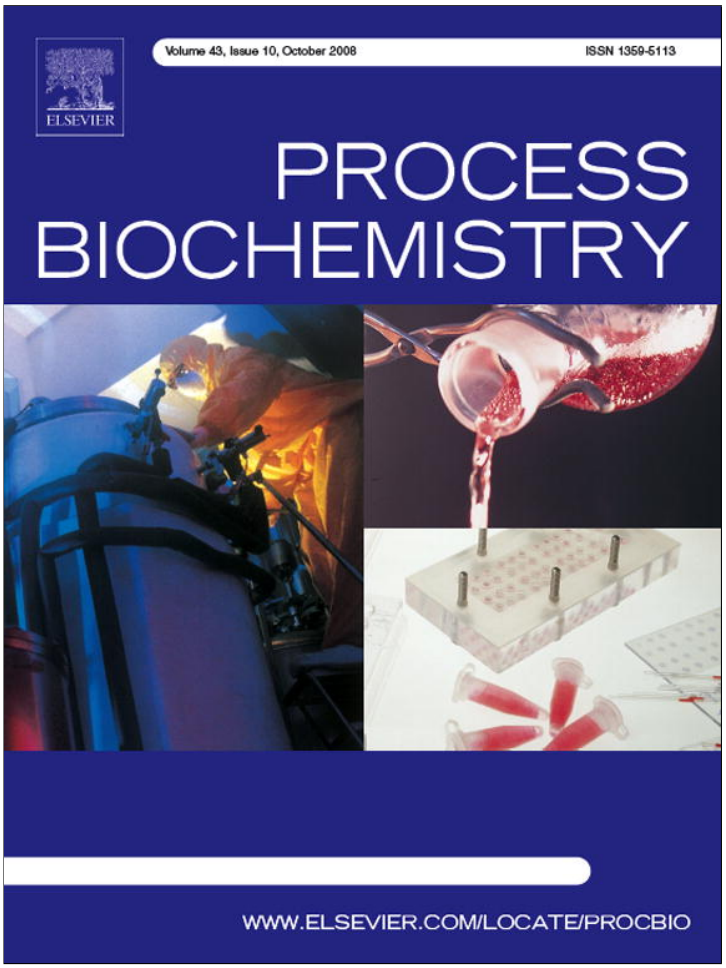

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Statistical optimization of medium components to improve the chitinase activity of *Streptomyces* sp. Da11 associated with the South China Sea sponge *Craniella australiensis*

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ABSTRACT

In this paper, statistical Plackett–Burman design and Box–Behnken Response Surface Methodology were applied to optimize the medium components to improve the chitinase activity of *Streptomyces* sp. DA11 associated with South China Sea sponge *Craniella australiensis*. Firstly, galactose and peptone were found to be the suitable carbon and nitrogen sources for the growth and chitinase activity by single factor Seriatim–Factorial test. Secondly, galactose, colloidal chitin and $MgSO_4{\cdot}7H_2O$ were proved to have remarkable effects on chitinase activity. Finally, an optimal medium was obtained by Box–Behnken methodology, which consisted of 5.00 g/L galactose, 2.62 g/L colloidal chitin, 0.10 g/L $MgSO_4{\cdot}7H_2O$ and 12.5 g/L peptone, 1.5 g/L PO_4^{3-} (KH_2PO_4 0.45 g/L, K_2HPO_4 1.05 g/L), 12.5 g/L powder chitin, 0.03 g/L $FeSO_4$ and 0.03 g/L $ZnSO_4{\cdot}7H_2O$ with artificial sea water (ASW). With this optimal medium, both the chitinase activity and cell growth were remarkably enhanced. The chitinase activity of 1559.2 U/g cell dry weight (36.43 U/mL) and the maximum cell dry weight of 23.3 g/L were reached after incubation of 72 h, which were 39.2-fold and 2.6-fold higher than that of the basic medium, respectively. The results suggest that *Streptomyces* sp. DA11 isolated from marine sponge might be a potential strain for the production of chitinolytic enzymes. To our knowledge, this is the first report of the statistical optimization of chitinase production from marine sponge-associated *Streptomyces*.

1. Introduction

Chitin is a kind of typical marine polysaccharides as well as abundant biomass resources in nature. It has been estimated that the worldwide annual recovery of chitin from the processing of marine invertebrates is 3.7×10^4 metric tons [1]. Chitinases play an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. Chitinases have received increasing attention because of their broad applications in the fields of medicine, agriculture, biotechnology, waste management and industrial applications, including antifungal, hypocholesterolemic, antihypertensive activity and as a food quality enhancer [2].

Microorganisms are good chitin hydrolysers [3,4]. The genus *Streptomyces* is widely distributed in nature and is a source of commercial enzymes and therapeutically useful bioactive molecules. Many novel bioactive compounds with several unique structures have been isolated from *Streptomyces* [5]. Marine microorganisms, with their unique nature differ very much from their terrestrial counterparts in many aspects, are known to produce diverse spectra of novel useful substances. The characteristic of higher pH tolerance, salinity tolerance of marine microbial chitinase may contribute to its special biotechnological potentiality. Marine chitinases from marine phytoplankton [6], marine invertebrates [7], estuarine waters [8] and sediments [9] have been investigated. ZoBell and Rittenberg [10] reported that many marine bacteria are capable of chitin depolymerization by chitinases. Some bioactive compounds have also been isolated from marine sponge-associated *Streptomyces* [11]. But, to our knowledge, study on the chitinase of marine *Streptomyces* remains very superficial.

The conventional method for medium optimization is one-factor-at-a-time approach, which is time consuming, expensive and unmanageable when large number of variables have to be investigated and cannot depict the combined effect of multiple factors involved. Optimizing the parameters by statistical

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experimental design can eliminate these limitations of single factor optimization process. Plackett–Burman is well established and widely used in the statistical designs for the selection of the medium components, which can screen the important variables as well as their significance levels [12]. Response Surface Methodology (RSM) has eased process development and has been of significant use at industrial level, among which Box–Behnken design methodology considers the interaction effects among the variables [13].

To date, study on the optimization of medium components of marine microbes for chitinase production using Plackett–Burman design and Box–Behnken RSM is rarely found except for few papers [13–15]. Marine sponge is known as an important source for marine natural products. Sponge-associated microorganisms could be the true source of some biologically active metabolites and involved in nutrients transfer [16]. Studies on sponge-associated *Streptomyces* are rare because of their low abundance in sponges and the difficulty of cultivation in vitro. To our knowledge, there is no report on sponge-associated microbial chitinase as well as *Streptomyces*.

We have isolated *Streptomyces* sp. DA11 with chitinase activity from South China Sea sponges [16,17]. The purpose of the present work was to attempt to optimize the medium components with statistical optimization strategy to increase the chitinase activity by *Streptomyces* sp. DA11 in order to evaluate the potential of *Streptomyces* sp. DA11 in chitinase production.

2. Materials and methods

2.1. Sponge and strain

Marine sponge *Craniella australiensis* (Porifera, Class Demospongiae, Order Choristida, Family Craniellidae) was collected by scuba diving at a depth of about 20 m in the South China Sea around Sanya Island and was identified by Prof. Jin-He Li in the Institute of Oceanology, Chinese Academy of Sciences [16,17].

DA11, isolated from Marine sponge *Craniella austrialiensis*, was identified as the genus *Streptomyces* by 16S rDNA sequencing (GenBank accession no. DQ180128) and proved to have chitinase activity [16,17].

2.2. Basic medium and culture condition

The seed medium was Gause I medium, containing soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5 g, $MgSO_4$ 0.5 g, $FeSO_4$ 0.01 g, ddH_2O 1000 mL and agar 18 g. The strain was incubated at 28 °C on the rotary shaker (180 rpm) for 3 days.

The basic medium used for medium optimization consisted of 1 g/L $(NH_4)_2SO_4$, 10 g/L powder chitin, 1 g/L PO_4^{3-} (KH_2PO_4 0.3 g/L, K_2HPO_4 0.7 g/L), $MgSO_4{\cdot}7H_2O$ 0.5 g/L, $FeSO_4$ 0.01 g/L and $ZnSO_4{\cdot}7H_2O$ 0.01 g/L [18]. Media were prepared with artificial sea water (ASW) [16] and pH was adjusted to 7.0. *Streptomyces* sp. DA11 was culture in flask (250 mL) with 100 mL of fermentation medium on the rotary shaker (180 rpm) at 28 °C.

2.3. Chitinase assay

Chitinase activity was analyzed by a dinitrosalicylic acid (DNS) method [19]. The absorption of the appropriately diluted test sample was measured at 540 nm using UV spectrophotometer (V-2102PCS, Shanghai, China) along with substrate and enzyme blanks. One unit (U) of the chitinase activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per min.

Cell growth was followed by measuring cell dry weight (CDW). The broth was put into a dried and weighed Ependorf tube, after centrifugation (8000 r/min) for 10 min, the supernatant was discarded. The Ependorf tube with cells was placed into an oven at 80 °C for 1 day and then weighed again. Unit per gram of cell dry weight (U/g CDW) was used to evaluate the microbial chitinase activity. The enzyme activity and cell dry weight was measured every 24 h, respectively. All measurements were performed in triplicates for each sample and results represent mean values.

2.4. Selection of carbon and nitrogen sources

The investigation for selection of carbon and nitrogen sources was carried out by Seriatim–Factorial test. For carbon selection, in the basic medium without powder chitin and $(NH_4)_2SO_4$, 5 g/L peptone was used as sole nitrogen source, 10 g/L lactose, galactose, glucose and powder chitin was used as carbon source, respectively. Similarly, in basic medium with the obtained optimal carbon source as sole carbon source instead of powder chitin, 5 g/L peptone, yeast extract, beef extract and NH_4SO_4, was used as nitrogen, respectively. Statistical software package SPSS 14.0 was used to analyze the bivariate correlation of the different concentration of carbon and nitrogen sources.

2.5. Statistical optimization of medium components

The optimization of medium constituents to improve chitinase activity of *Streptomyces* sp. DA11 was carried out in two stages, the identification of important nutrient components and the concentration of each important component, according to the enzymic activity of the cells after 96 h culture.

Firstly, eight variables including four inorganic components in basic medium, the selected carbon and nitrogen sources, colloidal chitin and powder chitin were investigated. The variables having most significant effect on chitinase activity were identified using a 2-level Plackett–Burman design.

Secondly, Response Surface Methodology was used to optimize the screened components to enhance chitinase activity using Box–Behnken design [12]. The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2$$

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

Statistical software package Design-Expert (Version 7.0, State-Ease, Minneapolis, MN, USA) was used. A 2^3 factorial design, with five replicates at the centre point with total number of 17 trials was employed.

3. Results and discussion

3.1. Selection of carbon and nitrogen sources

The comparison of chitinase activity under 10 g/L different carbon source was carried out by Seriatim–Factorial test. Galactose resulted in the highest chitinase activity of 81.02 U/g after 72 h culture compared with the other three carbon sources. The optimum concentration of galactose was 5 g/L among the tested concentration from 5 g/L to 20 g/L (w/v), and the maximum enzyme activity was 92.42 U/g (data not shown).

Of the four nitrogen sources (5 g/L) tested, peptone resulted in the highest chitinase activity of 101.7 U/g after 72 h culture, meanwhile, beef extract and yeast extract can also enhance chitinase activity. The following experiments were carried out under different concentrations of peptone supplementation ranging from 5 g/L to 20 g/L. The maximum chitinase activity 115.26 U/g was achieved at 10 g/L peptone concentration when galactose was 5 g/L (data not shown).

Carbon sources such as sucrose, glucose, maltose lactose and arabinose were not found to be significant in enhancing the chitinase production [3,20]. In this study, lactose and glucose were found to enhance the chitinase activity compare with powder chitin. Addition of glucose to medium repressed chitinase production in *Streptomyces* [15]. *Aspergillus* sp. S1-13 was found to have the maximum enzyme activity in a medium supplemented with peptone [21]. Similar observation was reported in the case of *Arthrobacter* [22] and *Trichoderma harzianum* [23]. These results are in constant with our result.

3.2. Screening of important medium components

To enhance the activity of chitinase, statistical Plackett–Burman method was used for the following medium optimization. According to the analysis with Statistical software package SPSS 14.0, the Pearson correlation between 5 g/L galactose and 10 g/L galactose was minimum (0.628), and the Pearson correlation between 10 g/L peptone and 15 g/L peptone was minimum (0.897) too. Therefore, 5 g/L galactose and 10 g/L galactose, 10 g/L peptone and 15 g/L peptone were used as the (−) value and (+) value in the following 2-level Plackett–Burman design, respectively. The (−) value and (+) value of the other six components are shown in Table 1. Table 2 represents the Plackett–Burman design for 12

Table 1
Values for the Plackett–Burman experiment

Variables	Medium components	+Values (g/L)	−Values (g/L)
X_1	Galactose	10	5
X_2	Peptone	15	10
X_3[a]	PO_4^{3-}	2	1
X_4	Powder chitin	15	10
X_5	Colloidal chitin	3	1
X_6	$MgSO_4{\cdot}7H_2O$	0.5	0.1
X_7	$FeSO_4$	0.05	0.01
X_8	$ZnSO_4{\cdot}7H_2O$	0.05	0.01

Note: X_3[a] was the mixture of KH_2PO_4 and K_2HPO_4 in proportion of 3:7.

trials with two levels of concentrations for each variable and corresponding chitinase activity. The variable X_1–X_8 represents the medium constituents and X_9–X_{11} represents the dummy variables/unassigned variables.

Table 3 shows the effect, standard error (S.E.), *T*, *P* and confidence level. The variables showing confidence level above 90% in the Plackett–Burman design were selected and further optimized [13,15]. According to Table 3, the confidence level of variables X_2 (peptone), X_3 (PO_4^{3-}), X_4 (powder chitin), X_7 ($FeSO_4$) and X_8 ($ZnSO_4{\cdot}7H_2O$) are below 90% and hence considered to be insignificant. The variables X_1 (galactose), X_5 (colloidal chitin) and X_6 ($MgSO_4{\cdot}7H_2O$) have confidence level above 90% and are considered to be significant. The chitin source has a significant effect on the type of chitinases in *S. lividans* [24]. Colloidal chitin was proved to be a better substrate for chitinase in *Microbispora* [25]. $MgSO_4{\cdot}7H_2O$ was reported to be an important factor for chitinase [26]. These phenomenona were observed in this study. Though peptone is a better nitrogen source for *Streptomyces* sp. DA11, its change in concentration has less effect on chitinase activity. In the study of chitinase production by *T. harzianum* [27], the use of high concentrations of chitin and ammonium sulphate and exclusion of peptone and urea from the medium resulted in higher level of enzyme production. Excess concentrations of phosphate will suppress the growth of some strains such as *Streptomyces viridochromogenes* [28]. Increased phosphate was reported to be inhibitory to chitinase production and morphogenesis in *Streptomyces* [15,29]. The above results indicated that the Plackett–Burman design is power tool for identifying factors, which have significant influence on chitinase activity.

3.3. Optimization of medium components

Three variables X_1 (galactose), X_5 (colloidal chitin) and X_6 ($MgSO_4{\cdot}7H_2O$), showing confidence level above 90% in the Plackett–Burman design were selected and further optimized using Box–Behnken design in RSM to determine the exact optimal values of the individual factors. The coded and uncoded values of the variables at various levels are given in Table 4. In Box–Behnken design, X_2 (peptone), X_3 (PO_4^{3-}), X_4 (powder chitin), X_7 ($FeSO_4$) and X_8 ($ZnSO_4{\cdot}7H_2O$) were set at their middle levels of 12.5 g/L, 1.5 g/L, 12.5 g/L, 0.03 g/L and 0.03 g/L, respectively.

Table 5 shows the Box–Behnken experimental design and the obtained chitinase activity. Data in Table 5 were analyzed by linear multiple regression using Design-Expert (Version 7.0; Stat-Ease, Inc.) and the following equation was obtained: $Y = +1253.34 + 24.24X_1 + 43.10X_2 - 53.89X_3 - 25.15X_1X_2 + 61.08X_1X_3 - 5.40X_2X_3 + 97.72X_1^2 - 54.96X_2^2 + 65.37X_3^2$ where Y is the predicted response and x_1, x_2 and x_3 are the coded values of galactose, colloidal chitin and $MgSO_4{\cdot}7H_2O$, respectively.

Isoresponse contour plots showing the effects of important factors $MgSO_4{\cdot}7H_2O$ and colloidal chitin, galactose and $MgSO_4{\cdot}7H_2O$, galactose and colloidal chitin are shown in Fig. 1a–c, respectively. The statistical optimal values of variables are obtained when moving along the major and minor axis of the contour and the response at the centre point yields maximum chitinase activity. Consequently, based on the contour plots, the optimal value for concentrations of galactose, colloidal chitin and $MgSO_4{\cdot}7H_2O$, were found to be 5.00 g/L, 2.62 g/L and 0.10 g/L, respectively.

Table 3
Effect estimates for chitinase activity from the result of Plackett–Burman design

Factors	Medium components	Effect	S.E.	*T*	*P*	Confidence level (%)
X_1	Galactose	33.13	6.5	2.55	0.084	91.6
X_2	Peptone	−4.07	6.5	−0.31	0.775	22.5
X_3	PO_4^{3-}	−10	6.5	−0.77	0.498	50.2
X_4	Powder chitin	18.2	6.5	1.4	0.456	54.4
X_5	Colloidal chitin	33.33	6.5	2.56	0.083	91.7
X_6	$MgSO_4{\cdot}7H_2O$	42.4	6.5	3.26	0.047	95.3
X_7	$FeSO_4$	−22.1	6.5	−1.7	0.187	81.3
X_8	$ZnSO_4{\cdot}7H_2O$	5.8	6.5	0.45	0.683	31.7

Table 4
Coded and real concentration values of the variables studied in Box–Behnken design

Coded value (level)	Real value of variables		
	Galactose (g/L)	Colloidal chitin (g/L)	$MgSO_4{\cdot}7H_2O$ (g/L)
−1	5	1	0.1
0	7.5	2	0.3
1	10	3	0.5

Table 2
2-Level Plackett–Burman design matrix with chitinase activity

Trial no.	Variables											Chitinase production (U/g cell dry weight)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	D_1	D_2	D_3	
1	1	1	−1	1	1	1	−1	−1	−1	1	−1	246.6
2	−1	1	1	−1	1	1	1	−1	−1	−1	1	146.8
3	1	−1	1	1	−1	1	1	1	−1	−1	−1	196.4
4	−1	1	−1	1	1	−1	1	1	1	−1	−1	162.6
5	−1	−1	1	−1	1	1	−1	1	1	1	−1	197.6
6	−1	−1	−1	1	−1	1	1	−1	1	1	1	142.8
7	1	−1	−1	−1	1	−1	1	1	−1	1	1	152
8	1	1	−1	−1	−1	1	−1	1	1	−1	1	187
9	1	1	1	−1	−1	−1	1	−1	1	1	−1	123
10	−1	1	1	1	−1	−1	−1	1	−1	1	1	111.8
11	1	−1	1	1	1	−1	−1	−1	1	−1	1	184.4
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	128.8

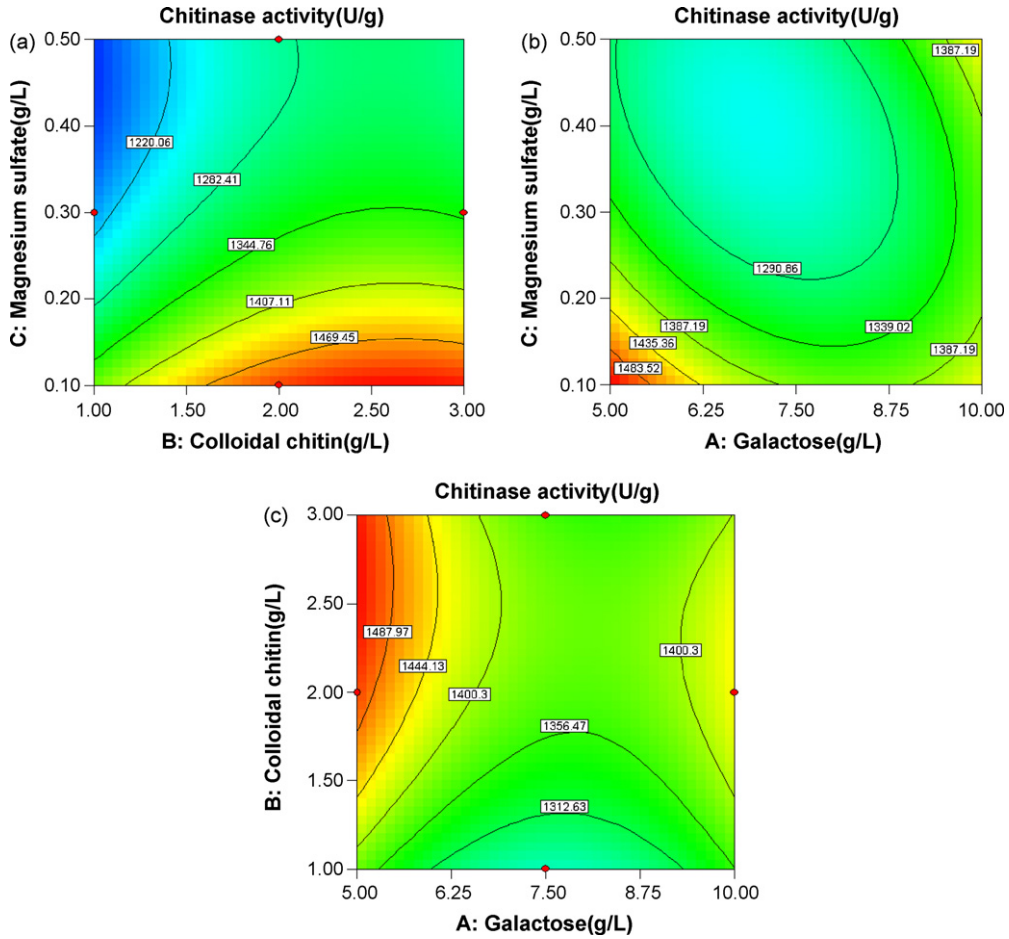

Fig. 1. Contour plots showing the effects of colloidal chitin and $MgSO_4{\cdot}7H_2O$ (a), galactose and $MgSO_4{\cdot}7H_2O$ (b), galactose and colloidal chitin (c) on chitinase activity, respectively.

To validate the regression coefficient, analysis of variance (ANOVA) for chitinase activity was performed as shown in Table 6. The values of Model F and Model $P > F$ were found to be 3.85 and 0.0446, respectively, which implies that the model is significant. Value of lack of fit F and lack of fit $P > F$ were found to be 0.50 and 0.6997, respectively, which implies that the lack of fit is non-significant. Non-significant lack of fit made the model fit. Here the value of correlation coefficient (R) is 0.9121 indicating a good agreement between experimental and predicted values of chitinase production.

3.4. Experimental validation of the optimized medium components

The maximal chitinase activity and the cell growth were compared among the basic medium, Seriatim–Factorial optimized medium with optimized carbon and nitrogen sources, the

Table 5
Box–Behnken design matrix with experimental and the predicted values of chitinase activity

Trial no.	Variables/levels			Chitinase activity (U/g CDW)	
	A: galactose (coded value)	B: colloidal chitin (coded value)	C: $MgSO_4{\cdot}7H_2O$ (coded value)	Experiment	Predicted
1	−1	−1	0	1165	1203.6
2	1	−1	0	1301.5	1302.4
3	−1	1	0	1341	1340.1
4	1	1	0	1376.9	1338.3
5	−1	0	−1	1538.6	1507.2
6	1	0	−1	1427.2	1433.5
7	−1	0	1	1283.5	1277.2
8	1	0	1	1416.4	1477.9
9	0	−1	−1	1276.3	1269.1
10	0	1	−1	1333.8	1366.1
11	0	−1	1	1204.5	1172.2
12	0	1	1	1240.4	1247.6
13	0	0	0	1136.2	1254.5
14	0	0	0	1276.3	1254.5
15	0	0	0	1290.7	1254.5
16	0	0	0	1312.3	1254.5
17	0	0	0	1251.2	1254.5

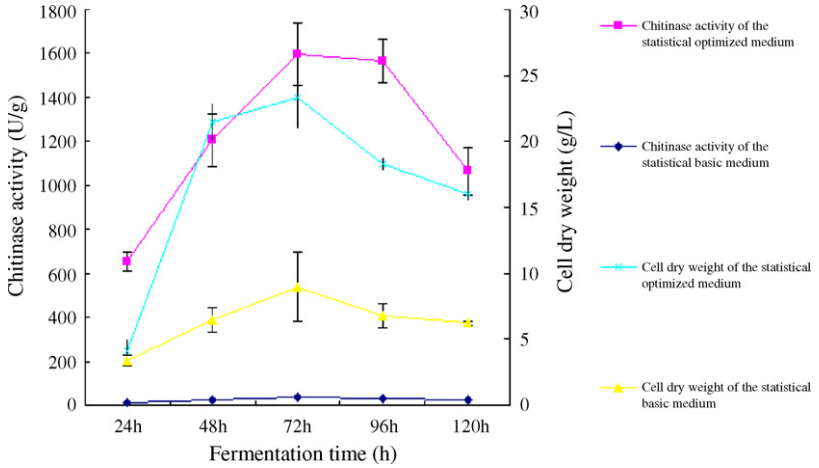

Fig. 2. The comparison of chitinase activity and cell dry weight between the statistical optimized medium and the basic medium.

statistically optimized medium using Plackett–Burman and Box–Behnken Response Surface Methodology in Fig. 2. 1559.2 U/g chitinase activity in the statistically optimized medium was achieved on the 72th h of cultivation, which was 12.5-fold higher than that of the optimized carbon and nitrogen sources medium, 39.2-fold higher than that of the basic medium, respectively. Meanwhile, *Streptomyces* sp. DA11 grows fastest in the statistically optimized medium, the maximum cell dry weight achieved 23.3 g/L on the 72th h, which was 2.6 times higher than that of the basic medium. The experimental result 1538.7 U/g on the 96th h was only 0.45% higher than the predicted 1531.6 U/g according to the equation from Design-Expert analysis, which indicated the proficiency of the model for optimizing chitinase activity.

With statistical optimization method, 141% increase in chitinase production by *A. xylosoxydans* [13], 4.21-fold increase in the marine isolate *Pantoea dispersa* [26], and 4.33-fold increase in chitinase production by *P. dispersa* [14] were achieved. In this study, 39.2-fold increase of chitinase activity was obtained, which indicated that the Plackett–Burman design and Box–Behnken Response Surface Methodology are powerful tools for identifying factors and optimizing medium components for chitinase production by *Streptomyces* sp. DA11. Compared with the chitinase activity of 0.34 U/mL of Nawani and Kapadnis [15], 36.43 U/mL chitinase activity was achieved, suggesting that *Streptomyces* sp. DA11 is a potential strain for chitinase production.

The characterizations and applications of chitinase were investigated [30]. The results showed that the chitinase from *Streptomyces* sp. DA11 belong to ChiC type, has a molecular weight of ca. 34 kDa, with antifungal activity against *Aspergillus niger* and *Candida albicans*. And this chitinase was found to be with thermal stability, alkaline tolerance and salinity tolerance, which may contribute to special application of this marine microbe-derived chitinase compared with terrestrial chitinases.

Table 6
Analysis of variance and regression analysis for the chitinase activity

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	$P > F$
Model	1.305×10^5	9	14497.5	3.85	0.0446
Residual	26359.98	7	3765.71		
Lack of fit	7234.41	3	2411.47	0.50	0.6997
Pure error	19125.57	4	4781.39		
Corrected total	1.568×10^5	16			

4. Conclusions

By Plackett–Burman design and Box–Behnken Response Surface Methodology, an optimized medium was obtained on the basis of basic medium, which was composed of 5.00 g/L galactose, 2.62 g/L colloidal chitin, 0.10 g/L $MgSO_4{\cdot}7H_2O$ and 12.5 g/L peptone, 1.5 g/L potassium hydrogen phosphate (KH_2PO_4 0.45 g/L, K_2HPO_4 1.05 g/L), 12.5 g/L powder chitin, 0.03 g/L $FeSO_4$ and 0.03 g/L $ZnSO_4{\cdot}7H_2O$ with ASW. The chitinase activity, maximum cell dry weights were 39.2-fold, 2.6-fold higher than that of the control basic medium, respectively. The chitinase yield of *Streptomyces* sp. DA11 after 72 h fermentation is 1559.2 U/g (36.43 U/mL). According to this study, Plackett–Burman design and Box–Behnken Response Surface Methodology are effective to optimize medium components, and *Streptomyces* sp. DA11 associated with South China Sea sponge *C. austrialiensis* is a potential strain for chitinase production.

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References

[1] Shaikh SA, Deshpande MV. Chitinolytic enzymes: their contribution to basic and applied research. Microbiol Biotechnol 1993;9:468–75.
[2] Bhattacharya D, Nagpure A, Gupta RK. Bacterial chitinases: properties and potential. Crit Rev Biotechnol 2007;27:21–8.
[3] Gupta R, Saxena RK, Chaturvedi P, Virdi JS. Chitinase production by *Streptomyces viridificans*: its potential in fungal cell wall lysis. Appl Bacteriol 1995;78:378–83.
[4] Uchiyama T, Katouno F, Nikaidou N, Nanaka T, Sugiyama J, Watanabe T. Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from *Serratia marcescens* 2170. J Biol Chem 2001;276:41343–9.
[5] Sanchez LJM, Martinez IM, Perez BJ, Fernandez PJL, Canedo HLM. New cytotoxic indolic metabolites from a marine *Streptomyces*. J Nat Prod 2003;66:863–4.
[6] Smucker RA, Dawson R. Products of photosynthesis by marine phytoplankton: chitin in TCA "protein" precipitates. J Exp Mar Biol Ecol 1986;104:143–52.
[7] Elyakova LA. Distribution of cellulases and chitinases in marine invertebrates. Biochem Mol Biol 1972;43:67–70.
[8] Smucker RA, Kim CK. Chitinase activity in estuarine waters. In: Chróst RJ, editor. Microbial enzymes in aquatic environments. Berlin: Springer-Verlag; 1991. p. 249–69.
[9] Xiao X, Lin J, You ZY, Wang P, Wang FP. Chitinase genes in lake sediments of Ardley Island, Antarctica. Appl Environ Microbiol 2005;71:7904–9.
[10] ZoBell CE, Rittenberg SC. The occurrence and characteristics of chitinoclastic bacteria in the sea. J Bacteriol 1938;35:275–87.

[11] Lee HK, Lee DS, Lim J, Kim JS, Im KS, Jung JH, et al. I inhibitors from the *Streptomyces* sp. strain KM86-9B isolated from a marine sponge. Arch Pharm Res 1998;21:729–33.
[12] Box GEP. Multi-factor designs of first order. Biometrika 1952;39:49–57.
[13] Vaidya R, Vyas P, Chhatpar HS. Statistical optimization of medium components for the production of chitinase by *Alcaligenes xylosoxydans*. Enzyme Microb Technol 2003;33:92–6.
[14] Gohel V, Trivedi S, Vyas PR, Chhatpar HS. Formulation of medium constituents by multiresponse analysis of central composite design to enhance chitinase production in *Pantoea dispersa*. Ind J Exp Biol 2004;42:1123–31.
[15] Nawani NN, Kapadnis BP. Optimization of chitinase production using statistics based experimental designs. Process Biochem 2005;40:651–60.
[16] Li ZY, Liu Y. Marine sponge *Craniella austrialiensis*-associated bacterial diversity revelation based on 16S rDNA library and biologically active Actinomycetes screening, phylogenetic analysis. Lett Appl Microbiol 2006;43:410–6.
[17] Li ZY, Hu Y, Huang YQ, Huang Y, Isolation. phylogenetic analysis of the biological active bacteria associated with three South China Sea sponges. Microbiology 2007;76:494–9.
[18] Reynolds DM. Exocellular chitinase from a *Streptomyces* sp. J Gen Microbiol 1954;11:150–9.
[19] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959;31:426–8.
[20] Ulhoa CJ, Peberdy JF. Regulation of chitinase synthesis in *Trichoderma harzianum*. J Gen Microbiol 1991;137:2163–9.
[21] Rattanakit N, Yang S, Wakayama M, Tachiki T. Saccharification of chitin using solid-state culture of *Aspergillus* sp. S1-13 with shellfish waste as a substrate. JBiosci Bioeng 2003;95:391–6.
[22] Morrissey RF, Dugan EP, Koths JS. Chitinase production by an *Arthrobacter* sp. lysing cells of *Fusarium roseum*. J Soil Biol Biochem 1976;8:23–8.
[23] Tronsmo A, Harman GE. Coproduction of chitinolytic enzymes and biomass for biological control by *Trichoderma harzianum* on media containing chitin. J Biol Control 1992;2:272–7.
[24] Vionis AP, Niemeyer F, Karagouni AD, Schrempf H. Production and processing of a 59-kilodalton exochitinase during growth of *Streptomyces lividans* carrying pCHIO12 in soil microcosms amended with crab or fungal chitin. Appl Environ Microbiol 1996;62:1774–80.
[25] Nawani NN, Kapadnis BP, Das AD, Rao AS, Mahajan SK. Purification and characterization of a thermophilic and acidophilic chitinase from *Microbispora* sp. V2. J Appl Microbiol 2002;93:965–75.
[26] Gohel V, Chaudhary T, Vyas P, Chhatpar HS. Statistical screenings of medium components for the production of chitinase by the marine isolate *Pantoea dispersa*. J Biochem Eng 2006;28:50–6.
[27] Kapat A, Rakshit SK, Panda dT. Optimization of carbon and nitrogen sources in the medium and environmental factors for enhanced production of chitinase by *Trichoderma harzianum*. Bioprocess Biosyst Eng 1996;15:13–20.
[28] Coleman RH, Ensign JC. Regulation of formation of aerial mycelia and spores of *Streptomyces viridochromogenes*. J Bacteriol 1982;149:1102–11.
[29] Smucker RA, Kim CK. Effects of phosphate on *Streptomyces griseus* chitinase production. In: Zikaki's JP, editor. Chitin, chitosan and related enzymes. New York: Academic Press; 1984. p. 397–406.
[30] Han Y, Yang BJ, Zhang FL, Miao XL, Li ZY. Characteristics investigation of antifungal marine chitinase from *Streptomyces* sp. DA11 associated with South China Sea sponge *Craniella australiensis*. Mar Biotechnol; doi:10.1007/s10126-008-9126-5.