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ORIGINAL ARTICLE

Optimization of chitinase production by a new *Streptomyces griseorubens* C9 isolate using response surface methodology

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Abstract The purpose of this article is to use statistical Plackett–Burman and Box–Wilson response surface methodology to optimize the medium components and, thus, improve chitinase production by *Streptomyces griseorubens* C9. This strain was previously isolated and identified from a semi-arid soil of Laghouat region (Algeria). First, syrup of date, colloidal chitin, yeast extract and K_2HPO_4, KH_2PO_4 were proved to have significant effects on chitinase activity using the Plackett–Burman design. Then, an optimal medium was obtained by a Box–Wilson factorial design of response surface methodology in liquid culture. Maximum chitinase production was predicted in medium containing 2% colloidal chitin, 0.47% syrup of date, 0.25 g/l yeast extract and 1.81 g/l K_2HPO_4, KH_2PO_4 using response surface plots of the STATISTICA software v.12.0.

Keywords Chitinase activity · *Streptomyces griseorubens C9* · Plackett–Burman · Response surface methodology · Box–Wilson

Introduction

Chitin is the second most represented polysaccharide in nature after cellulose (Gooday 1990; Whitman et al. 1998; Kaiser and Benner 2008), with at least 10 gigatonnes synthesized and degraded each year in the biosphere. It is a crystalline polysaccharide consisting of long linear chains containing more than 1000 units of N-acetyl-β-D-glucosamine linked by β-1,4 glycoside bonds. It may also be related to other structural elements, such as proteins or glucans (Attwood and Zola 1967; Schaefer et al. 1987; Merzendorfer and Zimoch 2003). It is widely distributed in nature, including eukaryote single-cell (yeast, amoeba, diatoms) and multicellular organisms (filamentous fungi, arthropods, nematodes, snails) (Ehrlich et al. 2007b). It is also present in some marine sponges and algae (Ehrlich et al. 2007a). However, it is not present in plants, vertebrates or prokaryotes (Funkhouser and Aronson 2007). Chitin can be degraded by chemical, physical or enzymatic methods, but the physical and chemical methods are limited by their low efficiency, high cost, and lack of specificity, even if they have been industrialized. On the other hand, enzymatic degradation of chitin is much more eco-friendly but relatively slow in kinetics (Gooday 1990). Therefore, scientists have been interested in improving this enzymatic method, in order to find chitinase-producer organisms and enhance their chitinolytic activity. Chitinases (EC 3.2.1.14) belong to the glycosyl hydrolases family and are present in a wide range of organisms that may not contain chitin but still play an important eco-physiologic role. Many chitinolytic enzymes have been identified in several *Streptomyces* species, including *S. antibioticus*, *S. griseus*, *S. plicatus*, *S. lividans*, *S. aureofaciens* and *S. halstedii* (Narayana and Vijayalakshmi 2009). These enzymes can be used as antibacterial and biocontrol agents in agriculture and medicine. They can also be used in the fields of industry (Usui et al. 1987) and biotechnology (Radford 1991). Studies on medium optimization for chitinase production using statistical methods reduce the time and expense because they are able to detect the real optimum level of factors in less time. In addition, the culture medium components have a major influence on the microbial production of

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extracellular chitinases. For this, Plackett–Burman design (PBD) and response surface methodology (RSM) are the most widely used statistical approaches (Montgomery 2008). In this study, we evaluate the effects of 15 factors on the production of chitinase enzyme by *S. griseorubens* C9. The variables that could affect the production of chitinase were identified statistically by PBD and central composite designs (CCD).

Materials and methods

Microorganism and culture conditions

The chitinase-producing bacterial strain C9 was isolated from a semi-arid soil surrounding the region of Laghouat (Algeria) and was identified as a member of the *Streptomyces* genus by 16S rDNA sequencing (GenBank accession no. LN864570). Colloidal chitin medium (CCM) was used for growth and chitinase production, containing the following constituents (g. l^{-1}): 1 g colloidal chitin, 0.7 g KH_2PO_4, 0.3 g K_2HPO_4, 4 g NaCl, and 0.5 g $MgSO_4 \bullet 7H_2O$, 1 mg $FeSO_4 \bullet 7H_2O$, 0,1 mg $ZnSO_4 \bullet 7H_2O$, 0,1 mg $MnSO_4 \bullet 7H_2O$, and 20 g agar. Colloidal chitin broth (50 ml) in 250-ml capacity Erlenmeyer flasks was inoculated with a 1% (v/v) spore suspension (adjusted to 0.8 OD_{600}) of the strain and incubated at 40 °C in a rotary incubator (150 rpm) for 7 days. After centrifugation (10,000*g*, 4 °C, for 20 min), the supernatant was collected for measurement of chitinase activity.

Chitinase assay

Chitinase activity was determined by a dinitro-salicylic acid (DNS) method (Miller 1959). This worked on the concentration of N-acetylglucosamine (NAG), which is released as a result of enzymatic action (Ulhoa and Peberdy 1991; Fenice et al. 1998). The 2-ml reaction mixture contained 1 ml of 0.1% colloidal chitin in acetate buffer (50 mM, pH 5.0) and 1 ml of crude enzyme extract. The mixture was then incubated in a water bath shaker at 50 °C for 1 h. The reaction was stopped by the addition of 3 ml DNS reagent to 1 ml of the filtrate, followed by heating at 100 °C for 5 min, and the absorption was measured at 540 nm using UV spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of N-acetylglucosamine per ml in 1 min. The colloidal chitin was prepared as described by Hsu and Lockwood (1975).

Screening of critical media components using a Plackett–Burman design

The PBD was used to select significant medium components affecting the production of chitinase. It is a two-factorial design that allowed the screening of n variables in an n+1 experiment (Plackett and Burman 1946). The variables considered for the design are listed in Table 1. A total of 19 variables (15 real and 4 dummy) in 20 different combinations were selected for this study. Table 2 shows the design matrix, including the 19 variables, to assess their effect on chitinase production; it also gives the response evaluated as a chitinase activity. All experiments were carried out in duplicate. Each independent variable was explored at high (+1) and (−1) low level. The PBD is based on the first-order model:

$$Y = \beta_0 + \Sigma \beta_i X_i + \varepsilon \quad (1)$$

Where Y is the experimental response (chitinase activity), β_0 the main effects of the factors, β_i is the regression coefficient, X_i is the level of the independent variable, and ε is a random error. This model does not describe the interaction between factors. It is only used to screen and evaluate the significant factors which have a great influence on the response (chitinase production). After regression variables analysis, these most significant factors were then optimized by response surface method (RSM) using central composite design (CCD) of Box-Wilson.

Optimization of medium with the response surface method

A CCD (Box and Wilson 1951) matrix was developed under the RSM to optimize the levels of the four most significant factors identified by PBD. Each factor in the design was

Table 1 Variables in real values, for screening by Plackett–Burman design

Variables	Levels		Units
	+1	−1	
A : pH	5	9	
B : Colloidal chitin	1	3	%
C : Date syrup	0	2	%
D : Dummy	–	–	–
E : Lactoserum	0	2	%
F : Peptone	10	15	g/l
G : Casein	0.1	0.4	g/l
H : Dummy	–	–	–
I : Tryptone	10	15	g/l
J : Yeast extract	0.1	0.4	g/l
K : Ammonium sulfate	0.1	0.4	g/l
L : Dummy	–	–	–
M : PO_4	1	2	g/l
N : Trace elements	0.5	1.5	ml/l
O : Crayfish shell	0	20	g/l
P : Dummy	–	–	–
Q : Mushroom	0	20	g/l
R : Shrimp shell	0	20	g/l
S : NaCl	5	10	g/l

studied at five different levels, Table 3 gives the actual and the coded levels of the variables tested. The factors were coded according to the following equation:

$$X_R = \frac{X_i - X_0}{d} \quad (2)$$

Where X_R is the coded level, X_i is the real value, X_0 is the real value of central point, and d is the value of step change of variable.

Table 4 gives the design matrix and the response evaluated (the averages of duplicate experiments) in terms of chitinase activity. Chitinase activity can be expressed as a function of independent variables by the polynomial equation of the second order:

$$Y = \beta_0 + \sum\beta_j x_j + \sum\beta_{jj} x_{j2} + \sum\beta_{jk} x_j x_k \quad (3)$$

Where Y is the response here in terms of chitinase activity, β_0 is the intercept, β_j, β_{jj}, β_{jk} are linear, quadratic and interactive coefficients, respectively.

Statistical analysis

The responses obtained were subjected to multiple non-linear regression analysis to obtain the coefficients. Estimates of coefficients with levels higher than 95% ($P < 0.05$) were included in the final models. The statistical significance of the polynomial model equation was carried out by an *F* test and the significance of the regression coefficients was tested by *t* tests. In addition, the coefficient of determination R^2 of the equation and the analysis of variance (ANOVA) were determined. STATISTICA software v.12.0 (Dell) was used for the experimental design and analysis of the experimental data.

Table 3 Coded and real values of variables selected for CCD

Variables	Unit	Levels				
		−2	−1	0	+1	+2
A: colloidal chitin	(%)	1	1.5	2	2.5	3
B: Syrup date	(%)	0	0.5	1	1.5	2
C: Yeast extract	(g/l)	0.05	0.15	0.25	0.35	0.45
D: PO_4	(g/l)	1	1.25	1.5	1.75	2

Results

Chitinase production

Initially, basal medium (CCM) was used for the production of chitinase. From the shake flask fermentation, *S. griseorubens*

Table 2 Plackett–Burman experimental design matrix with the observed response (chitinase activity)

Run	Variables																			Chitinase activity (U/ml)
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	
1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	7.476
2	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	2.239
3	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	7.053
4	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	7.043
5	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	6.544
6	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1.799
7	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1.973
8	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1.116
9	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	7.049
10	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	−1	3.244
11	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	1	1.861
12	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	−1	6.527
13	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	−1	1.934
14	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	−1	7.033
15	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	−1	1.986
16	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	1	1.924
17	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	1	2.042
18	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	−1	2.062
19	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	1	1	−1	−1	1	1	6.639
20	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	2.134

Table 4 Central composite design of variables (in coded levels), with extracellular chitinase activity as response

Run	Level				Chitinase activity (U/ml)	
	A	B	C	D	Observed	Predicted
1	−1	−1	−1	−1	7.421	6.368
2	1	−1	−1	−1	9.226	7.061
3	−1	1	−1	−1	9.358	8.204
4	1	1	−1	−1	9.785	8.758
5	−1	−1	1	−1	6.987	5.908
6	1	−1	1	−1	6.206	5.837
7	−1	1	1	−1	9.752	7.944
8	1	1	1	−1	9.456	7.732
9	−1	−1	−1	1	1.855	1.426
10	1	−1	−1	1	1.786	1.884
11	−1	1	−1	1	6.051	4.710
12	1	1	−1	1	6.104	5.029
13	−1	−1	1	1	1.487	0.804
14	1	−1	1	1	1.497	0.497
15	−1	1	1	1	4.275	4.287
16	1	1	1	1	4.498	3.841
17	−2	0	0	0	1.471	3.306
18	2	0	0	0	1.527	3.553
19	0	−2	0	0	1.763	3.171
20	0	2	0	0	5.897	8.351
21	0	0	−2	0	2.948	5.089
22	0	0	2	0	1.720	3.442
23	0	0	0	−2	7.775	11.033
24	0	0	0	2	1.596	2.200
25	0	0	0	0	1.546	1.557
26	0	0	0	0	1.563	1.557
27	0	0	0	0	1.582	1.557
28	0	0	0	0	1.537	1.557

C9 had maximum chitinase production of 0.058 U/ml after 6 days of fermentation time at 40 °C (Fig. 1).

Evaluation of significant variables using Plackett-Burman design

Fifteen variables supposed to affect chitinase production were evaluated under 20 experiments for the PBD (Table 1). Table 2 shows the responses obtained in terms of chitinase activity, estimated by DNS method. The responses were statistically evaluated and the variables with P value less than 0.02 and confidence levels above 98% were considered to have a significant effect on chitinase production. The regression coefficients and determination coefficients (R^2) for the linear regression model of the chitinase production were represented in Table 5. The model was highly significant ($P < 0.02$) and $R^2 = 0.98429$, meaning that 98.4% of the total variability in the response could be explained by this model. The P values of the important variables in the PBD as given below were the most significant variables affecting chitinase production by *S. griseorubens* C9 (Table 5): syrup of date ($P = 0.000552$), yeast extract ($P = 0.003029$), K_2HPO_4, KH_2PO_4 ($P = 0.016506$) and colloidal chitin ($P = 0.015921$). From the experimental data, these four variables could clearly affect the production of chitinase. Among them, syrup of date and K_2HPO_4, KH_2PO_4 had a positive effect on chitinase production, while the other two variables exerted negative effects. These optimum variables were further evaluated by RSM using the Box–Wilson design. Considering the results displayed in Table 5 and after exclusion of the insignificant model terms (based on their insignificant P values > 0.02) the reduced polynomial Eq. (1) may be written as follows:

$$Y = 3.984 - 1.22B + 3.06C - 1.95J + 1.21M \quad (4)$$

Where B = Colloidal chitin, C = syrup of date, J = yeast extract and M = K_2HPO_4, KH_2PO_4.

Optimization using RSM

Based on the identification of variables by PBD, a CCD experimental plan was carried out for variables that affected significantly chitinase production. Table 3 shows the real and the coded values of the levels of variables selected in the CCD. The predicted and observed values of the response (chitinase activity) generated in CCD are described in Table 4. A multiple regression analysis was applied to the experimental data, and a second order polynomial equation was found to explain the chitinase production by *S. griseorubens* C9. Only significant variables are shown in this equation as below:

$$Y = 2.93 + 1.295B - 2.208D + 0.362B{*}D + 0.822B^2 + 1.035D^2 \quad (5)$$

Where B = syrup of date and D = PO_4 (K_2HPO_4, KH_2PO_4). The experimental results revealed that this polynomial equation could satisfactorily explain the effects of the most significant variables concentration in chitinase production of *S. griseorubens* C9. Analysis of variance (ANOVA) for the reduced model of most significant variables with chitinase production as responses was generated. The Fisher's F test revealed a very low P value ($P < 0.0001$) which indicated that the model was highly significant (Table 6). The robustness of the model was determined by calculating the determination coefficient R^2 (0.7323), which suggested that it is a reliable model and that it is able to explain more than 73.23% of the total variations. Only 26.77% of the total variation of chitinase production was not explained by the model. The relatively high adjusted determination coefficient (R^2Adj = 0.6714) accounts for the significance of the model. Tests for the lack-of-

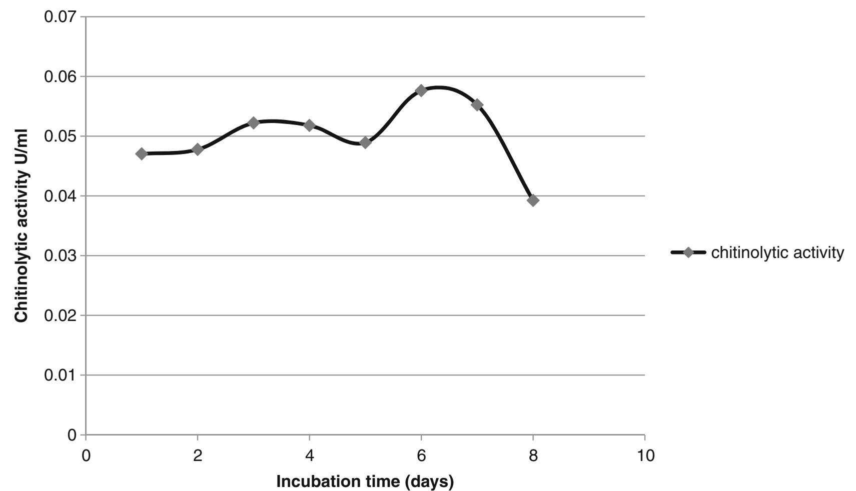

Fig. 1 The time-course of chitinase production by *Streptomyces griseorubens* C9 before optimization

fit of the model showed that the results were significant (Table 7). The 3D response surface plot described by the regression model was drawn to illustrate the effects of the most important independent variables, and their combined effect, upon the response variable (Fig. 2). The response surface showed a curvature along the syrup of date and PO_4. The concave shape of the plot indicated that we can find an optimum value for the response in the range of the studied variables, which could be due to the statistical significance of the quadratic coefficients of these variables. The response is expected to increase the syrup of date concentrations and decrease the K_2HPO_4, KH_2PO_4 concentrations.

Table 5 Effect estimates for chitinase activity from the result of the Plackett–Burman design

Factors	Effect	*t* value	*p* value	Coefficient
Intercept	3.98400	26.15835	0.000013	3.984000
A : pH	−0,96300	−3.16146	0.034136	−0.481500
B : Colloidal chitin	−1.22320	−4.01567	0.015921	−0.611600
C : Date syrup	3.06060	10.04772	0.000552	1.530300
D : Dummy	–	–	–	–
E : Lactoserum	1.08560	3.56394	0.023503	0.542800
F : Peptone	−.087400	−2.86928	0.045502	−0.437000
G : Casein	−0.36480	−1.19761	0.297183	−0.182400
H : Dummy	–	–	–	–
I : Tryptone	−0.67400	−2.21269	0.091352	−0.337000
J : Yeast extract	−1.95500	−6.41812	0.003029	−0.977500
K : Ammonium sulfate	−0.14760	−0.48456	0.653326	−0.073800
L : Dummy	–	–	–	–
M : PO_4	1.21000	3.97234	0.016506	0.605000
N : Trace elements	0.96360	3.16343	0.034072	0.481800
O : Crayfish	0.88980	2.92115	0.043194	0.444900
P: Dummy	–	–	–	–
Q : Mushroom	1.03940	3.41227	0.026971	0.519700
R : Shrimp	−0.08680	−0.28496	0.789822	−0.043400
S : NaCl	0.90320	2.96514	0.041343	0.451600

R^2 = 0.98429, Adj R^2 = 0.92537

Validation of the experimental design

Optimum levels of the tested factors were obtained by applying a regression analysis on Eq. 5 using STATISTICA software v.12.0 (Dell). The coded values of the most important factors were as follow: B = −1.064 and D = 1.252. When translating these coded values, the concentrations of syrup of date and K_2HPO_4, KH_2PO_4 were calculated as 0.47%, and 1.81 g/l, respectively, for the maximum chitinase activity of the 0.902 U/mL, produced by *S. griseorubens* C9 and predicted by the mathematical model.

The study of chitinase production by *S. griseorubens* C9 was performed on the optimized medium in shaken Erlenmeyer flasks (250 ml). The practical response of chitinase production was 1.53 U/ml (Fig. 3), which is in agreement with the model prediction. The yield of the chitinase production was enhanced 26.38 times using RSM optimization, in comparison with the basal medium (0.058 U/ml). This result showed that the experimental values obtained were in accordance with those predicted statistically and confirmed the authenticity of the model.

Table 6 Effect estimates and regression coefficient for chitinase activity from the result of CCD

Model term	Effect	*t* value	*P* value	Coefficient
Intercept	2.93170	5.0909	4.229e-05	2.93170
B	2.59024	3.4841	0.002103	1.29512
D	−4.41633	−5.9403	5.602e-06	−2.20816
B × D	0.72402	0.7952	0.435014	0.36201
B × B	1.64410	2.3311	0.029313	0.82205
D × D	2.07161	2.9372	0.007624	1.03580

Table 7 Analysis of variance (ANOVA) of chitinase activity for the reduced model

Model term	SS	*df*	MS	*F* value	*P* value
B	40.2562	1	40.2562	12.13898	0.002103
B × B	18.0203	1	18.0203	5.43391	0.029313
D	117.0238	1	117.0238	35.28771	0.000006
D × D	28.6104	1	28.6104	8.62727	0.007624
B × D	2.0968	1	2.0968	0.63228	0.435014
Residual	72.9581	22	3.3163		
Lack of fit	63.193	3	21.064	40.9865	1.708e-08
Pure error	9.765	19	0.514		

R^2 = 0.7323; Adj R^2 = 0.6714

df degrees of freedom; *SS* sum of squares; *MS* mean square;

Discussion

This study noted that the composition of the culture medium can significantly affect the production of chitinases. Similar studies were conducted for *Pseudomonas fluorescens* where changes in clear zones on chitin medium with variable compositions were distinguished (Nielsen and Sørensen 1999). Studies performed on *Streptomyces* sp. (Reynolds 1954) gave maximum activity after 6 days of incubation and decreased thereafter, which is consistent with our observations. It is well known that the conventional method for medium optimization like the one-factor-at-a-time approach is time-consuming, expensive and difficult when a large number of variables must be explored and the interactions between multiple factors involved cannot be detected. On the other hand, optimizing the parameters by statistical experimental design can eliminate these limitations. The statistical tool is used in many biotechnological processes, i.e. optimization of culture conditions (Huang et al. 2010), production of biomass (Yu et al. 1997), and ethanol (Ergun and Mutlu 2000); enzymes (Treichel et al. 2010) and also for optimizing the yield of recombinant products such as actinorhodin (Elibol 2004), lysozyme (Gheshlaghi et al. 2005), the alkaline protease (Adinarayana and Ellaiah 2002) and hirudin (Rao et al. 2000). In our study, the optimization of culture media was carried out in two stages: the first step was the selection of variables having a positive effect on the production of chitinase using PDB (Khan 2010), and the second step determined the optimum variables values selected by PBD, using central composite design. However, few studies were conducted for the production of chitinase using PBD and RSM (Singh et al. 2009).

PBD is well established and widely used in the selection of culture medium components. It can also screen the important variables as well as their significance levels (Box 1952). The results of PBD experiments revealed that colloidal chitin, syrup of date, PO_4 (K_2HPO_4, KH_2PO_4) and yeast extract had

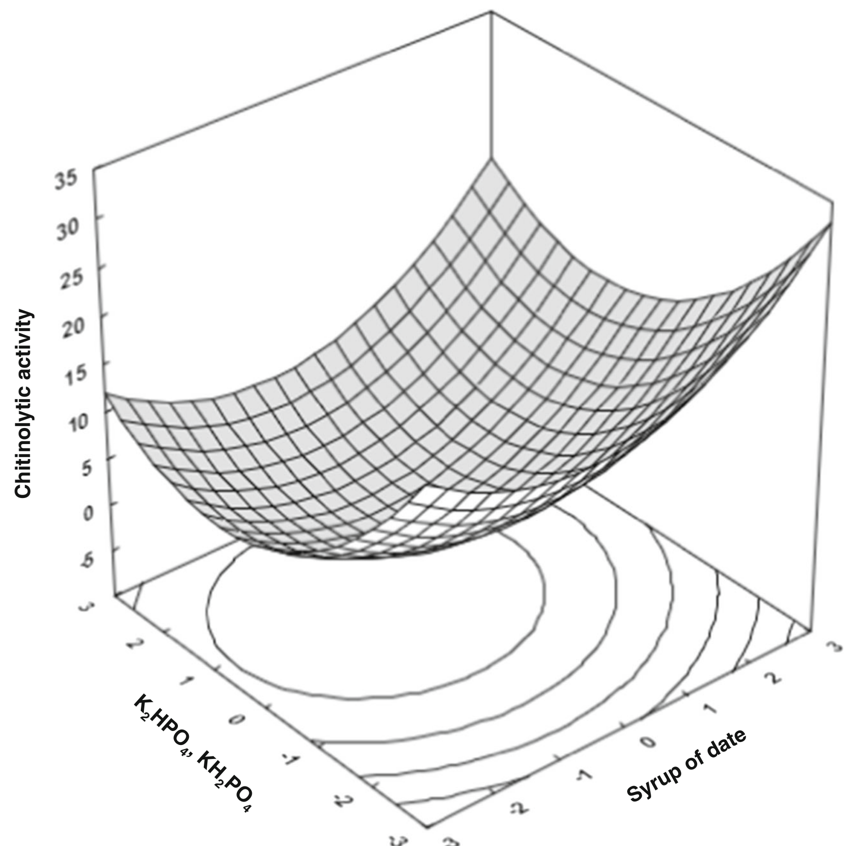

Fig. 2 Surface plot of chitinase activity of *Streptomyces griseorubens* C9 as a function of syrup date and K_2HPO_4, KH_2PO_4

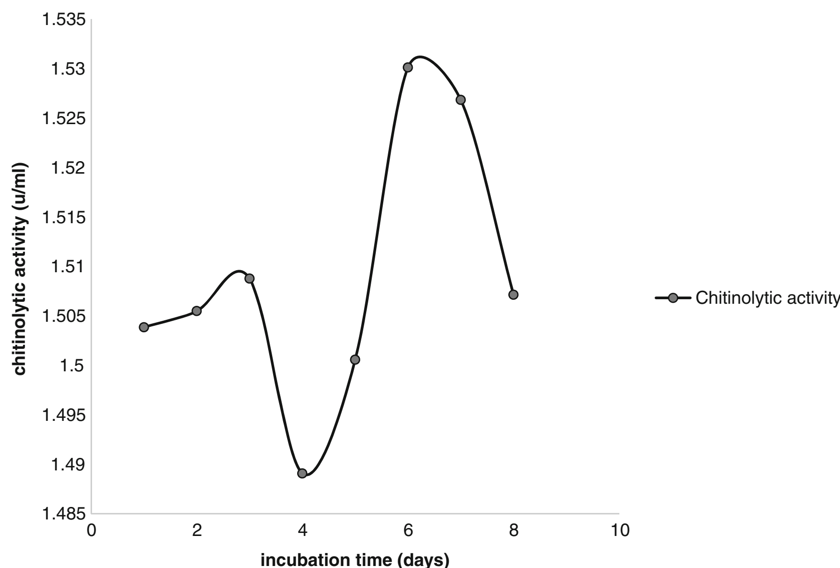

Fig. 3 The time-course of chitinase production by *Streptomyces griseorubens* C9 after optimization

significant effects on the production of chitinase by *S griseorubens* C9. Studies proved that colloidal chitin is the best substrate for chitinase production by *Microbispora* sp. (Nawani and Kapadnis 2005), Andronopoulou and Vorgias (2004) also reported that colloidal chitin was the best chitin source for chitinase production by *Thermococcus chitonophagus* (Andronopoulou and Vorgias 2004), which is in agreement with our observations. However, in the case of *Metarrhizium anisopliae*, good chitinase production was found using chitin flakes rather than colloidal chitin (St Leger et al. 1986). The importance of the nature of chitin in obtaining higher yields of chitinase was documented by Monreal and Reese (1969) in *Seratia marcescens*, and low chitinase production was seen on a mushroom or beetle chitin contrary to colloidal and swollen chitin. Syrup of date was used as another carbon source in this study and showed a significant positive effect on the production of chitinase. Dates were reported to be rich in carbohydrates (predominantly glucose and fructose) along with a range of minerals and vitamins, but low in protein content (1.5–3%, w/w) (Kamel 1979; Nancib et al. 2001). It was previously used to increase production of citric acid by fermentation (Roukas and Kotzekidou 1997), but never in chitinase production.

Nitrogen sources may also affect the production of chitinases. In our study, the addition of ammonium sulfate to the culture medium had no effect on the production of chitinases. However, the addition of yeast extract in the culture medium significantly affected the production of chitinase by *S. griseorubens* C9. In *Streptomyces* sp., Nawani and Kapadnis (2005) reported that decreased yeast extract and ammonium sulfate concentrations may promote chitinase production (Nawani and Kapadnis 2005). Other studies showed that the production of chitinases can be improved by adding yeast extract to *Serratia marcescens* (Monreal and Reese 1969), *Aspergillus carneus* (Sherief et al. 1991), *Alcaligenes xylosoxydans* and *Paenibacillus sabina* Strain JD2 (Vaidya et al. 2001; Patel et al. 2007). The addition of peptone and whey showed no significant effect on the production of chitinase. This is in agreement with the work of Singh et al. (2009), who discovered that the production of chitinase by *Paenibacillus sp.* D1 was reduced in the presence of peptone (Singh et al. 2009). Similar observations have also been described by Han et al. (2009) in *Streptomyces* sp. Da11 (Han et al. 2009), while Gohel and Naseby (2007) reported a significant effect of urea, yeast extract and peptone on the production of chitinase by *Pantoea dispersa* (Gohel and Naseby 2007).

Concentrations of PO_4 (KH_2PO_4, K_2HPO_4) positively regulated the production of chitinase by *S. griseorubens* C9. K_2HPO_4 was identified as the best phosphorus source for chitinase production by *Paenibacillus* sp. D1 (Singh et al. 2009). Nawani and Kapadnis (2005) described that low PO_4 levels were more favourable to the production of chitinase in *Streptomyces* when compared to high PO_4 levels, which were demonstrated in the CCD design in this study. The above results indicated that the PBD is an appropriate tool to examine the effect of culture medium constituents on the production of chitinases. Components with maximum contribution effects were then selected for RSM using Box–Wilson design.

RSM improved the development process and significantly used at an industrial level, among which Box–Wilson design methodology considers the interaction effects between the variables (Vaidya et al. 2003). The role of RSM in optimizing culture media is to define the optimal concentrations of significant variables previously determined by PBD and to find the relationship between more than one variable and a given response (Wang and Liu 2008; He et al. 2009). The RSM was used for the optimization of culture media for *Haematococcus pluvialis* growth (Gong and Chen 1997). It was also used for the production of hirudin from *Saccharomyces cerevisiae* (Rao et al. 2000).

The *P* value is used as a tool to determine the significance of each factor, which in turn is required to understand the structure of interactions between variables. The lower the *P* value, the more significant is the corresponding coefficient. The parameter estimates and their corresponding *P* values (Table 6) suggest that the linear terms of syrup of date had a significant, positive effect on the production of chitinase; however, the PO_4 showed a negative effect on chitinase production. At the same time, the square terms of syrup of date and PO_4 significantly affect the correlation between coefficients and their corresponding values, which were less than 0.05. No significant interactions were distinguished between the components.

The response surface curve was plotted to understand the interactions of the variables and to determine the optimum level of each variable to get maximum response. Figure 2 denotes the effect of the most significant variables on the production of chitinase by *S. griseorubens* C9, while the other variables were maintained at zero level. This 3D plot and its respective contour plot present a visual interpretation of the interaction between factors and eased the location of optimum experimental conditions. In Fig. 2, we observed an increase of chitinase production with increasing of syrup of date concentrations. The addition of PO_4 at low levels increases chitinase production. Nevertheless, high levels of PO_4 concentrations show a small decreasing in the production of chitinase, which is possibly due to the negative effect of increased cytoplasmic osmotic pressure (Pan et al. 2008). The circular shape of the contour plot indicated that no significant interaction exists between those variables (Fig. 2).

By PBD and Box–Wilson RSM, an optimized medium was obtained on the basis of basal medium. The model generated by RSM satisfied all arguments for their use in optimization. Another significant realization of this study is the selection of readily available components and a reduced cost of the medium. According to this study, the proposed model as designed by RSM proved to be significant in statistical terms and showed elevated production of chitinase in the presence of optimized medium (Fig. 3). Thus, *S. griseorubens* C9 is a potent strain for chitinase production.

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