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Superoxide Dismutase Activity and Ethanol Respiration in a Fungi Resistance to Ethanol *Monascus* sp. MM

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ABSTRACT

Monascus sp. MM was a contaminant fungus isolated from museum specimen preserved with ethanol 70 %. In order to verify role of superoxide dismutase (SOD) in protecting cell from ethanol toxicity during ethanol metabolism, SOD activities of *Monascus* sp.MM and a *Monascus* sp. NGK, which was isolated from fermented red rice (angkak), were compared. When fungus was grown with glucose, Cu/Zn-SOD activity of *Monascus* sp., MM was 7.1 times of that of *Monascus* sp. NGK. Whereas in ethanol medium, Cu/Zn-SOD activity of *Monascus* sp. MM was 24.6 times of that in *Monascus* sp. NGK. Induction of Cu/Zn-SOD *Monascus* sp. MM by ethanol was not observed. Compared with Mn-SOD, activity of Cu/Zn-SOD was markedly important (10 times of Mn-SOD when fungi grown with ethanol; 12 times when the fungi grown with glucose). The data indicated that Cu/Zn-SOD might play an important role in protecting cell from ethanol toxicity during ethanol metabolism. Ethanol respiration rate of *Monascus* sp. MM was also important since O_2 consumption and ethanol degradation rates were clearly higher than that of *Monascus* sp. NGK.

Keywords: *Monascus* sp., superoxide dismutase, respiration, ethanol resistance.

INTRODUCTION

Monascus sp. MM, was isolated as a contaminant fungi of museum specimen preserved in 70 % ethanol from Zoological Museum Bogoriense, Bogor - Indonesia. Reinoculation of the fungi on shrimp preserved with ethanol 70 % has been successfully done (Suharna & Rahayu, 2000). This extreme resistance of *Monascus* sp. MM to ethanol rises questions of how the fungal cell is protected from ethanol toxicity.

Based on study of 60 different cell types of bacteria, yeast, fungi, plant and animal, Jones (1989) suggested that biological effects of ethanol are reflection of ethanol metabolism rather than ethanol *per se*. Roles of NAD/NADH imbalance, acetaldehyde accumulation, the activation of replication processes are considered to be the central disorder mechanisms. Cells with Alcohol Dehydrogenas (ADH) and Aldehyde Dehydrogenas (AlDH) which are acetaldehyde tolerant are suggested to be ethanol tolerance. Membrane unsaturation that influences the resistance to autolysis or deactivation of replication has been also proposed as a mechanism for ethanol tolerance.

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Ethanol toxicity is also correlated with the productions of reactive oxygen species (ROS) (Chance *et al.*, 1979; Moradas-Ferreira *et al.*, 1996). Costa *et al.*, (1997) showed that in *Saccharomyces cerevisiae*, Mn superoxide dismutase (Mn-SOD) was essential for ethanol tolerance but not Cu/Zn-SOD. However, recently De Freitas *et al.* (2000) revealed that *S. cerevisiae* lacks of Cu/Zn-SOD (SOD) showed a series of defects, e.g. reduced rates of aerobic growth in synthetic glucose medium and reduced ability to grow in glycerol-rich medium. This indicated that SOD1 played an important role in protecting yeast cell from oxidative stress.

Superoxide dismutase was discovered in bovine red cells by McCord and Fridovich and can be classified as Cu/Zn-SOD, Mn-SOD, and Fe-SOD (McCord & Fridovich, 1969). So far, eukaryot cell contains SOD1 and SOD2 whereas Fe-SOD is exclusively found in prokaryot cell. The aim of this study was to investigated the role of SOD as one of mechanisms against ethanol toxicity in *Monascus* sp. MM. We analyzed activities of SOD1 or SOD2 in ethanol tolerant fungi *Monascus* sp. MM and *Monascus* sp. NGK, which was isolated from angkak. (Angkak or red rice are known as a fermented product which use for food and drink colorant in Far East Asia) as a control species. In addition, we also compared ethanol toxicity in both species of them as expressed by the rates of oxygen consumption and ethanol metabolism.

MATERIALS AND METHODS

Monascus strains and growth conditions

The *Monascus* sp. MM and *Monascus* sp NGK, are considered to be two different species based on morphological characteristic (Suharna, 1999). *Monascus* sp. NGK was used as a control species). The fungi were grown on glucose media containing 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, 0.01 g amphicilin, 10 ml trace elements solution, in 1 liter distilled water, at room temperature. Solution of trace elements consisted of 5 g $ZnSO_4.7H_2O$, 3 g $MnSO_4$, 2,8 g $CuSO_4$, in 250 ml distilled water). After 15 days of incubation, fungal biomass was harvested for further analysis.

Ethanol degradation rate

Fungal biomass was transferred from growth media to 40 ml ethanol media media containing 20 % ethanol (1,6 g KH_2PO_4, 1.6 g KNO_3, 0.8 g KCl, 0.8 g $MgSO_4.7H_2O$, 1 drop of $FeCl_3$ 28%, 10 ml trace elements solution, ethanol 20 %, in 1 l distilled water) in a 100 ml Erlenmeyer tube. After 2, 3, 4, and 5 days supernatants were collected for ethanol measurement. Ethanol content was analyzed in Gas Chromatograph Shimadzu 14B using Porapak Q column and FID detector. Temperature of column, injector, and detector were, 170°C, 190°C, and 200°C respectively. Pressure of nitrogen, hydrogen, and air were 300 k Pa, 70 kPa and 50 kPa respectively.

Oxygen uptake analysis

Harvested fungal biomass was homogenized in media containing ethanol with mortar. Homogenates were dissolved in media containing 1, 2, 3, 4, 5, and 6 % of ethanol. Oxygen consumption rate was measured with Dissolve Oxygen Meter Horiba. Dry matter assays were done after oxygen measurement. Fungal homogenates was filtered and dried at 105 °C for 24 h.

SOD (EC. 1.15.1.1) activity

Harvested fungal biomass was biomass was homogenized with mortar. Then the homogenate was put onto 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 were separated by microcentrifugation. Supernatants were collected and retained at 4°C until analysis. Fungal biomass homogenization and cell disruption were done on ice.

Proteins were assayed by Bradford (1976) method, using bovine serum albumin as a standard protein. SOD activity was estimated according to Winterbourn *et al.* (1975) and is based on the ability of superoxide dismutase to inhibit the reduction of nitro-blue tetrazolium (NBT) by superoxide. Into a series of cuvettes, 0.2 ml of 0.1 M Ethylene diamine tetraacetic acid (EDTA) containing 0 mM or 0.3 mM potassium cyanide (cyanide inhibits Cu/Zn-SOD but has no effect on the Mn-SOD), 0.1 ml of 1.5 mM NBT, 50 µl, or 100 µl, or 200 µl of sample, and 0.067 M Potassium phosphate buffer, pH 7.8 q.s. to 3 ml were mixed. The cuvette tubes were incubated in a box approximately 4' long X 8" X 6" with an internally mounted 18 W fluorescent bulb, Phylip for 10 minutes. Photoreaction was started by addition of 0.05 ml of 0.12 mM Riboflavin. Tubes were incubated in the light box for 7-9 minutes. Absorbency was then read at λ 560nm at 1 minute interval period. The percent inhibition versus amount of required enzyme was then plotted to determine the percent inhibition of NBT reduction. One unit is defined as that amount of required enzyme to inhibit half of the maximum inhibition of NBT reduction.

All Experiments were repeated three times.

RESULTS AND DISCUSSION

SOD activity

As shown in Table 1, activity of Cu/Zn- SOD in *Monascus* sp. MM grown with glucose was not significantly different from that of *Monascus* sp. MM grown with ethanol. This indicated that in this condition ethanol might not induce SOD activity. Whereas activity of Cu/Zn-SOD in *Monascus* sp. NGK grown with glucose was higher than that of *Monascus* sp. NGK grown with ethanol. However, Cu/Zn- SOD activity of *Monascus* sp. MM was remarkably higher than that of *Monascus* sp. NGK (7.1 times for fungi grown with medium glucose; 24.6 times for fungi grown with medium ethanol).

Activities of Mn-SOD in both *Monascus* sp. MM or NGK were presented in Table 2. Mn-SOD activity of *Monascus* sp. MM or *Monascus* sp. NGK grown with ethanol was slightly higher than that of both *Monascus* sp. grown with glucose.

Quantitative comparison between Cu/Zn-SOD activity, and Mn-SOD activity suggested that Mn-SOD might play a minor role in protecting cell from oxidative stress provoked by ethanol metabolism. Since Cu/Zn -SOD activity of *Monascus* sp. MM was remarkably higher than that of Mn-SOD (approximately10 times in media ethanol, 12 times in media glucose), we therefore suggest that Cu/Zn-SOD might have important role in ethanol resistance. Our suggestion is in accordance with Ma *et al.* (1998) who reported that Cu/Zn-SOD extracted from the red cells of healthy human, increased the recovery of hemopoietic stem cell stored at 4°C. Beside ethanol metabolism, a number of

factors are thought to be involved in ROS generation including low temperature (Ma *et al.*, 1998; Park, *et al.*, 1998), xenobiotic compounds (Lauterburg, *et al.*, 1983). In addition, De Freitas *et al.*, (2000) and Avery *et al.* (2000) showed role of Cu/Zn-SOD in protecting cell from ROS caused by similar phenomenon in *Saccharomyces cerevisiae*.

Ethanol respiration rate

Oxygen consumption rate may express the physiological state at mitochondrial level. Figure 1 showed the effect of ethanol on oxygen consumption rate in *Monascus* sp. MM or NGK. As presented in Figure 1, oxygen consumption rate increased when ethanol concentration increased. However, *Monascus* sp. MM appeared more resistance to ethanol than *Monascus* sp. NGK since oxygen consumption rate of *Monascus* sp. MM was clearly higher than that of *Monascus* sp. NGK. Furthermore, oxygen consumption rate in *Monascus* sp. NGK began to decrease when concentration of ethanol reached 5 % level. On the contrary, oxygen consumption rate in *Monascus* sp. MM still increased slightly at the same concentration of ethanol.

Table 1. Activities of Cu/Zn- SOD in *Monascus* sp. MM or *Monascus* sp. NGK

Monascus sp.	Cu/Zn-SOD activity (U/mg protein)	
	Medium Glucose	Medium Ethanol
MM	0.3817 ± 0.0723	0.3847 ± 0.0838
NGK	0.0538 ± 0.0047	0.0156 ± 0.0051

Table 2. Activities of Mn-SOD in *Monascus* sp. MM or NGK

Monascus sp	Mn-SOD activity (U/mg protein)	
	Medium Glucose	Medium Ethanol
MM	0.0312 ± 0.0057	0.0380 ± 0.0068
NGK	0.0217 ± 0.0040	0.0284 ± 0.0001

Oxidative utilization of ethanol exhibits secondary effects due to the products and consequences of ethanol metabolism, i.e., elevated levels of acetaldehydes and acetate with decreased levels of ATP and NAD and consequent loss of TCA function (see review of Jones, 1989; Cederbaum *et al.*, 1974; Thayer, 1989). However, the decreasing or slowing down of oxygen consumption rate at 5 % concentration of ethanol might not exclusively as consequence of ethanol

toxicity. Since both *Monascus* could metabolize ethanol 20 % (see Table 3), we should also consider the probable existence of regulation system of enzyme or enzymes involved in ethanol metabolism including TCA cycle, in order to avoid the excessive effect of ethanol metabolism. In a unicellular algae *Euglena gracilis*, unclear or even paradoxical mechanism concerning oxygen consumption rate and TCA cycle function was described by Thuillier-Bruston, *et al.* (1990) and Julistiono (1995). In this microalgae, oxygen consumption rate of cell grown with ethanol was higher than that of cell grown with lactate but the pool of some organic acids of TCA cycle decreased.

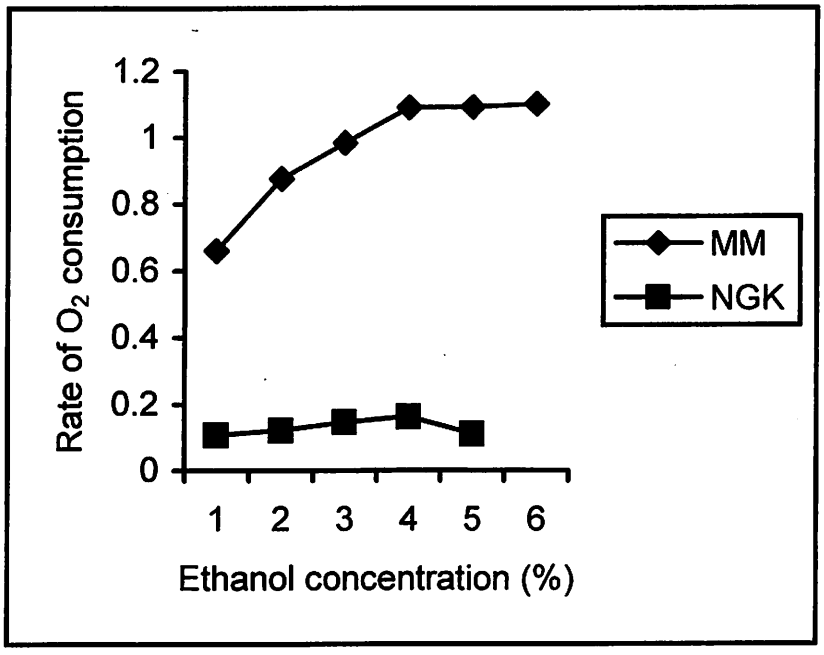

Figure 1. Effect of ethanol concentration on O_2 consumption rate.

Table 3. Rate of ethanol metabolization of *Monascus* sp. MM or NGK grown with ethanol 20%

Monascus sp.	Rate of ethanol degradation (% per dry weight per day)
MM	13.4 ± 2.1
NGK	6.3 ± 2.9

Table 3 showed the rate of ethanol metabolization in *Monascus* sp. MM or NGK. The data indicated that in ethanol 20 %, the function of enzymatic machinery involving ethanol metabolism in both of fungi still existed. Moreover, the rate of ethanol metabolization of *Monascus* sp. MM which is resistance to ethanol was distinctively higher than that of normal *Monascus* sp. NGK.

CONCLUSION

Based on enzymatic activities of *Monascus* sp. MM, Cu/Zn-SOD seem to play an important role in ethanol resistance rather than Mn-SOD. However, we could not exclude the role of Mn-SOD. Induction of Cu/Zn-SOD activity by ethanol was not observed. Ethanol resistance property of the fungi was also expressed by its ethanol respiration rate, which was clearly higher than that of *Monascus* sp. NGK, a fungus isolated from red rice (angkak). These two characters might be the reasons why *Monascus* sp. MM could survive in museum specimen preserved with ethanol 70%.

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