Xylitol Production from *Aspergillus niger* using Empty Fruit Bunches (EFBs) as a Substrate by Submerged Fermentation

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Abstract. Xylitol is a sugar alcohol that has five carbon chains and is one of the commercial products whose use is quite high in Indonesia, but its availability is still low. Xylitol is widely used in food, pharmaceutical, and cosmetic industries. The production of xylitol can be carried out through fermentation using microorganisms and pure xylose as a substrate, but the use of pure xylose is quite expensive so it will increase production costs. Xylose can be replaced by hydrolysate xylose from agricultural waste. Empty Fruit Bunches (EFBs) is one of the agricultural wastes which has a high hemicellulose content, which is 35%. The high hemicellulose content from EFBs can be used as a substrate in the production of xylitol by submerged fermentation. This research was conducted to determine the effect of variations substrate concentration on xylitol production, evaluate the effect of *Aspergillus niger* growth rate on xylitol production, and determine the concentration of xylitol produced during the fermentation process. The concentration variations of the substrate were 14.28%, 28.57%, and 42.85%. The result of this research indicated that the substrate concentration increase led to increasing cell concentration, cell growth rate, xylitol concentration, and xylitol production rate. The highest substrate concentration resulted in a cell concentration of 0.0078 g/ml, a cell growth rate of 0.00014 cells/hour, a xylitol concentration of 189.00 mg/ml, and a xylitol production rate of 2.861 mg/hour.

Keywords: *Aspergillus niger*, xylitol, empty fruit bunches, submerged fermentation

1. Introduction

Xylitol is a sugar alcohol consisting of five carbon atoms, which is commonly found in fruits and vegetables such as bananas, strawberries, apples, yellow plums, carrots, cauliflower, lettuce, and onions. Xylitol can be used in various industries, especially food and pharmaceutical industries [1,2]. The global xylitol market size by International Mining and Resources Conference (IMARC) expects increase the market up to 33.26% in during 2023-2028, exhibiting a Compounded Annual Growth Rate (CAGR) of 4.8% [3]. The demand for the xylitol in Indonesia is up to 850 tons per year and it is met mostly by imports. The potential production of xylitol from lignocellulose [4].

The xylitol production is available by chemical and biological process. The chemical processes require not only high energy and cost, but also produce high emission because of the use of pure xylose, metal as catalysts, high pressure and temperature for the production and purification process [5]. Therefore, scientists developed the process of xylitol production through fermentation methods with the aim of lowered cost, low energy requirement, and more environmentally friendly process using biomass

as substrate [6]. Biomass is one source of a renewable energy from agricultural residues. In Indonesia the potential production of xylitol production from lignocellulose which can be approximately 1.4 million tons per year has more than covered the need for xylitol in Indonesia, but it is limited by scale of production and economics, so this needs to be further developed and studied, especially in the utilization of the bioproduct [4].

The utilization of biomass has been widely applied, such as in the production of energy and chemical. In the production of energy, biomass can used as substrate to produce of biogas, syngas, bio-pellet [4], biofuels [7,8], biochemical, such as ethanol [9] and xylitol [2,10,11], bio-oil [12], and biomaterials [13]. Dasgupta [11] analyzed the material and energy consumption flows in xylitol production through microbial fermentation using corncobs as biomass with software application. The result showed that 0.502 kg of xylitol crystals could be generated from 3.5 kg of corncob biomass with the evaporators as the primary consumers of energy, which was almost 51.7%. The heat integration process could reduce the overall energy demand by 5.802 kW, which equals a reduction in greenhouse gas emissions of almost 14%. Using membrane-based systems (to concentratefeed) can be an alternative approach that may potentially result in both energy savings and lower emissions. Galan [10] informs that an increase in lignin content implies higher energy consumption in pretreatment and fermentation processes. Higher energy consumption leads to increased emissions. Thereverse engineering of biomass that reduces lignin will decrease investment cost until 0.198% and production costs up to 10.71%, and increase xylitol production until 58.96%.

The production of xylitol through fermentation method can utilize xylan which is the main composition of hemicellulose and is easily found in agricultural waste biomass. One of the agricultural biomass that has the potential to be utilized is the Empty Fruit Bunches (EFBs). A total of 51 million tons of EFBs are produced as biomass residues from the palm oil industry. EFBs consistingof 45.95% cellulose, hemicellulose 22.84%, and lignin 16.49% [12]. Hemicellulose is the component tobe used as the main raw material in the manufacture of xylitol because the monomeric sugar utilized forxylitol production is xylose obtained from hemicellulose degradation [14]. Production by submerged fermentation method is most often to production of xylitol [15,16].

Several studies have reported that xylitol production by submerged fermentation. Oh [17] informs that a concentration of xylitol using *Candida* of 3.98 g/L and 0.38 g/L using *Escherichia coli* recombinant [18]. In both studies, xylose was used as substrate. The other study has reported that a concentration of xylitol of 3.08 g/L using *Debaryomyces hansenii* ITB CC R85 and EFBs as substrate [19]. Based on the studies, xylitol can be produced by microorganisms, including fungi and bacteria. Fungi that are widely used in industry are the filamentous fungi, such as *Aspergillus oryzae*, *Trichoderma reesei*, and *Aspergillus niger*, because they produce xylanolytic enzymes that can degrade xylan [20]. Several studies have reported that the concentration of xylitol from *A. oryzae* dan *Trichoderma reesei* is lower [21,22]. Meanwhile, *A. niger* is a highly promising microorganism to construct strains for direct xylitol production from lignocellulosic biomass by metabolic engineering because *A. niger* can convert monosaccharides from biomass into energy and biomolecules [23,24]. Based on the previously mentioned research, it is important to observe the effect of substrate concentration variations on xylitol production, evaluate the effect of *A. niger* growth rate on xylitol production during the fermentation process, and determine the xylitol concentration resulting from the EFBs substrate fermentation process by *A. niger* using submerged fermentation with EFBs substrate. The purpose of this study was to evaluate the effect of substrate concentration variations on the growth rate of cell and the rate of xylitol production during the fermentation process produced by *A. niger* through submerged fermentation using EFBs as substrate.

2. Metodology

2.1. Preparation of substrate

The EFBs that used in this research was obtained from PTPN IV, Bah Jambi, North of Sumatera. The preparation procedure followed the procedures in the research of Mardawati [14]. EFBs were soaked for 24 hours, the fiber was collected, and then cleaned using distilled water to remove impurities. EFBswas dried for 5 hours in the oven at a temperature of 60°C. The pretreatment was carried out by mechanical method as the dried material was chopped into smaller particles and then sieved to 60 meshsize. In this study, 10 g of EFBs was blended with 4% sulfuric acid solution. Hydrolyzing was done using an autoclave at 121°C for 15 minutes. After that, the solution was cooled to room temperature andthen separated into two fractions. The hydrolysate was adjusted to the pH of 5.0 by applying 2 M NaOH.After reaching the pH level of 5, activated carbon was added 15 g/L and stirred for 60 minutes at 30°C.Then the activated carbon was separated by filtering it to get the purified hydrolysate. The purified hydrolysate was used for fermentation.

2.2. Preparation of inoculum

Inoculum in this study was purchased from The School of Life Sciences and Technology (SITH) ITB . *A. niger* cultures were cultivated into 400 mL of Yeast Peptone Glycerol (YPG) with the following ratio of 1:2:3 %. Then, the cultures were incubated at 37°C. The incubation time was controlled according to the cell growth time in exponential phase, which was at 30hours with a cell concentration of 0.0044 g/mL [25].

2.3. Preparation of xylitol standard curve

The xylitol standard curve was used to measure xylitol concentration and xylitol production rate during fermentation time. The solution of xylitol with five concentrations, then was measured for its absorbance at 492 nm. The linear regression equation applied was $y = 0.002x$, $R^2 = 0.996$ [26].

2.4. Preparation of fermentation media

The composition of the fermentation media per liter was 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 $MgSO_4$, and 200 µl trace element. The composition of the trace element per liter was 10 g EDTA, 4.4 g $ZnSO_4.7H_2O$, 1.01 g $MnCl_2.4H_2O$, 0.32 g $CoCl_2.6H_2O$, 0.315 g $CuSO_4.5H_2O$, 0.22 g $(NH_4)_6Mo_7.4H_2O$/, 1.47 g $CaCl_2.2H_2O$, and 1.0 g $FeSO_4.7H_2O$. The prepared fermentation media was sterilized by autoclaving it for 15 minutes at 121^{o}C [27].

2.5. Submerged fermentation

As much as 14.28, 28.57, 42.85 % v/v of EFBs substrate was put into each of 28.57% *A. niger* inoculum. The fermentation media was added until the working volume of each fermentation was 1400 ml and then incubated at 37°C with stirred at 200 rpm. The fermentation was stopped according to the cell growth time in death phase, namely in 56 hours. The fermentation process was carried out in duplo.

2.6. Sample analysis

Samples were taken every 2 hours to obtain the optimum variation of substrate concentration for cells and xylitol production. The sample taken each time was 6 ml. To analysis cells concentration, 1.5 mL of fermented samples was measured for its absorbance at 600 nm. The absorbance value was substituted to the linear regression equation of the cells standard curve, $y = 60.08x$, $R^2 = 0.998$. The cells standard curve refers to prosedures of Mussanto [16]. To analyze xylitol concentration,the supernatant of the fermented samples was centrifuged for 5 minutes at 1200 rpm was measured for the absorbance at 492 nm. The absorbance values were substituted in the linear regression equation ofy = $0.002x$, $R^2 = 0.996$. The analysis of samples was carried out in duplo.

3. Result and discussion

3.1. Cell growth curve

The growth curve is a curve that shows the profile of cells concentration during the fermentation time. The cells concentration was measured every 2 hours. The growth curve of *A. niger* was obtained from three variations in substrate concentration. The first variation was 14.28%, the second was 28.57%, and the third was 42.85% with working volume of 1400 ml. Figure 1 shows the growth curve of *A. niger* during the 56 hours fermentation process.

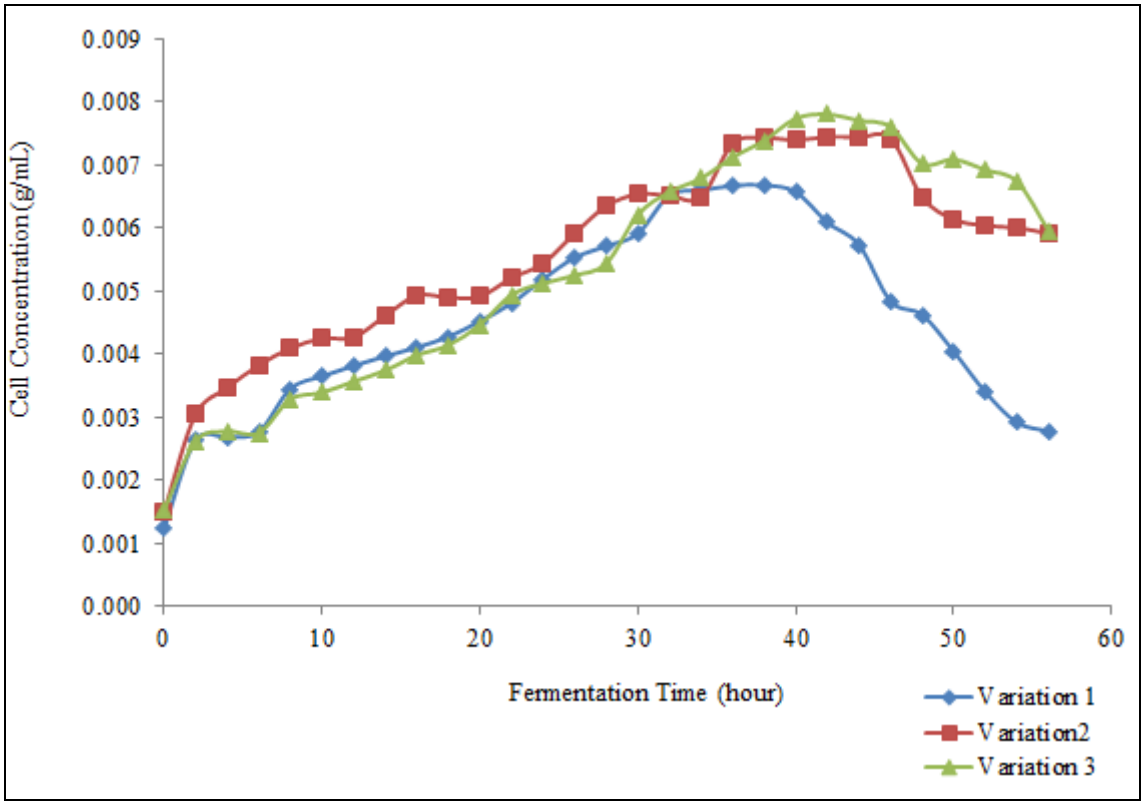

Figure 1. Cell growth curve.

Based on Figure 1, the adaptation phase of the second variation was the fastest of all variations, only 2 hours, whereas in the first and the third variations until 6 hours. The exponential or logaritmic and stationary phases at the second variation were the longest among all. The logarithmic phase at the first variation was 6-32 hours, the second was 2-36 hours, and the third was 6-40 hours, and the stationary phase at the first variation was 32-40 hours, the second was 36-46 hours, and the third was 40-46 hours. The death phase at the first variation was the fastest compared to the other variations, namely at the 40th hour, while in second and third variations at the 46th hour. The growth curve in this study was not significantly different to the growth curve in the study of Lhoas [28] that using diploid strain *A. niger* without substrate. Lhoas inform that the adaptation phase was 0-10 hour, the logaritmic phase was 10-25 hour, the stationer phase was 25-30 hour, and the death phase was 30-40 hour. Based on this study, the addition of substrate was able to extend the growth time of *A. niger*. It can be observed in this study, by the addition of substrate, *A. niger* was in the logarithmic and stationary phase at 30-46 hours, whereas without the addition of substrate at the 30th hour, *A. niger* in the death phase. Based on this study, it can also be observed that the variations of substrate concentration also affect the growth phases and cell concentrations of *A. niger*. The lower of the substrate concentration make the shorter of the logarithmic, stationary, and death phase. The lower of the substrate concentration make the lower of cell concentration, however a higher substrate concentration can decrease the cell concentration. In the second variation, cell concentration in adaptation and logarithmic phase was higher than the first and the third variations. This result was in line with the study that was informed by Pagarra [29]. Pagarra was informed that the high concentration of substrate and product could inhibit the growth of microbial.

The highest cell concentration in the first variation was produced at the 36th hour of 0.0067 g/ml, in the second variation it was at the 38th hour of 0.0074 g/ml, and in the third variation it was at the 42nd hour of 0.0078 g/ml. Microorganism growth was expressed as a specific growth rate (μ). The specific growth rate was calculated from cell concentration data during the logarithmic phase using Monod equation [25]. The specific growth rate of *A. niger* at the first variation was0.00012 cell/hour, the second was 0.00013 cell/hour, and the third was 0.00014 cell/hour.This study showed that increasing substrate concentration resulted in the increase in cell concentration and specific growth rate, although not significant. This result is in accordance with the study conducted by Septiani [30], which indicates that increasing substrate concentrations causes an increase in specificgrowth rate of microorganisms, both in bacteria and microalgae.

3.2 Xylitol production

Biomass conversion into biochemical products is one of the main approaches to resolve the current global challenges for waste mitigation through biorefinery mode. In this study the concentration of xylitol from *A. niger* using EFBs as substrat that was measured every 2 hours from three variations in substrate concentration are shown in Figure 2.

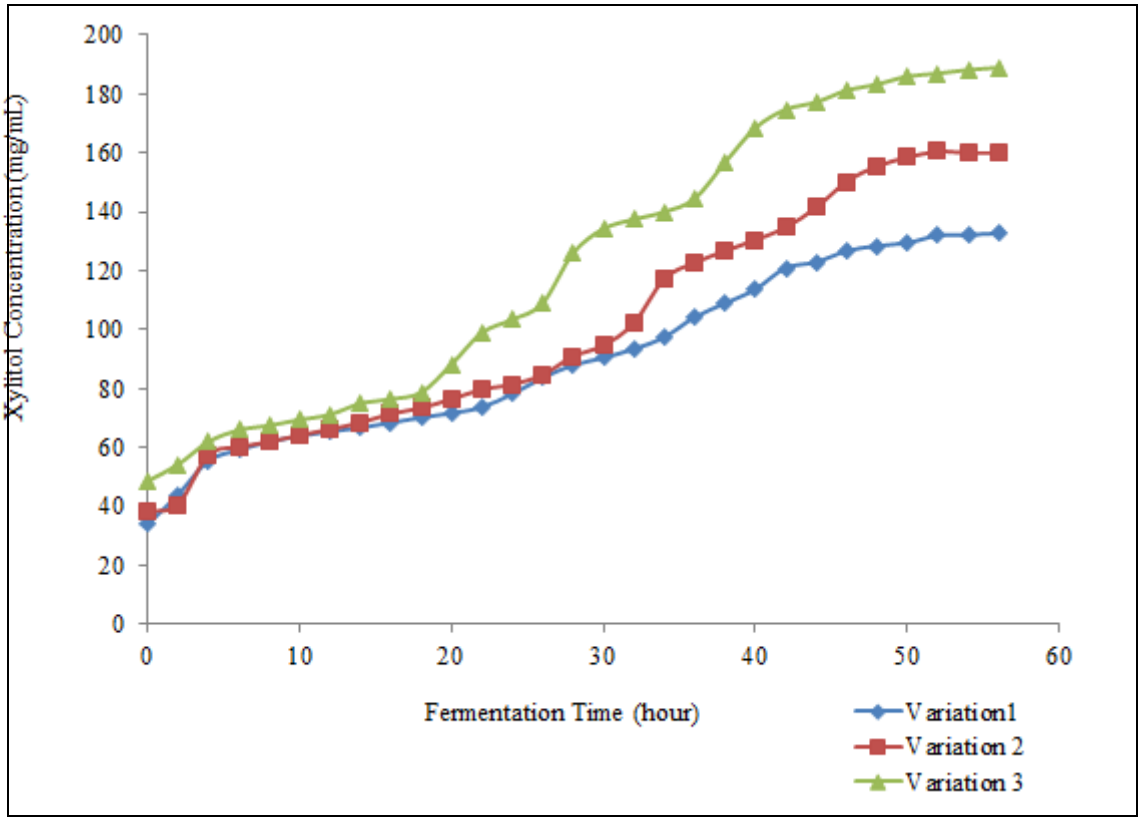

Figure 2. Xylitol production curve.

Based on Figure 2, an increase substrate concentration caused the increase in xylitol concentration and xylitol production rate. In variation 1, optimum xylitol concentration was obtained in the 56th hour. It was 132.75 mg/ml with the rate of 1.72 mg/hour. In Variation 2, optimum xylitol concentration was 160.50 mg/ml with the rate of 2.29mg/hour in the 52nd hour. In variation 3, it was 189.00 mg/ml with the rate of 2.86 mg/hour in the 56th hour. Figure 3 shows xylitol production by xylose metabolism using *A.niger* and agricultural wastes as the substrate.

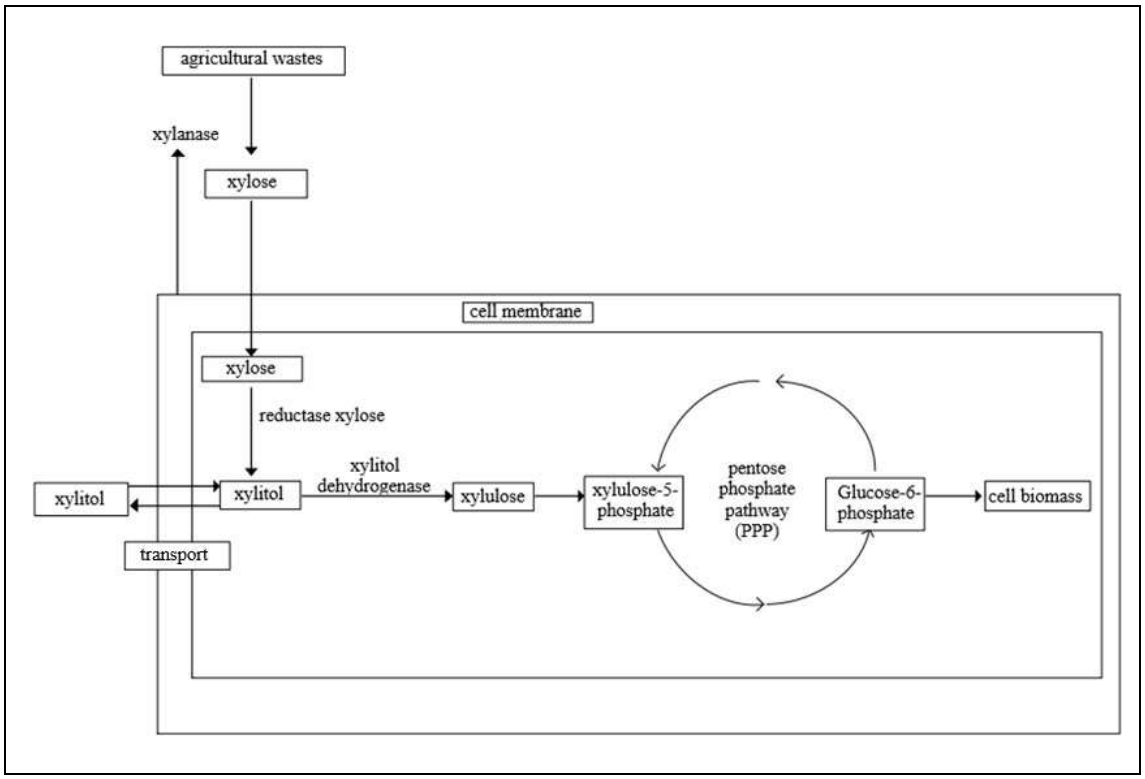

Figure 3. Modification xylose metabolism by *A.niger* from Tochampa and Meng [1,23]

Xylose was the primary sugar obtained from hemicellulose hydrolysate from pretreatment process. The pretreatment also produced other by-products such as glucose, acetic acid, and furfural. Xylose becomes the primary sugar obtained because the main component of hemicellulose is xylan [31]. Pretreatment can be conducted physically, chemically, and biologically. Physical pretreatment can be done by milling and thermal. Chemical pretreatment is conducted by an addition of chemical solution such as acid and alkali, while biological process can be done by adding enzymes. Different pretreatment methods showed different results of xylose concentration production. Beside the pretreatment method, the variation used in the pretreatment process such as the size of EFBs milling and the temperature used in physical process, the concentrations of alkyl or acid solution in chemical process, and the concentrations of enzymes in biological process also affected the results. Dilute acid released 90% hemicellulose, and steam explosion at 200 ^{o}C resulted in a higher of hemicellulose solubilising [14,31,32]. The pretreatment process will produce a xylose-rich hydrolysate [33]. To get pure xylose hydrolysate, activated carbon was added [14]. Mardawati [31] conducted pretreatment by physicochemical and physicobiological at 121 ^{0}C, and the result showed that the xylitol production by 15% enzymatic hydrolysis was higher than 12.5% acid hydrolysis although the xylose generated from acid hydrolysis was higher than that from enzymatic hydrolysis. Based on the data, it could be observed that using acid in hydrolysis may produce the highest concentration of xylose. Steam explosion at 121 ^{o}C is an effective pretreatment method by Kresnowati [32]. Mardawati [14] reported a higher concentration of xylose while applying lower concentration of sulfuric acid hydrolysis because higher concentration of sulfuric in hydrolysis caused an increase in decomposition reaction of xylose and more by-products. In this study, agricultural waste, EFBs were treated by physicochemical pretreatment using 4% sulfuric acid and steam explosion at 121 ^{o}C, but the result of xylose concentration was not measure in the pretreatment process.

Xylose was fermented by *A. niger* in the fermentation medium to produce xylitol. Besides, *A. niger* has the ability to produce xylanase that can hydrolise the hemicellulose in the EFBs subtrate used in the fermentation to produce xylose [34], so that more xylose could be converted into xylitol during the

fermentation process. Based on the metabolism pathway in Figure 3, utilising the enzyme xylose reductase, *A. niger* converted xylose into xylitol. To be utilised by *A. niger*, xylose must be transported into the cell using xylose transporter. In this study, no xylose transporter was used because Sloothaak [35] was informed that *A. niger* have three xylose transporters, namely XltA, XltB, XltC. The resulting xylitol was converted into xylulose using xylitol dehydrogenase. In Penthose Phosphate Pathway, Xylulose-5-phosphate was converted into glucose-6-phosphate and glucose-6-phosphate entered the glycolysis pathway so that it can be used for growth or increase cell biomass of *A. niger*. Besides for cell growth, xylitol was also transported out of the cells, into the fermentation medium as product fermentation. To determine the xylitol produced during the fermentation process, the concentration and production rate were measured. Along with the increase in substrate concentration, the concentration and production rate of xylitol produced also increased. In this study, the highest xylitol concentration was produced at 189.00 mg/ml and the production rate was 2.86 mg/hour at the highest substrate concentration of 42.85%v/v. This study result was in line with the study by Mardawati [31]. A high initial concentration of xylose is required to produce higher of xylitol, but the high xylose concentration retarded cell growth so to produce the maximum xylitol, a more time of fermentation is needed, and at spesific time. The optimum concentration was obtained at 200-300 g/L xylose for *C. guilliermondii*, while xylitol growth and production were limited at a xylose concentration of 400 g/L [36].

Previous studies examined xylitol production using different types of microorganism and agricultural wastes by fermentation. West [37] reviewed xylitol production by *Candida* species from hydrolysates of agricultural residues, such as apple pomace, banana leaves, chestnut shells, cocoa pod husks, corncob, cotton stalk, olive pomace, olive pruning, rapeseed straw, rice straw, sisal fiber sugarcane bagasse, sugarcane straw, water hyacinth leaves, wheat bran, and grasses. The xylitol concentrations were in the range of 3.5-109.5 g/L. Producing xylitol using *D. hansenii* and corn cobs as a substrate, Mardawati [31] found that the concentration of xylitol by acid hydrolysis was 0.100 g-xylitol/g-xylose and 0.216 g-xylitol/g-xylose by enzymatic hydrolysis. Hong [38] produced 10.86 g/L xylitol using *C. tropicalis* CCTCC M2012462 and corn stover as substrate. Ping [39] produced 38.8 g/L xylitol using *C. tropicalis* XK12K and corn cobs as substrate. Mohammad [40] produced 0.51 g/L xylitol using *C. tropicalis* and EFBs as substrate. Canilha [40] produced 0.81 g/L xylitol using *C. guiliermondii* and bagasse as substrate. The description shows that besides the pretreatment method and substrate concentration, the types of substrate and microorganisms also affect of the concentration of the xylitol produced.

The difference in the substrate used in fermentation has a great effect on xylitol production. Substrates from different agricultural wastes contain different cellulose, hemicellulose, and lignin composition. Hemicellulose-containing substrate becomes the basic material for xylitol synthesis. The higher hemicellulose is in substrate, the more xylitol is available because hemicellulose will be converted to xylose through pretreatment and then xylose will be converted to xylitol by microorganism through fermentation process. Different microorganisms used in the fermentation process affect xylitol production due to their ability to produce enzyme, different metabolic abilities and biochemical pathways to break down sugar into xylitol. In addition, microorganisms have different tolerances to environmental conditions such as temperature, pH, substrate concentration, and oxygen. During the fermentation process, microorganisms can also produce by-products that can affect xylitol concentration. Yeast *Candida* species, including *C. Tenuis, C. Guillermondii, C. Parapsilosis, C. Intermedia,* and *C. Tropicalis, Aspergillus* species, including *A. carbonarius, A. niger*, and *D. hansenii* have the ability to produce xylose reductase [35,42,43,44]. Xylose reductase is an enzyme that helps in the metabolism of xylose to xylitol. Xylitol reductase in microorganisms could lead to the genetic engineering of this enzyme to have a high affinity for the substrate that will result both in increasing xylitol production and reducing production cost [37]. The reduction of lignin from substrate during pretreatment process that also will results increasing xylitol production and reducing production costs, energy requirement, and emission [10,11].

4. Conclusion

The highest substrate concentration did not only increase the cell concentration and cell growth rate of *A.niger*, but also the concentration and production rate of xylitol. The optimum condition from this study was the third variation, 42.85% v/v substrate concentration. This condition produced 0.0078 g/ml cell concentration, 0.00014 cells/hour cell growth rate, 189.000 mg/ml xylitol concentration, and 2.861 mg/ml xylitol production rate. In future research, it is necessary to evaluate the substrate pretreatment process to obtain the optimal substrate pretreatment process that can increase the composition of hemicellulose that will be used in the fermentation process to production of xylitol. The fermentation time up to the xylitol concentration decrease. In addition, it is also suggested to measure the concentration of xylose and xylitol during the fermentation time so the yield coefficients used in microbial growth kinetics, such as the ratio of the number of cells formed to the amount of substrate consumed (Yx/s), the ratio of the number of product formed to the amount of substrate consumed (Yp/s), and the ratio of the number of products formed to the amount of cells formed (Yp/x). Xylitol concentration suggested to measure using HPLC method or D-sorbitol/ xylitol kit.

5. References

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