Optimization of On-Site Xylanase Production from Aspergillus niger via Central Composite Design (CCD)

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Abstract. Xylanases have stimulated considerable interest due to their potential application in several industries, especially in the bioethanol sector. Since the vitality of this enzyme is undeniable, this research is focused on optimization of on-site xylanase production from Aspergillus niger (A. niger). This initiative could reduce the dependence of commercial xylanase. Central Composite Design (CCD) was implemented in the process of xylanase production optimization. Incubation temperature and medium pH were two parameters selected to statistically optimized using Response Surface Methodology (RSM) in order to improve the xylanase production. From the data analyzed by Design of Experiment (DoE), maximal xylanase production was predicted to produce under condition of 32.67°C and pH 4.56 with desirability of 0.936. A validation test with triplicate was done to verify the predicted result. The maximum enzyme activity of 0.5638 U/mL was obtained from the validation test.

1. Introduction
Xylanase is the name given to a classification of enzyme which degrade the second most abundant linear polysaccharide, beta-1,4-xylan into xylose, as a consequence of breaking down hemicellulose, one of the primary components of plant cell walls [1]. Xylanase is critical for the efficient use of plant biomass to produce fuels, energy and other chemical compounds. At present scenario, the secondary bioethanol industry is known to be dependent on xylanase for the biofuel production [2]. To be precise, secondary bioethanol production is based on lignocellulosic biomass with the polysaccharide composition of cellulose and hemicellulose which could be converted into biofuel. Thus, this
conversion process, biomass requires the aid of cellulose to co-exist with xylanase and cellulase to be breakdown into reducing sugar and consecutively fermented into bioethanol [3].

Yet, the utilization of commercial hydrolases could be a major factor in the elevation of the project’s overall economics. With this, the credibility of the process in terms of being a cost-effective process will be in doubt [4]. Hence, on-site xylanase production was taken in consideration to reduce the expenditure on enzymes. filamentous fungi are widely known to be the essential industrial sources of xylanase [5]. Among these microbial sources, A. niger is chosen to be used in this study because it has a specifically interesting recognition. This filamentous fungus secretes these enzymes into medium and their xylanase levels are much higher than those observed in yeasts or bacteria [6].

Generally, production of high extracellular enzymes with excellent industrial quantities are dependent on environmental conditions such as pH, temperature, initial moisture, inoculum sizes, incubation period and the nutritive aspects of the growth medium [7]. In other words, optimization of process parameters is important to enhance the activity and the volumetric productivity of general metabolites which include enzymes such as xylanase [8]. With that, the objective of this study is to optimize selected process parameters for maximal xylanase production by A. niger using the CCD of response surface statistical optimization approach.

2. Material and Method

2.1. Materials

All the chemicals used in this research work were analytical grade. These chemicals were purchased from Sigma-Aldrich, St. Louis, MO USA except otherwise stated.

2.2. Inoculum Development

A 10 mL of 1% (v/v) Tween-80-sterile distilled water was added to a 5 days old pure A. niger culture on agar plate and the agar surface was rubbed with a sterile L-shaped spreader to obtain spore suspension. The suspension was sieved through Whatman no.1 paper to remove mycelia. After appropriate dilutions, inoculum sizes (2 x 10^6 spores/mL) were determined by direct microscopic observation using hemocytometer.

2.3. Submerged Fermentation (SmF)

Xylanase biosynthesis were performed in a 250 mL Erlenmeyer flask using SmF in a production medium containing 100 mL of Mandels and Sternburg’s basal medium and 5.0 g/L of xylan as carbon source. Composition of the growth medium includes: 2.0 g/L NH₄NO₃, 2.0 g/L K₂HPO₄, 1.0 g/L MgSO₄.7H₂O, 0.3 g/L CaCl₂, and 0.012 g/L trace elements: 5.0 mg/L FeSO₄.7H₂O, 1.6 mg/L MnSO₄.4H₂O, 3.45 mg/L ZnSO₄.7H₂O and 2.0 mg/L CoCl₂.6H₂O and 0.1% (v/v) Tween 80. Aliquot of 1 mL of standardized A. niger inoculum (2 x 10^6 spores/mL) was aseptically inoculated into the medium and fermentation was allowed to take place at different selected incubation temperature at 150 rpm for 5 days in an incubator shaker (Jeiotech, Korea). Crude enzyme was extracted with 0.05 M citrate buffer pH 4.8 (50 mL). After the addition of the buffer, the whole flask was kept in a rotary incubator at 150 rpm (32°C) for 15 min. The content was filtered through Whatman no.1 filter paper to extract the filtrate which was then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was collected as the crude enzyme for xylanase activity analysis [9].

2.4. Optimization Process of Xylanase Production

Response surface methodology was used for the optimization of conditions for xylanase production by A. niger using Central Composite Design (CCD) of the Design expert DX 11.0.4.0. A total of 11 runs including 4 factorial points, 4 axial points, and 3 center points in triplicates were independently done.
to verify the effects of variables on xylanase production. The variables studied in this model was incubation temperature ($X_1$) and medium pH ($X_2$). Each parameter was divided into three levels: low (-1), medium (0) and high (+1). Optimum incubation temperature and medium pH derived from CCD optimization were used as functions to generate model for enzyme activity (U/mL). The model was analysed based on ANOVA and regression analyses.

### 3. Result and Discussion

The overall design matrix of experiments on optimization of xylanase production from *A. niger* coupled with the experimental enzyme activity via CCD is shown in Table 1. pH and temperature of the cultivation medium were selected to be optimized value were the most significant variables for enzyme production by *Aspergillus* species [10]. Based on collected data, the enzyme activity was found varying in the range of 0.5237 U/mL to 0.3368 U/mL. The experimental run with highest enzyme activity (0.5237 U/mL) was achieved with run #1 (32.50°C, pH 5.00). In contrast, the lowest enzyme activity (0.3368 U/mL) was measured at run #7 (38.86°C, pH 5.00).

| Std | Run | Incubation temperature (°C) | Medium pH | Enzyme activity (U/mL) | Standard deviation |
|-----|-----|---------------------------|----------|------------------------|--------------------|
| 9   | 1   | 32.50                     | 5.00     | 0.5237                 | 0.0008             |
| 8   | 2   | 32.50                     | 7.83     | 0.3990                 | 0.0046             |
| 11  | 3   | 32.50                     | 5.00     | 0.5078                 | 0.0010             |
| 7   | 4   | 32.50                     | 2.17     | 0.4336                 | 0.0046             |
| 10  | 5   | 32.50                     | 5.00     | 0.4974                 | 0.0025             |
| 4   | 6   | 37.00                     | 7.00     | 0.4075                 | 0.0015             |
| 6   | 7   | 38.86                     | 5.00     | 0.3368                 | 0.0039             |
| 1   | 8   | 28.00                     | 3.00     | 0.4061                 | 0.0051             |
| 3   | 9   | 28.00                     | 7.00     | 0.3543                 | 0.0014             |
| 5   | 10  | 26.14                     | 5.00     | 0.3787                 | 0.0017             |
| 2   | 11  | 37.00                     | 3.00     | 0.4557                 | 0.0018             |

*The shaded rows indicated the maximum (run #1) and minimum (run #7) enzyme activity.*

The analysis of variance (ANOVA) was primarily used to identify the mathematical model that best suit for the condition. The model was selected based on the degree of significance. The significance of each coefficient was determined by whether it had p-value which less than 0.05. The F-value is the measurement of variation of the data about the mean. The high F-value and very low probability indicate that the model is in good prediction of the experimental results [11].
Table 2 tabulates the analysis of variance table for response quadratic model. Based on Table 2, it can be concluded that quadratic model was the best model to fit the response as the F-value was observed to be 7.36 implied that the model was significant. There was only a 2.36% chance that a “Model F-Value” could occur due to noise. The “Lack of Fit F-value” of 8.24 implied that the model is not significant relative to the pure error (more than 0.05). However, there is a 11.02% of chance that a “Lack of Fit F-value” could occur due to noise. Insignificant Lack of Fit indicated that the model was significant and fit. Besides, the accuracy of the fit of the model was checked by multiple correlation coefficients ($R^2$). The closest the correlation $R^2$ to value of 1.00, the higher the quality of the model. From the results of this study, the value of $R^2$ was found to be 0.8803 indicated that there was a high correlation of experimental value towards predicted value.

| Source           | Sum of Squares | df  | Mean Square | $F$  | $p$-value | Remark       |
|------------------|----------------|-----|-------------|------|-----------|--------------|
| Model            | 0.0344         | 5   | 0.0069      | 7.36 | 0.0236    | significant  |
| Residual         | 0.0047         | 5   | 0.0009      |      |           |              |
| Lack of Fit      | 0.0043         | 3   | 0.0014      | 8.24 | 0.1102    | not significant |
| Pure Error       | 0.0004         | 2   | 0.0002      |      |           |              |
| Cor Total        | 0.0391         | 10  |             |      |           |              |

*Cor Total= Corrected Total; df= Degree of freedom

Furthermore, second order equations were developed from the analysis of variance. The polynomial models from experimental results for enzyme activity were derived as shown in Equation 1 (actual factors terms) to assist future research works related to production of xylanase from *A. niger*.

Enzyme activity =

\[-3.44396 + 0.229322 \text{(Incubation temperature)} + 0.092287 \text{(Medium pH)} + 0.000099 \text{(Incubation temperature) (Medium pH)} - 0.003517 \text{(Incubation temperature)}^2 - 0.010483 \text{(Medium pH)}^2\]

(1)

Besides, the interaction of parameters was classified as A: B (Incubation temperature: Medium pH) and showed in specific via 3D surface plot. Figure 1 illustrated the 3D response surface plot of enzyme activity as a function of incubation temperature and medium pH. From Figure 1, it clearly shown that the optimized condition of the incubation process for maximum xylanase production for both parameters: incubation temperature and medium pH. The 3D response surface plot was observed to project upwards to a region of red colour. This designated that the optimized condition was achieved through this study. The optimized condition was identified allocated at point where the incubation temperature at 32.5°C and medium pH at 5.0 based on the analysis interpreted from the software. From the data interpreted between the interaction of two parameters shown in response surface plot, the maximal xylanase production was recorded at 0.5237 U/mL. Contrarily, the lowest xylanase production concentration is at 0.4000 U/mL.
Figure 1: 3D response surface plot of enzyme activity as a function of parameter
Design point above predicted value ( ), design point below predicted value ( )
(0.336776 0.523667 colour points by value of enzyme activity

Since the aim of optimization study was to find the conditions which gave the maximum enzyme activity production. Through the analysis of CCD in RSM, the goals for both the incubation temperature and medium pH were selected as “in range” condition, whereas, for enzyme activity, the goal was selected based on maximum in order to achieve the highest xylanase production. From the goals set, the software generated the optimum condition with incubation temperature at 32.67°C and medium pH of 4.56 that predicted will produced 0.5118 U/mL of enzyme activity with desirability of 0.936 from the incubation result.

Hence, validation test was carried using the given optimum condition for incubation to produce xylanase by \textit{A. niger}. A triplicate of optimum condition was done to increase the accuracy of result obtained. The actual enzyme activity was compared with the predicted enzyme activity for the error existed. In the experiment, 0.5638 U/mL was obtained under the stated optimum condition. The percentage error between actual enzyme activity (0.5638 U/mL) and predicted enzyme activity (0.5118 U/mL) is 10.16 \% which was considered low. Thus, the result obtained through actual enzyme activity under optimum condition for xylan as production was compatible with the predicted production. This indicated that the optimization achieved in this study was reliable.

4. Conclusion
In this study, the factors manipulated to be optimized through CCD under RSM were inclusive of incubation temperature and medium pH. Based on the analysis, the optimum condition for xylanase production from \textit{A. niger} was acknowledged at temperature of 32.67°C with a medium pH of 4.56 whereby the agitation speed and incubation period were statically set at 150 rpm and 5 days throughout the optimization process, respectively. From the validation done, the maximized xylanase activity was obtained at 0.5638 U/mL. Since through this research an optimized xylanase production condition via \textit{A. niger} was established. Hence, future researches could concentrate on substitution of the model xylan with the lignocellulosic waste to be consecutively used in bioethanol production. This alternative shall best suit the xylanase application in reality, besides reduce the cost spending on xylan.
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