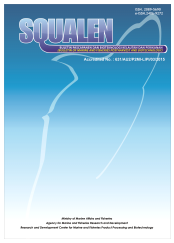

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THE OPTIMAL CONDITIONS OF XYLANASE PRODUCTION USING EMPTY FRUIT BUNCH RAW BIOMASS BY MARINE ISOLATE LBF-001

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Abstract

Several xylanases have already been studied. However, only a few xylanases derived from marine microorganisms has been reported. Marine bacterium LBF-001 was isolated from Pari Island Kepulauan Seribu, Indonesia. The purpose of this study is to identify isolate LBF-001 using 16S rDNA gene and to optimise the medium conditions for the xylanase production i.e. concentration of biomass, nitrogen source, pH and temperature. Based on 16S rDNA gene analyses, LBF-001 isolate has 99% similarity with *Bacillus pumilus* HNS70 (KF933667). Fermentation process to produce xylanases was conducted using several agricultural residues under solid-state fermentation (SSF). The optimum condition for xylanase production by *B. pumilus* LBF-001 was using a medium containing 2.5% empty fruit bunch and 0.6% lactose broth, at pH 6.5, temperature 30 °C, under submerged fermentation with shaking at 150 rpm for 48 h fermentation. The optimised condition resulted higher xylanase activity, i.e. 10.85 U/mL.

Keywords: empty fruit bunch, marine bacteria, optimisation, submerged fermentation, xylanase

1. Introduction

Many agricultural wastes contain xylan. Xylan composition in rice straw is 20 % of its weight (Dutta, Mukhopadhyay, Dasgupta, & Chakrabarti, 2014), in sugarcane bagasse is 9.6 % of its weight (Richana, Lestari & Irawadi, 2004), while in empty fruit bunch is 24 % of its weight (Rahman, Choudhury, Ahmad, & Kamaruddin, 2007). Xylan can be converted to food additives such as xylooligosaccharides (XOs). Lignocellulosic materials (LCMs) consist of three polymers: lignin (with phenolic nature), cellulose (a linear polymer made up of β-glucose units linked 1-4) and hemicelluloses (branched-heteropolysaccharides made up of a variety of monosaccharides, including xylose, arabinose, mannose, glucose and rhamnose) (Gibson, 2013).

Xylan has a commercial potential as biomass, where hemicelluloses are the second most abundant renewable resource, only exceeded by cellulose. Biodegradation of these xylans involves the action of several hydrolytic enzymes including xylanases (Collins, Gerday, & Feller, 2005). The complex structure of enzymes is required to degrade xylan, including endo-β-1,4-xylanases, which hydrolyze β-1,4-bonds between D-xylose residues in the main chain producing XOs, and β-D-xylosidases, which convert XOs to xylose (Den Haan & Van Zyl, 2003).

The applications of xylanase are wide in biotechnology research such as for improving digestibility of animal feed (Qiu et al., 2010), for improving food and beverage (Shah, Shah, & Madamwar, 2006), for bio-bleaching in paper (Nair, Sindhu, & Shashidhar, 2010), and for sizing of cotton and conversion to cellulosic bioethanol (Juodeikiene et al., 2011). Xylanases are also employed in green energy production, where it can assist in the conversion of biomass to bioethanol and biodiesel (Zhong, Lau, Balan, Dale, & Yuan, 2009).

Bacteria and fungus have been proven giving positive effect for xylanase production (Akpinar,

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Erdogan, Bakir, & Yilmaz, 2010; Du et al., 2013; Hung et al., 2011; Kallel et al., 2014; Kar, Mandal, Mohapatra, Mondal, & Pati, 2006; Kumar, Joshi, Kashyap, & Khanna, 2011; Li et al., 2012; Richana, Lestari, Thontowi, & Rosmimik, 2000; Yang et al., 2006). Present major applications are in the paper, feed and baking industries (Schoenlechner, Szatmari, Bagdi, & Tömösközi, 2013). Recently, some researcher discovered that marine bacteria, such as *Hahellachejuensis* (Lee, Kim, Kim, Choi, & Kim, 2013), *Bacillus subtilis* (Khandeparker, Verma, & Deobagkar, 2011) and *Thermoanaerobacterium saccharolyticum* (Hung et al., 2011) are positive in xylanase production. However, research on the use of xylanase produced by marine bacteria are still very limited (Crawford, Richardson, & Mather, 2005; Kiyohara et al., 2005; Khandeparker et al., 2011), yet marine bacteria have their uniqueness. Those bacteria are halotolerant due to their high salinity habitat.

Xylanase producing bacteria have a broad range of pH and temperature stability, which are preferred in the industries (Mohamed et al., 2011). Optimisation of xylanase production is important to fulfil the needs of industrial level. Several criterias need to be accomplished such as optimal fermentation conditions.

We have screened the ability of bacterial isolate LBF-001 to degrade xylan from beechwood using the simple procedure of Congo-Red method. Nevertheless, the information regarding optimisation of xylanase production by LBF-001 isolate is still needed. Hence, the purpose of this study is to identify the bacterial isolate using 16S rDNA gene and to optimise the medium conditions for xylanase productions i.e. biomass concentration, nitrogen source, pH value and temperature.

2. Material and Methods

2.1. Microbial Strains

The marine bacterium LBF-001 isolate is a collection of Laboratory of Biocatalyst and Fermentation, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI). The LBF-001 isolate was isolated from Pari Island, Kepulauan Seribu, Indonesia.

2.1.1. Bacterial identification of LBF-001 isolate

The analysis of the partial 16S ribosomal deoxyribonucleic acid (rDNA) was performed by amplifying the gene by Polymerase Chain Reaction(PCR) (Takara Bio Inc., Australia) using 9F and 1510R primers (IDT, California, USA) (Cavalca et al., 1999). The condition of PCR are 95 °C for 2 minutes (1 cycle); 95 °C for 30 sec, 65 °C for 1 min, 72 °C for 2 min (10 cycles); 95 °C for 30 seconds, 55 °C for 1 min, 72 °C for 2 min (30 cycles); and 72 °C for 2 min (1 cycle). The PCR products were analysed by electrophoresis (Voltronyx, UK) on 2% agarose gel.

2.1.2. DNA sequencing and sequence analysis

Partial 16S rDNA gene from bacterial isolate was sequenced using DNA sequencer ABI 310 (Pharmacia). Nucleotide sequences were analysed using Bio Edit and BLASTX (Altschul et al., 1997). Multiple alignment and phylogenetic analysis were produced by CLUSTAL X (Higgins & Sharp,1988), and a phylogenetic tree was visualised by the NJ plot program (Thompson et al., 1994) and MEGA 6 (Tamura et al., 2013).

2.2. Preliminary Test for Enzyme Degradation

Xylan agar was made using two layers agar plate method. The bottom layer consisted of 1.8 % agar purified powder (NACALAI TESQUE, INC., Kyoto, Japan) and the top layer consisted of 0.5% weight per volume (w/v) beechwood xylan (BWX) (SIGMA-ALDRICH, Missouri, USA). The amount of enzyme that spotted to each xylan agar was 5 µl. After 3 days of incubation, the agar plate was flooded with 0.25% Congo Red solution for 30 minutes. Subsequently, agar plate was rinsed using 1M NaCl solution until clear zone appeared. Finally, 5% acetic acid was added to visually show the clear zone.

2.3. Production of Xylanase from LBF-001 Isolate

The culture was refreshed using marine agar (Difco™ BD, MD, USA) and was incubated at 30 °C for 24 h. A single colony was then inoculated into the pre-culture medium. Pre-culture medium containing 38 g/l artificial sea water (ASW) (Marine Art® SF-1, Osaka, Japan), 1 g/l dried yeast extract (DYE) (NACALAI TESQUE, INC., Kyoto, Japan) and 5 g/l Bacto™ Peptone (BP)(BD, MD, USA) and 5 g/l BWX (SIGMA-ALDRICH, Missouri, USA) was employed after autoclaved sterilization at 121 °C for 15 min. Twenty (20) ml of preculture medium was prepared in 100 ml flask and incubated in an incubator shaker at 30 °C, 150 rpm for 24 h. Culture medium was composed of 38 g/l ASW, 1 g/l DYE, 5 g/l BP, and was fermented with addition of various biomasses i.e. empty fruit bunch (EFB), sugarcane bagasse (SCB), rice straw (RS) and 0.5 % beechwood xylan (BWX)

as a control parameter. The culture was incubated at 30 °C, 150 rpm for 48 h followed by cell growth measurement using spectrophotometer at a wavelength of 660 nm. Enzyme activities were measured by dinitro salicylic acid (DNS) method.

2.4. Optimisation of EFB Concentration

EFBs were prepared at 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; and 3.5 (%, w/v). Each of the prepared-EFB concentration was added into the culture medium. Cultures were fermented at 30 °C and 150 rpm for 48 h followed by cell growth measurement using spectrophotometer at a wavelength of 660 nm. DNS method was employed to determine the enzyme activities.

2.5. Optimisation of EFB by Different Nitrogen Source

Various nitrogen sources (6 g/l) were added to the medium. Nitrogen sources included BP (BD, MD, USA), BTC (BD, MD, USA), BTSB (BD, MD, USA), lactose broth (LB)(HIMEDIUM, Mumbai, India), beef extract powder (BEP) (HIMEDIUM, Mumbai, India), and dried yeast extract (DYE) (NACALAI TESQUE, INC., Kyoto, Japan). The culture medium consisted of 2.5% EFB and 38 g/l ASW. The culture was then inoculated in a 20 ml of culture medium and incubated at 30 °C, 150 rpm for 48 h followed by cell growth measurement using spectrophotometer at a wavelength of 660 nm. DNS method was employed to determine the enzyme activities.

2.6. Effect of Different pH Value

The effect of different pH value was conducted on 20 mL culture medium in 100 ml flasks containing 38 g/l ASW, 2.5% EFB, and 6 g/l LB, with a variation of pH values of 6.0, 6.5, 7.0, 7.5 and 8.0 in triplicate manner. The pH value was adjusted using 0.1N NaOH or 0.1N HCl. The culture was fermented at 30 °C and 150 rpm for 48 h followed by cell growth measurement using spectrophotometer at a wavelength of 660 nm. DNS method was employed to determine the enzyme activities.

2.7. Effect of Different Temperatures

The effect of different temperatures was carried out in 20 ml of culture medium in 100 ml flasks. The medium contains 38 g/l ASW, 2.5% EFB and 6 g/l LB with pH value adjusted to 6.5, according to the best result of the effect of different pH value. The fermentation was conducted at various temperatures, notably 20, 25, 30, 35, 40, 45 and 50 °C at 150 rpm for 48 h followed by cell growth measurement using spectrophotometer at a wavelength of 660 nm. DNS method was employed to determine the enzyme activities.

2.8. The Growth of the Cell in the Optimum Condition

The culture medium contained 38 g/l ASW and 6 g/l LB and was sterilised by autoclave at 121 °C for 15 minutes. The culture was fermented at 30 °C and 150 rpm for 48 h. Samples of the culture were taken at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h. Cell growth was examined using spectrophotometer at a wavelength of 660 nm, while the enzyme activities were examined using DNS method.

2.9. Enzyme Assay

As much as 250 μl of the enzyme was mixed with 250 μl of the substrate that containing 0.5% BWX along with 50 mM phosphate buffer and incubated for 15 minutes at 60 °C. The reaction was stopped by the addition of 500 μl of DNS. After that, the mixtures were mixed, boiled for 15 min at 100 °C and cooled quickly in ice for around 10 min. The enzymatic hydrolysis of xylanase was determined by measuring the absorbance at 540 nm. The blank and control were prepared by same procedure without the samples. The amount of xylanase activity was defined as the amount of an enzyme reducing sugars that equivalent to 1μmol of xylose per min. Xylanase activities were measured by DNS method (Miller, 1959).

3. Results and Discussion

3.1. Analysis of Phylogenetic

The LBF-001 isolate was identified based on 16S rDNA sequence analysis. Approximately 1,500 bp of LBF-001 isolate 16S rDNA gene was sequenced. Basic Local Alignment Search Tool (BLAST) analysis showed that LBF-001 isolate has 99% similarity with *Bacillus pumilus* HNS70 (KF933667) (Zheng *et al.* 2014). Fig. 1 shows the phylogenetic tree for LBF-001 isolate.

Based on BLAST analysis of the 16S rDNA gene, *Bacillus* LBF-001 isolate was identified as *B. pumilus. B. pumilus* is considered as a major component of marine bacterial communities (Berrue et al., 2009; Parvathi et al., 2009; Liu et al., 2013). Some papers

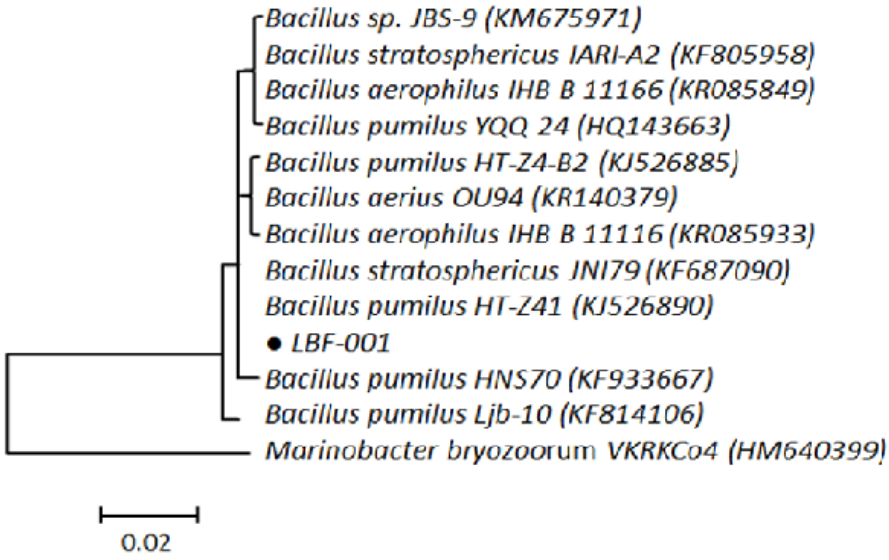

Figure 1. Phylogenetic tree derived from 16S rDNA gene NCBI sequence of LBF-001 isolate and sequences of closest phylogenetic neighbours obtained by NCBI BLAST(n) analysis. The NJ-tree was constructed using neighbor-joining algorithm with Kimura 2 parameter distances in MEGA 6.06 software. Bar, 2% estimated sequence divergence.

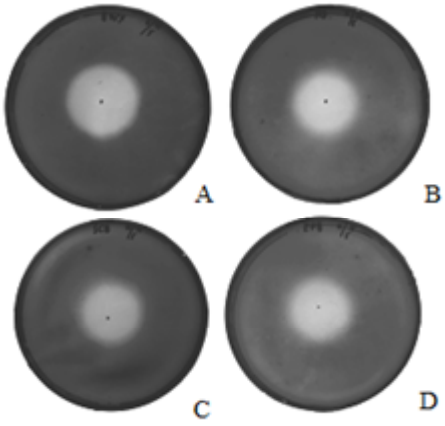

Figure 2. Degradation of xylan of various biomasses by xylanase enzyme from *B.pumilus* LBF-1-001. (A) BWX, (B) RS, (C) SCB, and (D) EFB. The result was documented after 48 h treatment.

reporting that *B. pumilus* has the capability to produce lipase (Faouzi et al., 2015), lipoamides, antimicrobial agents (Berrue et al., 2009), cellulose (Nagar et al., 2012), and xylanase (Moriya et al., 2007; Subramaniyan, 2012).

3.2. The Result of the Preliminary Test for Enzyme Degradation

To confirm that *B. pumilus* LBF-001 has the ability to degrade xylan, the production medium was substituted with agro-residues such as RS, SCB and EFB at 0.5% (w/v) concentration.

Figure 2 shows the xylanase activity of the *B. pumilus* LBF-001 enzyme. Each agro-residue of RS, SCB, and EFB at 0.5% (w/v) concentration was plated into two layers agar plate method. The clear zone in the plates indicated a positive result of various biomasses (xylans) (RS, SCB, EFB and positive control-BWX) degraded by the *B. pumilus* LBF-001 xylanase. The qualitative result showed that *B. pumilus* LBF-001 was able to produce xylanase in each of biomass tested. For further analysis, xylanase produced in several biomasses tested was quantitatively assayed using DNS method in order to know determine which agro-residues that can produce the highest enzyme activity.

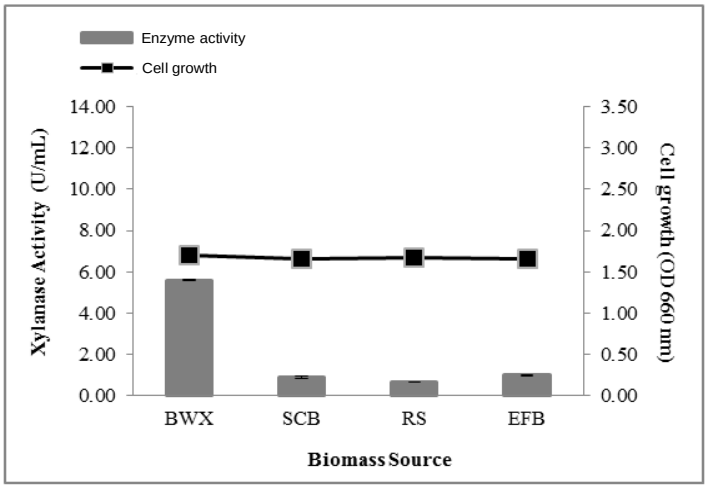

Figure 3. The effect of 0.5% (w/v) of SCB, RS, EFB and positive control-BWX biomass sources to xylanase activity.

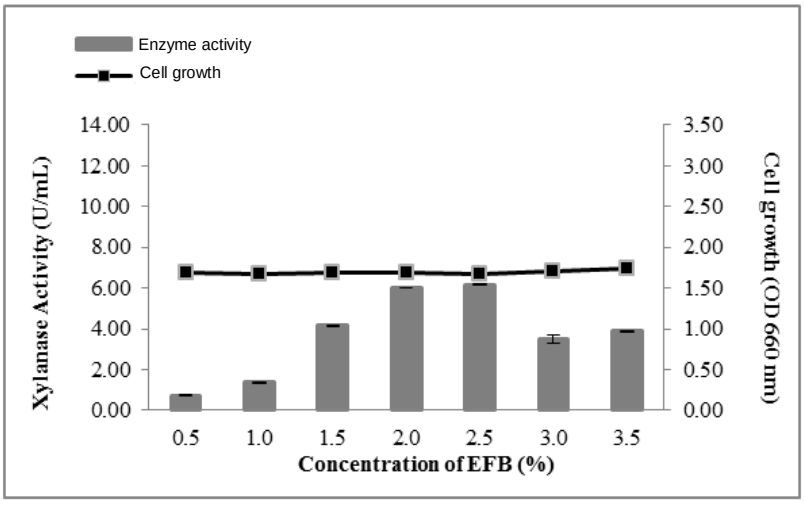

Figure 4. Effect of different concentrations of empty fruit bunch (150 RPM, 30 °C and 48 h) on xylanase activity.

Figure 3 showed that among 3 biomasses studied in this research, EFB has higher xylanase activity which indicated having higher xylan content compare to SCB and RS. As a comparison, xylan content in bagasse is equal to 21.1% weight (Wiselogel et al., 1997), in RS is equal to 13.9% weight (Moradi et al., 2013) and in EFB is equal to 24.01% (w/v) (Rahman et al., 2007). The xylanase activity of the positive control-BWX was 5.60 U/ml, while the xylanase activity of SCB, RS and EFB was 0.88 U/ml, 0.66 U/ml and 0.97 U/ml respectively. The control has the highest xylanase activity since BWX contained high xylan. The cell growth was measured by optical density using spectrophotometer at a wavelength of 660 nm. The cell growth did not show any effects on increasing xylanase activity due to the turbidity of the biomass in the cell culture.

Since among biomass sources tested in the experiment EFB produces slightly higher xylanase activity, the optimal concentration of EFB in the medium to produce a better xylanase activity was then studied further.

3.3. The Effect of Different EFB Concentration

The highest result for xylanase activity belongs to 2.5% concentration that equal to 6.17 U/mL with an optical density equal to 1.68 as shown in Figure 4.

The result shows that 2.5% concentration of empty fruit bunch gave the highest xylanase activity. This phenomenon is probably due to the effect of viscosity and transport of nutrient from the fermentation system. The lower or the higher concentration causes a negative effect on the uniform circulation of nutrient

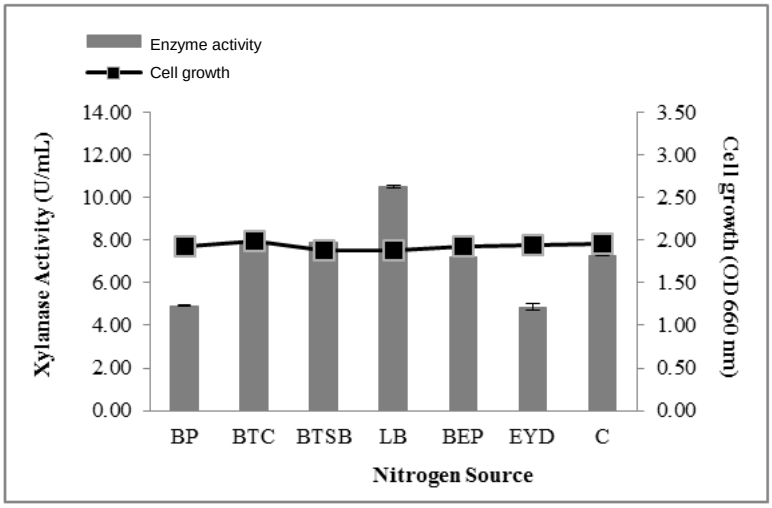

Figure 5. The effect of different nitrogen source on the xylanase production by *B. pumilus* LBF-001 (culture was incubated at 150 rpm, 30 °C for 48 h).
Note: Bacto™ Peptone (BP), Bacto™Tryptone Casein (BTC), Bacto™Tryptone Soya Broth (BTSB), Lactose Broth (LB), Beef Extract Powder (BEP), and Dried Yeast Extract (DYE) at a concentration of 0.6% (w/v) each, and Control (symbol C with containing 0.1% (w/v) (DYE) and 0.5% (w/v) BP).

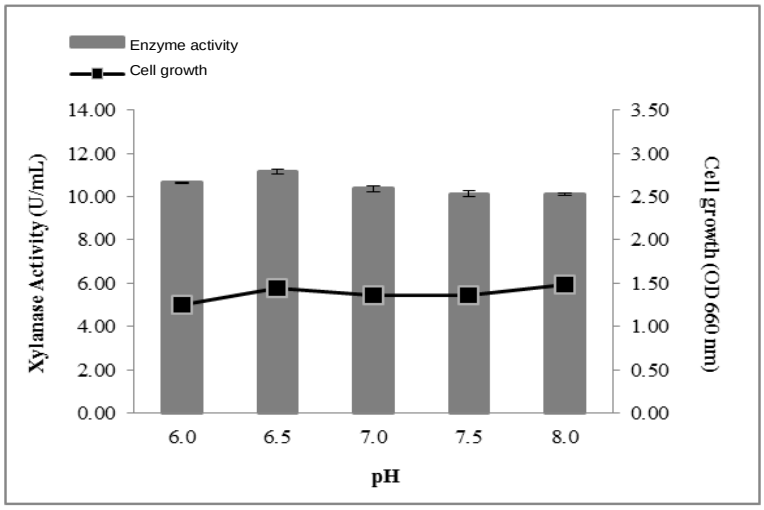

Figure 6. Effect of pH of the culture on the xylanase production by *B. pumilus* LBF-001 (culture was incubated at 150 rpm, 30 °C for 48 h).

and oxygen, and leads to the attenuation of microbial growth and ultimately decline the xylanase production (Bibi et al., 2014; Karim et al., 2014). The cell growth did not show any effect on the increasing xylanase activity due to the turbidity of the biomass in the cell culture, as described previously.

3.4. The Effect of Different Nitrogen Source

All of the tested nitrogen sources in Fig. 5 were able to support the production of xylanase from *B. pumilus* LBF-1-0001 isolate. The highest xylanase activity belongs to LB (10.49 U/mL) followed by BTSB (7.87 U/mL). Nitrogen source is a crucial component to the growth of many bacteria, where it is suggested to add a relevant concentration of desirable nutrient to improve the cell growth of bacteria. The effects of different nitrogen source on the production of xylanase by *B. pumilus* LBF-001 under submerge fermentation experiment giving the best result of using LB at 0.6 % concentration. This result is different from others xylanase researches previously reported (Nagar et al., 2012; Yang, 2006; Zhang & Sang, 2015) which confirmed that the best nitrogen source for xylanase production is yeast extract. In this research, the cell

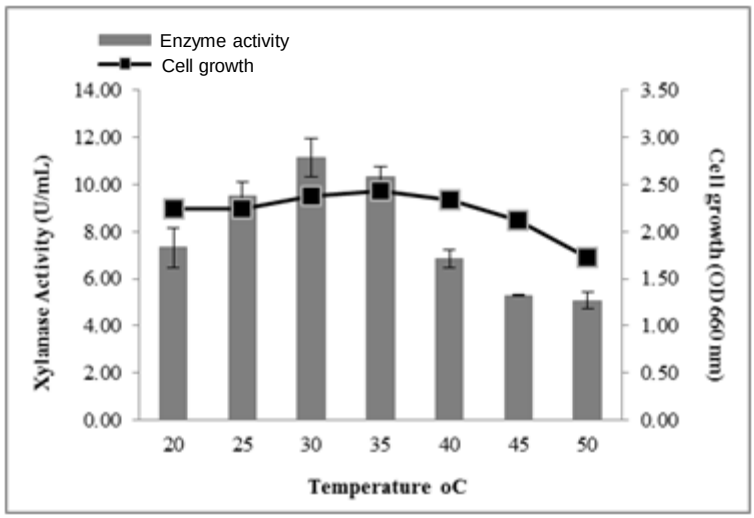

Figure 7.Effect of culture temperature (150 RPM, pH 6.5, and 48 h).

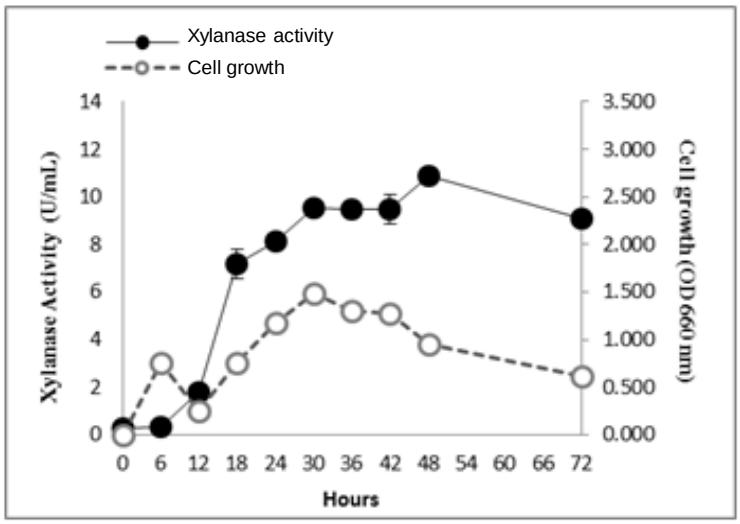

Figure 8.Empty fruit bunch optimum fermentation (150 RPM, 30 °C, pH 6.5, and 72 h).

growth did not show any effect on increasing xylanase activity due to the turbidity of biomass in the cell culture.

3.5. The Effect of Different pH Culture

To study the effect of different pH of the culture on the xylanase production, the pH of the culture values were tested at 6.0, 6.5, 7.0, 7.5 and 8.0. Figure 6 showed highest xylanase activity (11.15 U/mL) belongs to pH 6.5 treatment, when the cell growth was at 1.44 (OD 660 nm). The pH has affected the xylanase production by the *B. pumilus* LBF-001 on EFB medium. The pH of the medium strongly affects the enzymatic processes and transport in the system which then will affect various components across cell membrane (Kapoor et al., 2008). Kapoor et al. (2008) reported that *Bacillus pumilus* strain MK001 at 37 °C under shaking (150 rpm) conditions after 24 h of incubation produced the highest xylanase activity under pH 9. While Nagar, Mittal, Kumar, & Gupta (2012) reported that *Bacillus pumilus* SV-205 has the highest xylanase activity at pH 10.

3.6. Effect of Culture Temperature

The effect of the culture temperature on the xylanase production by *B. pumilus* LBF-1-0001 was investigated on 2.5% EFB medium. The culture temperatures were tested at 20, 25, 30, 35, 40, 45 and 50 °C. Nagar et al. (2012) reported that microbes are known to produce high enzyme titer at their

optimum growth temperature. The highest xylanase activity of 11.14 U/mL, with cell growth optical density of 2.38 was produced at culture temperature of 30 °C. It is indicated that optimum temperature for *B. pumilus* LBF-001 xylanase production was found to be 30 °C and occurred at 48 hours. This optimum temperature condition is similar to *Bacillus pumilus* SV-205 that has the highest activity at 37 °C (Nagar et al., 2012). The cell growth did not show any effect on increasing xylanase activity due to the turbidity of biomass in the cell culture.

3.7. EFB Optimum Xylanase Activity Fermentation

The culture medium was prepared at its optimum EFB concentration (2.5%), optimum pH (6.5) and incubated at its optimum temperature (30 °C) from previous experiment results. Figure 8 showed the effect of different culture incubation periods on the xylanase production by *B. pumilus* LBF-001. The highest xylanase activity was observed at 48 hours incubation period with xylanase activity of 10.85 U/mL and cell growth (optical density) equal to 0.89. The xylanase activity was not significantly decrease at longer incubation period, as shown in Figure 8.

As shown in Figure 8, the lag phase was occurred between 0-12 h, where in this phase, *B. pumilus* LBF-001 was gaining growth and adapted with the existing preculture medium. The log phase was at 12-30 h, where in this phase, *B. pumilus* LBF-1-0001 was able to double the cell (logarithmic condition). The stationary phase was between 30-48 h, where in this phase *B. pumilus* LBF-1-0001 was not able to grow further due to limitation or depletion of some nutrient. The death phase occurred after 48 h, where in this phase the *B. pumilus* LBF-001 was in the death period due to lack of nutrient. From the results, the highest xylanase activity was achieved at 48 h of incubation. Compared to the cell growth and the xylanase activity, it can be concluded that the increasing of the cell growth is consistent to the increasing of the xylanase activity and also the reduction. This phenomenon was described by Rath et al. (2014), where there is a connection between cell growth and enzyme activity.

4. Conclusion

Based on 16S rDNA gene analysis, LBF-001 isolate has been identified to have 99% similarity with *Bacillus pumilus* HNS70 (KF933667). The optimisation of xylanase production from LBF-001 isolate has shown that the highest xylanase activity of 10.85 U/mL was reached using culture medium containing 2.5% EFB, 0.6 % LB, cultured at pH 6.5, temperature 30 °C incubated for 48 hours. Further experiment for large scale production needs to be considered to meet the industrial needs.

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