Evaluation of Addition The Activated Charcoals and pH Adjustment in The Treatment of Lignocellulosic Hydrolisates for Xylitol Production

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Abstract. D-Xylitol is a five-carbon sugar alcohol, which can be obtained from xylose of agricultural wastes with microbial fermentation. The inhibitory chemical complexes (ICC) were found after biomass pretreatment step. The ICC in acid hydrolysate can inhibit microbial metabolism in the medium. Several methods for detoxification; adsorption by activated charcoal, by ion exchange resin or by using enzymes. This study aims to evaluate the effect of using charcoal in xylitol fermentation by using lignocellulosic hydrolysate. In this work, detoxification process by added 1% activated charcoal to reduce the inhibitors in corn cobs hydrolysate and sugarcane trash hydrolysate. The biomass hydrolysate was fermented by Meyerozyma caribbica Y67 to produce xylitol. It is expected that detoxification can reduce concentration of inhibitors, so that microbial growth is not inhibited and more product. The results showed that M. caribbica Y67 had not significant different between pure hydrolysate and detoxified hydrolysate with activated charcoal. We suggested that M. caribbica Y67 tolerance to ICC and potential for xylitol fermentation from lignocellulosic biomasses.

1. Introduction
Xylitol is a five sugar alcohol which has same sweetness as sucrose but low calories. Xylitol has health benefits such as oral health, reducing or preventing tooth decay, and medication benefits such as diabetes [1]. Xylitol is globally accepted as a natural sweetener approved by the Food and Drug Administration (FDA) and the American pediatric dentistry academy. More than 35 countries have agreed to use xylitol in the food, pharmaceutical and health products especially, gum, toothpaste, syrup and confectionery [2]. In the industrial scale, xylitol is produced through xylose hydrogenation using chemical catalysts at a temperature of 80 °C - 140 °C. This method is costly because requires extensive purification to comply with the food and pharmaceutical standards [3]. Whereas xylitol can be produced by the yeast fermentation of xylose from hemicellulosic materials such as agricultural wastes which more low energy and environmentally friendly than chemical process.
Xylose as a substrate fermentation released by biomass pretreatment. Several methods of pretreatment process is physical treatment by milling and grinding, chemical treatment using acids, alkali or organic solvents, physio-chemical treatment using liquid hot water or steam explosion and biological treatment using fungi [4]. The hydrolysis with acid is commonly used, because the acid pretreatment reaches 80 percents of hemicellulose degradation and below 10 percents of cellulose degradation according to pretreatment conditions [5]. The hydrolysis with acid released the inhibitory chemical complexes (ICC) like HMF and furfural [6]. The ICC are toxic to many microorganisms affecting for sugar utilization and product formation were limited. So, the lignocelullosic hydrolysate needs to be adjusted to be suitable for fermentation media. To reduce the concentration of the ICC, there are several methods for detoxification. One of detoxification methods with chemical by pH adjustment and added activated charcoal has been proven with good results [7]. The effectiveness can be achieved by added another variable in detoxification process. One of the variables that affect the effectiveness of detoxification using activated charcoal is pH fermentation. This study, aims to evaluate the effect of pH adjustment and added activated charcoal in xylitol fermentation by using lignocellulosic hydrolysate were used to remove the ICC from corn cobs hydrolysate, sugarcane trash hydrolysate, sorghum trunk hydrolysate and tobacco stalk hydrolysate.

2. Methods
2.1. Preparation of hemicellulosic hydrolysate
The hemicellulosic hydrolysate as a substrates fermentation are corn cobs hydrolysate and sugarcane trash hydrolysate which have been pretreated with Maleic acid 1.8% from Research Center for Biomaterial, LIPI. The freeze hydrolysates were defrosted in room temperature.

2.2. Detoxification methods
The hemicellulosic hydrolysates were separated into four fractions. Two of them were added activated charcoal powder food grade (Nourish). The first fractions, intial pH were adjusted to pH 5.5. The second fraction without pH adjustment and activated charcoal. The third fraction, pH increased up to 5.5, then treated with 1% w/v activated charcoal under agitation (150 rpm) at 30 °C for 1 h. The forth fraction, used in the formulation of the fermentation medium, was simply treated with 1% w/v activated charcoal under agitation (150 rpm) at 30 °C for 1 h. After incubation in 1 h, charcoal were remove by centrifuge 10.000 rpm at 4°C for 10 mins. Then, the hydrolysates sterilization with vacuum filtration through nitrocelullose filter 0,45 µm. The sterile hydrolysates ready to use as media fermentation.

2.3. Microorganism and inoculum preparation
Meyerozyma caribbica Y67 from Indonesia Culture Collection (InaCC)-LIPI was used in the study. Yeast Y67 strain was grown on yeast extract peptone glucose (YPD) agar at room temperature for 48 hr. The 1 loop inoculum was seeded into 500 mL erlenmeyer flasks containing 50 mL yeast extract peptone glucose (YPD) broth. The flask were incubated on a rotary shaker (150 rpm) at 30 °C for 18-20 hr. Afterward, the cells were centrifuged at 8000 rpm for 5 mins. Then, the cells were washed with MilliQ sterile twice and added 5 mL yeast nitrogen base (YNB).

2.4. Media and fermentation conditions
Comparison studies were made between four variation of pH adjustment and treated with activated charcoal each of hemicellulose hydrolysate. The M. caribbica Y67 was grown in 100 mL erlenmeyer flasks containing the hydrolysate and YM 10X with total volume is 12 mL. The inoculum was seeded into fermentation media at an intial density optical density at 600 nm of 3.0 with a dry cell weight of 0.8 g/L. The fermentation runs were carried out at 30 °C, 150 rpm for 48 hr fermentation.

2.5. Analytical procedures
The cells biomass was be measured optical density at 600 nm using Spectrophotometer (Genesys 10S UV-Vis) after 48 hr fermentation. The content of the sugars (glucose, xylose and xylitol), lactic acid, glycerol, acetic acid, ethanol, 5-HMF and furfural in the hydrolysate were analyzed by high performance liquid chromatography (Shimadzu Co. Ltd.; Kyoto, Japan) with an Aminex HPX-87 column (Bio-rad; Hercules, CA, USA). The flow rate of the mobile phase (5 mM H$_2$SO$_4$) was set at 0.6 mL/min at 60 °C with connection to a refractive index detector (RID-20A).

3. Results and Discussion

3.1. Effect of Detoxification with pH adjustment and activated charcoal on cell growth

Several microorganisms are inhibited by the inhibitor compounds from the pretreatment process of lignocellulose biomass. To investigate the effects of pH adjustment and combined activated charcoal in sugarcane and corn cobs hydrolysate fermentation by *M. caribbica* Y67, experiments were performed several conditions of pH adjustment and activated charcoal. The growth profiles on *M. caribbica* Y67 on sugarcane trash and corn cobs hydrolysate at four fractions of condition showed in Figure 1 and Figure 2. The cells biomass in sugarcane hydrolysate with treatment 1-4 showed at 24 hr continued 11.1, 3.1, 10.7 and 0.8 g/L, whereas in corn cobs hydrolysate showed continued 12.4, 0.7, 12.0 and 0.9 g/L. The cells were observed of both hydrolysate to grow well at pH 5.5 and with or without activated charcoal, whereas no significant cell growth was observed at pH 1.8 and with or without activated charcoal.

The results of the treatment hydrolysate with pH adjusted and activated charcoal led us to know that initial pH affected cell growth. At different initial pH, cells were observed to grow best at initial pH 5.5 in both of hydrolysate (Figure 1 and Figure 2). The maximum cell growth was observed at an initial pH 5.5 and without activated charcoal (Figure 1 and Figure 2). Mechanism of detoxification by pH adjustment and activated charcoal are pH adjustment to induce precipitation and/or instability of the toxic compounds, and the adsorption of these compounds on activated charcoal [7]. Under pH of medium low, concentration of acetic acid as inhibitor compound will diffuse into cell membrane and cause cytoplasmic acidification, which cause synthesis biomass was inhibited and the growth rate was stopped [8]. The insignificant cell growth between the addition of charcoal and without shows that the cells can grow on media containing inhibitor compounds.

![Figure 1. Growth profiles of *M. caribbica* Y67 at various treatment on sugarcane hydrolysate (treatment 1 = without charcoal; pH 5.5, treatment 2 = without charcoal; pH 1.8, treatment 3 = with charcoal; pH 5.5 and treatment 4 = with charcoal; pH 1.8)](image)

![Figure 2. Growth profiles of *M. caribbica* Y67 at various treatment on corn cob hydrolysate (treatment 1 = without charcoal; pH 5.5, treatment 2 = without charcoal pH 1.8, treatment 3 = with charcoal; pH 5.5 and treatment 4 = with charcoal; pH 1.8)](image)
3.2. Effect of detoxification with pH adjustment and activated charcoal on substrate consumption

Hemicellulose hydrolysis in sugarcane and corn cobs biomass produce xylose and glucose. Xylose and glucose are sources of carbon for yeast cells in the fermentation process. Both for growth and for being synthesized into products. The consumption of the substrates from the fermentation using the detoxified hydrolysates are shown in Figure 3. The consumption of both xylose and glucose as a substrate on sugarcane hydrolysate at 24 hr with treatment 1-4 showed successively 0.02, 16.3, 0.02 and 16.0 g/L, whereas glucose 13.3, 15.2, 13.2, and 15.0 g/L. Xylose was almost completely consumed when the hydrolysate was treated by pH adjustment with/or without activated charcoal from both hydrolysates, but when without treated by pH adjustment there is no consumption of xylose in both hydrolysates. Whereas, there is no glucose consumption in both hydrolysates of all treatments.

The results of the treatment hydrolysate with pH adjusted and activated charcoal led us to know that initial pH affected substrate consumption. The maximum substrate consumption was observed at an initial pH 5.5 and with/or without activated charcoal (Figure 3). The decrease in pH causes acidification of the cytoplasm which causes inhibition of enzyme activity and decreases the rate of biochemical energy synthesis. Decrease energy production together with increased use of energy to overcome cytoplasmic acidification which causes energy for biomass synthesis to be limited [8]. The substrate consumption between with/or without activated charcoal shows that cells can utilize substrate on media containing inhibitor compounds.

![Graphs showing substrate consumption](Image)

**Figure 3.** Substrate consumption during the fermentation of sugarcane and corn cobs hydrolysate in various treatment by *M. caribbica* Y67 (treatment 1 = without charcoal; pH 5.5, treatment 2 = without charcoal; pH 1.8, treatment 3 = with charcoal; pH 5.5 and treatment 4 = with charcoal; pH 1.8)

3.3. Effect of detoxification with pH adjustment and activated charcoal on xylitol production
Fermented substrate in the form of glucose and xylose is the result of hydrolysis of hemicellulose biomass from sugarcane and corn cobs which used by \textit{M. caribbica} Y67. The substrate consumption used by yeast for growth and xylitol production. Xylitol production from fermentation using the detoxified hydrolysates are shown in Figure 4. The xylitol production by \textit{M. caribbica} Y67 on sugarcane hydrolysates at 24 hr with treatment 1-4 showed successively 3.2, 1.1, 2.9, and 0.9 g/L, whereas xylitol production on corn cobs showed successively 3.4, 1.5, 3.2 and 1.4 g/L. The xylitol production were observed of both of hydrolysate maximum under pH 5.5 and without addition of activated charcoal, whereas minimum xylitol production under pH 1.8 and with addition of activated charcoal.

The results of the treatment hydrolysate with pH adjusted and activated charcoal led us to know that initial pH affected xylitol production by yeast. The maximum of xylitol production was observed at an initial pH 5.5 without activated charcoal (Figure 4). A decrease in pH can cause a decrease in xylitol accumulation caused by disruption of the redox balance of the bioreduction process, the change in intracellular pH which causes a decrease in the enzymatic rate of xylitol forming, affecting yeast cell permeability and the absence of micronutrient assimilation due to the occurrence of micronutrient precipitation \[9\]. Addition of activated charcoal can reduce sugar content around 11.5\% \[7\]. A decrease in sugar levels caused by activated charcoal can effect the substrate concentration and fermentation products, so that if the substrate is reduced then the product is getting smaller in amount. The substrates conversion into xylitol between with/without activated charcoal shows that cells can utilization substrates into products on media containing inhibitors compounds.

![Sugarcane Trash-Xylitol](image1)

![Corn Corb-Xylitol](image2)

**Figure 4.** Xylitol production during the fermentation of sugarcane and corn cobs hydrolysate in various treatment by \textit{M. caribbica} Y67 (treatment 1 = without charcoal; pH 5.5, treatment 2 = without charcoal; pH 1.8, treatment 3 = with charcoal; pH 5.5 and treatment 4 = with charcoal; pH 1.8)

4. Conclusion

This study showed that pH adjustment and activated charcoal affect the fermentation performance of \textit{M. caribbica} Y67 on sugarcane and corn cobs hydrolysate for xylitol production. It was found that pH adjusted and without addition of activated charcoal causes cells grew optimally and also gave the highest amount of xylitol production. The addition of activated charcoal can cause substrate reduction around 11.5%, so that the product of fermentation is getting smaller in amount. On the other hand, it was found that strain \textit{M. caribbica} Y67 is tolerance to ICC and potential for xylitol fermentation from lignocellulosic biomasses.

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