Emir. J. Food Agric. 2013. 25 (10): 790-797
doi: 10.9755/ejfa.v25i10.17004
http://www.ejfa.info/

REGULAR ARTICLE

Induction resistance of *Candida* sp. Y 390 to ethanol stress by kopyor coconut and virgin coconut oil

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Abstract

Coconut has been known to have many advantageous effects on human health and can be used to prevent and protect human body from oxidative damage. The aim of this research was to determine the effect of kopyor coconut and virgin coconut oil (VCO) on cellular defense mechanism against oxidative damage by ethanol stress 17.5% (v/v). The effect of kopyor coconut and VCO addition was analyzed by observing viability of yeast cells (*Candida* sp. Y 390) that had been given ethanol stress. In addition, acitivity of superoxide dismutase (SOD) was also measured. Results showed that addition of kopyor coconut and VCO into growth medium at early incubation stage increased viability of yeast cells under ethanol stress. SOD activity of yeast cells grown on medium containing kopyor coconut or VCO was found to significantly increase. SOD activity of *Candida* sp. Y 390 that given 0.84 g kopyor coconut, 37 µL VCO and 74 µL VCO was 135.66, 122.26 and 157.97 U/mg protein respectively. It was found that activity of MnSOD was higher compared to CuZnSOD enzyme. These results suggest that addition of kopyor coconut and VCO triggers *Candida* sp. Y 390 resistance to ethanol stress by induction of MnSOD activity.

Key words: Kopyor coconut, Virgin coconut oil, Superoxide dismutase, *Candida*, Ethanol stress

Introduction

Research on system of protection against the reactive oxygen species (ROS) in eukaryotic cells increased rapidly. ROS associated with oxidative damage that can lead to various types of degerative diseases and aging in higher organisms. Defense mechanism against the ROS in yeast is similar to cell from higher organisms. Complexity of antioxidant activity and oxidative stress mechanisms can be explained by the use of yeast cells (Moradas et al., 1996). Yeast cells therefore can be used as a model to study the mechanisms of cell resistance to oxidative stress through the induction of superoxide dismutase (SOD) activity. Oxidative stress is a state of unbalance amount prooxidant and antioxidants in the body. Under these conditions, the activity of free radicals or ROS molecules can cause cellular damage or genetic changes that influence the structure and function of cells that eventually leads to state of disease. SOD is one of the enzymes involved in the body's resistance to ROS. The presence of SOD in yeast cells has been proven by many researchers. As reported by Costa et al. (1993), a form of SOD, MnSOD plays an important role in cell defense against ethanol in postdiauxic phase yeast cells. Similar roles are also played by CuZnSOD (Park et al., 1998). Increased prevalence degerative diseases in Indonesia have motivated researchers to explore compounds derived from natural sources that can prevent and protect the human body from oxidative damages.

Indonesia is one of world's largest producers of tropical coastal plants, such as coconut (FAO 1995). Indonesian people have made use of coconut for food ingredient and other purposes. Based on the level of consumption, kopyor coconut is one of the popular coconut varieties (Rethinam, 2006). In addition to the combination of taste, aroma, and texture of the flesh that is more palatable, kopyor coconut also has higher nutrient content compared to common coconut oil. Kopyor coconut has higher mineral and vitamin content, lower fat, but with similar fatty acid content to common coconut oil (Santoso et al., 1998). Traditionally, coconut also

Received 21 March 2013; Revised 20 May 2013; Accepted 27 May 2013; Published Online 24 June 2013

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prepared in the form of virgin coconut oil (VCO) that has been known to have many benefits. VCO is known to have high lauric acid content, approximately 50% the total oil. Saturated medium chain fatty acids or medium chain fatty acids (MCFA) are easily to be arbsorbed by human body because their molecular size is relatively not large. Therefore, it can easily enter the energy metabolism and and is not dumped into fat tissue or cholesterol.

History has proven the consumption of VCO regularly can prevent many diseases, including heart disease, arteroskelorosis, stroke, and other degenerative diseases. However, studies on effects of nutrients in VCO and kopyor coconut on cellular defense mechanisms against oxidative damage have been limited. This research is a preliminary study that was aimed to determine the effect of kopyor coconut kernel and VCO on viability and defense mechanisms of yeast cells under conditions of oxidative stress by ethanol. The hypothesis of this study was that the nutrient content of kopyor coconut and VCO can increase the resistancy of *Candida* sp. Y 390 to the ethanol stress, thereby reducing the level of cell death. This study obtained was expected to provide scientific information on a effects from kopyor cocnut and VCO against oxidative damage at the cellular level thereby supporting the development of new compounds of natural origin for preventing oxidative damages.

Materials and Methods

Materials and equipments

This study using kopyor coconut from Serang, Banten, Indonesia (Figure 1), VCO from Bintang Kelapa Co. Ltd., Jakarta, Indonesia, and *Candida* sp. Y 390, a yeast culture collection of Indonesian Institute of Sciences (LIPI) isolated from oil contaminated-soil in the area of Ledok, Cepu, Central Java, Indonesia.

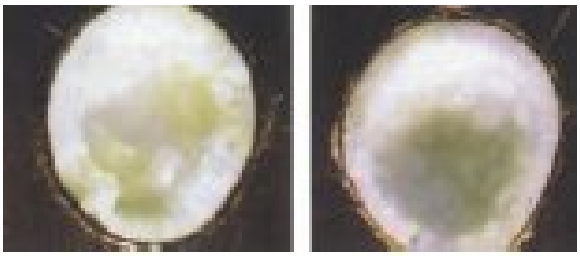

Figure 1. Kernel of kopyor coconut.

Methods

Stages carried out in the study included sample preparation, media preparation, preparation of working culture, observation of effect of kopyor coconut and VCO on yeast cell viability, measurement of SOD activity of *Candida* sp. Y 390 under ethanol stress.

Media preparation

Glycerol liquid medium was prepared by dissolving 2.4 mL of glycerol, 1.3 g KH_2PO_4, 0.15 g $MgSO_4.7H_2O$, 3 g yeast extract, 4 g bacto peptone, and 1 mL of solution X (5 g $ZnSO_4.7H_2O$, 3 g $MnSO_4$, 2.8 g $CuSO_4$ in 250 mL of distilled water) in 1 L distilled water. After all ingredients dissolved completely, the solution was aliquoted to 100 mL to 250 mL in Erlenmeyer flask, weighed, then sterilized by autoclaving for 15 minutes at 121°C and a pressure of 1 atm. Yeast malt agar (YMA) medium was used to grow yeast cells in petri dishes. YMA medium was prepared by dissolving 5 g of bacto peptone, 3 g of yeast extract, 20 g of bacto agar, 10 g of glucose, 0.5 g of $MgSO_4.7H2O$, and 1 g of $K_2HPO_4.3H_2O$ in 1 L distilled water. The mixture was heated during the dissolving process. After all ingredients dissolved completely, the medium was sterilized by autoclaving for 15 minutes at 121°C and pressure of 1 atm. Still in a warm temperature, about 10-13 ml YMA medium was poured into a petri dish aceptically.

Working culture preparation

Working culture was grown in liquid glycerol medium. *Candida* sp. Y 390 of stock culture on agar slant YMA was inoculated aceptically by transferring 2 loops of culture into liquid glycerol medium and then shaken for 48 hours. Culture was rejuvenated using a new liquid glycerol medium regularly.

Sample size determination

The amount of sample tested was determined by measuring the weight of 100 mL of liquid glycerol medium. Kopyor coconut kernel consumption in an adult human was assumed to be 250 grams per day, and consumption of VCO was assumed to be 3 tablespoons (± 22.5 mL) per day for adult human with 60 kg body weight. Example of calculation for the amount of kopyor coconut kernel added to the media was as follows**:** X g = **(**liquid glycerol medium weight x 250 g)/60 kg. Variations of sample size used was 1 x X g and 2 x X g.

Determination of effect of kopyor coconut and VCO on yeast cell viability

Samples at various sizes was added into liquid glycerol medium prior to inoculation with 1 mL culture and then shaken for 48 hours. Cell viability was measured by inoculating 0.1 mL of diluted culture onto plates YMA medium. Yeast cultures

were incubated at 30°C for 2 days. The number of growing colonies was counted or cell density was measured using turbidimeter.

Determination of kopyor coconut and VCO bioactivity

Effect of the addition of kopyor coconut and VCO was determined at various stages of yeast culture incubation, at the beginning of incubation, after 24 hours of incubation, and at the end of incubation. At the beginning of the incubation, sample was added to the 100 ml liquid glycerol medium that has been inoculated 1 ml of yest culture. Culture was then shaken for 48 hours. As much as 1 mL cell suspension was withdrawn and inoculated into 100 mL Erlenmeyer flask containing 9 mL of 17.5% ethanol and shaked for one hour. For viability measurements, 0.1 mL of cell suspension was taken and inoculated on to plates containing YMA medium. Cultures were incubated at 30°C for 2 days. The number of growing colonies was calculated. The same procedure of measurement was carried out on yeast culture after 24 hour of incubation and the end of incubation. Measurement of cell viability was also carried out on yeast grown on corresponding medium without kopyor coconut and VCO supplementation as a negative control.

Preparation of yeast cell lysate

Yeast cells were broken open to release its content. Yeast cell that had been treated and grown two days harvested by centrifugation at 5720 g for 5 min. Supernatant was discarded and pellet was dissolved in 5 mL phosphate buffer and then centrifuged again at the same speed for 5 minutes. Supernatant was discarded and pellet re-dissolved with 2 mL phosphate buffer and then vortexed vigorously until homogeneous. Cell suspension was transferred into Eppendorf tube containing glass beads. Cells were broken open by shaking the mixture for 2 hours using a mini-mixer in a cold temperture (in a refregerator). The mixture was centrifuged using a mini spin at the same speed, and the supernatant was collected to be used for analysis of total protein and SOD activity.

Preparation of standard curve for protein analysis

Standard curve constructed by using BSA solution 1.56 mg/mL and 1.5 mg/mL as the standard solution. BSA solution was put into a test tube in a volume of 10, 30, 50, 70 and 90 mL and then into the tube was added solution of NaCl 0.9% to a total volume of 100 mL per tube. Used as a blank was solution of NaCl 0.9% 100 mL, without the addition of BSA solution. Each tube was added with 2.9 mL Bradford reagent and then shaken with vortex. Before the measurement, the solution was allowed to stand for 5 to 7 minutes. Absorption of the standard solution was measured at a wavelength (λ) of 595 nm.

Total protein analysis

Total protein was determined using the method of Bradford (Bradford, 1976). A total of 100 mL sample was introduced into a test tube and then added with 2.9 ml of Bradford reagent and shaken. The solution was allowed to stand for 5 to 7 minutes. Absorption was measured at a wavelength (λ) 595 nm. If the value is too high, sample was diluted using 0.9% NaCl solution.

Analysis of SOD activity (Winterbourn et al., 1975)

Measurement of SOD activity was based on the ability of SOD in inhibiting NBT reduction by superoxide anion radicals. The reduced NBT forms blue purple formazan complex. The presence of SOD in the sample will inhibit the formation of the color complex. Enzyme activity was measured by placing a sample containing 10 mg of protein into a cuvette containing 67 mL of 0.1 M EDTA for measurement of total SOD or 67 mL of 0.1 M EDTA plus 0.3 mM KCN for measurement of MnSOD. As much as 33 mL of NBT 1.5 mM and 850 mL 0067 M phosphate buffer pH 7.8 were added. The solution in the cuvette was incubated in a light box that uses a 40 watt fluorescent light for 5 minutes followed by addition of 25 mL riboflavin 0.12 mM and incubated again in the box. Absorption measured at λ 560 nm in every 1 minute interval. The measured enzyme activity represented the specific activity, ie the number of enzyme units per milligram of protein, and one unit of enzyme was defined as the amount of enzyme that caused a half-maximum inhibition of the NBT reduction.

Statistical analysis

The data obtained were tested using ANOVA statistical analysis to determine the effect of treatment on the 95% confidence interval.

Results and Discussion

Effect of addition of kopyor coconut and VCO on growth of *Candida* sp. Y 390

These experiments were carried out to determine the effects of kopyor coconut and VCO on growth of *Candida* sp. Y 390 under normal condition after 2 days of incubation on growth medium supplemented with different levels of kopyor coconut or VCO. Results showed that

kopyor coconut promoted growth of *Candida* sp. Y 390 while VCO tended to inhibit growth of this yeast cells. Significant difference of yeast cell density was observed between the negative control and those supplemented with kopyor coconut (Table 1). These data suggest that kopyor coconut does not inhibit growth of *Candida* sp. Y 390. It on the other hand, stimulates growth of *Candida* sp Y 390 which may be due to its nutrient content. Kopyor coconut may enrich growth medium by provision of additional carbohydrate sources as the growth medium used contained only about 0.24% v/v of carbohydrate. In contrast to the effect of kopyor coconut, addition of VCO reduced population of *Candida* sp Y 390 cells (Table 2). The addition of 74 µL of VCO caused a significant decrease in cell density from 2.03 x 10^8 CFU / mL (negative control) to 1.5 x 10^8 CFU / mL. Addition of 37 µL of VCO, however, did not significantly reduce cell population. These results supported the study Pohl et al. (2011) which showed that 1 mM lauric acid, the major fatty acid in coconut oil, inhibits growth of *Helicobacter pylori* the bacterial pathogens that cause stomach ulcers (peptic ulcer) and trigger stomach cancer. In addition, Bergsson et al. (2001) also stated that caprylic acid, a fatty acid found in coconut oil, is effective and act fast in killing *C. albicans in vitro*. Visualization using transmission electron microscopy (TEM) shows the state of the cytoplasm is wrinkled and irregular (disorganized) due to damage of the plasma membrane of *C. Albicans*.

Table 1. Cell density due to the addition of kopyor.

Treatments	Cell density (10^8 CFU/mL)
Negative Control	0.98±0.02
Kopyor, 0.42 g	4.47±0.42*
Kopyor, 0.84 g	12.5±0.5*

*significantly different

Table 2. Cell density due to the addition of VCO.

Treatments	Cell density (10^8 CFU/mL)
Negative Control	2.03±0.15
VCO, 37 µL	1.87±0.15
VCO, 74 µL	1.50±0.10*

*significantly different

Effect of kopyor coconut and VCO on cell viability under ethanol stress condition at initial stage of incubation

Analysis of cell viability was aimed to determine the effect of the addition of VCO or kopyor coconut on cell ability to withstand ethanol stress. The results showed that the viability of *Candida* sp. Y 390 cells increased significantly on medium supplemented with kopyor (Table 3). Increased cell viability indicates that addition kopyor coconut at initial incubation time induces resistance of yeast cells to ethanol stress. Ethanol could lead to the formation of superoxide anion, a form of ROS. Study conducted by Yomes (2006), showed that addition of 17.5% ethanol for one hour caused lipid peroxidation in yeast cells. Damage on the lipid membrane of yeast cells may cause changes in osmotic pressure inside the cell that promotes cell death. The existence of stress is what drives the yeast cells to improve cell defense, one of which may be to increase the activity of enzymes SOD (Winterbourn et al., 1979; Harrison et al., 2005).

Table 3. Cell viability of *Candida* sp. Y 390 with addition of kopyor coconut.

Treatments	Viability (%)
Negative Control	2.09±0.18
Kopyor, 0.42 g	7.07±0.21*
Kopyor, 0.84 g	12.40±0.08*

*= significantly different

Therefore, when yeast cells were treated with 17.5% ethanol for 1 h, the SOD enzyme is ready to convert superoxide radicals into other compounds that do not turn off the cell so that the cell viability increased. The addition of VCO also affects the viability of yeast cells that were subjected to ethanol stress especially at high dose. This study showed that only addition of 74 µL of VCO gives significant improvement of cell viability (Table 4). The mechanism of VCO to increase the viability of yeast cells is not clear and it may be different from that of kopyor coconut since this study also showed that VCO reduced cell viability under conditions without ethanol stress.

Table 4. Cell viability of *Candida* sp. Y 390 with addition of VCO.

Treatments	Viability (%)
Negative Control	1.22±0.18
VCO, 37 µL	1.25±0.34
VCO, 74 µL	3.08±0.22*

*significantly different

Effect of kopyor coconut and VCO on cell viability under ethanol stress condition after 24 hours of incubation

After 24 hours of incubation, viability of the cells grown on medium supplemented with high

dose (0.84 g) of kopyor coconut increased significantly (Table 5). After 24 hours of incubation the addition of 0.24 g of kopyor coconut did not significantly improve cell viability although this dose was effective at initial stage of incubation. This indicated that the abilility of kopyor coconut in protecting cells from ethanol stress last longer when its concentration is increased. The difference in the number of colonies due to the addition of kopyor can be seen in Figure 2.

Table 5. Cell viability of *Candida* sp. Y 390 due to the addition of kopyor after 24 hours incubation.

Treatments	Viability (%)
Negative Control	0.18±0.04
Kopyor, 0.42 g	0.22±0.01
Kopyor, 0.84 g	7.23±0.23*

*significantly different

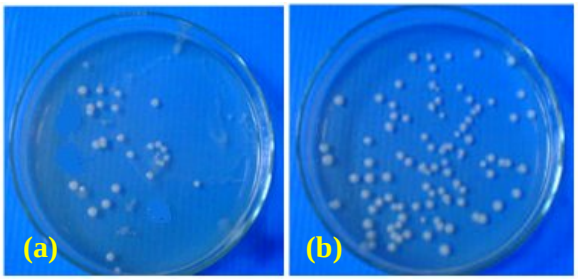

Figure 2. The difference in the number of colonies due to addition of kopyor.
(a) negative control; (b) added kopyor

After 24 hours of incubation, the addition of VCO was not able to increase the value of cell viability in medium with 17.5% ethanol (Table 6). The comparison of cell viability on medium with and without VCO supplementation is shown in Figure 3.

Table 6. Cell viability of *Candida* sp. Y 390 due to the addition of VCO after 24 hours of incubation.

Treatments	Viability (%)
Negative Control	5.08±0.68
VCO, 37 µL	2.73±0.53*
VCO, 74 µL	4.81±0.32

*significantly different

The addition of 37 mL of VCO tended to decrease cell viability after 24 hours of incubation under ethanol stress. Incubation period seemed to influence activity of VCO in protecting cells from ethanol stress. At initial stage of incubation VCO showed ability to improve cell viability.

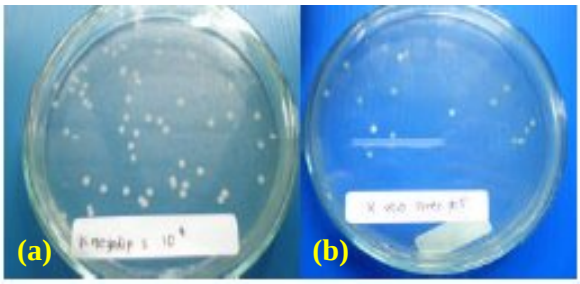

Figure 3. The difference in the number of colonies due to VCO addition.
(a) negative control; (b) with VCO addition

Effect of kopyor coconut and VCO on cell viability under ethanol stress condition after 48 hours of incubation

After 48 hours of incubation, the effect of the addition of kopyor or VCO on yeast viability was measured using turbidimetry since the use of direct plate count was not practical. The optimum wavelength for the measurement was found to be 450 nm (Figure 4). Results showed that under ethanol stress, cell viability was improved by addition of 0.84 g kopyor coconut to the medium. Addition of VCO did not significantly influence cell viability under ethanol stress after 48 hours of incubation (Figure 5).

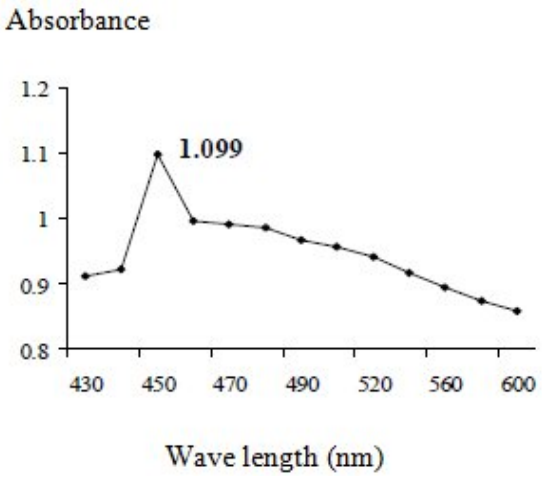

Figure 4. Absorbance of yeast cell at various wavelength.

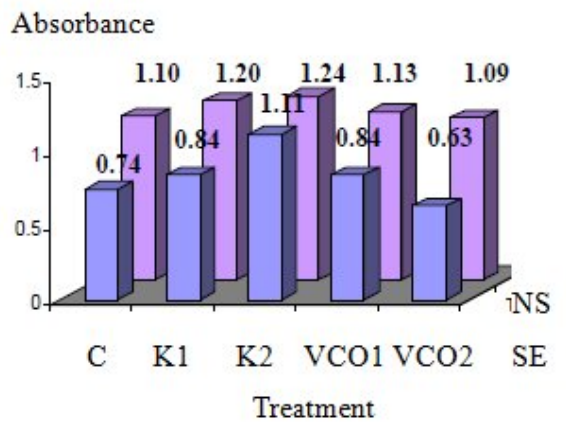

Figure 5. Absorbance value addition of VCO and kopyor.
(C=control, K=kopyor, VCO=virgin coconut oil, SE= stressed ethanol, and NS=Non stressed ethanol).

Effect of kopyor coconut and VCO on SOD Activity under ethanol stress

SOD activity was determined by using the method of Winterbourn et al. (1975), Hwang et al. (2003). Measurement was aimed to determine whether addition of kopyor or VCO influence SOD activity and hence affect the defense response of yeast cells to ethanol stress. The results showed addition of kopyor coconut increased total SOD activity of *Candida* sp. Y 390 (Figure 6). SOD activity of yeast cells grown on medium with o.84 kopyor coconut supplementation was significantly higher.

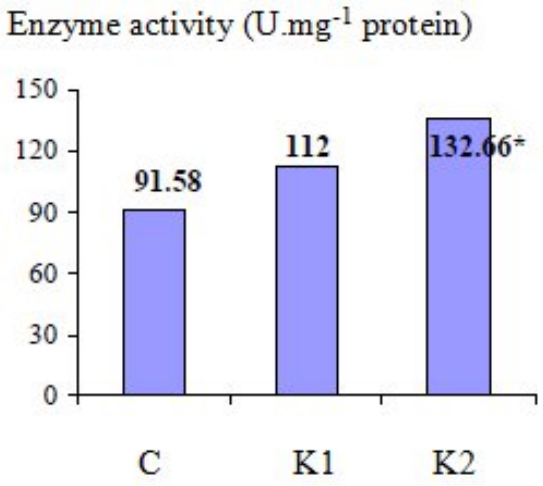

Figure 6. Total SOD activities due to the kopyor addition.
(*= significantly different, C=control, K1=kopyor 0.42 g, and K2=kopyor 0.84 g)

Similar results were observed on MnSOD activity (Figure 6). However, no significant increase in CuZnSOD activity was observed (Figure 7). Overall, MnSOD seemed to play better role in the resistance of cells of *Candida* sp. Y 390 to ethanol stress. The measurement results indicate that MnSOD enzyme activity is much higher than the activity of the enzyme CuZnSOD. The results also support the statement Costa et al. (1997) and Jamieson (1998) that in *Saccharomyces cerevisiae* the cell resistance to ethanol is due to MnSOD activity.

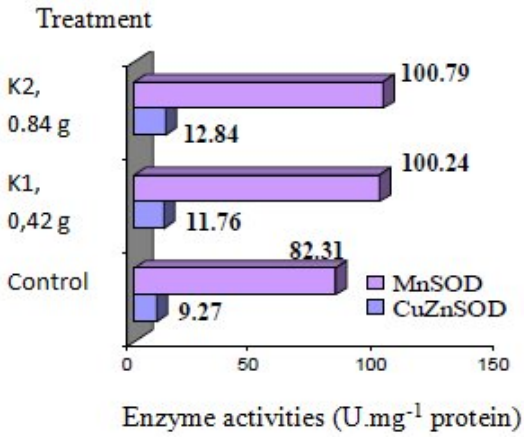

Figure 7. MnSOD and CuZnSOD activities due to addition of kopyor.

The increase in total SOD enzyme was more significant on cells grown on medium with VCO supplementation as presented in Figure 8. Both addition of 37 µL or 74 µL of VCO led to an increase in total SOD activity. Similarly, an increase in MnSOD activity was also observed (Figure 9). On the addition of vco MnSOD enzyme activity of yeast cells increased significantly. The increase in total SOD enzyme activity and MnSOD was higher than in the addition of kopyor treatment.

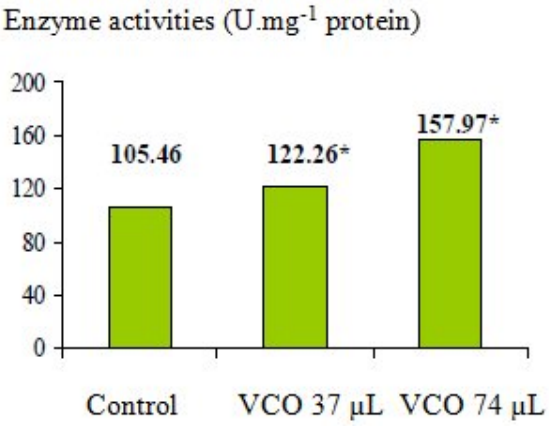

Figure 8. Total SOD activities due to the VCO addition.
(*significantly different)

In the yeast cells grown on medium with VCO supplementation, CuZnSOD activity was also found to be lower than that of MnSOD. Increased CuZnSOD activity was significant with VCO supplementation of 74 µL, while at lower dose (37 µL) no improvement of CuZnSOD activity was observed (Figure 9).

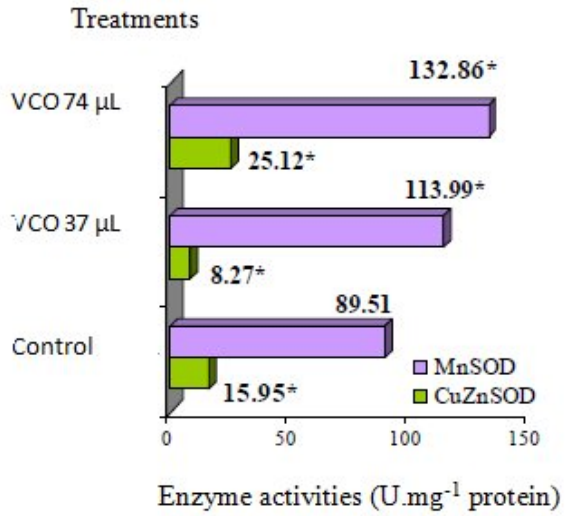

Figure 9. MnSOD and CuZnSOD activities of yeast cells grown on medium supplemented with VCO.

The results indicated that VCO may induce the genes that encode enzymes MnSOD and lead to increase MnSOD formation.

In general, this study showed that yeast cells grown on medium supplemented with an appropriate dose of kopyor coconut or VCO can result in an increase activity of SOD. MnSOD seemed to play a greater role in the resistance of cells to ethanol stress. According to Park et al. (1998) the difference in the role of different SOD enzymes against oxidative stress is dependent on the culture or the physiological state of cells and living creatures themselves.

Conclusions

The addition of kopyor coconut of 0.42 g, 0.84 g, or 37 µL of VCO did not affect the growth of *Candida* sp. Y 390 cells. While the addition of 74 µL of VCO reduced population of yeast cells.

The addition of VCO and kopyor coconut can increase the viability and SOD activity of yeast cells under ethanol stress. Similarly, the addition of VCO and kopyor coconut increase the MnSOD and CuZnSOD enzyme activity significantly.

Based on the results obtained, MnSOD plays a better role in inducing resistance of *Candida* sp. Y 390 cells to ethanol stress.

Acknowledgement

The authors are grateful for the financial support of the Director of Bintang Kelapa Co. Ltd., Indonesia. Thanks also to the Indonesian Institute of Sciences (LIPI), Cibinong, West Java, Indonesia, for kindly providing research materials (especially, *Candida* sp Y 390).

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