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Medium Optimization for Chitinase Production from *Trichoderma virens* using Central Composite Design

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Abstract Medium development for chitinase production by *Trichoderma virens* was first carried out using conventional method of one-factor-at-a-time. The medium was further optimized using Central Composite Design in which response surface was generated later from the derived model. An experimental design of four variables including various initial pH values, chitin, ammonium sulphate, and methanol concentrations were created using Design Expert® Software, Version 6.0. The design consists of 30 experiments, which include 6 replicates at center points. The optimal value for each variable are 3.0 g/L, chitin; 0.1 g/L, ammonium sulphate; 0.4% (v/v), methanol; and initial pH, 4.0 with predicted chitinase activity of 0.1495 U/mL. These predicted parameters were tested in the laboratory and the final chitinase activity obtained was 0.1471 U/mL, which is almost reaching the predicted value. The optimal medium design showed an improvement of chitinase activity of 80.9% compared to activity obtained from the original Absidia medium composition. © KSBB

Keywords: chitinase, Trichoderma virens*, response surface methodology, optimisation, experimental design, central composite design*

INTRODUCTION

Chitin is the second most abundant biodegradable polymer, which exists naturally in the biosphere as a structural polysaccharide of β-1,4-N-acetyl-D-glucosamine. Chitin can be found as part of fungi, plants, crustaceans, insects, arthropods, and algae components [1]. Chitinases are produced in vast amounts by fungi, yeasts, bacteria, plants, and in insects [2]. Highest chitinase production is obtained when chitin was used as carbon source [3-5]. Presence of nitrogen sources such as yeast extract or peptone could cause reduction in chitinase production. Ammonium sulphate is effective in increasing the amount of enzyme production while urea, peptone, and yeast extract had repressive effects [6]. Chitinase production is pH sensitive, in which pH influences enzyme stability [4,7]. pH value also affects growth and the release of chitinase into medium [8]. A stimulant to boost production is required. Methanol was first reported to be used as stimulant in citric and kojic acid productions from *Aspergillus niger and Aspergillus flavus,* respectively [9,10]. In this study, methanol was used as the stimulant to boost chitinase production.

Recent reports on the optimization of chitinase production focused on the conventional method of optimizing one-factor-at-a-time. However, this method is not very much preferred as numerous potential influential factors may be involved and their interactions could be missed [11]. A successful experimental factorial design and Response Surface Methodology (RSM) was already applied in other field of research and is well suited with the study of the main and interaction of the factor in bioconversion yield [12]. RSM is a concise way of describing and predicting response of a system of variables [13,14]. Factorial design of a limited set of variables is advantage in relation to the conventional method of the manipulation of a single parameter trial, because such approach frequently fails to locate optimal conditions for the process due to its failure to consider the effect of possible interaction between factors. Factorial ex-

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perimentation is highly efficient because each observation supplied information about all the factors included in the experiment.

MATERIALS AND METHODS

Culture Condition

T. virens was maintained on PDA. The spores were harvested from 6 days old fungal growth. Spores were suspended in 10 mL of 1.0% (v/v) Tween 80 aseptically. Spore suspension was then centrifuged at 4,000 rpm for 15 min. The pellets formed were once again suspended in distilled water in order to prepare spore count suspension with 10×10^{11} spores/mL as an inoculum.

Colloidal Chitin Preparation

Chitin flakes from crab shell (Fluka) were used in the experiments. Twenty grams of commercial crab chitin flakes were added to 200 mL of concentrated hydrocloric acid, stirred with a glass rod at 40ºC for 5 min. Excess amount of distilled water was added and the gelatinous white material formed was washed with tap water until the filtrate pH reading falls between pH 6.5~7.0. The drained retentate is the colloidal chitin, which yields a soft, pasty consistency with 98% moisture. The colloidal chitin was sterilized at 121ºC, 15 psi for 15 min and stored till further use.

Enzyme Production Medium

Absidia medium contains (g/L): chitin, 30; ammonium sulphate, 1.5; potassium sodium tartarate, 1.0; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2$, 0.1; and trace elements, 1.0 mL with pH 6.5. Medium compositions and initial pH value were varied according to the design. One mole per liter NaOH or 1.0 M HCl is used to adjust the initial pH values. Trace elements composition includes (g/L): $FeCl_3 \cdot 6H_2O$, 8.0; $ZnSO_4 \cdot 7H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.1; $Co(NO_3)_2 \cdot 6H_2O$, 0.1; and $MnSO_4 \cdot 5H_2O$, 0.1. Trace minerals were filter sterilized and added to the medium after being autoclaved.

Production of Chitinase in Batch Culture

Approximately 10% (v/v) of 1×10^{11} spores/mL spore suspension was transferred into 50 mL sterile production medium. Culture broth was incubated at 30ºC on a rotary shaker maintained at 200 rpm. Sample was withdrawn aseptically at optimum day 4 of fermentation. Withdrawn samples were centrifuged at 4,000 rpm for 10 min. The supernatant was used for chitinase assay and protein analysis.

Chitinase Assay

Chitinase activity was determined using 1.0% (w/v) colloidal chitin as a substrate. One milliliter of supernatant was mixed with 1.0 mL of 1.0% (w/v) colloidal chitin in 2.0 M phosphate buffer, pH 6.5. The mixture was incubated at 50ºC for 1 h. The final product of reaction was determined using DNS method. One unit (U) of chitinase activity is defined as unit of enzyme needed to catalyze the released of 1 µmol of N-acetyl-D-glucosamine under assay conditions [15].

Table 1. Coded and actual values of variables for optimization process

Variable	Component	Level				
		−2	−1	0	1	2
X_1	Colloidal chitin (% w/v)	0	1.0	2.0	3.0	4
X_2	Ammonium sulphate (% w/v)	0.05	0.10	0.15	0.20	2.5
X_3	Methanol (% v/v)	0.3	0.4	0.5	0.6	0.7
X_4	Initial pH	1.0	2.0	3.0	4.0	5.0

Experimental Design

Four variables that had significant effect on chitinase production were determined using conventional screening method. One variable was screened at a time. Chitin, ammonium sulphate, methanol concentrations, and initial pH values were important variables found to have significant effect in chitinase production. Experimental design was carried out using Design Expert® Software (State-Ease Inc., Statistic made easy, Minneapolis, MN, USA, Version 6.0.4). For the variables optimization, response surface method was employed. This method is capable of showing the statistical significance of chitin composition (carbon source), ammonium sulphate (nitrogen source), methanol (stimulant), and initial pH. In this study, a full central composite design was selected with 24 star points ($\alpha = 2.0$) and 6 replicates at center points, leading to a total of 30 experiments. The coded values of the low, middle, and high levels of each variable are given in Table 1.

The data obtained was fitted to a third-order polynomial equation as below. This result would include up to three interaction terms.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x^2_{ii} + \sum \beta_{iii} x^3_{iii} + \sum \beta_{ij} x_i x_j + \sum \beta_{ijk} x_i x_j x_k + \varepsilon \quad (1)$$

Where Y = predicted response for chitinase activity, β_0 = constant, β_i = linear effect, β_{ii} = quadratic effect, β_{iii} = cubic effect, β_{ij}, β_{ijk} = interaction effect, x = coded levels of the independent variables, and ε = random error.

The regression equation was solved using Design Expert® software. The statistical significance of the third-order model equation was determined by a significant F-value, an insignificant lack-of-fit F-value and a good multiple correlation coefficient, R^2 value. The design matrix for this experiment presented in Table 2.

Table 2. 2^4 full factorial central composite design matrix employed for chitinase production constituting of 30 sets of experiments including 24 star points and 6 center points

Experiment run	Variables (in coded value)			
	X_1	X_2	X_3	X_4
1	1	–1	1	1
2	–1	1	1	1
3	–1	–1	–1	1
4	1	–1	–1	–1
5	0	0	0	0
6	–1	–1	1	–1
7	–1	1	–1	–1
8	0	0	0	0
9	1	1	–1	1
10	1	1	1	–1
11	0	0	0	0
12	1	1	1	1
13	1	–1	–1	1
14	–1	–1	1	1
15	0	0	0	0
16	–1	1	1	–1
17	1	–1	1	–1
18	–1	1	–1	1
19	1	1	–1	–1
20	–1	–1	–1	–1
21	0	–2	0	0
22	0	0	0	–2
23	0	0	0	0
24	0	0	0	0
25	0	2	0	0
26	2	0	0	0
27	0	0	–2	0
28	0	0	2	0
29	0	0	0	2
30	–2	0	0	0

Table 3. Observed and predicted values for chitinase production from *T. virens*

Experiment run	Obtained value, Y (U/mL)	Predicted value, $\hat{Y}$ (U/mL)	Residual ($Y - \hat{Y}$)
1	0.095	0.12	–0.021
2	0.104	0.82	0.023
3	0.100	0.1	–0.00447
4	0.10	0.13	–0.028
5	0.12	0.084	0.034
6	0.16	0.14	0.018
7	0.100	0.084	0.016
8	0.071	0.084	–0.013
9	0.085	0.11	–0.023
10	0.100	0.1	0.000909
11	0.038	0.045	–0.0068
12	0.028	0.021	0.00763
13	0.15	0.12	0.035
14	0.047	0.059	–0.011
15	0.038	0.045	–0.0068
16	0.057	0.088	–0.032
17	0.12	0.11	0.012
18	0.047	0.056	–0.00897
19	0.066	0.052	0.015
20	0.043	0.047	–0.00423
21	0.090	0.089	0.000988
22	0.31	0.31	0.000988
23	0.100	0.1	–0.00395
24	0.100	0.1	–0.00395
25	0.081	0.08	0.000988
26	0.090	0.089	0.000988
27	0.17	0.17	0.000988
28	0.076	0.075	0.000988
29	0.076	0.075	0.000988
30	0.14	0.14	0.000988

RESULTS AND DISCUSSIONS

Central Composite Design

Central composite design (CCD) was employed in this study for optimization process of chitinase production. CCD would develop a mathematical correlation model between the significant variables for chitinase production from *T. virens* [16,17]. This design has unique star or axial points (α), which means that the star points are of equal distance and radially distributed making the design rotatable. A rotatable CCD is important in RSM as it would be able to provide good point predictions, at which the factorial and star points are of equal distant from the center [7]. With central composite design, different combination of chitin, ammonium sulphate, methanol concentrations, and initial pH values are being experimented. To guard against systematic trends in uncontrollable or unknown variables, experimental run was randomized during the execution of the designed model [13].

Model Development

At the initial stage of this experiment, the relationships among all the variables were not fully understood. Therefore, an empirical model was selected based on the highest coefficient determination to best describe the data. The data obtained was found to best fit the third-order polynomial compared to other polynomial models. Cubic model was reported to significantly fit better than quadratic models because higher order designs can appropriately model the surface much more precisely [14]. Responses obtained from

Table 4. Regression analysis (ANOVA) for the production of chitinase

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	P > *F*	R^2
Model	0.066	22	0.00298	9.15[a]	0.0100[b]	0.9766
Residual	0.001569	5	0.0003138	-	-	-
Lack of fit	0.0004437	2	0.0002219	0.59	0.60742[c]	-
Pure error	0.001125	3	0.0003751	-	-	-
Correlation total	0.085	29	-	-	-	-

[a] *F*-value is significant. [b] model is significant, with P > *F* less than 0.05. [c] model is fit due to insignificant *F*-value. Standard deviation is 0.028.

experiments were analyzed using analysis of variance (ANOVA). The regression equation obtained is as in equation 2 regardless of their significance. The generated equation is a third-order polynomial.

$$\begin{aligned} Y\,(\text{U/mL}) = {} & 0.077 + 0.012X_1 - 0.019X_2 + 0.008697X_3 \\ & + 0.012X_4 + 0.002323X_1^2 - 0.004793X_2^2 \\ & + 0.004102X_3^2 + 0.022X_4^2 - 0.009785X_1X_2 \\ & - 0.008599X_1X_3 + 0.001483X_1X_4 - 0.002076X_2X_3 \\ & - 0.001483X_2X_4 - 0.015X_3X_4 - 0.006029X_1^3 \\ & + 0.004052X_2^3 - 0.007808X_3^3 - 0.018X_4^3 \\ & + 0.003855X_1X_2X_3 - 0.007413X_1X_2X_4 \\ & - 0.006227X_1X_3X_4 + 0.016X_2X_3X_4 \end{aligned} \quad (2)$$

X_1 represents chitin concentration, while X_2, X_3, and X_4 represents ammonium sulphate, methanol concentration, and initial pH, respectively. This regression model was generated by Design Expert® Software, after considering all the variables. It consists of 1 offset term, 4 linear terms, 4 quadratic terms, 4 cubic terms, and 10 interactions. The predicted levels of chitinase production from *T. virens* at each experimental point using equation 2 were given in Table 3 along with experimental data.

Analysis of varians (ANOVA) for this model was presented in Table 4. The significant level for regression model was tested. It was found that the *P*-value obtained was 0.0100, which is much smaller compared to the desired significant level of $p = 0.05$. This indicates that the regression model was accurate in predicting pattern of significance for the production of chitinase from *T. virens*. Regression coefficient, R^2 value of 0.9766, suggests model adequacy and shows that the model is workable and can be accepted. With such regression coefficient, it shows that equation 2 represent the true relationship among the parameters studied. Here, all linear and quadratic terms were found to be significant except for X_2, in which it was significant when it is in cubic term. Only X_1X_4, $X_1X_2X_3$, and $X_2X_3X_4$ interaction terms were significant.

Response Surface Plot

Response surface methodology can be described as graphical representation of equations from data analysis [18]. RSM allow calculations of maximum production based on a few sets of experiments, in which all factors were varied in a close range [19]. It is a concise way of describing the behavior of a system of variables [14]. RSM is very useful in evaluating the significance of several factors especially that involves complex interactions. Complexity increases with number of variables [14,18]. Contour plot and response surface plot (Figs. 1 to 6) were generated based on equation 2. The plots represent interactions of two variables while keeping other two constant.

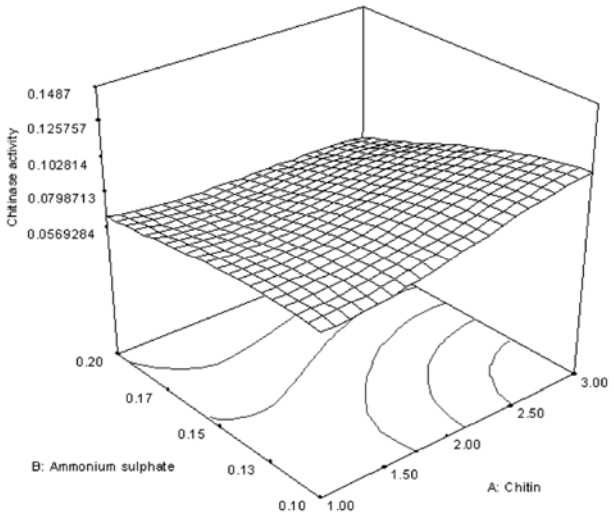

Fig. 1. Response surface plot of chitinase activity from model equation: effect of chitin and ammonium sulphate concentration.

In Fig. 1, the effect of both chitin and ammonium sulphate concentrations were studied with methanol concentration and initial pH kept as constant at the center point values. Chitinase activity increased proportionally with chitin concentration. However, as ammonium sulphate concentration decreases, chitinase activity increases. Production of chitinase is dependent mainly on chitin as carbon source [20]. The type of chitin used as substrate was colloidal chitin, which allows easy accessibility that lead to higher production compared to other chitin sources such as chitin flakes.

Nitrogen source that support initial fungal growth lead to better chitinase production [21]. In the aspect of enhancing chitinase activity, ammonium sulphate proved to be a better nitrogen source compared to other available sources such as peptone, urea, or yeast extract [20,21]. However, high concentration of this nitrogenous compound may lead to chiti-

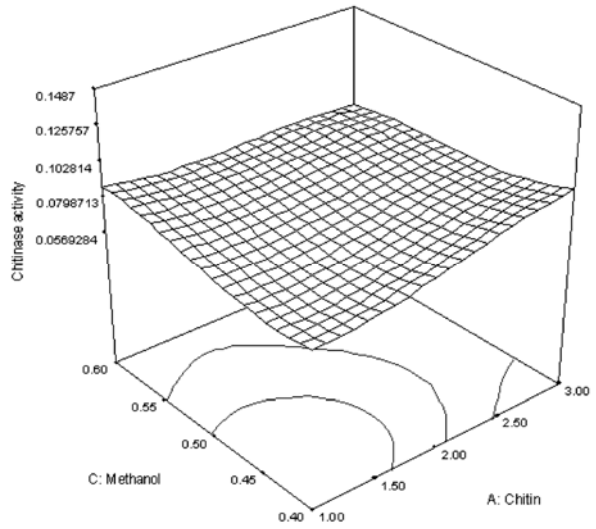

Fig. 2. Response surface plot of chitinase activity from model equation: effect of chitin and methanol concentration.

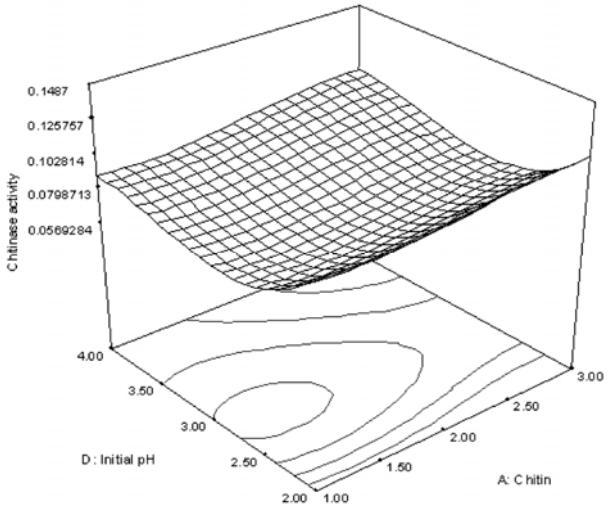

Fig. 4. Response surface plot of chitinase activity from model equation: effect of carbon concentration and pH value.

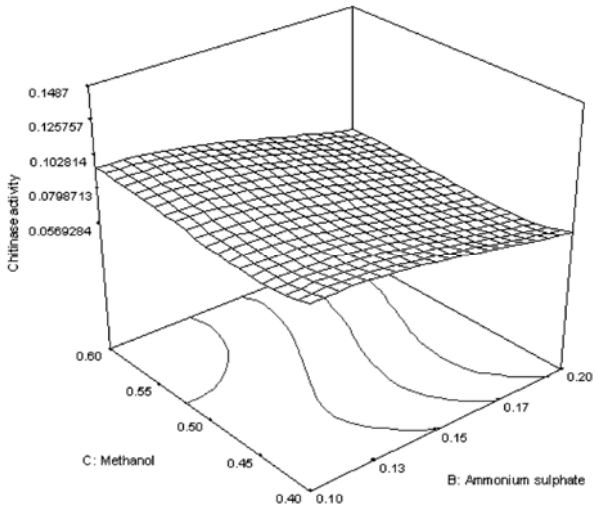

Fig. 3. Response surface plot of chitinase activity from model equation: effect of ammonium sulphate and methanol concentration.

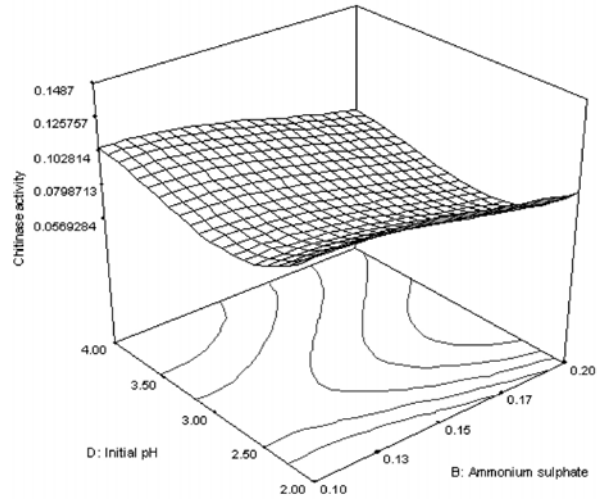

Fig. 5. Response surface plot of chitinase activity from model equation: effect of ammonium sulphate concentration and pH value.

nase repression. This proved that the production of chitinase is increased by the pressure of chitin alone [20]. It is assumed that the fungus may utilize chitin as its preferred carbon and nitrogen sources, while the addition of ammonium sulphate act as an inducer for chitinase production and supports fungal growth [6,22] .

Fig. 2 showed the increment of both chitin and methanol concentration, which lead to the increment of chitinase activity. Here, the ammonium sulphate concentration and initial pH value were set at center point. Meanwhile Fig. 3 illustrates the effect of ammonium sulphate and methanol concentration with chitin concentration and initial pH values being set at center points. Increment of methanol concentra tion enhances chitinase activity at low ammonium sulphate concentrations. Methanol improves membrane permeability, while increasing citric acid production making it an important stimulant in citric acid production from *A. niger* [9].

Chitinase activity was highly influenced by the initial pH values. Chitinase activity was found to be the highest at either high or low initial pH values. In Fig. 4, ammonium sulphate and methanol concentrations were kept constant. The cubic interaction was clearly illustrated. There were two optimal points, at which maximum chitin concentration, the higher the chitinase activity is predicted at very high or very low initial pH values. It can be concluded that initial pH values can severely affect chitinase production compared to other factors.

Similar to Figs. 1 and 3, Fig. 5 also shows that chitinase

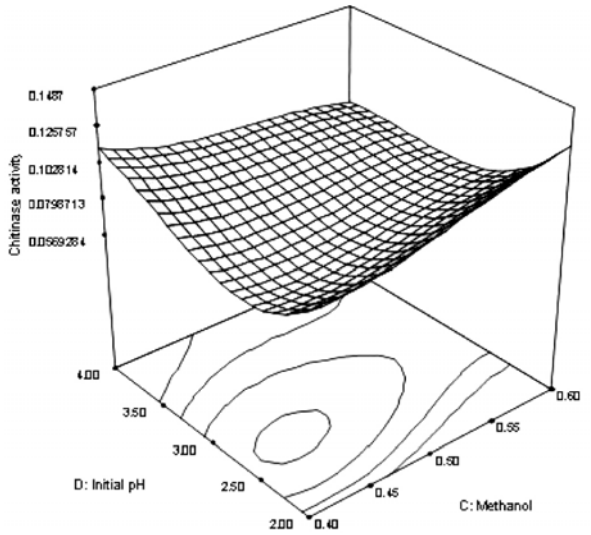

Fig. 6. Response surface plot of chitinase activity from model equation: effect of methanol concentration and pH value.

activity could be increased with lower concentrations of ammonium sulphate. Here the chitin and methanol concentrations were set at center points. The interaction of methanol concentration and initial pH value is as illustrated in Fig. 6. It was found that at high methanol concentration, chitinase activity could be increased.

Optimization of Medium

Optimization of enzyme production in fermentation technology through statistical analysis of factorial design and response surface methodology (RSM) is a common practice nowadays. This technique has been applied for the enhancement and optimization of culture conditions [23-26] and media composition [22] for various fermentation processes. The optimum medium condition was predicted by the Design Expert® Software. The optimum condition for chitinase production using 3.0 g/L, chitin; 0.1 g/L, ammonium sulphate; 0.4% (v/v), methanol; and initial pH, 4.0 were tested in the laboratory. The obtained value 0.1471 U/mL was found to be almost reaching the predictive chitinase activity value of 0.1495 U/mL. An experiment conducted on the non-optimized Absidia medium yields chitinase activity of 0.028 U/mL. Therefore, with the optimized medium, the improvement of chitinase production was 80.97% with 0.1471 U/mL obtained.

CONCLUSION

Central composite design (CCD) is useful in determining and predicting the optimum level of a composition that gives significant influence for chitinase production. A fitted model show suitable prediction response that indicates improvement of a model. From the study, it was proven that initial pH plays a crucial role in enhancement of chitinase activity. Decreasing the ammonium sulphate concentration would help to increase chitinase activity. On the other hand, increasing both chitin and methanol concentrations also gave a great impact towards chitinase activity.

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