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# Production of Beta-Glucan from *Saccharomyces cerevisiae* and Antioxidant Activity on t-BHP Induced Red Blood Cells

# (Produksi Beta-Glukan dari *Saccharomyces cerevisiae* dan Aktivitas Antioksidan pada Sel Darah Merah yang Diinduksi t-BHP)

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**Abstract:** Beta-glucan is a polysaccharide that can be found in cereals, yeast, bacteria, and algae. Beta glucan produced by *Saccharomyces cerevisiae* has the ability to enhance the body's defense system. The study aims to investigate the antioxidant activity of β-glucan produced by three strains of *Saccharomyces sp* BR 1, BR 2, and SC. Antioxidant activity assay was carried out *in vitro* on t-BHP induced red blood cells (RBC) by observing the level of catalase activity, SOD activity and MDA levels as lipid peroxidation product. The investigation was performed on 6 groups: normal control, negative control, positive control (RBC + t-BHP + vitamin E 100 IU), and samples (RBC+t-BHP) treated with 1.4 mg/mL β-glucan from *Saccharomyces* strain BR1, BR2 and SC respectively. *Saccharomyces cerevisiae* strain SC produced the highest β-glucan of 88.70% (glucose equivalent), while the BR1 and BR2 of 22.56% and 46.28% respectively. Antioxidant activity assay showed that the highest increase in catalase activity (581.48%) was given by the β-glucan from SC strain, the highest increase of SOD activity and decrease of MDA levels were given by the β-glucan from BR1 strain (359.17% and 48.18%) as compared to negative controls. Statistical test ($\alpha = 0.05$) showed antioxidant activity of β-glucan from *Saccharomyces sp* BR2 and SC did not differ significantly from the activity of vitamin E (100 IU).

**Keywords:** *Saccharomyces cerevisiae*, β glucans, antioxidant, red blood cells, t-BHP.

**Abstrak:** Beta glukan merupakan polisakarida yang dapat ditemukan pada sereal, khamir, bakteri, dan alga. Beta glukan yang dihasilkan oleh *Saccharomyces cerevisiae* memiliki kemampuan meningkatkan sistem pertahanan tubuh. Penelitian ini bertujuan untuk menguji aktivitas antioksidan β-glukan yang dihasilkan oleh tiga galur *Saccharomyces sp* yaitu BR1, BR2, dan SC. Uji aktivitas antioksidan dilakukan secara *in vitro* pada sel darah merah (SDM) yang diinduksi t-BHP dengan cara mengamati tingkat aktifitas katalase, aktifitas SOD, dan kadar MDA hasil peroksidasi lipid. Percobaan dilakukan pada 6 kelompok, yaitu: kontrol SDM normal, kontrol negatif, kontrol positif (+ vitamin E 100 IU), dan perlakuan dengan 1,4 µg/mL β-glukan dari masing-masing *Saccharomyces sp* BR1, BR2, dan SC. *Saccharomyces sp* galur SC ternyata menghasilkan β-glukan tertinggi sebesar 88,70% (ekivalensi glukosa), sedangkan galur BR1 dan BR2 masing-masing sebesar 22,56% dan 46,28%. Hasil uji aktivitas antioksidan menunjukkan bahwa peningkatan aktifitas katalase tertinggi (581,48%) diberikan oleh β-glukan dari *S. cerevisiae* SC, peningkatan aktifitas SOD dan penurunan kadar MDA tertinggi diberikan oleh β-glukan dari *Saccharomyces sp* BR1 (359,17% dan 48,18%) dibandingkan dengan kontrol negatif. Uji statistik ($\alpha = 0,05$) menunjukkan aktifitas antioksidan β-glukan *Saccharomyces sp* BR2 dan SC tidak berbeda nyata dengan aktifitas vitamin E (100 IU).

**Kata kunci:** *Saccharomyces cerevisiae*, β glukan, antioksidan, sel darah merah.

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## INTRODUCTION

POLYSACCHARIDES have been used extensively in food industry and medicine as an active ingredient or additive. Polysaccharide sources still rely on materials derived from plants or algae, with various disadvantages in term of supply and quality of raw materials. Production of polysaccharides derived from microbes can serve as a credible alternative, among others, derived from yeast. An advantage in producing polysaccharides from microbes is not affected by crop failure, climatic conditions, or pollution. Some of the polysaccharides produced by microbes to include: dextran, alginate, levan, fosfomanan, xanthan gum, and β-glucan[1].

Beta glucan can be produced by plants and microbes. One of microbes capable of producing β-glucan is *Saccharomyces cerevisiae* that is normally used in bread or "Indonesian tape". Beta glucan is homopoly-saccharides composed of glucose molecules. The glucose molecules on β-glucan is associated with β-glycosidic bond to form the polymer main chain. The wall of Saccharomyces cerevisiae cells contain two types of β-glucan, i.e. β-1,3-glucan and β-1,6-glucan. It has been reported that β-glucan can enhance the process of phagocytosis and interleukin formation in humans and animals, two processes that play an important role in the immune system. The benefits of β-glucan is essential to increase the body's defense system and to reduce the level of cholesterol in blood[2].

Blood consists of two parts, namely blood cells and blood plasma. Elements of blood cells namely red blood cells, white blood cells, and platelets suspended in plasma. Blood has a very important role in the immune system and oxygen transport. About 2% of oxygen consumed in the process of breathing will experience an incomplete reduction process to become reactive oxygen derivatives. Reactive oxygen derivatives can cause damage to cell components including proteins, DNA, and fat. To counter the oxidative attack, cells have enzymatic and non-enzymatic immune systems. The enzymatic immune system, to include glutathione reductase, superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH)[3].

Intake of oxygen into the body will activate the NADPH oxidase enzyme found in cell membranes and react to form $O_2$• radicals. Two molecules of $O_2$• radicals spontaneously reacts with 2H+ to form $H_2O_2$ catalyzed by cytoplasmic superoxide dismutase. $O_2$• and $H_2O_2$ are both oxidants, but the $H_2O_2$ is converted into $H_2O$ and $O_2$ by catalase enzyme:

$$NADPH + H+ + 2O_2 \rightarrow NADP+ + 2H+ + 2O_2\bullet$$

$$2O_2\bullet + 2H+ \rightarrow H_2O_2 + O_2$$

$$H_2O_2 \rightarrow H_2O + O_2$$

SOD in blood cells, is a form of cellular enzymatic defense against superoxide radicals ($O_2$• ). Increasing the amount of free radicals in the body will reduce the SOD activity. Malondialdehyde (MDA) is the product of lipid peroxidation as a result of excessive free radicals. Catalase is an enzyme that plays a role in catalyzing the decomposition of H2O2 and other peroxides into water and oxygen molecules[4,5]. Many factors causing the activity of these enzymes are insufficient, such as various foods additives or colourings and air pollutions; therefore intake of antioxidant substances into the body is necessary, which can maintain or rejuvenate the level of enzyme activities in cells. One of these antioxidant substances is β-glucan.

The study aims to determine the ability of β-glucan produced by three strains of *Saccharomyces sp*, as antioxidants in maintain ing the level of enzyme activity in blood cells. Parameters measured are catalase enzyme activity, SOD (superoxide dismutase) activity, and the levels of MDA (malondialdehyde) in sheep red blood cells which have been induced oxidative stress in vitro with t-BHP.

## MATERIALS AND METHODS

**MATERIALS.** *Saccharomyces cerevisiae*, local strains of BR1, BR2 and SC were obtained from Research Center for Biotechnology-LIPI; Sheep blood was obtained from the Faculty of Medicine University of Indonesia; tetra ethoxy propane (TEP) reference standard; vitamin E standard solution; t-butyl-hydroperoxide (t-BHP) solution; general chemicals were from E. Merck or Sigma; bovine serum albumin (BSA); thio barbituric acid (TBA) solution; trichloro acetic acid (TCA) solution; glucose reference standard.

**METHODS. Regeneration of *Saccharomyces sp.*** *Saccharomyces sp* in the study consists of 3 lines, namely BR1, BR2 and SC. One ose of each line of *Saccharomyces sp* from stock cultures, was inoculated into sterile regenera-tion medium compose of (w/v): 1% yeast extract, 2% peptone, 2% glucose, and 2% agar in a petri dish.

Cultures were incubated at 30°C for 2 days. One colony of each line was isolated and inoculated into similar medium in a test tube, then incubated at 30°C for 2 days.

**Characterization of *Saccharomyces sp*.** An ose culture of *Saccharomyces sp* BR1, BR2 and SC was wiped over one drop of water on an object-glass, fixation, then add safranin solution, leave for 2 minutes for complete absorbtion of the dye, and then rinsed with water and dried. Morphology of each *Saccharomyces sp* cell line was observed under a microscope with 1000x magnification.

**Production and extraction of β-glucan.** An amount of 1% fresh suspension of each *Saccharomyces* culture (OD~1) was inoculated aseptically into 150 mL of fermentation medium which composed of (w/v): 1% of yeast extract, 1% of peptone, and 2% of glucose. Subsequently the cell culture was incubated in an incubator-shaker at 150 rpm at room temperature for 5 days[5].

After 5 days, the above cell culture was extracted to obtain β-glucan as the followings: *Saccharomyces sp* cell culture was centrifuged at 6000 rpm at 15°C for 15 minutes. The supernatant was discarded, and the biomass was hydrolyzed with 15 mL of 0.75M solution of sodium hydroxide in a water bath for 6 hours at 75°C. Then subsequently suspension was centrifuged at 6000 rpm at 15°C for 15 minutes, then the supernatant were discarded. Biomass were washed with 15 mL of 0.5 M acetic acid heated at 50°C, and then were centrifuged at 6000 rpm at 15°C for 20 minutes, and supernatant were discarded. The washing step were carried out three times. The biomass then rinsed with water and centrifuged at 6000 rpm at 15°C for 15 minutes, then the supernatant was discarded. Rinsing was carried out twice. Finally, the bio-mass was added with 5 mL of ethanol and then centrifuged at 6000 rpm at 15°C for 5 minutes.

The supernatant was discarded while the biomass was collected for further investi-gation. Each biomass was dried in an oven at 50°C for approximately two days and then weighed as dry weight of crude β-glucan. Preparation of test solutions.

Sample of each crude β-glucan from *Saccharomyces sp* BR1, BR2, and SC was disolved in 0.75 M NaOH solution for the analysis of β-glucan levels (measured and calculated as glucose concentration). Glucose reference standard was used for the purpose. The solution was used also for the determination of protein concentration.

**Assay of β-glucan by HPLC[6].** Calibration curve was prepared as follows: ±10 mg of glucose reference standard dissolved in distilled water up to 10.0 mL. The solution was diluted to obtain dilution series of 200 ppm, 160 ppm, 120 ppm, 80 ppm, and 40 ppm. Half mL of each glucose standard solutions was diluted with 0.5 mL of concentrated sulphuric acid, heated on a boiling waterbath for 4 hours, then was analyzed with HPLC. Typical analysis condition as follows: Column Bondapak-carbohydrate (300 mm x 3 mm), mobile phase was water-acetonitrile (40:60), flow rate was 1 mL/min, detector was Refractive Index Detector (RID) and injection volume was 50 µL.

The data were calculated to obtain a regression line equation and correlation coefficient, to observe the relationship between concentration of reference standards or samples and peak areas in chromatograms. Precision test was carried out by five times 50 µL injections of 60 ppm of glucose reference standard solution into the HPLC, then the standard deviation and relative standard deviation were calculated.

One mL of each β-glucan test solution was added with 1.0 mL of concentrated sulfuric acid, then heated on a boiling water bath for 4 hours. Half milliliter of the test solution was added with 0.5 mL of distilled water and homogenized, and then analyzed by HPLC.

Subsequently, each sample concentration was calculated (as glucose concentration) by extrapolation of the sample peak area to the regression line equation.

**Preparation of blood substrates[7].** Some 10 mL of sheep blood was centrifuged at 3000 rpm at 5°C for 5 minutes to separate the plasma from the blood cells. Plasma will be used for the analysis of MDA levels.

Five mL of the above blood cells, washed with 15 mL of phosphate buffered saline solution (PBS) and then centrifuged at 3000 rpm at 5°C for 5 minutes. Washing was carried out three times. Red blood cells (RBC) will be used for the analysis of catalase and SOD activity[7]. Experiment was carried out on six groups of RBC or plasma accordingly (Table 1).

**Table 1. Experimental grouping.**

| Group | | Treatment |
|---|---|---|
| I | normal control | RBC or plasma only |
| II | negative control | RBC or plasma + t-BHP |
| III | positive control | RBC or plasma + vit E 100 IU + t-BHP |
| IV | test group $BR_1$ | RBC or plasma + 1.4 ug/mL β-glucan from $BR_1$ + t-BHP |
| V | test group $BR_2$ | RBC or plasma + 1.4 ug/mL β-glucan from $BR_2$ + t-BHP |
| VI | test group SC | RBC or plasma + 1.4 ug/mL β-glucan from SC + t-BHP |

**Measurement of MDA levels[8].** As much as 10.0 µL, 20.0 µL, 40.0 µL, 60.0 µL, and 80.0 µL of tetra ethoxy propane (TEP) solutions (1:80000) each was added with 250 µL of distilled water. 1.25 mL of 20% TCA, 0.5 mL of 0.67% TBA, and then homogenized. The mixture was heated for 30 minutes in aboiling-waterbath and was quickly cooled. Absorption of the solution was measured at $\lambda = 532$ nm. Concentration of TEP standard solutions and the corresponding absorptions were plotted to construct TEP standard curve, and the regression line equation was calculated.

A half milliliter of plasma was added 0.5 mL of each β-glucan solution, then incubated at room temperature for 15 minutes. The solution was then added with 1.0 mL of t-BHP solution and incubated at room temperature for 15 minutes, then centrifuged

for 5 minutes at 3000 rpm. A number of 0.25 mL of the supernatant was treated similarly as the TEP standard solution, and the absorption was measured at $\lambda = 532$ nm.

**Measurement of catalase activity[9].** A volume of 1.0 mL of red blood cells (RBC) was added with 1.0 mL of β-glucan test solution, then incubated at room temperature for 15 minutes. One milliliter of the mixture was added with 1.0 mL of t-BHP solution and incubated at room temperature for 15 minutes, then diluted with 2.0 mL of distilled water, then centrifuged for 5 minutes at 3000 rpm.

A volume of 100 µL supernatant from centrifugation (RBC supernatant) added with 1.0 mL of H2O2 0.059 M and 1.9 mL of 0.05 M phosphate buffer pH 7, and then the change in absorbance at 240 nm was recorded every 45 seconds for 2-3 minutes. Catalase activity at 25°C is defined as micromol of peroxide $H_2O_2$ that is consumed per minute per mL of sample. Catalase activity (min/mL) are calculated by using Equation 1:

$$= \frac{[\Delta \text{ Abs/min} \times 1000]}{43{,}6 \times [(\text{mL sample}) / (\text{mL total})]} \times \text{dilution factor}$$

**Measurement of SOD activity[7,9].** The blank in this assay was a mixture of 2900 µL of carbonate buffer (pH = 10.2), 50 µL distilled water, and 50 µL of 0.02 M epinephrin solution. Absorption was measured at $\lambda = 480$ nm in 30°C, one minute, 2, 3, and 4 minutes after mixing.

A volume of 1.0 mL supernatant from centrifugation (RBC supernatant) diluted with 7.0 mL of distilled water. One milliliter of the diluted supernatant was extracted with 1.0 mL of 96% chloroform-ethanol (3:5), then shaken at 2500 rpm for 10 minutes. Then 50 µL of the aqueous phase was mixed with 2900 µL of carbonate buffer pH 10.2 and 50 µL of 0.02 M epinephrin solution.

Absorption was measured at $\lambda = 480$ nm in 30°C, one minute, 2, 3, and 4 min after mixing.

Inhibition (%) was calculated using Equation 2 and SOD activity using Equation 3.

Equation 2

$$= \frac{\Delta \text{ Abs/min (blank)} - \Delta \text{ Abs/min (sample)}}{\Delta \text{ Abs/min (blank)}} \times 100\%$$

Equation 3

$$= \frac{\% \text{ Inhibition} \times \text{dilution factor}}{50\%}$$

## RESULTS AND DISCUSSIONS

**Morphology of *Saccharomyces sp.*** Sample of each *Saccharomyces sp* cells when added with safranin dye will appear pink. All cells of *Saccharomyces sp* BR1, BR2, and SC (reference) have similar oval shapes (Fig. 1).

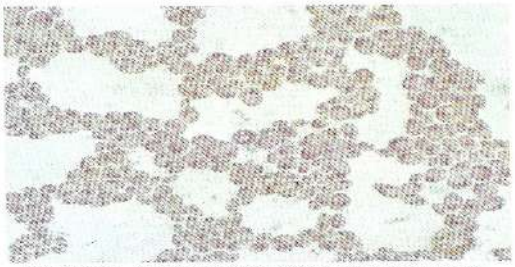

**Figure 1. Cells of *Saccharomyces* sp BR1.**

**The growth of *Saccharomyces sp.*** Observation of the growth phases of each *Saccharomyces sp* BR 1, BR 2 and SC was to determine the best timing for regeneration and harvesting of β-glucan. The growth rates were observed by measurement of optical density at 560 nm.

There were observed from OD values that the phase lag of all Saccharomyces sp were within 4-6 hour incubation period. The logarithmic phase were within 6-18 hours of incubation, and the stationary phase were observed up to 120 hours of incubation.

Therefore, regeneration of *Saccharomyces sp* with OD value ±1.0 were selected at around 10 hours of incubation, the β-glucan extraction from all Saccharomyces sp were performed on the stationary phase after ±120 hours of incubation.

**Dry Weight of Crude β-glucan.** Dry weight of crude β-glucan collected from Saccharomyces sp can be seen in Figure 2.

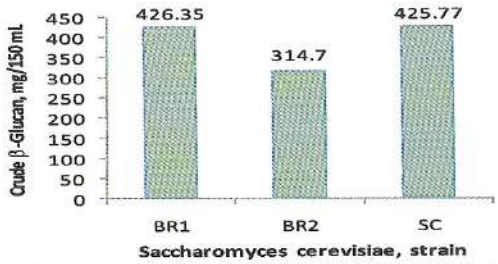


**Figure 2. Average weight of crude β-glucan collected from *Saccharomyces sp* BR1, BR2, SC.**

Figure 2 showed that the highest yield of crude β-glucan was collected from *Saccharomyces sp* BR1 (426 mg/150 mL), then from SC (425.8 mg/150 mL), and from BR2 (314.7 mg/150 mL) respectively.

**Beta-glucan and Protein Concentration.** Beta-glucan is homopolysaccharide composed from various number of glucose molecules. The best method to determine the β-glucan concentration, so far, is by HPLC method with RID detector, using glucose

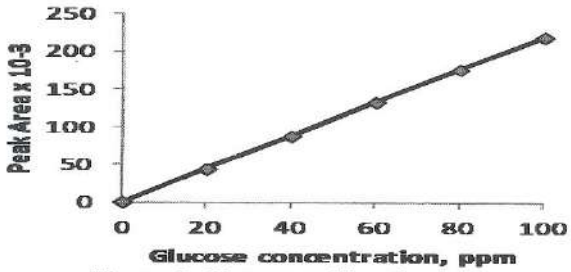


**Figure 3. Glucose calibration curve (y = 113.5528 + 2195.56 x; r = 0.9999).**

refence standard to generate a calibration curve. Precision of the method has been shown with RSD value of 1.15%.

The regression line of glucose standard was y = 113.5528 + 2195.56 x with a correlation coefficient of 0.9999, indicating a good linear relationship between peak areas with glucose concentrations (Figure 3).

Figure 4 showed that the highest β-glucan concentration obtained from *Saccharomyces sp* strain SC (88.70% of the dry weight), then from strain BR2 (46.28%), and BR1 (22.57%).

The presence of protein in a β-glucan product usually was kept as low as possible. Protein concentration was determined by the standard Lowry's method using BSA as reference standard, and absorption of visible light was measured in a spectrophotometer at λ = 540 nm. Results showed that β-glucan extracted from *Saccharomyces* SC has the lowest protein concentration (12.43%), then in β-glucan from BR2 (14.23%), and β-glucan from BR1 (15.47%) (Figure 4).

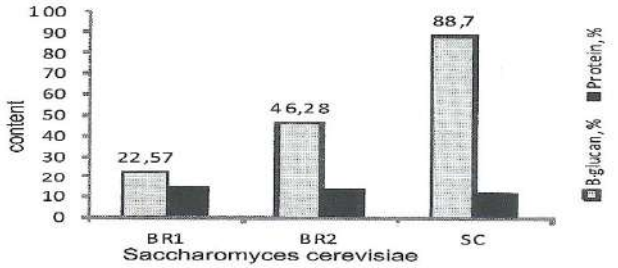


**Figure 4. Average of β-glucan and protein contents in each *Saccharomyces sp BR1*, BR2, and SC .**

**MDA levels.** The presence of free radicals in the body can be characterized by the presence of lipid peroxidation. Measurement of the amount of lipid peroxidation was carried out indirectly as secondary products, such as MDA. This product is formed as a result of reactions between free radicals with polyunsaturated fatty acids and can be measured by

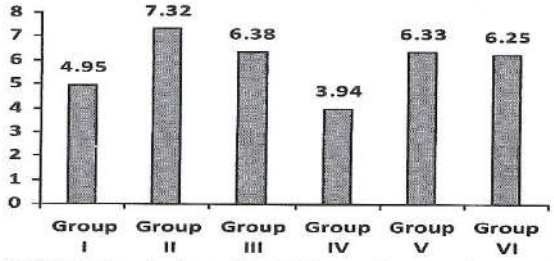


**Figure 5. MDA levels (nmol/mL) in each experimental group.**

spectrophotometry with the TBARS test method at λ 532 nm[8]. The reference standard in the method was tetra ethoxy propane (TEP). Block diagram of MDA measurement on each experimental group are shown in Figure 5.

In normal blood conditions the number of lipid peroxidation in the body is low enough as shown by Group I (Figure 5). With the increasing amount of free radicals, in this case plasma induced by t-BHP, will increase the occurrence of lipid peroxidation, resulting in increased formation of MDA product. Increased levels of MDA was seen in group II. The presence of vitamin E in Group III, as well as the presence of β-glucan in group IV, V, and VI, have been shown to inhibit the occurrence of further lipid peroxidation, therefore lower MDA level than in group II.

Statistical analysis on MDA levels was performed with a nonparametric method of Kruskal-Wallis and Mann-Whitney. Kruskal-Wallis test statistic obtained that P values was < 0.05, thus indicating that there is a significant difference between groups III, IV, V, and VI with group II. Group IV, V and VI showed lower MDA levels with the addition of β-glucan test solution.

**Analysis of Catalase Activity[9].** Analytical results and histogram of catalase activity in each experimental group are shown in Figure 6.

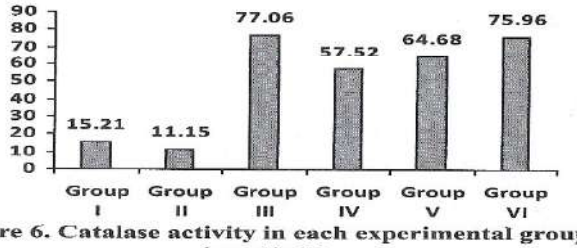


**Figure 6. Catalase activity in each experimental group (μmol/mL).**

Figure 6 shows that catalase activity in group II is lower than in group I, because the blood cells in group II has been induced with the t-BHP, therefore the catalase in the blood cells partly reacted with t-BHP,

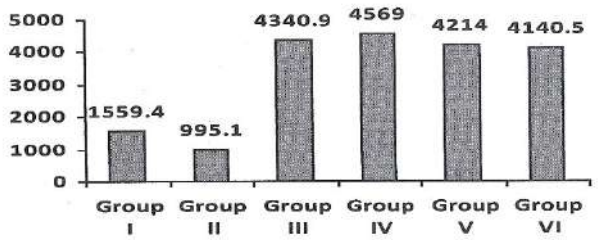


**Figure 7. SOD activity of each experimental group (μmol/mL)**

and the ability of catalase to neutralize peroxide $H_2O_2$ was lower[5]. The red blood cells in group IV, V, and VI with the presence of β-glucan solution (or group II with vitamin E) have significantly increased the catalase activity than in group I or group II, although t-BHP solution had been added afterward.

Catalase activity in group III, IV, V, and VI were 406.6%, 278.2%, 325.2%, and 399.4% respectively, greater than in group I. The Kruskal-Wallis statistical test obtained P values of $< 0.05$, thus indicating that there were significant difference between group I and groups III, IV, V, or VI. Group III, IV, V and VI showed much higher catalase activity in the presence of vitamin E or β-glucan solution than group I.

**Analysis of SOD activity[7,9].** Analytical results and histogram of SOD activity of each experimental group are shown in Figure 7.

The addition of t-BHP into the red blood cells can catalyze autooxidation of epinephrine become adenochrome, thereby the activity of the SOD in inhibiting autooxidation of epinephrine was reduced, as shown in group II.

The presence of β-glucan (in group IV,V,VI) and vitamin E (in group III) as antioxidant with the red blood cells will reverse the autooxidation of epinephrine, and therefore the observed SOD activity were higher than in group I.

The SOD activity in group III, IV, V, and VI respectively were 178.4%, 193%, 170.2% and 165.5% greater than group I. The Kruskal-Wallis statistical test obtained P values of $< 0.05$, indicating that there were significant diference between SOD activity in group I and in groups III, IV, V, and VI. Group III, IV, V and VI showed a higher SOD activity due to the addition of β-glucan solution, whereas group II showed a lower SOD activity due to the presence of t-BHP with the red blood cells.

## CONCLUSION

The highest β-glucan product was obtained from *Saccharomyces cerevisiae sp* SC (88.70%), followed by *Saccharomyces sp* BR2 (46.28%), and *Saccharomyces sp* BR1 (22.56%) (all calculated as glucose monomer). Application of 1.4 mg/mL of β-glucan obtained from these strains of *Saccharomyces* could significantly improve catalase activity or SOD activity in the blood cells, and could inhibit the lipid peroxidation into MDA.

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