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Medium optimization for pullulanase production from *Anoxybacillus* species using experimental design

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The aim of the present study was to improve pullulanase production from the thermophilic ***Anoxybacillus*** species strain SK3-4 by medium engineering. The strain was isolated from a Malaysian hot spring, Sungai Klah (SK). The optimization processes were performed in two stages using 2^4 full factorial design and central composite rotatable design (CCRD). Four variables were manipulated to improve pullulanase yield: initial culture ***p***H, and concentrations of pullulan, tryptone and ammonium chloride by using a 2^4 full factorial design. Following that a quadratic model of CCRD was then used to identify the optimum yield of pullulanase by manipulating all four of these significant parameters. The optimized medium composition was a ***p***H of 8.29, 0.79% (w/v) pullulan, 0.12% (w/v) tryptone and 0.39% (w/v) ammonium chloride, giving a final pullulanase activity of 3.86 U/mL and specific activity of 3.38 U/mg. The specific activity achieved was higher than the CCRD predicted value (3.00 U/mg). The application of two different experimental designs resulted in an improvement of pullulanase specific activity of approximately 73% from the original ***Thermus*** medium.

Keywords: ***Anoxybacillus***, central composite rotatable design, experimental design, optimization, pullulanase, response surface methodology

Introduction

Pullulanase (pullulan-6-glucanohydrolase, EC:3.2.1.41) is a debranching enzyme that hydrolyses the α-1,6-glycosidic bonds in pullulan and other branched polysaccharides, such as, starch[1]. This industrially important enzyme is generally used with other amylolytic enzymes, such as, α-amylase, β-amylase or glucoamylase, to increase the efficiency and yield of enzymatic reactions. For example, the quality of sugar syrup in the starch saccharification process can be enhanced by pullulanase[1]. In addition, the products of pullulan degraded by the pullulanase are frequently used in food and pharmaceutical industries[2]. Pullulanase also improves the production of cyclodextrins by pretreating the starch prior to the cyclodextrin glucanotransferase (CGTase) reaction[3]. Pullulanase also has applications in the textile, baking and detergent industries[1].

Several pullulanases were produced in vast amounts by mesophilic, thermophilic and hyperthermophilic bacteria[1]. Because pullulanase is an important industrial enzyme, several studies have been done to enhance enzyme production in cells by optimizing medium composition. The most common parameters are the types of carbon and nitrogen sources used in the fermentation medium. Pullulan[4], maltose[5] and starch[6] are types of carbon sources previously used to improve pullulanase production. Additionally, organic nitrogen sources, including yeast extract, peptone, tryptone, trypticase, casamino acids and corn steep liquor, were found to improve the pullulanase production[7].

An optimized fermentation medium is of critical importance because the composition of medium significantly affects the production yield. The major constraints in engineering a new medium formulation are that a large number of experiments are required and it is difficult to pinpoint the true optimal point for each medium component. The conventional one-factor-at-a-time (OFAT) approach is time consuming and, therefore, statistical experimental designs provide a better platform for optimization purposes. Experimental designs, such as, two-level full factorial design and central composite rotatable design (CCRD) were previously applied successfully to optimize pullulanase production[6-8].

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The present work deals with the outcome of an attempt to optimize pullulanase production by the newly isolated ***Anoxybacillus*** sp. strain SK3-4[9]. This strain is able to degrade pullulan, which is a rare characteristic among ***Anoxybacillus*** species[10]. Two statistical approaches were applied. First, 2^4 full factorial design was used to evaluate and screen four important variables: initial culture ***p***H, pullulan concentration (carbon source), tryptone concentration (organic nitrogen source) and ammonium chloride concentration (inorganic nitrogen source). Second, central composite rotatable design (CCRD) was used to find the optimum conditions for all significant variables.

Materials and Methods

Chemicals

All chemical reagents used were analytical grade. Pullulan was obtained from TSI Europe (Zwijndrecht, Belgium). Tryptone was a product of BD (Sparks, MD, USA). Ammonium chloride (NH_4Cl), sodium chloride (NaCl) and agar were obtained from Qrëc (New Zealand). Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) was from UNIVAR (Seven Hills, Australia). Peptone was purchased from Merck (Darmstadt, Germany) and yeast extract from Scharlau Microbiology (Barcelona, Spain).

Bacterial Strain and Inoculum Preparation

The thermophilic organism used for the study was ***Anoxybacillus*** sp. strain SK3-4 isolated from the Sungai Klah hot spring in Malaysia[9]. This organism was streaked on ***Thermus*** medium as previously described in Atlas[11] with slight modifications: 4.0 g/L of peptone, 4.0 g/L of tryptone, 4.0 g/L of yeast extract, 2.0 g/L of NaCl, 1.0 g/L of $MgSO_4 \cdot 7H_2O$ and 1.0% (w/v) pullulan. The medium was solidified with 1.5% (w/v) agar. The ***p***H was adjusted to 7.5 before autoclaving. All plates were incubated at 50ºC for 24 h.

For inoculum preparation, a single colony was cultured in 200 mL ***Thermus*** medium broth inside a 1.0 L conical flask. The inoculum culture was incubated at 55ºC with rotary shaking of 200 rpm for approx 4 h until the optical density at 600 nm reached 0.5, where the cells are in the middle of exponential phase[9].

Production of Pullulanase

A 10 mL inoculum (equivalent to 10% v/v) was aseptically transferred to a 500 mL conical flask containing 100 mL sterile ***Thermus*** medium. The flask was incubated at 55ºC in a shaker incubator at 200 rpm. After 12 h, the cultured broth was centrifuged at 8000× g for 15 min at 4ºC. The culture supernatant was assayed for pullulanase activity and protein content.

Analytical Procedures

Pullulanase activity was determined by measuring the amount of reducing sugars released from pullulan using the dinitrosalicylic acid (DNS) assay established by Miller[12] with slight modifications. A reaction mixture containing 0.1 mL supernatant and 1.0 mL of 1% (w/v) pullulan in 0.1 M potassium phosphate buffer (***p***H 7.5) was incubated at 60ºC for 15 min. The reaction was stopped by cooling on ice and 0.5 mL DNS reagent was then added into the mixture, followed by 50.0 µL of 0.1 N NaOH. The mixture was boiled for 5 min, cooled on ice and measured for absorbance intensity at 540 nm using a 100 UV-Vis spectrophotometer from BUCK Scientific Inc. (East Norwalk, Connecticut, USA). One unit (U) of pullulanase activity was defined as the amount of enzyme that generated 1 µmol of reducing sugar under the assay conditions.

Protein concentrations were determined using a modified Lowry assay as described by PIERCE[13] from Thermo Fisher Scientific Inc. (Waltham, USA). Bovine serum albumin was used as the standard protein. All DNS and protein assays were performed in triplicate, unless otherwise specified.

Statistical Analysis

Design Expert Software (Stat-Ease Inc., Statistic Made Easy, Minneapolis, MN, USA, version 6.0.4) was used for the design of the experiments and regression analysis of experimental data. Two experimental designs were used in this study: (1) 2^4 full factorial design, and (2) full central composite rotatable design. The data was compared to the pullulanase expression in the original ***Thermus*** medium.

Full Factorial Design

A 2^4 full factorial design was used to show the statistical significance of initial culture ***p***H, pullulan (carbon source), tryptone (organic nitrogen source) and ammonium chloride (inorganic nitrogen source) on the production of pullulanase. The range for the factors was based primarily on the literature and screening process (data not shown). A total of 16 sets

of experiments with 5 replicated centre points were employed in this study to determine the significant factors that affect the pullulanase specific activity (U/mg). The range and the levels of the variables investigated in this study are summarised in Table 1.

Central Composite Rotatable Design

After conducting full factorial analysis, the design was further expanded to a central composite rotatable design (CCRD), one of the designs in response surface methodology (RSM). The coding of variables was conducted according to equation (1)[14]:

$$\text{Coded value} = \frac{\text{Actual value} - (\text{high level} + \text{low level})/2}{(\text{high level} - \text{low level})/2} \quad \ldots (1)$$

Pullulanase specific activity (U/mg) was set as the response for this design and the significant term from the 2^4 full factorial design as the variable to be tested. All four variables were identified as the significant term by 2^4 full factorial design. A full CCRD with total of 16 sets of experiments, 8 axial points (coded value of –2 and +2) and seven replicated centre points was chosen, as shown in Table 2. The quadratic model for predicting the optimal point was expressed according to equation (2)[26], where $\boldsymbol{Y}$=predicted response for pullulanase specific activity (U/mg), β_0=constant, β_i=linear effect, β_{ii}=quadratic effect, β_{ij}=interaction effect, $\boldsymbol{x}$=coded level of the independent variables and ε = random error.

$$\boldsymbol{Y} = \beta_0 + \Sigma\beta_i \boldsymbol{x}_i + \Sigma\beta_{ii}\boldsymbol{x}_{ii}^2 + \Sigma\beta_{ij}\boldsymbol{x}_i\boldsymbol{x}_j + \varepsilon \quad \ldots (2)$$

The statistical significance of the second-order model equation was determined by a significant $\boldsymbol{F}$-value, an insignificant lack-of-fit $\boldsymbol{F}$-value and a good multiple coefficient of determination, $\boldsymbol{R}^2$.

Results and Discussion

Full Factorial Design

After analysis of variance (ANOVA) of 2^4 full factorial design, a regression equation as shown in equation (3) was obtained, where $\boldsymbol{X}_1$ is the initial culture ***p***H, $\boldsymbol{X}_2$ is pullulan concentration, $\boldsymbol{X}_3$ is tryptone concentration and $\boldsymbol{X}_4$ is ammonium chloride concentration. Table 3 shows the ANOVA results for 2^4 full factorial design.

Table 1—2^4 full factorial design and the results of experiment

Studied order	Actual variable level and coded variable level				Pullulanase specific activity	
	Initial culture ***p***H, $\boldsymbol{X}_1$	Pullulan % (w/v), $\boldsymbol{X}_2$	Tryptone % (w/v), $\boldsymbol{X}_3$	Ammonium chloride % (w/v), $\boldsymbol{X}_4$	Actual value (U/mg)[a]	Predicted value (U/mg)
1	7.00 (-1)	0.50 (-1)	0.10 (-1)	0.10 (-1)	1.37 ± 0.02	1.39
2	9.00 (+1)	0.50 (-1)	0.10 (-1)	0.10 (-1)	2.05 ± 0.04	2.01
3	7.00 (-1)	4.00 (+1)	0.10 (-1)	0.10 (-1)	1.03 ± 0.11	1.01
4	9.00 (+1)	4.00 (+1)	0.10 (-1)	0.10 (-1)	1.62 ± 0.07	1.63
5	7.00 (+1)	0.50 (-1)	1.00 (+1)	0.10 (-1)	0.56 ± 0.03	0.56
6	9.00 (-1)	0.50 (-1)	1.00 (+1)	0.10 (-1)	1.03 ± 0.01	1.05
7	7.00 (-1)	4.00 (+1)	1.00 (+1)	0.10 (-1)	0.73 ± 0.09	0.74
8	9.00 (+1)	4.00 (+1)	1.00 (+1)	0.10 (-1)	1.22 ± 0.01	1.24
9	7.00 (-1)	0.50 (-1)	0.10 (-1)	0.50 (+1)	1.32 ± 0.13	1.28
10	9.00 (+1)	0.50 (-1)	0.10 (-1)	0.50 (+1)	1.09 ± 0.12	1.15
11	7.00 (-1)	4.00 (+1)	0.10 (-1)	0.50 (+1)	1.19 ± 0.02	1.23
12	9.00 (+1)	4.00 (+1)	0.10 (-1)	0.50 (+1)	1.12 ± 0.13	1.10
13	7.00 (-1)	0.50 (-1)	1.00 (+1)	0.50 (+1)	0.82 ± 0.03	0.78
14	9.00 (+1)	0.50 (-1)	1.00 (+1)	0.50 (+1)	0.50 ± 0.02	0.52
15	7.00 (-1)	4.00 (+1)	1.00 (+1)	0.50 (+1)	0.60 ± 0.01	0.63
16	9.00 (+1)	4.00 (+1)	1.00 (+1)	0.50 (+1)	0.44 ± 0.01	0.38
17	8.00 (0)	2.25 (0)	0.55 (0)	0.30 (0)	1.32 ± 0.05	1.31
18	8.00 (0)	2.25 (0)	0.55 (0)	0.30 (0)	1.34 ± 0.07	1.31
19	8.00 (0)	2.25 (0)	0.55 (0)	0.30 (0)	1.28 ± 0.04	1.31
20	8.00 (0)	2.25 (0)	0.55 (0)	0.30 (0)	1.28 ± 0.02	1.31
21	8.00 (0)	2.25 (0)	0.55 (0)	0.30 (0)	1.33 ± 0.07	1.31

[a] Mean from triplicates; Values in bracket are coded variable level

Table 2—Central composite rotatable design and the results of experiment

Studied order	Actual variable level and coded variable level				Pullulanase specific activity	
	Initial culture ***p***H, X_1	Pullulan % (w/v), X_2	Tryptone % (w/v), X_3	Ammonium chloride % (w/v), X_4	Actual value (U/mg)[a]	Predicted value (U/mg)
1	8.00 (-1)	0.70 (-1)	0.10 (-1)	0.35 (-1)	2.20 ± 0.36	2.10
2	9.00 (+1)	0.70 (-1)	0.10 (-1)	0.35 (-1)	1.72 ± 0.27	1.64
3	8.00 (-1)	0.90 (+1)	0.10 (-1)	0.35 (-1)	2.73 ± 0.06	2.68
4	9.00 (+1)	0.90 (+1)	0.10 (-1)	0.35 (-1)	1.99 ± 0.12	2.05
5	8.00 (-1)	0.70 (-1)	0.20 (+1)	0.35 (-1)	1.38 ± 0.05	1.43
6	9.00 (+1)	0.70 (-1)	0.20 (+1)	0.35 (-1)	1.37 ± 0.05	1.42
7	8.00 (-1)	0.90 (+1)	0.20 (+1)	0.35 (-1)	1.79 ± 0.01	1.74
8	9.00 (+1)	0.90 (+1)	0.20 (+1)	0.35 (-1)	1.63 ± 0.01	1.55
9	8.00 (-1)	0.70 (-1)	0.10 (-1)	0.45 (+1)	2.37 ± 0.03	2.46
10	9.00 (+1)	0.70 (-1)	0.10 (-1)	0.45 (+1)	1.52 ± 0.04	1.55
11	8.00 (-1)	0.90 (+1)	0.10 (-1)	0.45 (+1)	2.51 ± 0.21	2.44
12	9.00 (+1)	0.90 (+1)	0.10 (-1)	0.45 (+1)	1.39 ± 0.05	1.36
13	8.00 (-1)	0.70 (-1)	0.20 (+1)	0.45 (+1)	1.98 ± 0.21	1.90
14	9.00 (+1)	0.70 (-1)	0.20 (+1)	0.45 (+1)	1.36 ± 0.02	1.43
15	8.00 (-1)	0.90 (+1)	0.20 (+1)	0.45 (+1)	1.52 ± 0.19	1.61
16	9.00 (+1)	0.90 (+1)	0.20 (+1)	0.45 (+1)	0.89 ± 0.03	0.97
17	7.50 (-2)	0.80 (0)	0.15 (0)	0.40 (0)	1.91 ± 0.04	1.97
18	9.50 (+2)	0.80 (0)	0.15 (0)	0.40 (0)	0.93 ± 0.04	0.88
19	8.50 (0)	0.60 (-2)	0.15 (0)	0.40 (0)	1.98 ± 0.09	1.96
20	8.50 (0)	1.00 (+2)	0.15 (0)	0.40 (0)	2.07 ± 0.01	2.09
21	8.50 (0)	0.80 (0)	0.05 (-2)	0.40 (0)	2.17 ± 0.16	2.24
22	8.50 (0)	0.80 (0)	0.25 (+2)	0.40 (0)	1.26 ± 0.03	1.19
23	8.50 (0)	0.80 (0)	0.15 (0)	0.30 (-2)	1.92 ± 0.01	2.03
24	8.50 (0)	0.80 (0)	0.15 (0)	0.50 (+2)	1.90 ± 0.01	1.80
25	8.50 (0)	0.80 (0)	0.15 (0)	0.40 (0)	3.00 ± 0.08	2.91
26	8.50 (0)	0.80 (0)	0.15 (0)	0.40 (0)	2.94 ± 0.16	2.91
27	8.50 (0)	0.80 (0)	0.15 (0)	0.40 (0)	2.89 ± 0.15	2.91
28	8.50 (0)	0.80 (0)	0.15 (0)	0.40 (0)	2.85 ± 0.10	2.91
29	8.50 (0)	0.80 (0)	0.15 (0)	0.40 (0)	2.97 ± 0.04	2.91
30	8.50 (0)	0.80 (0)	0.15 (0)	0.40 (0)	2.92 ± 0.02	2.91
31	8.50 (0)	0.80 (0)	0.15 (0)	0.40 (0)	2.83 ± 0.04	2.91

[a] Mean from triplicates; Values in bracket are coded variable level

$$\text{Pullulanase specific activity (U/mg)} = +1.04 + 0.092X_1 - 0.05X_2 - 0.31X_3 - 0.16X_4 - 0.031X_1X_3 - 0.19X_1X_4 + 0.059X_2X_3 - 0.082X_2X_3X_4 \quad \ldots (3)$$

This equation is based on the specific activity (U/mg) of pullulanase from ***Anoxybacillus*** sp. strain SK3-4 as a function of 4 independent variables: initial culture ***p***H, and pullulan (carbon source), tryptone (organic nitrogen source) and ammonium chloride (inorganic nitrogen source) concentrations. The ***F***-test with a very low probability value ($P_{model} > F < 0.0001$) and high ***F***-value ($F = 173.98$) demonstrates that this model is statistically significant. The insignificant lack-of-fit (***F***-value 2.93) implies that the model obtained is adequate for prediction within the range of variables employed. The coefficient of determination (R^2) of the model was calculated to be 0.9922. In other words, the model was able to comprehend 99% of the data variability. The higher value of predicted ***R***-squared, |***R***|, (0.9669) also presents a reasonable agreement between experimental and predicted values. The low value of CV (4.06) proves that the conducted experiments have high precision and reliability. In addition, the adequate precision value of 52.51 shows an adequate signal, indicating that the model is significant and acceptable.

According to Table 3 and equation (3), all four factors were significant. They either acted as an

Table 3—ANOVA for 2^4 full factorial design

Source model	Sum of squares	Degree of freedom	Mean square	***F***-value	***P***-value
	2.82	8	0.35	173.98	< 0.0001*
X_1	0.14	1	0.14	66.79	< 0.0001*
X_2	0.04	1	0.04	19.36	0.0011*
X_3	1.50	1	1.50	739.30	< 0.0001*
X_4	0.40	1	0.40	198.81	< 0.0001*
X_1X_3	0.02	1	0.02	7.75	0.0178*
X_1X_4	0.57	1	0.57	280.20	< 0.0001*
X_2X_3	0.05	1	0.05	27.05	0.0003*
$X_2X_3X_4$	0.11	1	0.11	52.56	< 0.0001*
Curvature	0.27	1	0.27	134.76	< 0.0001*
Residual	0.02	11	0.0020		
Lack of fit	0.02	7	0.0027	2.93	0.1578**
Pure error	0.036	4	0.0009		
Correlation total	3.12	20			

Standard deviation	0.045	***R***-squared, R^2	0.9922
Mean	1.11	Adjusted ***R***-squared	0.9865
CV	4.06	Predicted ***R***-squared, $\|R\|$	0.9669
PRESS	0.10	Adequate precision	52.51

* Significant ($P < 0.05$); ** Insignificant ($P > 0.05$)

inhibitor or enhancer of pullulanase specific activity. The coefficient factor for tryptone (X_3) was the greatest, and the negative sign in the equation means that tryptone was the major inhibitory factor. Tryptone is an organic nitrogen source that contains unknown proteins and thus it led to a higher protein content in the culture broth. However, tryptone consists of micronutrients that are essential for the production of pullulanase by this strain. Therefore, tryptone is one of the significant factors, as predicted by this model.

Conversely, the coefficient factor for pullulan (X_2) was the smallest and negative (–0.05), which indicates that pullulan had the least effect in this model. Nevertheless, in a separate experiment (data not shown), exclusion of pullulan from the culture medium inhibited the cells from producing the enzyme. This shows that the presence of pullulan as the carbon source is a strict requirement for pullulanase expression by ***Anoxybacillus*** sp. strain SK3-4. Although pullulan alone has no significant statistical effect based on equation (3), pullulan (X_2) exhibited a significant interaction role with tryptone (X_3) and ammonium chloride (X_4). In addition, the initial culture ***p***H coefficient (X_1) was the only factor showing a positive sign. This suggests that initial culture ***p***H is an enhancer of pullulanase production. The optimal condition for maximum pullulanase specific activity (U/mg) was obtained at ***p***H 9.00, 0.50% (w/v) pullulan, 0.10% (w/v) tryptone and 0.10% (w/v) ammonium chloride. The predicted pullulanase specific activity was 2.00 U/mg. The curvature ***P***-value of <0.0001 implies that there is a significant curvature in the design space. Thus, a continuation of the experiment using a second-order model should be performed to estimate the location of curvature and to obtain the optimum conditions. All four variables were further optimized using a central composite rotatable design.

Central Composite Rotatable Design

In the second statistical approach of this study, a central composite rotatable design (CCRD) was conducted to further optimise pullulanase production. Based on the preceding data, all four variables [initial culture ***p***H (X_1), and pullulan (X_2), tryptone (X_3) and ammonium chloride (X_4) concentrations] were significant factors affecting the production of pullulanase. At first, the same range used in full factorial for each significant factor was engaged in CCRD. The range was inappropriate, as the curvature was not identified (data not shown). It is a general practice to revise the range for each significant factor to locate the curvature if the original range was not suitable[14,15]. By changing the range, we managed to obtain the curvature (Table 2). The ANOVA results for CCRD are presented in Table 4. From the ANOVA, a multiple regression equation for pullulanase specific activity is shown as equation (4):

$$\text{Pullulanase specific activity (U/mg)} = +2.91 - 0.27X_1 + 0.03X_2 - 0.26X_3 - 0.056X_4 - 0.37X_1^2 - 0.22X_2^2 - 0.30X_3^2 - 0.25X_4^2 - 0.043X_1X_2 + 0.11X_1X_3 - 0.11X_1X_4 - 0.068X_2X_3 - 0.15X_2X_4 + 0.027X_3X_4 \quad \ldots (4)$$

The ***F***-value and ***P***-value for this model were 99.14 and <0.0001, respectively, indicating that the model was a good predictor of experimental results. The calculated value of R^2 was 0.9886, indicating that only 1.14% of the total variation in the experiment was not explained by the model. The experimental and prediction values were therefore very close. The lack-of-fit ***F***-value of 2.70 implies that the test is not significantly relative to the pure error.

Table 4—ANOVA for central composite rotatable design

Source model	Sum of squares	Degree of freedom	Mean square	***F***-value	***P***-value
	11.95	14	0.85	99.14	< 0.0001*
X_1	1.79	1	1.79	208.27	< 0.0001*
X_2	0.02	1	0.02	2.54	0.1306**
X_3	1.67	1	1.67	194.25	< 0.0001*
X_4	0.08	1	0.08	8.73	0.0093*
X_1^2	3.97	1	3.97	460.98	< 0.0001*
X_2^2	1.41	1	1.41	164.13	< 0.0001*
X_3^2	2.56	1	2.56	297.82	< 0.0001*
X_4^2	1.79	1	1.79	207.87	< 0.0001*
X_1X_2	0.03	1	0.03	3.39	0.0840**
X_1X_3	0.19	1	0.19	22.48	0.0002*
X_1X_4	0.21	1	0.21	24.28	0.0002*
X_2X_3	0.07	1	0.07	8.48	0.0102*
X_2X_4	0.36	1	0.36	41.36	< 0.0001*
X_3X_4	0.01	1	0.011	1.33	0.2664**
Residual	0.14	16	0.009		
Lack of fit	0.11	10	0.011	2.70	0.1181**
Pure error	0.03	6	0.004		
Correlation total	12.09	30			

Standard deviation	0.093	***R***-squared, R^2	0.9886
Mean	2.03	Adjusted ***R***-squared	0.9786
CV	4.58	Predicted ***R***-squared, |***R***|	0.9435
PRESS	0.68	Adequate precision	31.56

* Significant (***P*** < 0.05); ** Insignificant (***P*** > 0.05)

The 11.81% insignificant lack-of-fit is a good indication that an adequate model was developed for this experiment.

Interactions Among the Factors on Pullulanase Production

The response surface plots were generated based on equation (4) and shown in Fig. 1. All the response surface plots represent the interactions between two variables, while keeping the other two variables constant. From these plots, the trend of pullulanase specific activity under the experimental conditions could be clearly observed.

As observed in Figs 1 (a-c), the optimum initial culture ***p***H (X_1) for maximum pullulanase specific activity was 8.29. The ***Anoxybacillus*** sp. strain SK3-4 is an alkalophile, as it grew well at ***p***Hs between 6.0 and 10.0. The current experiment suggests that higher amounts of pullulanase are produced in alkaline conditions than at neutral ***p***H. Indeed, initial culture ***p***H is a crucial factor for microbial growth and enzyme production, and similar observations were made in other reports[16].

The optimum concentration of pullulan predicted by this model was 0.79% (w/v), as illustrated in Figs 1 (a,d,e). Pullulan concentration (X_2) was found to be significant as the interaction term. However, it appears that pullulan concentration was insignificant as a linear term. This proved that pullulan was required as one of the medium components. In this case, pullulan acts as the triggering factor for pullulanase production by this strain.

Figs 1(b-f) illustrate the optimum concentrations of tryptone (X_3, 0.12% w/v) and ammonium chloride (X_4, 0.39% w/v) that promote maximum pullulanase production. This work suggests that the combination of organic and inorganic nitrogen sources in certain amount could change the pullulanase production profile. In other studies, the concentration of tryptone was found to predominantly influenced RNase production by ***Bacillus firmus***[17]. The influence of combinations of organic and inorganic nitrogen sources on the production of other enzymes was also previously studied. For example, optimal CGTase production by ***Klebsiella pneumonia*** AS-22 was achieved with a combination of peptone, yeast extract (organic nitrogen source) and ammonium nitrate (inorganic nitrogen source)[18].

Verification of the Model

The Design Expert software predicted the optimal medium composition for each stage of the experimental design using the function of numerical optimization and desirability. A validation experiment was performed to certify the values given by each prediction. The experiment was conducted in a 500 mL conical flask containing 100 mL sterile engineered medium under conditions suggested by the software for both models (full factorial and CCRD, respectively). Table 5 summarises the comparisons in terms of the medium composition and the effect on production yield. The pullulanase specific activity increased from 0.91 U/mg in original ***Thermus*** medium to 2.81 U/mg in engineered 2^4 full factorial design medium. The engineered medium suggested by CCRD exhibited a specific activity of 3.38 U/mg, approximately 73% of the original ***Thermus*** medium (0.91 U/mg).

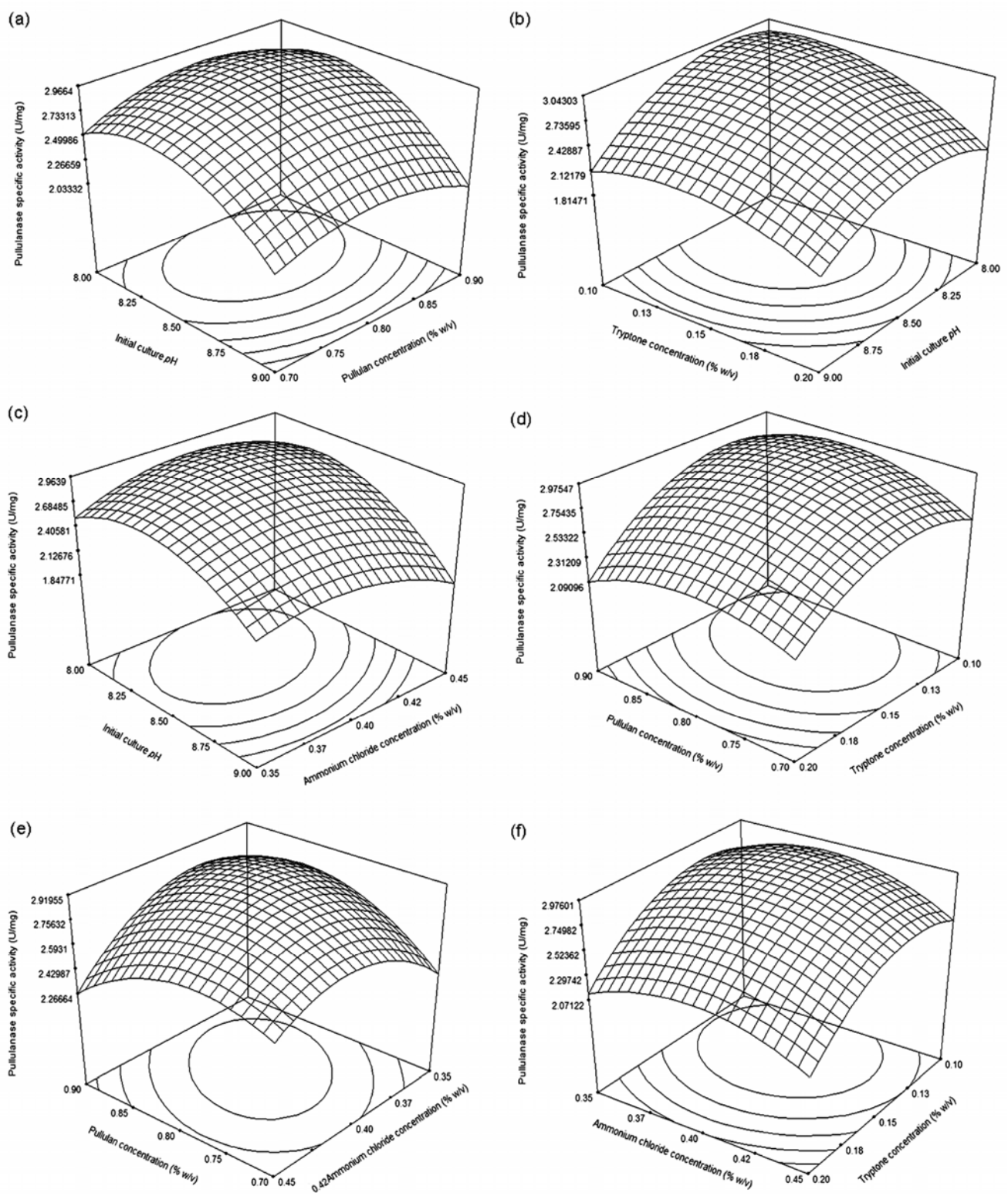

Fig. 1 (a-f)—Response surface plot of pullulanase specific activity from model equation: (a) Effect of initial culture ***p***H and pullulan concentration (% w/v); (b) Effect of tryptone concentration (% w/v) and initial culture ***p***H; (c) Effect of initial culture ***p***H and ammonium chloride concentration (% w/v); (d) effect of pullulan concentration (% w/v) and tryptone concentration (% w/v); (e) Effect of pullulan concentration (% w/v) and ammonium chloride concentration (% w/v); & (f) Effect of ammonium chloride concentration (% w/v) and tryptone concentration (% w/v).

Table 5—Summary of optimum condition for each variable in each design

Order	Variable				Pullulanase specific activity	
	Initial culture ***p***H	Pullulan conc.	Tryptone conc.	Ammonium chloride conc. (% w/v)	Actual value (U/mg)[a]	Predicted value (U/mg)
Original ***Thermus*** medium	7.50	1.00	0.40	-	0.91 ± 0.01	-
2^4 full factorial design medium	9.00	0.50	0.10	0.10	2.81 ± 0.03	2.00
Central composite rotatable design medium	8.29	0.79	0.12	0.39	3.38 ± 0.05	3.00

[a] Mean from triplicates

Fig. 2 shows the individual enzyme activity, protein concentration and specific activity for the original ***Thermus*** medium and other engineered recipes using different statistical designs. Interestingly, the results demonstrate that increment of pullulanase specific activity was achieved by (1) maintaining the total enzyme activity, and (2) decreasing the total protein present in the culture medium. As shown in Fig. 2, the total pullulanase activity for the first 2^4 full factorial design (3.57 U/mL) decreased compared to that of the original ***Thermus*** medium (3.90 U/mL). However, the total protein (organic nitrogen source) in the engineered 2^4 full factorial design medium was reduced significantly from 4.29 mg/mL to 1.27 mg/mL (Fig. 2). The subsequent CCRD medium rebound the pullulanase activity yield to 3.86 U/mL, which approximately the same level as in the original ***Thermus*** medium (3.90 U/mL). The total protein content in the CCRD medium was also slightly reduced.

The concept used in this work to optimize the production of pullulanase is particularly important for several reasons: (1) Because the organic source of the initial medium is expensive, reducing its use helps to cut costs[19]. (2) The organic nitrogen (protein) source is a fundamental burden in downstream processing. Purification becomes easier with less organic nitrogen base present. Sometimes, the background proteins in the medium reduce the purity of the recovery, as they may bind and elute from the chromatography columns at the same retention time as the target enzyme of interest. (3) Although reducing the use of the organic source is theoretically good, the total enzyme expression dropped slightly during the full factorial design. Therefore, further optimization is needed to improve the expression level for each enzyme of interest. In summary, the current CCRD has overcome these drawbacks.

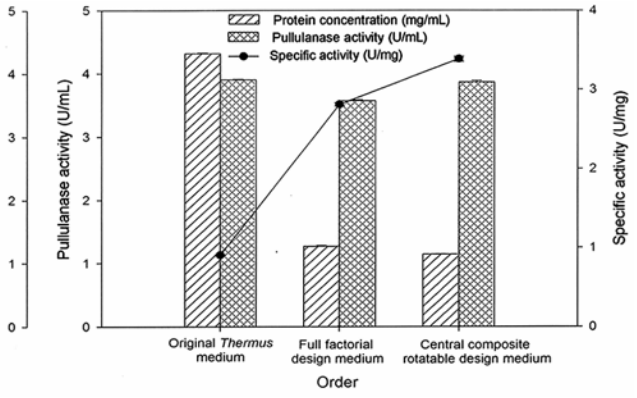

Fig. 2—Comparison in term of pullulanase activity (U/mL), protein content (mg/mL) and pullulanase specific activity (U/mg) for each model. Error bars indicate the mean±standard deviation of triplicates.

Conclusion

Full factorial design and central composite rotatable design were useful in determining and predicting the optimum composition that significantly influenced the production of pullulanase by ***Anoxybacillus*** sp. strain SK3-4. All experimental designs were employed successfully to enhance the pullulanase specific activity up to 73% from the original ***Thermus*** medium. Both selected experimental designs helped to limit the number of individual experiments required, which saves time, cost and material, while increasing the reliability and efficiency of the experiments. In addition, pullulanase has the potential for commercialisation and various industrial purposes.

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