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Statistical Optimization of Chitinase Production by *Pantoea dispersa* to Enhance Degradation of Crustacean Chitin Waste

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Abstract A novel chitinase-producing bacterial strain of *Pantoea dispersa* was isolated from the sea near Bhavnagar, India for efficient disposal of chitinous waste from the seafood processing industry. The medium components were optimized by using a cubic model in the central composite design for increasing chitinase production. The optimal concentrations for higher production of chitinase were (g l^{-1}) chitin, 10.0; urea, 0.35; $MgSO_4$ $7H_2O$, 0.08, and $CaCl_2$, 0.15. Here, peptone (0.05 g l^{-1}) was used as a constant variant in all trials. Using a statistical optimization method, the chitinase production was found to increase from 108 to 486.4 units ml^{-1}. Chitin was prepared from the crustacean waste, and Fourier Transform Infrared (FTIR) Spectroscopy was used to characterize the isolated chitin. Chitinous waste degradation was studied in terms of chitinase production.

Key words: Central composite design, chitinase, crustaceans waste, FTIR, *Pantoea dispersa*

Chitin is a linear homopolymer of β-1, 4-linked N-acetylglucosamine and has a broad-spectrum distribution in the biosphere. It is found to be present as an exoskeletal component of marine invertebrates, insects, and fungi. In 1993, the estimated worldwide annual recovery of chitin from the processing of marine invertebrates was 37,000 tonnes [14], which has increased to 80,000 tonnes in the year 2000 [10]. In India alone, 60,000 to 80,000 tonnes of chitinous wastes are produced annually, from which a lot of chitin can be recovered [16]. Conventionally, these wastes are disposed either by burning or land filling but these methods are harmful to the environment since burning releases carbon dioxide and carbon monoxide in the environment, which adds to global warming, while land filling is harmful as the waste is degraded very slowly and one of the product of degradation is ammonia which seeps through the soil, polluting the ground water [8]. Attempts are being made to find eco-friendly and economic methods to manage this seafood industrial waste to produce useful products like chitin and chitosan for use in sewage treatment, animal feed, food preservation, and formulations of biofungicides. The enzymes responsible for waste chitin degradation and modification are chitinases, which are found in a variety of organisms such as bacteria, actinomycetes, fungi, yeast, protozoans, coelenterates, nematodes, molluscs, arthropods, plants, and human [1]. In our efforts to study the chitinolytic organisms, we have optimized the medium statistically to enhance degradation of the crustacean chitin waste in terms of chitinase production by *P. dispersa*. The optimization of medium constituents for chitinase production is carried out in two steps; 1) screening of medium components, which are responsible in chitinase production and 2) optimization of these components by RSM (Response Surface Methodology). Central composite design is quite efficient as compared to other designs of RSM [4]. In the present study, we have used a cubic model in the central composite design to optimize the screened medium components, as the significance among all variables/ components at three levels of interaction was possible only by the cubic model but not through other models (mean, 2F1, linear and quadratic) in the central composite design. Optimized and unoptimized media were used to compare the degradation efficiency of crustacean chitin waste, sigma chitin, and Matsyafed chitin (Government of Kerala Undertaken, Neendaksya, Kollam-691 582, Kerala, India).

This report is an attempt to formulate a suitable medium using a cubic model in the central composite design that can substantially increase the chitinase production by *P. dispersa* for chitinous waste management.

Crude chitin was prepared from crustacean shell waste (collected from the local marine-food market) according to the method of No and Meyers [9]. Fourier Transform Infrared spectroscopy (FTIR) [Spectrum RX1, Perkin

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Elmer, U.K.] analysis of chitin was carried out as described by Marcin *et al.* [6] and Dani [2].

The culture *Pantoea dispersa* was isolated in our laboratory from sea dumps, near Bhavnagar. It was identified by Rapid ID - 32 E kit (Biomerieux Company, France). *Pantoea dispersa*, a Gram-negative rod, along with *Pantoea agglomerans* is the only members of the genus *Pantoea* which belongs to the family *Enterobacteriaceae* [2]. It was cultivated on chitin agar medium consisting of (g l^{-1}) acid swollen chitin, 5.0; yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.3; KH_2PO_4, 1.36, and agar 30. The pH of the medium was adjusted to 7.2. The medium was sterilized by autoclaving at 121°C for 15 min [7]. *P. dispersa* was grown in 50 ml liquid medium [7] in a 250 ml flask for 144 h at 30±2°C on a rotary shaker (180 rpm). After growth, the culture filtrate was collected by centrifugation at 8,000 rpm for 20 min and was used for the chitinase assay. Chitin was purchased from Sigma, Matsyafed, India, yeast extract from HiMedia, India and remaining chemicals from Qualigens Fine Chemicals, India.

The optimization of medium constituents for chitinase production by *P. dispersa* was carried out in two stages. First stage was screening of medium components by Plackett Burman design [12] at 5% level of significance. Second was to optimize the screened components through response surface methodology (RSM). The significant medium

Table 1. Central composite experimental design matrix of cubic model with experimental and predicted values of chitinase production at 144 h by *Pantoea dispersa* [here, peptone 0.05 g l^{-1} was used as a constant variant in all trials].

Run	A: Chitin (g l^{-1})		B:Urea (g l^{-1})		C: $CaCl_2$ (g l^{-1})		D: $MgSO_4 \cdot 7H_2O$ (g l^{-1})		Chitinase activity (Units ml^{-1})	
	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value	Experimental value	Predicted value
1	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	486.40	465.50
2	-1.0	5	1.0	0.50	-1.0	0.10	1.0	0.10	192.84	219.03
3	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	463.59	465.50
4	1.0	15	-1.0	0.20	1.0	0.20	-1.0	0.05	274.74	300.93
5	1.0	15	-1.0	0.20	-1.0	0.10	1.0	0.10	399.65	417.34
6	0.0	10	2.0	0.65	0.0	0.15	0.2	0.08	246.21	238.23
7	0.0	10	-2.0	0.05	0.0	0.15	0.2	0.08	191.32	193.34
8	-1.0	5	-1.0	0.20	-1.0	0.10	1.0	0.10	156.20	137.98
9	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	443.72	465.50
10	2.0	20	0.0	0.35	0.0	0.15	0.2	0.08	475.20	472.22
11	-1.0	5	1.0	0.50	1.0	0.20	1.0	0.10	327.31	289.09
12	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	473.06	465.50
13	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	465.12	465.50
14	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	445.43	465.50
15	0.0	10	0.0	0.35	-2.0	0.05	0.2	0.08	421.25	433.37
16	-1.0	5	-1.0	0.20	1.0	0.20	-1.0	0.05	349.50	321.28
17	1.0	15	1.0	0.50	1.0	0.20	-1.0	0.05	449.42	421.20
18	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	456.56	465.50
19	0.0	10	0.0	0.35	2.0	0.25	0.2	0.08	435.17	447.49
20	-2.0	0	0.0	0.35	0.0	0.15	0.2	0.08	38.52	40.54
21	-1.0	5	1.0	0.50	1.0	0.20	-1.0	0.05	327.42	353.61
22	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	465.94	465.50
23	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	473.47	465.50
24	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	471.55	465.50
25	-1.0	5	1.0	0.50	-1.0	0.10	-1.0	0.05	314.10	285.88
26	0.0	10	0.0	0.35	0.0	0.15	2.2	0.13	479.38	481.40
27	1.0	15	-1.0	0.20	-1.0	0.10	-1.0	0.05	446.41	418.19
28	1.0	15	1.0	0.50	-1.0	0.10	-1.0	0.05	320.37	346.56
29	0.0	10	0.0	0.35	0.0	0.15	-2.2	0.02	385.66	387.68
30	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	469.15	465.50
31	1.0	15	1.0	0.50	-1.0	0.10	1.0	0.10	380.66	362.44
32	0.0	10	0.0	0.35	0.0	0.15	0.2	0.08	462.23	465.50
33	-1.0	5	-1.0	0.20	-1.0	0.10	-1.0	0.05	281.66	307.85
34	-1.0	5	-1.0	0.20	1.0	0.20	1.0	0.10	314.60	330.79
35	1.0	15	1.0	0.50	1.0	0.20	1.0	0.10	391.98	418.17
36	1.0	15	-1.0	0.20	1.0	0.20	1.0	0.10	486.42	458.20

components such as chitin, urea, $MgSO_4 \cdot 7H_2O$, and $CaCl_2$ were screened by Plackett-Burman design. Here, peptone (0.05 g l^{-1}) was used as constant variant in all the trails. Response surface methodology was used to optimize these screened components to enhance the chitinase production using a cubic model in the central composite design. A 2^4 full factorial design (24 trials), with twelve replicates at the centre point with total number of 36 trials was employed as per Design-Expert (Version 6.0.10, State-Ease, Minneapolis, MN, U.S.A.). Here, peptone (0.05 g l^{-1}) was used as a constant variant in all trials. The variables were fitted in the software, which were selected on the basis of Plackett-Burman results (data not shown). The resulted coded value and actual value of the variables at various levels were obtained (Table 1). Chitin was used from a variety of samples like Sigma (C-7170, India), Matsyafed chitin (Government of Kerala Undertaken, Neendaksya, Kollam-691 582, Kerala, India), and chitin prepared from crustacean waste in both unoptimized [7] and optimized media. The optimized media is a result of the central composite design of this paper.

Chitinase was assayed as described by Vyas and Deshpande [18]. One unit of chitinase activity was defined as the amount of enzyme required to liberate 1 µmole of N-acetyl-D-glucosamine equivalent at 50°C per h.

All the experiments were done in triplicate and the values presented are the means of three independent determinations.

Thirty g of chitin was obtained from 500 g of crustacean waste. This chitin was identified by FTIR analysis. The IR band regions of different groups present in chitin are -OH, CH_3, -C=O and -NH which are detected in region 3,700-3,100, 3,000-2,500; 1,870-1,650 and 1,620-1,500 cm^{-1} [2]. Important chemical groups detected by IR also prove that the compound obtained from crustacean waste is chitin. By FTIR analysis, chitin from Sigma (c-7170, India) and chitin obtained from crustacean waste showed complete overlap of IR spectrum in the region 3,700-3,000 and 1,650-1,500 (Fig. 1).

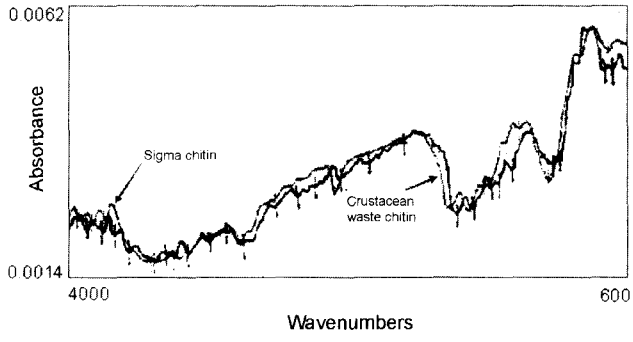

Fig. 1. FTIR spectra of chitin from Sigma (C-7170, India) and crustacean waste chitin.

P. dispersa produced 108 units ml^{-1} chitinase under unoptimized medium components. In the screening method of Plackett-Burman, nineteen different components such as chitin, glucose, peptone, yeast extract, urea, ammonium sulfate, ammonium nitrate, ferric citrate, NaCl, $MgCl_2 \cdot 6H_2O$, sodium sulfate, $CaCl_2$, KCl, Na_2CO_3, KBr, boric acid, $MgSO_4 \cdot 7H_2O$, KNO_3, and KH_2PO_4 were used. These medium components in the Plackett-Burman experiment were screened on the basis of their significance at 5% level in chitinase production. Out of nineteen components, four medium components such as chitin, urea, $MgSO_4 \cdot 7H_2O$ and $CaCl_2$ showed 5% level of significance, which was further used in central composite design. The result of experiment as well as central composite design summary obtained for chitinase production is shown in Table 1 (predicted value was calculated by substituting the factor values into the model equation). The center point in the design was repeated twelve times for estimation of error. Through the sequential analysis of the response surface, it was found that the cubic model was significant at 5% level of significance (p-value<0.05 and adjusted R-squared 91.09%). Data were analyzed using cubic model of central composite in the Design-Expert (Version 6.0.10; Stat-Ease, Inc.) software. ANOVA (Analysis of Variance) for chitinase production was performed. The values of Model F-statistics and Model p-value were found to be 17.25 and <0.0001 respectively, which implies that the model is significant. Here, the correlation coefficient (R^2) value of 0.9669 indicates a good agreement between experimental and predicted values of chitinase production. Thus, the cubic model is fitted on chitinase production for response. The fitted cubic model in terms of coded factor is:

$$\begin{aligned}\text{Chitinase} = {} & +465.50+38.82*A-3.46*B+31.99*C \\ & -18.01*D-52.28*A^2-62.43*B^2-6.27*C^2 \\ & -7.74*D^2-6.00*A*B-18.13*A*C+28.81*A*D \\ & +8.64*B*C-7.16*B*D+20.06*C*D+17.27*A^3 \\ & +3.67*B^3-7.12*C^3+10.36*D^3+17.20*A*B*C \\ & -10.78*A*B*D-2.66*A*C*D-22.13*B*C*D\end{aligned}$$

(* indicates multiplication)

The significant effect of individual variables/components as well as three levels of interactions among them in chitinase production was studied. The effect of components A: (Chitin), C: ($CaCl_2$), A^2: $(\text{Chitin})^2$, B^2: $(\text{Urea})^2$, AC: (Chitin and $CaCl_2$), AD: (Chitin and $MgSO_4 \cdot 7H_2O$), CD: ($CaCl_2$ and $MgSO_4 \cdot 7H_2O$), A^3: $(\text{Chitin})^3$, D^3: $(MgSO_4 \cdot 7H_2O)^3$ and BCD: (Urea, $CaCl_2$, and $MgSO_4 \cdot 7H_2O$) were significant on chitinase production at and below 0.05 % level of p-value. ABC: (Chitin, Urea, and $CaCl_2$) was at 0.06% significance level of p-value. The below 0.05 and at 0.06% level of p-value indicate that these components were essential in chitinase production by *P.dispersa*. Here A, C, A^2, B^2, A^3, and D^3 indicate their individual importance in the medium, whereas AC, AD, BCD, and ABC indicate the interaction

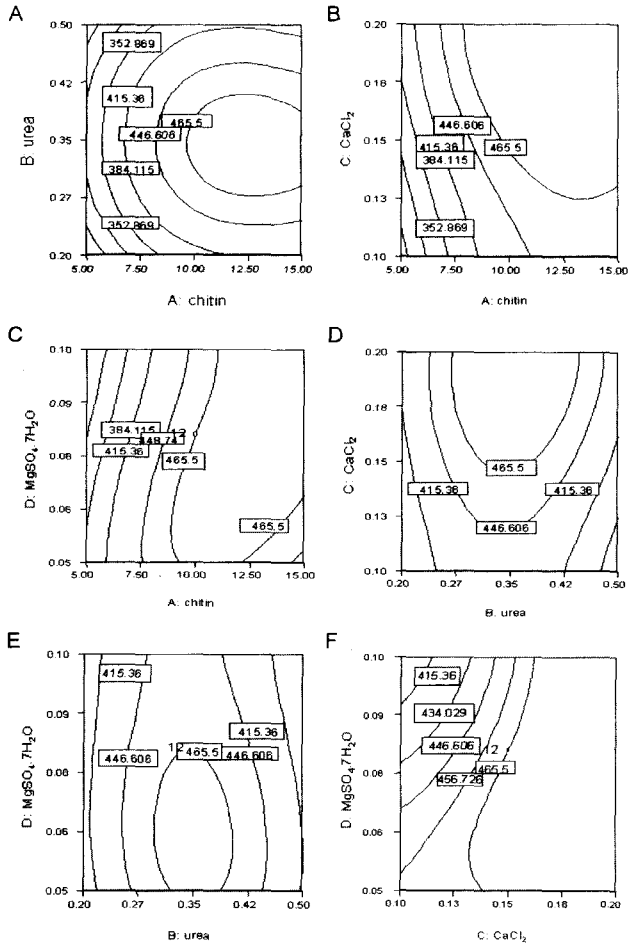

Fig. 2. Contour plots showing the effect of A) chitin and urea on chitinase production at concentration of 0.15 g l^{-1} $CaCl_2$ and 0.08 g l^{-1} $MgSO_4 \cdot 7H_2O$; B) chitin and $CaCl_2$ on chitinase production at concentration of 0.35 g l^{-1} of urea and 0.08 g l^{-1} $MgSO_4 \cdot 7H_2O$; C) chitin and $MgSO_4 \cdot 7H_2O$ on chitinase production at concentration of 0.35 g l^{-1} of urea and 0.15 g l^{-1} $CaCl_2$; D) urea and $CaCl_2$ on chitinase production at concentration of 10 g l^{-1} of chitin and 0.08 g l^{-1} $MgSO_4 \cdot 7H_2O$; E) urea and $MgSO_4 \cdot 7H_2O$ on chitinase production at concentration of 10 g l^{-1} of chitin and 0.15 g l^{-1} $CaCl_2$; F) $CaCl_2$ and $MgSO_4 \cdot 7H_2O$ on chitinase production at concentration of 10 g l^{-1} of chitin and 0.35 g l^{-1} of urea.

among them in the medium for chitinase production by *P. dispersa*.

The contour plots show the effect of chitin and urea, chitin and $CaCl_2$, chitin and $MgSO_4 \cdot 7H_2O$, urea and $CaCl_2$, urea and $MgSO_4 \cdot 7H_2O$, $CaCl_2$ and $MgSO_4 \cdot 7H_2O$, as shown in Fig. 2 (A-F) on chitinase production. The statistical optimal values of variables were obtained by moving along the major and minor axis of the contour and the response at the centre point yielding maximum chitinase production. From the study of the contour plots, the optimal values for concentration of chitin, urea, $MgSO_4 \cdot 7H_2O$, and $CaCl_2$ were found to be 10.0, 0.35, 0.08, and 0.15 g l^{-1}, respectively. Thus, the final concentractions of chitin, urea, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, and peptone were found to be 10.0, 0.35, 0.08, 0.15, and 0.05 g l^{-1}, respectively. At these concentration of variables, the statistically predicted value of chitinase 465.50 units ml^{-1} was obtained. Experimentally, it was found to be 486.42 units ml^{-1} by *P. dispersa* in the statistically optimized medium.

The utilization of chitin obtained from crustacean waste was comparable to commercially available chitin like Sigma chitin, Matsyafed chitin and crustacean waste chitin since the chitinase production was found to be 112±8.78, 99.33± 8.79 and 92.71±5.29 units ml^{-1}, respectively in unoptimized medium whereas 380.1±11.54, 346.98±5.3, 325.79±11.84 units ml^{-1}, respectively in optimized medium. Thus the production of the chitinase was found to be much higher in optimized medium as compared to unoptimized medium by *P. dispersa*. The production of chitinase obtained by *P. dispersa* at 144 h was comparable with other chitinase producers. Kole and Altosaar [5] reported that *Serratia marcescens* produced 60 units ml^{-1} of chitinase. Bhushan [1] reported that alkalophilic *Bacillus* sp. BG-11 produced 76 units ml^{-1} of chitinase. Vaidya *et al.* [17] reported that *Alcaligenes xylosoxydans* produced 44.11 units ml^{-1} of chitinase.

The chitin isolated from crustacean waste showed similar FTIR pattern with sigma chitin. In the IR spectrum, each functional group shows a band in a specific IR region, so this makes it possible to analyze the compound with respect to its specific functional group. Any change in the band pattern signifies a change in the chemical nature of the compound, which might not be visible. The complete overlapping of the spectra as per the Fig. 1 indicates that it is chitin. FTIR technique has been used to determine the purity of chitin obtained from insects and crustacea [4]. Crustacean chitin waste is produced to the tune of more than 80,000 tonnes annually by seafood processing industries, which is a major threat to environmental pollution. It is used for the chitinase production, which is economical and eco-friendly. This will ultimately reduce pollution problem caused by carbon dioxide, carbon monoxide, and ammonia, which are finally produced by either dumping or burning of chitinous waste.

The contour plots represent production of chitinase at fixed concentration of other two medium components. These contours were obtained when the data of chitinase production was fed into the design expert software, and analyzed by it. The software has the function by which we can predict the production of chitinase within the studied range of all four media components. Here, each contour plot represents the effect of two medium components at their studied concentration range and at fixed concentration of the third and fourth medium components. The value of third and fourth medium components was varied for that situation with the software and the optimum value was found out. The significance of fitting of the model is

dependent on lower *p*value and higher adjusted R-squared value, indicating a good agreement between experimental and predicted values of chitinase production as explained by the cubic model.

Using this methodology, 4.5-fold chitinase production was increased by *Pantoea dispersa*. By using the statistical optimization method, 35% increase in riboflavin production was reported in the UV mutant of *Eremothecium ashbyii* [12] and 35% higher recombinant hirudin production in *Saccharomyces cerevisiae* [13]. The degradation efficiency of crustacean waste chitin by *Pantoea dispersa* is higher in the optimized medium as compared to the unoptimized medium. *Alcaligenes denitrificans*, *Bacillus amyloliquefaciens*, *B. megaterium*, and *B. subtilis* have been shown to have potential to degrade shrimp-shell waste [14]. Chitinase produced using crustacean chitin waste may be used to control the plant pathogens. The new marine isolate *Pantoea dispersa* is reported for the first time for crustacean chitin waste management.

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