

Bioethanol Production by Using Detoxified Sugarcane Bagasse Hydrolysate and Adapted Culture of *Candida tropicalis*

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ABSTRACT

*Ethanol is considered as the most promising alternative fuel, since it can be produced from a variety of agriculturally-based renewable materials, such as sugarcane bagasse. Lignocellulose as a major component of sugarcane bagasse is considered as an attractive renewable resource for ethanol production due to its great availability and relatively low cost. The major problem of lignocellulose is caused by its need for treatment to be hydrolyzed to simple sugar before being used for bioethanol production. However, pretreatment using acid as hydrolyzing agent creates some inhibitor compounds that reduce ethanol production because these compounds are potential fermentation inhibitors and affect the growth rate of the yeast. Reduction of these by-products requires a conditioning (detoxification and culture starter adaptation). Thus, the aim of this study was to evaluate bioethanol production by fermentation with and without detoxified sugarcane bagasse acid hydrolysate using adapted and non-adapted culture of *C. tropicalis*. According to this study, the highest ethanol amount was obtained about 0.43 % (v/v) with an ethanol yield of 2.51 % and theoretical yield of 4.92 % by fermentation of sugarcane bagasse hydrolysate with detoxification using the adapted strain of *C. tropicalis* at 72 hours fermentation time. Furthermore, the addition of 3 % glucose as co-substrate on detoxified-hydrolysate media only achieved the highest ethanol concentration 0.21 % after 24 hours fermentation with the ethanol yield 0.69 % and theoretical ethanol yield 1.35 %, thus it can be concluded that the addition of glucose could not increase the ethanol production.*

Keywords: adapted culture, bioethanol, *Candida tropicalis*, detoxification, lignocellulose, sugarcane bagasse

1. INTRODUCTION

Bioethanol is one of the most important alternative sources for fuels that can aid fuel

supplies, and to some extent, substitute the use of crude oil by being mixed with fossil fuels (Papong and Malakul, 2010; Posada *et al.*,

2013). Ethanol is already commonly used for transportation fuels by mixing with gasoline, as a 10 % ethanol / **90 % gasoline blend**. Ethanol can also be used in adapted internal combustion engine vehicles at a blend of 85 % ethanol / 15 % gasoline (E85, in flexible fuels vehicles) or even 95 % ethanol (E95). Such nearly pure ethanol fuel provides a number of environment benefits, due to low pressure and reduced emissions into the atmosphere along with their clean-burning characteristics (Lynd *et al.* 1991).

About 90 % of all ethanol is derived from sugar or starch crops by fermentation of biomass; the rest is produced synthetically (Taherzadeh, 1999). On the other hand, Indonesia has an abundance of biomass wastes that can be used as renewable energy sources. One of the potential biomass products that has proven utilization to produce energy is sugarcane bagasses by fermentation with microorganisms. In this research, fermentation of sugarcane bagasses to produce ethanol was carried out by yeast (*Candida tropicalis*). The *C. tropicalis* was obtained from Indonesian Institute of Science. It is one of the yeasts in the culture collections of Indonesian local isolates. On the other hand, another variety of *C. tropicalis* (NBRC 0618) has been proven to produce ethanol using olive prunings (lignocellulose waste) as a carbon source (Martín *et al.* 2010).

One way to make use of the lignocellulose waste in ethanol production via fermentation is by hydrolyzing the main components: cellulose, hemicellulose and lignin. Sugarcane bagasse contains about 50 % cellulose, 25 % hemicellulose and 25 % lignin (Pandey *et al.* 2006). Hydrolysis of cellulose and hemicellulose components produces the monomers like pentoses (xylose and arabinose)

and hexoses (glucose, mannose and galactose). These compounds provide ideal substrate for microbial processes in ethanol production. The hydrolysis and fermentation of lignocellulose is more complicated than fermentation of sugar, because the process must meet the following requirements: (1) improve the formation of sugars; (2) avoid the degradation or loss of carbohydrate components (cellulose and hemicellulose); (3) avoid the formation of by-products inhibitory to hydrolysis and fermentation processes; (4) be cost effective (Sun and Cheng 2002).

In hydrolysis, the cellulosic part of the biomass is converted to sugars, and then fermentation converts these sugars to ethanol. To increase the yield of hydrolysis, a pre-treatment step is needed that softens the biomass and breaks down cell structures to a large extent to increase accessibility of microorganism towards substrate to produce ethanol efficiently (Carlo *et al.* 2005). In this research the pretreatment had been carried out by acid hydrolysis (1 % w/v sulphuric acid). The acid hydrolysis with diluted H₂SO₄ beside release sugars, resulting also in the generation of by-products such as acetic acid, furfural, hydroxymethylfurfural, phenols that are potential fermentation inhibitors and affect the growth rate of the yeast. Reduction of these side products requires a conditioning (detoxification and culture starter adaptation) process so as to optimize hydrolysate for fermentation, such as activated carbon addition (Pandey *et al.* 2006), overliming and Ca(OH)₂ addition (Dien *et al.* 2000), filtration with an ion exchange resins (Mancilha and Karim 2003), fermentation with several variety of yeasts (Martin and Jonsson 2003), and adaptation treatment for the starter culture (Rivas *et al.* 2003). A conditioning

process using ion exchange resins was developed to neutralize the pH or acid recovery by Carlo *et al.* 2005. In addition, Rao *et al.* (2006) and Huang *et al.* (2009) minimized the inhibitor concentration by an adaptation treatment and detoxification processes. Hence, in the present study the conditioning process was done by detoxification and starter culture adaptation (by cultivating the cells in the detoxified sugarcane bagasse hydrolysate medium before fermenting the sugar to produce ethanol). Detoxification was conducted with NaOH and phosphoric acid, continued by adding $\text{Ca}(\text{OH})_2$, active carbon and at the end NaOH was added to the hydrolysate until a pH of 5 was obtained. Thus, the aim of this study was to evaluate bioethanol production by fermentation with and without detoxified sugarcane bagasse acid hydrolysate using an adapted and a non-adapted culture of *C. tropicalis*. Besides that, the bioethanol production was evaluated with the addition of glucose as co-substrate.

2. METHODOLOGY

Preparation of Sugarcane Bagasse

The sugarcane bagasse was collected on-site after sugar production from P3GI (Indonesian Sugar Research Institute), Pasuruan, East Java, Indonesia. After an initial air-drying at room temperature the sugarcane bagasse samples were ground in a blade mill and then screened. The size of the fraction selected was between 20 and 40 mesh.

Preparation of Sugarcane Bagasse Hydrolysate

Hydrolysate was prepared based on acid hydrolysis of Alves *et al.* (1998) method. The ground bagasses was added 1

% (v/v) solution of H_2SO_4 . This hydrolysis was carried out in 300 mL Erlenmeyer flasks. After that, the system was heated to 120°C for 15 minutes. At the end of treatments the hydrolysates were obtained by filtration and total sugar was analyzed by refractometer and reducing sugar by the DNS method.

Hydrolysate Conditioning (Detoxification)

To increase the initial sugar concentration, the hydrolysates were treated with vacuum evaporation. Then, bagasse hydrolysates were detoxified to remove furfural compounds based on the Pandey *et al.* (2006) procedure being combined with sodium hydroxide in 300 mL of hydrolysates to pH 8.5 and then 27 % of phosphoric acid was added to pH 5-6. The hydrolysate was heated to a temperature 70°C and then 2 % of active carbon added. The process was continued by adding 4 M $\text{Ca}(\text{OH})_2$ for 1 h, followed by 2 % of active carbon with stirring for 30 min. At the end, the hydrolysates were treated with 10 M NaOH to a pH of 5.0 and then sterilized using a millipore membrane.

Starter Culture Preparation

All the fermentation experiments were done using *C. tropicalis* from Indonesian Institute of Sciences at laboratory scale in a batch-culture reactor. The culture starter preparation was carried out in 50 mL Erlenmeyer flasks. The single colony of the microorganism from a solid culture medium was inoculated into 12.5 mL of liquid medium composed of (in g L⁻¹): glucose 30, yeast extract 10; peptone 20; KH_2PO_4 0.5, K_2HPO_4 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, $(\text{NH}_4)_2\text{SO}_4$ 2 at pH 5. The starter culture was kept in a shaker at $30 \pm 1^\circ\text{C}$ with agitation speed at 120 rpm for 24 hours.

Culture Adaptation

Before fermentation, the starter culture was inoculated into 25 mL of adaptation medium which was carried out in 100 mL Erlenmeyer flasks. The composition of adaptation medium followed that of Rao *et al.* (2006) with some modifications, being (in g L⁻¹) KH₂PO₄ 0.5, K₂HPO₄ 0.5 and MgSO₄·7H₂O 0.5 were solubilized into 25 mL of detoxified and non-detoxified baggasse hydrolysates (pH of the medium was 5). The adaptation medium was sterilized by millipore membrane. After inoculation, the culture was kept in a shaker at 30 ± 1°C with agitation speed at 120 rpm for 24 hours. Adaptation of the yeast in the sugarcane baggasse medium were conducted for 40 days (20 cycles) and in each cycle of the process we added the starter cultures into the sterilized medium. After 5 cycles (10 days of incubation), the media were analyzed for both their optical density and reducing-sugar level. The optical density was measured on 660 nm absorbance.

Fermentation Procedure

There are two types of fermentation evaluation based on media: using bagasse hydrolysates (detoxified and non-detoxified) and using one of the best hydrolysate with adding co-substrate (glucose 3 % (w/v)) and without glucose. The composition of both media followed the procedure of Rao *et al.* (2006) with some modifications. The medium culture was composed of (in g L⁻¹): KH₂PO₄ 0.5, K₂HPO₄ 0.5, (NH₄)₂SO₄ 2 and MgSO₄·7H₂O 0.5 and solubilized into 100 mL aliquots of detoxified and non-detoxified baggasse hydrolysates (the pH medium was 5.0). The medium was sterilized through the use of the millipore



Figure 1 Sugarcane bagasse hydrolysate after conditioning (detoxification).

membrane. Fermentation was carried out in 125 mL Erlenmeyer flasks and kept in a shaker at 30 ± 1°C and with agitation speed 120 rpm for 96 h. After every 24 hours of incubation, the fermentation aliquot was taken and samples centrifuged at 2000 g for 15 min to measure optical density (OD, the absorbance 660 nm measured), reducing sugar and ethanol content.

Ethanol Analysis

The ethanol concentration was determined by a HPLC (model Shimadzu) using a capillary column (SHODEX SH1011). All HPLC runs used temperature programming 60 °C, 50 mM H₂SO₄ was used as carrier phase with velocity 0.6 µL/minute and ethanol retention time in the 25th minute. Ethanol concentration was determined from 20 µL samples. All determinations were done by means of standard curves. The ethanol yield was calculated by the modified formula (Huang *et al.* 2009).

$$\text{Ethanol yield \%} = \frac{\text{Ethanol produced (g/L)} \times 100 \%}{\text{Reducing sugar (g/L)}}$$

$$\text{Ethanol yield \% (theoretical)} = \frac{(\text{Ethanol yield \%})}{0.51}$$

The conversion factor of glucose to ethanol = 0.51.

Reducing Sugar Analysis

The concentration of reducing sugar was determined using 3,5-dinitrosalicylic acid (DNS) (Miller, 1959). The reaction mixture contained 1 mL of the sample and 3 mL of DNS and samples were incubated for 5 min at 100°C. After that, the mixtures were cooled to room temperature and the absorbance at 550 nm measured.

3. RESULTS

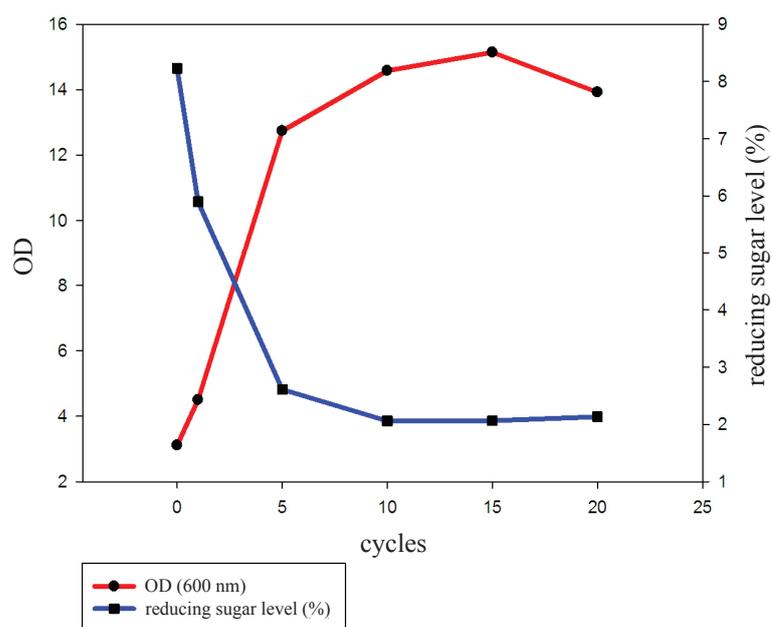
Sugarcane Bagasses Hydrolysate after Detoxification

Hydrolysate obtained from conditioning process was tested for pH. The pH of hydrolysate obtained from detoxification process was 5.0 and it had a black colour. A change of colour was caused by chemical reaction after adding Ca(OH)_2 , active carbon and NaOH. The content of sugar and reducing sugar in the sugarcane bagasse hydrolysate sample was 6.92 % and

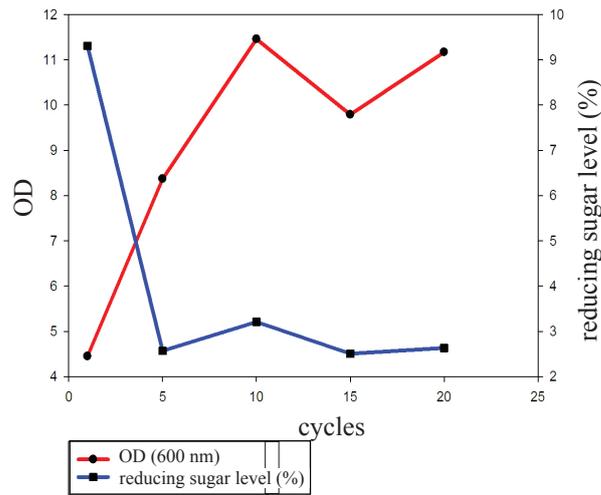
23 brixs, respectively. Sugarcane bagasses hydrolysate after conditioning by detoxification is shown in Figure 1. These hydrolysates were used as substrate in the adaptation of *C. tropicalis* cells and the production of bioethanol.

Adapted Cells of *C. tropicalis* in the Sugarcane Bagasse Hydrolysate Medium

The cells of *C. tropicalis* were grown in the sugarcane bagasse hydrolysate media both with detoxification (conditioning process) and without. The yeast growth pattern in each media can be seen in Figure 2. The data showed that *C. tropicalis* growth faster in the detoxified sugarcane bagasse hydrolysate than in the non-detoxified hydrolysate media. The OD value for detoxified medium on the start (0 hour) until the fifth cycle increased significantly from 3.11 to 12.74. The increase of the OD was slow after 5 cycles (the OD value just rose to 15.14 on the 15th cycle) and after that on the 20th cycle the OD value was decreased to 13.92. On the other hand, the OD value for non-detoxified medium



(a)



(b)

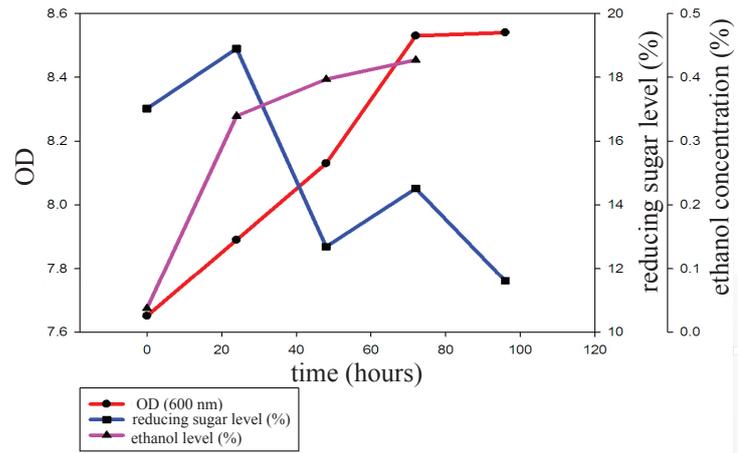
Figure 2 The growth curve of *C. tropicalis* and reducing sugar level which were adapted in the sugarcane bagasse hydrolysate medium (a) with detoxification and (b) without detoxification.

climbed substantially from the start (0 hour) to the 10th cycle (3.11 to 11.46). Based on the above results it can be concluded that the cells had been adapted well and grew faster on the detoxified media in the fifth cycle. In addition, the reducing sugar levels declined dramatically for both media in the start cycle until the fifth cycle. The reducing sugar level in the detoxified hydrolysate media decreased significantly from 8.23 % to 2.61 % then the decreasing of reducing sugar content continued low after 5 cycles (2.13 %). A similar pattern was found on the non-detoxified media.

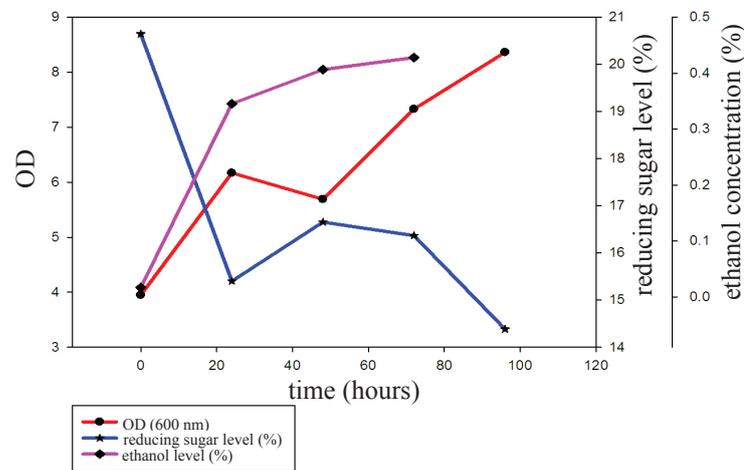
Optimum Condition for Bioethanol Production

Optimization of ethanol production was conducted as three parallel experiments (Figure 3). These are the cells of *C. tropicalis* had been adapted in the detoxified sugarcane bagasse hydrolysate medium were grown in the same medium (adapted cells-detoxified medium),

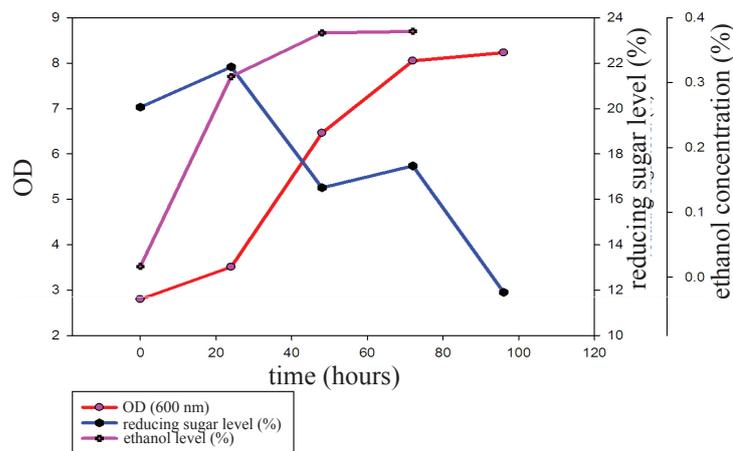
the cells which had been adapted in the non-detoxified sugarcane bagasse hydrolysate medium and were grown in the same medium (adapted cells-non-detoxified medium) and non-adapted cells were grown in the detoxified sugarcane bagasse hydrolysate medium (non-adapted cells-detoxified medium). The results showed that the best condition for ethanol production was adapted cells-detoxified medium after 72 hours of fermentation. The ethanol fermentation with this method had values of OD 8.53, reducing sugar level 14.51 %, ethanol concentration 0.43 %, ethanol yield 2.51 %, theoretical ethanol yield 4.92 % (Table 1). On the other hand, non-adapted cells-detoxified medium had the lowest of ethanol level and ethanol yield in the 72nd hour, respectively 0.38 % and 1.89 %. The patterns of reducing sugar level from all three experiments were shown to be fluctuate but with an overall descending trend in all cases.



(a)



(b)



(c)

Figure 3 The rate curves of *C. tropicalis* growth, bioethanol production and reducing sugar level with the treatments (a) adapted cells - detoxified medium, (b) adapted cells - non-detoxified medium and (c) non-adapted cells - detoxified medium.

Table 1 Bioethanol yield with the treatments: adapted cells-detoxified medium, adapted cells-non-detoxified medium and non-adapted cells-detoxified medium.

Treatments	Time (hours)	Bioethanol yield (%)	Theoretical bioethanol yield (%)
adapted cells-detoxified medium	0	0.22	0.43
	24	1.99	3.91
	48	2.33	4.57
	72	2.51	4.92
adapted cells-non-detoxified medium	0	0.08	0.16
	24	1.67	3.28
	48	1.97	3.86
	72	2.07	4.06
non-adapted cells-detoxified medium	0	0.08	0.16
	24	1.54	3.02
	48	1.88	3.68
	72	1.89	3.70

Bioethanol Production by Co-substrate Addition

The curve of bioethanol production and that yielded with adding the co-substrate glucose is shown in Figure 4. Based on the data, it could be seen that the addition of co-substrate did not influence bioethanol production, whereas the number of cell biomass did influence the

production. The values of OD, reducing sugar and bioethanol level in the 72 hours, respectively are 11.11, 38.55 %, and 0.06 %. At that time, the bioethanol yield was 0.20 % and theoretical ethanol yield was 0.37 % (Table 2). The highest bioethanol concentration was in the first 24 hours (0.21 %) with the ethanol yield 0.69 % and theoretical ethanol yield 1.35 %, however

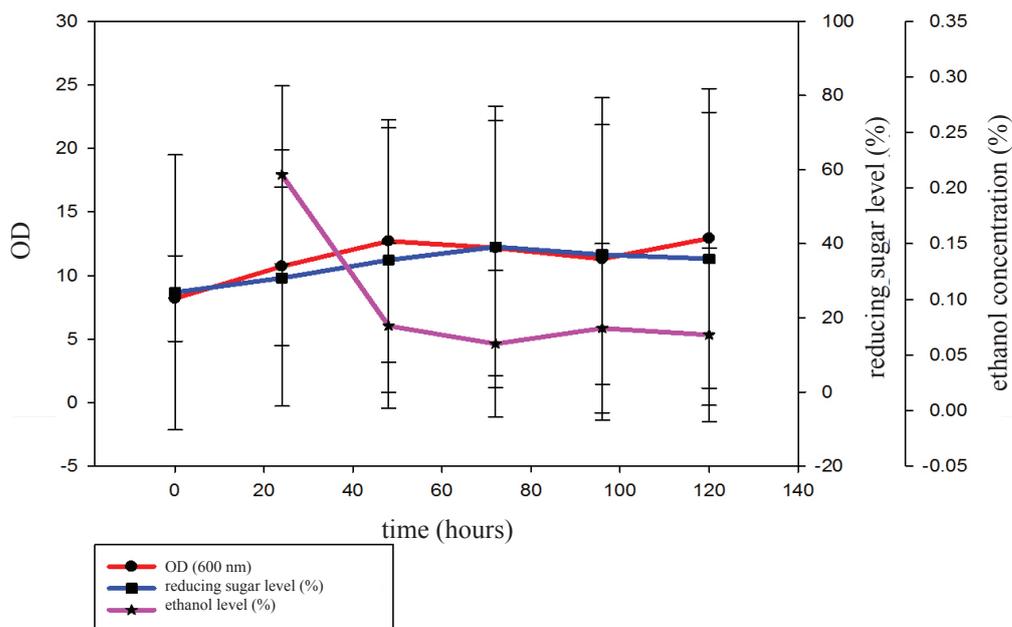


Figure 4 The rate curves of *C. tropicalis* growth, bioethanol production and reducing sugar level with adding co-substrate (adapted cells-detoxified medium).

reducing sugar level had the lowest level (32.00 %).

Table 2 Bioethanol yield with the adding co-substrate (adapted cells - detoxified medium).

Time (hours)	Bioethanol yield (%)	Theoretical bioethanol yield (%)
24	0.69	1.35
48	0.23	0.46
72	0.20	0.37
96	0.23	0.46
120	0.23	0.45

4. DISCUSSION

Before detoxification, the color of bagasse hydrolysate was black with pH 5, it is caused by the hydrolysis using 1 % (v/v) H_2SO_4 at a temperature of 121°C for 15 minutes. During hydrolysis, hemicellulose sugars may be degraded to weak acids, furan derivate and phenolic compounds. These compounds inhibit the later fermentation, leading to reduced ethanol yields. The production of these inhibitors increases when hydrolysis takes place at several conditions: higher temperatures and higher acid concentrations (Carlo *et al.* 2005). Hence, a conditioning process (detoxification and culture starter adaptation) needs to be done on the sugarcane bagasse hydrolysate to enhance fermentation yield. Detoxification consisted of an overliming process, adsorption and neutralization. The overliming process using $Ca(OH)_2$ and activated charcoal adsorption reduces the concentration of inhibitor compounds such as HMF, furfural, and organic acids. Reactions that occur in the process of adding $Ca(OH)_2$ is the formation of salts of acid-base reaction between H_2SO_4 and $Ca(OH)_2$. In addition, most of the Ca^{2+} ions react with sugars, HMF and furfural into compounds

having irreversible bonding (Purwadi 2006).

After conditioning, the detoxified bagasse hydrolysate was used as substrate for adaptation medium of *C. tropicalis*. Adaptation was done to adapt the cells to the sugarcane bagasse hydrolysate medium, so the growth of the cells in ethanol production medium would be optimum. Adaptation is able to shorten the lag phase time and increases the capability of microbes to produce primary and secondary metabolites (Huang *et al.* 2009). In addition, it was very important to know the yeast growth pattern in each media (Figure 2). The data show that *C. tropicalis* adapted well in the fifth cycle. The OD values of both medium with and without detoxification on the start cycle until the fifth cycle was rose significantly from 3.11 to 12.74 and the increasing of the OD was slow after 5 cycles (13.92). It means the cells had been adapted well in the fifth cycle. On the other hand, the reducing sugar levels were declined dramatically in the start cycle until the fifth cycle and the decrease of reducing sugar content was reduced only gradually after 5 cycles. The decrease of reducing sugar indicated that the *C. tropicalis* cells used the sugar for their biomass growth. It is also stated by Rao *et al.* (2006), that the adaptation of *C. tropicalis* for 20 cycles in the sugarcane bagasses and corn cob hydrolysate medium that able to raise the cell biomass further.

Optimization results of bioethanol production showed that the pattern of growth, production of bioethanol, and reducing sugars in the three treatments had similar patterns to each other. The growth pattern of *C. tropicalis* showed a lag phase in the start until the 24th hour on adapted cells-detoxified media, meanwhile the other two treatments showed no lag phase

because the cells had been adapted in advance for 5 cycles in both treatments. On the other hand, log phase could be seen on the start time until 72nd hour in the adapted cells-detoxified as well as adapted cells - non-detoxified media, whereas the non-adapted cells-detoxified medium occurred on the 24th to 72nd hour. During log phase the growth of *C. tropicalis* cells was very quick. However, the stationary phase is shown on from 72nd to 96th hour in the three kinds of treatments. For ethanol production, the best result was in the adapted cells-detoxified medium. Bioethanol production increased sharply from 0 to the 24th hour and began to decrease in the 24th until 72nd hour. This means that the ethanol production of *C. tropicalis* took place in the log phase. The highest of ethanol concentration production could be found on adapted cells-detoxified medium (0.43 %) with the highest ethanol yield was 2.51 % in the 72 hours of fermentation. On the other hand, non-adapted cells-detoxified medium had the lowest of ethanol concentration and ethanol yield, respectively 0.38 % and 1.89 % in the 72 hours of fermentation. To sum up, the adaptation could increase the ethanol production, whereas the detoxified medium was better than the non-detoxified one. Huang *et al.* (2009) evaluated that the ethanol yield climbed 0.44 g/g (87 %) in the hydrolysate medium with adaptation, overliming and neutralisation treatments. They explained also that the adaptation process pushed the lag phase time to faster and increased the capability of the microbial cells in the production of both primary and secondary metabolites as well.

The patterns of reducing sugar level from that three experiments were shown to be in fluctuation, but with an overall descending trend. The decreasing of reducing sugar was caused by

the metabolism activity of *C. tropicalis* which intake sugar for biomass growth and ethanol synthesis. On the other word, this shows that the metabolic process of ethanol fermentation that occurs in *C. tropicalis* is as the primary and secondary metabolism (Voet *et al.* 2009). The highest yield obtained from the adapted cells-detoxified treatment was selected for the next study, bioethanol production with adding co-substrate (glucose). The curve of bioethanol production with adding co-substrate (Figure 4) does not reflect the adaptation phase. This is because the cells were already adapted 5 times before growth in the fermentation medium. In addition, the exponential phases extend from times 0 h to 48 h, and from 72 h to 120 h. This is rationalized due to the presence of another carbon source (glucose), hence the exponential phases were doubled. On the other hand, there was no stationary phase in the model of cell growth.

The growth pattern is different from the production of bioethanol and reducing sugar pattern. Based on the data, it can be concluded that the addition of co-substrate did not influence bioethanol production, but it influenced the number of cells. Based on the data in Figure 4 and Table 2, it could be seen that the addition of glucose (3 %) as a co-substrate did not create an impact on the increase of bioethanol production by *C. tropicalis*. This phenomena were observed by Sanchez *et al.* (2004) as well as that the addition of co-substrate in the certain level of concentration, it could be the inhibitors to the fermentation.

5. ACKNOWLEDGEMENT

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