

## Induction of Superoxide Dismutase Activities and Ethanol Resistance by Sodium Chloride and Lead Acetate in Yeast *Candida* sp. Y390

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### ABSTRAK

**Aktivitas Induksi Superoksida Dismutase dan Resistensi Etanol oleh Sodium Klorida dan Plumbum Asetat pada Khamir *Candida* sp. Y390.** Untuk mengetahui sifat khamir *Candida* sp. Y390 dalam melindungi diri dari cekaman oksidatif, telah dilakukan riset kajian toleransi sel terhadap etanol dan hubungannya dengan enzim superoksida dismutase (SOD) pada khamir tersebut. Viabilitas sel yang ditumbuhkan pada media gliserol dan diinkubasi pada etanol 17,5 % selama 1 jam adalah  $0.30 \pm 0.09$  %, pada media gliserol dan 11,7 % natrium klorida adalah  $1.65 \pm 0.5$  % sedang pada media giserol dan 1 ppm plumbum asetat adalah  $1.16 \pm 0.7$  %. Aktivitas CuZnSOD pada sel yang diinduksi oleh natrium klorida atau plumbum asetat turun sedikit, yakni masing-masing 1,7 dan 1,9 kali. Aktivitas MnSOD pada sel yang diperlakukan dengan natrium klorida naik sedikit (sekitar 1,03 kali), namun pada sel yang diperlakukan dengan plumbum asetat naik secara menyolok (sekitar 3,6 kali). Data ini menunjukkan bahwa metabolisme etanol dapat menyebabkan *Candida* sp. Y390 mengalami cekaman oksidatif. MnSOD khamir mungkin berperan dalam melindungi sel dari kerusakan akibat cekaman oksidatif.

**Keywords:** *Candida* sp. Y390, yeast, ethanol metabolism, oxidative stress, SOD.

### INTRODUCTION

One of ethanol toxicity mechanisms could be correlated with the productions of reactive oxygen species (ROS) (Chance *et al.* 1979; Moradas-Ferreira *et al.* 1996). Costa *et al.* (1997) reported that in yeast *Saccharomyces cerevisiae*, Mn superoxide dismutase (Mn-SOD) was essential for ethanol tolerance but not Cu/Zn-SOD. However, De Freitas *et al.* (2000) and Park *et al.* (1998) revealed that other strain of *S. cerevisiae*,

Cu/Zn-SOD played an important role in protecting yeast cell from oxidative stress. In human or mammalian cells, principle of oxygen toxicity and its protection mechanisms is similar with that of yeast (Costa *et al.* 1997). Furthermore, yeast *Saccharomyces cerevisiae* can be used as a model of human cell (Cardenas *et al.* 1999) because of the similarity of calcineurin in yeast and human cells, since the partial steps of apoptosis of mammals cells could be reproduced in yeast, and the

easy manipulation of lipid in yeast, Manon (2004) proposed that yeast may be a powerful tool to understand of the role of lipid oxidation in programmed cell death in both mammals and yeast. We reported that various yeast isolated from several sources in Indonesia showed different of ethanol sensitivity and vitamin E, an antioxidant, in reducing death cells caused by ethanol (Yulinery & Julistiono 2003). However, we could not observe the activity of SOD in protecting cell from mortality caused by ethanol which is partially due to oxidative stress. Decreasing of ethanol sensitivity through SOD activity induction in *Candida* sp. Y390 is an important phenomenon indicating that one ethanol toxicity mechanism in *Candida* sp. Y390 is due to oxidative stress and its SOD is the enzyme that involve in defense system against oxidative stress.

In order to understand the activity of one of enzymic antioxidant defenses system in *Candida* Sp. Y390, we studied ethanol tolerance and CuZnSOD and MnSOD activities induced by heavy metals Pb and NaCl. Heavy metal and osmotic stress could induce oxidative stress in both yeast *S. cerevisiae* (Wisnicka *et al.* 1998; Koziol *et al.* 2005) and mammalian cell (Kitiyakara *et al.* 2003), as a result of redox imbalance state.

## MATERIALS AND METHODS

### Yeast strain

Yeast *Candida* sp. Y390 was obtained from LIPI Microbial Collection,

Microbiology Division, Research Center for Biology, LIPI Bogor. The yeast was isolated from the soil of oil spill contaminated area. Based on its viability to ethanol 17.5 %, *Candida* sp. Y390 was the most sensitive to ethanol compared with 19 yeast strains obtained from several sources in Indonesia (routine screening, unpublished data).

### Growth condition

Yeast was grown in glycerol medium (3.2 mM Glycerol, 0.3 % yeast extract, 0.4 % peptone, 0.95 mM  $\text{KH}_2\text{PO}_4$ , 0.6 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.07 mM  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.07 mM  $\text{MnSO}_4$ , 0.07 mM  $\text{CuSO}_4$ , pH 7. For Sodium chloride or lead cell treatment, 11.7 g/l Sodium chloride or 1 ppm lead acetate respectively was added, before inoculation. Cells were grown in erlenmeyer with volume 300 ml containing 100 ml medium, at room temperature, in an orbital shaker at 120 rpm. Experiments were performed when cultures reached 48 h of incubation. Data are expressed as mean values of at least three independent experiments.

### Living cell population measurement

Viability test was performed with pour plate method. Cells were grown on YA (3.0 g yeast extract, 5.0 g peptone, 20 g agar, 10 g glucose, 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 g  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , in 1 L distilled water) at 30 °C.

### Assay of cell viability treated with Ethanol

About  $9 \times 10^6$  cells were incubated in 9 ml ethanol 17.5 % for 1 hour at room

temperature with gentle shaking. After the incubation, 0.1 ml of cell suspension was then inoculated to the agar media for living cell counting. Cell viability was the ratio of living cells treated with ethanol and control.

### Lysate Preparation

After grew in glycerol medium, or glycerol with sodium chloride, or glycerol with lead acetate, cells were centrifused and the pellet was washed with aquadest. Then the pellet was put into small tube, which contained two third of glass bead then subjected to cell disruption by vigorous shaking for 1 hour at interval 4 min. and 1 min. on ice. Cells debris was separated by microcentrifugation. Supernatants were collected and retained at 4°C until analysis. Proteins were assayed by a method developed by Bradford (1976), using bovine serum albumin as a standard protein.

### Determination of Superoxide Dismutase Activity

SOD activity was estimated according to Winterbourn *et al.* (1975).

Mn-superoxide dismutase were measured by incubating protein fractions in reaction mixture consisted of 0.1 M ethylene diamine tetraacetic acid (EDTA) containing 0.3 mM sodium cyanide 0.2 ml, 1.5 mM nitroblue tetrazolium (NBT) 0.2 ml, 0.12 mM riboflavin and 0.067 M  $K_2HPO_4$  (pH 7.8). The reactions tube was placed in a light box with a 25 Watt bulb providing uniform light intensity. The tubes were incubated for 5-8 minutes. At zero time,

0.05 ml 0.12 mM riboflavin was added. Under these conditions, riboflavin was excited by a photon, and was able to oxidize an electron donor. This electron donation resulted a superoxide molecule ( $O_2^-$ ). The  $O_2^-$  molecule is able to reduce the NBT, giving an insoluble purple formazan. This color change can be measured spectrophotometrically at  $A_{560}$ . All tubes were incubated in the light box for 12 minutes and at timed intervals read  $A_{560}$ . Total (Mn and Cu/Zn-) SOD activity was measured by using the same procedure but with out sodium cyanide. Cu/Zn-SOD activity was calculated by the difference between total SOD activity and Mn-SOD activity. One unit of SOD is defined as the amount of enzyme necessary to produce a 50% inhibition of the maximum value of inhibition of NBT.

## RESULTS

### Effect of sodiutm chloride or lead acetate on viability of cell incubated in ethanol

Viability of cell previously grown with media containing 11.7 g/l sodium chloride or 1 ppm lead acetate incubated in ethanol 17.5 % for 1 hour is presented in Table 1.

Table 1 showed that viability of cell treated with high concentration of in ethanol was higher than that of untreated cell.

### Effect of sodium chloride or lead acetate on SOD activities

Activities of SOD in cell treated with sodium chloride or lead acetate

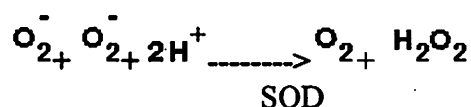
are presented in table 2.

Table 2 shows that the activity of salt slightly induced MnSOD was in contrast the activity of Cu-ZnSOD decreased slightly.

**DISCUSSION**

There are two kinds of SOD in general in yeast (Costa *et al.*, 1997). First, cytosolic Cu-ZnSOD and second mitochondrial MnSOD. When SOD is responsible for increasing ethanol tolerance in *Candida* sp Y390 in this

study, there was not appropriate data supporting Cu-ZnSOD as an enzyme which is responsible for ethanol tolerance. However, Mn-SOD could be an important enzyme that involve in protecting cell from oxygen toxicity. SOD is the first defense against the toxic intermediates by oxygen. It accelerates superoxide dismutation reaction as follow:



**Table 1.** Viability of cell previously grown with media containing Sodium chloride incubated in ethanol 17.5 % for 1 h (Values are mean ± SD of 5 cell cultures in each group )

Treatment	Cell viability (%)
Glycerol	0.30 ± 0.09
Glycerol+ 11.7 g/l NaCl	1.65 ± 0.5 *
Glycerol+ 1 ppm Pb acetate	1.16 ± 0.7 *

\* Significant, compared to untreated cell culutre (glycerol), p<0.05

**Table 2.** Activity of SOD enzyme in cell treated with Sodium chloride (Values are mean ± SEM of 3 cell cultures in each group)

Treatment	Enzyme Activity (unit/mg lysate protein)	
	Cu-ZnSOD	MnSOD
Glycerol	1.67 ± 0.1	1.91 ± 0.01
Glycerol+ 11.7 g/l NaCl	0.97 ± 0.08 *	1.97 ± 0.01**
Glycerol+ 1 ppm Pb acetate	0.86 ± 0.3 *	6.90 ± 0.1 **

\* and \*\* Significant, compared to untreated cell culutre (glycerol), p<0.05

Induction of MnSOD in *Candida* sp. Y390 followed with increasing of ethanol tolerance might indicate that ethanol toxicity is also correlated with the productions of reactive oxygen species (ROS). Moradas-Ferreira *et al.*, (1996) found that the role of SOD in protecting cell from ethanol toxicity in yeast *S. cerevisiae* was similar to that of mammalian cell in protecting from reactive oxygen species (Chance *et al.*, 1979). Furthermore, our study found that ethanol induced lipid peroxidation in *Candida* sp. Y390 (paper in preparation). Peroxidized lipid is an indicator of oxidative stress in cell. Moreover, Hernández *et al.* 2000 found that high concentration of salt is also responsible for induction oxidative stress in plants.

Induction pattern of lead acetate was similar to that induced by Sodium chloride. However, lead remarkably increased the MnSOD (about 3.6 fold). Metals and oxygen are chemically linked in biological systems. Metals and oxygen play important roles in enzymatic reactions, metabolism, and signal transduction; while react to form highly toxic oxygen-derived free radical species. Presence of heavy metal in human cell may produce reactive oxygen species. Marcusson *et al.* (2000) reported that the oxidative metabolism and, in particular, superoxide dismutase may perturbed in mercury-intolerant patients. Finding by Yang *et al.* (1997) indicated that oxidative stress caused by cadmium in human fetal lung fibroblasts followed with increasing of SOD activity.

This data supported suggestion that ethanol might be able to kill cell through

ROS production and MnSOD might play an important role in protecting cells from oxygen toxicity. Costa *et al* (1997) clearly found that ethanol tolerance is independent of cytosolic CuZnSOD but dependent of mitochondrial MnSOD. ROS Oxygen is produced in mitochondrion where respiration takes place. However, it has been found that yeast mutants deficient in cytosolic superoxide dismutase CuZnSOD are hypersensitive to ferrous iron. In contrast mutants that are deficient in catalases and cytochrome c peroxidase do not differ from the standard strain in this respect. These findings suggest that iron toxicity may depend on the redox status of the cell and the role of superoxide dismutases in preventing the toxic effects of oxygen (Wisnicka *et al.*, 1998). Presence of cytosolic Cu-ZnSOD and its regulation in *Candida albicans* was also reported by Gunasekaran *et al* (1998)

Weak induction of MnSOD could be caused by too high concentration of salt. In rat, high-Sodium chloride diets (2 and 4% NaCl) increased oxidative stress in the vasculature and kidney and induced kidney glomerulosclerosis and microalbuminuria (Dobrian *et al.*, 2002).

## CONCLUSION

This study indicated that ethanol metabolism in *Candida* sp Y390 might produce ROS and MnSOD which is inducible enzyme that plays a major role in protecting cells from oxidative damage. However, role of Cu-ZnSOD could not be excluded. Such physiological character of *Candida* sp

Y390 could be a basic reason to use the yeast as a model cell for studying the properties of traditional herbal medicine having antioxidant activity in living cells.

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