Bioprocess engineering of bioethanol production based on sweet sorghum bagasse by co-culture technique using *Trichoderma reesei* and *Saccharomyces cerevisiae*

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Abstract. The objective of this study was to investigate the technique to produce bioethanol by co-culture of *Trichoderma reesei* and *Saccharomyces cerevisiae* on sweet sorghum bagasse which has higher yield and productivity of bioethanol. Sweet sorghum bagasse contains 48.65±0.02% cellulose. Cultivation of *Trichoderma reesei* on sweet sorghum bagasse produced the highest concentration of sugar (10.90±0.05 g L⁻¹) at 36th hours of cultivation. The time at which highest concentration of sugar produced, is used to apply the bioprocess engineering to allow high production of bioethanol by *Saccharomyces cerevisiae*. The bioprocess engineering of co-culture technique was conducted by switching condition from aerobic to anaerobic and inoculation of *Saccharomyces cerevisiae* inoculums at 36th hours of *Trichoderma reesei* cultivation. As a comparison, bioethanol production was conducted using conventional technique in which co-culture of *T. reesei* and *S. cerevisiae* from the beginning of fully aerobic cultivation without switching conditions. The results showed that bioethanol production produced was higher than that of conventional technique (7.99±0.62 g L⁻¹ compared to 6.60±0.28 g L⁻¹). The yield of product formation (Yp/s) using this technique was 0.30±0.08 g bioethanol/g substrate which was higher compared to conventional technique (0.17±0.01 g bioethanol/g substrate). Bioethanol production rate using this technique was 1.48 ± 0.03 g L⁻¹ hour⁻¹ which was higher compared to conventional technique (1.04 ± 0.06 g L⁻¹ hour⁻¹).

1. Introduction

There is a great global interest in the use of bioethanol as a renewable energy alternative to petroleum, which is linked to the rapidly depleting petroleum resource and severe environmental impacts of climate change. Currently, numerous researches have been mostly focused on the second generation ethanol using inexpensive and abundant non-food lignocellulosic biomass (such as agricultural residue and crops). Sweet sorghum (*Sorghum bicolor* (L.) Moench) constitutes a promising crop since it is relatively easy to cultivate in diverse environmental
conditions. The lignocellulosic bagasse of the sweet sorghum could reach 20-30 ton/ha/year [1], but this abundant material is mostly utilized for production feed and compost. Furthermore, sweet sorghum is considered as a suitable raw material for bioethanol production due to its ease on cultivation aspects. The production of ethanol using lignocellulosic materials requires several steps, i.e. delignification, saccharification, and fermentation. In our research, NaOH and hydrogen peroxide (H₂O₂) are used for delignification. [2] has successfully removed 85% lignin in sweet sorghum bagasse using hydrothermal NaOH and hydrogen peroxide method.

Prior to fermentation by yeast, the saccharification of cellulosic material was carried out. Trichoderma reesei is reported capable of hydrolyzing the cellulose to form sugar since the yeasts unable to generate cellulase. One new method of producing bioethanol from starch or cellulose is conventional SSF (Simultaneous Saccharification and Fermentation) method. However, this technique seems to limit the microbial performance in producing bioethanol, which is considered as a serious disadvantage. As stated by [3][4][5][6], the modified technique through culture inoculation for saccharification and fermentation in a single reactor gradually improve the ethanol production compared to the conventional method. In our experiment, the bioprocess condition using co-culture was engineered through modifying aeration and inoculation of Saccharomyces cerevisiae.

2. Materials and Methods

2.1 Material Preparation

Sweet sorghum bagasse was supplied from PT. Samirana Surya Semesta, Jakarta, while TrichodermareeseiIPBCC.93.260 and Saccharomyces cerevisiaeIPBCC.Y. 05.544 was obtained from Culture Collection in Bogor Agricultural University (IPBCC). Delignification of sweet sorghum bagasse (SSB): SSB was dried, powdered, and passed through 40-mesh sieve. The SSB powder was then washed with hot water (1:10) and filtered using 100-mesh filter fabric three times. The washed SSB powder was oven-dried at 105 °C for 24 h. The dried SSB powder was dissolved with NaOH 2% (w/v) at 1:10 for 15 min in a covered baffle flask and sterilized in autoclave at 120 °C for 60 min. The sterilized sample was cooled at room temperature, washed to reach neutral pH, and dried. The dried sample was mixed with H₂O₂ 7.44% (w/v) at 1:10 and incubated at shaking incubator at 120 rpm and 40 °C. The pH was then adjusted to 11.5 by addition of NaOH 2 M [7,2].

2.2 Co-culture Preparation

Trichodermareesei cultures. T. reesei was refreshed in a reaction tube containing Potato Dextrose Agar (PDA) slant in a laminar air flow. T. reesei was then sub cultured to produce cellulase in a nutrition medium containing 1 L buffer citrate, 1.0 g yeast extract, 1.5 g bacteriological peptone, 1.4 g (NH₄)₂SO₄, 2.0 g KH₂PO₄, 0.005 g FeSO₄.7H₂O, 5 mL CMC (Carboxy Methyl Cellulose) 1%, and 1% SSB as an inducer. The nutrition solution was stirred to form homogenous solution.

Saccharomyces cerevisiae cultures. Saccharomyces cerevisiae isolate was sub cultured in PDA medium slant and incubated for 2 days. The isolate was then cultured in 50 ml YMGP (yeast malt
peptone-glucose) consisting of yeast extract (5 g/l), malt (5 g/l), glucose (10 g/l) and peptone (5 g/l) in 200-ml Erlenmeyer. The culture was incubated in shaking incubator at 125 rpm and 30 °C for 24 h.

2.3 Cultivation of Trichodermareesei

Cultivation of *Trichoderma reesei* was carried out to determine the microbial growth curve and find the higher sugar production time. The parameters cultivation process were used to identify the pattern of growth and the required time for producing the highest sugar production. Sweet sorghum bagasse with a concentration of 10% (w/v) was dissolved in 1 liter of distilled water and then 10% (v/v) inoculums of *Trichoderma reesei* was added to the substrate. The cultivation process was carried out at room temperature (±30 °C) by using 150 rpm agitation and 0.5-1vvm aeration. A sample was taken every 12 hours for 84 hours.

2.4 Conventional Technique of Bioethanol Production

Treated sweet sorghum bagasse with a concentration of 10% (w/v) was dissolved in 1 liter of distilled water. Then, 10% (v/v) inoculum was added to the substrate. *Trichoderma reesei* and *Saccharomyces cerevisiae* were inoculated from the beginning of cultivation. During the cultivation period, bioreactor was in aerobic conditions; with full agitation and aeration. The cultivation process used a 2-litres bioreactor using 1 liter working volume, and was performed with aeration of 0.5-1 vvm and agitation of 150 rpm for 72 h. The bioreactor was applied to obtain proper condition by controlling aeration and agitation. A sample was taken every 12 hours for 72 hours.

2.5 Co-culture Technique of Bioethanol Production and Kinetics of Cultivation Parameters

The cultivation of *Trichoderma reesei* produced maximum sugar concentration at the 36th hour. In this experiment, the medium (1.2 L, pH 5) was sterilized in autoclave at 121 °C for 20 min. The mixture was aseptically transferred into batch bioreactor previously sterilized at 121 °C for 45 min and inoculated with 10% (v/v) of *Trichoderma reesei* at initial stage of cultivation. The bioreactor was set under aerobic condition (1vvm) up to hour 36th, whiles the shifting condition to anaerobic system and inoculated with 10% (v/v) of *Saccharomyces cerevisiae* was provided from the 36th hour to 72th hour with constant agitation during cultivation. A sample was taken every 12 hours for 72 hours.

Samples were taken every 12 hours for 72 hours. Parameters measured and calculated as a performance indicator of cultivation process were Total biomass (X), Level of bioethanol produced (P), Residual of cellulose substrate still present in the media (S). Chromatography Gas (Agilent Tecnologies 7890A) was used to identify the bioethanol content of the samples. Yield of bioethanol per substrate (Yp/s) was calculated based on the slope of regression line between P-Po (ordinatee) and So-S (abscissa). Ethanol Productivity (g L⁻¹ h⁻¹) was calculated based on the value the slope of regression line between ethanol production (g L⁻¹) and sampling time (abscissa).
3 Results

3.1 Delignification of sweet sorghum bagasse and cultivation of Trichoderma reesei

Table 1. The Component of sweet sorghum bagasse

| Component     | Before Delignification | After Delignification |
|---------------|------------------------|-----------------------|
| Cellulose     | 31.72 ± 0.02           | 48.65 ± 0.02          |
| Hemicelluloses| 30.91 ± 0.01           | 27.63 ± 0.02          |
| Lignin        | 21.40 ± 0.03           | 7.47 ± 0.01           |

Delignification of sweet sorghum bagasse (SSB) needs to be carried out, which aims to convert the characteristics of the raw material specifically degrading lignin and hemicellulose and alleviating the cellulosic crystallinity.[8] found that high temperature and pressure during pretreatment of hydrothermal alkaline and H2O2 is useful to maintain cellulose, to dissolve hemicellulose and lignin, but not to conserve the dry weight of lignocellulosic material used. The increased cellulose concentration and decreased lignin after delignification could indicate optimization of hydrolysis process [9].

Figure 1. Cultivation of Trichoderma reesei
From the 12th to 36th h, the sugar concentration increased as the increasing growth of *Trichoderma reesei* since the mold could hydrolyze cellulose present in the sweet sorghum bagasse. The increment of sugar was indicated by the presence of monomers as the hydrolytic product of cellulose. The highest sugar production (10.90 g L⁻¹) was found at 36th h of cultivation, which is also observed as the end of exponential phase for growth of *Trichoderma reesei*. This cultivating time of 36 hours is regarded as the base for next stage in this experiment. The application of enzyme-producing microorganisms has been understood to have several advantages as they are inexpensive, easy to grow and control, as well as rapid growth and application.

3.2 Conventional process of bioethanol production

In this present work, ethanol was produced using conventional condition (full aeration) without any bioprocess modification. The maximum production of ethanol for 72 hours of cultivation was 6.60 g L⁻¹, *Trichoderma reesei* is used for producing cellulase which is capable of hydrolyzing cellulose in the sweet sorghum bagasse into monomeric sugars used for fermentation by *Saccharomyces cerevisiae*.

The aerobic condition for bioethanol synthesis is called Crabtree effect, which is associated with the inhibition of synthesis of respiratory enzyme. In a lower concentration of glucose, the synthesis of respiratory enzyme is still sufficient to breakdown glucose through respiration pathway. Nevertheless, when their population reached the log phase, the respiratory enzyme synthesis is insufficient to hydrolyze glucose due to action of glucose. This leads the switch from respiration to fermentation [10]. Under aerobic system, biomass was more produced by yeasts than ethanol since they utilizes more substrate for growth rather than ethanol. The growth of
Saccharomyces cerevisiae in the 12\textsuperscript{th} - 24\textsuperscript{th} hour was drastically higher compared to previous period. This suggested that, besides respiration, yeasts also promoted the conversion of sugars to bioethanol, which was indicated by a higher production of ethanol from 1.18 g L\textsuperscript{-1} to 3.02 g L\textsuperscript{-1} in the 12\textsuperscript{th} - 24\textsuperscript{th} hour. Total amount of ethanol is dependent on factors or parameters in the bioreactor, including the limitation of metabolic activity of the yeasts.

3.3 Bioprocess engineering of bioethanol production by co-culture technique

Co-culture for ethanol production was conducted by applying Trichoderma reesei and Saccharomyces cerevisiae. The use of both microorganisms exhibited desirable effects on the product as a result of combination of their properties and activities. Conversion of cellulose in sweet sorghum bagasse to monomeric sugars is required for fermentation. Trichoderma reesei is a widely used as cellulase producer. The cellulose hydrolysis was mediated by a combination of three main types of cellulases: (1) exo-1,4-\(\beta\)-D-glucanocellobiohydrolases (exoglucanases), hydrolyzing cellobiose units from the end of cellulose chains, (2) endo-1,4-\(\beta\)-glucan, hydrolyzing the internal glycosidic bonds, and (3) 1,4-\(\beta\)-D-glucosidase, hydrolyzing glucose units from cello oligosaccharide [11]. Saccharomyces cerevisiae is a facultative anaerobic microorganism, producing a higher level of ethanol under anaerobic condition with a limited oxygen concentration. With aerobic condition, the yeast utilizes substrate for formation and production of cells.

In order to optimize the role of each microorganism, the bioprocess was engineered through condition switching and inoculating of yeast at 36\textsuperscript{th} hour. The process was modified by stopping aeration and inoculation of Saccharomyces cerevisiae at 36\textsuperscript{th} hour. At this time, the highest production of sugar (10.90 g L\textsuperscript{-1}) was occurred. Although the aeration was stopped at 36\textsuperscript{th} hour, agitation was still provided till the end of cultivation, increasing the direct contact between the medium and cells.

Figure 3 show that ethanol produced from engineered bioprocess using co-culture was 7.4 g L\textsuperscript{-1}, which was higher than conventional one (6.60 g L\textsuperscript{-1}). The application of SSF needs to consider the microbial condition. When both microorganisms were added in the beginning stage, they competed for sugar consumption. Saccharomyces cerevisiae was inoculated after the sugar produced by Trichoderma reesei reached the highest level, followed by cessation of aeration at the same time. Inoculation of Trichoderma reesei started from the beginning of cultivation to induce saccharification of cellulose in SSB, causing production of sugar and cell growth. After reaching the highest sugar concentration at 36\textsuperscript{th} hour, aeration was stopped and Saccharomyces cerevisiae was inoculated simultaneously to ferment sugars.

The cessation of aeration caused Trichoderma reesei reaching the stationary phase, which led to the death phase at 60\textsuperscript{th} hour. This is clear since Trichoderma reesei is aerobic fungus that requires oxygen during growth and product synthesis. Even though reaching stationary phase at 36\textsuperscript{th} hour, the mold is still capable of degrading cellulose in SSB because of agitation and remaining
oxygen. In the absence of oxygen, *Saccharomyces cerevisiae* seems to have a stable growth and produces ethanol since the yeast is anaerobic facultative microorganism.

[1] stated that hydrolytic activity of sugars such as glucose by yeast could be present in both anaerobic (fermentation) and aerobic (respiration) system. However, hydrolysis by yeast under anaerobic condition is more recognizable for alcohol production. In absence of oxygen, yeast is able to ferment sugars for production of alcohol and CO₂. [12] studied the use of commercial yeast and found that higher ethanol was produced under anaerobic fermentation with limited oxygen concentration than that under complete aerobic condition. The different ethanol production in these two systems seems to depend on characteristics and types of the yeast used.

In anaerobic condition, the 2 ATP from glycolysis was used by yeast due to limitation of oxygen [13]. Fermentation constitutes the utilization of organic substances to generate energy without oxygen. The more sugar was used to produce ethanol under anaerobic fermentation. It is found that there is a decrease of total sugar from 36th h to 72th h. In fact, the sugar is not only converted into ethanol, but also used by yeasts to maintain their cellular metabolism.

![Figure 3. Results co-culture technique of bioethanol production](image)

**Figure 3. Results co-culture technique of bioethanol production**

### 4 Conclusions

Sweet sorghum bagasse is a favorable source of cellulose for production of bioethanol with presence of *Trichoderma reesei* and *Saccharomyces cerevisiae*. Cultivation of *Trichoderma reesei* under batch system for 84 hours was successfully carried out by using cellulose in sweet sorghum bagasse as substrate. This constitutes a preliminary stage that is used to determine
further process. At cultivation stage, *Trichoderma reesei* could produce the highest sugar concentration at 36th hour, i.e. 10.90 g L⁻¹. The use of conventional method for producing ethanol from sweet sorghum bagasse involved two microbial cultures, *Trichoderma reesei* as saccharifying agent and *Saccharomyces cerevisiae* as fermenting agent, yielded ethanol at level of 6.60 g L⁻¹. Furthermore, the combination of co-culture technique and engineered bioprocess could enhance ethanol production to 7.99 g L⁻¹ with production rate of 1.48±0.03 g L⁻¹ h⁻¹. In term of production aspect, this combined technique could raise the yield coefficient for product (Yp/s) from 0.17±0.01 g bioethanol/g substrate to 0.30±0.08 g ethanol/g substrate.

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