Optimization of Culture Process Conditions for Chitinase Production by a Soil Isolate *Streptomyces shandonggensis* CTI105 Using Response Surface Methodology

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ABSTRACT

Response surface methodology was used to optimize culture process conditions for chitinase production by *Streptomyces shandonggensis* CTI105. Firstly, a chitinase producing microorganism was screened and isolated from soft shell crab molted shell-enriched top soil from a nearby soft shell crab farming area in Chanthaburi province, Thailand. The most potent isolate, with the CZ/CS ratio of 2.08±0.14 at 7 days of incubation time at 35ºC, was identified as *Streptomyces shandonggensis* based on 16S rRNA gene sequence analysis. Secondly, the three important culture process parameters including colloidal chitin concentration (0.5-2.5%), culture pH (4-8), and culture temperature (25–45°C) were optimized to obtain the maximum response of chitinase activity using the statistical Box-Behnken design. The quadratic polynomial equation model developed incorporates three linear, three quadratic, and one interaction term (colloidal chitin concentration and pH). The predicted chitinase production obtained from the quadratic polynomial model using the optimum conditions of colloidal chitin concentration, pH and temperature (1.53% w/v, 6.06 and 34.89ºC respectively) was 36.39 Unit ml^{-1}. The result demonstrated that Box-Behnken design response surface methodology was an effective way to obtain optimal conditions for chitinase production by *Streptomyces shandonggensis*.

Keywords: chitinase, chitin, response surface methodology, Box-Behnken design

INTRODUCTION

Chitinases (E.C 3.2.2.14) are glycosyl hydrolases present in a wide range of organisms such as bacteria, fungi, yeasts, plants, actinomycetes, arthropods, and humans. Chitinases have the ability to degrade chitin, the second highest occurring biopolymer after cellulose, directly to low molecular weight chitooligomers, which serve a broad

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range of industrial, agricultural, and medical applications (Rathore and Gupta, 2015). Bacterial chitinases are active over a wide range of pH and temperatures, depending on the source of the bacteria from which they have been isolated (Hamid *et al*., 2013). Temperature and chitin supply have been reported as important environmental factors controlling both chitin hydrolysis rates and the chitinolytic community structure (Beier and Bertilsson, 2013). Chitin is a major carbon and nitrogen sources for many *Streptomycetes*, soil-dwelling mycelial bacteria, and these microorganisms have developed complex extracellular systems for chitin utilization (Hoang *et al*., 2010; Anne *et al*., 2014; Yandigeri *et al*., 2015). Chitinase production by *Streptomyces* sp. have been affected by many important process condition parameters, such as colloidal chitin concentration, culture pH, and culture temperature. Up to date, a wide range of chitin colloidal concentration, culture pH, and culture temperature have been studied (0.2-2.5% w/v, 6-12.5, and 20-60^{o}C respectively) for chitinase production from *Streptomyces* sp. (Joo, 2005; Kumaran *et al*., 2012; Brzezinska *et al*., 2013; Pradeep *et al*., 2014).

Response surface methodology, a collection of mathematical and statistical techniques for optimization of output variable which is influenced by several independent input variables in order to identify the reasons for changes in the output response, is a very useful tool for determining the optimal level of significant parameters and their interaction. Previous studies have demonstrated an effective way to obtain the optimum cultural process conditions for chitinase production by some other chitinase producing microbial strains using response surface methodology. For such instance, Box-Behnken design was used for optimization of cultural process conditions for chitinase production from *Citrobacter freundii* str. nov. haritD11 (Meruvu and Donthireddy, 2014), and *Bacillus pumilus* (Tasharrofi *et al*., 2011). The central composite design was used for optimization of cultural process conditions for chitinase production from *Serratia marcescens* JPP1 (Wang *et al*., 2014), and *Serratia marcescens* (Sudhakar and Nagarajan, 2011).

The objective of this present work was to optimize the culture process condition for chitinase production by the novel *Streptomyces shandonggensis* CTI105, isolated from soft-shell crab molted shell-enriched top soil. Box-Behnken design was used as a tool to determine the optimal level of significant parameters and their interaction; that is, colloidal chitin concentration, culture pH, and culture temperature for chitinase production.

MATERIALS AND METHODS

Preparation of chitin and colloidal chitin

Molted soft shell crab shells were collected from a waste deposit near a local processing plant in Khlung district, Chantaburi province, Thailand. The dried and ground material was demineralized using 1 N hydrochloric acid, and deproteinized using 1 N sodium hydroxide consecutively to yield chitin. The colloidal chitin was prepared according to Monreal and Reese (1969) with some modifications. Chitin powder (10 g) was slowly added with 100 ml of 85% (v/v) Phosphoric acid and stirred

at 180 rpm for 18 hr at room temperature (30 ±2°C). Chitin was precipitated as a colloidal suspension by adding it slowly to 1 l of water at 4 °C. The suspension was collected by centrifugation at 10,000g for 10 min at 4 °C, and washed 6 times with deionized water. The pH of the suspension was adjusted to 6 by 1 N Sodium hydroxide, and stored at 4 °C for further investigation.

Screening and isolation of chitinase producing microorganism

Soft-shell crab molted shell-enriched top soil samples were collected from a nearby local processing plant in Khlung district, Chantaburi province, Thailand. For screening of chitinase producing bacteria, the minimal medium with 2% w/v colloidal chitin was prepared according to Souza *et al*.(2009). The colonies showing clearance zones on a creamish background were considered as chitinase producing microorganism. After 7 days of incubation at 35ºC, plates were examined for the formation of clearing zones (CZ), and colony size (CS). Colonies with the highest ratio of CZ/CS were isolated and used for further investigation including strain identification on 16S rRNA gene sequence analysis.

Optimization of culture process conditions for chitinase production

The most potent isolate was inoculated into 50 mL of liquid minimal medium supplemented with colloidal chitin and incubated for 7 days with continuously agitation at 150 rpm. The samples were then centrifuged at 10,000 g at 4 ºC for 10 min, and assayed for chitinase activity.

Chitinase activity was determined by a modified colorimetric method for the estimation of N-acetylamino sugars (Reissig *et al*., 1955) using N-acetyl-D-glucosamine as a standard. One chitinase unit is the amount of enzyme which released one micromole of N-acetyl-D-glucosamine equivalent per ml of reaction mixture per min under the experimental condition.

Response surface methodology using 3 factor, 3 level Box-Behnken design was used to optimize the cultural process conditions for enhanced chitinase production with colloidal chitin (X_1), pH (X_2) and temperature (X_3). A total of 15 trials were employed with three replicates at the center point. The coded and actual values of the variables at various levels are given in Table 1. The model was represented by a quadratic polynomial equation as follows:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2 \quad (1)$$

where, Y was the predicted response,
β_0 was the offset term,
β_i is the linear offset,
β_{ii} is the squared offset,
β_{ij} was the interaction effect and
X_i is the dimensionless coded value of variables.

The statistical software package Minitab 14.0 (Minitab Inc., State College, PA) was used to analyze the experimental design. The response obtained was statistically evaluated and the model was built based on the variables with confidence levels more than 95%.

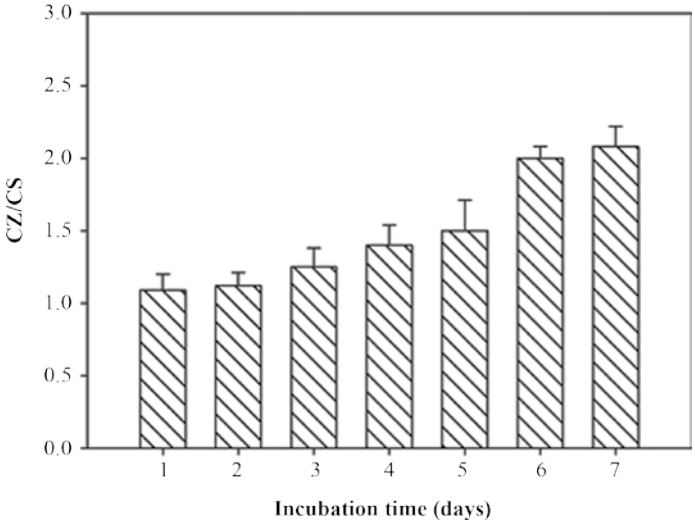

Figure 1. The ratio of clearing zones (CZ) and colony size (CS) produced by *Streptomyces shandonggensis* CTI105 incubated at 35ºC for 7 days.

RESULTS

Screening and isolation of chitinase producing microorganism

It was found that 4 out of 34 isolates (11.8%) were good chitinase producing strains, which were then classified on the basis of their CZ/CS ratio as good (CZ/CS> 2) or weak (CZ/CS<2) chitinase producers (Gooday, 1990; Vaidya *et al*., 2001; Faramarzi *et al*., 2009). Figure 1 demonstrates the ratio of clearing zones (CZ) and colony size (CS) produced by *Streptomyces shandonggensis* CTI105 incubated at 35ºC for 7 days. The most potent isolate from this present study demonstrated with the CZ/CS ratio of 2.08± 0.14 after 7 days of incubation time at 35ºC.

Optimization of culture process conditions for chitinase production

Table 1 shows the experimental values of chitinase activity produced by *Streptomyces shandonggensis* CTI105. Regression results from the data of Box-Behnken design experiments are presented in Table 2. Table 2 shows the significant linear coefficients X_1, X_2 and X_3, quadratic coefficients X_1^2, X_2^2 and X_3^2, and interactive coefficient X_1X_2 with confidence levels more than 95%. The estimated regression coefficients were fitted into the quadratic polynomial equation as follows:

$$Y = 36.3933+1.0725X_1+1.5500X_2-1.3550X_3+1.5325X_1X_2-16.0629X_1^2-17.1029X_2^2-16.3979X_3^2 \quad (2)$$

Where, Y is the response variable (chitinase activity)

X_1, X_2 and X_3 are colloidal chitin concentration, pH and temperature respectively.

The analysis of variance for the quadratic polynomial model is summarized in Table 3. The predicted values of chitinase activity calculated from the quardratic polynomial model are demonstrated in Table 1. The correlation between experimental, and predicted values of chitinase activity produced by *Streptomyces shandonggensis* CTI105 is illustrated in Figure 2. Figure 3 illustrates the three-dimensional response surface plot obtained by calculating from the model and the values taken by one factor where the second varies with constraint of a given Y value.

Table 1. Box-Behnken design plan in coded values, the experimental, and predicted values of chitinase activity produced by *Streptomyces shandonggensis* CTI105.

Trial	Variables/levels						Chitinase production ($U.ml^{-1}$)	
	Colloidal chitin (%) X_1		pH X_2		Temperature (^oC) X_3		Predicted	Experimental
	Coded Value	Actual Value	Coded Value	Actual Value	Coded Value	Actual Value		
1	0	1.5	-1	4	-1	25	4.48	5.16±0.49
2	0	1.5	-1	4	+1	45	1.24	1.75±0.61
3	0	1.5	+1	8	-1	25	6.09	5.59±0.17
4	0	1.5	+1	8	+1	45	3.91	1.86±0.39
5	-1	0.5	0	6	-1	25	1.79	1.79±0.23
6	-1	0.5	0	6	+1	45	0.89	1.06±0.19
7	+1	2.5	0	6	-1	25	6.70	6.53±0.31
8	+1	2.5	0	6	+1	45	2.19	2.19±0.15
9	-1	0.5	-1	4	0	35	2.14	1.46±0.08
10	-1	0.5	+1	8	0	35	1.22	1.73±0.12
11	+1	2.5	-1	4	0	35	2.17	1.66±0.25
12	+1	2.5	+1	8	0	35	7.38	8.06±0.11
13	0	1.5	0	6	0	35	36.39	37.01±2.80
14	0	1.5	0	6	0	35	36.39	36.11±0.17
15	0	1.5	0	6	0	35	36.39	36.06±0.18

The signs '+' and '-' represent the positive and negative directions respectively.

Table 2. Regression results from the data of Box-Behnken design experiments

Model parameter	Parameter Coefficient	SE Coefficient	*t*-value	*P*-value
β_0	36.3933	0.4844	75.132	0.000**
X_1	1.0725	0.2966	3.616	0.015*
X_2	1.5500	0.2966	5.225	0.003**
X_3	-1.3550	0.2966	-4.568	0.006**
X_1X_2	1.5325	0.4195	3.653	0.015*
X_1X_3	0.2625	0.1495	0.626	0.559
X_2X_3	-0.9025	0.4195	-2.151	0.084
X_1^2	-16.0629	0.4366	-36.789	0.000**
X_2^2	-17.1029	0.4366	-39.171	0.000**
X_3^2	-16.3979	0.4366	-37.556	0.000**

β_0 represents constant value, X_1, X_2, X_3 represent colloidal chitin concentration, pH, and temperature.
*Significant level at 5%, **Significant level at 1%.

Table 3. Analysis of variance (ANOVA) for a quadratic polynomial model

Source	Sum of squares (type III)	Degree of freedom	*F*-value	*P*-value
Model	2678.63	9	422.82	0.000
Linear	43.11	3	20.41	0.003
Square	2622.59	3	1241.91	0.000
Interaction	12.93	3	6.12	0.040
Residual error	3.52	5		
Lack-of-fit	2.95	3	3.44	0.233
Pure error	0.57	2		
Total	2682.15	14		

Coefficient of correlation (R^2) = 99.87%, R^2 (adj) = 99.63%

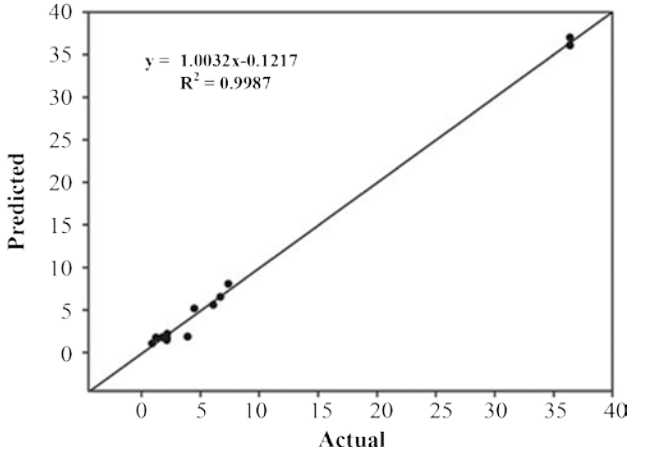

Figure 2. The experimental, and predicted values of chitinase activity produced by *Streptomyces shandonggensis* CTI105.

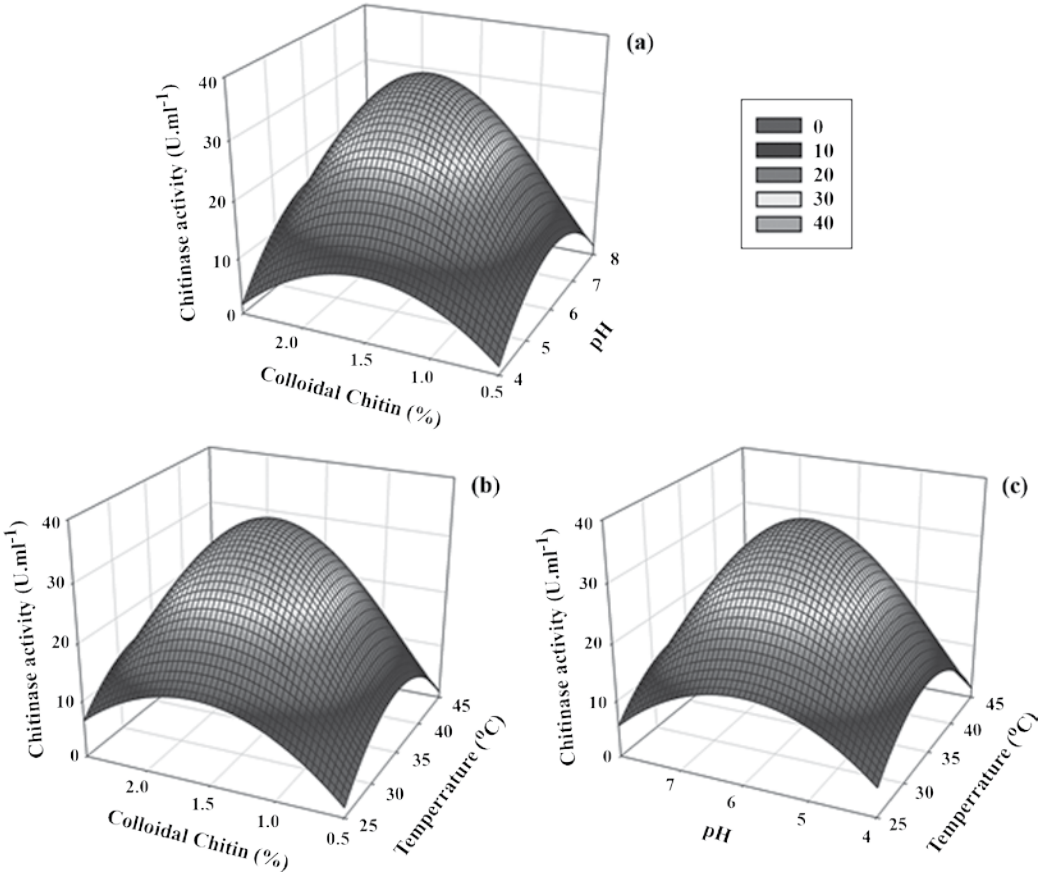

Figure 3. Three-dimensional response surface plot of chitinase activity produced by *Streptomyces shandonggensis* CTI105 affected by (a) colloidal chitin concentration and cultural pH, (b) colloidal chitin concentration and cultural temperature, (c) cultural pH and temperature.

DISCUSSION

Screening and isolation of chitinase producing microorganism

Soft-shell crab molted shell-enriched top soil samples were used due to the higher presence of chitin-containing organisms in the upper soil layers (Beier and Bertilsson, 2013). Similar studies have been reported by Faramarzi *et al.* (2009); that is, the 4 out of 18 isolates (22.2%) were classified as good chitinase producing strains isolated from soils in Tehran, Iran, and Vaidya *et al.* (2001); that is, the 13 out of 40 isolate (32.5%) were classified as good chitinase producing strains isolated from locations of Vadodara, Rurat and Mumbai, India. The most potent isolate was identified as *Streptomyces shandonggensis* based on 16S rRNA gene sequence analysis with 98% sequence similarity to *Streptomyces shandonggensis* strain NM UAF 227 (Accession number EF512276).

Optimization of culture process conditions for chitinase production

The calculation of regression analysis (Table 3) gives the value of the determination coefficient ($R^2 = 0.9987$), which indicates that the sample variation of more than 99.87% was attributed to the variables. The value of adjusted determination coefficient (Adj. $R^2 = 0.9963$) is also very high which indicates a high significance of the model. Figure 2 shows a satisfactory correlation between the experimental and predicted values wherein the points cluster around the diagonal line which indicates the good fit of the model, since the deviation between the predicted and experimental values was less. This great similarity between the predicted and the observed results reflects the accuracy and applicability of the Box-Behnken model in the optimization processes. In addition, value of lack of fit *F* value, and lack of fit *P* value were found to be 3.44 and 0.233 respectively, which implied that the lack of fit was insignificant relative to the pure error. Insignificant lack of fit made the model fit.

Response surface plots as shown in Figure 3 are a function of two factors at a time, maintaining all other factors at fixed levels and are helpful in understanding both the main and the interaction effects of these two factors. The interaction between colloidal chitin concentration and pH showed significantly effect on chitinase production as indicated by highest parameter coefficient of 1.5325 ($P<0.05$). The maximum predicted value is indicated by the surface confined in the response surface diagram. The optimum levels of the three examined independent variables as predicted from the model; that is, colloidal chitin concentration, pH and temperature were 1.53% w/v, 6.06 and 34.89°C respectively. At these conditions the predicted chitinase activity of 36.39 $U.ml^{-1}$ was achieved with a desirability of 0.961. A verification experiment was carried out at the optimum conditions and the experimental value was 36.11±0.55 $U.ml^{-1}$ which is almost similar to the value at the same conditions predicted. The similarity corroborates the validity and effectiveness of the model.

The effects of temperature and pH on chitinase production have been reported in terms of their important roles in cell growth and enzyme production (Kuddus and Ahmad. 2013). The colloidal chitin was used as a sole carbon and nitrogen source for

chitinase production (Kuddus and Ahmad. 2013). The optimum cultural process conditions achieved from this work (1.5% colloidal chitin concentration, pH 6, and 35°C) using response surface methodology were found to be almost similar to the values reported by previous work. Bacterial chitinases are active over a wide range of pH and temperatures, depending on the source of the bacteria from which they have been isolated (Hamid *et al*., 2013). For instance, the optimum conditions (1% colloidal chitin concentration, pH 6, and 35°C) for the laterite soil isolate *Streptomyces* sp. ANU6277 were obtained from step by step completely randomized designs (Narayana and Vijayalakshmi, 2009), and the optimum conditions (1% colloidal chitin concentration, pH 6.5-7, and 30-40°C) for *Streptomyces aureofaciens* CMUAc130 were obtained from step by step completely randomized designs (Taechowisan *et al*., 2003).

CONCLUSION

The optimum culture process conditions of chitinase production by *Streptomyces shandonggensis* CTI105 were effectively identified by Box-Behnken design. However, the culture medium compositions for a novel isolate *Streptomyces shandonggensis* CTI105 still needed to be optimized. Therefore, future studies will focus on medium optimization using response surface methodology, which will allow us to determine the optimal amount of significant medium compositions in order to maximize chitinase production from anovel *Streptomyces shandonggensis* CTI105.

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