Microbial Production of Xylitol from Oil Palm Empty Fruit Bunch Hydrolysate: Effects of Inoculum and pH

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Abstract. Considering its high content of hemicellulose, oil palm empty fruit bunch (EFB) lignocellulosic biomass waste from palm oil processing has the potential to be utilized as the raw material for the production of xylitol, a low calorie, low GI, and anti cariogenic alternative sugar with similar sweetness to sucrose. This research explored the possibility of converting EFB to xylitol via green microbial fermentation, in particular the effects of inoculum and initial pH on the fermentation performance. It was observed that the cell concentration in the inoculum and the initial pH affect cell growth and xylitol production. pH 5 was observed to give the best fermentation performance. Further, the fermentation tended to yield more xylitol at higher initial cell concentration. It was also observed that no growth or fermentation inhibitory compounds were found in the EFB hydrolysate obtained from enzymatic hydrolysis of EFB. Thus it can be used directly as substrate for xylitol fermentation.

Keywords: *cell concentration; cell growth; D. hansenii; fermentation; hydrolysate; inhibition; inoculum; pH; yield.*

1 Introduction

Only about 23% from every ton of oil palm fresh fruit bunch (FFB) result in crude palm oil (CPO) [1]. The rest ends up as biomass waste such as oil palm empty fruit bunches (EFB), fibers, and shells. At an EFB/CPO production ratio of 1.1, Indonesia produced 27.8 million tons of crude palm oil in 2013 [2] and also co-produced 30.6 million tons of waste EFB in the same year. The abundance of available EFB makes it a potential raw material for other industries.

Xylitol is a 5-carbon atom sugar alcohol. Naturally, xylitol can be found in small quantities in fruits and vegetables. This sugar has a similar level of sweetness to sucrose and is non-cariogenic. Because of its nature and characteristics, xylitol is widely used in the food and pharmaceutical industries. Xylitol is used as a coating on pharmaceutical products and can also be used as

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a sugar substitute for diabetics. In 2007, the world market of xylitol reached $340 million at a price of $ 4-5 per kg [3].

Xylitol can be produced through extraction, chemical synthesis, or bioprocesses (fermentation). The latter is a very interesting alternative production method for xylitol. Generally, biocatalyst based processes are conducted at moderate operating conditions. Another advantage is that this process does not need high-purity chemicals as raw materials. In most cases, the bioprocess can use waste as raw material.

EFB is mainly composed of lignin, cellulose, and hemicellulose. Hemicellulose is a heteropolymer mainly composed of xylose, a 5-carbon atom aldehyde sugar that can be used as raw material to produce xylitol. Xylose can contain 23.7 to 24.0 wt% of EFB [4]. Since EFB has a relatively high content of xylose and is abundantly available, EFB is considered a potential raw material for xylitol production. Currently, EFB is utilized for mulch and as raw material for organic fertilizer [5].

In most fermentation processes pH, temperature, aeration condition, substrate concentration, and inoculum are the most important process parameters. Each strain of microorganism has its own optimum condition for growth and product formation. Some aspects of fermentation for xylitol production have been studied. A previous research studied potential microbial strains for xylitol production along with the aeration condition and the initial substrate concentration [6]. *Debaryomyces hansenii* ITB CCR85 was suggested as one of the potential microbial strains for xylitol production and the suggested aeration condition was semi-aerobic.

Composition and concentration of the hydrolysate is another important variable. Hydrolysate of oil palm empty fruit bunches for use in xylitol fermentation consists of various components, such as acetic acid, which may act as inhibitor to the growth of microbes, in particular hydrolysate obtained from acid hydrolysis [4]. Enzymatic hydrolysis of EFB, however, is normally performed under milder conditions [7] and is thus expected to produce less compounds that are harmfull for the fermentation process.

The ultimate objective of this research was to utilize EFB hydrolysate for microbial production of xylitol. The specific research objective was to study the influences of the inoculum, or initial cell concentration, and the initial pH on the fermentation process on the production of xylitol using *Debaromyces hansenii* ITB CC R85. Further, the effect of using EFB hydrolysate obtained from enzymatic hydrolysis of EFB as the substrate for fermentation was also studied.

2 Materials and Methods

2.1 Strain and Media

The strain used in the experiments was yeast of *Debaryomyces hansenii* ITB CC R85 from the collection of Microbiology and Bioprocess Technology Lab., Department of Chemical Engineering, Bandung Institute of Technology. The strain was preserved in slant agar containing glucose, peptone, and yeast extract until further used.

2.2 Fermentation

Fermentations were performed in 1 L shake flasks with a working volume of 500 mL for the fermentation media, as described previously in [8], except for the carbon source. Unless indicated otherwise, synthetic media composed of 1.4 g/L glucose and 2 g/L xylose were used as the carbon source. This concentration was set based on the sugar concentration found in EFB hydrolysate [7]. EFB hydrolysate was prepared by enzymatic hydrolysis of EFB as described in [7].

Fermentations were performed at a controlled temperature of 30 ºC and under semi-aerobic condition by sparging the fermentation with an air and nitrogen mix at a ratio of 1:5. Samples were taken periodically from the fermentors until a stationary condition was obtained. Although the dissolved oxygen was not measured, we estimated that the maximum oxygen concentration in the fermentation broth was 1/5 lower than its oxygen concentration when the broth was in equilibrium with air. Thus, it was expected that the fermentation was in semi-aerobic condition.

The initial cell concentration and the initial pH of the fermentation were varied following a 2-factor full factorial experimental design. The best condition obtained with synthetic media was rerun on EFB hydrolysate. Every run was performed in duplicate.

2.3 Analysis

The samples were analyzed for biomass concentration with a spectrophotometer at 560 λm and by the gravimetric method. The fermentation broth was analyzed for residual glucose and xylose concentrations and the metabolic products, such as xylitol, ethanol, acetic acid and glycerol, by HPLC using a Bio-Rad Aminex HPX-87H column at 60 °C with 0.01 N H_2SO_4 as eluent at a flowrate of 0.6 mL/min. The peaks were detected using a refractive index detector (RID).

The capability of microorganisms to synthesize products was parameterized as the yield of product from substrate ($Y_{P/S}$) that was calculated following Eq. (1), in which P refers to products such as ethanol, xylitol, acetic acid, glycerol, or biomass, while S refers to the substrate concentration. Total substrate concentration, i.e. glucose and xylose, was used for the yield calculation, except for the yield of xylitol, which was assumed to be produced only from xylose and not from glucose.

$$Y_{P/S} = \frac{q_P}{-q_S} = \frac{C_P - C_P^0}{-\left(C_S - C_S^0\right)} \tag{1}$$

3 Results and Discussions

3.1 Effects of Initial pH and Initial Cell Concentration on Cell Growth

The growth profiles of *D. hansenii* on synthetic medium at various initial cell concentrations and initial pH values are presented in Figure 1. Cells were observed to grow well at initial cell concentration 2.10^7 cells/mL, whereas no significant cell growth was observed at initial cell concentration 6.10^7 cells/mL.

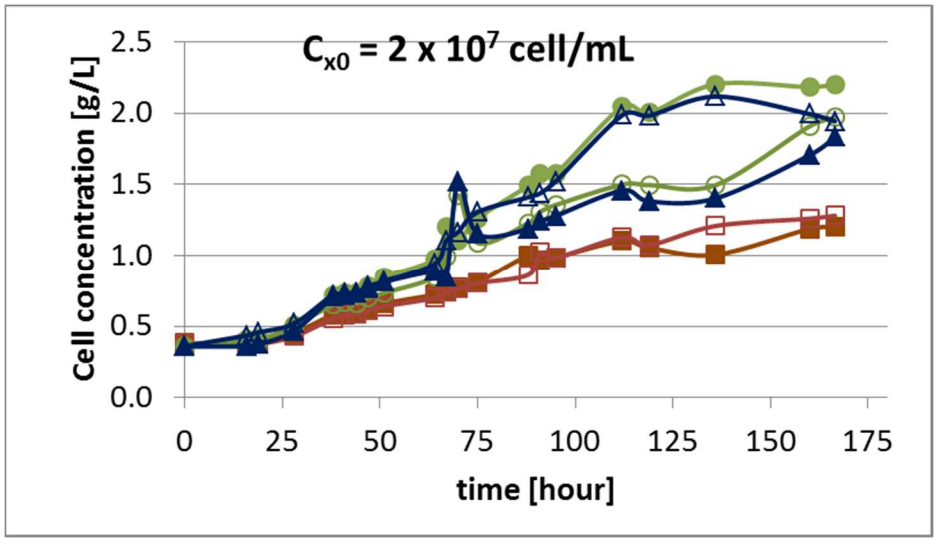

Figure 1 Growth profiles of *D. hansenii* at various initial cell concentrations (squares indicate initial pH = 4, circles indicate initial pH = 5, triangles indicate initial pH = 7, open and closed symbols represent the duplicate experiments).

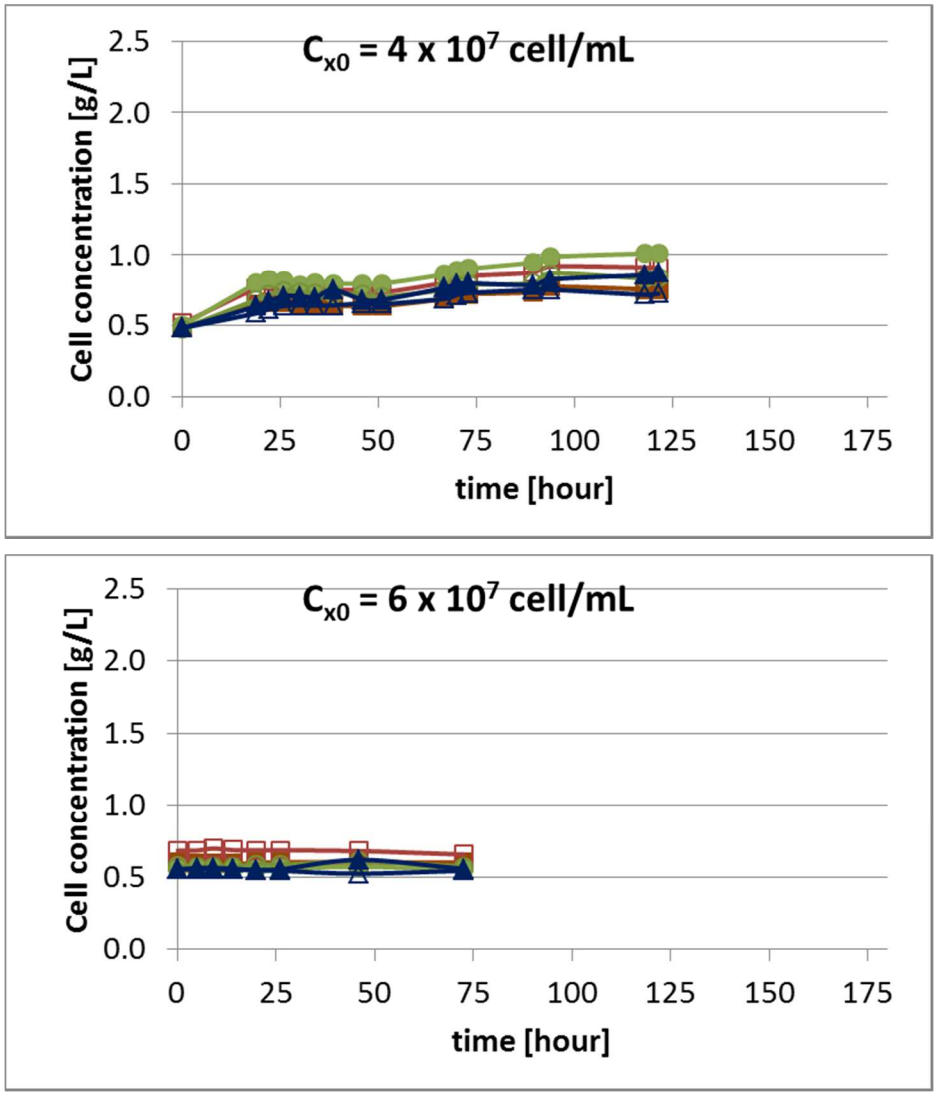

Figure 1 ***Continued***. Growth profiles of *D. hansenii* at various initial cell concentrations (squares indicate initial pH = 4, circles indicate initial pH = 5, triangles indicate initial pH = 7, open and closed symbols represent the duplicate experiments).

It was observed that an increase in initial cell concentration led to a decline in the specific growth rates. Limited available oxygen during the fermentation could be the cause of limited cell growth during the runs with a high initial cell concentration.

It was also observed that initial pH affected cell growth. At the same initial cell concentration, cells were observed to grow best at initial pH = 5 (Figure 1). Consistent observations were obtained from runs at initial cell concentrations of 2.10^7 cells/mL and 4.10^7 cells/mL.

The maximum cell growth was observed at an initial cell concentration of 2.10^7 cells/mL and an initial pH of 5 (Figure 1). It has been reported that optimum growth of *D. hansenii* is obtained at pH 5.5 [9]. The results obtained in this research thus showed a similar trend as those in the reference.

3.2 Effects of Initial Cell Concentration on Product Yield

Ethanol, xylitol, acetic acid, and glycerol were observed as the metabolic products of the fermentations. Neither xylitol nor ethanol was observed in the metabolic products of the fermentations with initial cell concentration 2.10^7 cells/mL. On the other hand, low concentrations of glycerol and acetic acid were observed at the end of these fermentations. Interestingly, negative values of yield were calculated for acetic acid (Table 1). This indicates that acetic acid was consumed during the fermentation. Its impact on the overall carbon balance, however, was further neglected. The low yields of metabolic products calculated for these fermentations correspond to the calculated high yield of biomass. Although the measured metabolic products and biomass in these experiments could only account for about 40% of the consumed substrate, the obtained results indicate that fermentation with low initial cell concentration is favored for biomass formation. A potential candidate for another carbon sink in this experiment would be CO_2 formation. However, this substance was not measured during the experiments.

More metabolic products were measured by the end of fermentation at higher initial cell concentrations, 4.10^7 cells/mL and 6.10^7 cells/mL. In addition, the measured metabolic product concentrations were observed to increase at higher initial cell concentration. Thereby, higher yields of metabolic products were calculated at higher initial cell concentrations. The yield of xylitol, for example, was calculated to be in the range of 0.055-0.102 (g xylitol/g xylose) at an initial cell concentration of 6.10^7 cells/mL, while they were calculated to be 0.006-0.059 (g xylitol/g xylose) at an initial cell concentration of 4.10^7 cells/mL. A similar trend was observed for the yields of the other metabolic products. In contrast, a lower yield of biomass was calculated at higher initial cell concentrations. This indicates that fermentation with high initial cell concentration is favored for metabolic product formation. Consistent with our previous observation, a limited oxygen concentration led to a fermentative metabolism for energy generation and an NADH recycling system, which also resulted in more metabolic product formation instead of cell growth.

The obtained results confirmed previous observations by Dominguez, *et al.*(1997) as reported by Parajó, *et al.* [10]. They reported that initial cell concentration affects the production of xylitol. An increase in xylitol productivity from 0.68 g/L.hr to 2.25 g/L.hr was observed along with the increase in initial cell concentration from 0.3 g/L to 3 g/L.

Interestingly, the carbon balances closed better at fermentation with high initial cell concentration. The carbon balances closed up to 40% at an initial cell concentration of 4 x 10^7 cells/mL, whereas the carbon balances closed up to 75% at an initial cell concentration of 6 x 10^7 cells/mL. A possible explanation for this may be that these fermentations produced metabolite products at a significant level that were measurable with the implemented analytical method, but at a low initial cell concentration they were produced at a very low level, i.e. below the threshold of the implemented analytical method.

Throughout these experiments we also found a hidden parameter that affected the fermentation performance, i.e. oxygen concentration. A limited oxygen concentration prevented cells from growing at a higher initial cell concentration, 2 x 10^7 cells/mL, or corresponding to less than 1 g dry weight/L, and thus opted for metabolic product formation through fermentative metabolism instead. Interestingly, we also observed that cells could still grow well until 2 g dry weight/L from the initial cell concentration of 2 x 10^7 cells/mL at a similar oxygen concentration. The aeration condition is an important question to be resolved for xylitol production via fermentation.

Table 1 Yield of metabolic products at various initial cell concentrations and pH values.

Initial cell concentration (cells/mL)	Initial pH	Yield of xylitol from xilose (g/g)	Yield of ethanol from substrate (g/g)	Yield of acetic acid from substrate (g/g)	Yield of glycerol from substrate (g/g)	Yield of biomass from substrate (g/g)
$2x10^7$	4	-	-	-	0.015 ± 0.001	0.261 ± 0.061
	5	-	-	-	-	0.390 ± 0.043
	7	-	-	-	0.002 ± 0.002	0.344 ± 0.009
$4x10^7$	4	0.006 ± 0.002	-	0.020 ± 0.020	-	0.137 ± 0.022
	5	0.059 ± 0.001	0.108 ± 0.010	0.103 ± 0.103	-	0.134 ± 0.017
	7	0.032 ± 0.001	0.046 ± 0.030	0.173 ± 0.065	0.081 ± 0.031	0.085 ± 0.005
$6x10^7$	4	0.074 ± 0.005	0.289 ± 0.008	0.106 ± 0.025	0.179 ± 0.009	-
	5	0.102 ± 0.007	0.372 ± 0.204	0.127 ± 0.072	0.164 ± 0.013	-
	7	0.055 ± 0.002	0.148 ± 0.081	0.406 ± 0.070	0.085 ± 0.057	-

3.3 Effects of Initial pH on Product Yield

Table 1 also shows that the initial pH of fermentation affected the product yield. The highest yields for most metabolic products were obtained at an initial pH of 5. At initial cell concentration 4.10^7 cells/mL, for example, the yield of xylitol was calculated to be 0.059 (g xylitol/g xylose) at initial pH 5, whereas it was calculated to be 0.006 and 0.032 (g xylitol/g xylose) at initial pH 4 and 7, respectively. Similar trends were observed for ethanol and glycerol. In contrast, the highest yield for acetic acid was observed at the highest pH, i.e. pH 7. It was observed that the yield of acetic acid increased as the initial pH increased.

It is also important to note that although xylitol was produced in these experiments, other metabolic products such as ethanol, acetic acid, and glycerol were also significantly produced at an even higher concentration than xylitol. This calls for a proper design of the downstream processes when this fermentation method is applied for xylitol production and also a proper design for strain improvement, so the yield of xylitol can be increased and the yield of other metabolic products can be lowered.

3.4 Comparison between Fermentations using Synthetic Medium and EFB Hydrolysate

EFB hydrolysate may contain various substances, including potential inhibitors of yeast growth and xylitol production. Acid hydrolysis of EFB, for example, produces acetic acid and furfural that have been reported to inhibit the subsequent fermentation process [4]. Some studies have been reported on xylitol fermentation using EFB hydrolysate obtained from acid hydrolysis of EFB [11-12]. These reports showed the need of a detoxification process before the EFB hydrolysate can be used as the substrate for xylitol fermentation. Enzymatic hydrolysis of EFB is normally conducted at milder conditions [7] and thus is expected to produce less compounds that are harmful for fermentation. The EFB hydrolysate obtained from enzymatic hydrolysis was used in further experiments to find out whether the enzymatic hydrolysis process also produces substances that potentially inhibit the subsequent fermentation process.

The experiments using EFB hydrolysate were conducted at initial pH 5 and initial cell concentration 4.10^7 cells/ml. This condition is not the optimum condition for xylitol production obtained in the previous section using synthetic media, i.e. at initial pH 5 and initial cell concentration 6.10^7 cells/mL, which yielded 0.102 g xylitol/g xylose (Table 1). However, at this condition we could explore the effects of using EFB hydrolysate on both cell growth and xylitol formation. Although the initial sugar concentration in the synthetic media and

the EFB hydrolysate were not exactly the same due to some technical problems in the enzymatic hydrolysis process, the overall fermentation performances were compared. The obtained results are presented in Table 2.

It was observed that the enzymatic hydrolysis of EFB also produced a significant amount of acetic acid, the level of which, after dilution, was indicated as the concentration of acetic acid at the beginning of the fermentation process (see Table 2). The presence of acetic acid was also observed at the beginning of the fermentation process using synthetic media, albeit at a much lower concentration. This indicates that acetic acid may also have been produced during the preparation of the yeast inoculum. It was observed that the increase in acetic acid at the beginning of the fermentation process did not lead to inhibition of yeast growth nor inhibition of xylitol production. Although a slower specific growth rate was calculated for fermentation with EFB hydrolysate, the obtained biomass and xylitol yields were significantly higher.

Table 2 Comparison of fermentation performance between fermentation using synthetic media and fermentation using EFB Hydrolysate.

Information	Fermentation medium	
	Synthetic Medium	**EFB hydrolysate**
Initial xylose concentration (g/L)	5.07 ± 0.20	2.16 ± 0.04
Final xylose concentration (g/L)	2.66 ± 0.20	0.90 ± 0.37
Xylose conversion (%)	47.6 ± 6	58.4 ± 16.6
Initial glucose concentration (g/L)	1.05 ± 0.31	0.56 ± 0.05
Final glucose concentration (g/L)	0.09 ± 0.09	0.47 ± 0.02
Glucose conversion (%)	91.6 ± 11.8	17.1 + 3.5
μ (1/hr)	0.0082 ± 0.001	0.0058 ± 0.000
Biomass yield from substrate (g/g)	0.13 ± 0.017	0.27 ± 0.09
Final xylitol concentration (g/L)	0.14 ± 0.03	0.11 ± 0.00
Xylitol yield from xilose (g/g)	0.059 ± 0.001	0.098 ± 0.029
Initial ethanol concentration (g/L)	0.00 ± 0.00	0.00 ± 0.00
Final ethanol concentration (g/L)	0.37 ± 0.12	0.00 ± 0.00
Ethanol yield from substrate (g/g)	0.108 ± 0.010	0.000 ± 0.000
Initial acetic acid concentration (g/L)	0.14 ± 0.14	1.51 ± 0.21
Final acetic acid concentration (g/L)	0.41 ± 0.41	0.69 ± 0.11
Acetic acid yield from substrate (g/g)	0.103 ± 0.103	-0.692 ± 0.391
Initial glycerol concentration (g/L)	0.00 ± 0.00	0.00 ± 0.00
Final glycerol concentration (g/L)	0.00 ± 0.00	0.00 ± 0.00
Glycerol yield from substrate (g/g)	0.000 ± 0.000	0.000 ± 0.000

The high initial concentration of acetic acid in the fermentation with EFB hydrolysate even led to acetic acid consumption as indicated by the negative value of the acetic acid yield (Table 2). Interestingly, no significant concentration of ethanol was observed in the fermentation with EFB

hydrolysate. It is suspected that the low glucose concentration in the media containing the EFB hydrolysate prevented the synthesis of ethanol and directed the metabolism more towards the utilization of xylose and correspondingly xylitol formation. Overall, the obtained results confirmed that there were no inhibitory substances in the EFB hydrolysate that would affect yeast growth or xylitol production. The obtained results showed that EFB hydrolysate obtained from enzymatic hydrolysis of EFB can be used directly as substrate for xylitol fermentation.

4 Conclusion

This research showed that both initial cell concentration and pH affect the fermentation performance of *D. hansenii* in producing xylitol. It was found that at pH 5 cells grew optimally and also gave the highest yield of xylitol. On the other hand, it was also found that a high initial cell concentration led to slow biomass growth and a low biomass yield, however, this condition also gave a high yield of xylitol. The results confirmed that there are no fermentation inhibitory compounds in oil palm EFB hydrolysate obtained from enzymatic hydrolysis of EFB that affect the activity of *D. hansenii* yeast, in particular for xylitol production. Thereby it can be used directly as the substrate for xylitol fermentation.

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