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Selection of Yeast Strains for Ethanol Fermentation of Glucose-Fructose-Sucrose Mixture

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Abstract

This study was aimed to compare the ability of some yeast strains to consume sugars (sucrose, glucose and fructose) and to convert them into ethanol during fermentation. The results of this comparison will be the basis of considerations in choosing the right strain to be used as a mixed culture to increase the production of ethanol from substrate containing a mixture of sucrose, glucose and fructose, such as juice of cane and sweet sorghum. The study was conducted using fermentation in substrate consisting of glucose, fructose, and sucrose separately, glucose-fructose mixture, and glucose-fructose-sucrose mixture using some yeast strains: FNCC3012, OUT7009, OUT7027, OUT7055, OUT7080, OUT7096, OUT7903, OUT7913, and OUT7921. Following the fermentation, analysis of the produced ethanol and the remaining sugar was conducted. The results of study indicated that the strains with the highest substrate consumption were OUT7921, OUT7096, OUT7055, OUT7027, and OUT7913 for glucose, fructose, glucose-fructose mixture, sucrose, and glucose-fructose-sucrose mixture, respectively. Strains that produced highest concentration ethanol were OUT7096 in glucose and sucrose substrates, OUT7921 in substrate of glucose-fructose mixture and sucrose, OUT7913 in substrate of glucose-fructose-sucrose mixture. Upon consideration of each strain capacity, both in consuming sugar and producing ethanol, the recommended strains for use in mixed culture in bioethanol fermentation using mixed substrate of glucose, fructose and sucrose are OUT7096, OUT7913, and OUT7921.

Key Words: *Yeast, fermentation, mixed culture, sugar, ethanol.*

Introduction

The use of yeasts in ethanol fermentation has been known for long time, even the term fermentation can be traced back to Latin word "*fervere*", which means boiling. Such a state refers to the boiling bubbles of air which is actually CO_2 formed during the conversion of sugar molecules into ethanol by yeast cells (Stanbury *et al*., 1995). Type of yeast widely used in the conversion of sugar-and-starch-based substrate into ethanol is *Saccharomyces cerevisiae* since this yeast is able to produce high amount of ethanol and has high tolerance to ethanol and other inhibitor compounds (Balat *et al*., 2008).

Ethanol fermentations in a high concentration of substrate containing a mixture of sugars in the form of glucose and fructose using *Saccharomyces cerevisiae* usually occurs not completely. At the end of the fermentation, the sugar, especially fructose, remains in the medium because it could not be completely converted into ethanol. In the wine-making industry, it is referred as *stuck fermentation* or *sluggish fermentation* by which the product has an unexpected sweet taste (Tronchoni *et al*., 2009). In the production of bioethanol, the incomplete

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conversion from sugar into ethanol has caused lower productivity of fermentation (Wu *et al*., 2010). Such imperfect conversion may occur because yeasts are more capable in converting glucose into ethanol than that of fructose into ethanol (Tronchoni *et al*., 2009; Berthels *et al*, 2004; Berthels *et al*., 2008; Guillaume *et al*., 2007). Yeasts will consume fructose when glucose has run out, during which the concentration of ethanol and other inhibitor compounds is high enough to poison the yeast cells so that their ability reduced significantly. Consequently, most of fructose molecules can't be converted into ethanol and remain in the broth at the end of fermentation (Wu *et al*., 2010). Therefore, to further increase the conversion of sugars into ethanol, yeast cultures with high capacity to convert fructose to ethanol, in addition to its ability to convert glucose and other sugars into ethanol, are required.

This research was aimed to compare the ability of some yeast strains to convert some types of sugars such as glucose, fructose, and sucrose, and glucose-fructose mixture into ethanol in the fermentation process. The results of this study can be used as a basis for selecting strains which can be used to improve the ethanol fermentation in substrate containing glucose, fructose, and sucrose mixture.

Materials and Methods

Yeast

There are nine yeast strains used in this study, namely FNCC3012, OUT7009, OUT7027, OUT7055, OUT7080, OUT7096, OUT7903, OUT7913, and OUT7921. The first was obtained from the Laboratory of Microbiology, Center of Food and Nutrition Study Universitas Gadjah Mada and the next eight were generously given by Prof. Satoshi Harashima, Dept. of Biotechnology, Faculty of Engineering, Osaka University, Japan. All yeast isolates were kept at a temperature of 4ºC in Malt Extract Agar (MEA) and sub-cultured every two month.

Fermentation media and chemicals

Fermentation media comprising of glucose, fructose, sucrose, yeast extract, peptone, $MgSO_4 7H_2O$, and K_2HPO_4. Sucrose was purchased from Difco; yeast extract and peptone from Himedia, while other chemicals were purchased from Merck.

Chemicals for analysis of glucose, fructose, sucrose and ethanol concentrations consisting of potassium carbonate, dinitrosalycilic acid, $K_2Cr_2O_7$, H_2SO_4, Na_2SO_3, phenol and potassium sodium tartrate were purchased from Merck.

Starter preparation

Starters were prepared by culturing each strain in a medium containing 15% glucose; 0.15% $MgSO_4 7H_2O$, 0.15% K_2HPO_4, 0.5% yeast extract, and 0.5% peptone in 250 ml Erlenmeyer flask with a working volume of 100 ml. Incubation was carried out on a shaker at 100 rpm, at temperature of 30ºC, for 12 to 24 h or until the cell density reached 10^6 cells/ml.

Ethanol fermentation

Ethanol fermentation of sugar containing substrates were performed anaerobically in a medium containing 10% of sugar, 0.15% $MgSO_4 7H_2O$; 0.15%K_2HPO_4, 0.5% yeast extract, and 0.5% peptone in 250 ml Erlenmeyer flask with a working volume of 100 ml. Incubation was carried out statically at 30ºC for 24 h. The fermented products were stored in a freezer for subsequent analysis of glucose and ethanol after the fermentation. In a mixed sugar media of glucose and fructose, the concentrations of the two substrates are both 5%, while in the glucose-fructose-sucrose mixture, the concentration of both glucose and fructose are 2.5% and that of sucrose is 5%.

Analysis of sugars and ethanol

Analysis of sugar remaining after the glucose and fructose fermentation was performed using *dinitrosalicylic* (DNS)

method, while the analysis of residual sugar after the fermentation of sucrose and mixed sugar was conducted with high performance liquid chromatography (HPLC) because DNS method could not differentiate the three compounds each other. Moreover DNS method could not be applied for analysis of non reducing sugar such as sucrose. Ethanol produced in the fermentations was analyzed also using HPLC.

Specification of instrument and conditions used in this analysis is HPLC Knauer 2500, column Aminex HPX-87C, detector RI, aquabidest as mobile phase at rate of 0.6 ml/min and column temperature is 85^0C.

Result and Discussion

Fermentation of glucose

The concentration of residual glucose and ethanol produced after fermentation can be seen in Table 1. The results show that residual glucose concentrations in the broth after fermentation using FNCC3012, OUT7009, OUT7027, OUT7096, OUT7903, and OUT7921 strains were among the lowest, while the highest one was found in the broth of fermentation using strain OUT7080. The higher the ability of a strain to consume a certain type of sugar, the lower the concentration of residual sugar left in the broth after fermentation. Conversely, the lower the ability of a strain to consume a certain type of sugar, the higher the concentration of residual sugar will be. Therefore, it can be expected that FNCC3012, OUT7009, OUT7027, OUT7096, OUT7903, and OUT7921 strains have high ability to consume glucose, while strain OUT7080 has the lowest one.

The results in Table 1 also show that OUT7009, OUT7055, and OUT7096 strains produce the highest concentration of ethanol. Other glucose-high consuming strains such as FNCC3012, OUT7027, OUT7903, and OUT7921 turned to be low in producing ethanol. This is possible since the consumed glucose was not entirely converted into ethanol but it was also used for cell growth, cell maintenance energy, and perhaps also to produce other compounds. Comparison between the produced ethanol concentration and the consumed glucose concentration demonstrated the efficiency in glucose to ethanol conversion. Strain OUT7055 was found to have the highest ethanol producing capability as well as the highest glucose conversion efficiency.

Table 1. Glucose and ethanol concentration and ethanol yield after glucose fermentation.

No	Strain	Glucose (%)	Ethanol (%)	$Y_{p/s}$ ($g.g^{-1}$)
1	FNCC3012	0.75	7.22	0.72
2	OUT7009	0.76	7.49	0.75
3	OUT7027	0.75	6.22	0.62
4	OUT7055	2.12	7.49	0.75
5	OUT7080	7.02	1.30	0.13
6	OUT7096	0.75	7.49	0.75
7	OUT7903	0.76	6.18	0.62
8	OUT7913	1.39	7.22	0.72
9	OUT7921	0.74	6.50	0.65

Fructose fermentation

The concentrations of produced ethanol and remaining fructose in the broth after fructose fermentation were shown in Table 2. The results show that residual fructose concentrations in the broth after fermentation using OUT 7096 and OUT7913 strains were the lowest, while the highest one was found in the broth of fermentation using

Table 2. Fructose and ethanol concentration and ethanol yield after fructose fermentation

No	Strain	Fructose (%)	Ethanol (%)	$Y_{p/s}$ ($g.g^{-1}$)
1	FNCC3012	1.60	7.52	0.75
2	OUT7009	1.62	7.75	0.77
3	OUT7027	4.57	5.31	0.53
4	OUT7055	6.15	5.86	0.59
5	OUT7080	7.51	3.62	0.36
6	OUT7096	0.97	8.42	0.84
7	OUT7903	1.15	8.14	0.81
8	OUT7913	0.97	8.38	0.84
9	OUT7921	2.23	6.81	0.68

strain OUT7080. These results suggest that OUT7096 and OUT7913 strains have the highest ability to consume fructose, while OUT7080 has the lowest one. The results also show that ethanol concentrations in the fermentation broth of OUT7096, OUT7903, and OUT7913 strains was among the highest. It appears that the ability of OUT7096 and OUT7913 strains to metabolize fructose was in accordance with their ability to convert them into ethanol

Sucrose fermentation

The concentrations of residual sucrose, glucose, and fructose, as well as produced ethanol in the broth of sucrose fermentation by the strains were shown in Table 3.The results show that FNCC3012, OUT7027, and OUT7903 were sucrose high consuming strains, while OUT7080 strain was the lowest one. The highest concentration of produced ethanol was found in the broth of OUT7921 strain, followed by OUT7027, and subsequently by other strains. It means that OUT7921 strain is more efficient in converting sucrose to ethanol compared to other strains.

Fermentation of glucose-fructose mixture

The concentrations of residual glucose and fructose, as well as produced ethanol found in the fermentation broth with glucose-fructose mixture as substrates were shown in Table 4.

The results shown in Table 4 show that with glucose-fructose mixture (1:1) as the substrate, OUT7055 strain has the highest ability in consuming the sugars, followed by OUT7921, OUT7913, and FNCC3012 strains, in descending order. If the residual glucose and fructose present in the mixture were to be differentiated, it turned out that the ones consumed the highest glucose and fructose were FNCC3012 and OUT7921, respectively.

Table 3. Sugars and ethanol concentration and ethanol yield after sucrose fermentation.

No	Strain	Sucrose (%)	Glucose (%)	Fructose (%)	Sugar (%)	Ethanol (%)	$Y_{p/s}$ ($g.g^{-1}$)
1	FNCC3012	0.13	0.11	0.26	0.50	6.22	0.62
2	OUT7009	0.10	0.38	0.47	0.95	5.67	0.57
3	OUT7027	0.06	0.02	0.19	0.27	7.54	0.75
4	OUT7055	0.11	0.43	0.71	1.25	3.61	0.36
5	OUT7080	0.34	5.41	4.15	9.90	1.27	0.13
6	OUT7096	0.13	0.44	0.73	1.30	4.23	0.42
7	OUT7903	0.16	0.04	0.23	0.43	4.94	0.49
8	OUT7913	0.12	1.00	1.04	2.16	7.17	0.72
9	OUT7921	0.12	0.40	0.66	1.18	8.03	0.80

Table 4. Sugars and ethanol concentration and ethanol yield after fermentation of glucose-fructose mixture

No	Strain	Glucose (%)	Fructose (%)	Sugar (%)	Ethanol (%)	$Y_{p/s}$ ($g.g^{-1}$)
1	FNCC3012	0.16	0.35	0.51	5.13	0.51
2	OUT7009	0.30	0.54	0.84	4.64	0.46
3	OUT7027	0.19	0.46	0.65	5.00	0.50
4	OUT7055	0.16	0.30	0.46	4.84	0.48
5	OUT7080	2.28	3.18	5.46	3.34	0.33
6	OUT7096	0.27	0.45	0.72	5.13	0.51
7	OUT7903	0.61	1.00	1.61	4.96	0.50
8	OUT7913	0.18	0.32	0.50	4.60	0.46
9	OUT7921	0.20	0.29	0.49	5.28	0.53

Table 5. Sugars and ethanol concentration and ethanol yield after fermentation of glucose-fructose-sucrose mixture.

No	Strain	Glucose (%)	Fructose (%)	Sucrose (%)	Sugar (%)	Ethanol (%)	$Y_{p/s}$ ($g.g^{-1}$)
1	FNCC3012	0.70	1.15	2.99	4.84	3.32	0.33
2	OUT7009	0.45	0.58	1.39	2.43	4.81	0.48
3	OUT7027	1.24	1.93	0.26	3.43	2.02	0.20
4	OUT7055	1.42	2.65	0.30	4.37	2.35	0.23
5	OUT7080	1.75	2.29	3.23	7.27	1.19	0.12
6	OUT7096	0.80	1.44	0.30	2.54	3.25	0.32
7	OUT7903	0.76	1.72	0.14	2.62	4.13	0.41
8	OUT7913	0.16	0.64	0.15	0.95	5.63	0.56
9	OUT7921	0.44	1.19	0.15	1.78	5.05	0.50

The highest ethanol concentration was generated by OUT7921, followed by OUT7096 and subsequently by other strains.

Fermentation of glucose-fructose-sucrose mixture

The concentrations of residual glucose, fructose, and sucrose, as well as produced ethanol found in the fermentation broth after fermentation of glucose-fructose-sucrose mixture (2.5%: 2.5%: 5.0%) were shown in Table 5.

The results show that when a mixture of glucose-fructose-sucrose was used as a substrate for ethanol fermentation, the lowest concentration of residual sugar was found in fermentation broth of OUT7913 strain, followed by that of OUT7921. The results also show that the highest concentration of ethanol was also produced by OUT7913, followed by OUT7921, and subsequently by other strains.

The ability of each strain in consuming sugar and producing ethanol was different due to differences in responding to the influence of some factors such as sugar concentration, ethanol concentration and temperature. The differences in responses were determined by the genetic and physiologic stability of each yeast strain (Souza *et al*., 2007).

From the results of those experiments, it can be noticed that the concentration of residual fructose after fermentation was almost always higher than that of glucose. This occurred to the fermentation of all five substrate used. The result is in accordance with those of earlier researches (Berthels *et al*., 2004, Tronchoni *et al*., 2009). While the *glucophylic* character of yeasts has been so far remains unclear as to the cause, there were hypotheses proposed by some authors. Guillaume *et al* (2007) assumed that differences in glucose and fructose consumption are due to the differences in transporting both compounds across cell plasma membranes, while Berthels *et al* (2008) supposed that it was caused by the differences in the kinetics of *phosphorylatio*n of both hexoses in cells. Both transporter and kinase for the two compounds are having different affinity and preference to glucose/ sucrose. While it is strongly assumed that the differences between fermentation of glucose and fructose lie in the transport and/or in the *phosphorylation* stages of the fermentation trajectory, it is not an impossibility that other causes existed. Yeast cells are known to have at least one glucose sensor protein in the plasma membrane, i.e. *GprI* which is known to have different affinities for glucose and fructose. It remains unclear whether yeast cells have a specific sensor for fructose. Other process differently affected by glucose and fructose is catabolite repression. With the availability of quickly fermentable sugars, such as glucose and fructose, yeast cells regulate the expression of genes involved in respiration, *gluconeogenesis,* and the metabolism of alternative carbon

sources. Maintenance of catabolite repression by glucose requires *Hxk2*, while fructose catabolite repression requires either *Hxk2* or *Hxk1*. Repression of fructose can be triggered by a mechanism somewhat different from that of glucose repression. Data suggest that differences in the fermentation of glucose and fructose may not only caused by one or more differences in the characteristics of the kinetics of transporters and enzymes of initial fermentation pathway (Berthels *et al*., 2004).

It can also be observed that the ability of a yeast strain to consume certain types of sugars is not directly related to its ability to consume the same type of sugar when it is mixed with other type of sugars. For example, OUT7921 strain with the highest capacity in metabolizing glucose does not necessarily mean that it will be the highest glucose metabolizer when the glucose present in a mixture. This phenomena can be related to differences in the preferences of each strain to glucose and fructose (Messias *et al*., 2008), and the ability to break down the sucrose molecules into glucose and fructose (Wang *et al*., 2004; Rolz and de Leon, 2011).

The amount of sugar consumed by each yeast strain is not necessarily proportional to the amount of ethanol produced during fermentation. For example, in glucose substrate, OUT7921 strain spent highest amount of glucose but does not produce the highest amount of ethanol. It turned out that the one produces the most ethanol is OUT7096 strain with slightly lower glucose consumption than OUT7921. This was the case since the consumed sugar is not merely converted into ethanol but is also used for other purposes such as to grow cells (Govindaswamy *et al.,* 2007) or to produce other substances (Matsushika and Sawayama, 2010).

The average concentrations of produced ethanol in glucose and fructose substrates were higher than those produced on the sucrose substrate. This indicated the influence of the *invertase* enzyme activity of yeast on the fermentation. In glucose and fructose substrates, the molecules can directly enter the *glycolytic* pathway for subsequent conversion into ethanol in the fermentation stage. This will be different if the substrate is sucrose. Sucrose molecules must first be hydrolyzed into glucose and fructose molecules and then entering the *glycolytic* pathway and subsequently converted into ethanol. This prolongs the mechanism of sucrose fermentation in yeast (Rolz and de Leon, 2011) and lead to a longer *lag* phase in sucrose substrates (Wang *et al*., 2004). With a longer *lag* phase, the conversion of substrates into products will be slower so that the results obtained are much less when the fermentation time is limited.

Ethanol produced in the fermentation of glucose-fructose mixture (1:1) was largely lower than those obtained in the sucrose substrates, while theoretically sucrose molecules will be split into molecules of glucose and fructose with the same number of moles. It seems that the osmotic pressure due to the high concentration of sugar known as Sugar Shock (Lefebvre, 2012) gives a considerable influence in this regard. Mixing a quantity of glucose and fructose directly into one solution will provide a greater osmotic pressure than that of dissolving sucrose with the same mass into one solution. Using equation $\pi = cRTi$ where π = osmotic pressure; c = molar concentration of dissolved substance; R = ideal gas constant; T = Kelvin temperature; and i = number of particle per dissolved molecular unit (Moore *et al*., 2011), it can be seen that 10% sucrose solution will provide an osmotic pressure of 7.27 atm at 30°C, while solution containing 5% glucose and 5% fructose at similar temperature will provide stronger osmotic pressure, which is 13.82 atm.

Based on the ability of each strain in consuming sugar in each of the above substrates, we made the following ranking: OUT7921 > OUT7913 = OUT7096 > OUT7027 = OUT7055 > FNCC3012 = OUT7903 > other strains. In ethanol production, the ranking can be as follow: OUT7921 = OUT7096 > OUT7913 > OUT7903 = OUT7027 > other strains. All

three strains ranked on the forefront order, both in sugar consumption and ethanol production, i.e. OUT7921, OUT7096 and OUT7913, are largely having the advantage in all types of substrate employed. Thus, the three strains can be considered for use as mixed culture in ethanol fermentation using sugar substrates consisting of glucose, fructose, and sucrose components.

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