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OPTIMIZATION PRODUCTION OF XYLANASE BY *Bacillus pumilus* IN EMPTY FRUIT BUNCH USING 3 L BIOREACTOR

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ABSTRACT

Indigenous biomasses, such as empty fruit bunch, contain high xylan and are useful to produce raw xylanase, where this biomasses are very abundant in Indonesia. The focus of this research is to scale up the fermentation product into 3 litter feeds and to record the highest enzyme activity. Fermentation process has been done by using stirred fermentor MBI Winpact One Fermentation System. The isolate marine bacterium *Bacillus pumilus* has been used to degrade empty fruit bunch to produce raw xylanase. The result for different agitation speed showed that at 150 rpm has the highest xylanase activity. The effect of different aeration flows gives at a rate of 4 VVM achieved the highest enzyme activity and equal to 7.61 U/mL with cell growth equal to 2.06. The Sigma Antifoam C has been successfully being used as deformer in the production fermentor and can be tolerated by *Bacillus pumilus*. The comparisons of different medium to generate different enzyme by *Bacillus pumilus* has achieved xylanase, mannanase, and cellulase, where xylanase has the highest enzyme activity among all of the enzymes produced.

Keywords: xylanase, enzyme activity, empty fruit bunch, and fermentor or bioreactor

ABSTRAK

Tandan kosong kelapa sawit yang sangat melimpah di Indonesia adalah biomassa yang memiliki kandungan kadar xilan yang tinggi dan sangat berpotensi dalam produksi xilanase baku. Penelitian ini berfokus pada peningkatan produk fermentasi menjadi 3 liter dan juga pencatatan aktivitas enzim tertinggi. Proses fermentasi telah dilakukan dengan menggunakan fermentor pengaduk bermerek MBI Winpact One Fermentation System. Isolat bakteri laut Bacillus pumilus *telah digunakan untuk mendegradasi tandan kosong kelapa sawit dan menghasilkan xilanase baku. Hasil untuk kecepatan agitasi yang berbeda-beda menunjukkan bahwa pada 150 rpm menghasilkan xilanase tertinggi. Aerasi yang dihasilkan pada kecepatan 4 VVM memiliki aktivitas enzim tertinggi yang sama dengan 7,61 U/mL serta dengan pertumbuhan sel yang sama dengan 2,06. Sigma Antifoam C juga telah sukses digunakan sebagai* defoamer *dalam fermentor produksi, di mana defoamer ini dapat ditoleransi oleh* Bacillus pumilus*.* Bacillus pumilus *juga dapat menghasilkan xilanase, mananase, dan selulase pada media yang berbeda, setelah dibandingkan didapatkan bahwa xilanase memiliki aktivitas enzim tertinggi dibandingkan enzim lainnya.*

Kata kunci: *xilanase, aktivitas enzim, tandan kosong kelapa sawit, dan fermentor atau bioreaktor*

INTRODUCTION

Many agricultural waste resources contain xylan as one of the polysaccharides. According to the previous studies on agricultural wastes, bagasse has 9.6% xylan, wheat bran has 12.3% xylan, corn cob has 12.9% xylan, rice husk has 6.3% xylan, nutshell has 6.3% xylan, skin cottonseed has 10.2 % xylan,[1] rice straw has 19.3% xylan,[2] empty fruit bunch has 20.7% xylan,[3] coconut cake has 6% xylan,[4] and Tobacco stalk has 22% xylan.[5]

Xylan is composed of a backbone of 1,4-linked D-xylose units, where the promising agricultural wastes are empty fruit bunch that contain xylan composition up to 24% weight.[6] Xylan hydrolysis needs xylanases as an enzyme, where many applications of xylanase were used by bioconversion of lignocellulosic materials, such as for fermentable sugars, textile, animal

feed, pulping, bread quality, clarification of juices and wine, improvement, and also bleaching process.[7,8,9,10,11]

Development lignocellulose as an abundant renewable resource has been studied recently. In Southeast Asia, huge biomass streams are produced as waste materials by the agricultural industries.[12] Biomass from agro-industrial waste is abundant in Indonesia where there are plenty of promising products that are useful for functional food. Functional food is used to add beneficial bacteria work to process into food which is very useful. One of the abundant agricultural industries in Indonesia is palm oil industry.[13] The main use of palm oil is for cooking oil which is produced by milling and refining, while one of the wastes of palm oil industries is an empty fruit bunch that can be converted to functional food by enzymatic hydrolysis. Enzymatic hydrolysis is a catalytic decomposition of a chemical compound by reaction with water, such as the conversion of cellulosic materials into fermentable sugars by the addition of specific enzymes. Enzymatic hydrolysis requires microbe that can convert the biomass into the desired product. One of enzymatic hydrolysis is xylanase in which this enzyme can convert xylan to xylooligosaccharide. One of the xylanases producing microbial is *Bacillus pumilus,* where previous research have reported the positive xylanase production for paddy husk in solid state fermentation.[14,15] Previous result reported that in lab scale 20 mL in 100 mL flasks where *Bacillus pumilus* has shown that the highest enzyme activity with the following conditions that are biomass empty fruit bunch in 2.5 % (w/v) concentration, lactose broth in 0.6 % (w/v), the pH values was 6.5, the temperature condition was 30^{o}C and given the xylanase activity 10.85 U/mL.[16]

This research aims to produce XOs in three litter batch fermentor in MBI Winpact One Fermentation System and to optimize the aeration and agitation condition, where it is an important factor to scale up the industry size need to optimise the power use in agitation and aeration.[17,18]

MATERIAL AND METHODS

a. Material and Medium

Empty fruit bunches from agro-waste lignocellulosic materials are provided from Tangerang and its surrounding areas. This material was dried at 40°C for twelve hours and then it was cut and reduced in size to 200 Mesh. The microorganism used in this research was *Bacillus pumilus* where it is a collection of laboratorium biocatalysts and fermentation, Biotechnology Research Center LIPI.

b. Pre-culture Medium

Pre-culture medium in 200 mL contains 38 g/L artificial sea water, 5 g/L peptone, 1 g/L yeast extract, and 0.5% (w/v) beechwood xylan. Pre-culture medium incubated at 30^{o}C and 150 rpm for 24 hours.

c. Culture Medium

The fermentor used was MBI Winpact One Fermentation System.[19] Culture condition using empty fruit bunch containing 2.5% (w/v) of biomasses in 200 Mesh, artificial sea water 38 g/L, lactose broth at 0.6% (w/v), and distilled water until 3 L. The process was finished in 36 hours according to previous literature.[16] During the fermentation process, if necessary, 3% of antifoam C emulsion from SIGMA-ALDRICH® was added to reduce the foaming inside the fermentor. The temperature condition was set to room temperature and left to occur naturally. A mixture of 0.1 M solution NaOH and 0.1 M solution HCl was used to stabilize pH at 7.

d. Reactor Operation MBI Winpact Fermentor

In this study, The MBI Winpact fermentor was carried out in a 5 L with working volume of 3 L. The fermentor has vessel with single wall, the agitation motor by Brushless motor, and the impeller type is Rushton, the aeration provided by orifice ring sparger and attached by air filter 0.2 µm PTFE Acro®50, whereas this conditions will sufficient to agitate properly. The fermentor was sterilized at 121^{o}C along with culture medium. After the cooling process for 24 hours, the

medium was inoculated with 200 mL inoculums from the pre-culture medium.

The pH was adjusted to 7 and the pH adjusters were prepared with NaOH 0.1 M and HCl 0.1 M. Anti-foam were added by Antifoam C Emulsion from SIGMA-ALDRICH® in 3% dilution with distilled water. In order to study the effect of the impeller speed on xylanase production, three different speed experiments were conducted, i.e. 100 rpm, 150 rpm, and 200 rpm at 4 vvm (vessel volume per minute) air flow in constant. The agitation rate was determined by using digital clamp meter MT 87 CAT II 600V EMC-LVD and it will be discussed further in Results and Discussion section. After the optimal impeller speed or agitation achieved, the next step is the aeration rate optimization on xylanase production in three different experiments, i.e. without air supply, using air supply 2VVM, 4 VVM and 6VVM. Figure 1 shows that fermentor MBI Winpact One Fermentation System is in progress for 150 rpm and 4 vvm aeration.

e. Analytical Techniques

Step one is procedure sampling and cell growth. At the specific time intervals, a sample in a volume of 10 mL from the fermentor was removed at 0, 6, 12, 28, 24, 30, and 36 hours. The cell growth samples were prepared by 1 mL sample and was measured by spectrophotometer at λ = 660 nm. The separation of crude enzyme and biomasses and others solid particle was carried out by using a centrifuge at 3.540 x *g* for ten minutes. After the enzyme has been harvested, it was stored in -20^{o}C for further analysis.

Figure 1. Fermentor MBI Winpact One Fermentation System for biomasses fermentation.

The xylanase activities were measured by dinitro salicylic acid (DNS) method.[20] Each sample has a mixture of 250 µl enzyme and 250 µl substrat containing 0.5% (w/v) xylan from beechwood SIGMA-ALDRICH® along with phosphate buffer 50 mM. The mixture was incubated for fifteen minutes at 60^{o}C and the reaction was stopped by the addition of 500µl DNS. After that, the reaction mixtures were vortex and were boiled for fifteen minutes at 100^{o}C and cooled quickly at 0^{o}C in ice for about ten minutes. The enzymatic hydrolysis of xylanase was determined by measuring the absorbance at 540 nm. The blank and control were prepared same as the samples procedure and the obtained data will decrease for gaining the actual of xylanase activity. The amount of xylanase activity was defined as the amount of an enzyme liberating reducing sugars that equivalent to 1µmol of xylose per minute.

The mannanase activities were measured by dinitro salicylic acid (DNS) method.[20] Each sample has a mixture of 250 µl enzyme and 250 µl substrate containing 0.5% (w/v) locus bean gum from ceratonia siliqua seeds SIGMA-ALDRICH® along with phosphate buffer 50 mM. The mixture was incubated for fifteen minutes at 60^{o}C and the reaction was stopped by the addition of 500 µl DNS. After that, the reaction mixtures were vortex and boiled for fifteen minutes at 100^{o}C and cooled quickly with 0^{o}C in ice around ten minutes. The enzymatic hydrolysis of mannanase was determined by measuring the absorbance at 540 nm. The blank and control were prepared same as the samples procedure and the obtained data will decrease for gaining the actual of mannanase activity. The amount of mannanase activity was defined as the amount of an enzyme liberating reducing sugars that equivalent to 1 µmol of xylose per minute.

The cellulase activities were measured by dinitro salicylic acid (DNS) method.[20] Each sample has a mixture of 250 µl enzyme and 250 µl substrate containing 0.5% (w/v) carboxymethyl cellulose sodium salt Nacalai tesque along with phosphate buffer 50 mM. The mixture was incubated for fifteen minutes at 60°C and the reaction was stopped by the addition of 500 µl DNS. After that, the reaction mixtures were vortex and were boiled for fifteen minutes at 100°C and cooled quickly with 0°C in ice for about ten minutes. The enzymatic hydrolysis of cellulose was determined by measuring the absorbance at 540 nm. The blank and control were prepared same as the samples procedure and the obtained data will decrease for gaining the actual of cellulase activity. The amount of cellulase activity was defined as the amount of an enzyme liberating reducing sugars that equivalent to 1 µmol of xylose per minute.

RESULTS AND DISCUSSION

a. Testing the 5 L MBI Winpact Fermentor

The MBI Winpact fermentor was used to culture the isolate marine bacterium *Bacillus* sp. LBF-001. The first test is to determine the agitation rate and power ratio by using digital clamp meter MT 87 CAT II 600V EMC-LVD in presented the Figure 2.

The power consumption test result shows that the fermentor was in steady state condition at 50–200 rpm and this can lead to efficiency and low power consumption. The agitation rate process is defined as Newtonian fluid where the viscous stresses arise from its flow, at every point, it is linearly proportional to the local strain rate. Figure 3 shows that the power consumption increased as well as the agitation speed. This phenomenon is similar to shaken bioreactors, in which power consumption is one of the most important criteria of successful scale up and has a direct influence on mass transfer as well as hydro-mechanical characteristics.[21] Agitation provided by the impeller in Rushton type which has three stable patterns of parallel, merging, and diverging flows.[22] The optimum agitation will be achieved by obtaining highest enzyme activity.[23] Furthermore, next step will be conducted on biomass culture for 100 rpm, 150 rpm, and 200 rpm agitation speed and compare it with the highest enzyme activity.

b. The Effect of Different Agitation Speed.

The agitation speeds are based on the power consumption result with the aeration of 4 vvm as

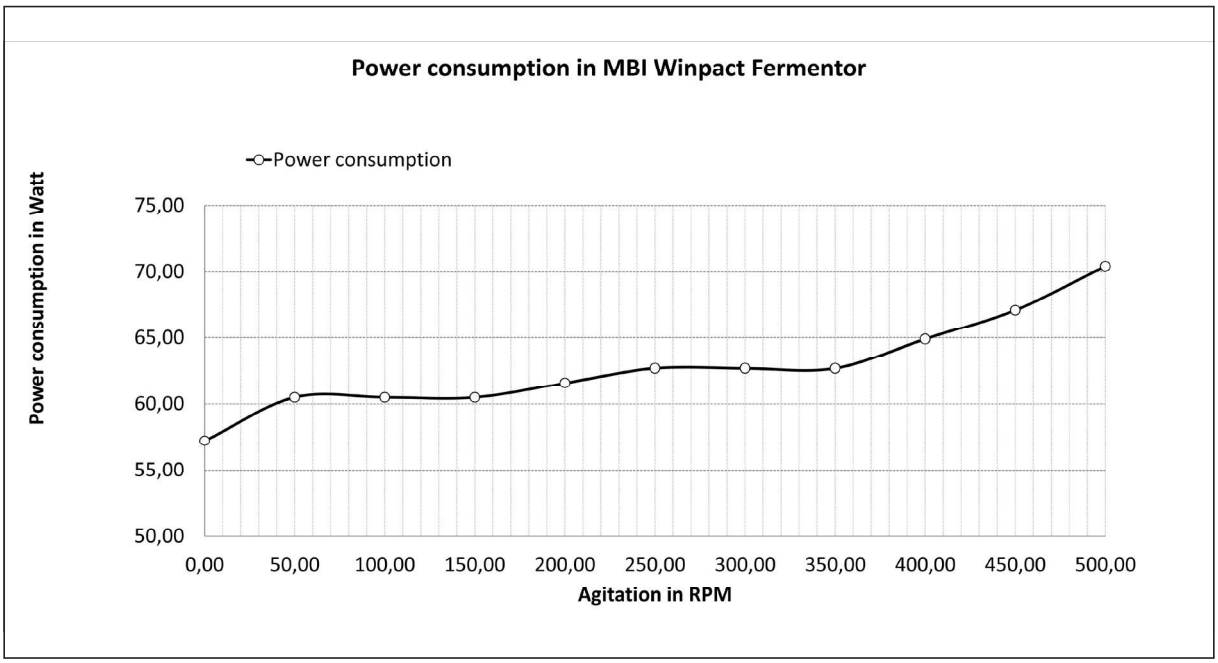

Figure 2. Power Consumption in Different Agitation at Stirred Tank 3 L MBI Winpact Fermentor

in Figure 3. The different agitation speed results showed that the highest xylanase activity at 150 rpm. The optical density do not show any effect compared to xylanase activity.

Based on the comparison between the cell growth and the xylanase activity, it can be concluded that the increasing of the cell growth is consistent to the increasing of xylanase activity and also the reduction. This phenomenon leads to a connection between the cell growth and enzyme activity.[24] Figure 4 shows there are no cell growth connections between 100 rpm, 150 rpm, and 200 rpm. The agitation speeds are based on the power consumption result with the aeration of 4 vvm as it was previously showed in Figure 3. The different agitation speed results showed that the highest xylanase activity was at 150 rpm that equal to 7.61 U/mL with cell growth equal to 2.06. The optical density do not show any effect compared to xylanase activity due to the turbidity different from the biomass and the difficulty in obtaining cell growth since the empty fruit bunch is not soluble in sea water.

c. The Effect of Different Aeration Flow

Studying the effect of different aeration flows is needed to optimize the enzymatic result.[25] Figure 4 shows the aeration flow effect in 2 vvm and 6 vvm with the data result of 4 vvm as comparison.

Figure 4 shows that 4 vvm of aeration is the best result for optimum xylanase activity and, as described previously, the cell growth do not show any effect on the xylanase activity due to the turbidity. The oxygen demand for the growth

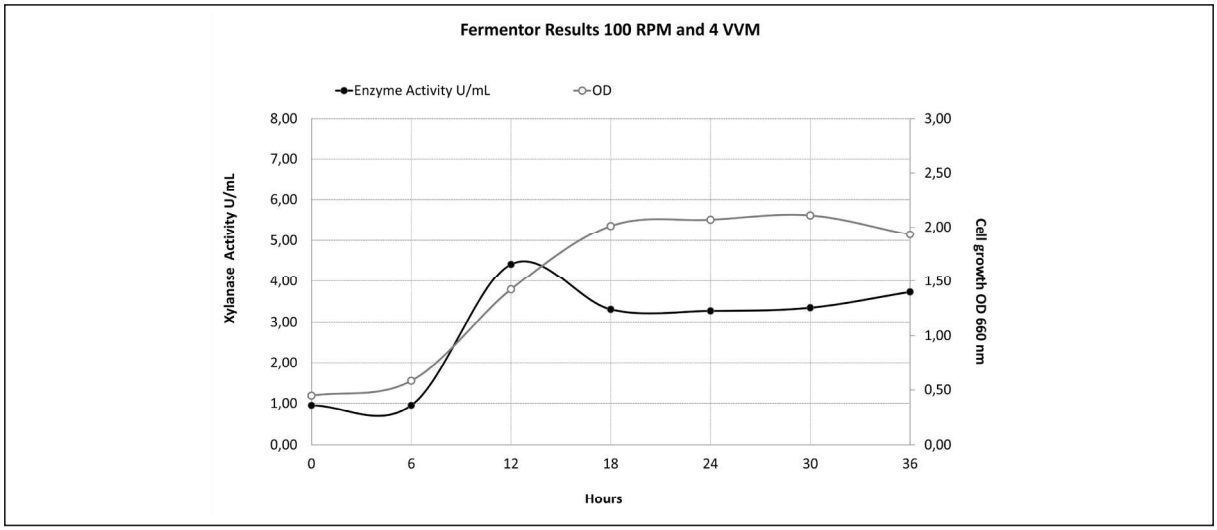

Figure 3. The Effect of Different Agitation Speed Result in 4 vvm and for 36 Hours

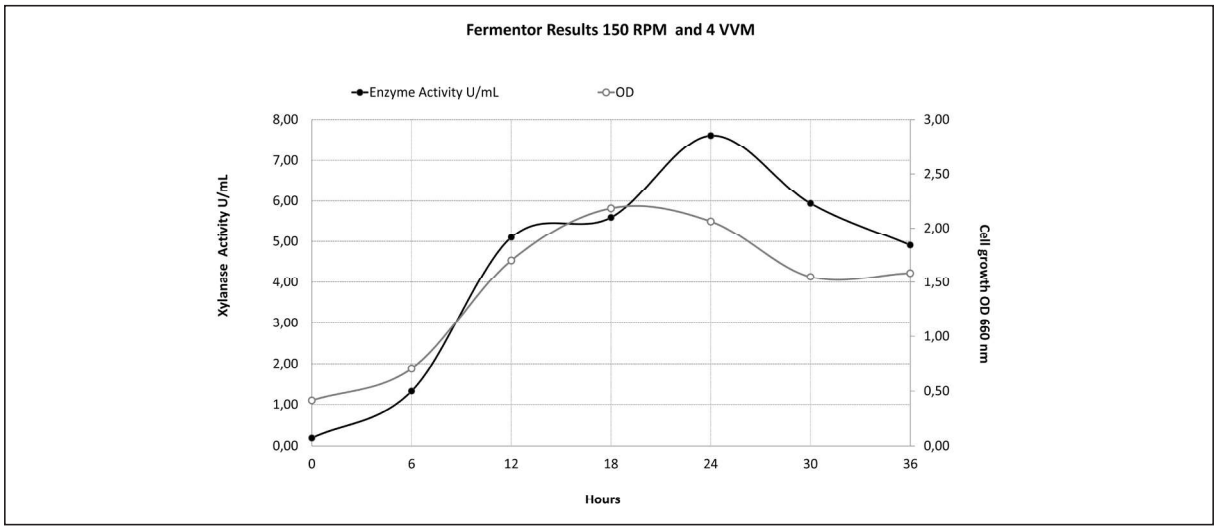

Figure 4. The Effect of Different Aeration Result at 150 rpm for 36 Hours

of microbial is essential, in which inducing or repressing several enzyme systems and activating the oxidative reactions to utilise for nutrient needs and to generate energy.[26] Comparing the results from Figure 3, Figure 4, and Figure 5, the agitation tendencies were stable state at every speed, but only at 150 rpm and 4 vvm the results give the highest enzyme activity, where this flow pattern in the form of complete dispersion.[27] Tinoco-Valencia et al. revealed another effect of agitation and aeration which is to demonstrate the energy dissipation or circulation function which is a valuable hydrodynamic parameter for evaluating the correlation between the mechanical shear stress and the growth rate or process productivity cultures. [28] The aeration has significant effect to produce xylanase rather than a static environment.[17] Agitation is necessary to maintain homogeneity and avoiding the formation of large and metabolically inactive pellets. Palma et al. mentioned that shearing forces may disrupt fragile microbial tissue and have a marked influence on xylanase production, called hydrodynamic stress.[29] The low enzyme activity at below 150 rpm dan below 4 vvm indicated that the dissipation or circulation was not at the highest contact, while higher than 150 rpm and higher than 4 vvm indicated that the isolate LBF-001 at hydrodynamic stress.

d. The Temperature Profile in Fermentor Operation.

In the fermentor MBI Winpact One Fermentation System, the temperature profile can be shown in Figure 6. The temperature was not set by the system and it occured normally depending on the reaction between the biomass and the microorganism itself. The temperature of 30°C in the middle of the process can be said as the almost stable condition. According to the small scale production by previous lab work,[16] the optimum growth and xylanase activity for LBF-001 is at 30°C .

e. pH Profile in the Fermentor.

Figure 7 shows the pH profile which was set in 7 by using pH adjusters NaOH 0.1 M and HCl 0.1 M, it can be shown that the pH tends to build up base form. This may indicate the reaction system in producing the base form condition where the bacteria produces xylanase at higher pH values and lead to the metabolism characteristic.[30,31] At the fermentation systems, the base form of reaction between bacteria and the substrate normally occurs as previously reported.[32] Some research reported that *Bacillus* sp. tolerates alkaline conditions and produces base or alkaline conditions.[33]

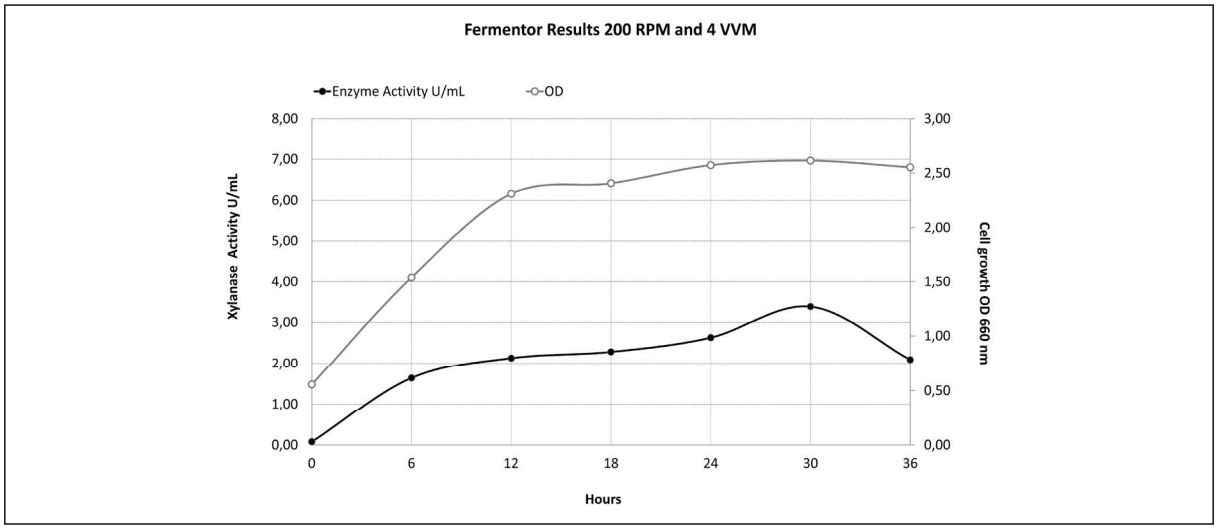

Figure 5. Agitation Profile in Fermentor MBI Winpact One Fermentation System

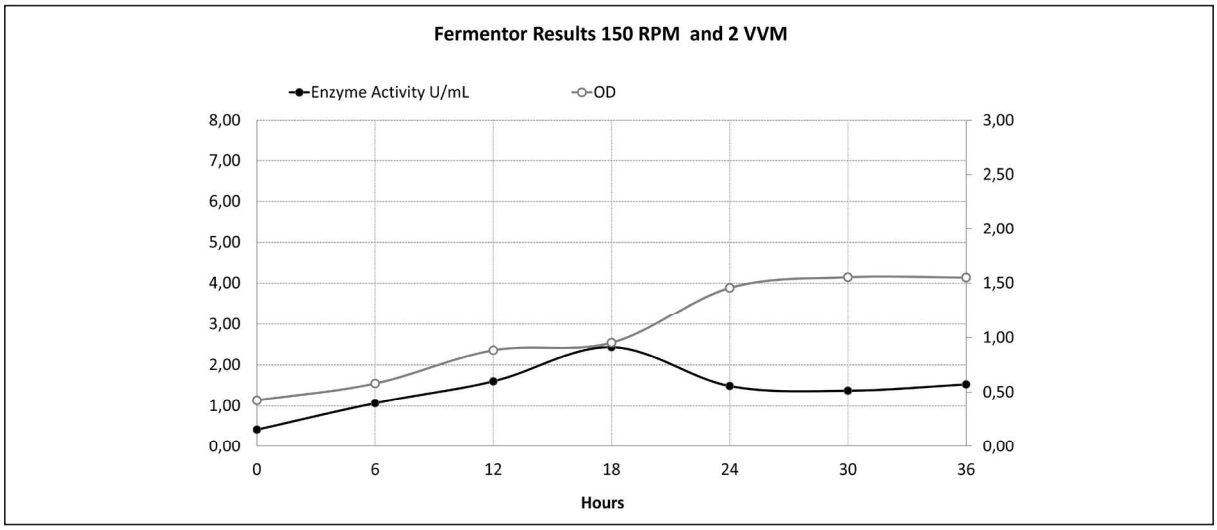

Figure 6. The Temperature Profile in Fermentor MBI Winpact One Fermentation System

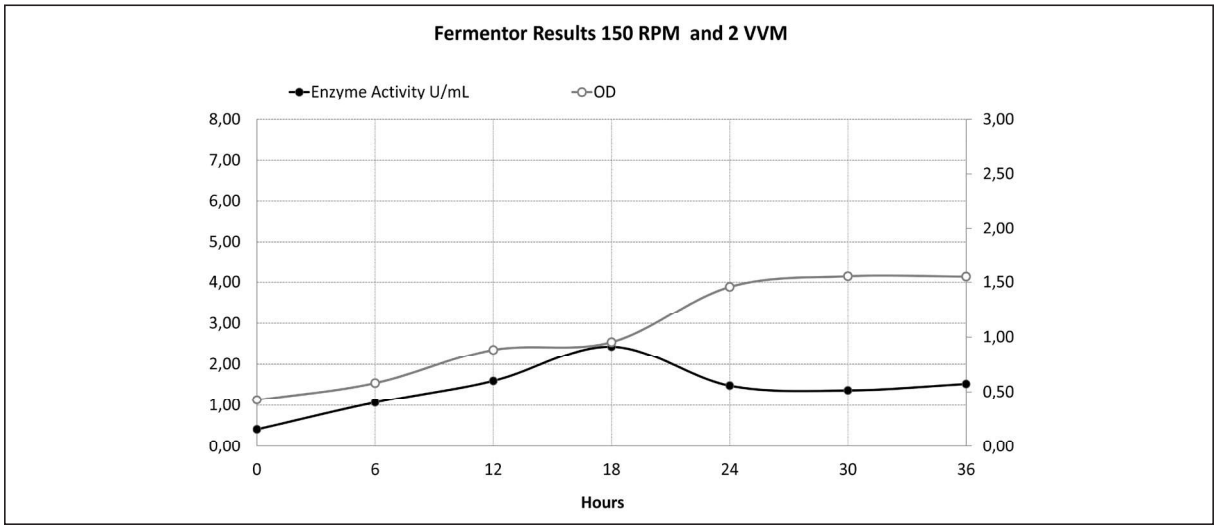

Figure 7. pH Profile in Fermentor MBI Winpact One Fermentation System

f. Acid, Base, and Defoamer Antifoam C Consumption

Figure 8 shows the increasing acid and base consumption when xylanase needs more acid stabilisation in optimum condition indicating the reaction tends to produce base form condition. On the contrary, the negative effect of xylanase production tends to produce acid condition. The foaming may occur during the production of enzymes.[34] The foaming tendency is one of crucial factors in bioreactor production where it can lead in reducing productivity process since bursting bubbles can damage proteins, while the foam that escapes the bioreactor would cause the sterility loss,[35] and also over-pressure condition can damage the systems.[36] The use of Sigma Antifoam C has no effect up to 8% on growth rate, while this research used 3% of dilution confirming that there was not any effect on isolate LBF-001 growth rate by using Sigma Antifoam C.[35] Comparing Figure 8 to Figure 3 and Figure 4, the foaming tendency would occur in line with the increasing of enzyme activity which led to the possibility of isolate LBF-001 to produce the xylanase along with foaming occurrence.

g. Comparisons of Enzyme Activity with Isolate LBF-001

Figure 9 is the comparison of isolate LBF-001 in single manner sampling. The test used a different medium to generate different enzyme and concluded that isolate LBF-001 has produced

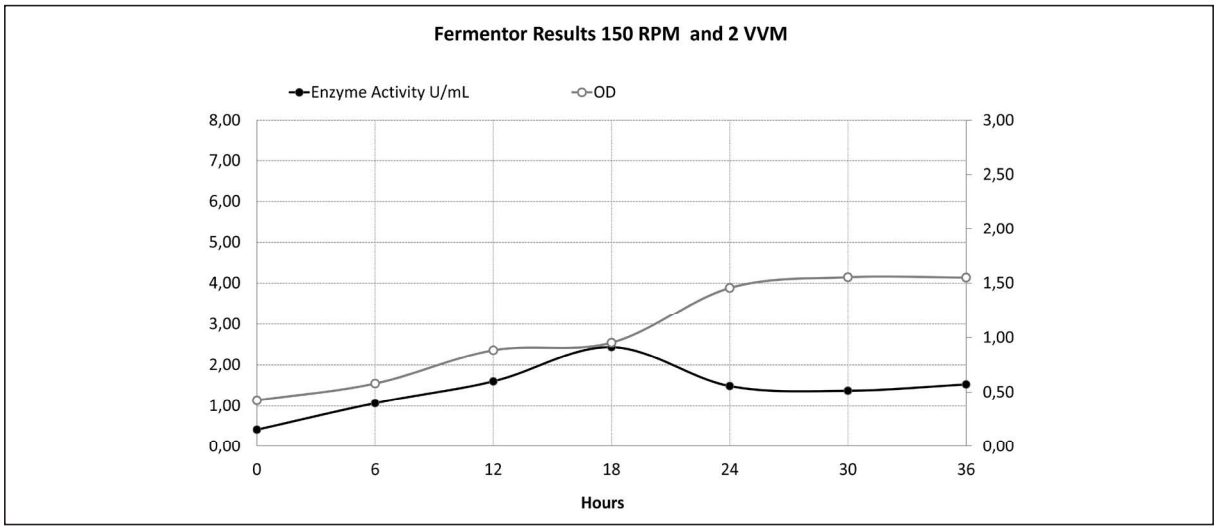

Figure 8. Acid, Base, and Defoamer Antifoam C Consumption

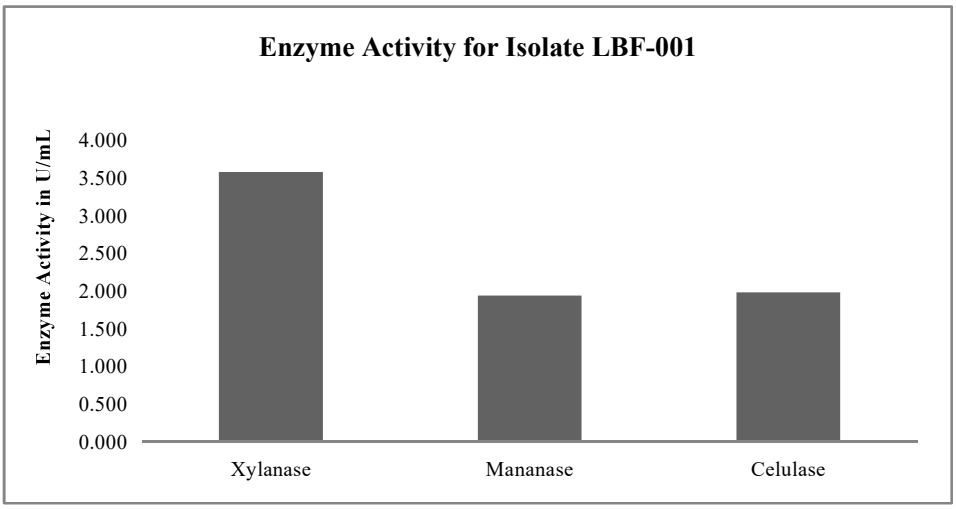

Figure 9. Comparisons of Enzyme Activity with Isolate LBF-001

three types of enzymes, i.e. xylanase, mannanase, and cellulase. The test was taken for about 36 hours in 150 rpm of rotational speed, 4 vvm of gas flow, and 30°C of temperature. The highest enzyme production are xylanase at 3.56 U/mL, mannanase at 1.93 U/mL, and cellulase at 1.97 U/mL. The product results indicate that LBF-001 has the potential to produce xylanase rather than any of other enzymes. *Bacillus pumilus* has reported its capability to produce mannan endo-1,4-β-mannosidase.[37] Cellulase production from *Bacillus pumilus* has been reported resulting degradation of cellulose materials effectively.[38] Other application *Bacillus pumilus* produced cellulase free and alkali stable xylanase in high levels are known to secrete high levels of extracellular xylanases which are either free or poor in cellulase activity.[31]

CONCLUSION

The fermentation with MBI Winpact by *Bacillus* sp. LBF-001 used an empty fruit bunch raw biomass in which the experiments were carried out under aerobic condition. The power consumption test result showed that the fermentor was in steady state condition at 50–200 rpm and could lead to efficiency and low power consumption. The different agitation speed result showed that the highest xylanase activity was at 150 rpm. The different aeration flows effect achieved the

highest enzyme activity at 4 vvm that is equal to 7.61 U/mL with cell growth equal to 2.06. The Sigma Antifoam C has successfully used as a defoamer in the fermentor production and can be tolerated by isolate LBF-001. The comparisons of isolate LBF-001 in using different mediums to generate different enzymes concluded the highest enzyme activity for isolate LBF-001 is xylanase activity and isolate LBF-001 can produce three types of enzymes, namely xylanase, mannanase, and cellulase. Further analysis for larger scale production needs to be considered for the industrial approach.

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