Microbial Production of Xylitol from Oil Palm Empty Fruit Bunches Hydrolysate: The Effect of Glucose Concentration

Efri MARDAWATI※1※2, Dwi Wahyudha WIRA※2, MTAP KRESNOWATI※1, Ronny PURWADI※1, and Tjandra SETIADI※1

(Received November 18, 2014)

Xylitol has beneficial health properties and can be found in nature albeit in small quantities. In commercial industries, xylitol is produced via chemical hydrogenation of xylose. This process, however, requires high purity of xylose as the raw material. Biotechnological process offers an alternative xylitol production process, using the hydrolysate of lignocellulosic material such as the agricultural waste oil palm empty fruit bunches (OPEFB) as raw material. This substances may contain glucose beside xylose. The presence of glucose as cosubstrate, in the fermentation medium is also a critical factor that regulates the xylitol production by yeasts. Glucose may repress the activity of the key xylose reductase enzyme involved in the xylose conversion into xylitol resulting in low yields of the product.

The purpose of this study was to explore the ability of microorganism to produce xylitol from OPEFB hydrolysate. This paper describes the effect of glucose as the co-substrate in xylitol production by Debaryomyces hansenii ITBCC R85 and further the use of OPEFB hydrolyasate as substrate in xylitol production. This research showed that addition of co-substrate glucose affected the fermentation performance of D. hansenii in producing xylitol. Glucose concentration of 2.5 g/L or concentration ratio of glucose to xylose of 25 % gave the highest yield of xylitol. The fermentation using OPEFB hydrolysate containing glucose to xylose ratio more than 25 % gave lower xylitol yield, addressing the hydrolysis of OPEFB to be optimized further.

Key Words
Fermentation, hydrolysate, Oil palm empty fruit bunches (OPEFB), Xylitol.

1. Introduction
Xylitol is a polyalcoholic sugar with five carbon atoms which is found naturally in fruits and vegetables albeit in small quantity. Xylitol has similar sweetness to sucrose, but it has lower calorie. Xylitol metabolism in the human is non-insulin mediated pathway, which makes the best sweetener substitute for the diabetics. It can be widely used in the drinking juice, coffee, milk, and bread.

Xylitol has no carbonyl group and thereby does not lead to maillard reaction when heated, which is interesting property for bakery products. In addition, xylitol has a strongly negative heat of solution which can give a cooling sensation. Another xylitol advantage is its anti-cariogenic property. This property, along with xylitol’s ability to pertain mixture moisture explains why xylitol has an important role as a toothpaste constituent.

In the world market, the demand for xylitol always increases along with the increasing utilization of xylitol in chemical, pharmaceutical and food industries. According to price trends in 2012, food grade xylitol is valued US $ 3.3-3.5/ kg and internet survey shows that China is the main xylitol producer in the world.

There are two methods of xylitol production. Xylitol can be obtained through solid-liquid extraction from fruits and vegetables such as lettuce, cauliflower, yellow plum, raspberry, strawberry, grape, and banana. However, the xylitol content in natural sources is very low (below 900 mg-xylitol/100 g-material), making the process not economically attractive.

Xylose reduction, or also known as hydrogenation,
can be executed either through chemical or biological processes \(^1\). Xylose reduction through chemical process is carried out by contacting liquid pure xylose with hydrogen gas, using noble metal-based catalysts, such as platinum, palladium, ruthenium, and nickel. This process is executed at non-atmospheric conditions, for example at temperature of 120-200 °C and hydrogen partial pressure of 2 MPa (20 bar) \(^3\)\(^4\).

Alternatively, xylose reduction can be executed through biological process, such as fermentation. Some yeast strains were reported to convert xylose to D-xylulose through oxido-reductive pathway, which consists of two consecutive reactions. In the first reaction, XR (xylose reductase) transforms D-xylose into xylitol using NADH and/or NADPH as cofactor. In the second one, XDH (xylitol dehydrogenase), which use NAD\(^+\) or NADP\(^+\) cofactor, converts xylitol into D-xylulose. Further D-xylulose can be either converted into ethanol through Embden Meyerhof Parnas or recovered into xylitol, depending on the utilized yeast strain. Consequently, xylose utilizing yeasts mostly accumulate xylitol, ethanol, or both, in the system \(^3\).

The advantage of the xylose reduction through biological conversion is the possibility of using not pure substances, such as hydrolysate as the raw material. Hemicellulose can be found in the lignocellulosic materials, which is available in huge quantity in nature. Utilization of lignocellulosic waste materials to value added product like xylitol is low cost, and renewable. The abundance of oil palm lignocellulosic waste, waste from crude palm oil industry may serve as a potential raw material. In the processing of crude palm oil approximately 20-22 % of OPEFB is generated as a waste product from each fresh fruit bunches processed \(^7\).

The OPEFB biomass comprises of cellulose (32-43 %), hemicellulose (23-25 %) and lignin (11-23 %) and other extractive \(^8\)\(^9\). Currently, many researches and development efforts were conducted to improve the saccharification of cellulose in lignocellulosic feedstocks to be further converted into ethanol. The potentials of hemicellulose and lignin are overlooked. Hemicellulose, which is a branched polysaccharide consisting of the pentoses (D-xylose and L-arabinose) and hexoses (D-galactose, D-glucose, and D-mannose) may be hydrolyzed to produce xylose for the production of xylitol.

Steps involved in the production of xylitol from OPEFB are the hydrolysis of hemicellulose to xylose, and reduction of xylose into xylitol. The hydrolysis process can be performed chemically, at high pressure and temperature using acid or alkaline as the catalyst or at ambient condition using biological xylanolytic enzyme as the catalyst \(^1\).

The OPEFB hydrolysate also contains other sugars including glucose. Therefore before utilizing OPEFB hydrolysate as substrate for xylitol fermentation, the effect of co-substrate glucose in the fermentation needs to be studied further. It is the ultimate goal of this research to study the utilization of hemicellulosic component of OPEFB to be converted to xylitol via biological processes. This paper focuses on studying the effect of glucose as the co-substrate for xylitol production by D.hansenii ITBCC R85 fermentation. Further, the results were validated by fermentation using OPEFB hydrolysate as substrate.

### 2. Experimental

#### 2.1 Raw materials

EFB was collected from palm oil mill in West Sumatera, Indonesia. Before used, the EFB was sun dried, cleaned, oven dried at 105 °C overnight and grinded. Only EFB of small size, max 80 mesh were used. The EFB composition has been previously determined to be 43.32 % cellulose, 23.67 % hemicellulose and 22.1 % lignin \(^8\)\(^9\).

#### 2.2 Production of crude xylanase

Xylanase was produced from solid state fungal fermentation using OPEFB as was reported previously \(^8\). The cultivations of Trichoderma viride ITB CC L.67 for xylanase production was conducted on optimum condition for the production of xylanase: 33 °C, substrate solid to liquid ratio 0.63 g-OPEFB/mL-(liquid medium) and 36 h cultivation time \(^8\). The crude enzyme extract was obtained by separating the solid (left over EFB and fungal) from enzyme solution by centrifugation.

#### 2.3 Enzymatic hydrolysis of OPEFB

The hydrolysate was produced from enzymatic hydrolysis of OPEFB using crude enzyme extract at optimum condition as was described previously: 45 °C and pH 4.7 \(^8\). The hydrolysate was obtained by separating the solid containing OPEFB waste from the solution by vacuum filtration and centrifugation.

#### 2.4 Fermentation

The yeast strain used in this study, D. hansenii ITB CCR85 was obtained from Microbiology and Bioprocess Technology Laboratory of Chemical Engineering ITB. The yeast was grown in glucose yeast extract agar (GYE) for 3 d at 30°C before further used.

To study the effect of cosubstrate glucose, fermentations were performed in shake flasks, with working volume of 500 mL on fermentation media as previously described \(^12\), except for the carbon source.
The synthetic media contain 10 g/L xylose whereas the glucose concentrations was varied to be 0, 2.5, and 5 g/L. Throughout the experiments the fermentation was temperature controlled at 30 °C and at semiaerobic condition by sparging with air and nitrogen mix at 1:5 ratios. Samples were taken periodically from the fermentors until the stationary condition was obtained.

Fermentation process of OPEFB hydrolysate was carried out in a 1.3 L Bioflow 115 (New Brunswick) bioreactor. The bioreactor is equipped with pH, temperature, level, dissolved oxygen (DO), foam and mixing control system. Batch fermentation was executed in 700 mL working volume and mixed at 450 rpm at pH 5 and temperature 30 °C. Inoculum used was 10 % of working volume, which was prepared using 15 g/L xylose.

The culture was sampled over time during the exponential growth and the stationary phases. The aeration condition was set as semiaerobic or limited oxygen condition by maintaining aeration level at 0.5 m3/(m3 min) during the fermentation process.

2.5 Analysis

Sample were analysed for glucose, xylose, biomass, xylitol and ethanol. Cell concentration was analysed by turbidometric-gravimetric method using UV-Vis spectrophotometer. The remaining xylose and glucose in the substrate, and the produced xylitol and ethanol concentrations were determined using high performance liquid chromatography (HPLC) with a type BioRad Aminex HPX-87H column and using 5 mM H2SO4 as eluent 9).

2.6 Data Interpretation

Growth of microorganisms was parameterised as the specific growth rate that was calculated from biomass concentration data during the logarithmic phase, following Eq (1).

\[
\frac{dX}{dt} = \mu X
\]  

In which \( \mu \) is the specific growth rate (h⁻¹).

Performance of the fermentation was parameterised by yield which is described as the ratio of formed product to used substrate and productivity following Eqs. (2) – (4).

\[
Y_{P,S} = \frac{(P - P_0)}{(S - S_0)} \quad (2)
\]

\[
Y_{X,S} = \frac{(P - P_0)}{(X - X_0)} \quad (3)
\]

\[
Q_p = \frac{P - P_0}{t} \quad (4)
\]

where:

\( P \) = xylitol product concentration (g/L)

\( t \) = total fermentation time (h)

3. Results and Discussion

3.1 Effect of co-substrate glucose on growth

In this set of experiments we varied the concentration of glucose as co-substrate while the initial concentration of main substrate xylose, was kept constant at 10 g/L. Overall, the effect of cosubstrate on the growth of \( D. hansenii \) are presented in Fig. 1.

Fig. 1 shows that cells grow well at initial glucose concentration of 5 g/L. Lower growth was observed at glucose concentration of 2.5 g/L, whereas no significant cell growth was observed with no glucose. Further, the specific growth rates at the glucose concentration of 2.5 and 5 g/L were calculated to be 0.055 h⁻¹, and 0.065 h⁻¹. This result indicated that the concentration of glucose as co-substrate significantly affected the specific growth rate. In general, the higher cosubstrate concentration resulted in the higher specific growth rate. It shows that glucose is needed for yeast growth.

![Fig. 1 Growth profile of yeast during the fermentation on 10 g/L xylose and varied glucose concentration](image-url)
3.2 Effect of cosubstrate glucose on xylitol formation and substrate utilization

Xylitol, ethanol and biomass were observed as the products of the fermentations as shown in Table 1. The yields were calculated from metabolic products, biomass, and substrate concentration profiles using equations (2)-(4) under assumption that xylitol can only be synthesized from xylose, whereas other products, which are ethanol and biomass, can be synthesized either from glucose or xylose.

It is interesting to ask whether both glucose and xylose were actually used during the fermentations. The residual glucose concentration at the end of \textit{D. hansenii} fermentation were found to be 0 g/L, which shows that glucose were fully consumed during the fermentations. On the other hand, not all xylose were consumed during the fermentations. At the end of \textit{D. hansenii} fermentations, the xylose utilization were found to be 7 \%, 28 \% and 49 \% (Table 1). The more glucose, the more xylose is utilized. Xylose was used for xylitol and biomass production, meanwhile glucose was used for ethanol and biomass production\(^3\).

Table 1 shows that neither xylitol nor ethanol was observed in the fermentations without co-substrate glucose addition. This indicates that xylose as the sole carbon source was only used for produced biomass, not adequate to produce metabolic products.

Table 1 also shows that the highest yield were obtained at the co-substrate concentration of 2.5 g/L that is \(Y_{x5}\) of 0.637 g xylitol/g xylose, and \(Y_{x5}\) of 0.011 g-ethanol/g-substrate. On the contrary, the yield of xylitol and ethanol decreased at the co-substrate concentration 5 g/L; \(Y_{x5}\) of 0.106 g/g and \(Y_{x5}\) of 0.08 g/g. This indicates that there is an optimum initial cosubstrate glucose concentration for xylitol production by yeast. The optimal concentration ratio of glucose to xylose concentration observed in this research was 25 \% (2.5 g/L of glucose and 10 g/L of xylose). Above the optimal ratio of glucose to xylose concentration, glucose may induce inhibition, decrease of the xylitol yield such as was reported in the literatures. The presence of hexoses, such as glucose, in the fermentation medium is a critical factor that regulates xylitol production by yeasts\(^3\).

Other research reported that xylitol fermentation using \textit{C. tropicalis}, the optimum ratio of glucose and xylose was 20 \%, while higher ratio may lead to a decrease in xylitol produced. The maximum xylitol volumetric productivity generated was 3.98 g/(L h) on the condition of 300 g/L xylose and glucose substrate 60 g/L (ratio of glucose and xylose was 20 \%)\(^1\).

The presence of glucose may repress the activity of the key xylose reductase enzyme involved in the xylose conversion into xylitol resulting in low yields of the product. On the other hand, at low concentrations, glucose is needed for the growth of yeast\(^1\) whereby xylose can be optimally converted into xylitol. Based on these results, it can be concluded that the presence of glucose content in OPEFB hydrolysate is required for yeast growth but the concentration must not be too high. It can cause a reduction in the formation of xylitol. This should be considered in utilizing OPEFB as xylitol fermentation substrate further.

3.3 Production of xylitol from EFB hydrolysate

The fermentation experiments using OPEFB hydrolysate was carried out for 96 h with two replicates. Growth profiles \textit{D. hansenii} in OPEFB hydrolysate is presented in Fig. 2. The lag phase was observed for 14 h, followed by the logarithmic phase until the fermentation time of 34 h. To calculate the specific growth rate, data along the logarithmic phase were used. The value of $\mu$

| Parameter | Glucose concentration (g/L) |
|-----------|----------------------------|
|           | 0                          | 2.5                          | 5               |
| $\mu$ (h\(^{-1}\)) | 0                           | 0.055                        | 0.065           |
| $Y_{x5}$ (g/g) | 0                           | 0.637 ± 0.390               | 0.106 ± 0.006   |
| $Y_{x5}$ (g/g) | 0                           | 10.16 ± 3.600               | 2.34 ± 0.15     |
| $Y_{x5}$ (g/g) | 0.047 ± 0.02                | 0.039 ± 0.010               | 0.029 ± 0.00    |
| $Y_{x5}$ (g/g) | 0.011 ± 0.020               | 0.008 ± 0.0009              |                |
| $Q_{x}$ (g/L h)\(^*\) | 0                           | 0.021 ± 0                   | 0.007 ± 0       |
| $Q_{x}$ (g/L h)\(^*\) | 0.006 ± 0.001               | 0.008 ± 0.001               | 0.008 ± 0.002   |
| $Q_{x}$ (g/L h)\(^*\) | 0.008 ± 0.001               | 0.040 ± 0.020               | 0.064 ± 0.008   |
| Xylose utilization (%) | 7 ± 2                       | 28 ± 14                     | 49 ± 11         |
| Glucose utilization (%) | 0                           | 100                         | 100             |

* $Y_{x5}$ = yield of ethanol from substrate
* $Y_{x5}$ = yield of biomass from substrate

![Fig. 2 Profile of substrate and products on xylitol fermentation from EFB hydrolysate](image-url)
was 0.045 h⁻¹, which is in range with that obtained from the fermentations with synthetic media which is 0-0.065 h⁻¹ (Table 1). It showed that yeast can grow well on the substrate OPEFB hydrolysate. This suggests that the hydrolysate provided sufficient carbon source for yeast growth. An overview on metabolic products produced during the fermentation is given in Table 2 and Fig. 2.

The fermentation was started with 19.6 g/L xylose and 6.8 g/L glucose mostly obtained from OPEFB. These sugars were used for yeast growth, biomass production and the metabolic products as well as the emissions released in the form of CO₂. By the end of fermentation experiment, 96 h, an average of 3.088 g/L xylitol was measured from 66 % xylose utilization, giving xylitol yield of 0.24 g-xylitol/g-xylose and xylitol volumetric productivity of 0.03 g/(L h). Fig. 2 shows that xylitol is produced during the logarithmic and stationary phase and thereby can be said to be mixed growth associated product.

The ratio of glucose and xylose concentration in OPEFB hydrolysate was 34 %, which was higher than the optimum co-substrate glucose to xylose ratio obtained previously, 25 %. It might be the presence of high glucose in the medium that inhibited the xylitol formation. Thereby lower xylitol yield was observed. The enzymatic hydrolysis process needs to optimize further in order to decrease the glucose contain in the hydrolysate. On the other hand the final xylitol concentration can still be increased by increasing the xylose utilization, for example by extending fermentation time.

When compared with similar studies, fermentation by D. hansenii from hydrolysated of sawdust produced about 0.5 to 9 g/L of xylitol from 18 g/L of xylose, giving a maximum yield of 0.79 g/g and xylitol productivity of 0.03 g/(L h) ¹⁰. Other researchers reported that xylitol fermentation of eucalyptus hydrolysates using yeast C. guilliermondii produced 19 g/L of xylitol, giving product yield of 0.2 g-xylitol/g-xylose and xylitol productivity of 0.1 g/(L h) ¹⁰. In this study high xylitol concentration was achieved by adding 60 g/L of synthetic xylose in the beginning of fermentation. The above literatures reported fermentation hydrolysate obtained from acid hydrolysis process. Literature also reported xylitol production from hydrolysate obtained from enzymatic hydrolysis of beechwood and walnut shell gave xylitol concentration of 2-3.5 g/L giving the xylitol yield of 0.5 g/g within a 72 h fermentation using yeast C. tropicalis ¹⁷. The result obtained in this study show a lower yield, despite similar xylitol concentration.

4. Conclusion

This research showed that the addition of glucose as co-substrate affected the fermentation performance of D. hansenii in producing xylitol. This research found that glucose concentration of 2.5 g/L or concentration ratio of glucose to xylose of 25 % gave the highest yield of xylitol. The high concentration ratio of glucose to xylose in OPEFB hydrolysate resulted in low xylitol yield and xylose utilization.

The result showed that OPEFB hydrolysate can be used as substrate for xylitol production via fermentation. The yeast can grow well, however the yield of xylitol is still low. The xylitol fermentation from OPEFB hydrolysate, giving xylitol concentration of 3.088 g/L and the yield was 0.24 g-xylitol/g- xylose. Xylitol was produced in the logaritmic and stationary phase so that it was categorized as mix growth associated product.

Acknowledgment

This research was funded by Directorate of Higher Education, Indonesian Ministry of National Education (DIKTI) under the scheme of National Strategic Research “The Production of ‘Green’ Xylitol: Integrated Convertion of Biomass Waste of Palm Oil into Bioethanol and Valuable Chemicals”.

References

1) Parajo, J. C.; Dominguez, H.; Dominguez, J. M., Bioresource Technology, 65, 191-201(1998)
2) Tochampa, W.; Sirisansaneeyakul, S.; Vanichsriratan, W; Srinophakun, P.; Bakker, H. H.; Chisti, Y., Bioprocess Biosyst Eng, 28(3), 175-83 (2005)
3) Sampaio, F. C.; Torre, P.; Passos, F. M. L.; Moraes, C. A.; Perego, P.; Converti, A., Applied Biochemistry and Biotechnology, 136, 165-181(2003)
4) http://Alibaba.com, (Last access: 2014.11.2)
5) Baudel, H.; Ahre, C.; Zaror, C., Journal of Chemical Technology & Biotechnology, 80(2), 230-233 (2005)
6) Millati, R.; Wikandari, R.; Trihandayani, E.; Cahyanto, M.; Taherzadeh, M.; Niklasson, C., *Agricultural Journal*, 6, 54-59 (2011)

7) Visvanathan, C.; Setiadi, T.; Herarth, G.; Shi, H., Eco-Industrial Clusters in Urban-Rural Fringe Areas, Asian Institute of Technology, Thailand, (2009)

8) Mardawati, E.; Stephanie, Arlene, Kresnowati, MTAP.; Setiadi, T., Proceeding, International Seminar on Biorenewable Resources Utilization for Energy and Chemicals, Bandung, Indonesia (2013)

9) Mardawati, E.; Kresnowati, MTAP.; Purwadi, R.; Setiadi, T., Proceeding, International Seminar on Mineral and Materials Processing, Bandung, Indonesia (2014)

10) Mardawati, E.; Werner, A.; Bley, T.; Kresnowati, MTAP.; Setiadi, T., *J. Jpn. Inst. Energy*, 93, 973-978 (2014)

11) Kresnowati, MTAP.; Ardina, B.; Oetomo.V. P., Proceeding 19th Regional Symposium of Chemical Engineering, Bali, Indonesia (2012)

12) Nobre, A.; Duarte, L. C.; Girio, F. M., *Appl Microbiol Biotechnol*, 59(4-5), 509-516 (2002)

13) Sillva, S. S.; Felipe, M.; Mancilha, I. M., *Applied Biochemistry and Biotechnology*, 70-72, 331-339 (1998)

14) Oh, D. K.; Kim, S. Y., *Appl Microbiol Biotechnol*, 50, 419-425 (1998)

15) Parajo, J. C.; Dominguez, H.; Dominguez H. M., *Food Chemistry*, 57(4), 531-536 (1996)

16) Canettieri, E. V.; Joao, B.; Almeida, E.; Silva, Maria, G.; Felipe, A., *Applied Biochemistry and Biotechnology*, 94, 159-168 (2001)

17) Tran, L. H.; Yogo, M.; Ojima, H.; Idota, O.; Kawai, K.; Suzuki, T.; Takamizawa, K., *Biotecnology and Bioprocess Engineering*, 9, 223-228 (2004)