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Characteristics of copper tolerance in *Yarrowia lipolytica*

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Abstract

We discovered that a mutant strain of the dimorphic yeast *Yarrowia lipolytica* could grow in the yeast form in high concentrations of copper sulfate. The amount of metal accumulated by *Y. lipolytica* increased with increasing copper concentrations in the medium. Washing with 100 mM EDTA released at least 60% of the total metal from the cells, but about 20–25 μmol/g DW persisted, which represented about 30% of the soluble fraction of cultured cells. The soluble fraction (mainly cytosol) contained only about 10% of the total metal content within cells cultured in medium supplemented with 6 mM copper. We suggest that although a high copper concentration induces an efflux mechanism, the released copper becomes entrapped in the periplasm and in other parts of the cell wall. Washing with EDTA liberated not only copper ions, but also melanin, a brown pigment that can bind metal and which located at the cell wall. These findings indicated that melanin participates in the mechanism of metal accumulation. Culture in medium supplemented with copper obviously enhanced the activities of Cu, Zn-SOD, but not of Mn-SOD.

Introduction

Copper is an essential element, as it functions as a cofactor for enzymes such as cytochrome c oxidase and Cu/Zn superoxide dismutase. However, copper is toxic to most organisms at elevated concentrations, largely through enzyme inhibition (Samarelli & Campbell 1983), the oxidation of membrane components, which might be related to the ability of copper to generate toxic hydroxyl radicals (Shi *et al*. 2003; Melo *et al*. 2004; Shanmuganathan *et al*. 2004) and membrane damage, resulting in the rapid leakage of ions and other low molecular weight compounds (Soares *et al*. 2003).

The fungal cell wall plays an important role as a site for heavy metal deposition through biomineralization (Kierans *et al*. 1991; Yu *et al*. 1996) or by binding metals *via* the amino, carboxyl and hydroxyl groups of cell surface proteins and carbohydrates (Brady & Duncan 1994; Brady *et al*. 1994; Kapoor & Viraraghavan 1997). The composition of the fungal cell wall changes during growth in medium supplemented with metal (Venkateswerlu & Stotzky 1986). Many reports have indicate that fungal melanins act as metal chelators and thus provide many potential binding or biosorption sites for metal ions as well as protection against irradiation, enzymatic lysis and desiccation (Gadd & de Rome 1988; Fogarty & Tobin 1996; Butler & Day 1998). Polyphenols have also been implicated in the binding of copper by *Neurospora crassa* and *Aureobasidium pullulans* (Gadd & Griffiths 1980; Suresh & Subramanyam 1998).

Because the dimorphic fungus *Y. lipolytica* can biodegrade many hydrocarbon compounds, it is used in the bioremediation of marine or soil environments contaminated by petroleum by-products (Margesin & Schinner 1997; Zinjarde & Pant 2002a, b). This fungus has also been used extensively to produce organic and amino acids due to its formidable excretory capacity (Barth &

Gaillardin 1997; Antonucci *et al*. 2001; Fickers *et al*. 2004). Furthermore, *Y. lipolytica* can survive extreme environments such as those containing high concentrations of NaCl or heavy metals (Andreishcheva *et al*. 1999; Zvyagilskaya *et al*. 2001; Butinar *et al*. 2005; Strouhal *et al*. 2003). However, little is understood about its tolerance mechanisms towards the metals and other environmental conditions. The strict aerobic yeast *Y. lipolytica* might have developed defense mechanisms to protect against metal-induced oxidative stress. One characteristic of *Y. lipolytica* is the ability to produce brown pigmented melanins that are located in and/or outside the cell wall (Carreira *et al*. 2001). Melanin production from *Y. lipolytica* is stimulated by Mn^{2+}, but not Cu^{2+} (Carreira & Loureiro 1998). The present study investigates some of the processes of copper tolerance in *Y. lipolytica* such as cellular accumulation and localization of copper as well as the roles of melanin and superoxide dismutase in scavenging intermediates of oxygen reduction.

The dimorphic fungus *Y. lipolytica* can grow as a filamentous, mycelia-producing fungus and/or as a yeast-like form, depending on the culture conditions (Ruiz-Herrera & Sentandreu 2002; Szabo & Stofaniková 2002). The mycelial and yeast-like forms of other dimorphic fungi such as *Mucor rouxii* and *Candida albicans* morphologically differ in cell wall composition, (Bartnicki-Garcia & Nickerson 1962; Elorza *et al*. 1988) and in metabolic activity (Mowll & Gadd 1984; Gadd & Mowll 1985). Copper activates some transcription factors involved in the morphogenesis of *Aureobasidium pullulans* (Gadd & Griffiths 1980) and of *Podospora anserina* (Borghouts & Osiewacz 1998). The coexistence of morphologically different cell types in culture considerably complicates the quantitation of growth, cell number and viability as well as other physiological responses. Therefore, we used a *Y. lipolytica* mutant that can only grow in the yeast-like form to examine the characteristics of copper tolerance.

Materials and methods

Strain and medium

The mutant strain of *Yarrowia lipolytica mhy 1-1* (*MAT A*, *ura3-302, leu2-207, lys8-11, mhy1-1*) that can grow only in the yeast form was donated by Dr. R. Rachubinsky, University of Alberta, Edmonton, Alberta, Canada (Hurtado and Rachubinski, 1999). Cells were agitated on a reciprocal shaker (120 rev./min) at 30 °C in liquid medium containing 20 g of glucose, 5 g of polypeptone, 4 g of yeast extract, 5 g of KH_2PO_4 and 2 g of $MgSO_4 \cdot 7H_2O$ in 1 l of distilled water. A solution of filter-sterilized copper sulfate was added to the liquid media to the desired final concentration. Growth was monitored by measuring the optical density at 600 nm.

Subcellular fractionation

The amount of copper on the cell surface was determined as follows. Cells were washed twice with distilled water and resuspended in 20 mM PIPES (pH 7.2) containing 100 mM EDTA. The cell suspension was agitated on a mixer for 1 min at 4 °C and then separated by centrifugation for 5 min at 5,000 *g*. Copper ions in the supernatant fraction were determined as being loosely bound to the cell surface.

After washing with EDTA, the cells were resuspended in cold 50 mM phosphate buffer (pH5.8) in glass tubes and mixer disrupted with glass beads (0.5 mm diameter) for 5 min at 4 °C. Intact cells were absent according to observation by light microscopy.

The homogenate was centrifuged at 15,000 *g* for 30 min and then cytosolic copper was determined in the supernatant fraction and that bound to the cell wall and membrane copper was determined in the pellets.

Assay of copper

Copper was determined by atomic absorption spectrometry. Samples (1 ml) were placed in test tubes containing 0.5 ml of 6 M HNO_3 and placed in a boiling water bath for 20 min. Undigested material was removed by centrifugation at 3,000 *g* for 5 min. The supernatants were diluted in distilled water and then the copper content was determined by atomic absorption spectrometry (Hitachi Z-5010). Samples obtained after the EDTA wash and from the supernatant fraction after disruptions were analyzed directly without further modification. Copper was quantified by comparison with a copper standard solution (Wako Chem.).

Assay of melanin

Melanin was extracted from the cells by an adaptation of the method described by Gadd and Griffiths (1980). Samples (10 ml) of liquid cultures were harvested by centrifugation (1,000 *g* for 5 min) in glass tubes and washed twice with distilled water. Absolute ethanol (5 ml) was added to tubes containing about 10 mg of dry weight of cells, and these were immersed in a water bath at 60 °C for 3 h. Samples were centrifuged and again washed twice with distilled water. After drying for 24 h at 95 °C, the washed pellets were resuspended in 1 ml of 6 M HNO_3 and heated for 3 h at 75 °C. Distilled water (5 ml) was added to each sample before centrifugation. The pellets were washed twice in distilled water and then melanin was extracted by boiling in 5 ml of 0.5 M NaOH for 20 min. The resulting solutions were passed through filter paper to remove any undigested debris and then the absorbance of the extracts at 430 nm was compared with that of commercially available melanin (MP Biomedicals, Inc).

Assay of superoxide dismutase (SOD) activity

Yeast cells were cultured for 24 and 48 h, washed twice with distilled water and then vigorously vortex-mixed with glass beads in 50 mM PIPES buffer (pH 7.0) containing 1 mM PMSF and 1 mM EDTA at 4 °C. The suspension was separated by centrifugation at 10,000 *g* for 30 min and then SOD activity was measured in the supernatant. Protein was determined using the Folin–Ciocalteau reagent with bovine serum albumin as the standard (Lowry *et al*. 1951).

The activity of SOD was determined on the basis of inhibition of the superoxide-dependent reduction of hydroxylamine by xanthine oxidase (Elstner & Heupel 1976). The reaction mixture (1 ml) contained 1 mM hydroxylamine, 0.1 mM xanthine, 0.05 mM EDTA, xanthine oxidase, the supernatant and 10 mM phosphate buffer (pH 8.2). The mixture was incubated for 30 min at 37 °C and then SOD activity was measured by spectrophotometry (Hitachi U-3000) at 550 nm. The amount of SOD required to inhibit the rate of hydroxylamine reduction by 50% was defined as 1 unit of activity. Cyanide-insensitive SOD activity was measured using the same reaction mixture containing 1 mM potassium cyanide.

Results

Effect of copper on yeast growth

The growth response of the yeast to copper toxicity was examined (Figure 1). In the absence of copper, *Y. lipolytica* started to grow after a lag of approximately 6 h and reached the stationary phase at 50 h. Copper concentrations from 2 to 4 mM did not significantly affect the growth rate at the logarithmic phase, but increased the lag period. During the prolonged lag phase, 6 mM copper sulfate did not decrease cell viability for at least 12 h, as shown by staining with methylene blue (data not shown). Therefore, the prolonged lag phase in medium containing copper appears to represent an accommodation period rather than selection of an extant sub-population of copper-resistant cells or *de novo* mutants.

Copper accumulation by Y. lipolytica

Figure 2 shows that the growth of *Y. lipolytica* cells cultured for 48 h in liquid medium containing increasing concentrations of copper was decreased and that the cells accumulated large amounts of copper. The concentration of copper required to inhibit growth by 50% was about 5 mM. In contrast, the copper content increased proportionally with increasing concentrations of copper over the range of 1–6 mM. The cellular copper content was 65 μmol/g DW, which was half the amount required to inhibit growth.

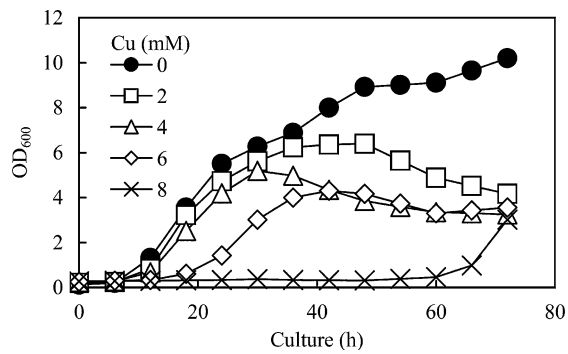

Figure 1. Growth of *Y. lipolytica* in the presence of copper sulfate. Cells were cultured at 30 °C. Values are means of two experiments. See Materials and methods for details.

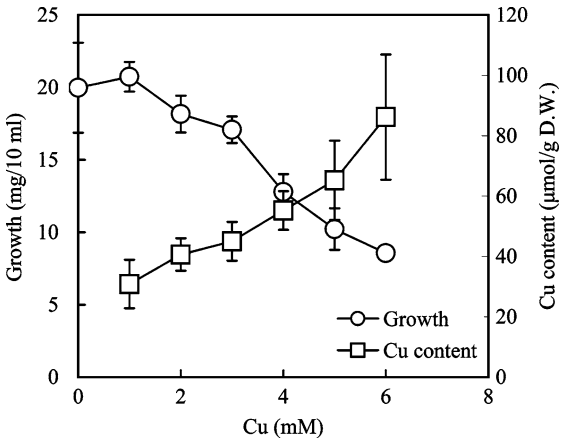

Figure 2. Effect of copper on growth and copper content of *Y. lipolytica*. Cells were cultured in medium containing various concentrations of copper sulfate at 30 °C for 48 h. Bars indicate standard deviation of 3 experiments. See Materials and methods for details.

Cellular distribution of copper

We examined the distribution of copper in the cell wall, cytosol and/or some organella to determine detoxification sites. Before analyzing the intracellular copper content, we estimated the amount of copper loosely bound at the cell surface after washing with 100 mM EDTA in HEPES buffer (pH 7.2).

Figure 3 shows the copper content in the released and retained fractions obtained after washing with EDTA. After 24 h of culture, the amount of copper removed by washing with EDTA increased in cultures incubated on media containing 1–6 mM copper sulfate (Figure 3a). Under these conditions, less copper accumulated in the EDTA resistant fraction. Thus, the ratio (%) of copper released from the cells was increased to about 25 and 45% of the total copper content (1 and 6 mM) of the media, respectively.

Figure 2 shows that after 48 h of culture, the amount of copper accumulated by the cells increased in proportion to the copper concentration in the culture medium. The amount of copper removed by washing with EDTA was 16.2 and 77.4 μmol/g DW in cells cultured in media containing 1 and 6 mM copper, respectively, that is, about 40% and 75% respectively of the total copper content (Figure 3b). In contrast, the amount of EDTA-resistant copper did not increase with increasing concentrations of copper in

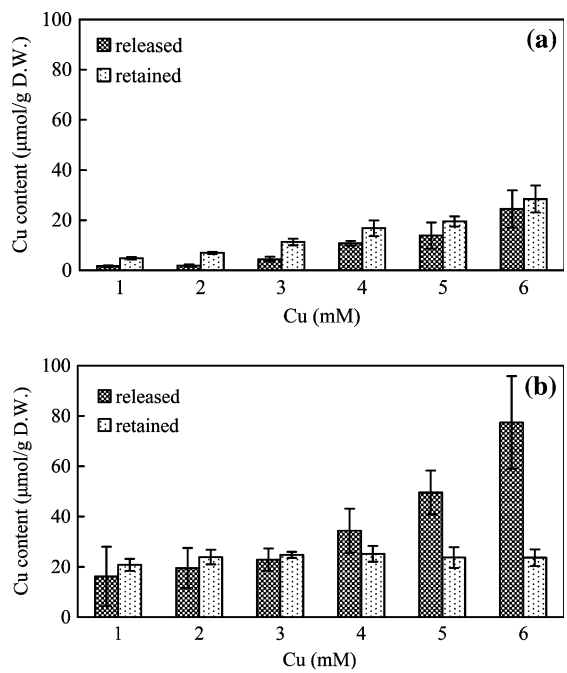

Figure 3. Release of copper ion from cell surface by washing with EDTA. Cells were cultured in medium containing various concentrations of copper sulfate at 30 °C for (a) 24 and (b) 48 h and then washed with 20 mM PIPES (pH 7.2) containing 100 mM EDTA. Bars indicate standard deviation of 3 experiments. See Materials and methods for details.

the medium. Interestingly, the EDTA-resistant cellular copper content remained constant at about 25 μmol/g DW in all cultured cells tested.

Following mechanical disruption of EDTA-washed cells and centrifugation, the copper content of the supernatant and the pellet fractions was measured to determine whether copper that accumulated in the cytoplasm was located in the vacuoles and organella or associated with the cell wall. Figure 4a shows the cellular distribution of copper in the soluble and pellet fractions of cells cultured for 24 h in liquid medium containing various concentrations of copper. The copper contents in the supernatant fraction were 3.2 and 10 μmol/g DW and those in the pellet were 1.3 and 11.7 μmol/g DW when the copper concentrations in the media were 1 and 6 mM, respectively. About 70% of the copper in cells cultured in medium containing 1 mM copper and then washed with EDTA was located in the supernatant fraction. This finding indicated that copper primarily localizes in the cytosol. Furthermore, Figure 4b shows that the subcellular distribution of copper in cells washed with EDTA was obviously lower in the supernatant than in the pellet fraction from cells were cultured for 48 h in medium containing increasing concentrations of copper. Moreover, the copper content remained constant at about 7 μmol and 17 μmol/g DW in the supernatant and pellet fractions of all cultured cells, respectively, regardless of the copper concentration in the growth medium (Figure 3b).

Washing cells in stationary culture with EDTA removed most of the cellular copper, leaving a constant amount of copper (20–25 μmol/g DW; Figure 3b) regardless of the copper sulfate concentrations in the medium. Furthermore, after cellular disruption and fractionation the soluble fraction (mainly cytosol) was equal to about 50% or less of the pellet fraction in all of EDTA-washed cells (Figure 4b). Consequently, when exposed to medium containing 6 mM copper, the estimated amount of copper in the soluble fraction represented only 10% of the total content.

Melanin content

Fungal melanins that can bind metal (Gadd & de Rome 1988) are located in the cell wall and *Y. lipolytica* produces this brown pigment. To

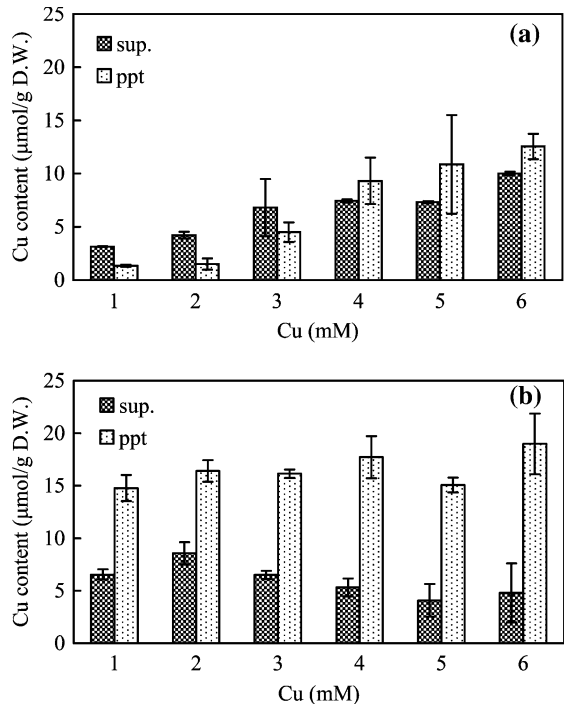

Figure 4. Distribution of copper to *Y. lipolytica* cell wall, membrane debris and cytoplasm. Cells were cultured in medium containing various concentrations of copper sulfate at 30°C for (a) 24 and (b) 48, washed with EDTA as in Figure 3, disrupted and separated into supernatant (sup) and pellet (ppt) fractions. Bars indicate standard deviation of 3 experiments. See Materials and methods for details.

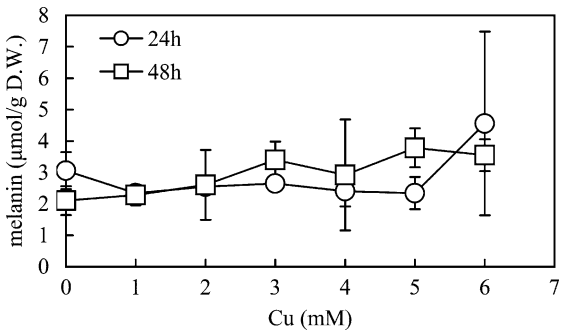

Figure 5. Melanin content of *Y. lipolytica*. Cells were cultured in medium containing various concentrations of copper sulfate at 30 °C. Bars indicate standard deviation of 3 experiments. See Materials and methods for details.

determine whether the cellular distribution of copper is affected by melanin, we measured the pigment content of *Y. lipolytica* cells grown in medium with or without copper (Figure 5). The melanin content did not increase in cells cultured in medium containing in copper for 24 h or in stationary phase cells at 48 h of culture. However, washing with EDTA released large amounts of copper from cells cultured with elevated copper concentrations (Figure 3b). Metal is initially adsorbed through a rapid and passive process involving reversible binding to negatively charged anionic sites in the cell wall. The *Y. lipolytica* cells grown in medium without copper for 48 h adsorbed only 3 μmol Cu/mg DW, when dipped in medium containing 2 mM copper and immediately removed from culture (data not shown). Therefore, the large amounts of copper released from cells by washing with 100 mM EDTA seemed to be gradually accumulated during growth if melanin contributes to its deposition or accumulation at the cell surface. Approximately 20% of total melanin was also liberated from cells by washing with 100 mM EDTA, together with the release of copper ions (data not shown). These results indicated that melanin is constitutively synthesized, and that the intracellular level is sufficient to bind large amounts of metal.

Superoxide dismutase (SOD) activity

We examined the role of SOD in the copper resistance of yeast. Table 1 shows that the amounts of SOD activity were 11.25 and 8.23 units/mg protein at 24 and 48 h, respectively. By contrast, when grown in medium containing 2 mM copper sulfate that does not inhibit cell growth, SOD activities at 24 and 48 h increased to 61.35 and 35.05 units/mg protein representing about 6- and 4-fold increases respectively, compared with the control. Furthermore, the increased SOD activity in cells cultured in the presence of copper was obviously diminished when 1 mM potassium cyanide was added to the enzyme reaction mixture. These results indicated that copper ions stimulate the activity of Cu, Zn-SOD, but not of Mn-SOD.

Discussion

Fungi have developed various survival mechanisms against toxic concentrations of metal ions. The present study found that a mutant strain of *Y. lipolytica* could thrive in high concentrations of copper sulfate. Furthermore, the amount of copper accumulated by *Y. lipolytica* increased with increasing copper concentrations in the medium. Many reports have detailed the mechanism of copper accumulation by fungi, particularly *S. cerevisiae*. With increasing amounts of copper in the external environment, the copper uptake system in *S. cerevisiae* is repressed by a transcription factor (Mac1p) (Yamaguchi-Iwai *et al.* 1997; Jensen & Winge 1998) that represses the *CTR1* gene encoding a high affinity copper transporter (Dancis *et al.* 1994). However, when copper ions further increase in the environment, they accumulate via a low affinity transport system (Yu *et al.* 1996). Therefore, when *Y. lipolytica* is cultured in medium containing elevated concentrations of copper, metal ions might enter the cells.

The present investigation showed that the intracellular copper content of *Y. lipolytica* progressively increased in direct correlation with

Table 1. SOD activity in *Y. lipolytica*.

	SOD activity	Unit/mg protein
Culture (h):	24	48
Control	11.2	8.3
+1 mM KCN	7.0	7.1
2 mM Copper Sulfate	61.6	35.1
+1 mM KCN	1.3	5.4

Cells were cultured in medium with or without 2 mM Copper Sulfate.Vales are means of two separate experiments.

increasing copper content in the medium. However, the cellular copper content in the cytosol and cell membrane of stationary phase cultures remained constant even in the presence of 6 mM copper. Furthermore, about 30% of the cellular copper in cells washed with EDTA was localized in the supernatant fraction. As a result, the estimated cytosolic copper content represented only 10% of the total amount of metal accumulated by *Y. lipolytica* cells cultured in medium containing 6 mM copper. One report has shown that the highest concentrations of heavy metals such as cadmium or nickel retained by dimorphic *Y. lipolytica* are located in the cell wall and membrane debris whereas the concentrations are lowest in the cytoplasm (Strouhal *et al*. 2003). However, that study did not morphologically determine whether mycelial-, yeast-like, or mixed populations of cells were responsible for the accumulation.

Riggle and Kimamoto (2000) reported that *Candia albicans* utilizes a copper-inducible P1-type ATPase (transporter) as the primary mechanism of copper resistance. Ramezani Rad *et al*. (1994) have also identified a putative P-type copper-transporting ATPase gene (*PCA1*) in *S. cerevisiae*. We found that a mutation of the *PCA1* gene confers cadmium resistance upon *S. cerevisiae* (Shiraishi *et al*. 2000). The fact that low levels of copper ions were maintained in the cytosol of *Y. lipolytica* cells is probably due to an inherent metal efflux system, which is induced by metal concentrations over a specific threshold.

Mutant *Y. lipolytica* cells cultured with copper accumulated metals near or at the cell wall. One of these mechanisms would be precipitation at the cell wall or the formation of copper complexes by cell wall components (Kierans *et al*. 1991; Yu *et al*. 1996; Suresh & Subramanyam 1998). We measured the content of melanin, an extracellular polymeric pigment with metal binding ability in the cell wall of *Y. lipolytica* (Gadd & de Rome 1988; Fogarty & Tobin 1996). The production of pigment by *Y. lipolytica* is independent of copper, which is a tyrosinase co-factor (Carreira & Loureiro 1998). We found that the melanin content did not increase in cells cultured in medium containing copper. However, large amounts of copper were loosely bound during growth, indicating that a high copper concentration induced an efflux mechanism. However, the copper was then deposited by entrapment rather than by adsorption in the periplasm and other membranes. This evidence indicates that melanin contributes to metal deposition at the cell wall (Fogarty & Tobin 1996). Moreover, another function of melanin in copper detoxification might be to prevent copper from entering cells (Butler *et al*. 1989).

When *Y. lipolytica* was cultured in medium containing copper, the activity of Cu,Zn-SOD, but not of Mn-SOD was enhanced. Copper ions stimulate SOD activity in *Candida famata* and in *S. cerevisiae* (Naiki 1980; Sarais *et al*. 1994; Manzano *et al*. 2000) and SOD-defective mutants are very sensitive to copper toxicity (Lapinskas *et al*. 1995; Lee *et al*. 1996). Therefore, we postulate that *Y. lipolytica* can avoid damage from toxic reactive oxygen species generated by transition metal ions such as copper.

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