Bio-ethanol production from sweet sorghum bagasse by engineered simultaneous saccharification and fermentation technology using Trichoderma reesei and Saccharomyces cerevisiae

K Syamsu, L Haditjaroko, and E A Syadiah

1Department of Agroindustrial Technology, Faculty of Agricultural Technology, IPB University, Indonesia
2Major Program of Agroindustrial Technology, Graduate School, IPB University, Indonesia

E-mail: khaswars@yahoo.com

Abstract. This study was performed on sweet sorghum bagasse as a medium for bio-ethanol production. The aim of this study is to design a method to produce bio-ethanol by Simultaneous Saccharification and Fermentation (SSF) technology using Trichoderma reesei and Saccharomyces cerevisiae at a higher yield. Pre-treatment stage was conducted by using sodium hydroxide (2%) hydrothermal and hydrogen peroxide (7.44%) for 6 h, at temperature of 40°C and agitation of 120 rpm. The experimental setup consisted of two treatments, namely Conventional SSF (CSSF) and Engineered SSF (ESSF). The results show that Conventional SSF using full agitation and aeration can produce bio-ethanol at 6.6 g L⁻¹, with the yield (Y p/s) of 0.17 g bio-ethanol/g substrate, and productivity of 0.09 g L⁻¹ h⁻¹. A better result is obtained using Engineered SSF which produces bio-ethanol at 10.73 g L⁻¹, with the yield (Y p/s) of 0.33 g bio-ethanol/g substrate and productivity of 0.15 g L⁻¹ h⁻¹.

1. Introduction

An increased demand in energy particularly coming from fossil fuels negatively correlated with availability of energy source. The is due to the fact that the decline of energy source is faster than the creation of energy alternatives. Bio-ethanol production is still considered less economically feasible compared to that of petroleum-based fuel. To deal with this challenge, two approaches are proposed: (a) exploitation of cheap and abundant substrates in nature and (b) use of technology to more effectively and efficiently produce bio-ethanol.

The sweet sorghum (Sorghum bicolor (L.) Moench) is one of the potential biomass resources for fuel ethanol production, because of its adaptability to any conditions and it has high fermentable sugar content. Furthermore, it has high juice concentration and high yield of green biomass. Alcohol produced from fermented molasses or starch is regarded as the first generation of bio-ethanol (G1). The residue after extracting the juice, called bagasse, can be hydrolyzed into sugar and can further be fermented to ethanol, and called as second generation bio-ethanol (G2) [1].

Previous studies have revealed that the production of cellulose-based bio-ethanol using a Simultaneous Saccharification and Fermentation (SSF) is more advantageous than a separate hydrolysis and fermentation (SHF). SSF can reduce the process and contamination time [2]. A new
approach of SSF called engineered SSF (ESSF), has been applied by [3] and [4]. This technique offers advantages such as a higher yield and productivity.

In this current work, Engineered SSF was applied to produce ethanol using sweet sorghum bagasse as a medium. Engineered SSF is a new approach that combines bio-process engineering techniques by switching the aerobic system to the anaerobic system at a right time. This shifting allows yeasts to change their metabolic activities by which cells are more produced under aerobic state, while more ethanol is produced under anaerobic state. Application of ESSF is expected to yield more ethanol compared to CSSF.

2. Materials and methods

2.1. Preparation of sweet sorghum bagasse as the medium

Sweet Sorghum Bagasse (SSB) was obtained from Samirana Surya Semesta (G19 and G20 varieties). It was then dried in the air and then ground to pass through sieve with 40-60 meshes. After that, it was washed using boiling water three times to substantially remove the major soluble sugars. SSB was heated using 2% NaOH solution in 500 ml Duran bottle at 121°C for one hour. The ratio of bagasse to water was 1:1 on a weight basis. After finished with the alkali hydrothermal process, 7.44% (w/v) hydrogen peroxide was mixed into the pre-treated bagasse at 40°C, for 6 h and agitated or 120 rpm [5]. The composition of materials component after pre-treatment was analyzed for cellulose, lignin, and hemicelluloses content.

2.2. Culture preparation of Trichoderma reesei and Saccharomyces cerevisiae

The isolate cultures utilized in this study (Trichoderma reesei IPBCC.93.260) and (Saccharomyces cerevisiae (IPBCC.Y.05.544) were obtained from the Institute Pertanian Bogor Culture Collection (IPBCC) at Bogor Agricultural University. Isolates of Trichoderma reesei were refreshed on a Potato Dextrose Agar (PDA) medium. The culture was incubated at 25°C, for 7 days. A loop of isolates Trichoderma reesei were grown in 100 ml buffer citrate nutrition medium consisting of 1 g L⁻¹ yeast extract; 1.5 g L⁻¹ bacteriological peptone; 1.4 g L⁻¹ (NH₄)₂SO₄; 2.0 g L⁻¹ KH₂PO₄; 0.005 g L⁻¹ FeSO₄.7H₂O in a 300 ml Erlenmeyer flask. Incubation was performed in a 125 rpm shake flask at room temperature (±30°C) for 7 days for producing cellulase enzyme before inoculation.

Isolates of Saccharomyces cerevisiae were refreshed on a PDA medium and incubated for 3 days. Isolates were grown in 100 ml of Yeast Malt Peptone Glucose (YMPG) propagation medium consisting 5 g L⁻¹ yeast extract, 5 g L⁻¹ malt, 10 g L⁻¹ glucose, and 5 g L⁻¹ peptone in a 200 ml Erlenmeyer flask. Incubation was performed in a 125 rpm Shake Flask at room temperature (±30°C) for 24-36 h before inoculation.

2.3. Cultivation of Trichoderma reesei

Cultivation of Trichoderma reesei was carried out to determine the microbial growth curve. The parameters of 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 L of distilled water and then 10% (v/v) inoculums of Trichoderma reesei were added to the substrate. The cultivation process was carried out at room temperature (±30°C) by using 150 rpm agitation and 0.5-1 vvm aeration. Samples were taken every 12 h for 84 h.

2.4. Conventional simultaneous saccharification and fermentation

Treated sweet sorghum bagasse with a concentration of 10% (w/v) was dissolved in 1 L of distilled water. Then, 10% (v/v) inoculums were added to the substrate. Trichoderma reesei and Saccharomyces cerevisiae were inoculated from the beginning of cultivation. During the cultivation period, the bioreactor was in aerobic conditions; with full agitation and aeration. The cultivation process used a 2-L bioreactor using 1 L 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.

2.5. Engineered simultaneous saccharification and fermentation (ESSF)

The growth curve of the previous *Trichoderma reesei* cultivation was used as a reference for implementing the engineered SSF (ESSF). After the highest sugar production had been reached, the aeration was stopped to allow anaerobic condition. Switching condition from aerobic to anaerobic condition is expected to shift the metabolism of the yeast *Saccharomyces cerevisiae* from respirative into fermentative state which would produce more bio-ethanol. In the meantime, *Trichoderma reesei* die out and reserved as additional nutrient for yeast which can grow under anaerobic condition. Therefore, by this metabolism shifting, it is expected to produce more bio-ethanol.

2.6. Kinetics of cultivation parameters

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 bio-ethanol produced (P), Residue of cellulose substrate still present in the media (S), and Maximum specific growth rate (μmax) indicated by slope of growth curve at exponential or logarithmic phase which was obtained by plotting lnX against time (hr).[3,4]. Chromatography Gas (Agilent Tecnologies 7890A) was used to identify the bio-ethanol content of the samples. Yield of bio-ethanol per substrate (Yp/s) was calculated based on the slope of regression line between P-Po (ordinate) and So-S (abscissa). Ethanol Productivity (g L⁻¹ h⁻¹) was calculated based on the value the slope of regression line between bio ethanol production (g L⁻¹) and sampling time (abscissa).

3. Results and discussions

3.1. Characterization of the sweet sorghum bagasse as the medium

For physical pre-treatment, sweet sorghum bagasse (particle size of 250-420μm) was washed using hot water to remove residual sugar remaining from the extraction of sweet sorghum. Furthermore, chemical pre-treatment consisted of alkali hydrothermal and hydrogen peroxide was conducted [5]. The delignification, by removing lignin and/or hemicelluloses, enables enzymes to degrade cellulose easily. The delignification using alkali could degrade ester and glycosidic chains, resulting in decreased lignin and increased cellulose in the material[6]. As reported by [7], the use of alkali pre-treatment did not require additional equipment as alkali is not a corrosive compound.

![Figure 1](image_url)

**Figure 1.** The lignocellulosic component on sweet sorghum bagasse before and after pre-treatment.
After treated with alkali pre-treatment, sample was immersed in hydrogen peroxide (7.44%) for 6 h at 40ºC. Hydrogen peroxide actively served at low temperatures (21-50ºC), indicating that process at low temperatures would result in a higher content of total sugar [8]. Figure 1 exhibits application of hydrothermal and enzymatic treatment.

The main component of delignified sweet sorghum bagasse was cellulose (41.65%), which was significantly higher compared to its original composition. Sweet sorghum bagasse cultured in United State, contains 36% cellulose, 17.8% hemicelluloses and 19.5% lignin prior to treatment[9]. In our study, cellulose content was slightly lower than previous studies, but was high after treated with delignification, which make it suitable as a substrate for bio-ethanol production and other derivative products of fermentable sugars.

3.2. Cultivation of Trichoderma reesei
Cultivation of sweet sorghum bagasse as a growth medium of Trichoderma reesei was to observe the growth of mold cells and the production of sugar which was required for Engineered SSF. The conventional SSF was modified by stopping aeration, thus—after reaching the highest content of sugar—Saccharomyces cerevisiae which is anaerobic facultative microbes which could yield a maximum production of bio-ethanol.

Figure 2 shows the growth of Trichoderma reesei based on dry weight biomass. The early phase of Trichoderma reesei started at the 0 h when the new culture was inoculated into the cultivation media. Trichoderma reesei growth was low due to the new media environment during the adaptation phase. The exponential phase occurred between the 12th to 36th h and the microbes grew at their maximum growth rate.

![Figure 2](image-url)  
**Figure 2.** The growth curve of Trichoderma reesei during cultivation.

Figure 3 shows the decrease of cellulose substrate may result from enzymatic activity of Trichoderma reesei at hydrolysis process, as represented by sugar produced. In accordance with [10], enzymatic hydrolysis of cellulose involved three types of enzymes, i.e. selobiohidrolase (exoglucanase), endoglucanase and β-glucosidase.
The remaining cellulose is present due to incapability of cellulase to hydrolyze cellulose encased in lignin and hemicelluloses. The effectiveness of hydrolyzing enzyme was lowered by the presence of lignin [11]. The remaining lignin present in the lignocellulosic material protects cellulose from depolymerisation, thus decreasing saccharification. It is also able to bind and inactivate the cellulolytic enzyme, reducing further fermentation [12].

The results show that sugar content does not remarkably increase within 12th h as *Trichoderma reesei* was at the adaptation phase. Meanwhile, the cellulase generated by *Trichoderma reesei* started to hydrolyze cellulose into sugars. The results exhibit that the highest level of sugar (10.9 g.L⁻¹) was achieved at 36th h of incubation, although it was then decreased at 48th h. This possibly displays the use of sugar by *Trichoderma reesei* for nutrition source. Interestingly, the level of sugar shows an increase at 60th h, since *Trichoderma reesei* was still capable of producing cellulase under aerobic conditions.

### 3.3. Simultaneous saccharification and fermentation (SSF)

SSF technique represents a direct conversion process. Hydrolysis and fermentation may be carried out simultaneously as long as glucose can be directly converted to ethanol, thus reducing the accumulation of selobiose and glucose and consequently accelerating cellulose hydrolysis into glucose [13]. In this study, by inoculating *Trichoderma reesei* and yeast of *Saccharomyces cerevisiae* which catalyse the hydrolysis of polysaccharides into sugar and ferment the sugar into bio-ethanol at the same time, the two processes occur simultaneously (simultaneous saccharification and fermentation, SSF).

Cellulose is used as substrate for *Trichoderma reesei* during saccharification by which cellulase enzyme hydrolyzes cellulose into simple sugars. Exponential phase of *Trichoderma reesei* was observed at 12th-36th h of incubation. The aerobic condition is suitable for the mold in maintaining cellular metabolic activities and producing metabolic products. Figure 4 shows that cellulose concentration decreases and it is consistent with growth of *Trichoderma reesei* and increased sugar production. However, in the simultaneous saccharification and fermentation, glucose or other simple sugars will be used directly by yeast to produce bio-ethanol or more cells [3]. As described in Figure 4, adaptation phase occurred within the first 12th h, then followed by exponential phase. At the same time, sugar is used by yeast to form more cells and bio-ethanol. For 72th h of cultivation, bio-ethanol
production reached 6.6 g L\(^{-1}\). Because the cultivation was performed under full aeration condition, the sugar was more utilized by yeast to produce cells instead of bio-ethanol. Aerobic fermentation involved a combination of respiration-fermentation, which was dependent on the presence of oxygen and cell concentration [4]. Sugar would be more converted into bio-ethanol in such following conditions as limited oxygen level, high cell number, and anaerobic condition.

![Graph](image.png)

**Figure 4.** Results of bio-ethanol production during Conventional SSF process.

### 3.4. Engineered simultaneous saccharification and fermentation (ESSF)

Engineered SSF produces more bio-ethanol in comparison with conventional SSF. As depicted in Figure 5, bio-ethanol concentration reached 10.7 g L\(^{-1}\) after 72\(^{th}\) h of incubation. The shifting metabolism from respiration to fermentation due to switching from aerobic to anaerobic condition remarkably raised bio-ethanol concentration, from 4.4 g L\(^{-1}\) at 36\(^{th}\) h to 10.7 g L\(^{-1}\) at 72\(^{th}\) h.

Within 36\(^{th}\) h of aerobic cultivation, yeast seemed to use the substrate and oxygen for respiration and production of more cells. The cellulose hydrolysis activity was high because mold was at the exponential growth within 0-48 h of cultivation. Meanwhile, the death phase of mold occurred within 48\(^{th}\)-72\(^{th}\) h of anaerobic condition. In absence of oxygen after 36\(^{th}\) h of incubation, the sugar was more converted into bio-ethanol by yeast as well-recognized that yeast was anaerobic facultative microbe. Bio-reactor containing high level of oxygen allowed the yeast to convert sugar into carbon dioxide and water. On the contrary, yeast is capable of converting sugar into ethanol and carbon dioxide under anaerobic system. However, a difference in ethanol yield produced under aerobic and anaerobic conditions was dependent on the type and character of the yeasts used.

In this work, the cultivation was carried out for 72\(^{th}\) h, and alcohol fermentation requires 30\(^{th}\)-72\(^{th}\) h [14]. The agitation was applied at 150 rpm which enables to facilitate the diffusion of oxygen into the medium, thus improving the contact between substrate and inoculums. Agitation promote a consistency of the microbial cell suspension and keep the medium still homogeneous from the beginning to the end of cultivation.

The remaining sugar in Engineered SSF is slightly lower than that of conventional SSF. This presumably reflects an enhanced conversion of sugar to ethanol, while sugar is more used for cellular respiration in conventional SSF. Application of Engineered SSF allowed *Saccharomyces cerevisiae* to produce more ethanol in comparison with conventional SSF (full aeration), as revealed by a different
level of remaining sugar, i.e. 0.25 g L\(^{-1}\) (Engineered SSF) and 0.64 g L\(^{-1}\) (conventional SSF) at the end of cultivation.

![Graph showing concentration of bio-ethanol, cellulose, and sugar over time]

**Figure 5.** Results of bio-ethanol production during Engineered SSF Process.

**Table 1.** The results of the kinetics of cultivation in conventional and engineered SSF.

| SSF Type     | \(\mu_{\text{max}}\) (h\(^{-1}\)) | Ethanol (g.L\(^{-1}\)) | Yp/s (g.g\(^{-1}\)) | Ethanol Productivity (g L\(^{-1}\) h\(^{-1}\)) |
|--------------|---------------------------------|------------------------|---------------------|-----------------------------------------------|
| Conventional | 0.02 ± 0.002                    | 6.60 ± 0.28            | 0.17 ± 0.01         | 0.09 ± 0.001                                  |
| Engineered   | 0.01 ± 0.001                    | 10.73 ± 0.62           | 0.33 ± 0.05         | 0.15 ± 0.002                                  |

The results demonstrate that the maximum specific growth rate of Engineered SSF was lower than that of conventional SSF (full aeration), which was 0.01/h. The maximum specific growth rate was low because it combined specific growth rates of yeast and mold. And at the Engineered SSF, specific maximum growth is lower than SSF because after switching to anaerobic, the growth of yeast cells slows down as it changes from respirative to fermentative.

The \(Y_{p/s}\) value of Engineered SSF was greater than that of conventional SSF, reaching 0.33 g of bio-ethanol/g substrate. Under Engineered SSF condition, more sugar was used by yeasts to produce ethanol. Only a small portion of the sugar was allocated by yeast for their growth and metabolic activity [16]. This presumably indicates that aerobic-to-anaerobic shifting allows the yeast to utilize the available substrate more for product (bio-ethanol) rather than for biomass (cells). Yeast is able to convert pyruvic acid into ethanol under anaerobic conditions.

### 4. Conclusions

The results of this study show that sweet sorghum bagasse is a viable medium for bio-ethanol production. Sweet sorghum bagasse has potential cellulose content as the substrate for *Trichoderma reesei*. The cultivation of *Trichoderma reesei* achieves a peak sugar production of 10.90 g L\(^{-1}\) during the 36 h of cultivation. Engineered SSF technique offers process which has higher product yield (Yp/s) than conventional SSF. The Conventional SSF using full agitation and aeration can produce bio-ethanol at 6.6 g L\(^{-1}\), with the \(Y_{p/s}\) of 0.17 g bio-ethanol/g substrate, and productivity of 0.09 g L\(^{-1}\) h\(^{-1}\).
A better result is obtained using Engineered SSF which produces bio-ethanol at 10.73 g L\(^{-1}\), with a Y p/s of 0.33 g bio-ethanol/g substrate and productivity of 0.15 g L\(^{-1}\) h\(^{-1}\).

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