters with respect to the antioxidant enzyme activities of *F. equiseti*. In this study, the increase in SOD and CAT activities and the decrease in LPO levels were 61.13, 316.46 and 59.07% at 10 g/L of peptone and 61.98, 130.09 and 30.96% at 15 g/L of glycine compared to the levels at 5 g/L of nitrogen sources respectively. However, although CAT activity decreased in the medium containing 15 g/L of glycine compared with 10 g/L of peptone, SOD and GSH-Px activities increased, and therefore LPO levels decreased. These results observed in *F. equiseti* growth with nitrogen sources showed coherence with the variations determined in *F. acuminatum*[29]. These results can be explained by assuming that besides the removal of $O_2^{\cdot -}$ by SOD, there is coperativity between CAT and GSH-Px activities in order to remove H_2O_2 in the protection of biological membranes from LPO[30−32]. It has been reported that because all nitrogen sources are eventually degraded to ammonia, the enzymes which control oxidative phosphorylation are related to the radical metabolism and are regulated according to the kind and the concentration of the nitrogen source present in the medium[33,34]. Our results show that antioxidant enzyme activities were also affected by this. Nevertheless, it is certain that the diffusion capacity and regulation of nitrogen sources, depending on the concentration in the metabolism, can play a significant role as observed in our results. In addition to ammonia uptake, *F. equiseti* has to adapt new nitrogen sources because the peptone has a rich amino acid composition and may need to produce the necessary enzymes for its utilization. However, these enzymes may not be expressed sufficiently in cultivation on peptone, causing decreases in antioxidant enzyme activities and increases in LPO levels. All the parameters appear to be important factors in the variations in antioxidant enzyme activities and membrane LPO levels in *F. equiseti*, depending on the nitrogen sources.

According to our results the lower antioxidant enzyme activities and the higher LPO levels caused by continuous generation of ROS in *F. equiseti* were found at higher carbon and nitrogen concentrations than the optimum values for maximum SOD and CAT activities. We may conclude that these enzymes were

suppressed in these higher concentration ranges.

LPO levels showed a negative correlation with SOD and CAT activities in glycerol (r = 0.457, 0.520 and –0.358) and in peptone (r = 0.646, 0.664 and –0.473) during 8-12 days and also in glycerol (r = -0.261, 0.598 and 0.293), in peptone (r = -0.469, 0.647 and 0.613) during 16-23 days. As a result in the increases of the inactivation caused by superoxide anion and H_2O_2 it may be that the aging process of *F. equiseti* starts after the 16^{th} day. It appears clear that the differences in ROS generation and in the protection system against oxidative stress have an important impact on the aging period of filamentous fungi[35].

The present study clearly demonstrates that SOD, CAT and GSH-Px enzymes, which protect the cells from reactive oxygen radicals and toxicity-initiated membrane LPO, are a pivotal component of the antioxidant defence network of the filamentous fungus *F. equiseti* cells which have an ability to respond to changes in the cell environment by using different carbon-nitrogen sources (glycine and peptone) and their concentrations; and the most suitable medium compositions were 15 g/L of saccharose as a carbon source and 20 g/L of glycine as a nitrogen source in the AFM medium.

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