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The Effects of Operational Conditions in Scaling Up of Xylanase Enzyme Production for Xylitol Production

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Abstract

The biological route to produce xylitol from Oil Palm Empty Fruit Bunches (EFBs) comprises of EFBs pretreatment, enzymatic hydrolysis, fermentation, and downstream separation of the produced xylitol. Due to the specificity in the hemicellulose composition of EFBs, a xylanase enzyme that has a high affinity to EFBs is required to hydrolyze the EFBs into xylose. In this research, the influences of aeration, humidity, and mixing in xylanase production were mapped. The xylanase production was performed by Aspergillus fumigatus ITBCCL170 in a solid-state fermentation using a tray fermenter with EFBs as the substrate. The optimal configuration was further scaled up into xylanase production using 1000 g of EFBs as the substrate. The results showed that the highest enzyme activity was 236.3 U/g EFB, obtained from the use of humid air airflow of 0.1 LPM, and mixing was performed once a day. The scaling up resulted in a lower xylanase activity and call for a better design of the fermenter.

Keywords: *aeration, humidity, mixing, OPEFBs, tray fermenter, xylanase, xylitol*

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INTRODUCTION

Indonesia is the world's largest producer and exporter of palm oil which controls 85-90% of the world market (Meryana, 2017). At the beginning of the semester 2018, Indonesia's palm oil production has reached 22.32 million tons and increased by 23% compared to 2017 in the same period (Sardjono, 2018). Crude Palm Oil (CPO) production process produces solid and liquid waste. Hasanudin *et.al* (2015) stated that for every one ton of Fresh Fruit Bunches that were processed into palm oil (CPO), solid waste consisting of 220-230 kg of Oil Palm Empty Fruit Bunches (EFBs), 120-130 kg of fiber, 50-60 kg of shells, and 5-6 kgs of boiler ash and liquid waste (POME) is 0.77 to 0.84 m^3 are produced.

EFBs consist of 11-23 % lignin, 25-35 % hemicellulose, and 35-42 % cellulose (Mardawati,

2015; Octavia, 2013; Rahman, Choudhury, & Ahmad, 2006; Shibata, Varman, Tono, Miyafuji, & Shiro, 2008). Octavia (2013) has reported that hemicellulose of EFBs is rich in xylan that overall it contains about 19.28 - 19.6% xylose. Thereby it can potentially be used as the raw material for xylitol production (Albuquerque *et.al*. 2014). The biological route for xylitol production from EFBs was proposed to be pretreatment, hydrolysis, fermentation, and downstream processing to give pure xylitol (Kresnowati, Mardawati, and Setiadi 2015). Due to the specificity in the hemicellulose composition of EFBs, a xylanase enzyme that has a high affinity to EFBs is required to hydrolyze the EFBs into xylose (Djakaria 2017).

The xylanase enzyme is a kind of enzyme that is used to hydrolyze the hemicellulose. The xylanase enzyme is divided into β-xylosidase, exo-xylanase, and endo-xylanase. β-xylosidase that hydrolyzes short-chain xylooligosaccharide into xylose. Exo-xylanase is classified as the xylanase enzyme group that breaks xylan, reducing-end polymer chains of xylose, into xylose as the main product and some short-chain oligosaccharides. Endo-xylanase is part of the xylanase enzyme that breaks β-1,4 chains of xylan regularly (Richana, 2002).

Xylanase has been produced by various kinds of substrates such as corncob using *Aspergillus foetidus* MTCC 4898, giving xylanase activity of 3,065 U/g substrate (Shah and Madamwar (2005)). This experiment was conducted at the scale of 5 g substrate used. Another substrate used was soybean waste using *Penicillium canescens* in a packed-bed reactor leading to xylanase activity of 7,900 U/g substrate (by Assamoi et.al (2008)). EFBs have been used to produce xylanase enzyme by Djakaria (2017) and giving xylanase activity of 3.54 U/g substrate. The latter research was conducted at a scale of 250 g EFBs substrate.

The aeration, humidity, and mixing were important to be evaluated in xylanase production using the SSF process because the main problems in SSF were heat and mass transfer difficulties (Chen, 2013). The challenges to scale-up production process through solid-state fermentation (SSF) include uneven air distribution, mycelia damage caused by substrate particle movement, the difficulty of pH control, and non-uniform bed temperature caused by metabolism heat of microbe (Mitchell, Berovič, & Krieger, 2006). Djakaria (2017) succeeded to acquire the optimal condition for solid-state fermentation of EFBs with an optimal size of EFBs of 1-2 cm, solid loading of 25%, and an incubation time of 2.5 days.

Lack of water content might inhibit microbe metabolism and even gradually stops because of a lack of diffused substrate or nutrition or higher concentration of inhibitory metabolite around the cells (Chen, 2013; Gervais & Molin, 2003). The role of aeration in solid-state fermentation is to supply air to fulfill oxygen (O_2) needs of aerobic microbe; control water content and water activity; eliminate volatile matter, carbon dioxide, and metabolism heat of microbe during fermentation, thus it can reduce heat developed inside tray (Chen, 2013). Temperature is an important factor that has a major impact on microbe growth and aerobic fermentation to release the heat during the growth phase of the microbe. Indirectly, aeration was able to reduce EFBs temperature inside the tray as the effect of metabolism heat of fermentation that increases the temperature previously. Another parameter that provides a meaningful effect on xylanase activity is mixing. In solid-state fermentation, bed depth affects the distribution of temperature, humidity, O_2, and carbon dioxide (CO_2). As fungal growth began, the particles inside the tray were about to agglomerate, and create chanel inside the bed dept. Therefore, it can inhibit O_2 and substrate diffusion inside the bed towards the microbe. Moreover, microbe metabolism releases heat that is kept inside the bed and vaporizes water from the bed then the humidity will decrease gradually (Chen, 2013). The humid air distributed inside the fermenter was to maintain humidity.

In scaling up of production scale, there are considerable factors that offer dominant influences towards the enzyme result such as aeration, humidity, substrate size, bioreactor size, and mixing. According to these challenges, the general purpose of this research is to map operational conditions in scaling up of xylanase enzyme production. The specific purposes are to map the influences of aeration, humidity, and mixing in xylanase production and evaluate the result of scaling up of xylanase production.

RESEARCH METHOD

Microbial culture and media

The microbe used in this research was *Aspergillus fumigatus* ITBCCL170, a collection of Microbiology and Bioprocess Technology Lab, Dept of Chemical Engineering, Institut Teknologi Bandung. *Aspergillus fumigatus ITBCCL170* that has been cultured in Potato Dextrose Agar (PDA) was then cultured in Potato Dextrose Broth (PDB) at 30 ° C for 5 days. This microbe was subsequently cultured in sterilized cooked rice then incubated at 30^{o}C for 5 days and dried in the oven at 37^{o}C. The immobilized dried spores of *Aspergillus fumigatus* were milled and stored at 4^{o}C before further used

EFBs were used as the carbon source for solid-state fermentation. The EFBs were kindly provided by PT. Perkebunan Nusantara VIII, Cikasungka, Bogor. The preparation of EFBs consisted of washing in running water; drying in the oven at 60^{o}C for 24 hours; and milling into 1-2 cm. Prior to the addition of inoculum, EFBs were sterilized at 121^{o}C for 15 minutes. In addition of EFBs as the carbon source, the other nutrients for the fermentation were provided by Prado Medium that containing $(NH_4)_2SO_4$ 1,5 g/L; KH_2PO_4 2 g/L; urea 0,3 g/L; $CaCl_2$ 0,03 g/L; and $MgSO_4.7H_2O$ 0,2 g/L (Alves-Prado *et.al*., 2010).

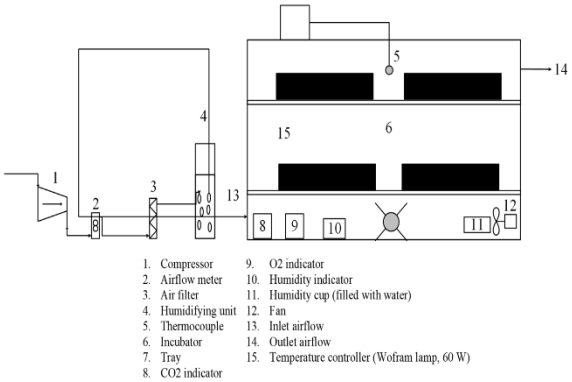

Figure 1. The configuration of tray fermenter for the solid-state fermentation

Fermentation and product extraction

The solid-state fermentations were conducted in a tray fermenter that was equipped with a temperature control system, aeration system, CO_2, O_2 and humidity indicators. The bed of media was placed in aluminum trays of 26x26x7 cm each. Each tray has 4 holes with 3 mm of diameter on its side to facilitate better air transfer. Figure 1 illustrated the configuration of the tray fermenter used.

Sterilized EFBs of 250 g were mixed with immobilized spores of *Aspergillus fumigatus ITBCCL170* at 10^7 spores/g EFB and sterilized Prado Medium to give solid to liquid ratio of 1:4. Cheesecloth was used to cover the tray during fermentation and the holes are kept open. The incubation was conducted for 3 days at 32ºC.

Xylanase (crude xylanase) as the product of fermentation was extracted from the fermentation using sterile citrate buffer 50 mM (pH of 5). at ratio 1:8 (mass of EFBs used as the substrate for fermentation/volume of buffer used for extraction) for 1 hour in an incubator shaker to ensure optimal enzyme extraction. Extraction product was separated from EFBs using cheesecloth then centrifuged at 4500 rpm for 30 minutes and stored at -4ºC (Lakshmi, Bhargavi, & Reddy Shetty, 2011) before further analysis/used.

Analysis

Xylanase activity was measured by the DNS method using 1% (w/v) beechwood xylan (Megazyme) as a substrate. The enzyme assay was performed at 50˚C for 15 minutes (Bailey, Biely, & Poutanen, 1992). Protein concentration was measured by the Bradford assay method (Bradford, 1976). The fermentation broth was analyzed to obtain total protein concentration. The supernatant obtained from the centrifugation process was analyzed as extracellular protein concentration.

Design of experiment

The effects of solid-state fermentation parameters such as aeration, humidity, and mixing on the produced enzyme activity were evaluated by following the factorial design of six variations

Table 1. Factorial design of experiment

Run	Aeration	Humidity
1	-	-
2	-	-
3	+	-
4	+	-
5	+	+
6	+	+

Aeration:
(+) 0.1 LPM airflow
(-) without airflow

Humidity:
(+) humidified air
(-) non-humidified air

Mixing:
(+) once a day
(-) without mixing

combinations as shown in Table 1. Each experiment was conducted by using 250 g EFBs as the substrate. All experiments were conducted in duplicate.

The best process parameters obtained at the previous set of experiments were applied in scaling up xylanase production by using 1 kg EFBs.

RESULTS AND DISCUSSION

The effects of aeration, humidity, and mixing in xylanase production.

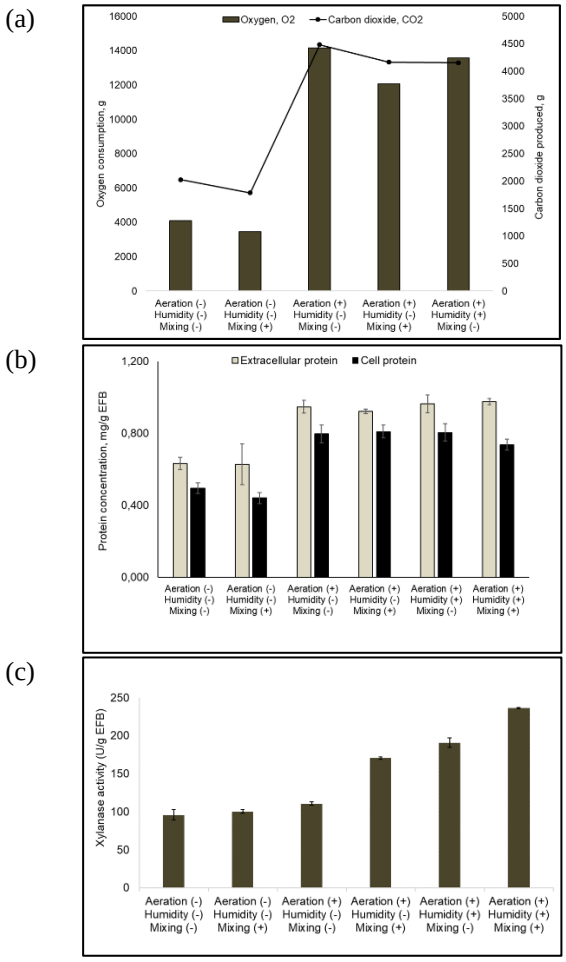

Figure 2. (a) Oxygen consumption and Carbon dioxide produced, (b) extracellular protein and cell protein produced, (c) xylanase activity during fermentation of xylanase production

The effect of aeration, humidity, and mixing in the O_2 and CO_2 profile was presented in Figure 2a. Providing better mixing on the fermentation system, giving the minimum O_2 consumption and CO_2 production. While the effect of aeration showed to be significant as the O_2 consumption and CO_2 production were higher than other combinations. However, there was an interaction effect between aeration, humidity, and mixing which resulted in a variation in O_2 consumption and CO_2 production. The humidity seemed to have a positive effect on aeration instead of mixing as can be seen in Figure 2a. The O_2 consumption was higher without mixing than with mixing but without humidity. This may be related to the fact that mixing, even intermittently, might break the fungal hyphae (Mitchell *et.al*., 2006).

The aeration effect by using 0.1 LPM aeration flowrate gave a higher CO_2 produced approximately 4,212 mg/g EFBs compared to the non-aerated process of 1,909 mg/g EFBs approximately. These results indicated that O_2 was effectively consumed by fungal to produce CO_2, and were confirmed in the protein analysis of the crude xylanase produced.

Oxygen consumption and CO_2 production corresponded to fungal growth, in particular for aerobic fungal such as *Aspergillus fumigatus.* To confirm this, protein analysis was performed to obtain the extracellular protein (excreted enzyme) dan protein cells, which represented an indirect microbe concentration measurement during the cultivation. The results are presented in Figure 2b.

The results show the O_2 supplied into the fermenter chamber was effectively consumed for fungal growth. The cell protein increased almost two-fold compared with the non-aerated process as well as extracellular protein as can be seen in Figure 2b. Thus, the use of aeration flow rate of 0.1 LPM into the fermenter chamber was sufficient to promote fungal growth and also extracellular metabolite (crude xylanase). In addition, aeration helps to decrease bed temperature as the effect of metabolism heat of microbe, thus microbe growth was well maintained to produce xylanase enzyme (Figueroa-Montero *et.al*., 2011).

The interaction effect of humidity and mixing to aeration in O_2 consumption and CO_2 production showed a similar trend in fungal growth. There were only slight changes in extracellular protein and cell protein among those varied conditions. However, the more process parameter used the higher the protein produced. This indicated that aeration, humidity, and mixing support each other in producing xylanase on tray fermenter. Furthermore, these effects must be confirmed whether it was positive or negative to xylanase activity.

The effects of aeration, humidity, and mixing in xylanase activity

Figure 2c showed the effect of these three process parameters: aeration, humidity and mixing on the produced xylanase activity.

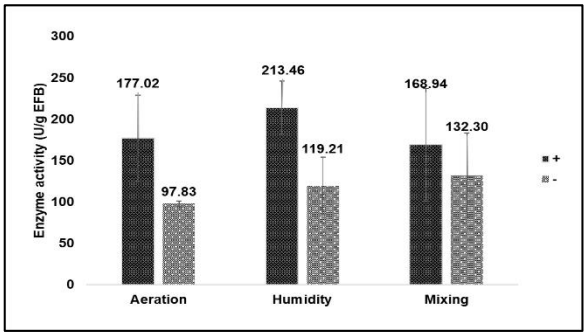

Figure 3. The main effects of aeration, humidity, and mixing on xylanase activity

The use of proper aeration, at flowrate 0.1 Lpm and using humid air and proper bed mixing provided a good environment for xylanase production in the SSF process using tray fermenter. The highest xylanase activity produced was 236.3 U/g EFB (which corresponds to 29.5 U/mL crude enzyme). This value was 2.8 fold of the enzyme activity obtained from the experiment without aeration, humidity condition, and mixing. In detail, the effect of each process variable was shown in Figure 3.

Figure 3 indicated that the effect of aeration on the produced xylanase activity was as important as the humidity and mixing were the least important. There was an increase of xylanase activity of 44.7% and 44.2% affected by aeration and humidity respectively. While mixing can only increase xylanase activity by 21.7%. The obtained results were in accordance with Perez-Rodriguez et.al. (2014) who reported that xylanase activity increased by approximately 80% by using humidified air of 0.1 LPM.

Xylanase activity was affected by humidity even though there was no significant difference in the humidity measured between humid air supplied and not supplied while incubation was conducted (data was not shown). This was because the measured humidity was the humidity inside the fermenter instead of the humidity inside the EFBs bed. Aeration and humidity allocated the most significant effect on xylanase activity that was shown in Figure 3. The results were in agreement with Assamoi et.al (2008) who reported that xylanase activity increased from 750 U/g substrate (non-humidified air) to 2,000 U/g substrate (0.1 LPM of humidified air).

The purpose of mixing in solid-state fermentation is to provide even distribution and diffusion of substrate and gases into microbe in the tray (Suryanarayan, 2003). Mixing contributes to the increase of xylanase activity; however, it is not overly significant as aeration and humidity as can be seen in Figure 3. The standard error of data obtained did not show a significant difference concluded that the effect of mixing was not significant as aeration and humidity. Besides, Lee et.al. (2011) reported that enzyme activity obtained by mixing every 24 h was higher than every 6 h mixing. This indicated that mixing was needed in solid-state fermentation, however, mixing frequency

should be set in order to avoid fungal mycelia from damage. However, this experiment was different since the size of the fermenter was much bigger than the earlier report. This indicated the importance of evaluation of scaling up in the xylanase production via SSF.

Scale up to 1000 g EFBs

In this experiment, we would like to evaluate the effect of scaling up the solid-state fermentation from using EFBs substrate of 250 g to 1000 g.

The production condition was at aeration of 0.1 LPM, intermittent mixing every 24 h, and humid air was constantly blown. The results were shown in Figure 4.

Figure 4 showed that there was a slight decrease in the protein content per unit of EFBs used when the fermentation scale increased from 250 g to 1 kg of EFBs used. The decreases were approximate 8.1% and 10.3% for extracellular protein and cell protein respectively. The specific xylanase activity per gram of EFBs used decreased by 44% from 236.3 U/g EFBs to 132.7 U/g EFBs, along with the increase in the fermentation scale from 250 g to 1000 g of EFBs used. These might be related to the change in environment for fungal growth, such as lack of water, oxygen, even the high temperature.

The fermenter was equipped with an aeration system and a humid air supply to meet the needs of fungal growth. As the fungus grew, the temperature gradient due to metabolic heat made the water evaporate (Chen, 2013).

(a)

(b)

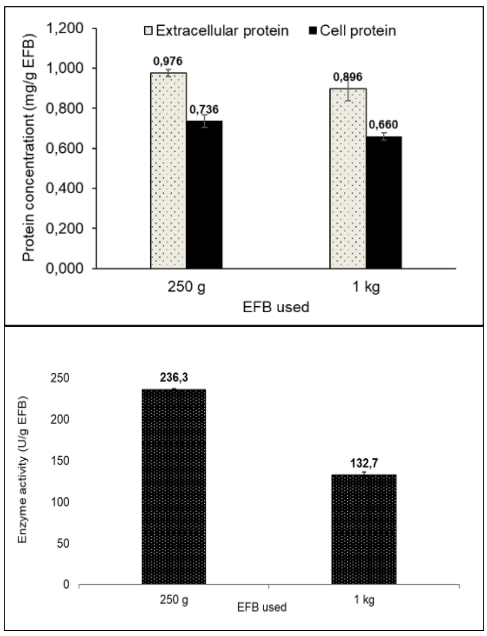

Figure 4 (a) Protein content profile and (b) xylanase activity as a function of changes in the bed mass.

The aeration flow rate of 0.1 LPM and the fan used to maintain even temperature distribution apparently was not sufficient. This was proven by a decreased amount of liquid medium on the tray. This condition led to an unfavorable environment to fungal growth which needed enough water to supply nutrients (Chen, 2013). As the consequences of that condition, the xylanase quality as the metabolite product also reduced. The result was shown in Figure 5. The fermenter should be designed as such to provide better heat distribution during the fermentation. Overall this showed that a better strategy was required to scale up the enzyme production via solid-state fermentation.

CONCLUSION

This research shows that EFBs can be potentially used as the production media for xylanase production via solid-state fermentation in a tray fermenter. The aeration, humidity, and mixing were shown to significantly affect the fungal growth during the fermentation and correspondingly and the enzyme activity produced in the fermentation. The best result was obtained from fermentation supplied with humid air, aeration and mixing, giving xylanase with the activity of 236.3 U/g EFB. The scale-up experiment led a significant decrease in the produced enzyme activity emphasized the needs of better scale-up strategy and proper fermenter design for xylanase production.

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