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Factorial experimental design for xylanase production by *Bacillus* sp. isolated from Malaysia landfill soil

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Abstract. Two-level full factorial design was applied to screen the important parameters for production of xylanase by newly isolated *Bacillus* sp. from landfill soil. Five production parameters were considered: initial pH media (pH 5–9), inoculum size (5%–10% v/v), incubation period (18–30 h), temperature (30–50 °C) and agitation speed (0–200 rpm). Xylanase activity was estimated using dinitrosalicylic acid (DNS) based on the xylose released under specified assay conditions. Based on the factorial analysis, it was observed that the significant parameters in the xylanase production were temperature, agitation speed and initial pH of media. Meanwhile, the interaction between temperature and initial pH of media gave the highest influence to the xylanase production. The model revealed that the highest xylanase activity can be achieved at 123.34 U/mL with initial pH media of 7.0, 30 h incubation period, 5% (v/v) inoculum size, agitation speed of 100 rpm at 40 °C. Confirmation run produced the highest experimental xylanase activity by *Bacillus* sp. at 123.10 U/mL with 0.17% of error than the predicted one. Hence, the model was reliably predicting the xylanase production.

1. Introduction

The substrate of xylanase: xylan [1] is the main carbohydrate component in hemicellulose that can be found in the plant cell walls. It comprises up to 20% to 35% dry weight of hardwood plants, softwood plants and agricultural waste [2]. Complete xylan degradation requires the operation of different xylanolytic enzymes include endoxylanase, β -xylosidase, α -glucuronidase, acetyl esterase and α -arabinofuranosidase. These xylanases are responsible for degradation of xylan into usable products such as xylooligosaccharides, xylotetrose, xylotriose, xylobiose, and xylose [3, 4].

Diverse genera and species of microorganisms for instance actinomycetes, bacteria, yeast and fungi have been recognized to be the rich sources of xylanase [5, 6]. There are many reports on xylanase production by microorganisms such as *Streptomyces thermovulgaris* [7], *Streptomyces* sp. [8], *Aspergillus foetidus* [9], *Aspergillus flavus* [10], *Aspergillus carneus* [11], *Coprinopsis cinerea* [12], *Pichia pastoris* [2], *Penicillium* sp. [13], *Bacillus amyloliquifaciens* [14], *Bacillus tequilensis* [15], *Bacillus pumilus* [16], *Bacillus subtilis* [17], *Micrococcus* sp. [18], *Acetobacter xylinum* and *Cellulomonas uda* [3]. Among the microorganism sources, bacteria are the most preferable because of their stability at high pH and temperature which assist the industrial purpose [14]. The family of *Bacillus* has been explored broadly among bacterial xylanase. *Bacillus* species are the suitable xylanase producer from the industrial perspective due to their potential to synthesis high level of proteins and extracellular enzymes and also because of their rapid growth rate [15, 17]. Huge scale



cultivation of actinomycetes and fungi is challenging due to their slow time of generation, inadequate oxygen transfer and production of high viscous byproducts such as polymers [19].

The biotechnological applications of microbial xylanase in various industries include the animal feed, food, detergents, textiles, paper and pulp, biofuel and chemicals production, fruit and vegetable processing, bread manufacturing, clarification of fruit juices and wines, and in waste treatment [1, 14]. These vast applications of xylanase in industries escalate the value and importance of the enzyme. The xylanase production was influenced by the microorganism type and its strain [20], nutrient type and its concentration and growth conditions [21]. Screening and optimizing the nutritional parameters are essential to determine the optimum production of xylanase [13]. Moreover, the successful industrial application of xylanase that requires cost effective in mass production can be achieved by optimizing the fermentation process and media formulation[9].

Statistical experimental design techniques for screening and optimizing the parameters are needed to provide statistical models which investigate several independent parameters simultaneously and to determine interactions effects between them [2]. Meanwhile, the one-factor-at-a-time (OFAT) technique having extensive of time which involve large number of experiments and analysis of many parameters as well as high reagents and materials consumption [22]. Therefore, the statistical techniques are better choice than OFAT technique in order to overcome these problems.

Hence, this study was aimed to screen the culture conditions for xylanase production by the landfill soil isolate, *Bacillus badius* using full factorial design. The studied culture conditions were agitation speed, inoculum size, temperature, incubation period and initial pH of media.

2. Methods

2.1. Microorganism and preparation of inoculum

Bacillus badius isolated from Malaysia landfill soil was obtained from Laboratory of Faculty of Chemical & Natural Resources Engineering, Universiti Malaysia Pahang (UMP). The bacteria were maintained on nutrient agar plate. The isolation and screening on the xylanolytic ability were previously described by Masngut *et al.* [23].

Inoculum of the *Bacillus* sp. was cultured by transferring a single loop of *Bacillus* sp. from nutrient agar plate into 100 mL broth medium containing (in g/L): glucose 10.0, peptone 10.0, ammonium sulphate 2.5, dipotassium phosphate 2.0 and magnesium sulphate 0.3, at initial media pH of 7.0. The mixture was incubated at 37 °C and agitated at 150 rpm. Culture with OD₆₀₀ 2.0 was used as the vegetative cells for inoculum source.

2.2. Experimental setup

The media contained (in g/L): xylan 10.0, peptone 10.0, ammonium sulphate 2.5, dipotassium phosphate 2.0 and magnesium sulphate 0.3. These media components were dissolved in buffer solutions. Three different buffer solutions according to desired pH of media were prepared. Sorensen's phosphate buffer (pH 7), Tris-HCl buffer (pH 8) and Glycine-NaOH buffer (pH 9) were used for this study. Medium was sterilized at 121 °C for 15 min.

The bacteria were grown in media supplemented with single carbon source which is corn core xylan. The submerged fermentation was carried out with a working volume of 50 mL in 250 mL of cotton plugged Erlenmeyer flasks. Fermentations were carried out according the designated run by Design-Expert®. Optical density at 600 nm and pH reading were taken at the beginning and the end of the fermentation. As fermentation ceased, the media were centrifuged at 10,000 rpm and 4 °C for 15 min. The clear supernatant was collected and used as a source of crude enzyme for xylanase assay.

2.3. Software used

The experiment design of five parameters in xylanase production were generated and analyzed with the aid of Design-Expert® (Version 7.1.6, Stat-Ease, Inc., Minneapolis, MN) software.

2.4. Design of experiment

The 2⁵ two-level full factorial design (FFD) was utilized to show the statistical significance of five parameters which were initial pH of media, incubation period, inoculum size, temperature, and agitation speed in the xylanase production by *Bacillus* species. Table 1 shows the designated parameters and levels to be employed in the experiments. In the experimental design, low and high factorial points were coded as -1 and +1, respectively, while the midpoint was coded as 0. The setting of range for parameters were based on the investigation of single parameters (screening process by OFAT method) as defined by Rosli *et al.* [24]. A total of 35 experimental runs were generated by the software as shown in table 2. The experiments were carried out in duplicates with xylanase activity (U/mL) as the response.

Table 1. Parameters and coded value for factorial design.

Parameter	Code	Level			Unit
		-1	0	+1	
Initial pH of media	A	7	8	9	pH
Incubation period	B	18	24	30	H
Inoculum size	C	5	10	15	% (v/v)
Temperature	D	35	37	40	°C
Agitation speed	E	100	150	200	rpm

2.5. Assay of xylanase activity

Estimation of xylanase activity was conducted by using recommended methodology by Kim *et al.* [25] with some modification. Activity of xylanase was determined by estimation of reducing sugars released from corn core xylan using dinitrosalicylic acid (DNS) reagent. A reaction mixture containing 0.2 mL of diluted crude enzyme, 0.5 mL of 1% xylan solution in 0.05 M phosphate buffer (pH 6.0) and 0.3 mL of phosphate buffer (pH 6.0) was incubated at 50 °C for 10 min. After that, the enzymatic reaction was stopped by adding 3 mL of DNS reagent, boiled in a capped glass tube at 100 °C for 5 min and quenched to room temperature for color stabilization. Subsequently, the absorbance was determined at 520 nm. All experimental works were performed in triplicates. A standard D-xylose plot was used as the reducing sugar expressed in this assay. One unit of xylanase activity was defined as the quantity of xylanase needed to release 1 μmol of xylose per minute under specified standard assay conditions, as in the equation 1, where *m* and *c* represent the slope and intercept from the D-xylose standard, respectively.

$$\text{Xylanase activity} \left(\frac{U}{mL} \right) = \frac{\text{Final absorbance} - c}{m} \times \frac{\text{dilution sample}}{\text{sample volume}} \times \frac{1}{\text{reaction time}} \times \frac{1000 \mu g}{1 mg} \times \frac{1 \mu mole}{150.13 \mu g} \quad (1)$$

Table 2. Full factorial design experimental runs.

Standard run order	Parameter					
	A	B	C	D	E	
1	-1	-1	-1	-1	-1	-1
2	+1	-1	-1	-1	-1	-1
3	-1	+1	-1	-1	-1	-1
4	+1	+1	-1	-1	-1	-1
5	-1	-1	+1	-1	-1	-1
6	+1	-1	+1	-1	-1	-1
7	-1	+1	+1	-1	-1	-1
8	+1	+1	+1	+1	+1	-1
9	-1	-1	-1	+1	+1	-1
10	+1	-1	-1	+1	+1	-1
11	-1	+1	-1	+1	+1	-1
12	+1	+1	-1	+1	+1	-1
13	-1	-1	+1	+1	+1	-1
14	+1	-1	+1	+1	+1	-1
15	-1	+1	+1	+1	+1	-1
16	+1	+1	+1	+1	+1	-1
17	-1	-1	-1	-1	-1	+1
18	+1	-1	-1	-1	-1	+1
19	-1	+1	-1	-1	-1	+1
20	+1	+1	-1	-1	-1	+1
21	-1	-1	+1	-1	-1	+1
22	+1	-1	+1	-1	-1	+1
23	-1	+1	+1	-1	-1	+1
24	+1	+1	+1	-1	-1	+1
25	-1	-1	-1	+1	+1	+1
26	+1	-1	-1	+1	+1	+1
27	-1	+1	-1	+1	+1	+1
28	+1	+1	-1	+1	+1	+1
29	-1	-1	+1	+1	+1	+1
30	+1	-1	+1	+1	+1	+1
31	-1	+1	+1	+1	+1	+1
32	+1	+1	+1	+1	+1	+1
33	0	0	0	0	0	0
34	0	0	0	0	0	0
35	0	0	0	0	0	0

A, B, C, D and E are the initial pH of media, incubation period, inoculum size, temperature and agitation speed, respectively. The transformation selection is None

3. Results and discussions

3.1. Screening of parameters affecting xylanase production

The screening result shows that the xylanase activity was ranged between 77.53 U/mL to 155.65 U/mL during the production. The highest activity was recorded at 155.65±0.00 U/mL with fermentation condition at the initial media pH value of 8, incubation period of 24 h, inoculum size of 10% (v/v), agitation speed of 150 rpm and temperature of 37 °C.

3.2. Model from two-level factorial

The regression model in term of coded parameter and actual parameter are shown in equation 2 and equation 3, respectively. These equations can be used as models for predicting response at different operation conditions.

Coded parameter:

$$Y = 100.36 - 6.33A + 1.17B - 1.53C + 5.72D + 4.90E - 5.80AD + 2.28AE - 5.05DE \quad (2)$$

where Y is referred as response, which is the xylanase activity. The main effects: A , B , C , D and E are indicated the initial pH of media, incubation period, inoculum size, temperature and agitation speed, respectively. The interaction effects are referred to AD , AE and DE .

Actual parameter:

$$\begin{aligned} \text{Xylanase activity} = & -819.32445 + 73.78616 \times \text{pH} + 0.19502 \times \\ & \text{incubation period} - 0.30626 \times \text{inoculum size} + 26.89954 \times \text{temperature} + \\ & 1.24768 \times \text{agitation speed} - 2.31884 \times (\text{pH} \times \text{temperature}) + 0.045632 \times \\ & (\text{pH} \times \text{agitation speed}) - 0.040394 \times (\text{temperature} \times \text{agitation speed}) \end{aligned} \quad (3)$$

3.3. Analysis of variance (ANOVA)

Analysis of variance (ANOVA) computed by Design-Expert® software was applied for the determination of significant parameter and tabulated in table 3. The parameters that affect the xylanase production were screened at the confidence level of 95% on the basis of their effects.

Table 3. ANOVA analysis for xylanase production.

Source	Mean square	F-value	p-value
Model	659.16	56.33	< 0.0001
A	1280.38	109.41	< 0.0001
B	43.81	3.74	0.0644
C	75.04	6.41	0.0180
D	1048.54	89.60	< 0.0001
E	767.66	65.60	< 0.0001
AD	1075.40	91.90	< 0.0001
AE	166.58	14.23	0.0009
DE	815.85	69.72	< 0.0001
Lack of Fit	8.82	0.20	0.9851

A, B, C, D and E are the initial pH of media, incubation period, inoculum size, temperature and agitation speed, respectively

The regression model was significant, accurate and well-fitted with the data of experiment, which indicated by the p -value that was less than 0.05. Model terms A (initial pH of media), C (inoculum size), D (temperature), E (agitation speed), AD (interaction between pH and temperature), AE (interaction between pH and agitation speed) and DE (interaction between temperature and agitation speed) were also significant.

The R -Squared is the multiple correlation coefficients that measure the amount of variation about the mean of the model. The closer the value of R -Squared to One, the better the model is. The R -Squared value was 0.9474 which is good considering that this model deals with biological component. The “Predicted R -Squared” of 0.8932 was in reasonable agreement with the “Adjusted R -Squared” of 0.9306.

The p -value for lack-of-fit that was at 0.9851 (shown in table 3) indicated that it was insignificant and thus showed that the model was valid. The lack-of-fit is defined as a measure of the failure of a model to represent domain experimental data at which data points were not included in the regression

model or variations in the models cannot be accounted by random error. If there is a significant lack-of-fit, the response is not fitted.

3.4. Main effect analysis

Main effect analysis is one of the elements investigated in the factorial analysis. Pareto chart interpreted by the factorial analysis as shown in figure 1 illustrate the parameter that has statistically significant effect to the xylanase production. This figure shows two reference lines. The lower line represents the effect of t -value limit; used to consider statistically significant at 95% confidence level for each individual effect, while the upper line is called the Bonferroni limit. Any parameters that exceed the upper line are significant and any parameters below the lower line are considered insignificant [26]. Therefore, from figure 1, the significant parameters; initial pH of media (A), temperature (D) and agitation speed (E) and significant interaction between parameters; AD, DE and AE exceed the Bonferroni-limit line. The remaining parameters which did not pass the Bonferroni-limit line were insignificant to the xylanase production.

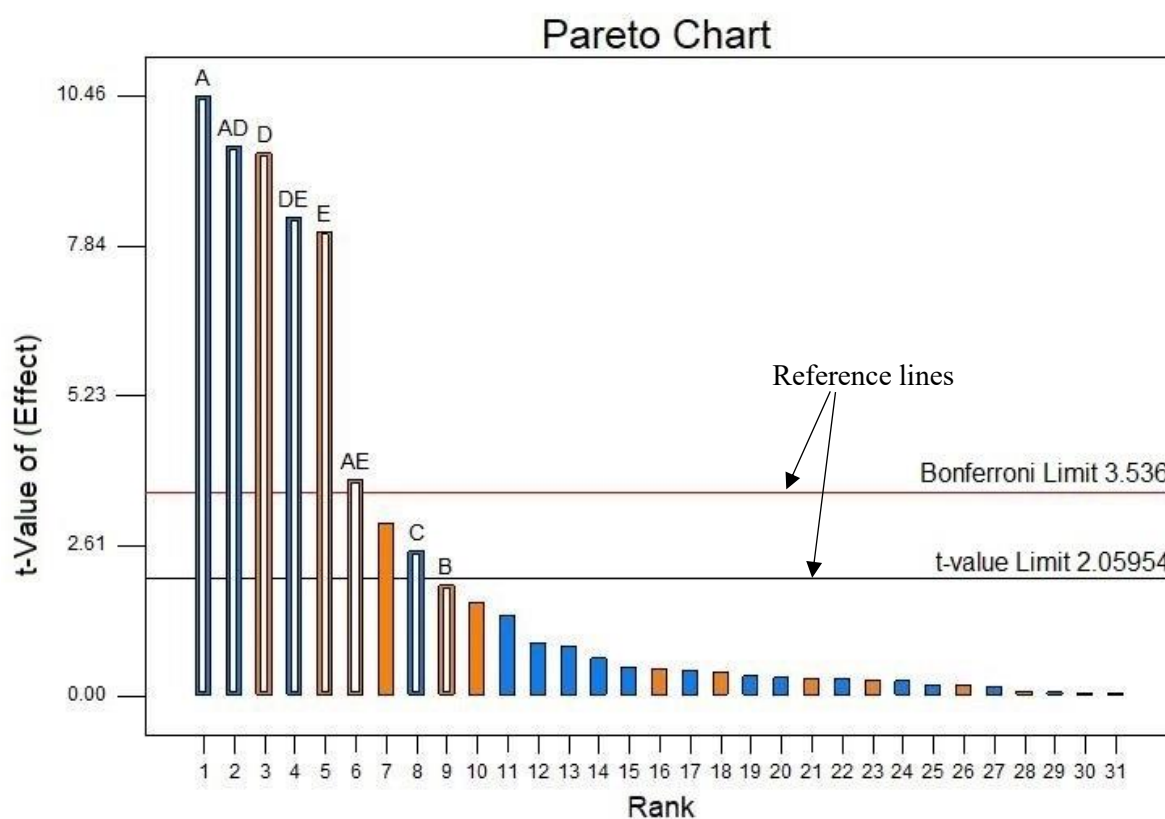


Figure 1. Pareto chart; A, B, C, D and E are the initial pH of media, incubation period, inoculum size, temperature and agitation speed, respectively. Half-coloured bars represent manually selected parameters; fully-coloured bars represent unselected parameters.

A study by Cunha *et al.* [9] on the xylanase production by *Aspergillus foetidus* via factorial design reported that the culture conditions were reduced from three parameters to only one significant parameter which was the pH. His study reported the highest xylanase activity at 13.98 U/mL. Meanwhile, study by Kaushala *et al.* [27] indicated that all investigated culture conditions were significance which were temperature, pH, inoculum size and substrate concentration. Their maximum xylanase activity by *Bacillus* sp. was recorded at 8.18 U/mL [27]. Besides, Tandon *et al.* [28] revealed that parameters such as pH, temperature and inoculum sizes were major significant culture conditions on xylanase production from *Bacillus atropheaus* (maximum xylanase activity: 85.16 U/g).

Several screening of culture conditions on xylanase productions by another method called Plackett-Burman factorial experimental design were reported by Ingale *et al.* [29], Ali *et al.* [30] and Gowdhaman *et al.* [31]. A study by Ingale *et al.* [29] revealed that wheat bran, pH and temperature were identified as statistically significant parameters on xylanase production which the xylanase synthesis by *Bacillus pumilus* was at 557 U/g. Furthermore, a report from Ali *et al.* [30] indicated that 14 parameters affecting the xylanase production were screened and they revealed that sucrose, xylan and CMC were the most influential culture conditions increasing the production. However, temperature parameter was reported to decrease the xylanase production [30]. The highest yield of xylanase by *Bacillus subtilis* was at 165 U/mL [30]. Other than that, a study by Gowdhaman *et al.* [31] published that, moisture content, nitrogen source, MgSO₄·7H₂O and substrate concentration were significant parameters from 9 parameters studied. Their maximum xylanase activity by *Bacillus aerophilus* was revealed at 45.9 U/gds.

The highest parameter contributing to the xylanase production was initial pH of media with the percentage contribution of 10.86%. The initial pH of the fermentation media may influence the cell growth and metabolic product formation. Moreover, the transport of various components across the cell membrane is also hugely impact by the initial pH of media [18].

The second highest parameter contributing to the xylanase production was temperature, which contributes 8.90%. The extracellular enzyme secretion was found to be affected by the incubation temperature, possibly by changing the physical properties of the cell membrane [18]. Nagar *et al.* [32] indicated that microorganisms have ability for high enzyme production at their optimum growth temperature. The optimum temperature recorded for the maximum growth of most *Bacillus* sp. for xylanase production ranging between 30 to 40 °C [15, 16, 33].

Another parameter that was considered during the production of xylanase was agitation speed with the percentage contribution of 6.51%. Agitation provides adequate mixing of the nutrients, promote good heat transfer, mass transfer, and enhancing dissolved oxygen in the fermentation medium [34]. Lower speed of agitation may cause insufficient oxygen contribution in the aerobic fermentation, cell clumping and improper mixing of media components, which affect the microbial growth. However, high speed of agitation may cause low production due to disruption of cells by shear forces and formation of vortex that caused poor mass transfer [34-36]. Therefore, it is essential to provide optimum agitation speed in the operation to obtain the maximum enzyme production.

The correlation between parameters highlighted was tested and as the outcome, three interactions provided the significant effect towards xylanase production. The highest percentage of contribution was between initial pH of media and temperature with contribution of 9.12%. Meanwhile, the interaction between initial pH of media with temperature and interaction between temperature and agitation speed gave 1.41% and 6.92% contribution, respectively. The least contributing parameter was the incubation period and inoculum size, which only contributes as much as 0.37% and 0.64%, respectively. Since these parameters were the least affecting parameter in this study, the values should be maintained in further optimization experiment.

3.5. Validation of the CCD model

Three runs of validation experiments were performed based on the conditions suggested by Design-Expert® analysis to validate the model. Comparison between the experimental results obtained and the predicted xylanase activity by the model was measured by their percentage of error. From the data in table 4, the calculated percentage error was between 0.17 and 3.29%.

Table 4. Validation runs.

Run	Conditions	Xylanase activity (U/mL)		Error (%)
		Predicted	Experimental	
1	pH 7, 30 h, 5% (v/v) inoculum, 40 °C, 100 rpm	123.34	123.10	0.17
2	pH 7, 29.75 h, 5% (v/v) inoculum, 40 °C, 100 rpm	123.24	125.74	1.77
3	pH 7, 29.33 h, 5% (v/v) inoculum, 40 °C, 100 rpm	123.05	127.70	3.29

This indicates the parameters that contribute to the xylanase production analyzed by the model are valid to be used. Therefore, three parameters (initial pH of media, temperature and agitation speed) were selected as significant parameters on xylanase production and will be furthered to the optimization study.

Conclusion

This study was designed and attempted to determine the significant parameters on the xylanase production via full factorial design (FFD). Using FFD, initial pH of media, incubation temperature and agitation speed were classified as the most influencing parameters on the production of xylanase with contribution of 10.86%, 8.90% and 6.51%, respectively. The least affecting parameters are inoculum size and incubation period with 0.64% and 0.37%, respectively. By application of the factorial design approach, the number of the experimental run can be minimized, and the selected significant parameters could be furthered for the optimization studies.

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