*Asian Journal of Chemistry; Vol. 23, No. 12 (2011), 5289-5294*

ASIAN JOURNAL OF CHEMISTRY

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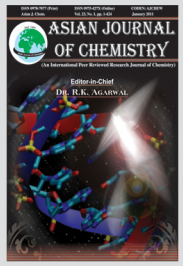

# Improvement of Chitinase Production from a Newly Isolated *Chitinolyticbacter meiyuanensis* SYBC-H1 Using Central Composite Design Technique

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(*Received*: 28 October 2010; *Accepted*: 12 August 2011) AJC-10271

The effect of physical processes such as rpm of the shaking incubator, initial pH of the medium and temperature on the production of chitinase from a newly isolated *Chitinolyticbacter meiyuanensis* SYBC-H1 was studied using central composite design technique. The individual optimum levels of rpm of the shaking incubator, initial pH of the medium and temperature were found to be 233 rpm, 6.44 and 26.21 ºC, respectively for chitinase production. As a result, maximum chitinase activity of 5.20 U/mL was obtained in the optimized medium, which was 15.5-fold higher than that in the basal medium.

**Key Words: Chitinase, Physical process parameters, Central composite design technique, *Chitinolyticbacter meiyuanensis*.**

## INTRODUCTION

Chitinases are widely distributed in nature and gaining much attention worldwide[1-4]. Because of immense interest due to their wide applications in such as generation of crustacean chitin waste management[5], fungal protoplasts[6], production of single cell protein[7], production of chitooligosaccharides (immune enhancers), chitooligosaccharides or N-acetyl-glucosamine for various applications[8] and specially in biocontrol of plant pathogens. Recently, microbial chitinases have attracted attention as one of the potential candidates for control of phyto-pathogenic fungi and insect pests[9]. A number of fungal, yeast and bacterial strains have been reported to be good producer of chitinase and much work has been done to enhance the activity of the chitinase, such as mutagenesis, constructing gene engineering strains, optimization of the culture and catalytic conditions and so on[10-14]. However, the chitinase production was not favorable for industrialization.

Chitinase production by microbes is influenced by number of factors such as pH, temperature, the nature of nitrogen sources and carbon, aeration present in the medium and various methods, including the optimization of culture conditions and medium composition to improve the enzyme yield. These factors have varied effects on different species[11,15,16]. Very less information is available on the factors which control the synthesis and release of extracellular chitinase. Since the chitinase production varies from organism to organism, the investigation on the nutritional and environmental factors controlling the chitinase production from this highly potent strain of *Chitinolyticbacter meiyuanensis* SYBC-H1 is required[17]. Response surface methodology (RSM) is a collection of statistical techniques for building models, designing experiments, evaluating the effects of factors and finding optimum levels of parameters for desirable responses[17]. This method has been successfully applied in many areas of biotechnology such as optimization of enzyme production[18,19]; culture conditions[20-22]; biomass production[23,24] and ethanol production[25,26]. It was also useful for optimizating the yield of recombinant products such asnisin[27]; actinorhodin[24]; alkaline protease[22]; lysozyme[28]; hirudin[29] as well as extracellular polysaccharides and biomass[30]. However, there have been few studies on the production of chitinase with effecting of physical parameters using RSM[31,32]. In this study, we reported a novel strain, which is a active producer of chitinase. We first adopted the physical processes including rpm of the shaking incubator, temperature and initial pH of the medium as factors to investigate the possibility for enhancement of chitinase production. Further studies on this topic would be useful not only to better characterize this genus, but also probably to identify enzymes with potential industrial applications.

## EXPERIMENTAL

**Microorganism:** The chitinase-producing bacterium was isolated in our laboratory from the soil samples collected from

Meiyuan Park (33º56'N, 120º18'E), Wuxi city, China. On the basis of physiological, biochemical and 16S rRNA gene sequence (GQ981314) study (data not presented), the isolate was identified as gen. nov., sp. nov. and named *Chitinolyticbacter meiyuanensis* SYBC-H1 which was deposited into the China General Microbiological Culture Collection Cente (CGMCC3438).

**Preparation of seed culture:** The stock culture was maintained on nutrient agar slants kept at 4 ºC. The seed culture was prepared by inoculating single colony in a plate containing the medium composed of 2 g/L glucose, 4 g/L peptone, 0.7 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L $K_2HPO_4$, 0.02 g/L $FeSO_4 \cdot 7H_2O$ and 2 g/L agar. In order to have stable strains, the cells were transferred into a fresh medium for several times. After incubated for 2 days at 30 ºC, a loop full of the colony from the flat plate is transferred to 50 mL seed medium composed of 2 g/L glucose, 4 g/L peptone, 0.7 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L $K_2HPO_4$ and 0.02 g/L $FeSO_4 \cdot 7H_2O$ in 250 mL shake flasks and incubated at 30 ºC for 12 h on a rotary shaker (200 rpm). The cell growth was monitored by absorbance measurement at 600 nm with a UV-visible spectrophotometer. For shake flask culture, the liquid medium was sterilized in the autoclave at 121 ºC for 20 min and pH was adjusted to 7 before autoclaving.

**Culture conditions:** Chitinase production was carried out by shake-flask culture in the optimum medium, inoculated with 4 % (w/w) seed. Samples were inoculated into 50 mL liquid medium in 250 mL shake-flasks and incubated for 72 h. Verification fermentation was carried out in a 4 L jar fermentor (BIOTECH- 4JS, China). The samples broth were centrifuged at 10,000 × g for 10 min at 4 ºC and the supernatant was used for chitinase assay.

**Assay of chitinase:** The chitinase activity was measured by reduction of 3,5-dinitrosalicylic acid, in the presence of the aminosugar N-acetyl-D-glucosamine (NAG) released by enzymatic hydrolysis of colloid chitin according to the method described by Rojas-Avelizapa *et al.*[29]. The absorbance was recorded at 530 nm. Readings were compared with a standard curve prepared with a series of dilutions of N-acetyl-D-glucosamine (0-5.0 mmol/mL) and 3,5-dinitrosalicylic acids. The chitinase activity was assayed in triplicate and the average enzyme activity with standard errors was measured using software of SPSS 11.5. One unit chitinase activity (U) was defined as the amount of enzyme required for producing 1 mmol of N-acetyl-D-glucosamine at 37 ºC/min.

**Physical characterization of cultured strain:** At the start of the experiment, we measured the variance of physical characteristics in the cultivated period, including the pH of medium, temperature and aeration (agitation speed). These physical characteristics could be applied to the experimental design for optimizing physical parameters on the production of chitinase from SYBC-H1. The effect of pH on chitinase production was carried out at pH 3.5, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5. Media pH was adjusted using 1M HCl and 1M NaOH. Temperature for production of chitinase was optimized by incubating the flasks containing the inoculated medium at 24, 27, 30, 33, 36, 39, 35 and 39 ºC.

**Experimental design and optimization:** The rpm of the shaking incubator, temperature and initial pH of the medium were considered in the present optimization process. Response surface methodology was used to study combined interactions among different physical variables on the production of chitinase. The central composite design (CCD) was used to improve Chitinase production from SYBC-H1. Considering optimized levels of variables, much data on suitable ranges were preliminarily determined and chitinase and cell growth of SYBC-H1 were taken as the responses. Design Expert version 7.1.5 statistical software (Stat-Ease, Minneapolis, MN) was used for the experimental design and regression analysis of the experimental data. According to CCD, the total number of treatment combinations was 17 ($= 2^k + 2k + 3$), where k is the number of independent variables[33]. Fourteen experiments were augmented with three replications at the center points to evaluate the pure error (Table-1). The relationship among the variables, *i.e.*, initial pH of the medium, temperature and rpm of the shaking incubator were expressed mathematically in

TABLE-1
EXPERIMENTAL DESIGN AND RESULTS OF THE CENTRAL COMPOSITE DESIGN

| Trial | Variables/levels | | | | | | Chitinase (U/mL) |
|---|---|---|---|---|---|---|---|
| | Shaking speed (rpm) | | Medium pH | | Temperture (ºC) | | |
| | Coded value | Actual value (g/L) | Coded value | Actual value (g/L) | Coded value | Actual value (mM) | Experimental |
| 1 | 0 | 200 | 0 | 7 | 0 | 29 | 4.591 |
| 2 | 1 | 250 | 1 | 8 | -1 | 26 | 3.472 |
| 3 | 1 | 250 | -1 | 6 | -1 | 26 | 4.483 |
| 4 | 1 | 250 | -1 | 6 | 1 | 32 | 3.096 |
| 5 | -1.68 | 116 | 0 | 7 | 0 | 29 | 2.148 |
| 6 | -1 | 150 | 1 | 8 | 1 | 32 | 1.945 |
| 7 | 0 | 200 | 0 | 7 | 0 | 29 | 4.743 |
| 8 | 1 | 250 | 1 | 8 | 1 | 32 | 2.316 |
| 9 | 0 | 200 | 0 | 7 | -1.68 | 24 | 4.748 |
| 10 | 0 | 200 | 0 | 7 | 0 | 39 | 4.38 |
| 11 | -1 | 150 | -1 | 6 | 1 | 32 | 3.675 |
| 12 | -1 | 150 | -1 | 6 | -1 | 24 | 4.119 |
| 13 | 0 | 200 | 0 | 7 | 1.68 | 34 | 2.952 |
| 14 | 1.68 | 284 | 0 | 7 | 0 | 29 | 5.16 |
| 15 | 0 | 200 | 1.68 | 8.68 | 0 | 29 | 1.905 |
| 16 | 0 | 200 | -1.68 | 5.32 | 0 | 29 | 4.654 |
| 17 | -1 | 150 | 1 | 8 | -1 | 26 | 2.557 |

the form of a quadratic model, which gave the response as a function of relevant variables (eqn. 1).

$$y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_i x_i^2 + \Sigma\beta_{ij} x_i x_j, \qquad i = 1, 2, \ldots, k \quad (1)$$

where Y is the response (enzyme production or cell growth), $\beta_0$ the constant coefficient, $X_i$ (i = 1-3) are non-coded variables (rpm of the shaking incubator: A, temperature: B and initial pH of the medium: C), $\beta_{is}$ are the linear, $\beta_i$ is are the quadratic and $\beta_{ijs}$ (i and j = 1-3) are the second-order interaction coefficients.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The quality of the polynomial model equation was judged statistically by the coefficient of determination $R^2$ and its statistical significance was determined by an F test. The significance of the regression coefficients was tested by a t test.

## RESULTS AND DISCUSSION

**Effect of pH and temperature:** In order to investigate the optimal pH for chitinase production, the initial pH of the culture medium was adjusted 3.5-9.5 using 1M HCl and 1M NaOH. The chitinase production for different pHs are shown in Fig. 1. The maximum amount of chitianse production occurred at *ca.* 6.2. The chitinase production at this pH was 1.17 U/mL.

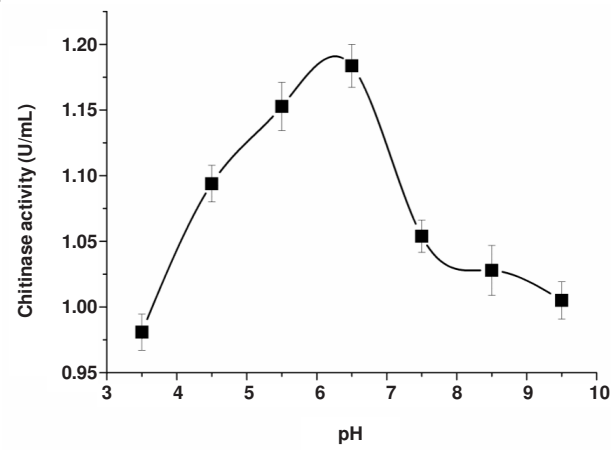


Fig. 1. Effect of pH on chitinase production by SYBC-H1

In order to investigate the effect of temterature on the chitinase production, culturing was carried out at pH 6.2 and 24 ºC to 39 ºC for 72 h, Fig. 2 shows the chitinase production for variout temperatures. According to the results, the optimal temperature was 28 ºC.

Statistical analyses were performed by SPSS software (version 11.5, SPSS). Test of significance were carried out using Tukey's test. Analysis of variance was conducted with one between-subjects variable (pH, temperature) and show that between different pHs or different temperature were significant, this is the reason to chose pH and temperature as the factors for response surface methodology.

**Optimization of physical parameters:** Experiments were performed according to the central composite design and the average chitinase activity was taken as the dependent variable or response (Y) are given in Table-1. The second order regression equation provided the levels of the production as a function of

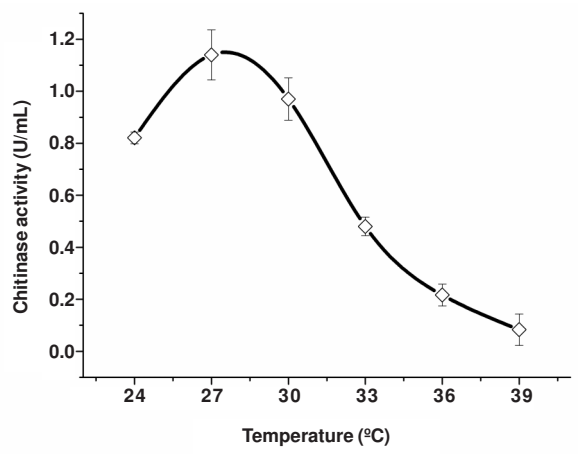


Fig. 2. Effect of temperature on chitinase production by SYBC-H1

rpm of the shaking incubator, temperature and initial pH of the medium, which can be predicted by the following equations:

$$Y = 4.59243 + 0.44934X_1 - 0.71072X_2 - 0.48470X_3 + 0.18763X_1X_2 - 0.18588X_1X_3 + 7.87500E - 003X_2X_3 - 0.39700X_1^2 - 0.52941X_2^2 - 0.32771X_3^2 \quad (2)$$

The analysis of variance (ANOVA) for chitinase production that the model is significant at $p < 0.0178$ (Table-2). The F values corresponding to production of the chitinase are 5.47. This shows that squared regression was significant. The value of $R^2$ (coefficient of determination) is the measure of the total variation of the observed values of responses about the mean explained by the fitted model, which is often expressed in percentage. In other words, $R^2$ describes the goodness of fit of the model. The values of $R^2$ corresponding to production of the chitinase is 87.56 %. This indicates the total variation of 87.56 % for the production of chitinase was explained by the model. The observed chitinase activity (the response) *versus* those factors from the empirical model eqn. 2 was shown in Fig. 3, which indicated that the predicted data of the response from the empirical model was in agreement with the observed ones in the range of the operating variables. The graphical representation provides a method to visualize the relationship between the response and experimental levels of each variable and the type of interactions between test variables to deduce the optimum conditions.

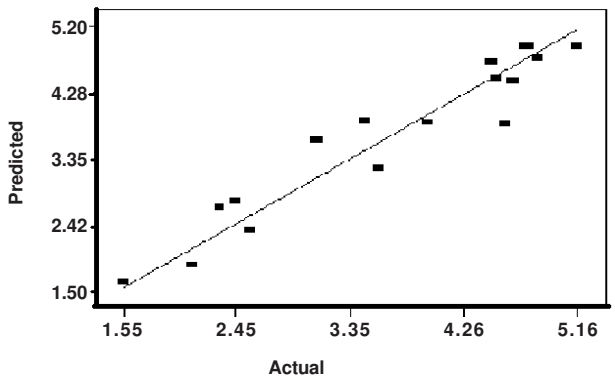


Fig. 3. Plot of predicted *versus* actual chitinase activity (U/mL values)

TABLE-2
ANALYSIS OF VARIANCE (ANOVA) FOR THE QUADRATIC MODEL

| Source | df | Coefficient estimate | Sum squares | Mean square | F ratio | Prob. level |
|---|---|---|---|---|---|---|
| Model | 9 | 4.59 | 17.47 | 1.94 | 5.47 | 0.0178 |
| $X_1$ Shaking speed (rpm) | 1 | 0.45 | 2.76 | 2.76 | 7.77 | 0.0270 |
| $X_2$ Medium pH | 1 | -0.71 | 6.90 | 6.90 | 19.45 | 0.0031 |
| $X_3$ Temperture (ºC) | 1 | -0.48 | 3.21 | 3.21 | 9.04 | 0.0197 |
| $X_1 \times X_2$ | 1 | 0.19 | 0.28 | 0.28 | 0.79 | 0.4025 |
| $X_1 \times X_3$ | 1 | -0.19 | 0.28 | 0.28 | 0.78 | 0.4067 |
| $X_2 \times X_3$ | 1 | 7.875E-003 | 4.961E-004 | 4.961E-004 | 1.399E-003 | 0.9712 |
| $X_1^2$ | 1 | -0.4 | 1.78 | 1.78 | 5.01 | 0.0602 |
| $X_2^2$ | 1 | -0.53 | 3.16 | 3.16 | 8.91 | 0.0204 |
| $X_3^2$ | 1 | -0.33 | 1.21 | 1.21 | 3.41 | 0.1072 |
| Residual | 7 | – | 2.48 | 0.35 | – | – |
| Lack of fit | 5 | – | 2.42 | 0.48 | 14.54 | 0.0656 |
| Total error | 2 | – | 0.066 | 0.033 | – | – |
| Corrected total | 16 | – | 19.96 | – | – | – |

R-Squared = 0.8756, Adj. R-Squared = 0.7156, CV = 16.61 %, Adeq precision ratio = 7.201.

In order to gain a better understanding of the effects of the variables on chitinase production, the predicted model was presented as a 3D response surface curve. Each 3D curve represents combinations of the two test variables, while the other variable maintained at the center level. An elliptical contour indicates that there is a perfect interaction between the independent variables, such as the rpm of the shaking incubator and temperature, as well as the rpm of the shaking incubator and initial pH of the medium. Fig. 3 showed a nearly circular contour, indicating significantly interaction between temperature and initial pH of the medium. The maximum predicted value is identified by the surface confined in the smallest ellipse in the contour diagram.

Fig. 4 represents the interaction of the rpm of the shaking incubator and initial pH of the medium on chitinase activity. The estimated *p*-value (0.4025) for the interaction of these two factors was high, which indicates low interaction. Lower and higher values of these two factors did not result in higher chitinase activity. Even at optimum conditions, but with low the rpm of the shaking incubator, the chitinase activity was also low. With increase in the initial pH of the medium range of 5.32-6.44, the chitinase activity was increased. But at higher initial pH of the medium, the activity of liberated enzyme was dropped, most probably due to catabolite repression. The maximum enzyme activity was obtained with 233 rpm and 6.44 of the rpm of the shaking incubator and initial pH of the medium, respectively. Fig. 5 shows the interaction of the rpm of the shaking incubator and temperature on enzyme production. The low *p*-value (0.4067) indicates approximately interaction between these variables. When the rpm of the shaking incubator was varied from 150-250 rpm, the optimum temperature was shifted from 26 to 32 ºC. At 32 ºC, the effect of the rpm of the shaking incubator on chitinase production was more significant than other the rpm of the shaking incubator. The response surface curve for the interaction of initial pH of the medium and temperature is represented in Fig. 6. Temperature had greater influence on chitinase activity. The *p*-value (0.9712) for the interaction of these two factors shows low interaction. So, temperature had an insignificant effect on the optimum value for initial pH of the medium. The change of temperature from 26 to 32 ºC resulted in a slight shift in optimum initial pH of the medium (6.44-7.0).

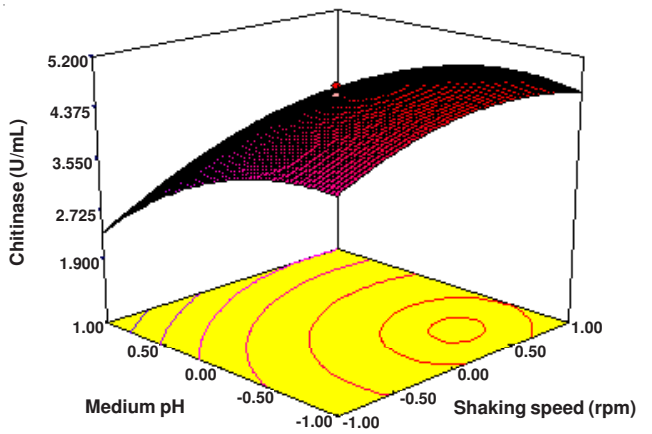


Fig. 4. 3D response surface curve for effects of shaking speed (rpm), medium pH and their mutual interaction on chitinase production by SYBC-H1. Other variables held at their zero level

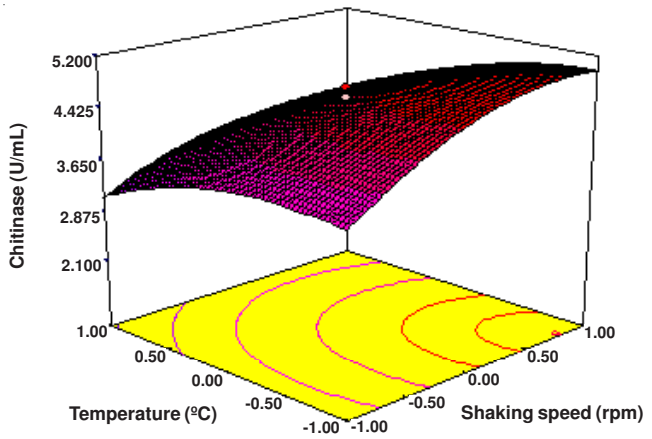


Fig. 5. 3D response surface curve for effects of shaking speed (rpm), temperture (ºC) and their mutual interaction on chitinase production by SYBC-H1. Other variables held at their zero level

In this case $X_1$ (0.0270), $X_2$ (0.0031) and $X_3$ (0.0197) are significant model terms show that the production of chitinase was strongly influenced by the rpm of the shaking incubator (dissolved oxygen), pH of the medium and temperature. Oxygen is essential to aerobic growth and metabolism. As one factor of environment, dissolved oxygen has effect on the metabolism of bacteria directly, been absorbed and utilized

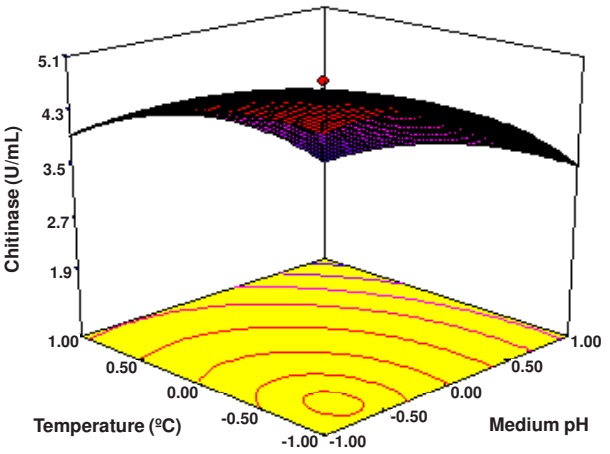


Fig. 6. 3D response surface curve for effects of medium pH, temperture (ºC) and their mutual interaction on chitinase production by SYBC-H1. Other variables held at their zero level

by aerobic bacteria and participate in the process of metabolism, oxygen can be seen a basis of trophism substrate. Different species of bacteria, pathway of metabolism, density of thalli, temperature, elements and densitry of medium, different oxygen utilization ratio[16,34]. Process of fermentation, to the growth of thalli, the form of production and cell metabolism, the participation of oxygen especially to the aerobic bacteria is very important. When the dissoloved oxygen is not enough, the situation is not only harmful to the growth of thalli, but also decrease the speed of production development. So the participation of oxygen has great effect on fermentation.

Microorganism's metabolism is maintained mainly by depending on a series of enzyme catalyses, the pH value 's influence on production of enzyme is mainly the pH change, because the pH can change the environment of the system enzyme and the nutrients' metabolism, causes the inductor and the growth factor changes between activeness and passiveness. Thus influenting the speed of thalli's growth and product formation, the lower pH value is advantageous to thalli's growth, in the experiments scope, when the pH is 6.44, the speed of mycelium's growth is highest.

**Conclusions and model verification:** From the study of plots, the maximum production of chitianse was obtained as: the rpm of the shaking incubator 233 rpm, initial pH of the medium 6.44 and temperature 26.21 ºC. The maximum value of chitianse production predicted from this model was 5.16 U/mL. The triplicate verification experiments were performed under the optimized nutrients levels, the mean value of the chitinase activity was 5.20 ± 0.32 U/mL, which is well agreed with the predicted value (5.16 U/mL). As a result, the models developed were considered to be accurate and reliable for predicting the production of chitinase by *Chitinolyticbacter meiyuanensis* SYBC-H1. To validate predicted production, a verification fermentation in the 4-L jar fermentor was also carried out with the optimum level of the rpm of the shaking incubator 233 rpm, initial pH of the medium 6.44 and temperature 26.21 ºC. The maximum value of chitianse production predicted from this model was 5.20 U/mL by *Chitinolyticbacter meiyuanensis* SYBC-H1. Therefore, the strain has great potential for industry applications.

Fermentation time courses under optimized (by shake-flask culture and by 4-L jar fermentor) and non-optimized conditions were given in Fig. 7. These time courses showed disparity fermentation process trend. Initially, chitinase production was very low and after a lag phase of near 48 h, chitinase production gradually increased. After about 84 h of incubation, chitianse reached the maximum yield. The time course showed that the optimized fermentation conditions change the chitinase producing process. After the enzyme activity reached maximum, the production of chitinase reduced rapidly, this was similar to the previous reports[34]. The reduction of chitinase might be caused by proteinase degradation or inactivation of the chitinase by unclear mechanisms. In many submerged fermentation processes, when the products reached the highest yield, further increase in incubation time could decrease the yield due to the degradation of metabolites[32].

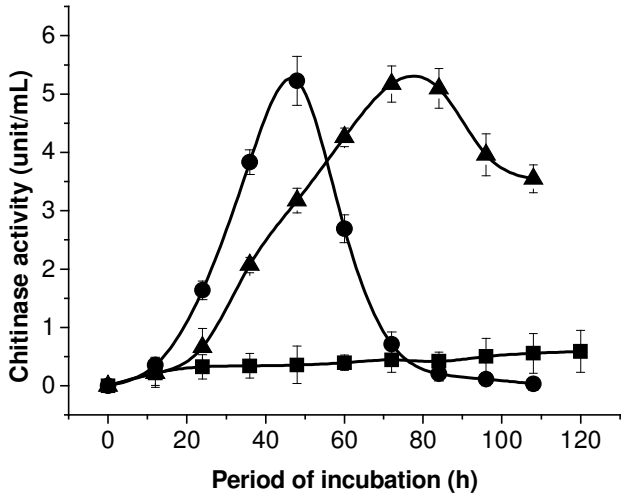


Fig. 7. Time-course of chitinase production by SYBC-H1 before and after the optimization (unoptimized: ■, optimized (by shake-flask culture): ▲ and optimized (by 4-L jar fermentor) ●)

## ACKNOWLEDGEMENTS

This work was financially supported by the National High Technology and Development Program of China (863 Program, Grant No. 2010AA101501).

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