Evaluation of Glutathione Production Method using
*Saccharomyces cerevisiae*

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**Abstract.** Industrial interest of glutathione, as a pure substance or accumulated in yeast, has been prominent as a result of various applications of glutathione in food and pharmaceutical industries. Glutathione production by fermentation process is easier and more economical compare to production by chemical and enzymatic reaction. This study aims to evaluate environmental condition to the fermentation process producing glutathione in three different strategies. In the following work, stress conditions and cysteine addition coupled with fed-batch fermentation of *Saccharomyces cerevisiae* ITBCC R58 for glutathione accumulation have been investigated. Stress conditions applied on this study included temperature shift from 30 to 50°C and 27°C, pH shift from 5 to 1.2 and 8.8, and osmotic stress by addition NaCl to the solution. Another strategy was to add cysteine as one of glutathione precursors into the fermentation medium. Later, the oxidative state of glutathione harvested was also checked. Osmotic stress showed the best result amongst stress variations applied on this work, however combined cysteine addition with fed-batch fermentation stood out as the best strategy in this study. In all experiments, oxidized-state glutathione was identified, indicating the need of a specific method development to harvest glutathione in reduced state.

1. **Introduction**
A current expansion of yeast global market has been expected to grow 7.1% between 2016 to 2022 [1] implying that varieties of yeast-derived products are of great interests. Yeast autolysates are commonly used in wide range of application from laboratory uses as an excellent medium for growth of microorganisms to flavor enhancers. Yeast extract was also reported to generate unique flavors, known as umami and kokumi-taste affected by different \(\gamma\)-glutamylcysteine derivatives such as glutathione, a tripeptide \(\gamma\)-glutamyl-L-cysteinylglycine [2]. On the other hand, intact yeast cells are widely used as fermentation starter in food industries, for example bread. Glutathione-enriched intact yeast cells are also considered to be able to improve dough extensibility and decrease mixing time in baking [3]. Further, yeast extract containing oxidized form of glutathione has been proven to enhance the gas-retaining capacity of gluten-free rice batter [4]. Glutathione-enriched yeast is priced 4 $/kg which doubles the normal yeast price. As an intracellular product, glutathione in glutathione-enriched yeast benefits from its no-need of purification, hence being very convenient and easy to manufacture.
Pure glutathione finds applications in pharmaceutical and cosmetic industry as a skin whitening agent [5] and scavenger for free radicals and heavy metals thus required in good purity and resulting in the high pricing of glutathione powder. Inside the cell glutathione serves to prevent cell damages caused by reactive oxygen species (ROS) [6]. Other roles of glutathione inside cells range from protecting protein during stress condition [7] to detoxifying heavy metals by generating glutathione conjugates [8]. Intracellular glutathione content in wild-type yeast is varied between 0.1 and 1%-w/w with a ratio of reduced form (glutathione) to oxidized form (GSSG) of 30-100:1 [9].

Industrial interests for glutathione served as motivation for a number of studies aiming to increase glutathione production by yeast cells. Using strain improvement method, it has recently been possible to generate yeast strains containing up to 3.9%-w/w of intracellular glutathione content [10]. Yet, despite promising scientific progresses, target-engineered strains are still hardly accepted in food industry due to authority issues [11]. Other proposed methods are controlling the microenvironment of the yeast cultivation, for example by applying stress condition such as sudden pH change [12], temperature drop [13], osmotic stress [14], magnetic fields [15], high-pressure [16] and H2O2 stress [17], addition of amino acid precursor [18], optimization of growth medium, as well as fed-batch mode to achieve high cell density [19]. In order to get the oxidized form of glutathione, reacting the reduced form of glutathione with oxidizers such as potassium bromate, benzoil peroxide and hydrogen peroxide has been suggested [20].

This study aims at evaluating the potential of local yeast, Saccharomyces cerevisiae ITBCC R58, in producing glutathione. Three different strategies to enhance glutathione production such as applying stress conditions (pH, temperature, or osmotic stress), addition of precursor, and implementing fed batch fermentation configuration were tested. Further the specific state of produced glutathione (whether the reduced or the oxidized state of glutathione) was also analysed.

2. Methods

2.1. Microorganism and Cultivation

Yeast Saccharomyces cerevisiae ITBCC R58 was obtained from the culture collection of Laboratory of Microbiology and Bioprocess Technology, Department of Chemical Engineering ITB. The inoculum was prepared by culturing the yeast at 100 mL YPD media containing glucose 20 g/l, peptone 10 g/l, and yeast extract 5 g/l at 30°C and pH of 5.0 for 16 h on a 150-rpm shaking incubator. The culture was then inoculated into the main fermentation broth. Unless stated otherwise, the medium for glutathione fermentation in batch cultures contained glucose 50 g/l, yeast extract 15 g/l, peptone 5 g/l; salt solution: (NH4)2HPO4 10 g/l, MgSO4 0.5 g/l; trace element solution: ZnSO4 10 mg/l, FeSO4 12 mg/l, CuSO4 12 mg/l, and MnSO4 10 mg/l; and vitamin solution: biotin 0.01 mg/L, pantothenic acid calcium salt 0.02 mg/L, and pyridoxine hydrochloride 0.2 mg/l. Main fermentation was carried out in 3 l stirred bioreactor (New Brunswick BioFlo 110) with working volume of 2 l at 30°C and pH of 5.0 using an aeration rate of 1.25 vvm and an agitation rate of 150 rpm. Right after reaching stationary phase, fermentation broth was split into several 250-ml Erlenmeyers with 200-ml working volume in which the three different strategies were applied. This aimed to generate a uniform starting condition for all variations.

2.2. Stress Condition, Cysteine Addition and Fed-Batch Fermentation

Glutathione fermentations were held in 8 different conditions in duplicate. The variations were as follows: low-pH stress from 5.0 to 1.2 by addition of HCl 5 M, high-pH stress from 5.0 to 8.8 by addition of KOH 5 M, low-temperature from 30 to ambient (27°C), high-temperature from 30 to 50°C, osmotic stress by addition of NaCl salt (80 g/l), cysteine addition by adding L- cysteinumchloride (8 mmol), fed-batch by addition of glucose (50 g/l), and combined fed-batch with cysteine addition by adding glucose (50 g/l) and L- cysteinumchloride (8 mmol). All variations were applied right after system reached stationary phase and subsequently held for 3 h in which sample was taken every hour.
2.3. Analysis
Biomass concentration was monitored by turbidimetry using spectrophotometer UV/VIS at 560 nm and gravimetry. Glucose and glutathione concentration were measured by using HPLC. For glucose analysis, Aminex HPX-87H column and RID detector were applied, using H_2SO_4 5 mM as the mobile phase. For glutathione analysis, intracellular glutathione was first extracted following the works of [21]. Analysis was conducted by applying μBondapak C18 column and UV detector, using NaH_2PO_4 25 mM as the mobile phase. In order to differentiate the specific state of glutathione, an HPLC analysis method modification of [22] was applied. The same HPLC system was applied, however, the mobile phase used was mixture of water, methanol (2.5 v/v), and phosphoric acid 25 mM, buffered at pH 3.

The measured glutathione concentration in the extract was used to calculate the overall glutathione concentration in the fermentation broth (C_{glu} [= g-glutathione/l]) as well as the intracellular glutathione content (C_{glu,in} [= g-glutathione/g-cell Dry Weight]). The glutathione yield on glucose as the substrate (Y_{S/glu} [= g-glutathione/g-glucose]) and the cell yield on glucose as the substrate (Y_{S/X} [= g-cell Dry Weight/g-glucose]) was calculated following eq 1 and 2, correspondingly.

\[
Y_{S/glu} = \frac{C_{glu,t} - C_{glu,0}}{C_{S,0} - C_{S,t}}
\]

\[
Y_{S/X} = \frac{C_{X,t} - C_{X,0}}{C_{S,0} - C_{S,t}}
\]

In which C_{S} and C_{X} is the glucose substrate concentration [=g-glucose/l] and the cell concentration [=g-cell Dry Weight/l] in the fermentation broth, whereas the subscripts t and 0 indicates the timing of the samples, final and initial, respectively.

3. Results and Discussion

3.1. Glutathione Production on Different Stress Conditions
Effects of five stress conditions applied on fermentations are shown in Figure 1. The stresses were applied right after system reached stationary phase, and thereby were slightly different for different batches. There was observed a trend of decrease in biomass concentration (Figure 1(a-b)) for all variations after cells were exposed to stress conditions. This phenomenon was taken as an indirect marker of stress conditions undergone by cells since for unicellular microorganisms, stress is defined as any conditions or states that could lead to the decrease in cell growth [23].

Figure 1 (c) and (d) show that the applied stress conditions positively affected glutathione production, except for the variations of low pH stress and high temperature stress. High-pH, osmotic, and low-temperature stress enhanced glutathione production and resulted in intracellular glutathione contents which were 56.3, 52.4, and 26.5% higher than initial condition, respectively. However, only the osmotic stress resulted in an increase value of glutathione yield on glucose, from 0.0011 to 0.0023 g glutathione/g glucose while the others did not noticeably improve the glutathione yield. Osmotic stress seems to be a good strategy to enhance glutathione production which in this study, resulted in 1.284%-w/w of final intracellular glutathione content (Table 1).

Glutathione is involved in oxidative stress response and is responsible to fight reactive oxygen species (ROS) by transfer electron via hydrogen for systems which are dependent on thiol such as peroxidase [24]. Even though osmotic stress does not fully associate with oxidative stress response, there has been a study finding that a fraction of oxidative stress response shared the same components with osmotic stress [25].

The high-pH variation resulted in an increase of intracellular glutathione content (Figure 1(d)) but not in the glutathione yield (Table 1). This implies that high-pH favors the glutathione accumulation inside yeast cells yet it is not efficient to convert substrate to glutathione. This is probably a result of the extreme condition that cells underwent in high-pH environment that reduced a great number of intact cells. A study performed by [26] showed an increase of glutathione production in Candida utilis.
by 42% when pH was brought up from 5 to 6. In the opposite, a decrease of glutathione production was recognized when pH was shifted from 5 to 4. The reason behinds the enhancement of glutathione production in high-pH condition was explained from a research by [27]. Yeast cells exposed to alkaline stress accumulate ROS in relatively short time. This ROS accumulation induces the expressions of genes involved in oxidative stress such as glutathioneI and glutathioneII that are responsible in catalyzing the biosynthesis of glutathione. Another finding of this current study is the reduction of pH resulted in a slight decrease of glutathione production (Figure1(d)). On the contrary, Nie, et al. [12] reported an increased glutathione production was observed from the fermentation of C. utilis with low-pH stress (pH of 1.2). However, almost all intracellular glutathione was extracted out of cells for inexplicable reasons. Regrettably, this phenomenon could not be observed in this current study since we did not analyze extracellular glutathione due to operational limitations.

A slight temperature shift from 30 to 27°C in this study showed an increase in glutathione content (Figure1(d)). This result is in accordance with [13] that applied similar strategy on Candida utilis. They found a temperature shift from 30 to 26°C increase glutathione content up to 19.04%. Similar to yeast stress response on alkaline stress, growing yeast cells at a suboptimal temperature also raises the intracellular ROS and promotes an oxidative response involving glutathione [28]. Although heat shock is expected to trigger stress response involving glutathione, we found that high temperature did not seem to be good strategies to increase glutathione accumulation (Figure 1(d)).

Figure 1 Effects of stress conditions on glutathione production on (a) Cell concentration, (b) Cell concentration relative to initial condition, (c) Intracellular glutathione content (d) Intracellular glutathione content normalized to the initial condition.
glutathione content relative to initial condition. The black arrows indicate the time when the stress was applied, data are mean values ± SD from duplicate experiments.

Table 1 Overview of batch fermentations with various strategies to improve glutathione content

| Variation            | Final glutathione concentration [g/l] | Final intracellular glutathione content [%w/w] | Glutathione yield on glucose [g-glutathione/g-glucose] | Cell yield on glucose [g-cell DW/g-glucose] |
|----------------------|--------------------------------------|----------------------------------------------|------------------------------------------------------|------------------------------------------|
| Control              | 0.0528                               | 0.426                                        | 0.0011                                               | 0.3675                                   |
| Low-pH               | 0.0693                               | 0.370                                        | 0.0015                                               | 0.4628                                   |
| High-pH              | 0.0397                               | 0.415                                        | 0.0007                                               | 0.1605                                   |
| High-temperature     | 0.0337                               | 0.455                                        | 0.0010                                               | 0.1950                                   |
| Low-temperature      | 0.0572                               | 0.299                                        | 0.0012                                               | 0.3475                                   |
| Osmotic              | 0.1112                               | 1.284                                        | 0.0023                                               | 0.1724                                   |
| Fed-batch            | 0.1160                               | 0.912                                        | 0.0013                                               | 0.1425                                   |
| Cysteine             | 0.0781                               | 0.984                                        | 0.0080                                               | 0.1688                                   |
| Fedbatch +cysteine   | 0.1511                               | 1.695                                        | 0.0017                                               | 0.0984                                   |

3.2. Effects of Cysteine Addition and Fed-Batch Mode on Glutathione Fermentation

Effects of cysteine addition and fed-batch mode on glutathione fermentations are shown in Table 1 and Figure 2. Cysteine and glucose were added right after system reached stationary phase, and thereby were slightly different for different batches. Figure 2 shows that cell concentration of fed-batch variation was elevated from 10.73 (control) to 12.72 g DCW/l while the cysteine addition did not affect cell concentration. According to [18], cysteine inhibits cell growth even though it enhances glutathione production. Spike timing which is at stationary phase was appropriately chosen to deter the negative effect of cysteine on cell growth.

Not only did the fed-batch mode improved cell concentration, intracellular glutathione content was also increased up to 16.03%. This overall improvement may be caused by the increase of chemical energy (ATP) supplied by glucose addition that could support glutathione biosynthesis which is dependent on ATP.

An elevation of glutathione production is also observed in cysteine addition strategy. The addition of cysteine alone elevated intracellular glutathione content up to 112.9% higher than initial condition. This result is in harmony with many former studies of cysteine addition in glutathione fermentation. Wei et al. reported an increase of glutathione content 106.5% higher than control due to cysteine addition [18]. A refined explanation of this result is not well understood, yet it is believed that cysteine addition enhances the conversion of \( \gamma \)-glutamylcysteine reaction.

Based on the above results, combined fed-batch and cysteine addition is strongly believed to boost glutathione production way more effective than their individual effect. In this study, this variation resulted in an increase of glutathione production and showed the best result over other variations. glutathione intracellular content is elevated from 0.137% to 1.695%-w/w. However, there is no significant increase in cell concentration although fed-batch fermentation had been applied. This could become some improvements in future studies to improve the method of fed-batch fermentation and timing for cysteine addition to achieve better high cell density as well as the high content of glutathione. Lorentz, et al. [3] successfully performed fed-batch fermentation with RQ control and periodic cysteine spike resulting an increase of intracellular glutathione content from 0.42 (control) to 1.74%-w/w.
3.3. Glutathione Yield on Substrate at Different Variations
Besides intracellular glutathione content, the glutathione yield on substrate (glucose) is also an important parameter for evaluating performances of each variation in the effort to enhance glutathione production. According to glutathione yield for all variations shown in Table 1, cysteine addition stood out as the best strategy to enhance glucose conversion into glutathione. The yield of control that is only 0.0011 g glutathione/g glucose was brought up to 0.0080 g glutathione/g glucose when cysteine addition was applied. In addition, amongst stress variations, osmotic stress showed a noticeable increase in glutathione yield reaching 0.0023 g glutathione/ g glucose. When the expected outcome is pure glutathione, a strategy that enhances glutathione yield is more favorable since it will improve the fermentation efficiency. However, if the expected product is glutathione-enriched yeast, a variation that improves intracellular glutathione content is more relevant.

3.4. Oxidative State of Glutathione
As aforementioned above, glutathione has two different oxidative states, reduced glutathione (GSH) and oxidized glutathione (GSSG). The reduced state glutathione serves as antioxidant and will transform into the oxidized state after fighting against ROS. However, due to the recycle system in cells, GSSG will transform into glutathione again to maintain a normal ratio between GSH and GSSG.

Figure 2 Effects of cysteine addition, fed-batch, and combined cysteine with fed-batch on a) Cell concentration, b) Cell concentration relative to initial condition, c) Intracellular glutathione content, and d) Intracellular glutathione content relative to initial condition. The black arrows indicate the time when the cysteine or glucose was added, data are mean values ± SD from duplicate experiments.
which is within a range of 30-100:1. An analysis to determine oxidative state of glutathione has been performed. Only GSSG was recognized in the fermentation product while GSH was completely unidentified. Since there is no oxidizing agent was introduced into the fermentation product that contained glutathione, it is assumed that glutathione was oxidized automatically into GSSG in the presence of oxygen in air. Due to the handling of glutathione during experimentations from glutathione extraction to its analysis, it is highly possible that GSSG is formed from glutathione. Therefore, a better harvesting system is needed to prevent glutathione from getting oxidized.

4. Conclusion
Various stress conditions, cysteine addition, and fed-batch strategy have been applied on fermentation of Saccharomyces cerevisiae in this study. Except for the high pH and high temperature stresses, all strategies have been shown to improve glutathione concentration. The obtained results shed a light of effective strategies for glutathione production since the industrial interest of glutathione, as a pure substance or accumulated in yeast, is still prominent. If the expected product is glutathione-enriched yeast cells, it is favorable to apply the strategy that yield the highest final intracellular glutathione content or give the highest final glutathione concentration. In this case, fed batch mode and cysteine addition stood up as the best strategy, giving final glutathione concentration of 0.151 g/l (1.695% w/w). If the pure glutathione product is expected, it is favorable to apply the strategy that increase the overall yield of glutathione on substrate. In this case, cysteine addition stood up as the best strategy, giving glutathione yield of 0.0080 g-glutathione/g-glucose. Further, since genetically modified organisms are barely accepted in food industries, uses of a non-modified food grade strain in this study show the potential in glutathione production.

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