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# The Effect of the *SOD2* and *SOD3* in *Candida albicans* on the Antioxidant System and its Potential as a Natural Antioxidant*

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## Abstract

Oxygen is necessary to sustain life, but reactive oxygen species (ROS) produced by oxygen metabolism can cause mutations and toxicity. ROS can damage cellular macromolecules, leading to oxidative stress, which can accelerate cell death and aging. ROS generated in food affect the taste, color, and aroma of food, and high levels of ROS in meat can cause spoilage. Superoxide dismutase (SOD) plays an important role in scavenging ROS in food and reducing their toxicity to organisms. SOD exerts its antioxidant effect by catalyzing the breakdown of $O_2$-• to $H_2O_2$. As a natural antioxidant, SOD has the ability to regenerate and maintain its activity over a long period of time without depletion, unlike chemical antioxidants that may have side effects or stability issues. This antioxidant effect of SOD has great potential in a variety of industries, and in the food industry it can be utilized to improve product quality and provide safe and healthy products to consumers. By disrupting the SOD2 and SOD3 genes in *Candida albicans*, we studied the effects of SOD2 and SOD3 genes on the antioxidant system, suggesting its potential as a natural antioxidant.

**Keywords:** Reactive oxygen species, Superoxide, Superoxide dismutase, *Candida albicans*

**Major Classifications**: Food industry, Food biochemistry, Probiotics, Food Science **(**Food Nutrition, Healthy Food)

## 1. Introduction

Oxygen is an essential element for life, but oxygen metabolism can lead to mutations and toxicity due to the generation of reactive oxygen species (ROS) (Buonocore et al., 2010). ROS are highly reactive oxygen-containing products that can cause oxidative stress by damaging cellular macromolecules that are highly reactive in normal cellular metabolism due to their high oxygen content (Bafana et al., 2011). They contain unpaired electrons and can react with other molecules, making them unstable and leading to chain reactions (Menvielle-Bourg, 2005). Specifically, free radicals can accelerate aging by damaging DNA, essential proteins in cells, and inducing cell death (Wu & Cederbaum, 2003).

ROS generated in food can cause oxidation and spoilage, affecting the taste, color, and aroma of food. High levels of ROS in meat can lead to quality degradation, loss of protein functionality, and depletion of essential amino acids (Falowo et al., 2014). Superoxide dismutase (SOD) plays a critical role as a natural antioxidant in removing ROS, such

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as superoxide (O2--), from foods and mitigating toxicity to organisms (Liang et al., 2023). SOD catalyzes the breakdown of O2-- to hydrogen peroxide (H2O2) (Younus, 2018) (Fig. 1.). Unlike chemical antioxidants, which may raise concerns about side effects or stability, natural antioxidants such as SOD have an inherent regenerative ability, suggesting that SOD can maintain catalytic activity for extended periods of time without depletion (Gogliettino et al., 2022). SOD is found in a variety of organisms and may have different forms and functions in each organism or tissue (Wang et al., 2018). In general, SOD is classified into three major different forms, of which SOD 2 is a manganese-dependent enzyme found in mitochondria and plays a role in protecting mitochondria by neutralizing radicals within them (Palma et al., 2020). SOD3 is an enzyme found primarily in the extracellular matrix and plays an important role in protecting cells from external oxygen radicals (Fattman et al., 2003).

Due to its antioxidant effects, SOD has significant potential in various industries, including food, pharmaceuticals, cosmetics, and agriculture. With the increasing interest in healthy foods, the demand for natural antioxidants has increased, highlighting the need for natural antioxidants alongside concerns about chemical antioxidants. In addition, the addition of antioxidants to foods can increase the body's antioxidant capacity, suggesting that antioxidants may not only improve the stability of foods but also have positive effects on the body. Therefore, research and technological development are needed in the food industry to improve product quality, provide consumers with safe and healthy products, and reduce food waste. *Candida albicans* has a mold-like shape and usually forms spherical cells. This fungus has cell walls (Kojic & Darouiche, 2004). *C. albicans* is a useful strain for studying its ability to adapt to various stressful situations for survival because it has the ability to adapt well to changes in the environment and grow (Davis, 2003). Therefore, in this study, we investigated the effects of *C. albicans* strains on the antioxidant system by destroying the *SOD2* and *SOD3* genes and suggested its potential as a natural antioxidant.

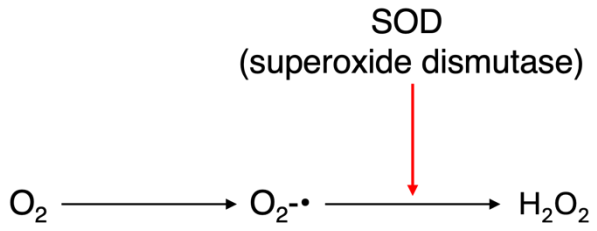


**Figure 1:** SOD catalyzes the destruction of the $O_2$-• free radical.

## 2. Materials and Methods

### 2.1. Organisms and Culture Conditions

The *C. albicans* strains and plasmids used in this study are listed in Table 1. The strains were primarily cultured at 30 °C on YPD (1 % yeast extract, 2 % peptone, 2 % D-glucose) medium. Cells carrying plasmids or disrupted genes were cultured in synthetic D-glucose (SD) medium containing 0.67 % yeast nitrogen base without amino acids (Difco), 2 % D-glucose, and appropriate supplements. Solid media were prepared by adding 1.8 % agar to liquid broth. *Escherichia coli* DH5*α* was used for most plasmid construction and maintenance, and cells of this strain were grown at 37 °C in Luria-Bertani medium (LB) supplemented with 100 mg/ml ampicillins.

**Table 1:** Strains, primers, and plasmids used in this study.

| Strain, primer, or plasmids | Genotypes, sequence, or description | Source or reference |
|---|---|---|
| Strain | | |
| *C. albicans* | | |
| SC5314 | Wild type isolate | Fonzi and Irwin, 1993 |
| CAI4 | *Δura3::imm434/ Δura3::imm434* | Fonzi and Irwin, 1993 |
| HY204, homozygous *Δsod2* | *Δura3::imm434/ Δura3::imm434 Δsod2::hph/Δsod2::hph* | This study |
| HY304, homozygous *Δsod3* | *Δura3::imm434/ Δura3::imm434 Δsod3::hph/Δsod3::hph* | This study |
| HY404, heterozygous *Δsod2/Δsod3* | As HY304, but *Δura3::imm434/ Δura3::imm434 Δsod2::hph/Δsod2::hph* | This study |
| Primer | | |
| HJ2a-SacI | 5′-GAGCTCGATTTAAGGAAAATGGAAAGATTA-3′, *SacI* site of pYJ2F | This study |
| HJ2b-KpnI | 5′-GGTACCCTTTAATGTCAATTGAAGTGAAAA-3′, *KpnI* site of pYJ2F | This study |
| HJ2c-SalI | 5′-GTCGACGTCAAGTACATATTTAAATCCAAT-3′, *SalI* site of pYJ2R | This study |
| HJ2d-HindIII | 5′-AAGCTTGATACCTATTTCAAGAAACCCTAC-3′, *HindIII* site of pYJ2R | This study |
| HJ3a-SacI | 5′-GAGCTCAAGAGGTTAAACAATATCTACTGG-3′, SacI site of pYJ3F | This study |

| | | |
|---|---|---|
| HJ3b-KpnI | 5′-GGTACCCAGAGAACTGGCAATGGGCTTTTA-3′, *KpnI* site of pYJ3F | This study |
| HJ3c-SalI | 5′-GTCGACGTGCGCCAAAAAAACAAAAAAATA-3′, SalI site of pYJ3R | This study |
| HJ3d-HindIII | 5′-AAGCTTTTTCTGGTTCGATTTCCAATAACG-3′, HindIII site of pYJ3R | This study |
| Plasmid | | |
| pQF181 | pUC18 containing *hph-URA3-hph* (forward) from pQF86 | Hwang *et al*., 2003 |
| pQF182 | pUC18 containing *hph-URA3-hph* (reverse) from pQF86 | Hwang *et al*., 2003 |
| pYJ2F | pGEM-T Easy vector containing HJ2a-HJ2b fragment, upstream region of *SOD2* | This study |
| pYJ2R | pGEM-T Easy vector containing HJ2c-HJ2d fragment, downstream region of *SOD2* | This study |
| pYJ3F | pGEM-T Easy vector containing HJ3a-HJ3b fragment, upstream region of *SOD3* | This study |
| pYJ3R | pGEM-T Easy vector containing HJ3c-HJ3d fragment, downstream region of *SOD3* | This study |

## 2.2. Disruption of *C. albicans SOD2* and *SOD3*

The *SOD2* and *SOD3* genes were disrupted as described previously (Fonzi and Irwin, 1993) with some modifications. The plasmids and primers used are shown in Table 1. The 3.5 kb *BamH*I fragment containing an *hph-URA3-hph* disruption cassette isolated from pQF86 (Feng et al., 1999) was ligated with *BamH*I, yielding pQF181 and its reverse construct pQF182, which can be excised with *Sac*I/*Hind*III (Hwang et al., 2003). *SOD2* and *SOD3* were disrupted using the URA blaster method using pQF181 and its derivative pQF182, respectively, to remove the 705 bp *SOD2* and 681 bp *SOD3* coding region (Feng et al., 1999; Fonzi & Irwin, 1993; Hwang et al., 2003). The 524 bp *Sac*I/*Kpn*I (HJ2a-HJ2b) and 366 bp *Sal*I/*Hind*III (HJ2c-HJ2d) digestion fragments from the pYJ2F and pYJ2R of the upstream/downstream flanking regions of the SOD2 open reading frame (ORF) were inserted into the SacI/KpnI and *Sal*I/*Hind*III sites of hph-URA3-hph from pQF181 or pQF182, respectively. Likewise, the 534 bp *Sac*I/*Kpn*I (HJ3a-HJ3b) and 382 bp *Sal*I/*Hind*III (HJ3c-HJ3d) digestion fragments from the pYJ3F and pYJ3R of the upstream/downstream flanking regions of the SOD3 ORF were inserted into the *Sac*I/*Kpn*I and *Sal*I/*Hind*III sites of *hph-URA3-hph* from pQF181 or pQF182, respectively (Fig.2.). The completed construct was digested with *Sac*I/*Hind*III, transformed into CAI4 (Ura3− derivative of wild-type strain SC5314) (Fonzi & Irwin, 1993), and selected by Ura3 maker gene. Spontaneous Ura3− derivatives of the heterozygous disruptants were selected on SD medium containing 5-fluoroorotic acid (625 μg/ml) and uridine (100 μg/ml) (FoA SD medium). This procedure was repeated once more to generate a homozygous *sod2/sod2* mutant strain (HY204) , *sod3*/*sod3* mutant strain (HY304), and *sod2*/*sod3* mutant strain (HY404).

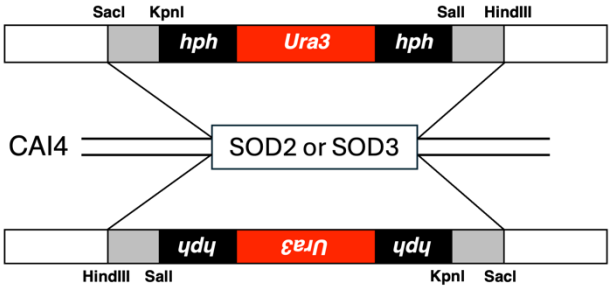


**Figure 2:** Schematic representation of the disruption strategy. Restriction map of the SOD2 and SOD3 locus and insertion of the his-Ura3-his cassette into the indicated *SacI*/*HindIII* sites of the SOD2 coding sequence.

## 2.3. Activity Staining for Detecting *SOD2* and *SOD3*

The activity staining was performed some modifications (Hwang et al., 2002). The crude extract of *C. albicans* cells was prepared by vigorously creaking the cell suspension with glass beads for 10 seconds, followed by resting on ice for 15 seconds, repeated 5 times, in 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM EDTA. The protein concentration in the crude extract was determined using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, USA), with bovine serum albumin as the reference protein. 50 μg of total protein was loaded onto an 8% native polyacrylamide gel (PAGE) in which SOD activity was detected by negative staining (Manchenko, 1994). The gel was incubated in 50 mM phosphate buffer (pH 7.5) for 10 minutes, in nitro blue tetrazolium solution (1 mg/ml) for 10 minutes, and then in 50 mM phosphate buffer (pH 7.5) containing riboflavin (0.2 mg/ml) and N, N, N', N'-tetramethylethylenediamine (10 mg/ml) for 15 minutes at 30°C with gentle shaking. Bands were observed in areas of SOD activity, but no reaction occurred in areas where activity was lost.

## 3. Results and Discussion

ROS damage cells, causing oxidative stress that accelerates aging. To prevent this, SOD, the primary intracellular antioxidant, exerts its antioxidant effect by catalyzing the decomposition of O2-•, one of the ROS, to hydrogen peroxide. To investigate the effects of SOD2 and SOD3 on the antioxidant system in *C. albicans*, we sequentially disrupted SOD2 and SOD3 using the Ura3 maker gene. Therefore, PCR amplicons were generated using upstream primers (HJ2a/HJ2b and HJ3a/HJ3b) and downstream primers (HJ2c/HJ2d and HJ3c/HJ3d) of SOD2 and SOD3, respectively. Each amplicon was inserted into the pGEM-T Easy vector for screening with the ampicillin resistance gene to generate pYJ2F, pYJ2R, pYJ3F and pYJ3R. The HJ2a-HJ2b and HJ2c-HJ2d digestion fragments from the pYJ2F and pYJ2R of the upstream/downstream flanking regions of the SOD2 ORF and HJ3a-HJ3b and HJ3c-HJ3d digestion fragments from the pYJ3F and pYJ3R of the upstream/downstream flanking regions of the SOD3 ORF were inserted into the *SacI*/*KpnI* and *SalI*/*HindIII* sites of *hph-URA3-hph* from pQF181 or pQF182, respectively. The completed construct was digested with restrict enzyme *SacI*/*HindIII*, transformed into CAI4 (Ura3− derivative of wild-type strain SC5314) (Fonzi & Irwin, 1993), and selected by Ura3 maker gene (Fig 1). Spontaneous Ura3− derivatives of the heterozygous disruptants were selected on FoA SD medium. This procedure was repeated once more to generate single disruption mutants HY204 and HY304, and double disruption mutant HY404.

To confirm the disruption of SOD2 and SOD3 in HY204, HY304 and HY404, the activity of SOD was checked using cell crude extract. The activity of SOD was confirmed by staining with N,N,N,N',N'-tetramethylethylenediamine and riboflavin on native PAGE. The results showed that the active sites of SOD2 and SOD3 were not stained, confirming that they were completely disrupted (data not shown).

When SOD is disrupted, cells may have a greatly reduced ability to protect against oxidative damage. SOD plays an important role in neutralizing harmful superoxide radicals produced in cells, preventing them from reacting with other biological molecules to cause cellular damage and protecting cells by reducing oxidative stress (Wang et al., 2018). A deficiency of SOD can lead to increased oxidative damage, cell damage and cell death in cells exposed to oxidative stress. Specifically, a deficiency of SOD can lead to increased damage to the delicate components of cells, such as DNA, proteins, and lipids (Chaves & Silva, 2012). This can interfere with the physiological function of the cell, triggering cell death and reducing the function of the tissue or organ.

SOD is a natural antioxidant that is important in food, pharmaceuticals, cosmetics and agriculture, and with the increasing demand for healthier foods, there is growing concern about chemical antioxidants. Adding antioxidants to foods can improve the body's antioxidant capacity, which can have a positive impact on health. Therefore, the potential of SOD as a natural antioxidant supports research and technology development in the food industry to improve product quality, provide safer products and reduce food waste. Therefore, in order to explore the potential of SOD as a natural antioxidant in real food, future research aims to further study the molecular mechanism of SOD to develop new antioxidant therapy based on it, evaluate the stability and efficacy of SOD in different food matrices to investigate its applicability in real food, and the effect of SOD on the nutritional value of food as well as food stability.

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