Optimization of moistening solution concentration on xylanase activity in solid state fermentation from oil palm empty fruit bunches

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Abstract. Xylanase is an enzyme used in the industrial world, including food industry. Xylanase can be utilized as a 1,4-β-xylosidic endo-hydrolysis catalyst of xylanase, a hemicellulose component for obtaining a xylose monomer. This study aims to determine the optimum concentration of the fermentation medium using Response Surface Method (RSM) in the production of xylanase enzyme from oil palm empty fruit bunches (OPEFB) through solid state fermentation process. The variables varied in this study used factor A (ammonium sulphate concentration 1.0-2.0 g/L), B (concentration of potassium dihydrogen phosphate 1.5-2.5 g/L) and C (urea concentration 0.2 – 0.5 g/L). The data was analysed by using Design Expert version 10.0.1.0 especially CCD with total 17 running including 3 times replicated of canter point. Trichoderma viride was used for the process production of xylanase enzyme. The ratio between substrate and moistening solution used was 0.63 g / mL with temperature of 32.8°C, 60 h incubation time. The analysis of enzyme activity was done by DNS method with 1% xylan as substrate. Analysis of protein content in enzyme was done by Bradford method. The optimum of moistening solution concentration in this fermentation was obtained. They are, the ammonium sulphate concentration of 1.5 g/L, potassium dihydrogen phosphate 2.0 g/L and urea 0.35 g/L with activity of 684.70 U/mL, specific activity enzyme xylanase 6261.58 U/mg, protein content 0.1093 U/mg, the model was validated using experiment design with perfect reliability value 0.96.

1. Introduction

The usage of enzyme in Indonesia reaches 2500 tons with an import value of $ 14.0 millions. Many food and non-food industries have used enzyme. One of the enzyme that has high economic value in the industry is xylanase that can be used for bioleaching in paper industry, cleansing and improving aroma of juice and wine, improving the quality of bread and animal feed, waste processing and composting. Xylanase is a group of enzymes that have the ability to hydrolysed hemicellulose in this case is xylene or polymer of xylose and xylo-oligosaccharide [1]. Xylan which is a substrate for xylanase enzymes is found in many annual crops and especially agricultural wastes such as corncobs, bagasse,
rice straw, wheat bran, and cottonseed. Oil palm empty fruit bunches (OPEFB) are the main waste of the palm oil processing industry. The percentage of OPEFB waste is 23% of fresh fruit bunches, while the percentage of fibre and shell of kernels are 13% and 5.5% respectively of fresh fruit bunches [2]. The main components of palm solid waste are cellulose and lignin so that this waste is also called lignocellulosic waste. The results also showed similar results, OPEFB containing cellulose 43-43.7%, hemicellulose 22.93-23.67% and lignin 21.28-22.10% [3].

The high content of hemicellulose in OPEFB has the potential to produce xylanase by microorganisms that produce xylanase by utilizing xylan. Xylanase can be produced from microorganisms such as fungi and bacteria, one of them is Trichoderma sp. through the fermentation process [3]. Also reported that the best activity of xylanase was obtained from \textit{Trichoderma viridae} incubated at 32.8°C compared with \textit{Aspergillus niger} and \textit{Penicillium sp.} on OPEFB substrate [4].

In the solid-state fermentation, the moisture obtained from the addition of moistening solution, the addition of moistening solution is intended to increase the number of cells and assist cell adaptation to new environmental conditions [5]. Metabolism of microorganisms requires macronutrients such as nitrogen, phosphorus, potassium, sulphur, and micronutrients such as chromium, cobalt, selenium, tungsten, magnesium, iron, zinc, molybdenum, etc. [6]. Mineral salt solutions are important for enzyme production because some enzymes such as xylanase, cellulase and proteinase are very sensitive to the moistening solutions used [7].

Salt solutions containing a mixture of essential minerals such as Mg, K, and P need to be added with a certain concentration into the fermentation medium because of the addition of one or several combinations of mineral solution concentration to the fermentation process and metabolite products produced by microbes. Based on the background, it is necessary to conduct further study to find out the optimum concentration of moistening solution.

2. Materials and Methods
2.1 Raw Material Preparation

OPEFB was collected from PTPN VIII Cikasungka, Bogor, Indonesia. OPEFB fibre was washed using tap water and then sun dried for 12 h and oven dried at 105°C overnight. Dry OPEFB was grinded using a disc mill and sieved using Tyler 60 mesh.

2.2 Fungal Preservation

The fungal strain was tested during the experiment is \textit{T. viridae} ITB CC L.67 was preserved in potato dextrose agar (PDA) slants. The spore of \textit{T. viridae} were preserved as immobilized spores on dried rice pellets. Inoculum was prepared by suspending the fungal spores in sterile distilled water to give a final spore count of 1×10^6 spores/mL.

2.3 Preparation of Cultivation Medium

The media for solid state fungal cultivation was prepared by mixing dried OPEFB as the carbon source for the fungal cultivation with liquid moistening solution which was adapted from Aves-Prado et al., (2010). The moistening solution contained (NH$_4$)$_2$SO$_4$, KH$_2$PO$_4$, urea, CaCl$_2$.2H$_2$O and MgSO$_4$.7H$_2$O. The media was first sterilized in an autoclave at 121°C for 15 minutes before use.

2.4 Solid State Fermentation
2.4.1 Evaluation of cultivation time

In the process of determining the optimal cultivation time for each of the \textit{T. viridae}, 6.3 g OPEFB was mixed with 10 mL of mineral medium (moistening solution) to give the solid to liquid substrate ratio of 0.63 g substrate/mL moistening solution. The fungal cultivation of 1×10^6 spores/mL were carried out in 250 mL shake flasks at 32.8°C and were performed over a duration of 60 h. Sampling was done 60 h incubation and the obtained samples were used for the analysis of xylanase activity, protein content and xylanase activity specific. Each run was performed in Duplo.
2.4.2 Evaluation of OPEFB particle size

The drying process was done before using the raw material of palm stem, the reduction and uniformity of the size to 40-60 mesh to increase the surface area and increase the solubility in water. The reaction rate increases with time to particle size. The smaller particle size gives an increasingly fast cooking time.

2.4.3 Experimental design for optimization of parameters using RSM

The production of xylanase was optimized using the method of response surface methodology, which is the Central Composite Design (CCD) experimental design with application Design Expert version 10.1.0.0, on three variables: ammonium sulphate concentration (1.0–2.0 g/L), calcium dihydrogen phosphate concentration (1.5–2.5 g/L), and urea concentration (0.1–0.5 g/L). The cultivation temperature was varied at 32.8°C, whereas the substrate ratio applied were in 0.63 (g OPEFB/mL moistening solution).

The total amount of treatment variation is 17 running including 3 times the repetition of the value of centre (central point). Furthermore, in a near-response state, two or more order models are usually required to approximate the response because of the curvature on its surface expressed by (1):

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j + \epsilon \]  

where \( Y \) is the predicted response, \( x_i, x_j, \ldots, x_k \) are the input variables, \( \beta_0 \) is the intercept state, \( \beta_i \) (\( i = 1, 2, \ldots, k \)) is the influence of linear, \( \beta_{ii} \) (\( i = 1, 2, \ldots, k \)) is the influence of squared, \( X_{ij} \) (\( i = 1, 2, \ldots, k; j = 1, 2, \ldots, k \)) is the influence of interaction and \( \epsilon \) is a random transgression.

2.5 Harvesting of Enzymes

Enzyme harvesting process was conducted according to [8]. Enzymes were extracted from the fungal cultivation by adding 40 mL distilled water (four times volume of liquid medium) to the cultivation solution. Subsequently the solution was stirred with a sterile glass stick and then shaker at 100 rpm for 1 hour at room temperature (25°C). The solution was then vacuum filtrated to remove solid and particulates. The filtered solution was further centrifuged at 10,000 rpm for 12 minutes at 4°C. The obtained supernatant (the crude enzyme) could be used for enzyme activity analysis.

2.6 Analytical Methods

2.6.1 Lignocellulosic material composition

Analysis of chemical components of raw materials aims to determine the chemical composition contained in the raw material, consisting of moisture content (b/b) gravimetric method, ash content and lignocellulosic levels.

2.6.2 Enzyme Activity

The xylanase activity was determined using larch wood xylan (Sigma Co., USA) as substrate [9]. In brief, the reactant containing 0.5 mL of crude enzyme and 0.5 mL of 1% (w/v) larch wood xylan (pH 5.0 acetate buffer) were incubated in a water bath at 40°C for 15 min. The reaction was stopped by adding 1.5 mL of dinitro salicylic (DNS) acid solution. After incubation in a boiling bath for 5 min, the liberated reducing sugars were measured with spectrophotometer at 540 nm wavelength. The reducing sugars produced were quantified by the dinitro salicylic acid method using D-xylose as standard. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose per minute under the assay conditions.

2.6.3 Protein Content
The measurement of protein content in the sample was determined by Bradford method with Bovine Serum Albumin (BSA) as the standard. A total of 100 μL filtrate (rough xylanase enzyme) was added with 2 mL of the Bradford reagent inserted into the test tube. The reaction tube was vortex and incubated at room temperature (25°C) for 5 minutes and the measured absorbance at 595 nm. The protein content of the sample was determined from the standard BSA solution curve with a concentration of 0.2 - 1.0 mg/L.

2.6.4 Enzyme Specific Activity

The specific activity of crude extract of xylanase enzyme states a number of μmol substrates which can be converted into products within a minute by 1 milligram of enzyme under optimum conditions. The higher the value of the specific activity of the enzyme, the better the ability of xylanase to utilize the substrate. Calculation of specific activity of crude extract of xylanase enzyme was done by comparing the value of activity of crude extract of xylanase enzyme with protein content.

3. Results and Discussions

3.1 Characterization of OPEFB

Characterization of chemical composition of oil palm empty bunches carried out include testing the levels of cellulose, hemicellulose, lignin, water, and ash. The results are presented in Table 1.

| Component       | Percentage (5)       |
|-----------------|----------------------|
| Hemicellulose (db) | 17.31 ± 0.5586       |
| Cellulose (db)    | 39.99 ± 0.7354       |
| Lignin (db)       | 23.96 ± 0.9970       |
| Water (wb)        | 4.60 ± 0.1131        |
| Ash (db)          | 4.78 ± 0.1626        |

Hemicellulose levels need to be analysed to find out how big the potential of empty bunches of palm oil to be used as raw materials in the production of xylanase enzymes. Hemicellulose level in OPEFB used was 17.31%. The main component of hemicellulose is needed, xylan contained in hemicellulose. Xylan acts as an inducer for xylanase production in the hope that xylanase production will increase.

In addition, OPEFB contained quite high carbon because they contain cellulose of 39.99%. The component of cellulose as a carbon source for microorganisms. Lignin prevents the entry of enzymes in breaking down polysaccharides into monosaccharides [10]. Based on the results of analysis known that the lignin content of 23.96%. This indicates that empty bunches of palm oil can be used as substrate because it contains lignin ≤25% [11].

Water content needs to be analysed to determine whether OPEFB have long or no shelf life. High water levels cause rapid OPEFB damage due to the growth of microorganisms. If the water content is known then it can be adjusted raw material storage conditions to minimize the possibility of the growth of microorganisms. In addition, knowing the initial moisture content of the material can be used as a reference to the addition of a fermentation medium (moistening solution) so that the water content in the material (substrate) is not more than 20% so it can be referred to as solid state fermentation (SSF).

Ash content indicates the content of organic substances or mineral elements of a material. Ash content is influenced by type, age of material, and others. Based on the analysis, OPEFB have ash content of 4.78%.

3.2 Optimization production of enzyme xylanase

Parameters for SSF optimization to be considered are carbon and nitrogen sources, adaptation of inoculum, pH, temperature, incubation time, water content [12]. In addition, the fermentation medium is also an important factor in the state fermentation process for enzyme production because some
enzymes such as xylanase, cellulase and proteinase are very sensitive to the composition of the fermentation medium (moistening solution). Response to each of the moistening solution concentrations is presented in Table 2 and the analysis of ANOVA is presented in table 3.

**Table 2. Response on Overall Condition of Optimization**

| Run | Concentration (g/L) | Xylanase Activity (U/mL) | Protein content (mg/mL) | Xylanase Activity Specific (U/mg) |
|-----|---------------------|--------------------------|-------------------------|-----------------------------------|
| 1   | 1.50 2.00 0.35      | 712.71 ± 43.21          | 0.1076 ± 0.0017         | 6624.53                           |
| 2   | 1.50 2.00 0.60      | 562.46 ± 97.23          | 0.0506 ± 0.0025         | 11114.87                          |
| 3   | 2.00 2.50 0.50      | 554.82 ± 21.60          | 0.0718 ± 0.0008         | 7732.44                           |
| 4   | 2.00 2.50 0.20      | 535.53 ± 2.88           | 0.1123 ± 0.0133         | 4769.37                           |
| 5   | 2.34 2.00 0.35      | 450.10 ± 5.76           | 0.1011 ± 0.0025         | 4450.98                           |
| 6   | 1.00 1.50 0.50      | 542.09 ± 32.41          | 0.0671 ± 0.0025         | 8084.49                           |
| 7   | 2.00 1.50 0.20      | 524.26 ± 43.21          | 0.0923 ± 0.0033         | 5679.17                           |
| 8   | 1.00 2.50 0.20      | 590.47 ± 0.00           | 0.0571 ± 0.0017         | 10347.16                          |
| 9   | 1.00 2.50 0.50      | 593.02 ± 68.43          | 0.1440 ± 0.0017         | 3905.75                           |
| 10  | 1.50 2.00 0.35      | 733.08 ± 7.20           | 0.0988 ± 0.0008         | 7421.75                           |
| 11  | 1.50 2.84 0.35      | 598.11 ± 3.60           | 0.0970 ± 0.0017         | 6165.31                           |
| 12  | 1.00 1.50 0.20      | 565.01 ± 14.40          | 0.1229 ± 0.0017         | 4598.78                           |
| 13  | 1.50 2.00 0.10      | 577.74 ± 32.41          | 0.0876 ± 0.0050         | 6594.20                           |
| 14  | 2.00 1.50 0.50      | 536.99 ± 90.03          | 0.0923 ± 0.0050         | 5817.11                           |
| 15  | 1.50 1.60 0.35      | 536.46 ± 82.83          | 0.0588 ± 0.0008         | 9561.01                           |
| 16  | 1.50 2.00 0.35      | 692.34 ± 0.00           | 0.1187 ± 0.0008         | 5830.30                           |
| 17  | 0.66 2.00 0.35      | 557.37 ± 104.44         | 0.0894 ± 0.0042         | 6236.22                           |

**Table 3. ANOVA for Optimisation of Xylanase Enzyme Activity Resapons**

| Source                          | Sum of Squares | df | Mean Square | F Value | p-value | Prob > F |
|---------------------------------|----------------|----|-------------|---------|---------|----------|
| Model                           | 79047.49       | 9  | 8783.05     | 25.47   | 0.0002  | significant |
| A-Ammonium sulphate             | 7467.38        | 1  | 7467.38     | 21.65   | 0.0023  |           |
| B-Potassium dihydrogen phosphate| 2004.51        | 1  | 2004.51     | 5.81    | 0.0467  |           |
| C-Urea                         | 14.08          | 1  | 14.08       | 0.041   | 0.8456  |           |
| AB                              | 279.54         | 1  | 279.54      | 0.81    | 0.3979  |           |
| AC                              | 343.09         | 1  | 343.09      | 0.99    | 0.3518  |           |
| BC                              | 128.24         | 1  | 128.24      | 0.37    | 0.5613  |           |
| A²                              | 56340.70       | 1  | 56340.70    | 163.36  | < 0.0001 |           |
| B²                              | 21411.40       | 1  | 21411.40    | 62.08   | 0.0001  |           |
| C²                              | 25181.38       | 1  | 25181.38    | 73.01   | < 0.0001 |           |
| Residual                        | 2370.08        | 7  | 338.58      |         |         |           |
| Lack of Fit                     | 1584.31        | 5  | 316.86      | 0.76    | 0.6596  | not significant |
| Pure Error                      | 829.87         | 2  | 414.94      |         |         |           |

R² 0.9704

### 3.2.1 Response to Xylanase Enzyme Activity

The ANOVA analysis showed that the F-values of 25.47 and p-values (Prob > F) were less than 0.05 (0.0002), indicating that the (quadratic) model was significant at 95% confidence level (Table 3). This means that the independent variable (X) in the model has a significant effect on the response (Y).
xylanase enzyme activity. In addition, non-significant Lack of Fit values also indicate that the model is good to implement. Lack of Fit is a model that has not been exact or there is no match between the data and the model.

Based on Table 3, it is known that the $F_{\text{count}}$ value is more than 0.05 at the $\alpha = 5\%$ level so that the model is suitable or the suitability between the model and the data is appropriate. In Table 3, it can also be known the value of coefficient of determination or also known as $R^2$ of 0.9710 which means the influence of independent variables on the change of dependent variable (xylanase enzyme activity) is 97.10% while the rest of 2.90% is affected by other factors outside the independent variables used in this study. In general, a regression that has a coefficient value of determination over 0.90 indicates a very strong correlation. The value of the adjusted determination coefficient (Adjusted R-Squared) is also fairly high (0.9336). This is sufficient to prove that the equation model is significant.

Checking the suitability of the model can also be done using a normal probability plot of residual and residual plots on the response prediction. Plot The normal probability to residual is considered to be satisfied when the residual value (the difference between the actual value and the response prediction value) is in the vicinity of a straight line. The residual value residing in the straight line on the curve indicates that the model's non-conformance to the response data is still normally distributed. Meanwhile, the residual plot of the response prediction is considered to be satisfactory when the model in the sample is drawn at random and the result is around a straight line.

![Figure 1. Prediction vs Actual Curves for Xylanase Enzyme Activity Response](image)

The predictive and actual plot shows that residuals are drawn at random but there are some points far enough away from the straight line. The point that is quite far from the line still can be said to meet the model if it is still inside the graph area (not missing from the picture). The optimization equation in the form of the actual value to predict the response of the xylanase enzyme activity of factor A (Ammonium sulphate), B (potassium dihydrogen phosphate) and C (urea) where Y is the predicted response activity xylanase enzyme, can be seen in equation (2):

$$Y = -846.85 + 818.55A + 738.55B + 1246.12C - 23.65AB + 87.32AC + 53.38BC - 282.87A^2 - 174.38B^2 - 2129.51C^2$$ (2)
Figure 2. Contour (a) and 3D plots (b) Effect of interaction between Ammonium sulphate and Potassium dihydrogen phosphate to Xylanase Enzyme Activity

Fig. 2a contour shows the condition of both factors where optimum response value at ammonium sulphate concentration 1.5 g/L, concentration of potassium dihydrogen phosphate 2.0 g/L and the region is marked with red colour. While in Fig. 2b (3D surface) can be seen more clearly form model that resembles parabola, this is considered good because this graph shows existence of maximal value before finally declining. Based on this research, it can be seen that optimum xylanase enzyme activity at ammonium sulphate concentration 1.5 g/L and concentration of potassium dihydrogen phosphate 2.0 g/L while urea concentration 0.35 g/L.

Studies stated that higher xylanase enzyme activity uses ammonium sulphate ((NH$_4$)$_2$SO$_4$) as an inorganic nitrogen source at 0.14% (1.4 g/L) concentration of fermentation medium compared to sodium nitrate (NaNO$_3$) and ammonium nitrate (NH$_4$NO$_3$) [13]. Also stated that the highest enzyme activity of xylanase used ammonium sulphate ((NH$_4$)$_2$SO$_4$) at 0.12% - 0.15% (1.2 – 1.5 g/L) concentrations [14]. The interaction between ammonium sulphate and urea can be seen in Figure 3.

Figure 3. Contour (a) and 3D plots (b) effect of interaction between ammonium sulphate and urea on xylanase enzyme activity

As has been reported [13] the activity of xylanase enzyme is higher with activity of 760.0 U/mg in fermentation medium which added urea compared with medium without urea with activity of 711.5 U / mg. Also that the highest production of xylanase enzyme from T. reesei RUT-C30 is on urea containing
medium and producing xylanase enzyme from Melanocarpus albomyces states that the highest activity occurs on urea containing medium [8]. The produced of xylanase enzyme from Aspergillus niger XY-1 increases with increasing ammonium sulphate and urea concentrations. The interaction between potassium dihydrogen and urea can be seen in Figure 4.

![Figure 4](image)

**Figure 4.** Contour (a) and 3D plots (b) effect of interaction between Potassium dihydrogen phosphate and urea on xylanase enzyme activity

The graphs in Fig. 4a and 4b show the relationship between potassium dihydrogen and urea. The contour graph displays the conditions of the two factors where the optimal response value, the region is marked with red colour. While the 3D surface can be seen more clearly form a model that resembles a parabola, this is considered good because this graph shows the maximum value before it eventually declines. Based on the result of the research, it can be seen that the optimum xylanase enzyme activity is obtained at the concentration of 2.0 g/L potassium dihydrogen phosphate and urea concentration 0.35 g / L.

Potassium dihydrogen phosphate (KH$_2$PO$_4$) is one of the largest components present in the growth medium of T. viridae. In the solution potassium dihydrogen phosphate will decompose into potassium and phosphorus is needed microbe for metabolism. Phosphorus (P) required microbes as part of the formation of nucleic acids, phospholipids and coenzymes. Potassium acts as an inorganic cation in the cell and as a cofactor of some enzymes.

**3.2.2 Protein content and specific activity xylanase enzyme**

High protein levels allegedly indicate a high enzyme as well, but the enzyme formed cannot be ascertained is xylanase. The protein of the xylanase can be determined by knowing the activity of the xylanase first. The relationship between xylanase activity and the resulting protein is expressed by specific activity. Based on Table 4, the measurement of xylanase activity in some treatments showed that the highest enzyme activity was 711.90 U/mL. High enzyme activity is not always followed by high specific activity, and vice versa.

Enzymes are the most common group of proteins in living cells and have an important function as catalysts of biochemical reactions that collectively form metabolisms-intermediates from cells. The protein content is used as one of the tests because the solid state fermentation causes the cells to mix with the substrate so it is difficult to calculate the number of dry weight cells. The protein testing process, similar to enzyme activity, was applied to T. viridae with a temperature of 32.8°C and a substrate ratio of OPEFB with a moistening solution of 0.63 g substrate/mL moistening solution.
3.2.3 Optimization of conditions and response

The optimum value (maximization) of the xylanase enzyme activity response through solution provided by Design Expert software version 10.0.1.0. The solution provided is proven by way of experimenting again with the concentration of each factor that has been given. The results of confirmation of the equations and solutions can be seen in Table 4.

| Factor Optimization Solution | Factor Concentration (g/L) | Xylanase Enzyme Activity Prediction (U/mL) | Results (U/mL) | Validation (%) |
|------------------------------|----------------------------|------------------------------------------|----------------|----------------|
| Ammonium sulphate            | 1.50                       |                                          | 711.91         | 684.70         |
| Potassium dihydrogen phosphate | 2.00                     |                                          | 96.18          |                |
| Urea                         | 0.35                       |                                          |                |                |

The accuracy of an optimization prediction model to predict the actual value can be measured by the degree of reliability. Software Design Expert version 10.0.1.0 defines that:

- Comparison validation > 0.90 then reliability is perfect
- Comparison of validation between 0.70 - 0.90 then high reliability
- Comparison of validation between 0.50 - 0.70 then moderate reliability
- Comparison of validation <0.50 then reliability is low

Based on RSM model validation data in Table 4, it can be seen that the accuracy of RSM model can be said perfect because the value of validation comparison of experimental results with optimization model prediction value on all responses above 90%.

4. Conclusions

The optimum concentration of moistening solution for the production of xylanase enzyme was at ammonium sulphate concentration of 1.5 g/L, concentration of potassium dihydrogen phosphate 2.0 g/L and urea concentration 0.35 g/L with perfect reliability value (0.96). The optimum condition of fermentation using oil palm empty fruit bunches as substrate were autoclave pretreatment with substrate ratio 0.63 g substrate/mL moistening solution, temperature 32.8°C with fermentation time for 60 h.

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