SCREENING OF ALCOHOL-TOLERANT YEAST OF *Saccharomyces Cerevisiae*

Wita Dewi Sondari, Achmad Ali Syamsuriputra*, Tjandra Setiadi*
Laboratory of Microbiology and Bioprocess Technology,
Chemical Engineering Department, Faculty of Industrial Technology, ITB
Jl. Ganesa 10, Bandung 40132
E-mail: WietaDS@Yahoo.com

Abstract

In order to obtain culture of Saccharomyces cerevisiae which has the highest ethanol tolerance and can produce high yield of ethanol as well a study of mutation has been begun. Mutation experiment conducted by continuous adaptation on a chemostat was initiated with a preliminary study of screening of alcohol-tolerant yeast. The procedures of screening of alcohol-tolerant yeast continued by optimization of substrate concentration and determination of its critical pH. Recently, the Laboratory of Microbiology and Bioprocess Technology Faculty of Industrial Technology ITB has various kind of yeasts that have been obtained or isolated from various sources. The best culture for mutation has been chosen as the most ethanol tolerant one. By screening them on two types of experiment, has been obtained that culture Saccharomyces cerevisiae R-60 gave the highest external ethanol and internal ethanol as well. External ethanol means the ethanol that was purposely added to the cultivation media, while the internal ethanol means the ethanol that was resulted from fermentation of the yeast. As preparation for mutation experiment, the determination of optimum substrate concentration which can give the highest amount of Saccharomyces cerevisiae cells has been carried out. In order to set up the control point of culture viability on chemostat, the critical pH of chosen culture have also been obtained. The result of the experiment gave optimum glucose concentration of 18.6% and critical pH of 4.5 to 3.8, were to be applied in the mutation process.

Keywords: Cultivation; Fermentation; *Saccharomyces cerevisiae*; Screening; Yeast

Abstrak

Penelitian untuk mendapatkan kultur Saccharomyces cerevisiae yang mempunyai toleransi etanol yang tinggi dan dapat menghasilkan perolehan etanol yang juga tinggi telah dilaksanakan. Percobaan mutasi dilakukan dengan proses adaptasi secara kontinyu dalam chemostat yang diawali dengan suatu studi pendahuluan yang dinamakan skrining ragi tahan etanol. Prosedur skrining ragi tahan etanol ini dilanjutkan dengan optimasi kandungan substrat dan penentuan pH kritis-nya. Pada saat ini Laboratorium Mikrobiologi dan Teknologi Bioproses Fakultas Teknologi Industri ITB telah memiliki berbagai jenis ragi yang berasal dari berbagai sumber. Kultur terbaik untuk mutasi dipilih sebagai kultur yang paling toleran terhadap etanol. Melalui percobaan screening ragi tahan etanol yang dilakukan dalam dua jenis percobaan, diperoleh bahwa kultur Saccharomyces cerevisiae R-60 memiliki toleransi etanol eksternal dan internal paling tinggi. Etanol eksternal adalah etanol yang sengaja ditambahkan pada media kultivasi ragi, sementara etanol internal adalah etanol yang dihasilkan dari fermentasi oleh ragi tersebut. Dalam mempersiapkan percobaan mutasi, penentuan konsentrasi substrat optimum yang dapat menghasilkan jumlah sel Saccharomyces cerevisiae terbesar telah dilakukan. Selain itu titik tetap viabilitas kultur dalam chemostat yang berupa pH kritis kultur pilihan juga telah ditentukan. Dari percobaan pendahuluan mutasi tersebut diperoleh konsentrasi glukosa optimum sebesar 18.6% dan pH kritis kultur R-60 adalah 4.5 dan 3.8. Data tersebut akan diterapkan pada percobaan mutasi.

Kata Kunci: Kultivasi; Fermentasi; Pre-mutasi; Ragi; *Saccharomyces cerevisiae*

1. Introduction

Recently, development of bio-ethanol production in Indonesia is in accelerating period. The government of Indonesia notifies and promotes the use of bio-ethanol as blending component of gasoline to be as 15%wt by the year of 2007. The use of bio-energy was agreed to be an alternative solution in overcome the non renewable energy sources beside give other benefits to economy, environmental and agricultural sectors.

The use of bio-energy is widely known as "environmentally friendly" and it could also increase vehicles performance. The production of bio-ethanol also can be directly beneficial to the agriculture because it can utilize some agricultural crops such as cassava, corn, potato, sago, taro, and talas. The sugar content or other compound that can be converted into sugar on such crops can bring them into fermentation process to produce ethanol.

The fermentation process of ethanol production is commonly done by yeast. The carbon synthesis internal the yeast cells produce ethanol and carbon dioxide. This process simply occurs when yeast is breaded in media that contain carbon sources such as glucose under anaerobic conditions. While under aerobic conditions the metabolism path of yeast can direct to the growth and regeneration (cultivation) of cells.

There are so many kind of yeast can be used in fermentation. Some species of *Saccharomyces*, *Shizosaccharomyces*, *Torula*, *Willia*, *Yarrowia*, etc have different characteristic and productivity in ethanol production (Gray, 1941). They also have specific media for their optimal growth. Some factors known might give different effect to the growth of yeast cells and to the production of ethanol by the yeasts. Temperature, pH, substrate composition, even the produced ethanol itself could influence each species of yeast in different magnitude.

So far, it has been determined literally and experimentally that ethanol could inhibit the yeast cell activity. Considering batch fermentation process, the rate of ethanol production could be decreased as the declining in cells growth. The severe of inhibition process by ethanol concentration could be different for different kind of yeasts depends on their ethanol tolerance (Nagodawithana and Streinkraus, 1976; Andreishcheva, et al., 1999). But the impact of this factor might be continued by the next step of ethanol production.

As the low yield of ethanol produced, the separation process by distillation and absorption might have some problems. Especially in the high utilization of energy can make this process inefficient. Therefore some effort should be taken

to increase the ethanol yield. One way is by increasing the capability of yeast to tolerate ethanol content in the media.

The process of increasing ethanol tolerance of yeast can be done by some mutation technique. Varied from quite simple adaptation process to the sophisticated process of genetic manipulation can give different result as well as different risk to be taken (Nagodawithana and Streinkraus, 1976; Jimenez and Benitez, 1987). Particularly genetic manipulation can give significant results compared to the adaptation. But the risk of "creating" damaged or even harmful living things is other thing to be considered.

The purpose of these studies was to prepare a strain of yeast for the mutation process by continuous adaptation in the *chemostat*. The preparation experiments for the mutation process consisted of yeast strain screening, determination of optimum substrate composition, and the determination of critical pH.

The screening experiment has been done to choose the best culture of *Saccharomyces cerevisiae* for mutation process that had the highest tolerance of internal and external ethanol. The optimum substrate composition and critical pH would be applied in mutation experiment in order to get the highest cells inoculums. In the other hand, the critical pH would be applied as set point and the control of cells viability in adaptation process of yeast on continuous fermentation media in a *chemostat*.

2. Fundamental

The model of *chemostat* that would be applied in the mutation process was adapted from Jimenez and Benitez (1988). It consisted of closed-stirred tank reactor with agitation, filtered oxygen supply, continuous ethanol input, and feedback-mode media supply controlled by pH. Some of the parameter conditions for the *chemostat* operation were determined in this preliminary study of mutation process. By using this model of reactor, the cultivation of the yeast could be kept continuously as the increase of ethanol content on the media during about 16 days cultivation.

On the batch operation of yeast cultivation, there are five phases used to be held. They are adaptations, logarithmic, exponential, stationary, and death or declining phases. Each phase could be identified by the rate of cells growth which defined by the cells amount. When the phases were to be identified, the cells amount should be counted and plotted into a graph. The identification of growth phases in a batch operation or moreover in continuous operation would need large data of cells amount. This kind of measurement method could

give some difficulties if the samples were taken frequently and in quite long period.

Jimenez and Benitez, 1987 reported the relationship between the amount of cells and the phase occurring on the pH of the media and cultures. By making an acidification curve during cultivation they found that the pH of culture tends in inverse trends with the cells growth rate. In the beginning of cultivation, the pH was decreasing as the cells were increasing. At the time of declining phase, the pH was increasing while the cells were decreasing. The decreases in pH happen as the production of CO_2 during the carbon synthesis on cells growth. While the increase of pH after the stationary phase happen as the production of NH_4^+ during the protein synthesis on cells growth. The yeast start to metabolize protein since the glucose in the media was consumed. This phenomenon was applied on the principles of viability cells on the continuous adaptation in a *chemostat* as was mentioned above.

The cultivation of yeast in continuous and semi-continuous (feed-batch) operation is quite different with those in the batch operations which could apply continuously input or (and) output to the fermenting reactor. Overall, the main objective of cultivation is the same to produce as much yeast cells as possible.

In this study, the yeast cells for adaptation process should have not only large quantity but also high ethanol tolerance. This could be obtained by determining and setting the optimum conditions for cultivation of yeast and by screening their ethanol tolerance (Gray, 1941, 1946, 1947).

Cultivation and fermentation process of ethanol production using yeast such as *Saccharomyces cerevisiae* optimally can be carried out in pH of 4.0-5.0 and temperature of about 30°C (Supiarma & Halomoan, 1985). The fermentation reaction can be written as follows:

Beside temperature and pH, glucose concentration also can influence the yield of ethanol. Glucose is a carbon sources for growing of yeast cells which have role as bio-catalyst and also as a substrate for the fermentation process. The optimum substrate composition could be specific for the different kind of yeast. As reported by Aiba (1973) *Saccharomyces cerevisiae* generally has optimum glucose concentration ranges between 10-18%. Higher glucose concentration (above 18 %) can inhibit the performance of the yeast. Even at more extremely concentration (above 35%) can kill the yeast. The death of yeast is caused by high glucose concentration which making water emerge

through the cells wall.

3. Metodology

This study was initiated with choosing yeasts that consisted of five kind of *Saccharomyces cerevisiae* and one *Schizosaccharomyces pombe* (Table 1) and was prepared by some screening experiments. These experiments were divided into two different method of experiments. One is to determined the external ethanol tolerance and the other is to determine it internally.

Table 1. Yeasts Strain for Sreening

Code	Type	Source
R-28	<i>Sacch. cerevisiae</i>	Isolated from traditional beer
R-58	<i>Sacch. cerevisiae</i>	American yeast
R-60	<i>Sacch. cerevisiae</i>	Isolated from sugar pabric
R-61	<i>Sacch. cerevisiae</i>	Isolated from rotten fruit
R-62	<i>Sacch. cerevisiae</i>	Isolated from sugar pabric
R-86	<i>Schizosacch. pombe</i>	Isolated

In experiment of external ethanol tolerance, certain composition of ethanol was set up on the same media. The six type of yeasts were cultivated in 10% glucose concentration of GYE (Glucose-Yeast-Extract) media with various ethanol composition (ranges between 6 to 15% vol) under aerobic condition. The parameter examined was the glucose utilization after 24 hours cultivation. The indication of un-tolerant yeast was from the high differences of glucose utilization (over 15%) compared to them on the previous or lower ethanol content media.

The internal ethanol tolerance has been done by fermenting GYE media that contain 2.5% glucose in Erlenmeyer 100 mL using all yeast strains. They were put into the media in the same culture volume and were covered with sulfuric acid filled-swan neck pipe to create aerobic condition. The released CO_2 was calculated as the samples weight loss during fermentation which was weighed every 24 hours. By converting the CO_2 released from fermentation process to the ethanol yield using Gay-Lussac fermentation reaction, can be seen the ability of each yeast in producing ethanol as well as their tolerance to ethanol that they have produced.

The determination of optimum substrate composition was carried out by cultivating the same volume of same culture sources in the same media but with different glucose concentration. The glucose content were varied from 2.5 % to 20%. The parameter examined was the amount of

cells during the cultivation process in aerobic condition that was measured using counting chamber method. The optimum substrate composition would be measured as the composition at which can give the highest amount of culture as well as the highest growth rate.

The determination of critical pH was carried out by determining the pH in the initial of cultivation and in the beginning of stationary phase. Selected yeast was put into media with optimum substrate concentration and then was cultivated until the stationary phase was achieved. The procedure of experiment was similar to the making of growth characteristic curve with additional measurement of pH during the measurement of cells amount. The cells amount was measured using counting chamber method and pH was measured directly using pH-probe on digital pH-meter.

4. Results and Discussion

The screening of external ethanol tolerance has been done. It can be seen from Figure 1 that the glucose utilization of R-60 is the highest one. At ethanol concentration of 10%, it still gave high glucose utilization (about 98.60%) while the others have fallen to under 80% glucose utilization. This meant that R-60 has the highest external ethanol tolerance.

Compared to the ethanol tolerance of *Saccharomyces cerevisiae* that selected by other researchers (Gray, 1941; Benitez, 1983), R-60 can be classified into high tolerance yeast strain (around 10% ethanol concentration). The ethanol tolerance of yeasts is very typical. It depends on the place of each yeast were isolated.

Figure 1. Screening of external ethanol tolerance

Figure 2. Screening of internal ethanol tolerance

Figure 2 shows the ethanol produced by each yeast strains for about 150 hours. It can be seen that R-60 has the highest ethanol yield. While the others began to decline in producing ethanol, it still can ferment several fold higher. The less ethanol yield of others strains meant that they became not endurance to the increasing of ethanol content on the media.

Gray (1941), reported that the production of CO_2 during the fermentation process or the produced ethanol were directly influenced by the ethanol tolerance of yeast. Compared to his experimental results, R-60 can be classified also as a high tolerance of ethanol tolerance yeast which can still produce large amount CO_2 and ethanol even until quite long period (150 hours).

In order to get the largest amount of yeast cells, the optimum substrate composition had to be determined. The experiment was set up by cultivating 5 mL of starter-culture R-60 in 45 mL of GYE media on Erlenmeyer 100 mL with various glucose concentrations. The cells amounts were counted every 3 hours using counting chamber method until stationary phases were obtained.

From polynomial regression on cells amount data in Figure 3 can be seen that each sample has reached their stationary phase. The amounts of cells tend to increase as the raise in glucose contain in the media. The highest cells amount of each samples were collected and then were plotted on graph as shown in Figure 4. After doing differentiation on polynomial regression equation, it has been obtained that the largest amount of yeast cells was obtained at glucose content of 18.6%.

Figure 3. Yeast Cells Growth on Various Glucose Content

Figure 4. Optimum Glucose Content

From both graph (Figure 3 & 4) can be seen that the amount of cells on fermentation tend to be lower than cultivation. It could happen because in fermentation on anaerobic conditions cells had lean to convert glucose into ethanol instead of using it for cells growth. In the other hand, the pH of fermentation broth leaned to be higher than those cultivation broths. This could be caused mostly by the fewer amounts of cells in the media. Ethanol content might give some affect but as reported by Jimenez and Benitez (1987) that the influence of ethanol content resulted from fermentation on the media was quite small.

The critical pH of strain R-60 was obtained as the initial pH of cultivating broth and the pH at which stationary phase was began. So that its determination should be combined with the determination of growth characteristic curve using

the amount of cells as an experimental parameter. From Figure 5 can be seen that the stationary phase began after 30 hours for cultivation and after 28 hours for fermentation. At both time, the stationary pH for cultivation and fermentation were 3.8 and 3.9 respectively (Figure 6).

Figure 5 & 6. Determination of Critical pH

5. Conclusions

The main experiment of mutation process that will continue this study would apply *Saccharomyces cerevisie ITBCC-R60* that was obtained from the screening experiments as the most ethanol tolerant one. It would be cultivated continuously in GYE media with substrate (glucose) concentration of 18.6%. The set point of pH controlled-*chemostat* would be set at 4.5 to 3.8.

Acknowledgement

This mastery research project was funded by LPPM ITB through PP-PSDAPL ITB (Center of Research on Natural Sources and Environmental Conservation, ITB).

References

- [1] Andreishcheva, E. N., et al., (1999), Adaptation to salt stress in a salt-tolerant strain of the yeast *Yarrowia lipolytica*, *Biochemistry-Moscow*, vol. 64. No. 9
- [2] Benitez, T. et al., (1983), Selection of wine yeast for growth and fermentation in the presence of ethanol & sucrose, Departamento de Genetica, Facultad de Biologia, Universidad de Seville, Spain
- [3] Gray, W. D. (1941), *Studies on the alcohol tolerance of yeast*, Department of Botany, Miami University, Oxford, Ohio
- [4] Gray, W. D. (1946), *The acclimatization of yeast to high concentrations of glucose: The subsequent effect upon alcohol tolerance*, Department of Botany, Iowa State College, Ames, Iowa
- [5] Gray, W. D. (1947), *Further studies on the alcohol tolerance of yeast: its relationship to cell storage products*, Department of Botany, Ohio State University, Columbus, Ohio
- [6] Hockenhull, D. J. (1964), The Selection, Improvement, and Preservation of Micro-organisms, *Progress in Industrial Microbiology*, volume V, A Heywood Book, London
- [7] Jimenez, J. and Benitez, T. (1987), Adaptation of yeast cell membrane to ethanol, *Departamento de Genetica*, Facultad de Biologia, Universidad de Seville, Spain
- [8] Jimenez, J. and Benitez, T. (1988), Adaptation of yeast to ethanol, *Departamento de Genetica*, Facultad de Biologia, Universidad de Seville, Spain
- [9] Nagodawithana, T. W. And Streinkraus, K. H. (1976), Influence of the rate of ethanol production and accumulation on the viability of *Saccharomyces cerevisiae* in "Rapid Fermentation", *Applied and Environmental Mikrobiology*, Vol. 31. No. 2
- [10] Norris, J. R. and Ribbons, D. W. (1970), Improvement of Micro-organisms by mutation, hybridization and Selection, *Methods in Microbiology: Chapter VII*, Vol. 3A, Academic Press, London & New York