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Glutathione from *Saccharomyces cerevisiae* as By-Product of Second Generation Bioethanol from Oil Palm of Empty Fruit Bunch Fiber

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Abstract. Glutathione is an antioxidant agent can be applied in various fields such as pharmacy, cosmetic, foodstuff and another field. The objective of this research is to obtained glutathione from *Saccharomyces cerevisiae* in fermentation waste as by product of second generation of bioethanol (G2). After bioethanol process for 72h, centrifuge method was used to separate the yeast cells from residual fermentation broth of bioethanol. The yeast cells were extracted by maceration method using variation solvent with 25% ethanol solvent, 40% ethanol in water, H₃PO₄ 0.1 M and Phosphate Buffer pH 7.2. Glutathione concentration was tested with alloxan method using Spectrophotometer UV-Vis and characterization using LC-MS. The result showed fermentation of oil palm empty fruit bunch (OPEFB)15% obtained ethanol 39 g/L and total cell after 72 h showed 2.15×10^8 . The highest glutathione concentration had obtained from phosphate buffer pH 7.2 with extraction time 90 min which is equal 1449.52 mg/L.

1. Introduction

The oil palm empty fruit bunch (OPEFB) is one of the most attractive lignocellulosic biomass resources for second generation bioethanol production. In Indonesia, OPEFB was considered as a potential feedstock, with estimated the palm oil industry produces 1.1 to 1.5 tones OPEFB for every tone Crude Palm Oil produced. The OPEFB contain 36.59% of cellulose, 24.97% of hemicellulose and 26.53% lignin. Cellulose and hemicellulose content can be converted to glucose so that fermentation can be done to second generation of bioethanol [1].

Bioethanol is one of the biofuels that currently popular to replace petroleum, their existence significantly contributes to the reduction of crude oil consumption and environmental pollution. The



production process second generation of bioethanol generally there are four steps: pretreatment, enzymatic scarification, fermentation, and distillation. Pretreatment aims to overcome the recalcitrance of biomass and improve the yield of liberated sugars in the following enzymatic hydrolysis step. After pretreatment, biomass undergoes enzymatic hydrolysis for conversion of polysaccharides into monomer sugars, such as glucose and xylose. Subsequently, sugars are fermented to ethanol by *Saccharomyces cerevisiae*. Fermentation processes produced ethanol and remained broth from the fermentation process which still contain yeast of *S. cerevisiae*. According Wu *et al.*, [2], glutathione reduces (GSH) compound was found in *S. cerevisiae*. Genetically, *S. cerevisiae* can produce glutathione compounds by 3-8% [3]. The glutathione is a tripeptide consisting of glutamic acid, cysteine, and glycine.

Glutathione compounds have important pharmaceutical and cosmetic functions and pharmaceuticals such as heavy metal detoxification [4]. Glutathione also acts as a skin whitening agent and antioxidant in free radicals [5]. In the field of food is used as food additives [6]. Widely, glutathione has potential in the cosmetics industry [7]. Glutathione compounds can be obtained by fermentation, chemical synthesis, enzymatic catalysis, and metabolic or genetic techniques [2]. The waste of the fermentation product is not directly disposed of, because in the waste there is still a microorganism agent that play the role of changing ethanol with fermentation, that is *S.cerevisiae* yeast containing the important compound that is glutathione [8]. This work has been performed in effort to get a novel bio refinery sequence that can be optimized across a variety of co-products, thereby demonstrating the potential of co-production of ethanol and other value-added products using OPEB a low-cost starting feedstock. Therefore this study have focused on isolation and characterization of reduced glutathione (γ -glutamyl-L-cysteinylglycine, GSH) from *S.cerevisiae* as by product second generation of bioethanol fermentation.

2. Materials and Method

2.1. Materials

Biomass waste used is Oil Palm of empty fruit bunch (OPEFB) were obtained from palm oil mill in Riau, Indonesia. Reagents such as NaOH, ethanol, H₂SO₄, phosphate are of commercial grade from Sigma Aldrich, used without further purification. Yeast extract, KH₂PO₄ (Merck), L-Cysteine, Glycine, Alloxan monohydrate (Sigma Aldrich), L-Glutathione Reduced Standard (Sigma Aldrich). Enzymes of Cellic® Ctec2 and Cellic® Htec2 from Novozymes, and commercial dry yeast of *Saccharomyces cerevisiae*.

2.2. Second generation bioethanol process

2.2.1. Pretreatment

OPEFB was chopped and milled into particle size 2-3 mm, with moisture content was about 10%. After that pre-treated using 10% of NaOH solution. The pre-treatment process was carried out at temperature 150°C, 4 bars of pressure, and processed for 30 minutes. After the pre-treatment process, OPEFB was washed by water until neutral pH then dried in an oven at 50°C for 24 hours prior use in the further process of scarification and fermentation.

2.2.2. Simultaneous Scarification and Fermentation (SSF)

15% of pretreated OPEFB were incorporated into SSF media. The SSF media is a modification of the medium for fermentation performed by Cha *et al.* [9], which consists of 0.05 M citrate buffer that added with 0.06% of KH₂PO₄ nutrition, 1% of yeast extract, 0.05% of L-Sisteina and 0.05% of Glycin. The fermentation performed by SSF method used cellulose enzymes (Cellic® Ctec2 and Cellic® Htec2) and dry yeast *S. cerevisiae* which are added simultaneously at the beginning of fermentation.

The cellulose enzymes were added about 30 FPU/g (Cellic® Ctec2), and 20% of the β -glycosidase enzyme (Cellic® Htec2) and 1% (g/mL) the yeast was put into the medium. SSF process used incubator shaker 150 rpm, temperature 32 °C for 72 hours. The remaining yeast from the fermentation process was separated from the fermentation solution by using 10,000 rpm of centrifuge for 10 minutes.

2.3. Extraction

The extraction process was performed, dried yeast cells were placed into the test tube and extracted with 25% ethanol solvent, 40% ethanol in water, H_3PO_4 0.1 M and Phosphate Buffer pH 7.2. In the extraction process, the variations time of sonication process were 60, 90, 120 and 150 min, using sonicator (Ultrasons, Selecta, 40 kHz). The extracted sample was then centrifuged at 10,000 rpm for 10 minutes to get the fraction of supernatant (filtrate) containing glutathione sulphhydryl (GSH), then analyzed. All experiments were run in duplicate for the extraction.

2.4. Glutathione analysis

Glutathione analysis used a method performed by Wen et al. [10]. 1 ml of extracted sample was added 1 ml of 1000 mg/L alloxan solution, 3.5 mL of phosphate buffer solution pH 7.52 and 0.5 mL of glycine solution 0.1 M. After that it was homogenized using a vortex and then allowed for 20 minutes at room temperature. Glutathione concentration was measured with a UV-Vis spectrophotometer at a wavelength of 305 nm. Glutathione concentration was calculated based on the linear regression equation obtained from the standard solution.

3. Results and Discussion

3.1. Calculation cell growth of *S. cerevisiae* during Fermentation

To find out the growth of *S. cerevisiae* during fermentation process, calculation of *S. cerevisiae* cells and ethanol content was performed at hours 0, 24, 48, and 72. The cell count calculations were performed using a hemocytometer, the results shown at Figure 1.

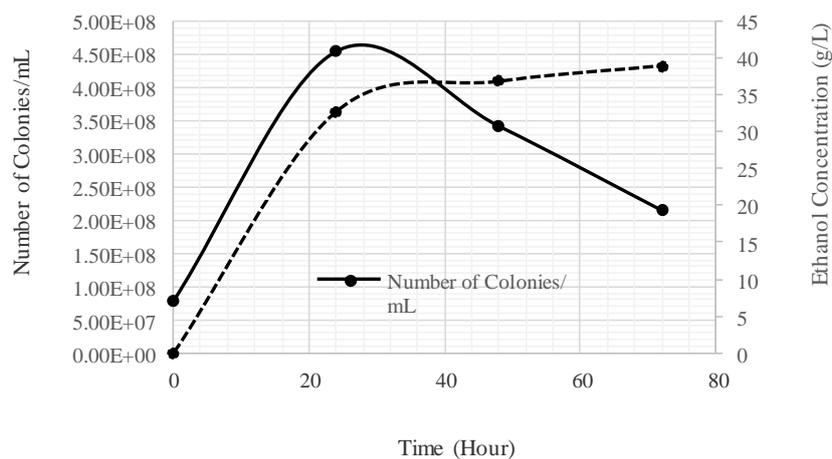


Figure 1. Number of colonies from *S. Cerevisiae* and ethanol concentration in variation of time

Based on the Figure 1, at 0 hours fermentation the yeast cell count was 7.83×10^7 , after 24 hours of fermentation yeast cell count was 4.55×10^8 . This indicates that the initial process of fermentation by yeast occurs very quickly. After fermentation for 48 hours, resulting ethanol content was 36.9 g/L and the yeast cell count was decrease to 3.42×10^8 . This indicates that decrease in the number of cells caused by nutrients in the medium has been reduced. At 72 hours, the resulting ethanol content is as much as 39 g/L and the yeast cell count is 2.15×10^8 . The number of cells in the 72nd hour decreases

and enters the phase towards cell death which is marked by the fall of the growing curve. According to Suprihatin [11], this occurs because the nutrients and energy reserves in the cell is reduced and the presence of alcohol that can be obstruct of cell life.

3.2. Glutathione Extraction

Glutathione can be produced by extraction from natural biomass or via chemical synthesis. However, at present study, glutathione is produced by fermentation using *Saccharomyces cerevisiae*. The main problem in the production of GSH, however, lies in the separation of GSH from fermentation broth, which may limit the extensive applications of GSH. An advanced separation technique is essential to obtain highly-purified GSH with high recovery rate. Several methods have been suggested to separate GSH from fermentation broth, including copper-salt method, ion-exchange chromatography, and affinity chromatography [8-10].

The precipitated residue of fermentation which has been separated by centrifugation then washed two times with distilled water to separate others impurities fore extraction and to minimize errors in the next process. After washed, the sample is dried to constant weight to obtain dry yield and can be used for extraction and fractionation.

The extraction of glutathione was done by using immersion and ultra-sonication method. The solvent used was ethanol 25%, ethanol 40%, buffer phosphate pH 7.2 and H₃PO₄ 0.1 M. The use of some solvent aims to know which solvent extract the highest glutathione. The filtrate of the extraction determined its glutathione content by the alloxan method. Glutathione measurements used a uv-vis spectrophotometer at a wavelength of 305 nm. Analysis of glutathione levels using the alloxan method utilizes a reaction between alloxan and glutathione incubated for 20 minutes. The alloxan will react with an amino acid having a thiol group such as L-cysteine or GSH. The reaction produces superoxide radicals and hydrogen peroxide. Dialuric acids work as redox couples that are limited by the presence of alloxan, reducing agents, and electron acceptor (Oxygen). Thus, in the presence of oxygen, there is a change in the pH of the solution containing alloxan from pH 3,0 to 7.2 thus affecting the rapid measurement at 305 nm wavelength [12].

The glutathione compound reacts with the alloxan to form a detectable compound at a wavelength of 273 nm. The presence of glutathione causes a shift in measurements to 305 nm [13]. This is caused by the shift of the cylinder because there are more C-O and C-N bonds in the compound that have binds to glutathione.

The use of different solvents in the extraction process results in different concentrations of glutathione as shown in Figure 2. A, B, C and D. The results showed that time and solvent have an influence on glutathione concentration. The resulting glutathione levels range from 270-1449 mg/L. The lowest glutathione concentration obtained from extraction with ethanol 40% for 60 minutes. The highest glutathione concentration with a value of 1449.52 mg/L was obtained from extraction with phosphate buffer pH 7.2 at 90 minutes.

High glutathione obtained by phosphate buffer pH 7.2 these possibly due to the glutathione compound is soluble under pH ± 7 [14], the results was higher than the research conducted by Wen et al [10] by batch of 329,3 mg/L. Further research conducted by Wen et al [10] by fed-batch has higher levels than the obtained level of 2190 mg/L, but the study is more complicated and requires more media. The difference between a batch and fed-batch method lies in the time or amount of adding something to the medium. The batch method is from start to finish fermentation of fixed medium quantities whereas fed batch can occur reduction and addition during fermentation.

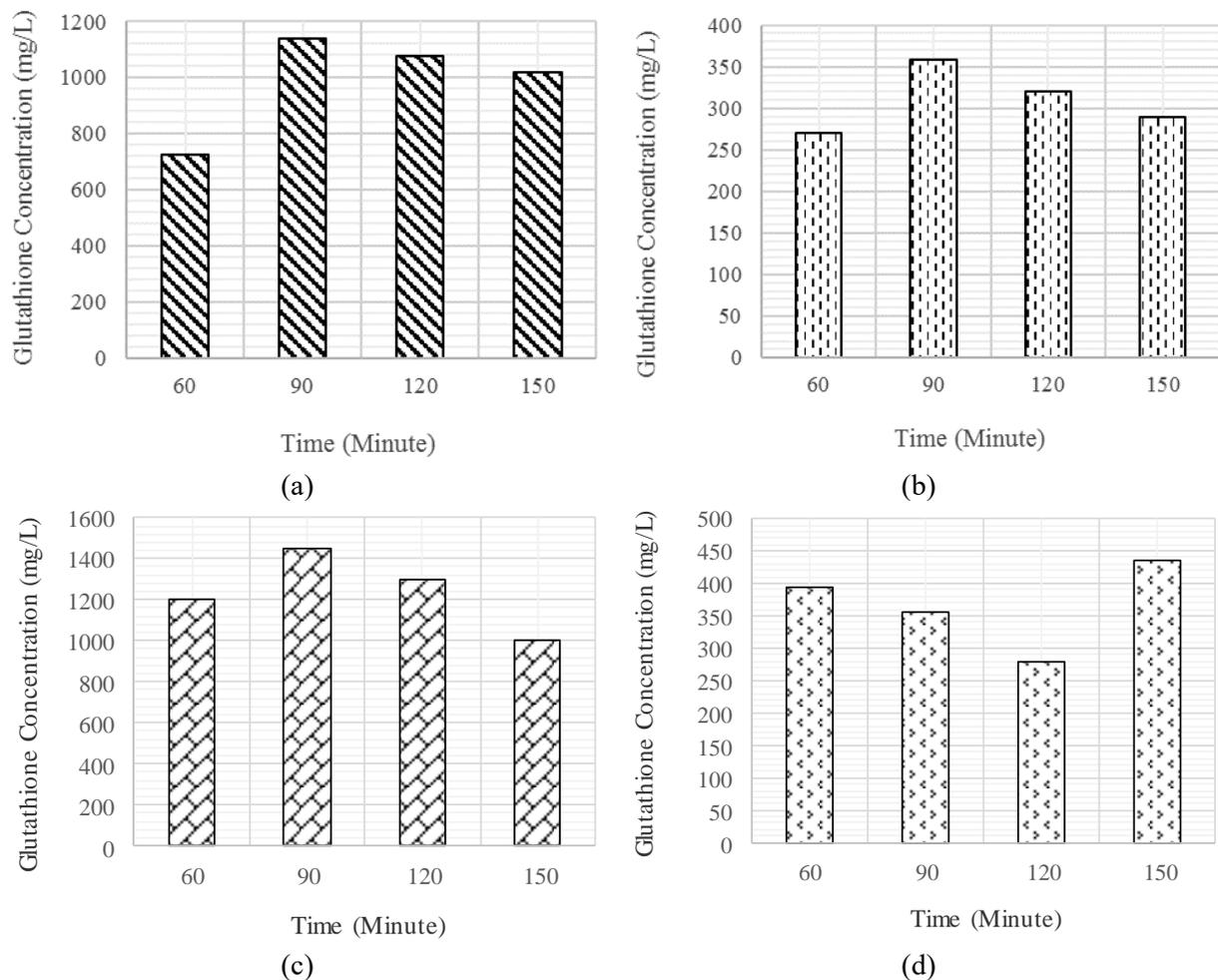


Figure 2. Glutathione concentration in variation of time and solvent
 (A). Ethanol 25%, (B). Ethanol 40%, (C) Phosphate Buffer pH 7.2, (D). H₃PO₄ 0.1 M

4. Conclusion

Ethanol, Phosphate and H₃PO₄ can be used to extract glutathione in *S. cerevisiae* remaining from waste of fermentation second generation bioethanol. The concentrations of solvent and time of extraction process had an effect on the result of extractions. The highest glutathione concentration reached 1449.52 mg/L was obtained by using Phosphate buffer pH 7.2 for the 90 minutes of maceration followed by 15 minutes of sonication extraction process.

5. Acknowledgement

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