Xylanase Production via Aspergillus niger: Effect of Carbon Source and Composition

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Abstract. Xylanases are secondary metabolite product of variety organisms from various agriculture wastes. Xylanase demands in industrial level are increasing. Precisely, the necessity of enzyme such as xylanase to breaks down the xylan into reducing sugar for biofuel production is inevitable. However, the production of xylanases is insufficient to support the market demand. Hence, Aspergillus niger is used as the xylanase producer in this research. In this study, effect of carbon sources (corn cob-based xylan (CCX) and empty fruit bunches (EFB)) and concentrations of carbon source (2.5g/L to 4.0g/L) on xylanase production through One-factor-at-time (OFAT) experimental technique were investigated. The optimum fermentation period of 5 days determined by using mycelial dry cell mass and Bradford protein concentration growth profile is 5 days was set as the incubation period. Among these data, both carbon sources show the maximum value at concentration of 3.5g/L. CCX showed a higher xylanase concentration (0.882±0.005μg/mL) compared to EFB (0.533 ± 0.006μg/mL). Hence, among these data analysis CCX has a better performance compared to EFB. Hence, among these data analysis CCX has a better performance compared to EFB.

1 Introduction

Biofuel is a well-established renewable energy source in the fuel industry. Recently, lignocelluloseous feedstock rich in hemicellulose has gained recognition for biofuel production [1,2]. However, lignocellulosic biomass has a complex structure, thus it is very hard to access the xylan structure for enzymatic hydrolysis. Furthermore, the heterogeneous structure of hemicellulose requires several types of backbone and side-chain-cleaving enzymes to degrade it [3]. Xylanase is the main enzyme used to break down the bonds that hold the fibers together inside hemicellulose by cleaving the beta-1,4 molecules backbone into a simpler sugar called xylose can be further converted in bioethanol [4]. Besides, xylanase have great potential application in several industries such as biotechnological industrial [5].

Yet, implementation of exorbitant commercial xylanase could be a bottleneck to the overall process. Since, xylanase is a bio-enzyme that can be produced by fungi, bacteria, marine algae, protozoans, snails, crustaceans, insect, seeds, and others. Hence, researchers put on-site xylanase production into practice. Filamentous fungi are advantageouse xylanase producers during fungi secondary metabolite process. Aspergillus species fungi is the main xylanases producer used in industrial field. They have advantages of high producing levels of extracellular enzymes and easy to cultivate [6]. Thus, Aspergillus niger (A. niger) is selected as the xylanase producer in this study. There are numerous parameters that can affect the xylanase production from fungus, A. niger. In specific, xylanases production is usually dependent on the nature and concentration of the carbon source in the nutrient medium during fermentation [7].

Commonly, xylan is widely considered as a model carbon source to aid xylanase production [8]. In this study, the effective performance of empty fruit bunches (EFB) from oil palm industry was compared with the corn cob-based xylan (CCX). EFB had been gaining recognition for its abundance and it was taken into consideration as lignocellulosic biomass. Lignocellulosic materials are mainly made up of cellulose, hemicellulose, lignin and other minor components. The cellulose and hemicellulose contribute to be potential of this biomass to be used as carbon source for xylanase production process [9]. Moreover, EFB wastes are traditionally burnt and thus lead to environmental pollution. Thus, application of EFB as carbon source would be a solution to the waste disposal problem [10].

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With that, the effect of concentration of carbon sources and compositions on xylanase synthesized from *A. niger* is investigated via one-factor-at-time (OFAT) technique in this research work. Growth profiles of *A. niger* was presented by using EFB as the carbon source by mycelial dry cell mass and Bradford protein method. Concentration of CCX and EFB are the chemical factor on chemical composition effect study. Comparison of biomass, enzymatic activity and xylanase production between both carbon sources at different concentrations were further discussed. The methods used to analyse are mycelial dry cell mass, Bradford method and 3,5-dinitrosalicylic (DNS) method.

### 2 Material and Methods

#### 2.1 *A. niger* Maintenance

*A. niger* was obtained from Universiti Tunku Abdul Rahman, UTAR. The strain was sub-cultured periodically on autoclaved PDA slant and incubated at 30°C for five days. Then, it was maintained at 4°C for preservation before used [11].

#### 2.2 *A. niger* Growth Profile Construction

Potato dextrose broth (PDB) was prepared by dissolving 24 g of PDB powder in 1 L of distilled water then autoclaved. Aliquot of 1 ml fungal spores suspension isolates with 2 x 10⁶ spores/mL inoculum size was inoculated with 100 mL of PDB in 250 mL of Erlenmeyer flasks. The fungus was incubated at room temperature and 150 rpm for 9 days. Totally 10 flasks per set including control were incubated, and sampling was done by collecting one flask from rotary shaker every day. The flasks were removed on daily basic to determine the mycelial dry cell mass and protein concentration which aided the establishment of the *A. niger* growth profile.

#### 2.3 Preparation of Carbon-Based Substrate

CCX and EFB were selected as carbon sources for the growth of *A. niger*. CCX was chosen since it is available in the market and was efficient in xylanase production as reported by Javed et al. [8]. CCX is initially in dry powder form. Hence, there was no preparation needed for CCX before fermentation. While dried EFB was obtained from Universiti Tunku Abdul Rahman, UTAR and initially in large size. To obtain the maximum growth of *A. niger*, the size of EFB must be reduced into smaller size by using grinding machine. After grinded, the EFB powder was washed by distilled water twice before autoclaved. The ratio of EFB to distilled water is 1:2 which means 1 L of EFB was washed by 2 litres of distilled water. After rinsed, the EFB was autoclaved and dried overnight in oven at 70°C. The EFB was kept inside a container under room temperature before used.

#### 2.4 Submerged Fermentation

Xylanase biosynthesis was performed in a 250 mL Erlenmeyer flask using submerged fermentation. *A. niger* was submerged in 100 mL of Mandels and Sternberg basal medium with 2.5 g/L of solid substrate (CCX/EFB) to support and enhance the fungus growth. 20 g/L of yeast extract is required as the main nitrogen source in the growth medium only when EFB is used as substrate [11]. A plug of *A. niger* on PDA agar was transferred into each autoclaved Erlenmeyer flask which containing the growth medium and solid substrate. After the plugs are transferred, the mouth of flask was plugged with the cotton plunger. The flasks which containing the fungus were agitated at 150 rpm inside incubator shaker at 28°C for 5 days.

#### 2.5 Chemical Factors Affecting Xylanase Production

Chemical effect in xylanase production was studied by using OFAT experiment, whereby one factor at one time and other factors were remain unchanged. Two chemical parameters were selected to study the optimization of xylanase production from *A. niger*. The manipulated variables are type of carbon sources and concentrations of carbon source. The carbon sources selected to study are CCX and EFB. Yeast extract is needed when EFB is used as the carbon source to provide the nitrogen nutrient for *A. niger* to grow. The concentrations studied for both carbon sources were 2.5 g/L, 3.0 g/L, 3.5 g/L and 4.0 g/L. The xylanase production was evaluated in terms of xylanase activity, xylanase concentration and mycelial cell dry weight.

#### 2.6 Analytical Methods

##### 2.6.1 Mycelial Dry Cell Mass

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After fermentation, the broth was filtered using Whatman Grade 1 Qualitative Filter Papers. The mycelial dry mass was determined after drying at 60°C for 24 hours in the oven [12].

2.6.2 3,5-Dinitrosalicylic Analysis Method

DNS method was used to analyse the xylanase activity. One unit of enzyme activity (U) is defined as the amount of enzyme required to liberate 1μmol of reducing sugars per minute. A 0.2 mL of sample from each concentration was dispensed into labelled centrifuge tubes and incubated at 50°C water bath for 30 minutes. 0.6 mL of DNS reagent was added into each tube after incubation and boiled for 5 minutes in water bath. After boiled, 4.2 mL of distilled water was added and the absorbance was measured at 550nm by using microplate reader. The concentration of released xylose sample was determined by interpolation from absorbance values versus xylose concentration standard curve. Equation 1 was used to quantify the xylanase activity [13].

$$\text{Xylanase Activity} = \frac{\text{Xylose Concentration} \times \text{Incubation Time}}{\text{Absorbance}} \times \text{Dilution Factor}$$

(1)

2.6.3 Bradford Analysis Method

Bradford’s method was used to determine the xylanase per volume concentration. 0.1 mL of fermentation sample was dispensed into labelled centrifuge tube. Then, 5.0 mL of Coomassie dye reagent was added into each tube and vortex immediately. The mixture was incubated at room temperature for 10 minutes. After incubation, the mixture was vortex again and the absorbance value was measured at 595 nm by using microplate reader. The concentration of the sample was determined by interpolation from Bovine Serum Albumin (BSA) standard curve [14].

3 Results

3.1 Growth Profile Construction of A. niger

This research involved the study of xylanase produced using the xylanase synthesized from A. niger. Initially, growth profile was used to determine the optimum fermentation period of A. niger to produce the maximum amount of xylanase. The growth profile bar chart of dry mycelial cell mass and protein concentration against fermentation period was drawn in Figure 1. From the mycelial dry cell mass and protein concentration growth profile, the optimum period found is 5 days. Hence, 5-day incubation is used for the effect study.

Fig 1. Growth profile of A.niger

3.2 Effect of Selected Parameter on Xylanase Production

The type and concentration of carbon sources are selected as the manipulated variable to study the effect on xylanase activity, xylanase concentration produced and mycelial dry cell mass. The effects of CCX and EFB at different concentrations are shown in Table 1 and Table 2, respectively. To diminish the analytical error in data collected, three identical samples with same condition were prepared for each experiment.

From the chemical effect study, the value of enzyme activity, xylanase concentration and mycelial dry cell mass were increased when the concentration of both carbon sources was increased from 2.5 g/L to 3.5 g/L. The maximum value of
enzyme activity, xylanase concentration and mycelial dry cell mass are obtained when the concentration of both carbon sources is 3.5 g/L. However, the values start to decrease when the concentration was increased from 3.5 g/L to 4.0 g/L due to substrate inhibition. Overall, CCX shown a higher value in enzyme activity, xylanase concentration and mycelial dry cell compared to EFB.

| CCX Concentration (g/L) | Xylanase Activity (U/mL) | Xylanase Concentration (µg/mL) | Mycelial Dry Cell Weight (mg/mL) |
|-------------------------|--------------------------|-------------------------------|---------------------------------|
| 2.5                     | 0.597 ± 0.009            | 0.678 ± 0.006                 | 0.374 ± 0.004                   |
| 3.0                     | 0.675 ± 0.010            | 0.789 ± 0.011                 | 0.383 ± 0.004                   |
| 3.5                     | 0.789 ± 0.012            | 0.882 ± 0.005                 | 0.482 ± 0.003                   |
| 4.0                     | 0.753 ± 0.009            | 0.802 ± 0.005                 | 0.401 ± 0.003                   |

| EFB Concentration (g/L) | Xylanase Activity (U/mL) | Xylanase Concentration (µg/mL) | Mycelial Dry Cell Weight (mg/mL) |
|-------------------------|--------------------------|-------------------------------|---------------------------------|
| 2.5                     | 0.291 ± 0.006            | 0.216 ± 0.009                 | 0.066 ± 0.002                   |
| 3.0                     | 0.302 ± 0.004            | 0.324 ± 0.005                 | 0.168 ± 0.003                   |
| 3.5                     | 0.409 ± 0.005            | 0.533 ± 0.006                 | 0.370 ± 0.002                   |
| 4.0                     | 0.404 ± 0.004            | 0.529 ± 0.006                 | 0.269 ± 0.003                   |

4 Discussion

4.1 Growth Profile Construction of A.niger

Growth curve is a useful method to study the growth features of filamentous fungi in different nutrient media and determine the optimal period for filamentous fungi to grow. Generally, there are five phases in growth of filamentous fungi which are lag phase, first transition period, log phase, second transition period, and stationary phase. Among these five phases, log phase had the smallest variability in growth rates. Hence, the optimal period is obtained by determining the time used for the fungus to reach the log phase [15].

In this study, the kinetic growth of A. niger was monitored by mycelial dry cell mass (biomass) and Bradford protein concentration growth profile (Figure 1). From the dry mycelial cell mass growth profile, the lag phase is shown in the first 24-hour incubation. The growth rate of A. niger is very low. An optimum growth rate obtained from day 1 to day 3 in the Figure 1. A. niger took 2 days to reach the stationary phase after the optimal growth. Highest mycelial mass is shown on day-5. After day-5, the mycelial cell mass was decreasing due to lack of nutrients and carbon source in medium to support A. niger to grow.

In terms of protein concentration growth profile, the protein concentration of the samples is collected from each day are determined by BSA method and the unit of the protein concentration is µg/mL. Protein growth curve with protein concentration versus fermentation period was plotted in Figure 1. The protein concentration was increase from day 1 to day 5 and started to reduce after day 5. This shown that the growth of A. niger is terminated at day 5. The lowest concentration increment was found during the first 24 hour, this is the lag phase of A. niger growth. The concentration of protein is rise speedily from day-1 to day-3 and it takes 2 days to reach stationary phase (day-4 to day-5). Highest protein concentration is determined on day-5 then the protein concentration is started to reduce. This is because the nutrient in the medium is not enough to support the fungus to grow after day 5.

4.2 Effect of Selected Parameter on Xylanase Production

OFAT technique was applied to study the chemical effect on xylanase activity, xylanase production and mycelial dry cell mass of A. niger by only changing one variable and others is fixed at constant. Mycelial dry cell mass is used to determine the growth of A. niger; while BSA method and DNS method is used for the xylanase assay. To increase the data accuracy, 3 set of samples is prepared from each experiment.

Two parameters were selected to study the chemical effect which are type of carbon sources (CCX and EFB) and concentration of carbon source (2.5 g/L to 4.0 g/L). 20 g/L of yeast extract is needed when EFB is used. The crude enzyme is collected from the sample under a fermentation condition of 28°C, pH 5.0 and agitated at 150rpm after 5 days incubation.

CCX was used as the carbon source in Mandels and Sternberg basal medium to support A. niger growth. The concentration of CCX was adjusted from 2.5 g/L to 4.0 g/L. The samples from each concentration were collected after 5 days incubation. The effect of CCX concentration on enzymatic activity is shown in Table 1. According to the Table 1 the enzyme activity, xylanase concentration per volume and mycelial dry cell mass are increasing when the concentration of CCX is increased from 2.5 g/L to 3.5 g/L. Among these studies, maximum values are obtained when the concentration

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of CCX is 3.5 g/L. However, the enzyme activity, xylanase concentration and mycelial dry cell mass are decreased when the concentration of CCX is increased to 4.0 g/L.

Subsequently, EFB was used as the carbon source and 20 g/L of yeast extract was added into Mandels and Sternberg basal medium as nitrogen sources to support A. niger growth. The concentration of EFB was adjusted from 2.5 g/L to 4.0 g/L. The samples from each concentration were collected after 5 days incubation. The effect of EFB concentration enzymatic activity is tabulated in Table 2. From the table, the enzyme activity, xylanase concentration per volume and mycelial dry cell mass are increasing when the concentration of EFB is increased from 2.5 g/L to 3.5 g/L. Maximum values are obtained when the concentration of EFB is 3.5 g/L. However, the enzyme activity, xylanase concentration and mycelial dry cell mass are decreased when the concentration of EFB is increased from 3.5 g/L to 4.0 g/L.

As can be clearly observed in Table 1 and 2, the enzyme activity, xylanase concentration per volume and mycelial dry mass are increasing when both carbon sources concentration increased from 2.5 g/L to 3.5 g/L. But the values were dropped at concentration of 4.0 g/L which means that the excess of carbon source will limit the xylanase production due to substrate inhibition. The optimum concentration of the carbon source determined from this OFAT experiment is 3.5 g/L. Carbon source is the main element for fungus growth and crude enzyme production. However, excess of carbon source will limit the fungus secondary metabolism process which only occurs at limited carbon concentration. Xylanase is the product of the secondary metabolites, hence excess of carbon concentration will restricted the xylanase production [16].

Furthermore, CCX shows the higher xylanase concentration (0.882±0.005 μg/mL) compared to EFB (0.533±0.006 μg/mL). Besides, CCX also has higher values in enzyme activity and mycelial dry cell compared to EFB. According to Patel & Shukla [17], CCX is a corn cob-extracted simple carbon which is easier for A. niger to digest and produce xylene compared to EFB. While EFB used in this study is a direct lignocellulosic waste from palm oil industry and without any pre-treatment. EFB was washed, autoclaved and grinded into suitable size before it is used as the substrate in growth medium for A. niger to grow. Besides, yeast extract is also required as the main nitrogen source when EFB is used as substrate. By comparing all analysis, CCX has been proven to be a more feasible carbon source to synthesize xylanase from A. niger compared to EFB.

5 Conclusion

From the chemical effect study, the value of enzyme activity, xylanase concentration and mycelial dry cell mass were increased when the concentration of both carbon sources was increased from 2.5 g/L to 3.5 g/L. The maximum value of enzyme activity, xylanase concentration and mycelial dry cell mass are obtained when the concentration of both carbon sources is 3.5 g/L. However, the values start to decrease when the concentration was increased from 3.5 g/L to 4.0 g/L due to substrate inhibition. Corn cob-based xylan (CCX) shown the higher value in enzyme activity, xylanase concentration and mycelial dry cell as compared to empty fruit bunches (EFB). This is due to CCX is a corn cob-extracted simple carbon and easier to digest by fungus. The optimum concentrations from chemical effect for CCX and EFB are 3.5 g/L were used to grow A. niger in submerged fermentation. By comparing all analysis, CCX has better performance compared to EFB. Hence CCX is the better carbon source to grow Aspergillus niger which has a higher xylanase production compared to EFB.

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