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The Effect of Hydrolysis Time with *Trichoderma Reesei* on Soluble Sugar Concentration in Sweet Sorghum Stalk Pulp and Alcohol Concentration of Its Fermentation Results

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Abstrak- It has been done a study on the use of *Trichoderma reesei* in the hydrolysis of sweet sorghum stalk pulp continued to fermentation by *Saccharomyces cerevisiae* to produce bioethanol. The purpose of this study was to examine the effect of hydrolysis time by *T. reesei* on the sugar content of sweet sorghum stalk pulp and the ethanol concentration obtained after its fermentation using *Saccharomyces cerevisiae*. The sweet sorghum stalks were cut into small pieces, blended until crushed, incubated with *T. reesei* at various times. and then fermented using *S. cerevisiae* for 24 hours after the incubation period was complete. The results showed that the soluble sugar concentration in the sweet sorghum stalk pulp increased after the incubation, which occurred from the 8th to the 24th hour. Furthermore, it continued to decrease until the longest incubation time. The alcohol concentration produced after the fermentation period changed according to the pattern of changes in sugar concentration in the substrate. These results suggest that to increase bioethanol production from sweet sorghum stalk, it will be better to incubate the pulp of stalk first with *T. reesei* for approximately 24 hours before fermented with *S. cerevisiae*.

INTRODUCTION

One of the plants with very good prospects as a source of raw material for making bioethanol is sweet sorghum (*Sorghum bicolor (L) Moench*), because it has several advantages such as short life, less water requirements, and low cost of crop management compared to sugarcane and sugar beet [1]. The composition of sweet sorghum stalks includes water about 70% wet weight, soluble carbohydrates about 58.2% dry weight and insoluble carbohydrates including cellulose and hemicellulose at 22.6% dry weight [2].

The most part of the sorghum that has been used to produce bioethanol is sap from the stems [3]. As for the fruit, it is more profitable if it is used as food or animal feed [4], while the bagasse is also more often used as animal feed [5], [6]. Actually, the bagasse can also be converted into bioethanol but the process is longer and complicated and requires more resources [7], [8]. The bagasse must first be pre-treated and hydrolysed before being fermented into ethanol.

The conversion of sap and bagasse to ethanol separately requires a lot of process and of course resources. The conversion of sap consisting of the squeezing stage of the sorghum stalks to produce sap and then the fermentation of sap to ethanol. The conversion of bagasse consists of grinding the bagasse into pulp, hydrolysis the pulp, and the fermentation of hydrolysate into ethanol. So there are five stages in total. In this study, we converted the two materials (sap and bagasse) in one process consisting of three continuous stages, namely grinding sorghum stalks, followed by hydrolysis, then fermentation. In this way, it is expected that the bioethanol yield obtained from sweet sorghum will be higher than if it is only converted from sap.

During the conversion of carbohydrates to ethanol using a single culture of *S. cerevisiae*, only soluble sugars (sucrose, glucose and fructose) are converted to ethanol while cellulose and

hemicellulose are not converted therefore, the ethanol yield achieved is not optimal. In order to obtain a higher yield, cellulose and hemicellulose needs to be converted to ethanol, which is carried out by breaking down cellulose and hemicellulose into glucose and xylose molecules, respectively. Furthermore, to break down these components, cellulolytic organisms such as *Trichoderma reesei* are needed because this fungal species produce cellulolytic enzymes that hydrolyzes lignocellulose material into soluble sugars in the form of glucose and xylose [9].

This assumption is based on the theoretical framework that *T. reesei* plays a role in hydrolysing insoluble carbohydrates into soluble sugars and *S. cerevisiae* in converting soluble sugars into ethanol. The soluble sugar converted to ethanol is obtained from sorghum stalks and hydrolysis with *T. reesei*.

Cellulose is a homo polysaccharide with glucose as its monomer which are connected to one another through β (1-4) *glycosidic* bonds therefore, its hydrolysis will produce glucose as in Eq. (1).

$$\underset{\text{Cellulose}}{(C_6H_{10}O_5)n} \xrightarrow[\textit{cellulases}]{H_2O} \underset{\text{glucose}}{n\ C_6H_{12}O_6} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Hemicellulose is a hetero polysaccharide with a complex structure containing glucose, xylose, mannose, galactose, arabinose, *fucose*, *glucouronic* acid and *galactouronic* acid in varying amounts, depending on the source of material [10]. Therefore, the results of the hemicellulose hydrolysis reaction are different, depending on the type of monomer in the polymer structure.

In the fermentation stage, the reaction generating ethanol is written as in Eq. (2).

$$\underset{\text{glucose}}{C_6H_{12}O_6} \xrightarrow[\text{fermentation}]{\textit{S. cerevisiae}} \underset{\text{ethanol}}{2\ C_2H_5OH} + 2\ CO_2 \quad \ldots\ldots\ldots\ldots \quad (2)$$

The important point of this research is to show whether the combination of the two processes of converting sweet sorghum stalks into ethanol can work well. The success of a fermentation process is driven by many factors, one of which is the time or duration of fermentation. Therefore, in the first study, researchers examined the effect of fermentation time on the concentration of dissolved sugars and ethanol produced.

RESEARCH METHODS

Research materials

Substrate: Sweet sorghum stalks were obtained from farm area in Tarus, Kupang Regency. **Microorganism**: *T. reesei* were purchased from the Laboratory of Food and Nutrition Department, Gadjah Mada University and *S. cerevisiae* was isolated from commercial yeast Fermipan. **Solid medium** : potato dextrose agar (PDA) was purchased from Merck. **Nutrient solution**: citric buffer solution, yeast extract, bacteriological peptone, $(NH_4)2SO_4$, KH_2PO_4, $FeSO_4.7H_2O$, carboxy methyl cellulose (CMC) and glucose were purchased from Merck.

Refreshing the T. reesei

The *isolates* of *T. reesei* were taken using loop needles that had been sterilized with Bunsen flame. Furthermore, the fungus was inoculated by means of zigzagging scratches on the surface of PDA media and incubated at 37°C for 7 days. All the processes were carried out aseptically in laminar air flow.

Preparation of nutrient solution

The nutrient solution was prepared by mixing a citrate buffer solution pH 3.5 with 1.0 g of yeast extract, 1.5 g of Bacteriological peptone, 1,4 g of $(NH_4)2SO_4$, 2,0 g of KH_2PO_4, 0,005 g of $FeSO_4.7H_2O$ and 5 ml of 1% CMC (Carboxy methyl cellulose) solution [11]. Furthermore, the solution was stirred using a magnetic stirrer until homogeneous.

Preparation of T. reesei culture

The wire loop was annealed in a bunsen flame and used to extract *T. ressei* isolates. Furthermore, the *T. reesei* cells or spores attached to the end of the wire loop were put into an Erlenmeyer flask containing a nutrient solution. The wire loop was shaken several times in the nutrient solution to ensure that all the yeast cells enter the solution. Subsequently, the Erlenmeyer flask was covered with cotton and incubated at room temperature for 7 days until the fungal mycelium grew.

Preparation of S. cerevisiae culture

Five grams of glucose, 0.5 grams of yeast extract, 0.5 grams of peptone, 0.15 grams of $MgSO_4.7H_2O$ and 0.15 grams of KH_2PO_4 were dissolved with distilled water to a solution volume of 100 mL in Erlenmeyer flask. Furthermore, the solution was sterilized using an autoclave at 121 ^{0}C for 15 minutes and was cooled to room temperature. After cooling the medium, 1 gram of baker's yeast (Fermipan, France) was added, stirred until homogeneous, clogged with cotton and shaken for 20 hours until the cell concentration reached 10^6 - 10^7 cells/mL.

Making of Sweet Sorghum (Sorghum bicolor L) Stalk Pulp

The stalks of 115 days old sweet sorghum were felled, the leaves and fruit were separated and cut into small pieces (±1 cm). The pieces then were added with water in ratio of 1:1 and blended to became pulp. Before used, this pulp was stored in a cooling machine hence the sugar content was preserved.

Bioethanol Production

Hydrolysis Stage

In this stage, 35 g of sorghum stalk pulp was put into a 200 mL bottle, added by 60 mL of distilled water and autoclaved at 121^{o}C for 15 minutes. After cooling at room temperature, the mixture was added aseptically with 8 mL of *T. reesei* culture and then placed on a shaker at a speed of 100 rpm. This experiment was carried out in 8 groups with varying times (length) of hydrolysis on shakers, namely 8, 16, 24, 32, 40, 48, 56 and 64 hours. Each group consisted of 3 replications, hence the total was 24 units. After the time was over, 10 mL of samples were taken from each bottle and filtered with *Whatman* filter paper and the clear solution obtained was measured for its sugar concentration using a refractometer.

Fermentation Stage

The results of hydrolysis were continued to the fermentation stage by opening a bottle in laminar airflow and adding 8 mL of *S. cerevisiae* culture, shaking slowly until the mixture became homogeneous, then incubated at 30^{o}C for 24 hours. This was carried out for all hydrolysis products, hence the total was 24 fermentation units.

Determination of Alcohol Content

The fermentation results were filtered, 80 mL of this filter was added with 80 ml of distilled water, and the mixture was then distilled until 80 mL of distillate was obtained. Furthermore, the concentration of alcohol in the distillate was measured using an alcoholmeter.

Data Analysis

Data of sugar content in sweet sorghum stalk pulp from hydrolysis were analysed using one-way ANOVA to determine the difference in sugar concentration as a result of variations in hydrolysis time. Furthermore, it was followed by the Tukey test to discover which data were

significantly different. In addition, the two steps of analysis were also carried out on the fermented alcohol content data.

RESULTS AND DISCUSSION

Sugar concentration of hydrolysate

The sugar concentration of sweet sorghum stalk pulp before and after hydrolysis using the *T. reesei* culture are seen in Table 1.

Table 1. Sugar content (% Brix) of sweet sorghum stalk pulp before and after hydrolysis with *T. reesei*.

No	Hydrolysis time (hours)	[sugar], % Brix
1	0	7.54 ± 0.33 b
2	8	8.51 ± 0.41 bc
3	16	9.05 ± 0.33 c
4	24	9.84 ± 0.00 c
5	32	8.05 ± 0.52 b
6	40	5.95 ± 0.63 ab
7	48	4.05 ± 0.83 a
8	56	3.71 ± 0.42 a
9	64	3.46 ± 0.55 a

Data followed by the same letter are not significantly ($p > 0.05$) different.

Based on the data in Table 1, it was observed that the sugar content before (hydrolysis time = 0 hours) and after hydrolysis with varying times, differed from one another. Therefore, it was stated that the hydrolysis time using *T. reesei* have an effect ($P>0.05$) on the sugar content in the sweet sorghum stalk pulp. Furthermore, Tukey test showed that there are significant differences among the data of sugar levels. The highest sugar content was obtained by hydrolysis for 24 hours.

The sugar content before hydrolysis (hydrolysis time = 0 hours) of 7.54% Brix was relatively low when compared to data from other sources. Jasman [12] provided average Brix from 7.42 to 14.9% for several cultivars in the sorghum bicolor variety, while Noerhartati and Rahayuningsih [13] provided average Brix from 11.3 to14.7 % for the same variety. However, differences in the above data were not a problem because the variation of sugar content in sweet sorghum stalks are depend on many factors such as soil type and fertilization [14], [15], planting density [15], planting time [16], storage techniques [17] and varieties of the sorghum [16].

Furthermore, after hydrolysis using *T. reesei* culture for 8 to 24 hours, there was an increase in sugar content in the sweet sorghum stalk pulp. The hydrolysis time was directly proportional to the sugar content, which suggested that the longer the hydrolysis time, the more cellulose components were hydrolyzed into simple sugar molecules (monosaccharides and disaccharides) as written in Eq. (1). The hydrolysis mechanism of cellulose to glucose was that the endo-β (1→4) *glucanase* enzyme cuts the cellulose chain from the inside, followed by the *exoglucanase* enzyme which cuts the short-chain of cellulose from near the end to produce *cellobiose* and was finally cut by β-glucosidase to become glucose [18], [19].

When the hydrolysis time reached 24 hours, the sugar concentration in the mixture achieved the maximum value, which indicated that the amount of sugar molecules produced from the hydrolysis reaction has also reached the maximum. The additional time after this period no longer added to the sugar content due to several possibilities. Firstly, the cellulose and hemicellulose levels in the medium may had ran out, secondly, cellulose and/or hemicellulose may still present but not readily to be hydrolyzed, thirdly, the *cellulase* enzyme produced by *T. reesei* may be inhibited or denatured, and the lastly, the *T. reesei* cells have died hence inhibited the production of *cellulase* enzyme. However, the most possibility was that cellulose and hemicellulose were still present but not available for hydrolysis. The reason was that the structure of

lignocellulose involving inter-chain hydrogen bonds between the molecular chains of cellulose and hemicellulose were wrapped in lignin, making it difficult for enzymes to access [20], [21]. The lignocellulose material consists of defensive inner structure which has contributed to the hydrolytic stability and structural robustness of the plant cell walls and its resistance to microbial degradation. The presence of cross-link between cellulose and hemicellulose with lignin via ester and ether linkages leads to the biomass recalcitrance [22]. In order for cellulose and hemicellulose in lignocellulose materials to be hydrolyzed easily, these materials needed to be given physical, chemical, biological or combination pre-treatment [23]. The pre-treatment carried out in this study was only physical, where the sorghum stalks were ground but not to perfectly fine pulp. Another reason was that if the lignocellulose content in sweet sorghum stalks was around 11.72% [24] and half of it was cellulose [25], the conversion of all the cellulose into glucose would increase sugar content by approximately 5.5%. However, the data showed that the increase in sugar content (% Brix) was only 2.3%. This indicated that a lot of cellulose was still not hydrolyzed.

When the hydrolysis time was more than 24 hours, the sugar content continued to decrease until the hydrolysis time was 64 hours. This raised the suggestion that the activity of *T. reesei* was no longer able to hydrolyze cellulose, hence it switched to using glucose as a carbon source for its growth [26].

Alcohol concentration

The alcohol content in the result of fermentation is seen in Table 2.

Table 2. The alcohol concentration of fermented hydrolysate (fermentation time = 24 hours).

No	Hydrolysis time (hours)	[alcohol], % m/v
1	0	4.17 ± 0.29 bcd
2	8	4.33 ± 0.29 cd
3	16	5.33 ± 0.29 de
4	24	6.17 ± 0.29 e
5	32	4.33 ± 0.58 cd
6	40	4.00 ± 0.50 bcd
7	48	3.83 ± 0.29 abc
8	56	2.83 ± 0.29 ab
9	64	2.50 ± 0.50 a

Data followed by the same letter are not significantly (p > 0.05) different.

Table 2 showed that there are significant differences (P> 0.05) in the alcohol content among the fermented materials hydrolysed with *T. reesei* in different time periods. Furthermore, it was observed that the pattern of changes in the concentration of fermented ethanol followed that of the concentration of sugar in sweet sorghum stalk pulp. This is consistent with the prediction that the ethanol yield is directly proportional to the sugar content in the substrate. This indicates that the fermentation process is running normally. The table also showed that the highest alcohol content was obtained from the substrate which had been hydrolyzed for 24 hours.

The pattern of changes in alcohol content of all treatments seemed to follow the pattern of changes in sugar level obtained from the previously hydrolysis stages. This was reasonable according to predictions that the sugar content in the sample was directly proportional to the alcohol content of the fermentation results [27]. However, when the sugar content in the medium was too high, the alcohol content produced during fermentation was not as it should be because of the death of yeast cells as a result of high osmotic stress and ethanol inhibition [27], [28].

CONCLUSION

It was proven that the hydrolysis use *T. reesei* may increase soluble sugar content in the sweet sorghum stalk pulp. The hydrolysis time with *T. reesei* have a significant effect on hydrolyzed sugar and fermented alcohol content. The highest concentration of soluble sugar in

sweet sorghum stalk pulp was obtained after incubation with *T. reesei* for 24 hours, however it decreased after that. The pattern of changes in the concentration of fermented alcohol was same with that of sugar concentration in the hydrolysis product.

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