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Sweet Sorghum Bagasse Bioethanol (Sweet Sorganol) Production by Simultaneous Saccharification and Fermentation by *Trichoderma reesei* and *Saccharomyces cerevisiae*

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ABSTRACT

Bioethanol production from renewable resources has received great attention because of the increasing petroleum shortage. Demands of renewable energy from agricultural field residues increase because high population growth nowadays. Sweet sorghum bagasse (SSB) is one of the main agricultural residues in Egypt. So this study was performed on sweet sorghum bagasse as a resource for production of bioethanol. Pretreatment method for sweet sorghum bagasse by sodium hydroxide (2%) hydrothermal and hydrogen peroxide (7,44%) in 6 hours with 120 rpm. Pretreated sweet sorghum bagasse was further enzymatically treated throughout simultaneous saccharification and fermentation (SSF). In previous studies, the hydrolysis and fermentation processes were performed separately using a separated hydrolysis and fermentation (SHF) technique. This study processes via a SSF technique which produced higher substrate efficiency, cell yield, and product yield compared to the SHF process. The characterization process showed that pretreated SSB contained higher cellulose than untreated cellulose. Sugar from the cellulose was produced by batch system cultivation for 84 hours using *Trichoderma reesei*. The highest sugar production was achieved during the 36 hours. The production of bioethanol by SSF using *Trichoderma reesei* and *Saccharomyces cerevisiae* for 60 hours was 4,08 g/L of bioethanol, with a yield of bioethanol per substrate use (Y_p/s) of 0,30 g ethanol/g substrate and a substrate conversion efficiency of 89,8%.

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SWEET SORGHUM BAGASSE BIOETHANOL (Sweet Sorganol) PRODUCTION BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION BY TRICHODERMA REESEI AND SACCHAROMYCES CEREVISIAE

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Abstract

Bioethanol production from renewable resources has received great attention because of the increasing petroleum shortage. Demands of renewable energy from agricultural field residues increase because high population growth nowadays. Sweet sorghum bagasse (SSB) is one of the main agricultural residues in Egypt. So this study was performed on sweet sorghum bagasse as a resource for production of bioethanol. Pretreatment method for sweet sorghum bagasse by sodium hydroxide (2%) hydrothermal and hydrogen peroxide (7,44%) in 6 hours with 120 rpm. Pretreated sweet sorghum bagasse was further enzymatically treated throughout simultaneous saccharification and fermentation (SSF). In previous studies, the hydrolysis and fermentation processes were performed separately using a separated hydrolysis and fermentation (SHF) technique. This study processes via a SSF technique which produced higher substrate efficiency, cell yield, and product yield compared to the SHF process. The characterization process showed that pretreated SSB contained higher cellulose than untreated cellulose. Sugar from the cellulose was produced by batch system cultivation for 84 hours using *Trichoderma reesei*. The highest sugar production was achieved during the 36 hours. The production of bioethanol by SSF using *Trichoderma reesei* and *Saccharomyces cerevisiae* for 60 hours was 4,08 g/L of bioethanol, with a yield of bioethanol per substrate use (Y_p/s) of 0,30 g ethanol/g substrate and a substrate conversion efficiency of 89,8%.

Keywords: Bioethanol, *Trichoderma reesei*, *Saccharomyces cerevisiae*, Simultaneous saccharification and fermentation, sweet sorghum bagasse.

I. Introduction

Biofuel, as an alternative renewable energy source, has been taken attention worldwide due to the negative effect of fossil fuel reserves and the rapid accumulation of atmospheric greenhouse gases.

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 in. Furthermore it has high juice concentration and high yield of green biomass.

The juice can be readily fermented to alcohol, known first generation bioethanol (G1). The residue after extracting the juice, it called bagasse, can be hydrolyzed into sugar and further can be fermented to ethanol [1], and called as second generation bioethanol (G2) [2]. Nowadays, the production of the second generation cellulosic ethanol from those abundant and

inexpensive nonfood lignocellulosic biomass materials, such as agricultural residues and herbaceous energy crops, has become the focus of research because of its potential advantages [3].

After extracting process of the juice, the bagasse from the sweet sorghum stalks is a lignocellulosic material, which formed by celluloses, hemicelluloses and lignin. During simultaneous saccharification and fermentation (SSF), the sugars detached from the lignocellulosic biomass during hydrolysis by biocatalyst such as cellulolytic enzymes from fungi. During the SSF process, the sugar content, dry cell weight (dcw) of biomass, and the ethanol yield are measured for data analytical requirement.

The aim of the study to gain the accessibility and cellulose content of the substrate, a pretreatment method such as NaOH hydrothermal and H_2O_2 was

investigated in this paper. Performance improve hopefully can be increased in hydrolysis process. Taking SSB pretreated with simultaneous saccharification and fermentation (SSF) to produce Sweet Sorghum Bagasse Bioethanol (Sweet Sorganol) was researched in this study.

II. Literature review

Sweet sorghum bagasse, like other lignocellulosic materials, contains of considerable amount of carbohydrate polymers (cellulose and hemicellulose) and lignin. The carbohydrates can be hydrolyzed to fermentable sugars by acids or enzymes and then fermented to ethanol. Cellulose and hemicellulose can be converted to bioethanol involves enzymatic hydrolysis of the biopolymer soluble oligosaccharides followed by their fermentation process to ethanol. by the yeast. A further advantage of SSF over the Separate Hydrolysis and Fermentation (SHF) is the cost reduction resulting from use of only bioreactor.

Simultaneous saccharification and fermentation (SSF) integrates enzymatic hydrolysis and microbial fermentation into one step: the sugars produced from enzymatic hydrolysis are shortly consumed by the fermentative microorganism for ethanol production in this case that *Saccharomyces cerevisiae*.

Trichoderma reesei is moldy filament that well known as cellulolytic organisms and produce cellulolytic enzymes, including enzymes selobiohidrolase, endoglucanase and β -glucosidase [4]. The advantages of *Trichoderma reesei* besides producing complete cellulolytic enzymes, as well produces xyloglucanolytic enzyme [5]. The presence of this enzyme will make it easier cellulolytic enzyme in breaking cellulose.

Saccharomyces cerevisiae is the most prevalent microorganism for ethanol production from sugars (hexoses). However, comprehensive ranges of sugars, e.g., pentoses, are available in lignocellulosic substrates, which cannot assimilate to ethanol by *S. cerevisiae* some

advantages compared to other ethanol-producing microorganisms.

III. Research Methods

3.1 Preparation of Sweet Sorghum Bagasse as Media

Sweet sorghum bagasse (SSB) was obtained from Samirana Surya Semesta G19 and G20 varieties. SSB was 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, present in the liquid phase stalk. The ratio of bagasse to water was 1:1 on a weight basis. At last, it was dried at 60°C and stored in plastic bag at room temperature to avoid possible interference in the evaluation of the enzymatic hydrolysis of the cellulose [6].

Ten grams dry sweet sorghum bagasse was slurried with 100 mL 2% (w/v) sodium hydroxide solution for 5 min in a 500 mL flask with a silicone stopple, and then autoclaved at 121°C for 60 min. The 7,44% (w/v) hydrogen peroxide was mixed into the pretreated slurry at 40°C and agitation for 120 rpm [7,8]. The residues were centrifuged and washed with distilled water until neutral pH was achieved and dried at 60°C.

3.2 Preparation Cultures of the *Trichoderma reesei* and *Sacharomyces cerevisiae*

The isolated cultures were used in this research (*Saccharomyces cerevisiae* (IPBCC.Y.05.544) and *Trichoderma reesei* were obtained from the Institut Pertanian Bogor Culture Collection (IPB CC) at Bogor Agricultural University. Isolates of *Trichoderma reesei* was refreshed on a Potato Dextrose Agar (PDA) medium (Bratachem, Indonesia). The culture was ncubated at 25°C, for 7 days before inoculation. Isolates of *Saccharomyces cerevisiae* were refreshed on a PDA medium and incubated for 3 days. Isolates were grown on 50 ml of Yeast Malt Pepton Glucose (YMPG) propagation medium consisting of 5 g/L yeast, 5 g/L malt,

glucose 10 g/L, and 5 g/L pepton in 200 ml erlenmeyer flask. Incubation was performed in a 125 rpm shaker at room temperature ($\pm 30^\circ\text{C}$) for 24 hours before inoculation. Isolates *Trichoderma reesei* were grown at Nutritional medium for producing cellulase enzymes. The medium contains 2% $(\text{NH}_4)_2\text{SO}_4$, 2% KH_2PO_4 , 3 ml Urea 10%, 3% CaCl_2 in 1 L distilled water. To produce cellulase enzyme was added 1% Pretreated Sweet Sorghum Bagasse and 1% Polypeptide. This culture was propagated in 8 days and CMC-ase and FP-ase was measured.

3.3 Cultivation of *Trichoderma reesei*

For determining the microbial growth curve, so it was cultivated the *Trichoderma reesei*. Biomass yield, residual cellulose, total sugar and also Total Plate Count (TPC) from the cultivation process were used to identify the curve of pattern growth and the requires time for highest sugar production reached.

Sweet sorghum bagasse with a concentration of 10% (w/v) was dissolved in 1 liters of distilled water and then 10% (v/v) inoculum was added to the substrate. The cultivation process was carried out at room temperature by using 160 rpm agitation and 2 vvm aeration. A sample was taken and analyzed every 12 hours for 84 hours.

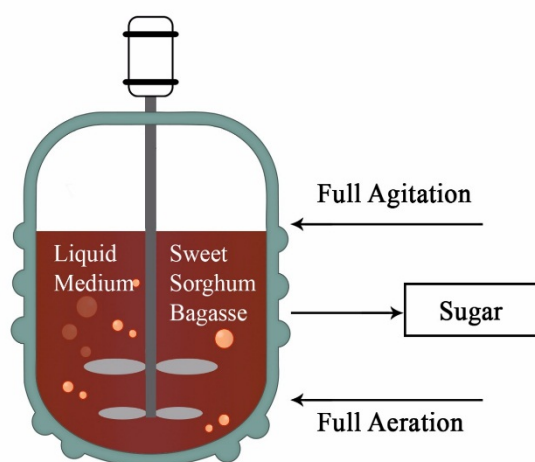


Figure 1. Cultivation of *Trichoderma reesei*
The cultivation of *Trichoderma reesei* is shown at figure 1. It shows that full

agitation and full aeration. Liquid medium is prepared for this cultivation. So, the sugar production is expected in this process using *Trichoderma reesei* in sweet sorghum bagasse medium.

3.4 Simultaneous Saccharification and Fermentation

Sweet sorghum bagasse with a concentration of 10% (w/v) was dissolved in 1.2 liters of liquid media. Then, 10% (v/v) inoculum was added to the substrate. *Trichoderma reesei* and *Saccharomyces cerevisiae* were inoculated at the beginning of cultivation. Aeration was set at 1 vvm, while agitation at a speed of 160 rpm at room temperature. SSF was carried out for 72 hours for bioethanol production.

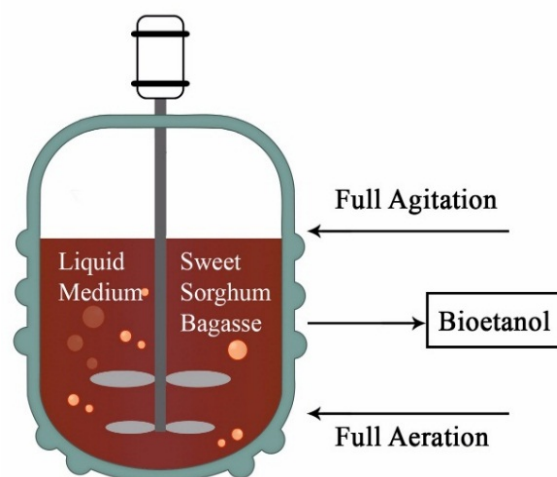


Figure 2. Simultaneous Saccharification and Fermentation of SSB using *Trichoderma reesei* and *Saccharomyces cerevisiae*

Simultaneous saccharification and fermentation process condition is shown at figure 2. It shows the process ocured at full agitation and aeration condition, cultivation at aerobic conditions. SSF was kept produce bioethanol as expected using *Trichoderma reesei* and *Saccharomyces cerevisiae*.

3.5 Measurement Parameters (Kinetic of cultivation)

Samples from bioreactor were taken and analyzed every 12 hours for 72 hours. And the parameters measured and calculated from the samples as performance indicators of the cultivation process such as:

- Total biomass produced (X)
- Level of bioethanol produced (P)
- Residual of cellulose substrate in the media (S)
- Maximum specific growth rate (μ_{max}): slope of curve was obtained by plotting $\ln X$ against cultivation time (h) [10].
- Mathematical Model:

$$\frac{dx}{dt} = \mu x$$

Yield of biomass and bioethanol per substrate used:

$$Y_{x/s} = \frac{X-X_0}{S_0-S} \quad Y_{p/s} = \frac{P-P_0}{S_0-S} \quad (1)$$

Yield of product formation of biomass:

$$Y_{p/x} = \frac{P_t - P_0}{X - X_0} \quad (2)$$

Substrate efficiency (Se):

$$\left(\frac{\Delta S}{S_0}\right) = \frac{S_0 - S}{S_0} \times 100\% \quad (3)$$

IV. Results and Discussion

4.1 Characterization of Sweet Sorghum Bagasse



Figure 3. Pretreated sweet sorghum bagasse using NaOH hydrothermal with H_2O_2

To figure 3 as shown that sweet sorghum bagasse has been pretreated using NaOH hydrothermal with H_2O_2 . Theoretically, high ethanol production depends on a high cellulose concentration. Increases in cellulose content are best influenced by removal of non-cellulose components by pretreatment. The chemical compositions of sweet sorghum bagasse (SSB) between untreated and treated were shown in Fig. 4. The percent hemicellulose content of native SSB is 31,80%, and decreased to 19,35%. Not only that, percent lignin

content decreased from 18,15% to 4,35% after NaOH Hydrothermal and H_2O_2 pretreatment. in contrast, after pretreatment the percent of cellulose content increased from 38,33% menjadi 56,93%.

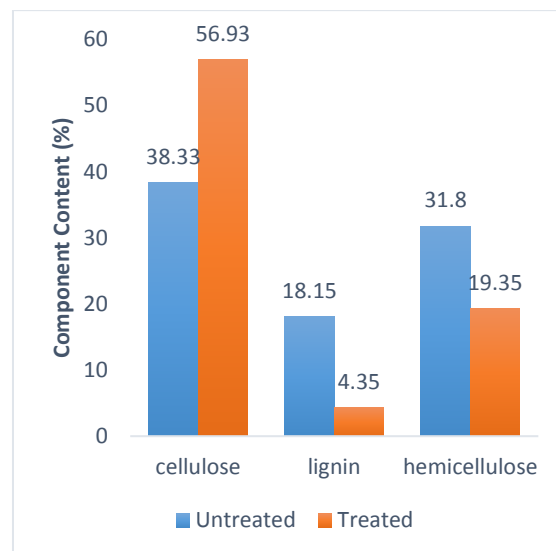


Figure 4. chemical components of untreated and treated of Sweet Sorghum Bagasse

Lignin has been implicated as a competitive cellulase adsorbent that can reduce the amount of cellulase available to catalyze cellulose hydrolysis. Therefore, excision of lignin has a significant effect on enzymatic hydrolysis. NaOH with hydrothermal process as an effective agent for lignocellulose pretreatment, and especially for increasing cellulose content.

In figure 4 shows that treated cellulose concentration has increased than untreated cellulose. Lignin content will be mostly degraded and cellulose with hemicellulose will be retained at perfect pretreatment. But for the best pretreatment method will occur usually depend mainly in the type of lignocelluloses. For enzymatic hydrolysis process has been facilitated the increase of cellulose and the decrease of lignin contents. Compared to Dahnum *et al.* (2015) which did pretreatment using NaOH 10% for EFB resulted only 38% lignin was removed, the results for this research clearly showed higher lignin removal.

4.2 Cultivation of *Trichoderma reesei*

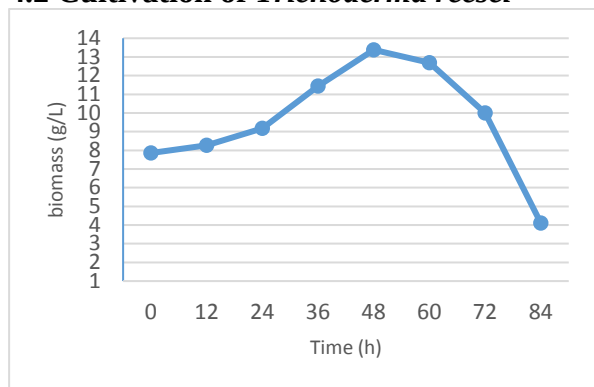


Figure 5. Growth pattern of *Trichoderma reesei* during cultivation

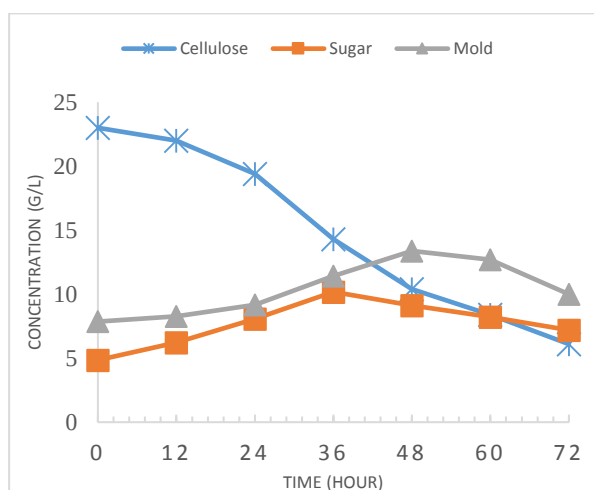


Figure 6. Cultivation results by the batch system of Sweet Sorghum Bagasse using *Trichoderma reesei*

Figure 6 shows the growth of *Trichoderma reesei* based on dry weight biomass. The early stage of *Trichoderma reesei* began at the 0 hour when the new culture was inoculated into the cultivation media on the process. *Trichoderma reesei* got into the adaptation phase after inoculation. The microbial growth performance was low because of the new media condition during the adaptation phase. Furthermore, after the adaptation phase there is lag phase of microbial growth curve the exponential phase come about 24th and 48th hours. During this exponential phase, the microbes grew at their maximum growth rate. During hours 0-12 for the adaptation phase, sugar production using *Trichoderma reesei* increased slowly. Throughout this adaptation phase, the endo- β -1,4-glucanase and exo- β -1,4-high glucanase enzymes

formed as biocatalyst for cellulose hydrolysis [11]. Sugar production rise up summarily along with the growth of *Trichoderma reesei* from the 12th hour until the 36th hour.

The endo- β -1,4-glucanase and exo- β -1,4-high glucanase enzymes were produced by *Trichoderma reesei* and break down the polymer chains of cellulose into simple monomer units. Cellulose concentration exssicate along with mold growth as sugar production increased. *Trichoderma reesei* growth reached the end of exponential phase at the 48th hour.

During the cultivation process, the content of residual cellulose decrease due to the *Trichoderma reesei* has metabolic activity that produces hydrolisis enzymes such as endo- β -1,4-glucanase and exo- β -1,4-high glucanase [12,13]. According to the performance of the process, cellulose of sweet sorghum bagasse an be used as a carbon source for the growth of *Trichoderma reesei* to produce sugar. Furthermore, *Trichoderma reesei* can also convert any other lignocellulosic material to produce sugar.

4.3 Simultaneous Saccharification and Fermentation (SSF)

SSF was conducted using NaOH hydrothermal and H₂O₂ Pretreated. To evaluate the ability to produce biethanol from these compounds by fermentation with *Trichoderma reesei* and *Saccharomyces cerevisiae*. Bioethanol was produced from sweet sorghum bagasse via a SSF process that used *Trichoderma reesei* and *Saccharomyces cerevisiae*. The *Trichoderma reesei* functioned as a saccharification agent to convert cellulose into sugar; then this sugar converted through fermentation into bioethanol by the *Saccharomyces cerevisiae*.

In accordance with [14] that simultaneous saccharification and fermentation is a method can be increased enzymes performance due to decrease of product's inhibition. Bioethanol production from affluent raw materials can be acquired

through the cultivation time, with lower cost and shorter processing times that increase productivity.

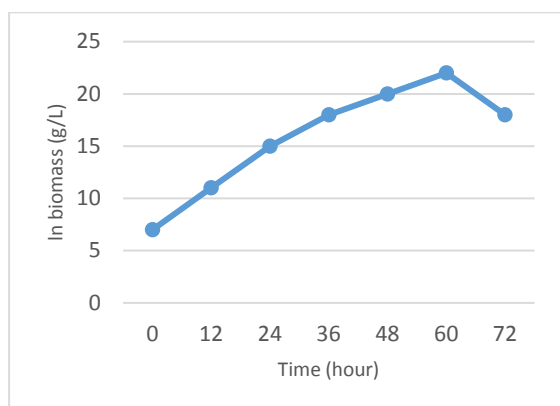


Figure 7. Growth of Microbial biomass during SSF

According to figure 7 which the dry cell weight of two microorganisms of *Trichoderma reesei* and *Saccharomyces cerevisiae*. For this SSF process, aeration is needed for a group of molds and yeasts to produce cells. The major factor which has substantial impact on microorganism's survival is the presence of oxygen in aerobic cultivation condition. The aeration process cannot be detached from the agitation process. The air flow from the compressor entered the medium to sustain aeration and agitation.

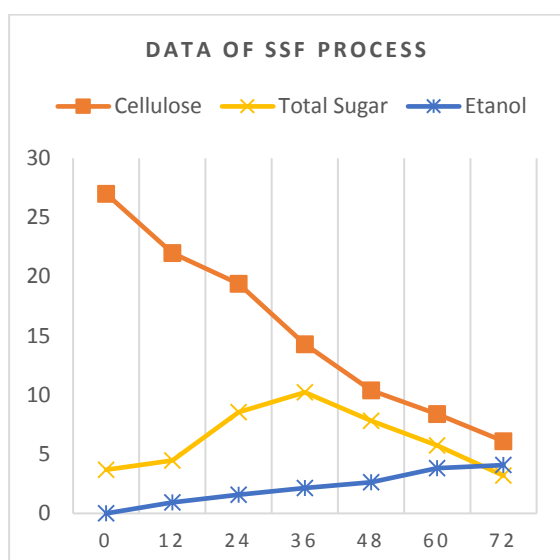


Figure 8. Trendline of analyzed component of Simultaneous Saccharification and Fermentation Process

The maximum bioethanol concentration, which was obtained during the 72nd hour, was 4,08 (g/L). *Saccharomyces cerevisiae* converted sugar into bioethanol by fermentation in addition to respiration. After the 36th hour, the sugar production met decline phase while the yeast growth started to increase. This condition shows that the sugar substrate has been consumed by the mold and yeast for cell production and by the yeast for bioethanol production.

Accordingly, sweet sorghum bagasse has been considered as a particularly important energy plant for the production of bioethanol fuel [15]. As a lignocellulosic biomass from agricultural residues, it is potentially available at \$60 ton for conversion into >20 billion gallons of cellulosic biofuels [16]. Lignocellulosic biomass is fundamentally composed of cellulose (35-50 wt%, dry basis), hemicellulose (15-30%), pectin (2-5%), and lignin (12-35%). Cellulose and hemicellulose which make more than 50% of total mass can be potentially converted to sugars for their conversion to bioethanol [17].



Figure 9. TPC SSF Process

Total plate count in SSF process at 60 h is shown in Figure 9. It explains the yeast grew dominantly for producing bioethanol.

4.5 The kinetics of cultivation in SSF

SSF process was duplicated and has been cultivation kinetics calculated, its used for measuring specific growth rate (μ). If maximum specific growth rates was relatively low, probably because of culturing microbes in a low concentration of medium.

The results was shown at table 1, yield of substrate used into biomass ($Y_{p/s}$) for SSF process was 0,30 g product/g substrate. While the efficiency of substrate by 89,8%. It shows that sweet sorghum bagasse used as a medium for bioethanol production by SSF technique using the two microbes since the effeciency is quite high. And the SSF technique as additional advantages because polysaccharides which have been converted into monosaccharides are not returned into polysaccharides again, but can directly fermented into ethanol.

Time	$Y_{p/s}$ (g/g)	$Y_{x/s}$ (g/g)	Se (%)
12	0,19 ± 0,02	0,01 ± 0,01	89
24	0,27 ± 0,01	0,03 ± 0,00	90
36	0,32 ± 0,00	0,04 ± 0,02	91
48	0,39 ± 0,02	0,03 ± 0,01	91
60	0,31 ± 0,01	0,03 ± 0,00	90
72	0,30 ± 0,01	0,02 ± 0,01	88
ave	0,30	0,27	89,8%

Table 1. Kinetic Parameters

V. Conclusion

Sweet sorghum bagasse (SSB) clearly show as the potential materials for bioethanol production. SSB has potential cellulose content of more than 56%. *Trichoderma reesei* is effective as saccharification agent and catalyst for producing sugar

A number conclusion can be made from this study, the combination of NaOH solution autoclaving and H_2O_2 immersing pretreatment was effective in preparing sweet sorghum bagasse for ethanol production. The processes of bioethanol production from sweet sorghum bagasse was previously carried out in Separate Hydrolysis and Fermentation (SHF). In response to SSF method was considered as

a better process than SHF due to fastly ethanol production and the highest concentration of produced ethanol. Beside that, the SSF process reduced the time process than SHF process. The implementation of SSF process using *Trichoderma reesei* as the saccharification catalyst and *Saccharomyces ceevisiae* as the fermentation catalyst simultaneously produced 4,08 g/L bioethanol.

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