

Medium Optimisation of Chitinase Enzyme Production from Shrimp Waste Using *Bacillus licheniformis* TH-1 by Response Surface Methods

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Abstract: The optimization of fermentation medium for the production of chitinase by *Bacillus licheniformis* TH-1 was carried out using Response Surface Methodology (RSM) based on the two level factorial design. This procedure limited the number of actual experiments performed while allowing for possible interactions between 5 components. RSM was adopted to derive a statistical model for the effect of chitin, Yeast Extract (YE), peptone, NaNO₃ and K₂HPO₄ on chitinase production. The p-value of the coefficient for linear effects of chitin, peptone and YE was 0.0001, suggesting that this was the principal experiment variable, having the greatest effect on the production of chitinase. The optimal combinations of media constituent for maximum chitinase production are determined as 10 g L⁻¹ chitin, 0.5 g L⁻¹ YE, 0.5 g L⁻¹ peptone, 2.55 g L⁻¹ NaNO₃ and 1.55 g L⁻¹ K₂HPO₄. The optimization of the fermentation medium resulted not only in a 5.4 fold increase of enzyme activity compared to unoptimized medium but also a reduced amount of the required medium constituents. The response surface analysis provided a useful tool for the optimization of a low cost enzyme producing medium for potential use on an industrial scale.

Key words: Chitinase, NAG, response surface methodology

INTRODUCTION

Chitinases are the enzyme responsible for biological hydrolysis of chitin to its monomer N-acetyl D-glucosamine (Sahai and Monacha, 1993) and have been found to be produced by a number of microorganisms. Microbial chitinases attracted the attention as one of the potential enzyme for applications in agriculture, pharmaceutical, waste management, biotechnology and industry. Their high demand and wide potential uses, has led to the discovery of new strains of microorganisms that capable to produce enzymes with novel properties and the development of low cost industrial media formulations. Microorganisms such as *Paenibacillus* sp. CHE-N1 (Kao *et al.*, 2007), *Penicillium chrysogenum* (Patidar *et al.*, 2005), *Serratia marcescens* (Nawani and

Kapadnis, 2001), *Bacillus cereus* (Pleban *et al.*, 1997), *Aspergillus carneus* (Sherief *et al.*, 1991) and *Aeromonas* sp. (Ueda *et al.*, 2003) are capable to produce chitinase.

Studies on medium optimization for chitinases production are the worthwhile technique for multifactor experiments because it is less time consuming and capable of detecting the true optimum of the factor. In addition, medium compositions greatly influence the microbial production of extracellular chitinase and their interaction play an important role in the synthesis of this enzyme. On the other hand, medium optimization is very important not only to maximize the yield and productivity, but also to minimize the production cost (Park *et al.*, 2005). In most cases, chitin (colloidal chitin, chitin flakes or chitin powder) was utilized as a carbon source in the production of chitinase (Gohel *et al.*, 2006; Nawani and Kapadnis,

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2005; Vaidya *et al.*, 2003; Huang *et al.*, 1996; McCormack *et al.*, 1991). Studies on the medium optimization for chitinase production using the statistical approach have been done by Gohel *et al.* (2005, 2006), Nawani and Kapadnis (2005) and Andrade *et al.* (2003). The commercial interest of utilizing chitin and its derivatives to produce various products lead to the need of inexpensive, reliable source of active and stable chitinase preparations. Moreover, there is a growing interest in the production of monomers, such as N-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) from chitin hydrolysis (Ramirez-Coutinho *et al.*, 2006). Hence, the objective of this study was to optimize the fermentation medium by applying response surface methodology for the production of chitinase from laboratory to the pilot scale. In addition, response surface methodology using the fractional factorial central composite design experiment can be used to develop a mathematical correlation between chitin, nitrogen and mineral salt for the optimum chitinase production by *B. licheniformis* TH-1.

MATERIALS AND METHODS

Microorganisms and culture conditions: The bacterium used in this study, *Bacillus licheniformis* TH-1 was supplied by research collaborator from UTM Skudai; Johore, Malaysia. The strain was kept as glycerol stock at -81°C . The microbe was grown on modified chitinase-detection agar (CHDA) prior to their use for inoculum preparation. Basically, the medium used in this study consisted of colloidal chitin as a carbon source and a mixture of yeast extract and peptone as a nitrogen source. The medium 4 consisted of 2 g L^{-1} colloidal chitin, 3.5 g L^{-1} bacteriological peptone (Oxoid), 1.5 g L^{-1} yeast extract (Oxoid), 1.6 g L^{-1} NaNO_3 (Unilab), 1 g L^{-1} K_2HPO_4 (R and M), 0.5 g L^{-1} KCl (Univar), 0.5 g L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Riedel de Haen), 0.01 g L^{-1} $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Univar) (Kawachi *et al.*, 2001). The final pH of the fermentation medium was adjusted to 7.0 before the inoculation. The 10% (v/v) culture with 0.5 OD_{600} was used as an inoculum. The fermentation process was carried at 150 rpm and temperature at 45°C for 24 h. Preliminary study using optimized culture condition was conducted in 500 mL of shake flask to investigate the performance of chitinase enzyme production by *Bacillus licheniformis* TH-1. Samples were taken at certain time intervals and centrifuged at 7000 rpm for 5 min. The supernatant was then used for residual NAG (N-acetyl D-glucosamine) and chitinase enzyme assays.

Table 1: Variables in real values, for screening by the 2 level fractional factorial design

	Variable (g L^{-1})	Low level (-1)	High level (+)
A	Chitin	1.0	10.0
B	Peptone	0.5	8.0
C	Yeast extract	0.5	5.0
D	NaNO_3	0.1	5.0
E	K_2HPO_4	0.1	3.0

Analysis: Enzyme activity was determined by an adaptation of the chitinase assay established by Rojas-Avelizapa *et al.* (1999) by detecting the amount of reducing sugar liberated from the hydrolysis of the chitin polymer to the simpler forms of N-acetyl-D-glucosamine monomers (NAG). One unit of chitinase activity is defined as the amount of enzyme required to form $1\text{ }\mu\text{mol}$ of NAG in an hour at 50°C . The reducing sugar released (as NAG) was measured by the DNS method (Miller, 1959) and the amount of monomer released was extrapolated from the standard graph of NAG.

Experimental design: A two-level fractional factorial design was employed to determine the maximum chitinase production at optimum levels for chitin, yeast extract, peptone, NaNO_3 and K_2HPO_4 . Five variables, which were expected to have a significant effect on chitinase production, were identified by preliminary experiments. The minimum and maximum range of variables considered for the design are shown in the Table 1. The full experimental plan with respect to their actual and coded forms which contains a total of 20 experimental trials involving 4 replicates at the centre points are shown in the Table 2. The design was employed by selecting chitin, yeast extract, peptone, NaNO_3 , K_2HPO_4 and 1 response of chitinase activity. Each independent variable was investigated at three different levels of a high (+1), center points (0) and a low (-1) level. Runs of center points were included in the matrix and statistical analysis was used to identify the effect of each variable on chitinase production. The runs were randomized for statistical reasons (based on fractional factorial design results obtained using the statistical software package Design Expert 6.0.1, Stat-Ease Inc., Minneapolis, USA). The variables having major effects on chitinase production were identified for the isolates on the basis of confidence levels above 95% ($p < 0.05$). The response surface graphs were obtained using the statistical software to understand the effect of variables individually and in combination and to determine their optimum level for maximal chitinase production. The optimize medium compositions will be further used in fermentation process to scale-up the chitinase production.

RESULTS AND DISCUSSION

Chitinase production: Initially, the basal medium for chitinase production containing (in g L⁻¹) chitin, 2; yeast extract, 1.5; peptone, 3.5; NaNO₃, 1.6, K₂HPO₄, 1.0; KCl, 0.5; MgSO₄·7H₂O, 0.5 and FeSO₄, 0.01 were used for the production of chitinase. From the shake flask fermentation, *B. licheniformis* TH-1 was found to produce maximum chitinase activity 0.215 U mL⁻¹ after 10 h of fermentation time.

Effect of medium components on chitinase production:

For optimization of the chitinase production, the combinations of design experiments with the observed responses of 20 formulations including four center points were determined. A fractional factorial design was applied to derive a statistical model for the effects of the medium formulations on chitinase production by *B. licheniformis* TH-1 and to identify the combination of factors that would lead to the enhancement of chitinase yield. The concentration ranges of the medium components were established on the basis of the data reported by Gohel *et al.* (2006) and Nawani and Kapadnis (2005). The results showed that the chitinase yields varied within the range of 0.242 to 1.163 U mL⁻¹. The highest chitinase activity (1.163 U mL⁻¹) was obtained from medium formulation No. 8 (Table 2) which contained (in g L⁻¹): chitin, 10; peptone, 0.5; yeast extract, 0.5; NaNO₃, 5 and K₂HPO₄, 3, while the lowest chitinase concentration (0.242 U mL⁻¹) was obtained in medium formulation no. 10 which contained (in g L⁻¹): chitin, 10; peptone, 0.5; yeast extract, 0.5; NaNO₃, 5 and K₂HPO₄, 0.1.

Medium supplemented with low level of yeast extract and peptone and high level of NaNO₃ and K₂HPO₄ produced the highest chitinase activity. After treatments combinations, the response data for chitin, yeast extract, peptone, NaNO₃ and K₂HPO₄ yielded significant terms. Therefore, the level of each of five factors needs to be optimized for maximum response.

Once the variables statistically showed significant influence towards chitinase activity, the responses were identified and Central Composite Design (CCD) was performed to determine the optimal level of medium constituents and their interaction (Box and Wilson, 1951). A CCD with 6 replicates at the centre points leading to a total of 22 experiments was employed. Table 3 shows the design and results of experiments carried out by the CCD design. According to the response surface of five variables, peptone and yeast extract gave the most significant effect toward chitinase production. Yeast extract contains nitrogenous compounds, several growth factors and oligomers of NAG, so its addition in low concentrations can have a stimulating effect on cell growth (Nawani and Kapadnis, 2005).

All the linear and quadratic terms of chitin were included in the model since these were significant terms based on the value of p<0.01. Sequential F-tests were performed, starting with a linear model and adding terms (2FI and quadratic). The F-statistic is calculated for each type of model and the highest order model with significant terms normally would be chosen. The chitinase production by *B. licheniformis* TH-1 can be expressed in terms of the following regression equation (coded factors):

Table 2: Two level fractional factorial design for the optimisation of chitin and four nutrients for maximum chitinase activity during fermentation of *B. licheniformis* TH-1, as well as the experimental values of chitinase activity

Run	Block	Real (g L ⁻¹) and coded values					Max. chitinase activity (U mL ⁻¹)
		A	B	C	D	E	
1	1	10.00 (1)	0.50 (-1)	5.00 (1)	0.10 (-1)	3.00 (1)	0.654
2	1	1.00 (-1)	0.50 (-1)	5.00 (1)	0.10 (-1)	0.10 (-1)	0.297
3	1	10.00 (1)	8.00 (1)	5.00 (1)	5.00 (1)	3.00 (1)	0.478
4	1	10.00 (1)	8.00 (1)	0.50 (-1)	5.00 (1)	0.10 (-1)	0.750
5	1	1.00 (-1)	8.00 (1)	0.50 (-1)	5.00 (1)	3.00 (1)	0.540
6	1	5.50 (0)	4.25 (0)	2.75 (0)	2.55 (0)	1.55 (0)	0.593
7	1	5.50 (0)	4.25 (0)	2.75 (0)	2.55 (0)	1.55 (0)	0.757
8	1	10.00 (1)	0.50 (-1)	0.50 (-1)	5.00 (1)	3.00 (1)	1.163
9	1	10.00 (1)	8.00 (1)	5.00 (1)	0.10 (-1)	0.10 (-1)	0.693
10	1	1.00 (-1)	0.50 (-1)	0.50 (-1)	5.00 (1)	0.10 (-1)	0.242
11	1	1.00 (-1)	8.00 (1)	5.00 (1)	0.10 (-1)	3.00 (1)	0.506
12	1	1.00 (-1)	8.00 (1)	5.00 (1)	5.00 (1)	0.10 (-1)	0.510
13	1	5.50 (0)	4.25 (0)	2.75 (0)	2.55 (0)	1.55 (0)	0.617
14	1	10.00 (1)	0.50 (-1)	5.00 (1)	5.00 (1)	0.10 (-1)	0.700
15	1	10.00 (1)	0.50 (-1)	0.50 (-1)	0.10 (-1)	0.10 (-1)	0.949
16	1	5.50 (0)	4.25 (0)	2.75 (0)	2.55 (0)	1.55 (0)	0.788
17	1	1.00 (-1)	0.50 (-1)	5.00 (1)	5.00 (1)	3.00 (1)	0.437
18	1	1.00 (-1)	0.50 (-1)	0.50 (-1)	0.10 (-1)	3.00 (1)	0.313
19	1	10.00 (-1)	8.00 (1)	0.50 (-1)	0.10 (-1)	3.00 (1)	0.678
20	1	1.00 (-1)	8.00 (1)	0.50 (-1)	0.10 (-1)	0.10 (-1)	0.418

A: Chitin, B: Peptone, C: Yeast extract, D: NaNO₃, E: K₂HPO₄

Table 3: Central composite design for the optimization of chitin and four nutrients for maximum chitinase production during fermentation of *B. licheniformis* TH-1, as well as the experimental values of chitinase activity

Run	Real values (g L ⁻¹)					Max. chitinase activity (U mL ⁻¹)
	A	B	C	D	E	
1	5.25	4.25	2.75	2.55	1.55	0.593
2	10.00	8.00	5.00	0.10	0.10	0.693
3	5.25	4.25	2.75	2.55	1.55	0.757
4	1.00	8.00	0.50	0.10	0.10	0.418
5	10.00	8.00	0.50	0.10	3.00	0.678
6	1.00	0.50	0.50	0.10	3.00	0.313
7	10.00	0.50	5.00	0.10	3.00	0.654
8	10.00	0.50	0.50	5.00	3.00	1.163
9	1.00	8.00	0.50	5.00	3.00	0.540
10	1.00	8.00	5.00	0.10	3.00	0.506
11	10.00	0.50	5.00	5.00	0.10	0.720
12	10.00	8.00	0.50	5.00	0.10	0.750
13	1.00	0.50	0.50	5.00	0.10	0.242
14	5.25	4.25	2.75	2.55	1.55	0.617
15	10.00	8.00	5.00	5.00	3.00	0.478
16	5.25	4.25	2.75	2.55	1.55	0.788
17	1.00	0.50	5.00	0.10	0.10	0.297
18	1.00	8.00	5.00	5.00	0.10	0.510
19	1.00	0.50	5.00	5.00	3.00	0.437
20	10.00	0.50	0.50	0.10	0.10	0.949
21	5.25	4.25	2.75	2.55	1.55	0.654
22	5.25	4.25	2.75	2.55	1.55	0.703

A: Chitin, B: Peptone, C: Yeast extract, D: NaNO₃, E: K₂HPO₄

Table 4: Analysis of variance for the reduced quadratic model

Term	SS	df	F-value	Prob>F
A	0.498	1	56.8	<0.0001
B	0.00255	1	0.291	0.598
C	0.0359	1	4.10	0.0611
A ²	0.0446	1	5.09	0.0395
AB	0.154	1	17.6	0.000776
AC	0.0949	1	10.8	0.00496
Model	0.83	6	15.8	<0.0001
Lack of fit	0.101	10	1.68	0.295
Error	0.0302	5	-	-
Total	0.962	21	-	-

SS: Sum of Squares, df: Degree of freedom

$$Y = 0.14 + 0.14A + 0.0287B + 0.0208C - 0.00499A^2 - 0.00582A*B - 0.0076A*C \quad (1)$$

where, A is the chitin, B is the peptone and C is the yeast extract.

The quadratic model in Eq.1 with 6 terms contains 3 linear terms, 1 quadratic term and 2 two factorial interactions.

The ANOVA analysis of the optimization study indicated that the model terms A, A², AB and AC are significant model in terms of chitinase production (prob>F is less than 0.05). The results of variance analysis are shown in Table 4. The quadratic models derived from RSM can be adequately used to describe the medium concentrations and the chitinase yield (Y) under a wide range of operating conditions. Thus, a reduced quadratic model was selected for the analysis. The model F-value is 15.79 and lack of fit F-value is 1.68, (the lack of fit is not

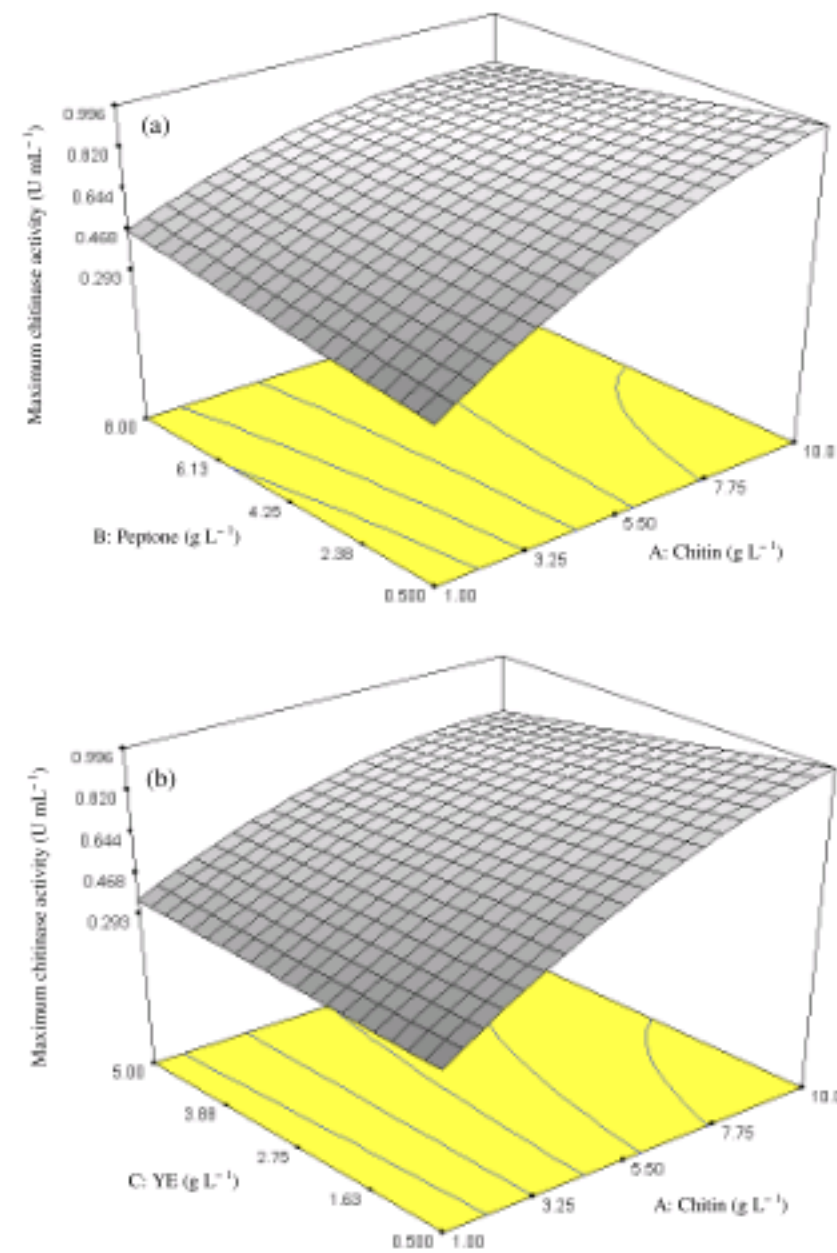


Fig. 1: Effects of (a) chitin and peptone (A and B) and (b) chitin and yeast extract (A and C) and their interactive effect on chitinase production

significant relative to the pure error). The fitness between developed model and experimental data can be determined based on coefficient value (R²). High F-value and non-significant lack of fit indicate the model is a good fit. In this case, the R² value (multiple correlation coefficient) is 0.863 (a value >0.75 indicates aptness of the model). It is an estimate of the fraction of overall variation in the data accounted for by the model can explain 90.42% variation in the response. The value of adjusted R² and predicted R² values are 0.809 and 0.685, respectively. For a good statistical model, R² value should be close to 1.0 and all the five factors should be positive and close to each other. Also, the model has an adequate precision value of 13.3, which suggests that the model can be used to navigate the design space.

The fitted response for the above regression model was shown in Fig. 1. The 3D response surface curve shown the variation of chitinase activity, as a function of concentrations of two medium components (peptone and yeast extract) with the other two (NaNO₃ and K₂HPO₄) being at their constant levels (obtained through analysis of variance). It is easy and convenient to understand the interactions between two nutrients and also to locate their

optimum levels. Combination of high chitin concentration and low yeast extract concentration are the key factors that influence chitinase activity. An increase in chitin concentration in medium supplemented with low yeast extract can further enhance chitinase production was also suggested by Nawani and Kapadnis (2005).

The predicted optimum levels of tested variables (chitin (A), peptone (B), yeast extract (C), NaNO_3 and K_2HPO_4) were obtained by using regression analysis of Eq. 1. The optimal levels for the variables were as follows: $A = 10 \text{ g L}^{-1}$, $B = 0.5 \text{ g L}^{-1}$ and $C = 0.501 \text{ g L}^{-1}$ with the corresponding $Y = 0.864 \text{ U mL}^{-1}$. To validate this model, an experiment was conducted using optimal medium with an addition of $2.55 \text{ g L}^{-1} \text{ NaNO}_3$ and $1.55 \text{ g L}^{-1} \text{ K}_2\text{HPO}_4$ and the activity values were measured. The maximum chitinase activity was found to be 0.844 U mL^{-1} where the model predicted a value of 0.996 U mL^{-1} under the same conditions. This result corroborated the validity and the effectiveness of this model.

CONCLUSION

A highly significant quadratic polynomial obtained from CCD was very useful for determination the optimal concentrations of constituents that gave significant effects on chitinase production. Under the optimal condition, 0.864 and 0.844 U mL^{-1} of chitinase activity could be produced in theory and practical experiment, respectively. Medium No. 8 which formulated was superior as compared to other medium in terms of original compositions for enhancing chitinase production. Linear model obtained from experimental data showed chitin, peptone, yeast extract, NaNO_3 and K_2HPO_4 gave positive effect on chitinase production. The methodology used in this work proved to be adequate for the design and optimization of fermentation process for production of potential valuable product such as chitinase from chitinous waste generated from aquaculture waste.

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