

OPTIMIZATION OF PROCESS PARAMETERS AND SCALE-UP OF XYLANASE PRODUCTION USING CORN COB RAW BIOMASS BY MARINE BACTERIA *BACILLUS SUBTILIS* LBF M8 IN STIRRED TANK BIOREACTOR

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Article received 24.5.2018, Revised 24.8.2018, Accepted 3.9.2018

ABSTRACT

Optimization parameter for production several xylanases have already been studied, but only a few derived from marine bacteria especially *Bacillus* sp. Focus on this research is optimization of process parameters for xylanase production and scale-up production in stirred tank bioreactor using corn cob raw biomass as a substrate. Maximum xylanase production was obtained at 1,5% corn cob concentration, pH medium 6, temperature fermentation 30°C, and agitation rate 200 rpm. For production using agricultural waste, production with corn cob (2,963 U/mL) is higher than rice straw (1,938 U/mL) and sugarcane bagasse (1,728 U/mL). Optimization process parameters increased xylanase activity from 1,864 U/mL at 96 h to 2,963 U/mL at 24 h incubation. The scale-up of the fermentation process up to 2 L stirred tank bioreactor with the optimum condition at flask scale, significantly increased xylanase activity to 3,519 U/mL.

Keywords: Optimization, *Bacillus subtilis* LBF M8, xylanase, corn cob, stirred tank bioreactor

INTRODUCTION

Xylanolytic enzymes are enzymes that play a role in the process of hydrolysis of xylan which is the main component of hemicellulose and the second most abundant renewable resource on earth. In the xylan hydrolysis process, xylanase (endo-1,4 β -xylanase) has a more important role than other xylanolytic enzymes such as β -D-xylosidase, acetyl xylan esterase, arabinase and α -glucuronidase. The structure of hemicellulose complex in xylan composed of β -1,4-linked xylose residues of short-side chains of o-acetyl, α -L-arabinofuranosil, D- α -glucuronic, and residues of phenolic acids cause physical barriers that limit the xylan degradation process. Xylanase will cut the internal bonds of β -1,4 xylose and convert xylan into food additives such as xylooligosaccharides. Utilization of xylanase in industrial applications like food, feed, pharmaceutical and for sustainable production of fuels and chemicals has increased, but production xylanase with commercial xylan at industrial scale is too expensive. Production cost determines the success of an industry processes, produce xylanase from inexpensive substrates be the ideal thing for commercial application (Heck *et al.*, 2005; Chakrit *et al.*, 2006; and Rozanov *et al.*, 2015).

Xylan which is the main constituent of hemicellulose found in plant cell walls, causes a lot of agricultural waste to contain high xylan. This makes agricultural waste into a potential raw material that can be used as a more economical xylanase production substrate. Agricultural waste produced on land and processing sites, mostly dis-

posed of as waste, creates environmental problems if not handled properly (Rahmani *et al.*, 2014; and Wang *et al.*, 2016). The use of cheap raw materials such as agricultural waste in industrial scale production can reduce production costs and avoiding environmental pollution caused by agricultural waste (Sharma *et al.*, 2013; and Silva *et al.*, 2015). The production uses agricultural waste can be reduced the cost of the growth substrate and increased bioconversion of the biomass to value-added compounds by economically feasible ways (Yegin *et al.*, 2016). According to the previous studies on agriculture waste, corn cob consist of 28% xylan (Van Dongen *et al.*, 2011), which higher than rice straw (19,3%) (Sasaki *et al.*, 2016), empty fruit bunch (20,7%) (Zhang *et al.*, 2012), and tobacco stalk (22%) (Akpinar *et al.*, 2010), making corn cobs as a potential material for xylanase production.

Microorganisms such as bacteria, fungi and actinomycetes are alternative sources for enzyme production. In industrial-scale production, enzyme production using microorganisms has more stable results. Xylanase production using bacteria is mostly done because production using fungi and actinomycetes has a slow generation time, produces many byproducts, and poor oxygen transfer. Many bacteria naturally produce xylanase from various biological sources, including sea and land. The use of bacteria isolated from the land area has been done a lot while the bacteria isolated from the oceans are still not much done. The character and uniqueness of marine bacteria such as halo-

toleran and high salinity, making industrial interest require new microbial strains for various increased enzyme production in marine bacteria. Mostly bacteria especially genus *Bacillus* has a potential ability to produce many enzymes one of which is xylanase. Xylanase production from marine bacteria especially by *Bacillus* sp. not much done. (Akpinar *et al.*, 2010; Annamalai *et al.*, 2009; Wijaya *et al.*, 2016). Xylanase by marine *Bacillus* sp. has been reported, such as *B. subtilis* (Annamalai *et al.*, 2009; and Khandeparker *et al.*, 2011), *B. pumillus* (Menon *et al.*, 2010), and in Indonesia, *B. safencis* (Rahmani *et al.*, 2014), *B. pumillus* (Wijaya *et al.*, 2016), and *B. tequilensis* (Yopi *et al.*, 2017). Several studies have already been performed on the utilization of corn cob for xylanase production by *Bacillus* sp., for example, Irfan *et al.*, (2015) examined the potential of corn cob on xylanase production using by *B. subtilis* and *B. megaterium*. Xu *et al.*, (2017) reported potential xylanase production by *Bacillus* sp. BS-5 using alkali pretreatment corn cob as a substrate.

Production in laboratory scale bioreactors at first is important factors before going through the larger scales. Increased production using a stirred tank bioreactor can determine the effect of dissolved oxygen concentration and high heat and mass transfer rates on the productivity of xylanase-producing bacteria (Yegin *et al.*, 2016). For the cultivation of microorganisms the conditions in the bioreactor need to be adjusted to the specific needs of microorganisms in order to obtain high productivity from microorganisms so as to produce the desired product (Gonciarz *et al.* 2016). Utilization corn cob and marine bacteria especially *Bacillus* sp. from Indonesia, and optimization process parameter i.e biomass concentration, pH medium, temperature, agitation rate and scale-up xylanase production in stirred tank bioreactor is the aim of this research.

MATERIALS AND METHODS

Bacterial strain: The bacterial strain *Bacillus subtilis* LBF M8 is a collection of Biocatalyst and Fermentation Laboratory (LBF), Research Center for Biotechnology, Indonesia Science Institute (LIPI). Isolate *B. subtilis* LBF M8 were locally isolated from seawater in Pari island, Indonesia.

Substrates preparation: Corn cobs come from local plantations in the city of Bogor, Indonesia. Corn cobs that will be used as substrate for xylanase production are dried at 60°C for twelve hours and then cut and reduced in size to 100 mesh.

Qualitative assay: The qualitative assay was conducted using congo red methods (Meddeb-Mouehi *et al.*, 2014). A loopful strain *B. subtilis* LBF

M8 were patched onto xylan agar plates containing (w/v) 3.8% artificial seawater (Marine Art[®] SF-1, Osaka, Japan), 0.1% yeast extract (Bacto[™] USA), 0.5% peptone (Bacto[™] USA), 0.5% beechwood Xylan (SIGMA-ALDRICH, USA) and 1.5 % agar (Bacto[™] USA). then incubated at 30°C for 48 hours. After the incubation process is complete, the plate is soaked with 0.25% congo red for 1 hour, then 5% acetic acid for 5 minutes and washed twice using 1 M NaCl.

Fermentation technique: Fifty millilitres pre-culture medium (g/L: 3.8 artificial sea water, 0.5 peptone, 0.1 yeast extract, and 0.5 beechwood xylan) was sterilized in 500 mL flask at 121°C for 15 min. After sterilization, media was inoculated with a loopful of 48 h old strain *B. subtilis* LBF M8 and incubated at 30°C for 24 h with the agitation speed of 150 rpm. For culture medium contain the same material just 0.5g/L Beechwood xylan replaced by 0.5 g/L corn cob. After sterilization, the culture medium was inoculated with 10% (v/v) pre-culture medium and incubated at 30°C with the agitation speed of 150 rpm. The cell growth measurement using spectrophotometer at a wavelength 660 nm.

Optimization of culture condition: Optimization of xylanase production by *B. subtilis* LBF M8 was carried out on several parameters such as, corn cob concentration (0.5, 1, 1.5, 2, and 2.5% : w/v), pH medium (5, 6, 7, 8, and 9), incubation temperature (20, 30, 40, and 50°C), and agitation rate (0, 50, 100, 150, and 250 rpm). The fermentation broth was separated from the bacterial cell by centrifugation at 4°C, 11000 rpm, 15 minutes. After centrifugation, the supernatant is used as a source of enzymes which will be tested for xylanase activity.

Xylanase assay: Xylanase activity was measured by the DNS method (Bailey *et al.*, 1992). As much as 500 µL of the crude enzyme was mixed with 500 µL substrate that containing 0.5% beechwood xylan along with 0.05 M phosphate buffer pH 7 and incubated for 30 min at 60°C. The addition of 1 mL of DNS and heated to 95°C for 15 minutes, aims to stop the reaction. After that, the solution was cooled in ice for 10 min and incubated at room temperature for 10 min. The concentration of reducing sugar was measured by absorbance at 540 nm using D-xylose (Wako Pure Chemical Industries, Ltd) as a standard. The blank and control were prepared by same procedure (blank and substrate control without enzyme, and enzyme control without substrate).

Xylanase production using different materials: Based on the optimum conditions to produce xyla-

nase using corn cobs, xylanase will be produced using beechwood xylan, rice straw, and bagasse. Beechwood xylan as a comparison with the agricultural waste used. preparation for rice straw and bagasse as same as corn cobs. Xylanase activity was measured as described before.

Effect of xylan concentration on xylanase activity: The effect of xylan concentration on xylanase activity was tested using the DNS method with different Beechwood xylan concentrations (0.5-2.5%) with a regular interval of 0.5%.

Scale-up production in bioreactor: The bioreactor used is a 2 L lab scale stirred tank bioreactor (New Brunswick Scientific, New Jersey, USA) with a working volume of 1 L. The conditions of the production parameters used were optimum conditions on the flask scale. The fermenter was sterilized at 121°C for 15min.

RESULTS AND DISCUSSION

Qualitative analysis: A qualitative test was conducted to see the potential of Isolate *B. subtilis* LBF M8 in producing xylanase enzyme through the clear zone formed. Isolates of *B. subtilis* LBF M8

include marine bacteria isolated based on their ability to produce mananase enzymes (Djohan 2014). *B. subtilis* LBF M8 has the ability to produce mananase enzymes with a crude extract enzyme activity of 9.5 U/mL (Yopi *et al.*, 2017). The ability of *B. subtilis* LBF M8 to produce xylanase was detected by using congo red solution. Basically, the enzyme will bind to its specific substrate, so that the xylanolytic ability of the bacteria will be seen when grown in the medium containing xylan which is a polysaccharide organic compound consisting of linear (1-4) chains related to xylopyranose residues. The hydrolyzed xylan will form a clear zone around the growing colonies of bacteria. The formed zone will be more clearly visible after coloring with congo red. Xylopyranose residues will bond strongly with congo red which produces results in the formation of red areas in xylan media and clarify clear zones as shown in Fig 1, which shows clear zone because xylopyranose residues have been hydrolyzed and will not bind to red congo (Meddeb-Mouelhi *et al.*, 2014 and Rahmani *et al.*, 2014).

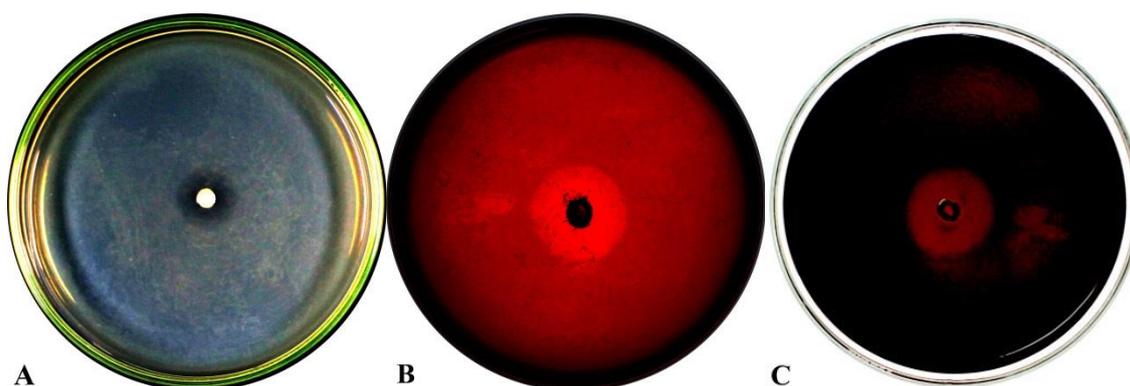


Fig. 1: Detection of xylanase activity from from *B. subtilis* LBF M8 on xylan agar plates. (A) plates before destaining by Congo red solution (B) plates flooded with Congo red, (C) plates flooded with Acetic acid.

Effect of corn cob concentration for xylanase production: Corn cob is a carbon source for xylanase production in this research. This parameter must be optimized in the microbial fermentation medium, because of his main role in cellular growth and metabolism (Motesafi *et al.*, 2016). 0.5-2.5% Corn cob concentration was conducted to study the optimum concentration to produce concentration. Effect of corn cob concentration for xylanase production was shown in Fig 2A. The xylanase activity on all substrate concentration has increased from 0 to 96 h and decreased in 120 h, this result indicates a dding more substrate does not show any significant improvement for xylanase production by *B. subtilis* LBF M8. Corn cob concentration 1.5% showed the highest xylanase activity (1.864 U/mL) and the lower at 0.5% corn

cob concentration (0.852U/mL) therefore, cob concentration 1.5% as the optimum condition for production xylanase by *B. subtilis* LBF M8. The highest xylanase activity by *B. subtilis* LBF M8, is similar to the highest xylanase activity (1.8 U/mL) of the results obtained Tork *et al.*, (2013) that produces xylanase using corn cob by marine bacteria *B. subtilis* XP10. These results are the same as those obtained by Rahmani *et al.*, (2014) was produce xylanase using 1.5% substrate by marine bacterium *B. safencis* P20. Irfan *et al.*, (2015) reported *B. substillis* to exhibit maximum xylanase production with 2% corn cob concentration and 1.5% for *B. megaterium*.

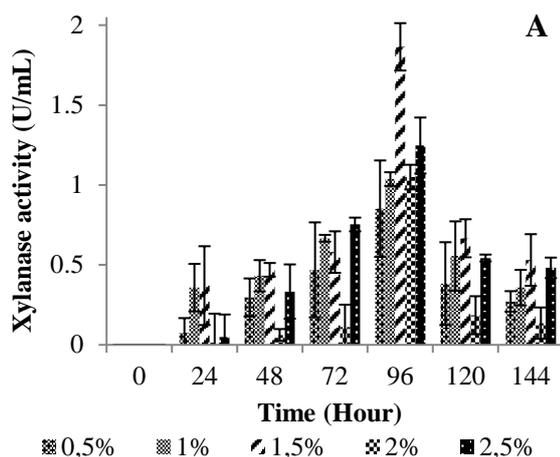


Fig. 2A: Optimization of Process Parameters for xylanase production by *B. subtilis* LBF M8. Effect of corn cob concentration and fermentation period for xylanase production.

The longer the fermentation time can reduce the production of enzymes produced. The production of toxic metabolites and the decreasing nutrient sources such as carbon during microbial growth can inhibit enzyme synthesis (Irfan *et al.*, 2015). Before the optimization process, the fermentation time at 96 hours showed the highest xylanase activity and became the optimum time for xylanase production by *B. subtilis* LBF M8, this result indicates *B. subtilis* LBF M8 takes a long time to hydrolyse xylan from corn cob because corn cob was still raw materials. The further fermentation process, the medium can be incubated for 96 h. Gupta *et al.*, (2009) reported maximum xylanase production by *Bacillus* sp. after 72 h and Tork *et al.*, (2013) at 4 days 96 h incubation using corn cob as a substrate.

Effect of initial pH medium for xylanase production: The pH medium strongly affects the enzymatic processes and transport in the system which then will affect various components across the cell membrane and an unfavourable pH may limit the growth and consequently xylanase production by substrate inaccessibility (Nagar *et al.*, 2012; and Kumar *et al.*, 2017). To study the effect of different pH medium for xylanase production, the pH of the medium was tested at 5-9. Fig 2 (B) showed highest xylanase activity at pH 6 (2.358 U/mL) and lower at pH 5 (1.741 U/mL) for 24 h incubation. The pH optimum for xylanase production by *B. subtilis* LBF M8 was found to be in the acidic range, this result indicates *B. subtilis* LBF M8 tolerant to acidic conditions. The xylanase activity obtained at various pHs did not significantly indicate the pH condition of the production media affecting xylanase activity by *B. subtilis* LBF M8. The optimum pH 6 found in this study

was similar to the xylanase from *B. Amyloli- quifaciens* Sh8 (Kumar *et al.*, 2017). Some marine bacteria have been reported to have various pH range for xylanase production of such pH 6 by marine bacteria *B. subtilis* cho40 (Khandeparker *et al.*, 2011), pH 6.5 by marine bacteria *B. Pumi- llis* (Wijaya *et al.*, 2016), pH 7 by marine bacteria *B. safencis* P20 (Rahmani *et al.*, 2014), pH 8 by marine bacteria *B. subtilis* XP10 (Tork *et al.*, 2013).

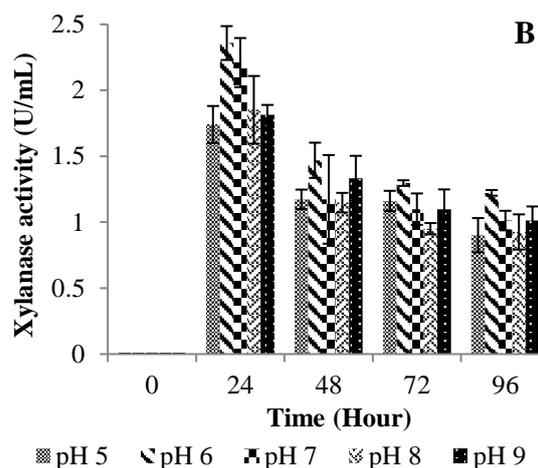


Fig. 2B: Optimization of Process Parameters for xylanase production by *B. subtilis* LBF M8. Effect of initial pH medium for xylanase production,

Effect of incubation temperature for xylanase production: To produce high enzyme titers in enzyme production using microbes requires optimal growth temperatures, because temperature has a major influence on enzyme activity. enzyme production processes on an industrial scale often face problems at optimum temperatures needed for enzyme production (Nagar *et al.*, 2012, Kumar *et al.*, 2017). To study the optimal incubation temperature, production was carried out at several different temperatures (20-50°C) to see the maximum xylanase production. The results (Figure 2C) show that at an incubation temperature of 30°C (2.235 U/mL) the optimum production temperature and at a temperature of 20 and 50°C the bacteria do not grow well, resulting in a decrease in enzyme production up to 0.556 U/mL. The highest xylanase activity at 30°C and 40°C did not have a significant difference which showed that the optimum temperature for xylanase production by *B. subtilis* LBF M8 was between 30-40°C incubation temperature. This optimum temperature condition is similar to the results obtained by Irfan *et al.*, (2015) which showed 35°C temperature is the optimum condition for xylanase production by *B. subtilis*. At an incubation temperature 50°C significantly decreases xylanase activity. Tork *et*

al., (2013) reported a decrease in xylanase activity at 50°C and obtained an optimum temperature of 40°C for xylanase production by *B. subtilis* XP10.

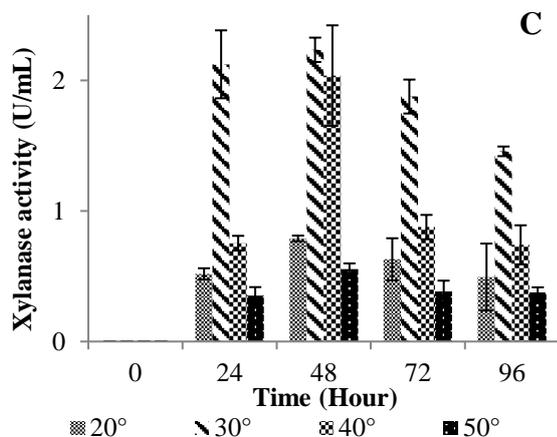


Fig. 2C: Optimization of Process Parameters for xylanase production by *B. subtilis* LBF M8. Effect of incubation temperature for xylanase production

Effect of Agitation rate for xylanase production: Optimization of agitation rate in flask scale is very rare, whereas agitation rate affects the microorganism growth, increases the oxygen transfer, and mixing of the production broth that fermentation condition is homogenous. Some agitation rate increases the hydrolysis rate, but excessive mixing can deactivate the enzyme and reduce the conversion yield (Kumar *et al.*, 2017). The effect of agitation on xylanase production was examined by growing cultures at different agitation rates (0-250 rpm). Fig 2 (D) showed the highest xylanase activity at 200 rpm (2.963 U/mL) and the lower at 0 rpm (1.593U/mL). Lower activity at 0 and 50 rpm shows the importance of agitation rate, lack of stirring causes low bacterial cell growth and non-homogeneous conditions of medium fermentation. At 100 rpm has a greater than 150 and 250 rpm, this result showed the high rate of agitation can lead the bacteria growth not optimal and affects the amount of enzyme produced (Luis *et al.*, 2003). Result described fermentation with 200 rpm was optimum for xylanase production by *B. subtilis* LBF M8. This result indicates at 200 rpm agitation rates can lead to homogeneous fermentation conditions, increased cell growth, and the amount of dissolved oxygen.

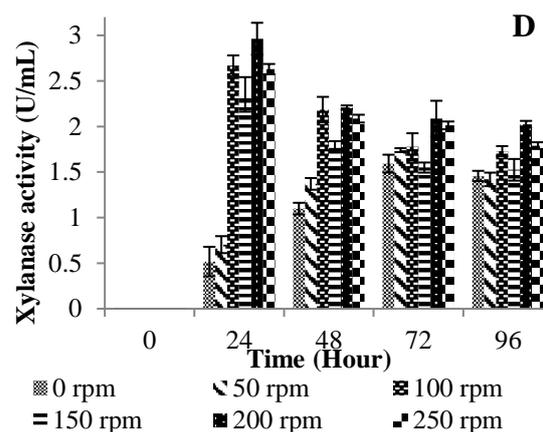


Fig. 2D: Optimization of Process Parameters for xylanase production by *B. subtilis* LBF M8. Effect of agitation rate for xylanase production.

Effect of different material for xylanase production: Different materials were tested for production xylanase as a substrate and carbon source. Additional carbon source in the fermentation medium was further enhanced xylanase production. Based on the optimum condition, *B. subtilis* LBF M8 will be produced xylanase by using beech wood xylan, rice straw, sugarcane bagasse, and corn cob as a carbon source. Fig 3 exhibit maximum xylanase production by using beechwood xylan as carbon source (3.790 U/mL). For production using agricultural waste, production with corn cob (2.395 U/mL) is higher than rice straw (1,938 U/mL) and sugarcane bagasse (1.728 U/mL). This result was higher than was obtained by Tork *et al.*, (2013) produced xylanase by *B. subtilis* XP10. The highest enzyme production was produced using xylan is 2.82 U/ml and 1.8 U/ml using corn cob. Irfan *et al.*, (2012) reported maximum xylanase production by *B. subtilis* using various agricultural wastes were observed in sugarcane bagasse and corn cob showed the lower activity, but in this research, corn cob has the higher activity over sugarcane bagasse.

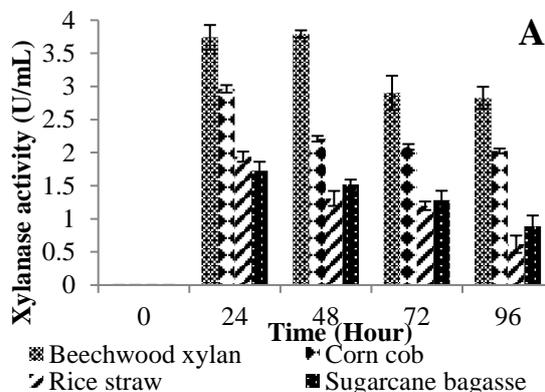


Fig. 3A: Xylanase production using different material. The effect of using beechwood xylan, rice straw, sugarcane bagasse, and corn cob as a substrate

Effect of xylan concentration on xylanase activity: Enzyme activity was found to be greatly affected by substrate concentration. Different xylan concentration ranging from 0.5 to 2.5% was tested to check the optimum on xylanase activity. Maximum xylanase activity obtained from 24-hour incubation reduce by activity at 0 h. Fig 3B showed 2.5% beechwood xylan concentration was found maximum xylanase activity using beechwood xylan, rice straw, sugarcane bagasse, and corn cob. Xylanase activity shows a gradual increase of beechwood xylan concentration 0.5% -2.5% (4.062 to 13.321 U/mL), rice straw (2.259 to 8.506 U/mL), sugarcane bagasse (2.049 to 8.346 U/mL) and corn cob (3.099 to 7.086U/mL). These results indicate the higher the xylan concentration used will increase the xylanase activity. This result similar with Kumar *et al.*, (2017) who tested the effect of different concentration of xylan in the range of 0.2%, 0.4% and up to 2.0%, the enzyme increases from 0.2% to 1.8% and contact at 2.0%. In general, an increase in substrate concentration can increase the speed of enzyme reactions at a certain substrate level, but an increase in substrate concentration can also show a substrate saturation point, which then leads to an increase in negligible nil or enzyme activity (Luis *et al.*, 2003). Different xylan concentration for xylanase activity was reported 0.5% (Annamalai *et al.*, 2009; and Menon *et al.*, 2010), 1% (Yopi *et al.*, 2017, Kumar *et al.*, 2017).

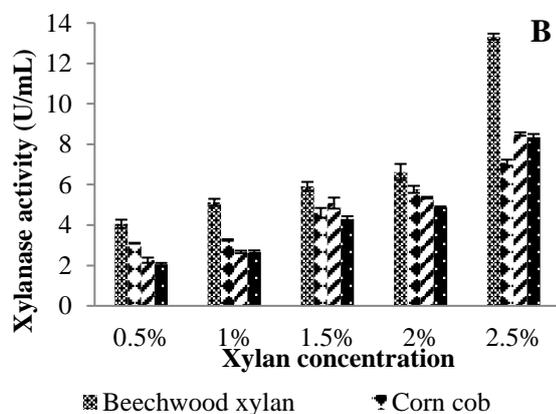


Fig. 3B: Xylanase production using different material. The effect of xylan concentration on xylanase activity.

Scale-up for xylanase production in stirred tank bioreactor: Increased production to a larger scale is expected to have higher production activities than the flask scale. Xylanase production is increased on a large scale using a 2L stirred tank bioreactor with 1L working volume. In bioreactor-scale xylanase production using corn cobs, it showed higher xylanase activity compared to production on the flask scale with the same production parameters. Increased xylanase activity on the biore-

actor scale can be caused by efficient aeration rates, better mixing and heat transfer (Kumar *et al.*, 2017). Time production up to 72h is done because the industrial process requires a fast production time and *B.subtilis* LBF M8 has the optimum time of production at 24 h.

Fig 4 showed the comparison xylanase production at flask scale and scale-up bioreactor. Scale-up production in a stirred tank bioreactor shows higher activity (3.519U/mL) than flask scale production (2.395U/mL) on the same of production parameters condition (1.5% corn cob concentration, pH medium 6, 30°C fermentation temperature, and 200 rpm agitation rate). These results are the same as those obtained by Kumar *et al.*, (2017) was scaleup xylanase production in stirred tank bioreactor. Measurement of bacterial cell growth was carried out using spectrophotometry at a wavelength of 660 nm. Based on the comparison between the cell growth and the xylanase activity, it can be concluded that the increase of the cell growth is consistent to the increasing of xylanase activity (Rahmani *et al.*, 2014). The optical density does not show any effect compared to xylanase activity due to the higher OD 660 on flask scale production did not show higher xylanase activity more than scale-up production in stirred tank bioreactor.

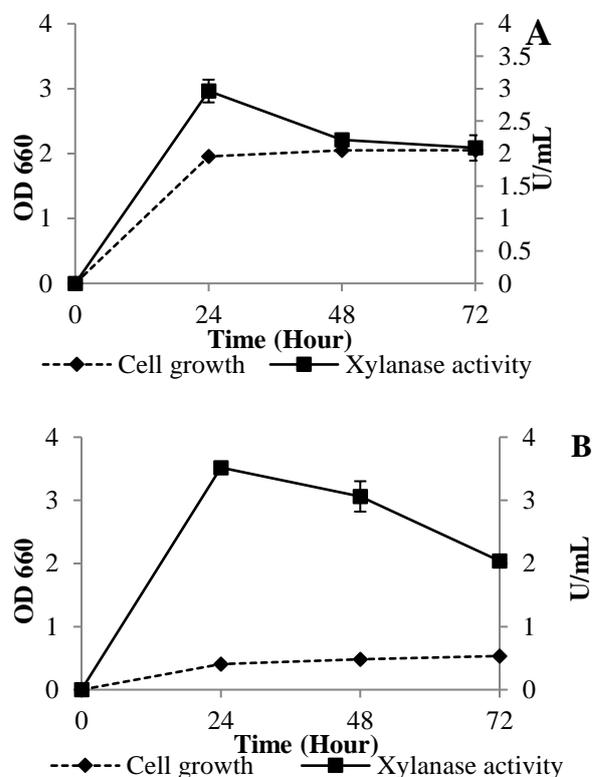


Fig. 4 Comparison xylanase production at flask scale and scale-up bioreactor. (A) Cell growth (OD 660) and xylanase activity at flask scale production and (B) Cell growth (OD 660) and xylanase activity at scale-up production in stirred tank bioreactor.

CONCLUSION

Based on the qualitative analysis, the diameter of clear zone indicates *Bacillus subtilis* LBF M8 have xylanolytic activity, although isolated based on manolitic abilities. The optimization parameter process increased xylanase production by *Bacillus subtilis* LBF M8 using corn cob as a substrate. Before optimization, the highest xylanase activity is 1.864U/mL for 96 h and after optimization increased to 2,395 U/mL for 24 h incubation time. In the comparison of production substrates, production using corn cobs has a higher activity than other agricultural wastes. Scaleup production in 2L stirred tank bioreactor increased xylanase activity to 3.519 U/mL with the optimum condition at flask scale production. The final results showed that the optimization process of production parameters and increased production at the bioreactor scale could increase xylanase production by using *B. subtilis* LBF M8.

Acknowledgment

This research was supported by Biocatalyst and Fermentation Laboratory (LBF), Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong, Bogor, Indonesia.

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