Process optimization studies on xylanase production and bioethanol fermentation

Tan Chia Zhi[1*], *Ivory* Thien Sze Chen[1], *Teoh* Yi Peng[1], *Lee* Kok Chang[2], *Kunasundari* Balakrishnan[3] and *Ooi* Zhong Xian[4]

[1]Department of Petrochemical Engineering, Faculty of Engineering and Green Technology, Universiti Tunku Abdul Rahman (UTAR), Kampar Campus, Jalan Universiti, Bandar Barat, 31900 Kampar, Perak, Malaysia.
[2]Department of Biological Science, Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), Kampar Campus, Jalan Universiti, Bandar Barat, 31900 Kampar, Perak, Malaysia.
[3]Department of Chemical Engineering Technology, Faculty of Engineering Technology, Universiti Malaysia Perlis, Kampus UniCITI Alam, Sungai Chuchuh, Padang Besar, 02100, Perlis, Malaysia.
[4]Department of Chemical Science, Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), Kampar Campus, Jalan Universiti, Bandar Barat, 31900 Kampar, Perak, Malaysia.

Abstract. Xylanase is one of the hydrolytic enzymes with a broad industrial application in several industries. Bioethanol can be synthesised from lignocellulosic biomass by using xylanase and other hydrolytic enzymes. A filamentous fungus, which is *Aspergillus niger* produces xylanase under submerged fermentation when oil palm empty fruit brunches were used as carbon sources. This study aimed to optimize the operating conditions (medium pH and incubation temperature) of xylanase production process using the OFAT analysis technique. From the data obtained, the highest xylanase production was 0.508 U/mL at pH 5.0 and 0.524 U/mL at an incubation temperature of 32°C, respectively. *S. cerevisiae* yeast was added into the fermentation supernatant for bioethanol fermentation. The concentration of bioethanol produced by xylanase enzyme from *A. niger* at optimum operating condition was 15.54±0.47 g/L. This study proved that *A. niger* is one of the filamentous fungi which show the potential of hydrolysing lignocellulosic material to carbon sources and subsequently to bioethanol production.

1 Introduction

Malaysia generates significant agricultural waste, such as oil palm empty fruit bunches (EFB), kernel cake, sugarcane bagasse, rice straw, and rubber wood dust. Efficient and cost-effective waste management is essential to minimize environmental impact and transform these materials into valuable industrial resources. These wastes can be used as growth medium for microorganisms, which produce important industrial enzymes like cellulases, xylanases, lipases, β-glucosidases, mannanases, phytases, proteases, and lignin [1].

Xylanase is a hydrolytic enzyme that breaks down β-1,4 xylans by cleaving β-1,4 glycosidic bonds. Xylans are linear polymers of D-xylose linked by β-1,4 bonds, found in

*Corresponding Author: tcz_95@hotmail.com

hardwoods and annual plants, and are the most common non-cellulosic polysaccharides. Xylanase degrades hemicellulose in plant cell walls and has various industrial uses, including bio-bleaching of wood pulp, papermaking, food and beverage processing, animal nutrition, and bioethanol production [2], [3].

Recently, fungal pathogens, particularly *Aspergillus* species, have become prominent in xylanase production. Research shows that filamentous fungi, like *Aspergillus*, produce significantly more xylanase compared to bacteria and other microbes, making them superior for this purpose [4], [5]. Several factors, including incubation temperature, medium pH, agitation speed, incubation time, initial moisture, and the nutritive components of the medium can affect the production of xylanase enzyme during the fermentation [6].

To date, there is less reports study on the performance of *Aspergillus niger* for the xylanase production, particularly under submerged fermentation system. This study used a fungal strain from UTAR's laboratory culture and employed the one-factor-at-a-time (OFAT) method to evaluate the Mandels and Sterburg's basal salt (MSBS) medium and other factors. Additionally, OFAT was used to assess how physical parameters like pH and incubation temperature affect xylanase production.

2 Methodology

2.1 Substrate preparation

Oil palm EFB was used as a carbon source for enzyme production. EFB was ground, washed with distilled water to remove contaminants, autoclave at 121ºC for 20 minutes, and then dried in an oven at 90ºC for 3 days. After drying, it was stored at room temperature until use.

2.2 Microorganism Maintenance

The fungal strain identified as *Aspergillus niger* (*A. niger*) was obtained from Universiti Tunku Abdul Rahman (UTAR). The strain was maintained on Potato Dextrose Agar (PDA) slants by monthly transfer and was stored at 4°C for storage.

2.3 Submerged Fermentation

Xylanase production was carried out under submerged fermentation. A conical flask was prepared with 100 mL of Mandels and Sternberg basal medium and 2.5 g of EFB. The medium contained 2.0 g/L NH_4NO_3, 2.0 g/L K_2HPO_4, 1.0 g/L $MgSO_4.7H_2O$, 0.3 g/L $CaCl_2$ and 0.012 g/L trace elements which included 5.0 mg/L $FeSO_4.7H_2O$, 1.6 mg/L $MnSO_4.4H_2O$, 3.45 mg/L $ZnSO_4.7H_2O$ and 2.0 mg/L $CoCl_2.6H_2O$. Three fungal plugs of *A. niger* from a 7-day potato dextrose agar culture were added. Fermentation was agitated at 150 rpm and room temperature for 9 days, with 1 mL samples taken daily for xylanase assays.

2.4 Process Parameters Optimization

Optimization on process parameters that influences production of xylanase from *A. niger* was carried out using OFAT experimental design. The manipulated variables were pH and incubation temperature. The pH used in this study was varied at 3.0, 5.0, 7.0, and 9.0 while the remaining variables were kept constant. After that, the incubation temperature used was varied at 25ºC, 28ºC, 32ºC and 37ºC.

2.5 Determination of growth profile

The kinetic growth of *A. niger* was examined by monitoring using the incubation period and the optimum period of *A. niger* growth on a PDB medium using OFAT analysis techniques. The analysis was conducted by measuring the absorbance using the Bradford method for protein concentration and the DNS method for reducing sugar determination.

2.6 Determination of xylanase activity

Xylanase activity was assessed using 1% xylan in 0.05 M citrate buffer (pH 5.0). Reducing sugars were quantified with the 3,5-dinitrosalicylic acid (DNS) method and a xylose standard curve. The assay involved mixing 50 mg of xylan in 1.0 mL sodium acetate buffer with 1.0 mL of crude enzyme supernatant, incubating at 50°C for 30 minutes. Reducing sugars were detected by developing color with DNS, and absorbance was measured at 540 nm using a UV/vis spectrophotometer.

2.7 Determination of Ethanol concentration using high-performance liquid chromatography (HPLC)

Triplicate samples from bioethanol fermentation were centrifuged at 10,000 rpm for 10 minutes. The supernatants were diluted 5-fold with 5 mM H2SO4, filtered through a 0.45 μm membrane filter, and 1 mL was injected into an HPLC column. Each series of samples included blank and control solutions for accuracy. The column was run at 60°C with a 0.6 mL/min flow rate of 5 mM H2SO4, and each analysis took 25 minutes. Ethanol concentration was determined using a standard curve [7]. In this study, the standard equation obtained was $y = 3148.1x - 15953$ with $R^2 = 0.98$, where y indicates the HPLC peak and x indicates the ethanol concentration.

3 Results and discussion

3.1 Growth curve of *A. niger*

Based on the Figure 1, *A. niger* has shown a very slow-moving growth rate which is indicated as the lag phase of the *A. niger* from day 0 to day 1. This is because the *A. niger* was introduced into the fresh culture medium at early stage. During this period, *A. niger* become more adapted to the environment, start to secrete enzyme and acclimatize to the culture medium condition. Then, the growth curve started entering its log phase after 24 hours incubation. According to the Figure 1, the combined graph shows a steeply sloped straight line from day 1 to day 2. This is because the microbes in the broth medium were started to reproduce rapidly through binary fission under optimum nutritional and physical conditions.

After that, it took 2 days to reach the stationary phase (day 4 to day 5) due to the nutritional limitation. The population growth was slowed since the essential nutrient becomes depleted. The other reason for this situation is that the fermentation occurred in a closed system. However, it showed a decrement caused by the mycelium cell's mass and protein concentration reduction from day 5 to day 9. The fungus stepped into the death phase due to changes in the dentrimental environment. This is because the nutrient was depleted and the toxic products started accumulating, which can kill or inhibit the isolates. There is possibility that the new cell will consume the old cell in the broth media [8].

Based on the observation above, it can conclude that *A. niger* has an optimal growth and optimal protein concentration from day-1 to day-3 of incubation day when Mandels and Sternberg basal medium added with EFB is used as the growth medium.

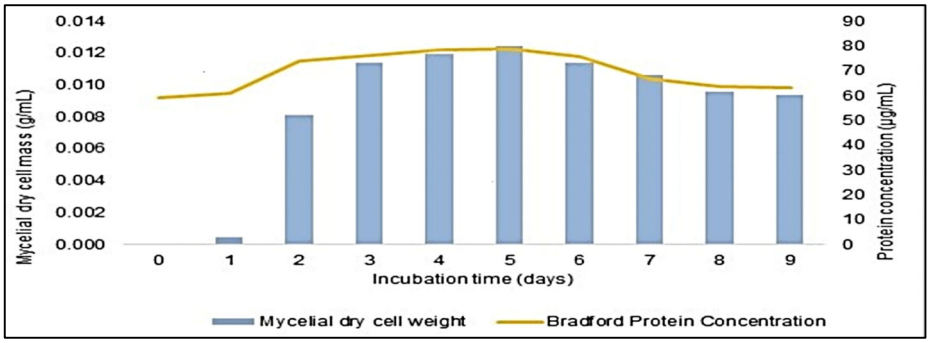

Fig. 1. Mycelial Dry Cell Mass and Protein Concentration Profiles of *A. niger* Using Bradford Method.

3.2 Physical parameters on the Xylanase activity

This study used two physical parameters, incubation temperature and medium pH, to optimize the xylanase production. Xylanase activity was studied at different pH ranges from 3 to 9 when the incubation temperature (32ºC) and agitation speed (150 rpm) were fixed at the same reading for 5 days. Based on the Figure 2, the optimum enzyme activity was found at pH 5.0. The results indicated that the maximal enzyme activity was 0.508 U/mL, with mycelial dry mass of 0.093 mg/mL, as shown in Figure 3. The enzyme activity significantly decreased when the pH reached 9.0.

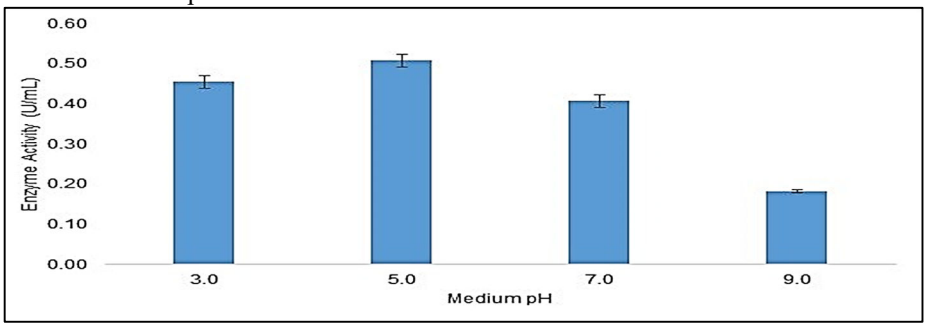

Fig. 2. Effect of Medium pH on Xylanase Activity.

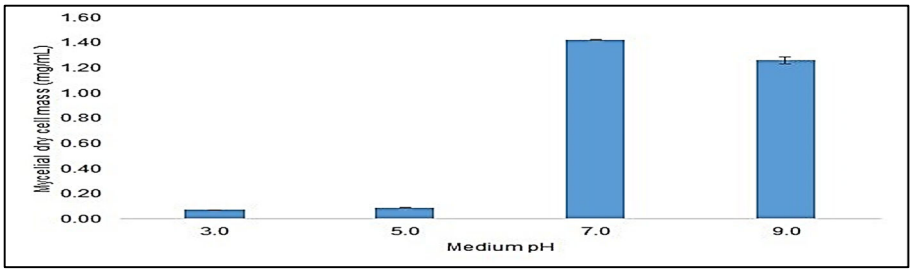

Fig. 3. Effect of Medium pH on Mycelial Dry Cell Mass.

Research prove that the permeability of microbial cells, changing the stability of the enzyme and cause denaturing by deactivating the enzyme activities and membrane transport protein can be influenced by varying the medium pH [9]. Moreover, the enzymes activity can be affected by the ionic components of the substrate changes and the substrate charge when changing the medium pH. On the other hand, the optimum pH for xylanase production by *A. niger* has been reported by Cunha et al. (2018) [6] at around pH 7.

For the purpose of determining the impact of temperature on the production of xylanase from *A. niger*, the incubation temperature was examined within the range of 25°C to 37°C. At the same time, the pH was set at pH 5.0 and agitation speed was fixed to 150 rpm for 5 days incubation.

Based on Figure 4, the maximal enzyme activity was (0.524 U/mL) obtained at 32°C while the minimum (0.406 U/mL) was observed at 25°C. Raising the incubation temperature from 25ºC to 28ºC increased enzyme activity from 0.406 U/mL to 0.510 U/mL, but further increasing it to 37ºC decreased the activity. Moreover, mass for the mycelial dry cell was detected showing a similar trend. From Figure 5, mycelial dry mass obtained was 0.074 mg/mL at 25°C. After that, it continued to rise to 0.270 mg/mL at 32°C. As the temperature adjusted to 37°C, it showed a sharp decline to 0.093 mg/mL.

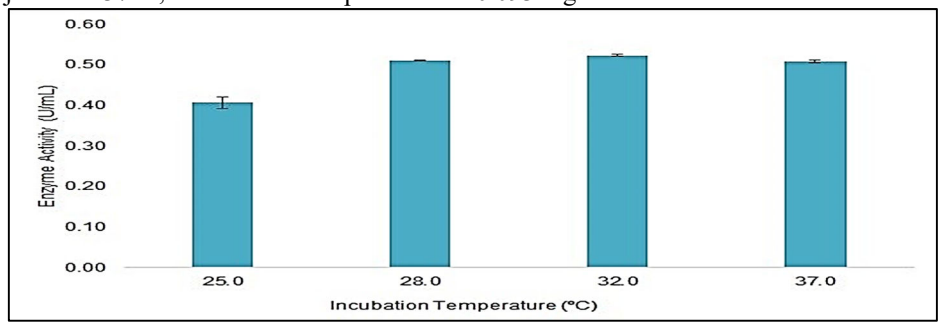

Fig. 4. Effect of Incubation Temperature on Xylanase Activity.

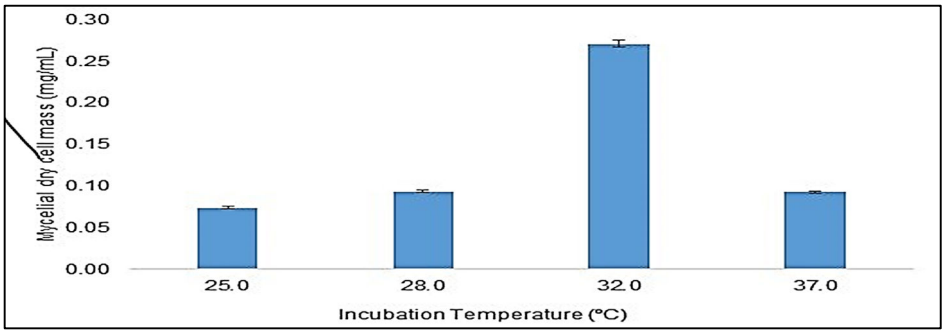

Fig. 5. Effect of Incubation Temperature on Mycelial Dry Cell Mass.

A researcher reported that incubation temperature can significantly affect the growth rate, as it influences the capacity for delignification. It suggested that higher temperatures might enhance enzymatic activity or microbial growth, leading to more effective delignification [10]. From the observation above, more enzymes were produced at temperature 32°C. Hence, it can be concluded that the temperature of 32°C significantly influenced enzyme production using SmF, which shows consistency with maximal production of mycelial dry mass. A research report investigated xylanase production from *Aspergillus niger* ATTC 6275 in palm oil mill wastes and concluded that maximum production can be achieved at a growth temperature of 30°C [11]. It appears that optimal xylanase production by *Aspergillus niger*

can be achieved consistently at around 30°C, whether using submerged or solid-state fermentation techniques.

3.3 Bioethanol production

Bioethanol produced by xylanase synthesized from *A. niger* at 32°C, pH 5.0, 150 rpm and incubated for 5 days since it determines the optimum condition for the enzyme and xylanase production. The concentration of bioethanol produced by xylanase synthesized from *A. niger* at optimum condition after studied the physical effect of xylanase production was 15.54±0.47 g/L.

Based on the study of Batori et al. (2015) [12], there was 7.6 g/L of ethanol concentration and 5.8 g/L of biomass concentration for the *Neurospora intermedia* and *Aspergillus oryzae* in two-stage cultivation whole stillage. Furthermore, ethanol production reached 58.0 g/L using the on-site multienzyme produced by *Aspergillus awamori* fungus, along with the addition of *S. cerevisiae*. [13]. By comparing those researches, the bioethanol concentration obtained at optimum conditions is higher than the ethanol concentration obtained in two-stage whole stillage cultivation. However, it showed quite large differences compared to the amount of ethanol produced by the on-site multienzyme from *Aspergillus awamori* fungus with *S. cerevisiae*. This may be due to *Aspergillus awamori,* which can produce many kinds of hydrolytic enzymes known as glucoamylases producer. Hence, it can synthesise more enzymes that can used for bioethanol production. According to Tran et al. (2019) [14], a combination of pretreatment methods must be carried out before any production in order to increase bioethanol yield.

4 Conclusion

There are two parameter that been studied for this research which were effect of incubation temperature and medium pH in xylanases production from *A. niger* using Mandels and Sternburg's basal medium cultivated in the submerged fermentation system. Based on the study of effect of the medium pH, the maximum highest amount of xylanase production (0.508 U/mL) was shown when adjusted to pH 5.0. On the other side, the study effect of incubation temperature showed that the optimal enzyme activity was 0.524 U/mL obtained at 32°C. Hence, the optimum medium pH at pH 5.0 and incubation temperature at 32°C was chosen to use for incubating *A. niger* to produce bioethanol. The concentration of bioethanol produced by xylanase synthesized from *A. niger* at optimum conditions after studying the physical effect of xylanase production was 15.54±0.47g/L. This can prove that *A. niger* is one of the filamentous fungi which shows the potential of utilizing crude fungal enzymes to further hydrolyse lignocellulosic material and can be applied in the bioethanol industry.

The authors would like to acknowledge the support from the Universiti Tunku Abdul Rahman for the financial aid through the UTAR Research Fund (UTARRF) with the project number IPSR/RMC/UTARRF/2019-C1/T05. Furthermore, the authors are also grateful to the Fundamental Research Grant Scheme (FRGS) under the grant number FRGS/1/2017/TK02/UNIMAP/02/7 from the Ministry of Education Malaysia.

References

1. P.P. Kheng, D. Ibrahim, L. Poppe, G. Szackacs, I.C. Omar, Production of cellullolytic enzymes by a newly isolated, *Trichoderma* sp. FETL c3-2 via solid state fermentation grown on sugar cane bagasse: Palm kernel cake as substrates. Pak. J. Biol. Sci. 9 (8), 1430–1437 (2006). https://doi.org/10.3923/pjbs.2006.1430.1437

2. P. Bajpai, Sources, Production, and Classification of Xylanases (Xylanolytic Enzymes, Elsevier, 2014)
3. R.D. Kamble, A.R. Jadhav, Isolation, purification, and characterization of xylanase produced by a new species of bacillus in solid state fermentation. Int. J. Microbiol. **2012**, 683193 (2012). https://doi.org/10.1155/2012/683193
4. U.A. Okafor, V.I. Okochi, O. Okerenta, N. Chinedu, Xylanase production by *Aspergillus niger* ANL 301 using agro-wastes. Afr. J. Biotechnol. **6** (14), 1710–1714 (2007)
5. M.L.T.M. Polizeli, A.C.S. Rizzatti, R. Monti, H.F. Terenzi, J.A. Jorge, D.S. Amorim, Xylanases from fungi: Properties and industrial applications. Appl. Microbiol. Biotechnol. **67** (5), 577–591 (2005). https://doi.org/10.1007/s00253-005-1904-7
6. L. Cunha, R. Martarello, P.M. Souza, M.M. Freitas, K.V.G. Barros, E.X.F. Filho, M.H. Mello, P.O. Magalhaes, Optimization of xylanase production from *Aspergillus foetidus* in soybean residue. Enzyme Res. **2018**, 6597017 (2018). https://doi.org/10.1155/2018/6597017
7. D. Mikulski, G. Kłosowski, Efficiency of dilute sulfuric acid pretreatment of distillery stillage in the production of cellulosic ethanol. Bioresour. Technol. **268**, 424–433 (2018). https://doi.org/10.1016/j.biortech.2018.08.005
8. J.M. Wiley, L.M. Sherwood, C.J. Woolverton, Microbial growth (McGraw-Hill Higher Education, New York, 2008)
9. A.K. Ajijolakewu, C.P. Leh, W.N. Wan Abdullah, C.K. Lee, Optimization of production conditions for xylanase production by newly isolated strain *Aspergillus niger* through solid state fermentation of oil palm empty fruit bunches, Biocatal. Agric. Biotechnol. **11**, 239–247 (2017). https://doi.org/10.1016/j.bcab.2017.07.009
10. B. Tsegaye, C. Balomajumder, P. Roy, Microbial delignification and hydrolysis of lignocellulosic biomass to enhance biofuel production: an overview and future prospect, Bull. Natl. Res. Cent. **43**, 51 (2019). https://doi.org/10.1186/s42269-019-0094-x
11. P. Prasertsan, A. Kittikul, A. Kunghae J. Maneesri, S. Oi, Optimization for xylanase and cellulase production from *Aspergillus niger* ATTC 6275 in palm oil mill wastes and its application. World J. Microbiol. Biotechnol. **13**, 555–559 (1997). https://doi.org/10.1023/A:1018569426594
12. V. Batori, J.A. Ferreira, M.J. Taherzadeh, P.R. Lennartsson, Ethanol and protein from ethanol plant by-products using edible fungi *Neurospora intermedia* and *Aspergillus oryzae*. Biomed. Res. Int. **2015**, 176371 (2015). https://doi.org/10.1155/2015/176371
13. E. Uçkun Kiran, Y. Liu, Bioethanol production from mixed food waste by an effective enzymatic pretreatment. Fuel **159**, 463–469 (2015). https://doi.org/10.1016/j.fuel.2015.06.101
14. T.T.A. Tran, T.K.P. Le, T.P.M. Mai, D.Q. Nguyen, Bioethanol production from lignocellulosic biomass. Xiandai Huagong/Mod. Chem. Ind. **31** (2), 40–44 (2011). https://doi.org/10.5772/intechopen.86437