Optimization of Colloidal Chitin and Inoculum Concentration in Chitinase Production by *Streptomyces* sp. PB2 Using Response Surface Methodology

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Abstract. Chitinase is an enzyme capable of catalyzing the hydrolysis of chitin. Bacteria is one of the sources for chitinase and the modification process of production are continuously developed. *Streptomyces* sp. is one of Actinomycetes group that shows high activity in hydrolyzing chitin. This study aimed to find the optimal conditions of *Streptomyces* sp. PB2 for producing chitinase at various colloidal chitin (0.5%, 1%, 1.5%) and inoculum (0.5%, 1%, 1.5%) concentration in the chitin broth medium using Respons Surface Methodology (RSM). The examined parameters included Total Plate Count (CFU/ml) and chitinase activity (U/ml). Chitinase activity was statistically analyzed by MiniTab 17 to obtain a mathematical model, then were validated. The mathematical model of chitinase activity was $Y = -0.000075 + 0.00056 K + 0.00067 I$, with the optimum colloidal chitin concentration (K) of 1.5% and inoculum concentration (I) of 1.5%. The highest chitinase activity of 0.0019 U/ml in day-2 fermentation. The validation test showed that the mathematical model had a low accuracy with the SSE value of 0.5306. This study shows that colloidal chitin and inoculum concentration are important factors to be optimized. However, further model is needed to be developed for a better estimation of process.

1 Introduction

Chitinase is an enzyme catalyzing the hydrolysis of β -1,4-glycosidic bond of chitin. It is also well-known enzyme having capability to produce value-added product derived from shrimp shell waste such as chitin and/or chitosan oligomers, N-acetylglucosamine, and glucosamine which can be applied in food, cosmetics, and biomedical industries [1] [2]. Basically, hydrolysis of chitin is carried out through the enzymatic or chemical process. However, enzymatic hydrolysis, in which chitinolytic microorganism plays an important role in this process, is preferable compare to the chemical process as it offers an eco- friendly process, more affordable, and easier to be handled [3].

Approximately 90-99% chitinolytic bacteria population belongs to the group of Actinomycetes [4]. The Department of Fisheries, Faculty of Agriculture, Universitas Gadjah

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Mada have collection of chitinolytic bacteria, including *Streptomyces* sp. PB2 that previously isolated from shrimp pond-sediment [5]. Further molecular identification revealed that PB-2 was 100% similar to *Streptomyces misionensis* based on the 16s rRNA analysis[6]. During investigation, *Streptomyces* sp. PB2 showed a chitinolytic index and chitinase activity of 1.30 and 0.0007 U/ml, respectively, after the fourth day of incubation at 37°C [5]. Further study on the optimization of chitinase activity produced in the culture medium of *Streptomyces* sp. PB2 revealed that the highest activity was achieved at the incubation condition of 30°C and pH 7 (0.0017 U/ml). It indicates that environment modification helps to increase chitinase activity in bacterial culture. Another factors that needed to be optimized to increase the chitinase activity in bacterial culture were the level of bacterial inoculum and concentration of colloidal chitin in the medium [7] [8].

Chitinase is an inducible enzyme, which produced by the bacteria when the inducer presents. Chitin stimulates microorganism for releasing chitinase to help the breakdown of the complex polymer of chitin into a simple sugar that will be further uptake as the nutrition by the cell [9]. Beside of the substrate, the number of initial inoculum also affects bacterial enzyme production. Inoculum becomes the critical factor in optimizing chitinase activity by *Streptomyces* sp. as it relates to lag phase period [10]. High concentration of inoculum may result in non-optimal enzyme formation which caused by competition between bacteria cell dispersed on medium [9]. Hence, substrate concentration and inoculum are two important factors that have to be considered to optimize the bacterial chitinase production.

Factorial experimental design is commonly used to analyze the interaction between two factors in statistical analysis. However, this design requires many experimental units because of the considerable number of treatment combination. Box and Wilson in 1951 conveyed modified method to determine both of experiment effect and optimal point from multifactor experiment known as *Response Surface Methodology* or RSM [11]. RSM offers an effective and efficient experimental unit in determining the effect and interaction between each factor. This study aimed to determine the optimal combination of colloidal chitin and inoculum concentration for *Streptomyces* sp. PB2 in producing chitinase using RSM.

2 Materials and methods

2.1 Medium preparation for chitinase production

Colloidal chitin medium was used for chitinase production. Chitin was extracted from shrimp shell [12] and formed into colloidal chitin [13]. Colloidal chitin medium consisted of KH₂PO₄ (0.03%), K₂HPO₄ (0.07%), colloidal chitin (0.5; 1; 1.5%), MgSO₄.5H₂O (0.05%), ZnSO₄ (0.0001%), and MnCl₂ (0.0001%) [14].

2.2 Inoculum preparation

Streptomyces sp. PB2 was cultivated in 7 ml nutrient broth (NB) medium on 37°C and incubated for 2 days to reach late log phase (approximately 10 log CFU/ml (10⁵ - 10⁸). Culture was centrifuged at 3,000 rpm for 1 min and pellet was collected, followed by addition of 1 ml sterile distilled water. The mixture was served as the inoculum stock. The variation of inoculum concentration was prepared by diluting inoculum stock with certain volume of sterile distilled water (Table 1). The concentration on initial cell concentration (inoculum size) in production medium was counted based on aerobic plate count method grown in nutrient agar (NA) at 37°C for 18-24 hours.

Table 1 Formulation of inoculum concentration

Treatment (%)	Inoculum Stock (μl)	Sterile Distilled Water (μl)	Total volume of inoculum* (μl)	Bacteria on production medium (CFU/ml)
0.5	50	450	500	2.9×10^3
1	100	900	1,000	1.9×10^5
1.5	150	1,350	1,500	3.8×10^5

*in 100 ml of production medium

2.3 Optimization of colloidal chitin and inoculum concentration using RSM

There were 2 treatments used in this experiment, colloidal chitin concentration (K) and inoculum concentration (I). Both factors were set as an independent variable. Three level of inoculum concentrations (0.5%; 1%, and 1.5%) were assigned for each factor (Table 2).

Table 2. Independent variable and treatment code

Independent Variable	Symbol	Range & Level		
		-1	0	+1
Colloidal chitin concentration (%)	X1	0.5	1	1.5
Inoculum concentration (%)	X2	0.5	1	1.5

According to the range and level, 8 experimental units were obtained from MiniTab 17 (Table 3). Those experimental units were applied into 100 ml colloidal chitin medium and incubated at 30°C using 100 rpm agitation speed for 2 days. Samples were collected every 24 hours for chitinase activity assay.

Table 3. Combination of treatment

Treatment	X1	X2
A	-1	-1
B	-1	+1
C	+1	-1
D	+1	+1
E	0	0
F	0	0
G	0	0
H	0	0

2.4 Chitinase activity assay

Preparation of sample for chitinase activity assay followed the method of Wang et al. [15]. Briefly, 1 ml culture medium was harvested and centrifuged at 10,000 rpm for 2 min at 4°C. The amount of 0.5 ml cell-free supernatant obtained from centrifugation was mixed with 1 ml colloidal chitin 1.3% (in buffer phosphate 50 Mm pH 7.4), incubated for 30 min at 37°C, and boiled for 30 min in boiling water to stop the reaction. After cooled down to reach room temperature (28°C), mixture was centrifuged at 10,000 rpm for 3 min. The reaction mixture was measured for its N-acetylglucosamine (GlcNAc) content.

GlcNAc content was measured by spectrophotometry [16]. Briefly, 0.25 ml enzymatic reaction mixture was added with 0.05 ml kalium tetraborate (pH 9.1), incubated in boiled

water for 3 min, added with 1.25 ml p-dimethylaminobenzaldehyde (DMAB), and incubated at 37°C for 30 min. The absorbance of the mixture was measured by UV-Vis spectrophotometer measurement at the wavelength of 540 nm. Negative control and GlcNAc standard were also measured. One unit of chitinase activity was defined as amount of enzyme releasing 1 µmol of GlcNAc from chitin hydrolysis reaction each minute under mentioned incubation condition.

2.5 Data analysis

Data obtained from this study was analysed statistically. Normality test and homogeneity test were performed using R-Studio software to ensure the data met the Anova assumption requirement. For RSM analysis, the statistical test was performed sequentially as followed lack of fit, sum of square and R-square analysis using MiniTab 17 software. The mathematical model was obtained, and the validation test was performed. To measure the discrepancy between the data and an estimation model, sum of squared error (SSE) was calculated.

3 Results and discussion

Three concentrations of colloidal chitin 0.5%; 1%; 1.5% were used. Previous study [10] reported that 1% chitin concentration helped to produce optimum chitinase activity of *Streptomyces* sp. ANU6277. Optimum chitinase activity by *S. aureofaciens* CMUAc130 was also achieved using 1% colloidal chitin [17]. Another studies reported that the addition of 0.25% to 1.5% colloidal chitin in medium helped to improve bacterial chitinase production [18,19]. Thus, the level of colloidal chitin of 0.5%, 1%, and 1.5% were chosen as the treatments. Meanwhile, for the inoculum concentration of 10³ – 10⁶ CFU/ml were used for the production of chitinase by *Streptomyces* sp. MBRL10 [20]. Three levels from 2 treatment factors were then combined for further optimization using *response surface methodology*.

Table 4. Chitinase activity (U/ml) of *Streptomyces* sp. PB2 cultured at 30°C, 100 rpm, 2 days

Treatment Combination Code	Colloidal Chitin Concentration (%)	Inoculum Concentration (%)	Chitinase Activity (U/ml)
A	0.5	0.5	0.0001
B	0.5	1.5	0.0004
C	1.5	0.5	0.0003
D	1.5	1.5	0.0013
E	1	1	0.0003
F	1	1	0.0005
G	1	1	0.0004
H	1	1	0.0006

The chitinase activity produced after 2 days incubation shows that treatment D, a combination of 1.5% colloidal chitin and 1.5% inoculum concentration, resulted in the highest chitinase activity (Table 4). The analysis of Anova assumption (normality and homogeneity test) and goodness of fit (lack of fit test, sum of square and R-square test) were employed using MiniTab 17 to obtain the mathematical model. The shapiro normality test

showed the p-value of 0.09, meanwhile the homogeneity test resulted in p-value of 1. The p-value higher than 0.05 indicates that normality and homogeneity assumption of the data was achieved and the analysis of statistical model was proceeded.

Table 5. Anova linear model of chitinase activity (combination of colloidal chitin/ K) and inoculum concentration/ I)

Source of Variant	Df	SS	MS	F-Value	P-Value*
Linear	2	0.000001	0.000000	10.52	0.016
K	1	0.000000	0.000000	8.83	0.031
I	1	0.000000	0.000000	12.21	0.017
Error	5	0.000000	0.000000		
Lack of Fit	2	0.000000	0.000000	3.11	0.186
Pure Error	3	0.000001	0.000000		

*Significance level 95%
H0 : Model is not fit (P-value > 0.05)
H1 : Model is fit (P-value < 0.05)

Analysis of the goodness-of-fit showed that the p-value obtained from the linear model was 0.018 (Table 5). P-value of less than 0.05 showed that linear model fitted the mathematical model for the chitinase activity produced by *Streptomyces* sp. PB2. Furthermore, lack of fit analysis was done to measure the fitness of the model to the first order. A good mathematical model is a model without lack of fit [20]. Hypothesis used to conclude this analysis was:

H₀: No lack of fit on the model (P value > 0.05)
H₁: There was lack of fit on the model (P-value < 0.05)

P-value obtained (0.354) was higher than 0.05 (Table 5). Therefore, H₀ was accepted, meaning that no lack of fit in the model. Based on the Anova regression calculation, the R-square value was 0.80 nearly to 1. The R-square value of nearly to 1 indicates a good model as it fits the data and explains all the variability of data response [20]. R-square of 0.80 showed that chitinase activity was affected by colloidal chitin and inoculum with 0.80 probability.

According to statistical analysis, a linear model was obtained as a mathematical model of RSM for chitinase activity towards colloidal chitin and inoculum concentration as follows:

$$Y = - 0.00075 + 0.00056 K + 0.00067 I$$

Y was chitinase activity; K was colloidal chitin concentration; whereas I was inoculum concentration. From this model, a contour plot of chitinase activity resulted from the combination of various concentrations of colloidal chitin and inoculum was obtained (Figure 1).

Fig. 1. Contour plot of various concentrations of colloidal chitin and inoculum against chitinase activity

Figure 1 explained that the higher the colloidal chitin and inoculum concentration added to the production medium, the higher the chitinase activity obtained. Our finding revealed that 1.5 % colloidal chitin concentration and 1.5% inoculum resulted in the highest chitinase activity (0.0013 U/ml). The same ratio of colloidal chitin concentration and inoculum concentration resulted to the highest chitinase activity in this study.

A lower chitinase activity was obtained from a combination of colloidal chitin and inoculum in a lower concentration than 1.5%. The combination of 0.5% colloidal chitin and 0.5% inoculum resulted to the chitinase activity of 0.0001 U/ml, meanwhile 0.5% colloidal chitin and 1.5% inoculum produced chitinase activity of 0.0004 U/ml. Low concentration of colloidal chitin may caused an inadequate nutrition to support cell growth as colloidal chitin served as carbon and energy source for bacteria [21]. Low nutrient availability in the medium may result to the competition between bacteria. Furthermore, the combination of higher colloidal chitin (1.5%) and low concentration of inoculum (0.5%) was also resulted to the low chitinase activity. Higher concentration of substrate compared to inoculum concentration may lead to the accumulation of organic acid that decrease the medium pH and further inhibit cell growth and interfere enzymatic reactions [22].

Validation test with 2 replications was done to observe the goodness-of-fit between mathematical model and observation data. Table 6 shows the comparison of chitinase activity obtained from observation with the value obtained from the calculation using mathematical model.

Table 6. Mathematical model validation of chitinase activity from the combination of colloidal chitin and inoculum

Colloidal chitin (%)	Inoculum (%)	Calculated (U/ml)	Observation (U/ml)	SE
1.5	1.5	0.0010	0.0019	0.2244
1.5	1.5	0.0010	0.0017	0.1696
2	2	0.0017	0.0024	0.0851
2	2	0.0017	0.0022	0.0517
SSE				0.5306

Table 6 shows that the observed value of chitinase activity was higher than the calculated chitinase activity obtained from the model. The high value of SSE indicated that the discrepancy between the data obtained from experiment was far from the estimated value using the model obtained from the RSM analysis. This result indicated that the development of other model was required.

The optimum chitinase activity in this study was reached at day-2 with the value of 0.0019 U/ml. The highest chitinase activity of 0.0017 U/ml was obtained from *Streptomyces* sp. PB2 at day-6 of incubation before the optimization process. using the combination of 2 % colloidal chitin and 1% inoculum [23]. This study shows that optimization of colloidal chitin and inoculum concentration in the medium accelerated the period of chitinase production by *Streptomyces* sp. PB2. However, chitinase activity produced by *Streptomyces* sp. PB2 from this study was lower compared to other report on *Streptomyces* sp. chitinase. Chitinase activities of *Streptomyces* sp. NK1057, *Streptomyces* sp. NK528, and *Streptomyces* sp. NK951 were reported upon the optimization of colloidal chitin and inoculum concentration along with other components of medium using RSM. Optimum chitinase activities from the highest to the lowest were 325 mU/ml by *Streptomyces* sp. NK1057, 194 mU/ml by *Streptomyces* sp. NK528, and 132 mU/ml by *Streptomyces*. sp. NK951 with the ratio of colloidal chitin concentration of 20.96, 15, and 15 g/l, respectively, with the same inoculum desnity of 0.15 [24]. Further optimization of growth condition for *Streptomyces* sp. PB2 is needed to achieve higher chitinase activity of the culture.

4 Conclusion

Chitinase activity of *Streptomyces* sp. PB2 was optimized by the combination of colloidal chitin and inoculum concentration in the medium. Statistical analysis using RSM obtained the mathematical model for chitinase activity of $Y = - 0.00075 + 0.00056 K + 0.00067 I$. With the optimum colloidal chitin concentration (K) of 1.5% and inoculum concentration (I) of 1.5%. This study shows that colloidal chitin and inoculum concentration are the important factors to be optimized in order to reach an optimal production of bacterial chitinase.

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