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Optimization of Culture Media for Enhanced Chitinase Production from a Novel Strain of *Stenotrophomonas maltophilia* Using Response Surface Methodology

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Chitinase is one of the most important mycolytic enzymes with industrial significance. This enzyme is produced by a number of organisms including bacteria. In this study, we describe the optimization of media components with increased production of chitinase for the selected bacteria, *Stenotrophomonas maltophilia*, isolated from soil. Different components of the defined media responsible for influencing chitinase secretion by the bacterial isolate were screened using Plackett–Burman experimental design and were further optimized by Box–Behnken factorial design of response surface methodology in liquid culture. Maximum chitinase production was predicted in medium containing 4.94 g/l chitin, 5.56 g/l maltose, 0.62 g/l yeast extract, 1.33 g/l KH_2PO_4, and 0.65 g/l $MgSO_4 \cdot 7H_2O$ using response surface plots and the point prediction tool of the DESIGN EXPERT 7.1.6 (Stat-Ease, USA) software.

Keywords: Chitinase, *Stenotrophomonas maltophilia*, response surface methodology, Plackett–Burman, Box–Behnken, DESIGN EXPERT.

A number of bacteria have been reported to produce chitinases. Chitinase-producing bacteria have been isolated from a number of sources including soil. *Stenotrophomonas maltophilia* strains have been isolated from a variety of natural sources, mainly, rhizospheres, soil, and aquatic habitats [10]. Palleroni and Bradbury [15] introduced the species *Stenotrophomonas maltophilia* of genus *Stenotrophomonas* in order to accommodate *Xanthomonas maltophilia*, which was formerly described as *Pseudomonas maltophilia.* It has been reported that *S. maltophilia* strains are used in biological control of plant diseases owing to their production of a number of antifungal metabolites and enzymes such as chitinase [10, 13]. *Stenotrophomonas maltophilia* is a common microorganism in the rhizosphere of cruciferous plants, and has also been found in association with corn and beets [7]. Chitinase secretion by this bacteria may possibly be behind its action against pathogenic fungi such as *Aspergillus* and *Fusarium*. The action of chitinolytic enzymes against chitin-containing pathogenic fungi makes it a powerful defens agent in the area of biological control, and hence most of the studies in the same regard have been diverted towards the mechanism of action of chitinolytic enzymes [4, 12, 17]. The optimization of different components of defined media is a difficult task because of the involvement of multivariable process parameters. Primarily major factors were screened in this process, followed by optimization of those major factors using different techniques [1, 11]. Introduced by Plackett Burman in 1946, these designs are two-level partial factorials most commonly employed for identifying important factors for further investigation. Designs are available for up to 99 factors in 100 runs, and the results can be analyzed on a spreadsheet. Theoretically, the Plackett–Burman design should be used only when factor effects are additive and there are no interactions; otherwise, the apparent effect of a factor can be enhanced or masked by other factors. In practice, provided the levels of factors are chosen correctly, these designs can produce useful results. Box and Wilson [2] developed a comprehensive methodology employing factorial designs to optimize chemical production processes. The Box–Wilson methodology, now known as response surface methodology (RSM), employs several phases of optimization [1]. It is a three-factor design that explains the relationship between one or more measured dependent responses with a number of input (independent) factors [6]. In this model linear or quadratic effects of experimental variables construct contour plots, and a model equation, fitting the experimental data. This facilitates the determination of the optimum value of

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factors under investigation, and the prediction of response under the optimized condition [5]. The objective of the present study was to optimize the different components of defined media for secretion of chitinase by *Stenotrophomonas maltophilia*, by the culture shaking method. RSM has been utilized to optimize the different components of the defined media, which were screened by Plackett–Burman design.

Materials and Methods

Microorganism

The bacterial culture of *Stenotrophomonas maltophilia* used in this study was isolated from a soil source and identified by sequencing (Accession No. EU492391) of the amplified product of 16S rRNA. The bacterial culture was inoculated in media containing chitin (5 g/l), yeast extract (0.5 g/l), KH_2PO_4 (1.36 g/l), and $MgSO_4 \cdot 7H_2O$ (0.6 g/l) and then incubation at 37°C O/N. The cultures were stored as glycerol stock at −70°C and subcultured every 30 days.

Screening for Chitinase Secretion

Preparation of colloidal chitin. Commercially available chitin powder (TITAN Biotech Pvt. Ltd, India) was used to prepare the colloidal chitin [9]. Commercial chitin (40 g) was weighed and taken in a 2-l beaker; 500 ml of conc. hydrochloric acid was added, followed by continuous stirring at 4°C. After stirring for 1 h, the hydrolyzed chitin in the beaker was washed several times with distilled water to remove the acid completely and hence bring the pH into the range of 6–7. Once this pH was obtained, the colloidal chitin was filtered using Whatman filter paper. The filtered colloidal chitin was then collected and stored in the form of a paste at 4°C. This colloidal chitin was used at 5% of the composition of the medium as the sole carbon source with other minimal salts and agar.

Freshly grown bacterial culture was spot inoculated at five equidistant points on a colloidal chitin agar plate, followed by incubation at 30°C for 3 days. Plates were then observed for zone of hydrolysis around the inoculated area. The selected bacterial isolate was found to produce chitinase, as observed by a hydrolyzed area around the inoculation.

Design of experiment. Best three carbon sources along with chitin were selected on the basis of their role in chitinase secretion enhancement, and three nitrogen sources were also selected on a similar basis. Magnesium sulfate and potassium dihydrogen phosphate were also considered as growth nutrients that form the most important part of defined media.

The design was formulated according to the Box–Behnken tool of RSM using DESIGN EXPERT 7.1.6 (Stat-Ease, USA) for the selected media components. An optimum value of the factors for maximum secretion of chitinase was determined by the point prediction tool of the software.

Chitinase Activity

Biochemical quantification of chitinase. The chitinase activity in the culture supernatant was estimated as described previously using hydrolyzed chitin as the substrate [18]. The reaction mixture for the chitinase assay contained 1 ml of 5% acid swollen chitin, 1 ml of 50 mM acetate buffer, pH 5.0, and 1 ml of enzyme solution, and was incubated at 50°C for 1 h. The reaction was then stopped after boiling it for 15 min. The mixture was centrifuged at 5,000 rpm for 20 min and the concentration of released GlcNac (*N*-acetyl D-glucosamine; the repeating units of chitin) produced was assayed at 530 nm spectrophotometrically, with colloidal chitin as substrate [14], from the aliquots following a DNS sugar estimation test using GlcNac as the standard.

Results

Stenotrophomonas maltophilia exhibited clearance zones around its inoculation areas on colloidal chitin agar plates as observed after proper incubation. After carrying out optimization studies for chitinase secretion by the bacteria, the carbon and nitrogen sources that produced maximum chitinase activity were screened further, along with the other components of the defined media, to assess the best variable and hence their percentage contribution in chitinase secretion. The Plackett–Burman design employs a design that allows testing of the largest number of factor

Table 1. Chitinase activity of the bacterial isolate by experimental run of the Plackett–Burman model.

S.N.	Chitin	Maltose	Galactose	Fructose	L-Asparagine	Sodium nitrate	Yeast extract	KH_2PO_4	$MgSO_4 \cdot 7H_2O$	CA[a]
1	8.00	8.00	2.00	8.00	1.50	1.50	0.00	0.36	0.60	0.152
2	2.00	8.00	8.00	2.00	1.50	1.50	1.50	0.36	0.00	0.121
3	8.00	2.00	8.00	8.00	0.00	1.50	1.50	0.36	0.00	0.144
4	2.00	8.00	2.00	8.00	1.50	0.00	1.50	2.36	0.00	0.112
5	2.00	2.00	8.00	2.00	1.50	1.50	0.00	2.36	0.60	0.166
6	2.00	2.00	2.00	8.00	0.00	1.50	1.50	2.36	0.60	0.076
7	8.00	2.00	2.00	2.00	1.50	0.00	1.50	0.36	0.60	0.092
8	8.00	8.00	2.00	2.00	0.00	1.50	0.00	2.36	0.00	0.166
9	8.00	8.00	8.00	2.00	0.00	0.00	1.50	2.36	0.60	0.048
10	2.00	8.00	8.00	8.00	0.00	0.00	0.00	0.36	0.60	0.156
11	8.00	2.00	8.00	8.00	1.50	0.00	0.00	2.36	0.00	0.168
12	2.00	2.00	2.00	2.00	0.00	0.00	0.00	0.36	0.00	0.239

[a]Chitinase activity (U/ml).

Table 2. Effect of each component on the secretion of chitinase as calculated by Plackett–Burman design.

Media components	Percentage effect
Chitin	2.95
Maltose	4.99
Galactose	0.34
Fructose	0.17
L-Asparagine	0.030
Sodium nitrate	0.085
Yeast extract	0.30
KH_2PO_4	8.33
$MgSO_4 \cdot 7H_2O$	19.96

effects with the least number of observations. Five of the media components (chitin, maltose, yeast extract, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$) screened by Plackett–Burman design (Table 1) were chosen for further study on the basis of their maximum percentage contribution in chitinase secretion (Table 2). Experimental design of 46 runs containing 6 center points as generated by the Box–Behnken tool for response surface design for the five selected components of the defined media followed. The individual and interactive effects of these media components were studied by carrying out the chitinase assay run at different randomly selected levels (Table 3) of all five different components. The response was measured in terms of chitinase activity. The results of the experimental data and simulated values have been depicted in Table 4. The data obtained for chitinase activity in each run were analyzed using the software DESIGN EXPERT 7.1 and fitted into

Table 3. Levels of different media components used in the experiment.

Media components (g/l)	Levels		
	-1	0	+1
Chitin	2	5	8
Maltose	2	5	8
Yeast extract	0	0.5	1
KH_2PO_4	0.36	1.36	2.36
$MgSO_4 \cdot 7H_2O$	0	0.3	0.6

Table 4. Chitinase activity from the experimental design for the Response Surface Quadratic Model (RSM).

Run	Chitin	Maltose	Yeast extract	KH_2PO_4	$MgSO_4 \cdot 7H_2O$	Chitinase activity (U/ml)	
						Actual	Predicted
1	2	2	0.5	1.36	0.65	0.036	0.049
2	8	2	0.5	1.36	0.65	0.034	0.038
3	2	8	0.5	1.36	0.65	0.058	0.060
4	8	8	0.5	1.36	0.65	0.116	0.110
5	5	5	0	0.36	0.65	0.110	0.110
6	5	5	1	0.36	0.65	0.027	0.022
7	5	5	0	2.36	0.65	0.014	0.016
8	5	5	1	2.36	0.65	0.037	0.029
9	5	2	0.5	1.36	0	0.019	0.049
10	5	8	0.5	1.36	0	0.111	0.110
11	5	2	0.5	1.36	1.3	0.005	0.005
12	5	8	0.5	1.36	1.3	0.117	0.110
13	2	5	0	1.36	0.65	0.015	0.026
14	8	5	0	1.36	0.65	0.019	0.030
15	2	5	1	1.36	0.65	0.024	0.041
16	8	5	1	1.36	0.65	0.027	0.024
17	5	5	0.5	0.36	0	0.047	0.044
18	5	5	0.5	2.36	0	0.018	0.016
19	5	5	0.5	0.36	1.3	0.013	0.046
20	5	5	0.5	2.36	1.3	0.011	0.046
21	5	2	0	1.36	0.65	0.113	0.110
22	5	8	0	1.36	0.65	0.011	0.013
23	5	2	1	1.36	0.65	0.027	0.041
24	5	8	1	1.36	0.65	0.066	0.023
25	2	5	0.5	0.36	0.65	0.008	0.019
26	8	5	0.5	0.36	0.65	0.019	0.026
27	2	5	0.5	2.36	0.65	0.03	0.026
28	8	5	0.5	2.36	0.65	0.019	0.026
29	5	5	0	1.36	0	0.008	0.019

Table 4. Continued.

Run	Chitin	Maltose	Yeast extract	KH_2PO_4	$MgSO_4 \cdot 7H_2O$	Chitinase activity (U/ml)	
						Actual	Predicted
30	5	5	1	1.36	0	0.088	0.054
31	5	5	0	1.36	1.3	0.02	0.025
32	5	5	1	1.36	1.3	0.002	0.001
33	2	5	0.5	1.36	0	0.116	0.110
34	8	5	0.5	1.36	0	0.018	0.022
35	2	5	0.5	1.36	1.3	0.038	0.043
36	8	5	0.5	1.36	1.3	0.020	0.039
37	5	2	0.5	0.36	0.65	0.093	0.064
38	5	8	0.5	0.36	0.65	0.016	0.006
39	5	2	0.5	2.36	0.65	0.045	0.034
40	5	8	0.5	2.36	0.65	0.027	0.044
41	5	5	0.5	1.36	0.65	0.014	0.038
42	5	5	0.5	1.36	0.65	0.003	0.017
43	5	5	0.5	1.36	0.65	0.019	0.026
44	5	5	0.5	1.36	0.65	0.029	0.019
45	5	5	0.5	1.36	0.65	0.097	0.065
46	5	5	0.5	1.36	0.65	0.065	0.020

multiple nonlinear regression models proposed by the following equation (in the coded factor) for chitinase activity:

Chitinase (U/ml)=0.11−8.938E−003A+0.011B−0.012C −5.188E−003D+1.250E−003E−0.010AB−3.750E−003AC +3.500E+003AD−3.000E−003AE−1.000E−002BC+1.250E −003BD−0.750E−003BE−9.250E−003CD−4.000E−003CE +0.018DE−0.042A^2−0.037B^2−0.046C^2−0.046D^2−0.036E^2,

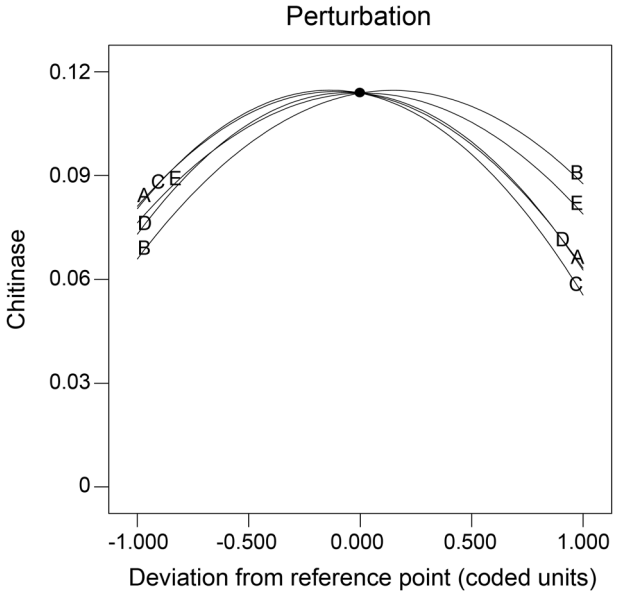

Fig. 1. Perturbation of plots showing the effects of media components on chitinase activity.
A, B, C, D, and E represent chitin, maltose, yeast extract, potassium dihydrogen phosphate, and magnesium sulfate respectively.

where A, B, C, D, and E represent chitin, maltose, yeast extract, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$, respectively, in grams per liter. The effects of all components of the defined media on chitinase activity were compared with the help of a perturbation plot (Fig. 1). The lines in the graph represent the influence and sensitivity of respective factors for chitinase activity. This model generates 12 response surface graphs. A few of the response plots of the calculated model for chitinase activity are shown in Fig. 2A and 2B. The analysis of variance of regression for chitinase activity has been summarized in Table 5, and the *p*-value analysis of each component has been summarized in Table 6. In the case of chitinase activity, this calculated model was able to explain 93.9% of the results. Since it was quite difficult to analyze all the response surfaces/contours simultaneously, point prediction of the DESIGN EXPERT software was used to determine the optimum values of the factors for maximum chitinase activity. Finally, the optimum values of chitin (4.94 g/l), maltose (5.56 g/l), yeast extract (0.62 g/l), KH_2PO_4 (1.33 g/l), and $MgSO_4 \cdot 7H_2O$ (0.65 g/l) were determined. These values predicted 0.116 U/ml of chitinase activity. These optimized values of medium components were validated further in a duplicate culture shake flask study, and an average value of 0.109 U/ml of chitinase activity was obtained. This showed the high validity of the predicted model. The point prediction tool of the DESIGN EXPERT software was used to determine the optimum values of the factors for chitinase secretion. At the obtained optimum values, the predicted value of 0.116 U/ml for chitinase activity was validated by carrying out the experiment comprising the normal components in the defined media and the activity was assessed. An

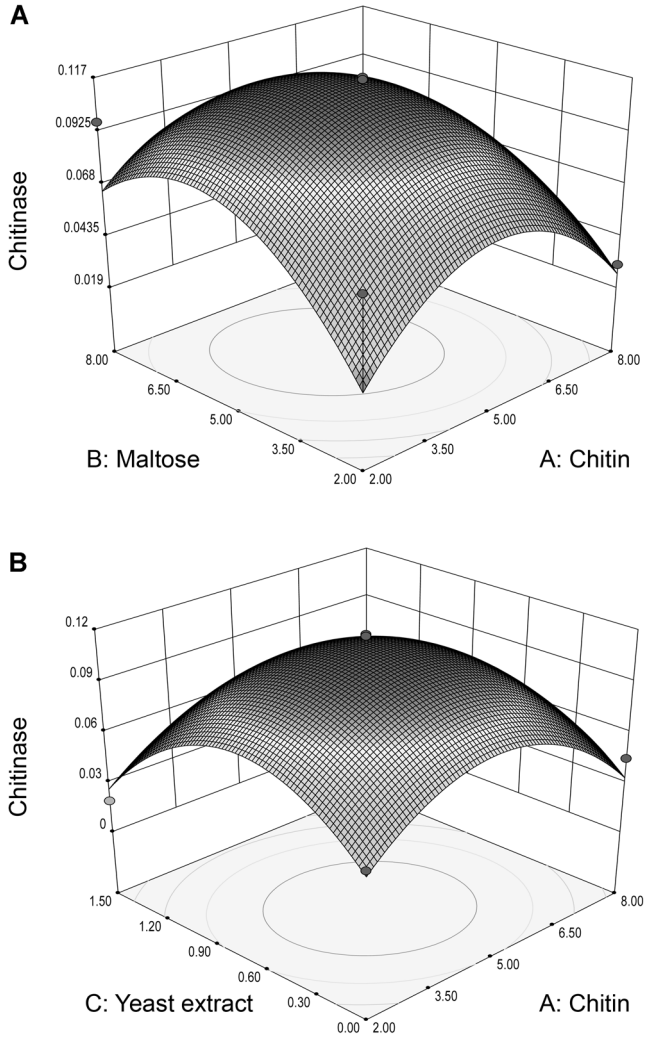

Fig. 2. 3D response plots of some of the models, displaying interaction between different components.

average value of 0.109 U/ml was obtained. This shows a 93.9% validity of the predicted table. The 3D response surface plots described by the regression model were drawn to illustrate the effects of independent variables, and the combined effect of each independent variable, upon the response variable (Fig. 2).

DISCUSSION

Various scientists have published their work on the application of statistical experimental methodology for the optimization of xylanase production by members of genera *Aspergillus* and *Trichoderma* [3]. The Plackett–Burman model generates the maximum contribution effect of different components of defined media from which the components with maximum contribution were selected further for response surface study. RSM generates the model consisting of media components with maximum contribution in chitinase secretion. From the model it became quite clear that $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 exerts positive effects on chitinase secretion in the presence of chitin, whereas the other components such as yeast extract were found to be negatively significant. The proposed model equation illustrates the interaction between two factors. From the equation, it was observed that chitin, maltose, and $MgSO_4 \cdot 7H_2O$ interacted positively with KH_2PO_4, whereas yeast extract showed negative interaction with selected components with respect to chitinase secretion. The relative effect of medium components on chitinase secretion while keeping other components constant has been shown in response surface (three-dimensional) graphs. From Fig. 2, it is evident that an increase in maltose and chitin concentrations caused enhancement in chitinase secretion, followed by a decrease in its secretion. Other components such as $MgSO_4 \cdot 7H_2O$, KH_2PO_4, and yeast extract showed maximum chitinase secretion at lower concentration levels. Adequate precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratio of 7.813 in this experiment indicates an adequate signal. This proves that the proposed model can be used to navigate the design space. The model F-value of 4.54 implies the model is significant. Values of Prob>F less than 0.0500 indicate model terms are significant. In this case, the validity of the model was found to be 93.9%. A

Table 5. Analysis of variance of the calculated model for chitinase secretion.

Regression	
Sum of squares	0.046
df [a]	20
Mean squares	0.002
F ratio	4.54
P	0.0002
Residual	
Sum of squares	0.013
df [a]	20
Mean squares	0.0006
Correlation coefficient (R^2)	0.784058
Coefficient of variation (CV, %)	53.85153

[a]Degrees of freedom.

Table 6. P-Value analysis of different components of the proposed model.

Component	P-Value
Chitin	0.3927
Maltose	0.0768
Yeast extract	0.1395
KH_2PO_4	0.0195
$MgSO_4 \cdot 7H_2O$	0.7726

response surface study for lovastatin production by *Monascus purpureus* showed the validity of the model to be 93% [16]. The proposed model as designed by the Box–Behnken tool of the DESIGN EXPERT software has proved to be significant in statistical terms, and showed elevated secretion of chitinase in the presence of selected components of the defined media. Therefore, the proposed model can be utilized to screen the possible role of selected components and hence their interactive effects in enhancing the chitinase activity.

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