Increasing Bioethanol Yield from Fermentation of Sweet Sorghum (Sorghum bicolor L. Moench) Sap by Mixed Culture Composed of Two Yeast Strains

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Abstract

Objectives: To evaluate the performance of the mixed culture of yeast strains to increase ethanol fermentation yield of sweet sorghum sap. Methods/Statistical Analysis: The yeast strains used were OUT7096, OUT7913, and OUT7921 while sweet sorghum saps utilized were KCS105 and FS501. Fermentation was performed in batch process and concentration of ethanol and sugars were determined by HPLC. Performance of each culture was indicated by ethanol yield and sugar conversion efficiency. The best culture was indicated by the best ethanol yield determined based on the one way ANOVA and Least Significant Difference test. Findings: The results showed that the composition of sugars and minerals in the two sweet sorghum saps are good for ethanol fermentation but supplementation with a bit of nitrogen and phosphorus may necessary for optimum yield. On the fermentation of KCS105 sap, all the three mixed culture especially OUT7913/OUT7921 (1:1) provided higher performance than when used in single. On the fermentation of FS501 sap, the mixed culture of OUT7096/OUT79013 (1:1) showed the highest performance among all of the cultures used, despite the data are not significant. The high value of SCE in both fermentation of KCS105 and FS501 saps suggested the occurrence of sugars other than sucrose, glucose and fructose. It was also found that mixed culture of OUT7913/OUT7921 (1:1) was more glucophilic than the mixed culture of OUT7906/OUT79013 (1:1). On the contrary, OUT7913/OUT7921 (1:1) culture was more fructophilic than the culture of OUT7913/OUT7921 (1:1). Applications/Improvements: Mixed culture may improve ethanol yield of fermentation of substrate containing types of sugar like sweet sorghum sap.

Keywords: Bioethanol Yield, Fermentation, Mixed Culture, Sweet Sorghum, Yeast Strains

1. Introduction

Oil production of Indonesia decreases from year to year since 1996. At the same period, the need of energy increases continually because of population and industrial growth and transportation. In global context, the stock of fossil energy such as coal, petroleum, and natural gas are decreasing continually. On the other hand, economic growth in a number of big countries for example China and India will encourage the needs of a larger energy sources. At the same time, the world community pays more attention to the environment issues, including the contribution of petroleum for the global warming. For this reason, we are urged to seek the new and renewable as well as low pollution energy sources. The sources of alternative energy produced from biomass include bioethanol and biodiesel. Bioethanol produced from plants is an interesting fuel because of its good quality of burning. Bioethanol is ethanol obtained from conversion of carbohydrates in the biomass to ethanol via fermentation using microorganisms. The most widely microorganisms used in bioethanol fermentation is Saccharomyces cerevisiae.

One of the plants having a good prospect for bioethanol production is sweet sorghum (Sorghum bicolor (L)
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Moench). However, several studies showed that ethanol concentration and sugar conversion efficiency resulted from fermentation of sweet sorghum sap actually is still lower than the maximum level could be reached, hence this present study try to increase ethanol yield of the process.

Sugars composition of sweet sorghum sap are mainly influenced by type of cultivar and planting time and age of the crop. Generally, sucrose has the highest concentration followed by glucose and fructose respectively. There are some features of sweet sorghum sap related to the sugar composition may affect the yield of ethanol fermentation:

- The high concentration of fructose (equal to glucose) and the nature of yeasts which prefer glucose to fructose cause the conversion of sugar into ethanol;
- When the sugars concentration of juice is too high, yeast cells will suffer from osmotic stress and substrate inhibition, resulting in incomplete of fermentation; and
- the most sugar in the sweet sorghum juice is sucrose, thus requires yeast strains having high activity of invertase.

Many factors affecting the success of ethanol fermentation process, several of which are yeast strains, sugars concentration, and the nature of substrates. In order to obtain an optimal result, the fermentation must be held at the best conditions of every factor. Yeast strains used should be chosen which have a great ability in converting all kind of sugar in the medium to ethanol.

Strains of Saccharomyces used for ethanol fuel production so far are effective in using glucose but less effective for fructose. On the contrary, the strains used in making wine, particularly for dry wine, more effective in changing fructose to ethanol compare to the most strains used in making bread and beer. These features suggest that different species or strains of yeast may give different result (yield or composition) of sugar fermentation. Regarding sweet sorghum sap is mainly consisted of a blend of sugars (sucrose, glucose, and fructose), it was supposed to be better to fermenting it using mixed culture of strains yeast.

Based on previously study, there are three isolates recommended: OUT7096, OUT7913, and OUT7921. OUT7096 is a strain of Saccharomyces chevalieri isolated from wine; OUT7913 is a strain of Saccharomyces steineri isolated from Kwass, a kind of alcoholic drink made from honey, hops, water, and berry juice; and OUT7921 is a strain of Saccharomyces steineri isolated from Florilyin yeast, a kind of active dry yeast using for making bread.

From the three strains, then the formula of mixed culture recommended are OUT7096/OUT7913, OUT7096/OUT7921, and OUT7913/7921. (Note: OUT7096/OUT7913 means that the culture composed of OUT7096 strain and OUT7913 strain with a ratio of amount of cells 1:1. This explanation is also applied to the two other mixed culture. This recommendation is based on our previous study.

2. Material and Method

2.1 Microorganism, Medium, and Chemicals

2.1.1 Yeast

Strains of yeast used in this project are: OUT7096, OUT7913, and OUT7921 obtained from Microbiology Laboratory, Faculty of Agriculture, Gadjah Mada University. The isolates were cultured on media of Malt Extract Agar, stored in refrigerator at 4°C, and refreshed every two months.

2.1.2 Sweet Sorghum Sap

Sweet sorghum saps used in this study are obtained from cultivar of KCS105 and FS501 planted in Kricak Village, Subdistrict of Tegalrejo, Yogyakarta City.

2.1.3 Chemicals and Media

Glucose, fructose, magnesium sulphate (MgSO4·7H2O), potassium hydrogen phosphate (K2HPO4), and malt extract agar (MEA) were obtained from Merck; sucrose from Difco; yeast extract and peptone were from Himedia.

2.2 Apparatus and Instruments

2.2.1 Apparatus

A 1000 ml bottle of Duran, rubber stopper, small plastic tube, microscope, Hemositometer, laminar air flow, peristaltic pump, micropipette, shaker, and freezer.

2.2.2 Instrument

A set of HPLC (Knauer smartline RI detector 2300, Germany; column Aminex HPX-87C 300 x 7,8 mm Bio-Rad, USA at temperature of 85°C).
2.3 Procedure

Mainly, the procedure in this study were involved analysis of sorghum sap composition, fermentation of sweet sorghum sap using mixed culture, and analysis of ethanol and residual sugars in the results of fermentation.

2.3.1 Analysis of Sweet Sorghum Sap Composition

Components of sucrose, glucose, and fructose were analyzed using HPLC method, nitrogen by Kjeldahl method, while phosphor and potassium were determined by wet oxidation method using HNO₃ and HClO₄.

2.3.2 Inoculum Preparation

Inoculum were prepared by culturing the three strains of yeast separately on a media containing a blend of sucrose, glucose, and fructose with composition 5.0, 2.5, and 2.5% respectively; both MgSO₄·7H₂O and K₂HPO₄ were 0.1%; and both peptone and yeast extract were 0.5%. Incubation was carried out on an orbital shaker at 100 rpm for 20 hour or until the density reach 10⁶ cell ml⁻¹.

2.3.3 Fermentation of Sweet Sorghum Sap

The liquid of sap was filtered using steril cloth and then pH was adjusted to 5.0. After sterilization and cooling to room temperature, the media in experimental group were fermented using three mixed culture namely: OUT7096/OUT7913 (1:1), OUT7096/OUT7921 (1:1), and OUT7913/7921 (1:1). Media in control group were fermented using single cultures of OUT7096, OUT7913, and OUT7921. Cell's concentration at initial fermentation is 3.5 x 10⁷ cell ml⁻¹ and working volume of fermenter is 800 ml. Incubation was performed at 30°C, static, and anaerobe condition for 72 hour. Upon finishing the incubation, the broth was then be freeze at -20°C in refrigerator to stop the fermentation process while waiting for analysis. Analysis of ethanol and sugars residual were then carried out using a set of HPLC equipped by Knauer smartline RI detector 2300, Germany and column Aminex HPX-87C 300 x 7.8 mm Bio-Rad, USA at temperature of 85°C. Mobil phase utilized is double-distilled water at flow rate of 0.6 ml per minute.

3. Results and Discussion

3.1 Composition of Sweet Sorghum Sap

Composition of sweet sorghum sap used in this study including sugars, nitrogen, phosphorus, and potassium is shown in Table 1. Based on the data in Table 1 it can be seen that composition of sugars, nitrogen, phosphorus, and potassium in the two cultivar are different. Totally, sugars content of KCS105 sap are higher than that of FS501 sap. This data is different from the data obtained before that sugars content of FS501 sap are slightly higher (1.1 times) than that of KCS105 sap. From Table 1 it also can be seen that distribution of the three types of sugar are variously between the two type of cultivar. In the sap of KCS105, fructose content is 1.1 times higher than glucose content, on the contrary, in the sap of FS501, glucose concentration is 1.5 times higher than that of fructose.

| No | Cultivar  | Parameter | Composition (%) | No | Cultivar  | Parameter | Composition (%) |
|----|----------|-----------|----------------|----|----------|-----------|----------------|
| 1  | KCS105   | Sucrose   | 9.75           | 2  | FS501    | sucrose   | 7.64           |
|    |          | Glucose   | 4.90           |    |          | glucose   | 5.52           |
|    |          | Fructose  | 5.40           |    |          | fructose  | 3.70           |
|    |          | N         | 0.04           |    |          | N         | 0.05           |
|    |          | P₂O₅      | 0.08           |    |          | P₂O₅      | 0.06           |
|    |          | K₂O       | 1.27           |    |          | K₂O       | 0.94           |
|    |          | water and others | 78.56     |    |          | water and others | 82.09     |
|    |          | Total     | 100.00         |    |          | Total     | 100.00         |
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while sucrose content is higher than the others in both the sap. Distribution of glucose and fructose in the sap of KCS105 in this study are in line with the data obtained previously but differ in the sap of FS501.20 Other research showed that sucrose content in all variety of sweet sorghum is the highest among the three sugars, and glucose content is higher than fructose content.21 The existence of these differences suggest that sugars content in different sweet sorghum sap is affected by many factors, for example type of cultivar, planting time, temperature, crop age, planting space, and fertilizer.2,22,23

The maximum rate of ethanol production can be reached at sugar concentration of 150 g.l⁻¹ or 15%.24 When sugar level more than 15%, catabolite inhibition will occur and the rate of conversion of sugar to ethanol become lower.25 This fact indicate that sugars level of FS501 sap is ideal for ethanol fermentation, whereas sugar’s level of KCS105 sap is higher than the ideal concentration. This is not means that KCS105 sap will produced lower concentration of ethanol, instead it need a longer time to complete the conversion of its sugar to ethanol.

Beside sugars, other components playing important role in convert sugar to ethanol using yeast are nitrogen, phosphorus, potassium, vitamins and other minerals. Nitrogen is necessary for protein and nucleic acid synthesis during cells growth, phosphorus is needed for synthesis of nucleic acid, ATP, phospholipid, and other compounds containing phosphorus, while potassium is one of several metal elements which essential for microorganism growth.26 Potassium is also play an important role in osmoregulation, charge equilibrium, regulation of absorption of doubled-charged ions and phosphate in to the cell. Low potassium and high sodium condition will cause intoxication on yeast cells.27 A previously study, show that sugar content and the abundant of mineral elements make sweet sorghum sap a good and economical substrate for ethanol production.28 The study also show that maximum ethanol formation rate is 119.12 g.l⁻¹ hour⁻¹ could be reached using sweet sorghum sap containing sugar 180.7 g.l⁻¹, nitrogen 2.15 g.l⁻¹, and phosphorus 0.77 g.l⁻¹. Another study could produce 135 g.l⁻¹ ethanol from high sugar sweet sorghum sap (300 g.l⁻¹) supplemented with 0.8 g.l⁻¹ nitrogen in the form of CO(NH₄)₂.29

Based on the data in Table 1 and the discussion above, it can be conclude that from the point of sugar content, the two type of sweet sorghum sap are good for ethanol production. Supplementation with a bit of nitrogen and phosphorus sources may necessary for optimize the fermentation process.

### 3.2 Fermentation of KCS105 Sap

Fermentation of KCS105 sap give data showed in Table 2. It can be seen that mixed culture of OUT7913/OUT7921 (1:1) get the highest value of ethanol concentration, ethanol yield, and sugar conversion efficiency (SCE). This data is in line with the result of fermentation on sucrose-glucose-fructose mixture using mixed culture.19 Fermentation on KCS105 sap containing 19.59% of sugar using mixed culture of OUT7913/OUT7921 (1:1) provide average of ethanol concentration 11.59%. This value is 1.21 to 1.52 times higher than ethanol produced by two others mixed culture and all single culture. Ethanol yield obtained by this culture is 1.21 to 1.48 times higher than those of reached by other culture. This result indicates that the mixed culture of OUT7913/OUT7921 (1:1) is the most powerful in converting sweet sorghum sap of KCS105 to ethanol. This result also higher than those of obtained in several previous study.30,31

The fermentation using mixed culture of OUT7913/OUT7921 (1:1) provide high efficiency in converting sugar

| No. | Initial sugar (% w/v) | Ethanol (%) | Ethanol yield | SCE (%) | Culture       |
|-----|----------------------|-------------|---------------|--------|---------------|
| 1   | 19.596               | 9.588 ab    | 0.489 ab      | 97.080 | OUT7096/OUT7913 (1:1) |
| 2   | 19.596               | 7.828 a     | 0.398 a       | 79.260 | OUT7096/OUT7921 (1:1) |
| 3   | 19.596               | 11.590 b    | 0.591 b       | 117.350| OUT7913/OUT7921 (1:1) |
| Controls | 19.224               | 7.974 a    | 0.413 a       | 82.300 | OUT7096 |
| 5   | 19.224               | 7.616 a     | 0.398 a       | 78.605 | OUT7913 |
| 6   | 19.224               | 7.896 a     | 0.405 a       | 81.495 | OUT7921 |
to ethanol. This is indicated by the value of SCE (sugar conversion efficiency) reaching 117.35%. This high value is in accordance with the result of a research which show that the use of mixed culture of F. Oxysporum and S. cerevisiae on bioethanol production from sweet sorghum using saccharification and simultaneously fermentation provide the higher SCE than theoretical value based on dissolved sugar. The very high SCE value (>100%) turning up a suggestion of the occurrence of any other sugar than the three (sucrose, glucose, and fructose) considered before in the sap of sweet sorghum. Possibility of existence of other sugars is supported by FAO which shows that the types of sugars exist in sweet sorghum sap are xylose, ribose, arabinose, fructose, sorbose, galactose, manose, sucrose, polyglucose, and glucose.

Figure 1 shows the increasing of ethanol concentration since the start of fermentation and reach maximum at the 72nd hour. The graph of ethanol increasing almost linear, there is no any surge and stagnation at a certain period. This graph suggests that ethanol formation continually progress with almost constant rate until the 72nd hour. This pattern and the pattern of reduction of total sugar concentration are similar but their orientation are different each other.

Figure 1. The alteration process of sucrose, glucose, fructose, ethanol, and biomass during the fermentation of KCS105 sap at static condition, temperature of 30°C, and initial pH 5.0.

Although the concentration of sucrose at the start of fermentation is drastically decrease, the glucose was just a bit decrease even fructose was increase. Thus, over all, profile of sugar concentration decline almost linear. Both the graph of ethanol increasing and sugar decreasing show that the rate of fermentation of KCS105 sap is slower than that of fermentation of sucrose-glucose-fructose mixture. This difference may be caused by the difference of sugar and others compound concentration in the medium. Possibility of inhibitor compound as the main problem has been ignored because previous study had showed that sweet sorghum sap of KCS105 did not indicate the presence of the inhibitor. Thus the slow fermentation may be caused only by high concentration of sugar in the substrate. Despite the fermentation of KCS105 juice go slower than that of sucrose-glucose-fructose mixture, actually the rate of fermentation of KCS105 juice is fairly fast because it could produce ethanol about 48 g.L⁻¹ (4.8%w/v) at 16th hour. As comparative data, in fermentation of sorghum RSSV9 juice, production of ethanol only reach 26.19 g.L⁻¹ at 15th hour and 43.42 g.L⁻¹ at 18th hour. In fermentation of sorghum CSH22SS, ethanol production only reach 31.25 g.L⁻¹ at 15th hour.

3.3 Fermentation of FS501 Sap

On the fermentation of FS501 sap, mixed culture of OUT7096/OUT79013 (1:1) gave ethanol concentration, ethanol yield, and SCE 6.54%, 0.57, and 110.47% respectively. The highest ethanol concentration and SCE were achieved by OUT7096/OUT7913 (1:1) followed by OUT7913/OUT7921 (1:1) and single culture OUT7921. Nevertheless, the difference of ethanol yield among the three mixed cultures and the single culture are not significant based on LSD test with α=0.05. Therefore, it can be said that all of culture used in this fermentation provide almost the same result. Comparing to others study suggest that ethanol yield from fermentation of FS501 sap using mixed culture OUT7096/OUT7913 (1:1) is high enough. For example, fermentation of sorghum Keller-40 sap by single culture S. cerevisiae only provide ethanol yield 0.47; fermentation of sorghum sap of AM-4 using single culture S. cerevisiae obtain ethanol yield only 0.48. Comparing the data to the results obtain in fermentation of KCS105 sap, it can be seen that mixed culture of OUT7913/OUT7921 (1:1) and OUT7096/OUT7913 (1:1) consistently provide the highest yield on the fermentation of the two juice. Over all, from fermentation of mixed sugar, the most consistent culture to provide the best results is the mixed culture of OUT7096/OUT7913 (1:1).

During the fermentation process, the rate of fructose consumption is more than that of two others sugar but all of the three diminished at 52nd hour.
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The change of ethanol concentration appeared here is different from that occur on the fermentation of KCS105 juice. On this fermentation, the maximum ethanol concentration achieved around the 52<sup>nd</sup> hour, faster 1.4 times than that of fermentation of KCS105 juice. This is probably because the concentration of sugar in FS501 juice is so lower that consumed faster by yeast cells. A slight increasing of ethanol concentration after 20<sup>th</sup> hour indicated that sucrose and glucose were still existing in the broth though fructose had been used up.

Figure 2 shows that the consumption of glucose is not faster than the consumption of fructose. The concentration of fructose is always lower than that of glucose during the fermentation proceed. The profile of this fermentation is different from the profile of fermentation of sugar mixture and the fermentation of sweet sorghum KCS105 sap. During the fermentation of sugar mixture, the rate of diminution of glucose concentration from the start of fermentation until 20<sup>th</sup> hours, is 1.40 times greater than that of fructose concentration. Furthermore, on the fermentation KCS juice, from the start until 20<sup>th</sup> hours, the rate of glucose concentration decreases at 0.08 % hour<sup>-1</sup>, while fructose concentration on the contrary, increase at 0.03% hour<sup>-1</sup>. This feature indicates that in both previously fermentation, the consumption of glucose was greater than that of fructose. This result imply that the culture used on the fermentation of sugar mixture and fermentation of KCS105 juice namely OUT7913/OUT7921<sub>(1:1)</sub> was more *glucophylic* than the culture used on the fermentation of FS501 juice. On the other hand, culture used on the fermentation of FS501 juice, namely OUT7096/OUT7913<sub>(1:1)</sub> was more *fructophylic* than the culture used on the fermentation of sugar mixture and fermentation of KCS105 juice. The *fructophylic* character appeared to be supported by data of fructose fermentation in previously study which showed that the most powerful strain to consume fructose is OUT7096 followed by OUT7913 is shown in Table 3.<sup>15</sup>

![Figure 2. The alteration process of sucrose, glucose, fructose, ethanol, and biomass during the fermentation of FS501 sap at static condition, temperature of 30°C, and initial pH 5.0.](image)

### Table 3. Ethanol concentration, ethanol yield, and SCE obtained from fermentation of FS501 Sap

| No. | Initial sugar (% w/v) | Ethanol (%, w/v) | Ethanol yield | SCE (%) | Culture                  |
|-----|-----------------------|------------------|---------------|---------|--------------------------|
| 1   | 11.749                | 6.541 a          | 0.566 a       | 110.471 | OUT7096/OUT7913<sub>(1:1)</sub> |
| 2   | 11.717                | 5.827 a          | 0.478 a       | 98.673  | OUT7096/OUT7921<sub>(1:1)</sub> |
| 3   | 11.933                | 6.207 a          | 0.518 a       | 103.213 | OUT7913/OUT7921<sub>(1:1)</sub> |
| 4   | 12.901                | 6.460 a          | 0.506 a       | 99.350  | OUT7096                  |
| 5   | 12.901                | 5.051 a          | 0.388 a       | 77.688  | OUT7913                  |
| 6   | 12.901                | 6.620 a          | 0.496 a       | 101.819 | OUT7921                  |

Control

Based on the whole results, it can be concluded that ethanol fermentation of sweet sorghum sap using mixed culture may produce higher ethanol yield than that of using single culture, although the results may also be influenced by sugar composition of the sap.

### 4. Acknowledgements

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