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Statistical optimisation of medium components for chitinase production by *Paenibacillus sabina* strain JD2

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Abstract - *Paenibacillus sabina* strain JD2, a chitinolytic marine bacterium, was isolated from sea dumps collected at Sultanpur near Bhavnagar, India and its nutritional requirement for chitinase production was defined using statistical optimisation method. Effect of 8 different medium components on chitinase production by *Paenibacillus sabina* strain JD2 was screened by Plackett-Burman design. The screened medium components were further used in central composite design where chitinase production, pH and biomass responses were used in different models to evaluate fit ones. After performing power transformation, quadratic model was found to be fit for chitinase response and 2F1 model was found to be fit for biomass response. Whilst for pH response, quadratic model was found to be fit without any requirement of power transformation. In multiresponse analysis, medium formulation consisting of (g/l): chitin 18, yeast extract 0.50, and $CaCl_2$ 0.08, was found to predict 82.93 U/ml of chitinase with overall highest desirability of 0.842 as compared to other formulations. The selection of model was done on basis of high adjusted R^2 value and lowered *p*-value for each model in individual analysis of each response. Through desirability analysis, it was found that biomass and pH played an important role in increasing the chitinase production by *Paenibacillus sabina* strain JD2. Through statistical optimisation method, 2.74-fold increase in chitinase production was achieved as compared to unoptimised medium.

Key words: biomass, chitinase, medium components and modelling, *Paenibacillus sabina* strain JD2, pH, Plackett–Burman, R-Squared value, response surface design, *p*-value.

INTRODUCTION

Production of chitinase is widespread in variety of organisms such as bacteria, fungi, actinomycetes, yeast, plants, protozoans, coelenterates, nematodes, molluscs, arthropods and humans (Bhushan, 2000; Tan, 2000). Several medium components such as chitin, yeast extract, ammonium sulphate, trace elements, Tween-20, magnesium sulphate, ammonium chloride, potassium nitrate, diammonium hydrogen phosphate, sodium nitrate, ammonium nitrate, sodium chloride, potassium bromide, L-glutamine, L-asparagine, peptone, potassium nitrate, potassium dihydrogen phosphate and urea have been reported to influence chitinase production by bacteria (Monreal and Reese, 1969; Bhushan, 2000; Tan, 2000; Vaidya *et al.,* 2001; Gohel *et al.,* 2004b; Nawani and Kapadnis, 2005).

Productivity of microbial metabolites can be increased by manipulating nutritional requirements, physical parameters and genetic make up of the producing strain (Greasham, 1983; Chauhan, 2006). Development of economical medium requires selection of carbon, nitrogen, phosphorous, potassium and trace element sources. Nutritional requirements can be manipulated by the conventional or statistical methods. There are large numbers of reports on optimisation of carbon and nitrogen source using classical method of medium optimisation by changing one independent variable while fixing all the others at certain level. This can be extremely time consuming and expensive for a large number of variables. Conventional practice of single factor optimisation by maintaining other factors at an unspecified constant level do not depict the combined effect of all the factors involved (Monreal and Reese, 1969). The method requires a large number of experiments to determine optimum levels, which are unreliable. Optimising all the effective parameters can eliminate these limitations of a single factor optimisation process collectively by statistical experimental design (Gohel *et al.,* 2006).

The statistical experimental design method has found broad applications in many disciplines. In fact, we may view experimentation as part of the scientific process and as one of the ways of learning how the systems or processes work. This experimental design is a critically important tool in the engineering world for improving the performance of a manufacturing process (Taguchi, 1987; Gohel *et al.,* 2004c). It also has extensive applications in the devel-

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opment of new media constituents. In a characterisation experiment, we are usually interested in screening media components and then determining, which formulation of media, affect the response. Logical steps are to screen, optimise the important screened components and verify the statistically obtained medium that lead to the best possible response (Gohel *et al.,* 2004c; 2006).

Plackett-Burman design is well established and widely used statistical design technique for the screening of the medium components in shake flask. The Plackett-Burman experimental design is most common saturated design (Plackett and Burman, 1946). A saturated design is one in which the number of design points is equal to one more than the number of factor effects to be estimated. Saturated fractional factorial designs allow unbiased estimation of all main effects with smallest possible variance/components.

Moreover, the design is orthogonal in nature, implying that the effect of each variable worked out pure in nature and not confound with interaction among variables (Simpson *et al.,* 2001). In present Plackett-Burman screening experiment, eight different components including chitin, glucose, yeast extract, peptone, ammonium sulphate, magnesium sulphate, calcium chloride and potassium dihydrogen phosphate were used for the selection for better chitinase production.

Next logical step after screening of medium components is optimisation. There are number of designs available for optimisation of process, from these central composite design is one of the most important experimental designs used in the process optimisation studies (Montgomery, 1997). The Response Surface Methodology is a collection of mathematical and statistical techniques that are useful for the modelling and analysis of problems in which a response of interest is influenced by several variables and objective is to optimise this response (James, 1976). During the response surface analysis, we simultaneously studied model behaviour of the response variables chitinase and biomass in *Paenibacillus sabina* strain JD2 as a function of input variables such as chitin, yeast extract and $CaCl_2$ within some region of interest. The model adequacy checking was also carried out, which is necessary to examine the fitted model to ensure that it provides an adequate approximation to the true system and to verify that none of the response surface model assumptions are violated. Optimisation of chitinase response was generally done either by graphical (counter plot) analysis or desirability function approach. The desirability function approach is an analytical technique based on the concept of utility or desirability associated with a given response function (Myers and Montgomery, 1995; Hill and Hunte,r 1996). The graphical analysis was not found to be suitable in comparison with desirability approach as only two components have to be considered (x, y) in response to their effect on chitinase (z) in counter plot to predict the medium components' concentration. Furthermore, only two components concentrations could vary in single time whilst other two components concentration have to be fixed at particular concentration, resulting in increased number of graphs with different combinations of components in response to chitinase production for media formulation. Due to increasing number the graphs in response to chitinase production, the error proficiency on designing the exact concentrations of these components together for enhancing the chitinase productions was increased (Gohel *et al.,* 2005; Gohel, 2006).

The present report was an attempt to screen and formulate a suitable production medium using statistical optimisation method that can substantially increase the chitinase production by *Paenibacillus sabina* strain JD2.

MATERIALS AND METHODS

The bacterial strain and culture conditions for growth. A chitinolytic marine bacterium, identified as *Paenibacillus sabina* strain JD2 by 16S rDNA method, was isolated in our laboratory from sea dumps collected at Sultanpur near Bhavnagar, India. The culture was cultivated on chitin agar medium consisting of (g/l): acid swollen chitin 5.0, yeast extract 0.5, $(NH_4)_2SO_4$ 1.0, $MgSO_4 \cdot 7H_2O$ 0.3, and KH_2PO_4 1.36. The pH of the medium was adjusted to 7.2 and sterilised by autoclaving at 121 ºC for 15 min (Monreal and Reese, 1969). The method for preparation of acid swollen chitin was same as reported earlier (Hackman, 1962).

Medium used for chitinase production. Initially, Monreal and Reese's medium was used as an unoptimised medium for chitinase production by *Paenibacillus sabina* strain JD2.

Assay of chitinase activity. Chitinase was assayed by the slightly modified method of Vyas and Deshpande (1989). The assay system consisted of 10 mg of acid-swollen chitin, 50 µmol of phosphate buffer (pH 6.0), and 300 µl of enzyme in a total volume of 3.0 ml. After incubation at 40 °C for 5 min, the *N*-acetyl-D-glucosamine products were estimated by the Nelson method (1994). One unit of chitinolytic activity was defined as the amount of enzyme required to liberate 1 µmol of *N*-acetyl-D-glucosamine equivalent per h at 40 °C.

Determination of biomass. Biomass was estimated as total protein content by the method of Waterborg and Matthews (1996). Cell culture (1.0 ml) was palleted out, and hydrolysed with 1.0 ml 1 N NaOH in a boiling water bath for 20 min. The suitable aliquots of cell free supernatant were taken in a total system of 1.0 ml and protein was assayed by the method of Lowry *et al.* (1951)

Optimisation procedure.

Screening of important nutrient components. Total of eight components (variables, $k = 8$) were selected for the study with each variable being represented at two levels, high (+) and low (−) and three dummy variables in 12 trials as shown in Tables 1 and 2. The role of dummy variables in Plackett-Burman design helps to find out the experimental error. The number of positive and negative signs per trial are $(k + 1)/2$ and $(k - 1)/2$, respectively. Each column should contain equal number of positive and negative signs. Each row represents a trial and each column represents an independent/assigned or dummy/unassigned variable (Plackett and Burman, 1946). The effect of each variable was determined by following equation:

$$E_{(Xi)} = 2(\Sigma Mi_{+} - Mi_{-})/N$$

where $E_{(Xi)}$ is the concentration effect of the tested variable, Mi_{+} and Mi_{-} are the chitinase activities from the trials where the variable (X_i) measured was present at high and low concentrations, respectively, and N is the number of trials i.e. 12. Experimental error was estimated by calculating the variance among the dummy variables as follows:

$$V_{eff} = \Sigma(E_d)^2/n$$

where V_{eff} is the variance of the concentration effect, E_d the concentration effect for the dummy variable and n is the number of dummy variables. The standard error (S.E.) of the concentration effect is the square root of the variance of an effect and the significance level (p value) of each concentration effect is determined using student's t test:

$$t_{(Xi)} = E_{(Xi)}/\text{S.E.}$$

where $E_{(Xi)}$ is the effect of variable X_i.

TABLE 1 - Variables showing medium components used in Plackett-Burman design

Variables	Medium components	+ Values (g/l)	– Values (g/l)
X_1	Chitin	5.0	0.50
X_2	Glucose	0.5	0.05
X_3	Yeast extract	0.5	0.05
X_4	Peptone	0.5	0.05
X_5	$(NH_4)_2SO_4$	0.3	0.03
X_6	$MgSO_47H_2O$	0.1	0.01
X_7	$CaCl_2$	0.2	0.02
X_8	KH_2PO_4	1.36	0.14

Optimisation of screened components. The multiresponse analysis of response surface design using desirability approach was used to optimise the screened components for chitinase production with respect to biomass and pH. Multiresponse analysis involves first building an appropriate response surface model for each response and then trying to find a set of operating conditions that in some sense optimised all responses or at least keeps them in desired ranges. The input concentration (-1, 1) of chitin (5, 15), yeast extract (0.1, 0.5) and $CaCl_2$ (0.04, 0.15) was selected initially in central composite design. The resulted coded and actual value of the variables at various level where obtained for the optimisation experiment (Table 3). The combination of the two designs leads to five levels of each factor, with ($-\alpha$, -1, 0, +1, $+\alpha$) coordinates expressed as coded values. The coded value for chitin, yeast extract and $CaCl_2$ with (-2.364, -1, 1, 3, 4.364), (-1, -0.778, 0.022, 1, 1.667) and (-1.178, -1.044, -0.867, -.0.556, -0.333), respectively was obtained. Whilst the actual value of chitin, yeast extract and $CaCl_2$ with (1.59, 5, 10, 15, 18.41), (0.05, 0.1, 0.28, 0.5, 0.65) and (0.01, 0.04, 0.08, 0.15, 0.20), respectively was obtained (Table 3). In central composite design (Table 3), there was no equidistance in the centre point of yeast extract and $CaCl_2$ indicate the curvature of response surface in the region of the design.

Furthermore, this design is central composite design with six replicates at centre point with total number of 20 trials obtained as follows:

$$N=2^n+ (nx2) + \text{centre point}$$

where N is total number of run and n is the number of input variables.

To explain the behavior of the whole process, first, each individual response, i.e. chitinase production and biomass was modelled. Different models are available in central

TABLE 2 - Plackett-Burman experimental design matrix with experimental values of chitinase production by *Paenibacillus sabina* strain JD2

Trail	Assigned variables								Dummy variables			Chitinase production (U/ml)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	D_1	D_2	D_3	
1	+	+	–	+	+	+	–	–	–	+	–	55.86
2	–	+	+	–	+	+	+	–	–	–	+	1.54
3	+	–	+	+	–	+	+	+	–	–	–	27.20
4	–	+	–	+	+	–	+	+	+	–	–	13.91
5	–	–	+	–	+	+	–	+	+	+	–	8.40
6	–	–	–	+	–	+	+	–	+	+	+	5.87
7	+	–	–	–	+	–	+	+	–	+	+	19.43
8	+	+	–	–	–	+	–	+	+	–	+	38.49
9	+	+	+	–	–	–	+	–	+	+	–	13.10
10	–	+	+	+	–	–	–	+	–	+	+	3.43
11	+	–	+	+	+	–	–	–	+	–	+	37.53
12	–	–	–	–	–	–	–	–	–	–	–	15.27

Here, the X_1, X_2, . . ., X_8 are different variables that are shown in Table 1. D_1, D_2, and D_3 are dummy variables; the sign '+' is for the high concentration of variable and '–' is for the low concentration of variable.

composite design of statistical software package Design Expert (State-Ease Inc., Minneapolis, USA). They are (1) linear (2) 2F1 and (3) quadratic, which were used in the present study. The model we selected for chitinase production Y_1 as a function of chitin (X_1), yeast extract (X_2) and $CaCl_2$ (X_3) is a second order (quadratic) polynomial model. It is of the form:

$$Sqrt(Y_1) = Sqrt(\beta_0 + \sum\beta_i X_i + \sum\beta_{ii} X_i^2 + \sum\beta_{ij} X_i X_j\ \varepsilon);\quad i = 1,2,3,4$$

where x_i represents the coded values of X_i, the squared terms (X_i^2) represent the curvature in the response surface and the multiplicative terms (X_iX_j) represents the interaction term. Also we assumed that $\varepsilon \sim N(0, \sigma^2)$ and X_i's are independent.

The model we selected for response biomass (Y_2) and response pH (Y_3) as a function of chitin (X_1), yeast extract (X_2) and $CaCl_2$ (X_3) is a linear and quadratic model, respectively. They are of the form:

$$Ln\ Y_2 = Ln(\beta_0 + \sum\beta_i X_i + \varepsilon);\quad i = 1,2,3,4$$
$$Y_3 = \beta_0 + \sum\beta_i X_i + \sum\beta_{ii} X_i^2 + \sum\beta_{ij} X_i X_j + \varepsilon;\quad i = 1,2,3,4$$

where, the terms in models have usual meaning as described earlier with same assumptions. After building the appropriate models, model adequacy checking was performed, which checks whether all the assumptions of fitted models are satisfied.

Model diagnostics. The diagnostics includes a) Normal probability plot of residuals, b) Plot of studentized residuals versus predicted values, and c) Box- Cox plot for power transformation.

a) Normal probability plot indicates whether the residuals follow normal distribution and in that case the points will follow a straight line;

b) the plot of studentized residuals versus predicated values indicates whether the assumption of constant variance for residuals is satisfied and in that case points will show random pattern;

c) the Box-Cox plot is a tool, which helps in determining the most appropriate power transformation to apply to response data. Most data transformation can be described by the power function:

$$\sigma = fn\ (\mu\alpha)$$

where σ (sigma) is the standard deviation and m (mu) is the mean and the a (alpha) is the power.

If the standard deviation associated with an observation is proportional to the mean raised to the a power, then the transforming the observation by 1-α (or λ) power gives scale satisfying the equal variance requirements of the statistical model. Commonly used transformation are λ = -1 inverse, λ = 0 natural log, λ = 0.5 square root, λ = 1 no transformation. The lowest point in the Box-Cox represents the λ value, which results in the minimum residual sum of

TABLE 3 - Central composite design with experimental values of chitinase production, biomass and pH by *Paenibacillus sabina* strain JD2

Run No.	Chitin (X_1) (g/l)		Yeast extract (X_2) (g/l)		$CaCl_2$ (X_3) (g/l)		Chitinase production (U/ml)	Biomass (protein mg/ml)	pH
	Actual value	Coded value	Actual value	Coded value	Actual value	Coded value			
1	10.00	1.000	0.65	1.667	0.08	−0.867	15.54	0.17	7.42
2	15.00	3.000	0.50	1.000	0.01	−1.178	26.67	0.17	7.61
3	10.00	1.000	0.28	0.022	0.08	−0.867	7.41	0.14	7.34
4	15.00	3.000	0.05	−1.000	0.01	−1.178	20.34	0.15	7.40
5	10.00	1.000	0.28	0.022	0.08	−0.867	10.66	0.16	7.31
6	5.00	−1.000	0.50	1.000	0.01	−1.178	2.98	0.11	7.49
7	10.00	1.000	0.28	0.022	0.04	−1.044	12.66	0.17	7.38
8	18.41	4.364	0.28	0.022	0.08	−0.867	84.79	0.3	7.27
9	10.00	1.000	0.28	0.022	0.08	−0.867	6.96	0.15	7.23
10	10.00	1.000	0.28	0.022	0.08	−0.867	10.03	0.12	7.22
11	15.00	3.000	0.50	1.000	0.15	−0.556	40.05	0.18	7.25
12	5.00	−1.000	0.50	1.000	0.15	−0.556	17.08	0.09	7.30
13	10.00	1.000	0.28	0.022	0.08	−0.867	16.81	0.2	7.35
14	15.00	3.000	0.05	−1.000	0.15	−0.556	26.56	0.19	7.32
15	5.00	−1.000	0.05	−1.000	0.01	−1.178	12.47	0.35	7.67
16	10.00	1.000	0.28	0.022	0.08	−0.867	16.45	0.15	7.25
17	10.00	1.000	0.28	0.022	0.20	−0.333	23.95	0.18	7.35
18	5.00	−1.000	0.05	−1.000	0.15	−0.556	17.99	0.06	7.17
19	1.59	−2.364	0.28	0.022	0.08	−0.867	31.64	0.06	7.28
20	10.00	1.000	0.10	−0.778	0.08	−0.867	12.02	0.15	7.25

squares in the transformed model. The plot shows the minimum λ values as well as lambda as the 95% confidence range. If the 95% confidence interval around this λ includes one, no transformation is required.

Desirability analysis. After fitting the models and residual analysis of all three responses, for optimisation of these multiresponses simultaneously an optimisation techniques popularised by Derringer and Suich (1980) was used. Their procedure makes use of desirability functions. The general approach is to first convert each response y_i into an individual desirability function d_i that varies for the range $0 \leq d_i \leq 1$, where as if the response y_i is at its goal or target then $d_i = 1$, and if the response is out side and acceptable region $d_i = 0$. Then the design variables are chosen to maximise the overall desirability:

$$D = (d_1 * d_2 * \text{———} * d_n)^{1/n}$$

where, n is number of responses. Statistical software package Design Expert (Version 6.0.10, State-Ease Minneapolis, MN, USA) was used to design and analyse experiment.

Verification experiment. Does the statistically predicted medium formulation actually produce similar amount of chitinase with respect to pH and biomass as predicted by statistically. For that verification experiment was conducted by inoculating *Paenibacillus sabina* strain JD2 in statistically obtained medium as described previously.

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

RESULTS AND DISCUSSION

Production profile of chitinase

The production of extracellular chitinase was monitored during the growth of *Paenibacillus sabina* strain JD2. The total cell protein was also determined as a parameter to monitor the growth of this isolate. Initially, chitinase assay was estimated without any modification in assay system as described by Vyas and Deshpande (1989). The production of chitinase and growth was found to be maximal at 96 h (Fig. 1). The production of chitinase has been reported to be maximal at 144 h for *Serratia marcescens* (Monreal and Reese, 1969), at 96 h for *Bacillus circulans* no. 4.1 (Wiwat *et al.,* 1999), at 72 h for *Alcaligenes xylosoxydans* (Vaidya *et al.,* 2001), at 120 h for *Enterobacter amnigenus*, whereas at 144 h for *Pseudomonas* sp. and *Pantoea dispersa* (Gohel *et al.,* 2004a).

The optimum conditions for chitinase activity of *Paenibacillus sabina* strain JD2 was found to be 0.3 ml of enzyme, 50 µmol of phosphate buffer (pH 6.0) and 40 ºC for 5.0 min enzyme substrate incubation period (data not shown). Since optimum chitinase activity was observed at pH 6.0 and 40 ºC for 5.0 min, subsequent chitinase assays were carried out at these conditions.

Screening of important medium components

Paenibacillus sabina strain JD2 produced 29.72 ± 2.53 U/ml chitinase by Monreal and Reese's medium, which is considered as basal medium/unoptimised medium in present investigation. To enhance the chitinase production by *Paenibacillus sabina* strain JD2 statistical method for medium optimisation was performed. This statistical method was carried out in three different stages include screening, optimisation and verification experiments.

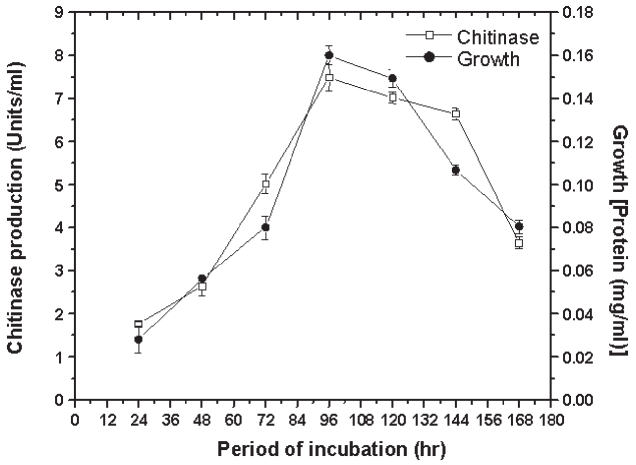

FIG. 1 - Production profile of chitinase and growth of *Paenibacillus sabina* strain JD2.

The assumption made initially for medium screening experiment was that all effects of interaction among variables were negligible provided that not much information was available on the physiology of *Paenibacillus sabina* strain JD2 with respect to chitinolysis, Plackett-Burman design was preferred as screening design in the present study. Table 2 represents the Plackett-Burman experimental design for 12 trials with two levels of concentrations for each variable and corresponding chitinase production. The variables X_1–X_8 represent the medium constituents and D_1–D_3 represents the dummy variables/unassigned variables. Table 4 represents the effect, standard error, $t_{(Xi)}$, p and confidence level of each component from the result of chitinase production.

The confidence level of components glucose, peptone, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 were below 95% in chitinase production and hence were considered insignificant. The rest of the components chitin, yeast extract and $CaCl_2$ showed confidence level at or above 95% and were considered to be significant (Table 4). Interestingly among twelve runs of Plackett-Burman experiment, the chitinase production was observed more where chitin concentration was more that is because of the inducible nature of the chitinase in presence of chitin substrate.

The above results indicated that the Plackett-Burman design is a powerful tool for identifying factors, which had significant influence on chitinase production. The exact optimal concentration of the significant components was determined by the subsequent central composite design.

Optimisation of screened medium components

Multiresponse analysis

Individual response in each model of central composite design

The concentration range of these screened components for central composite experimental design was selected on the basis of their confidence level at or above 95% and their

TABLE 4 - Statistical analysis of medium components on chitinase production from the results of Plackett-Burman design of Table 2

Factors	Medium coomponents	Effect	S.E.	$t_{(xi)}$	*p*	Confidence level (%)
X_1	**Chitin**	**+23.86**	**2.25**	**10.58**	**0.009**	**99.10**
X_2	Glucose	+2.10	2.25	0.93	0.449	55.10
X_3	**Yeast extract**	**–9.61**	**2.25**	**4.25**	**0.050**	**95.00**
X_4	Peptone	+7.93	2.25	3.51	0.072	92.80
X_5	$(NH_4)_2SO_4$	+5.55	2.25	2.46	0.133	86.70
X_6	$MgSO_4 \cdot 7H_2O$	+5.77	2.25	2.56	0.124	87.60
X_7	**$CaCl_2$**	**–12.99**	**2.25**	**5.76**	**0.028**	**97.20**
X_8	KH_2PO_4	–3.05	2.25	1.35	0.308	69.20

effects (either positive or negative) in Plackett-Burman experiment. If the component shows at or above 95% confidence level and its effect is negative, it indicates that the component is effective in chitinase production but the amount required is lower than the indicated low (–) concentration in Plackett-Burman experiment, and if the effect is positive, a higher concentration than the indicated high value (+) concentration in Plackett-Burman experiment (Plackett and Burman, 1946; Gohel, 2006). For example in present investigation, chitin has positive effect but it shows above 95% confidence level in Plackett-Burman experiment and hence low (5 g/l) and high (15 g/l) were selected in central composite design of response surface methodology. Whilst yeast extract and $CaCl_2$ showed at or above 95% confidence level but their effects found to be negative and hence low (0.05 g/l and 0.01 g/l), high (0.5 g/l and 0.15 g/l) for component yeast extract and $CaCl_2$, respectively were selected in central composite design. These low and high values of each component were feed in the design expert software using formula number four (as descried in Materials and Methods) of the central composite design resulting 20 trails were obtained with centre points repeated for six times for estimation of error (Table 3). The initial pH of the medium of each trial was adjusted to 7.2. By using Design Expert software different models were fit on a) chitinase, b) biomass, and c) final pH.

Chitinase response

For modelling of chitinase as a function of chitin, yeast extract and $CaCl_2$, quadratic model was found to be significant (*p*-value, Prob > *F* = 0.0001, Adjusted R-squared = 84.59%, and R-squared = 91.88%). The estimated quadratic model in terms of coded factors is:

$$\text{Sqrt(chitinase)} = + 3.44 + 0.99 * X_1 + 0.10 * X_2 + 0.69 * X_3 + 0.47 * X_1 * X_2 - 0.16 * X_1 * X_3 + 0.28 * X_2 * X_3 + 1.30 * X_1^2 - 0.088 * X_2^2 - 0.088 * X_3^2$$

and in terms of actual factors is:

$$\text{Sqrt(chitinase)} = +6.66176 - 0.91839 * \text{chitin} - 4.17439 * \text{yeast extract} + 12.51362 * CaCl_2 + 0.41681 * \text{chitin} * \text{yeast extract} - 0.46744 * \text{chitin} * CaCl_2 + 17.69716 * \text{yeast extract} * CaCl_2 + 0.051937 * \text{chitin}^2 - 1.74339 * (\text{yeast extract})^2 - 17.95540 * (CaCl_2)^2$$

where * indicate multiplication.

In the Analysis of Variance (ANOVA) through quadratic model for chitinase production the contribution of factors X_1: chitin, X_3: $CaCl_2$, $X_1 * X_2$: chitin*yeast extract and X_1^2: $(\text{chitin})^2$ were found to be significant in explaining the behavior of chitinase at 5% level of significance. The significance of X_1^2: $(\text{chitin})^2$ indicates that there is a curvature in the response surface of chitinase. A high value of adjusted R-squared of 84.59% was observed, which indicates a good agreement between experimental and predicted values of chitinase production as explained by quadratic model. But the lack of fit test was also found to be insignificant (*p*-value = 0.1698) which indicates the absence of block effect in the experiment.

Biomass response

In the case of the biomass response, 2FI model was significant (*p*-value Prob > *F* = 0.0382, adjusted R-squared = 53.90%, and R-squared = 68.46%). The fitted linear model in terms of coded factors is:

$$\text{Ln(Biomass)} = -1.89 + 0.30 * X_1 - 0.041 * X_2 - 0.13 * X_3 + 0.11 * X_1 * X_2 + 0.28 * X_1 * X_3 + 0.17 * X_2 * X_3$$

and in terms of actual factors is:

$$\text{Ln(Biomass)} = -1.15135 - 0.029885 * \text{chitin} - 2.00997 * \text{yeast extract} - 12.95044 * CaCl_2 + 0.093883 * \text{chitin} * \text{yeast extract} + 0.80636 * \text{chitin} * CaCl_2 + 11.09500 * \text{yeast extract} * CaCl_2$$

In the ANOVA though 2FI model for biomass response, effect of X_1: chitin, $X_1 * X_3$: Chitin*$CaCl_2$ were found to be significant at 5% level of significance. Here the insignificance of lack of fit test (*p*-value) again indicated the absence of block effects in the experiment.

pH response

For modelling of pH as a function of chitin, yeast extract and $CaCl_2$ quadratic model was found to be significant (*p*-value Prob > *F* = 0.0148, adjusted R-squared = 61.19%, and R-squared = 79.58%). The estimated quadratic model in terms of coded factors is:

$$\text{pH} = +7.28 - 5.111E\text{-}003 * X_1 + 0.019 * X_2 - 0.14 * X_3 + 0.024 * X_1 * X_2 + 0.031 * X_1 * X_3 + 3.833E\text{-}003 * X_2 * X_3 - 3.577E\text{-}003 * X_1^2 + 0.032 * X_2^2 + 0.100 * X_3^2$$

and in terms of actual factors is:

pH = +7.72100 - 0.011115 * chitin - 0.49685 * yeast exract - 6.18026 * $CaCl_2$ + 0.021134 * chitin * yeast extract + 0.089286 * chitin * $CaCl_2$ + 0.24337 * yeast extract * $CaCl_2$ - 1.43077E - 004 * chitin^2 + 0.63664 * (yeast extract)^2 + 20.35005 * ($CaCl_2$)^2

In the ANOVA through quadratic model for pH the contribution of factors X_3: $CaCl_2$ and X_3^2: ($CaCl_2$)^2 were found to be significant in explaining the behaviour in changing final pH at 5% level of significance. The significance of X_3^2: indicates that there is a curvature in the response surface of final pH changing. But the lack of fit test was found to be insignificant (*p*-value = 0.2793) which indicates the absence of block effect in the experiment.

Model diagnostics

Model adequacy checking was also performed, which is (an) important part of data analysis procedure. These diagnostics include a) Normal probability plot of residuals, b) Plot of studentized residuals versus predicted values, and c) Box-Cox plot for power transformation.

Normal probability plot of residuals

Chitinase and pH response for quadratic models and biomass response for 2FI model, normality assumption for residuals were loosely satisfied since the pattern of the residuals creating a little bit S-shaped curve (Fig. 2A, 2B and 2C).

Plot of studentized residual versus predicted values

The pattern of the plot showed the random distribution of studentized residuals in all the responses indicating that the assumption of constant variance was true (Fig. 3A, 3B and 3C).

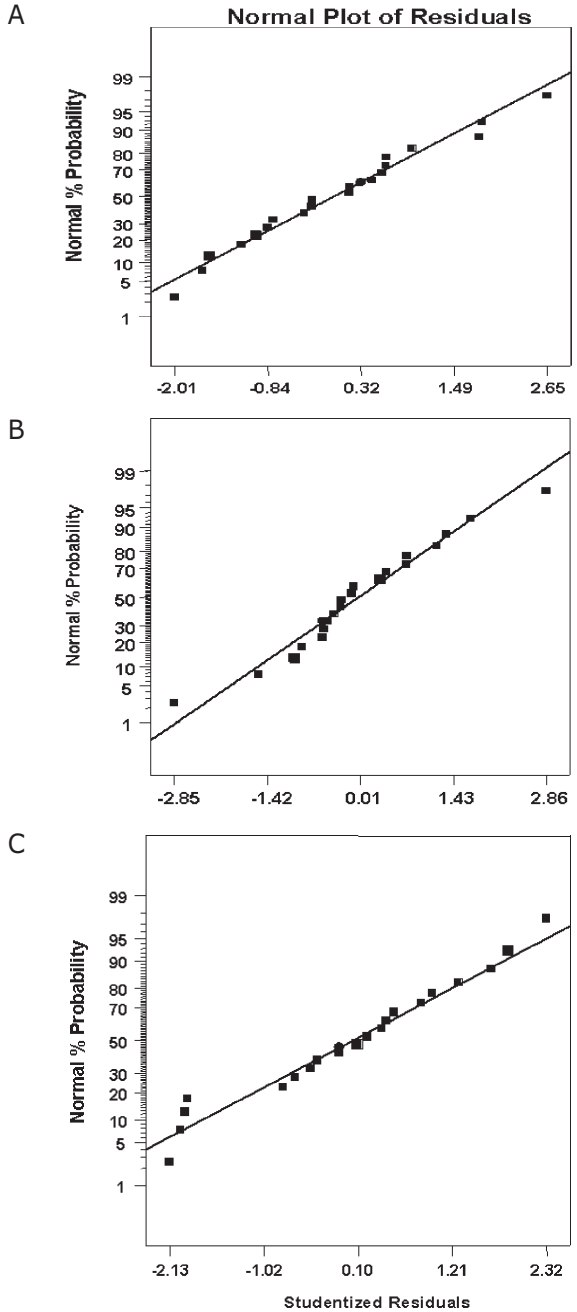

FIG. 2 - Normal probability plot of residuals of A) chitinase response, B) biomass response, and C) pH response.

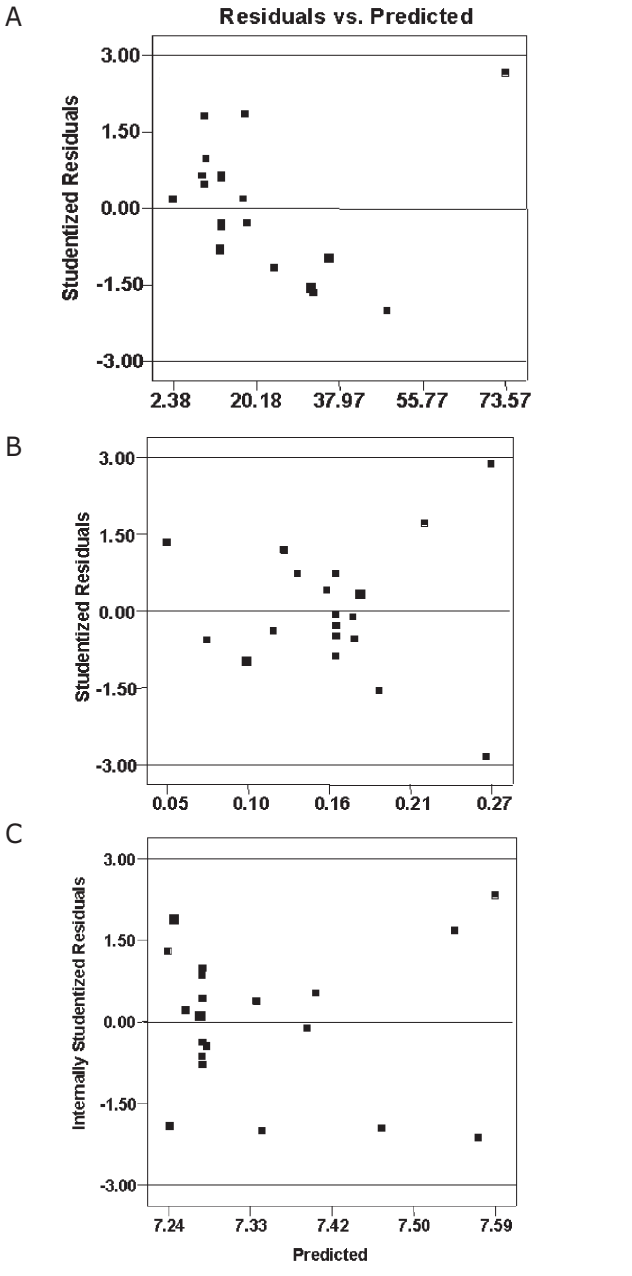

FIG. 3 - Plot of studentized versus predicted values of A) chitinase response, B) biomass response, and C) pH response.

Box-Cox plot

The Box-Cox plot of chitinase, biomass and pH responses was suggested lowest value of 0.5, 0 and 1, respectively which was one and also included in the 95% confidence interval as shown in Fig. 4A, 4B and 4C, respectively. Thus, as per the above discussion, no power transformation was required for pH response whilst square root and natural log transformation required for quadratic and 2FI model for chitinase and biomass responses, respectively before formulation of medium through desirability approach.

Hence, these model diagnostic plots suggested that assumptions for all these three fitted models for each response were satisfied and they fitted well.

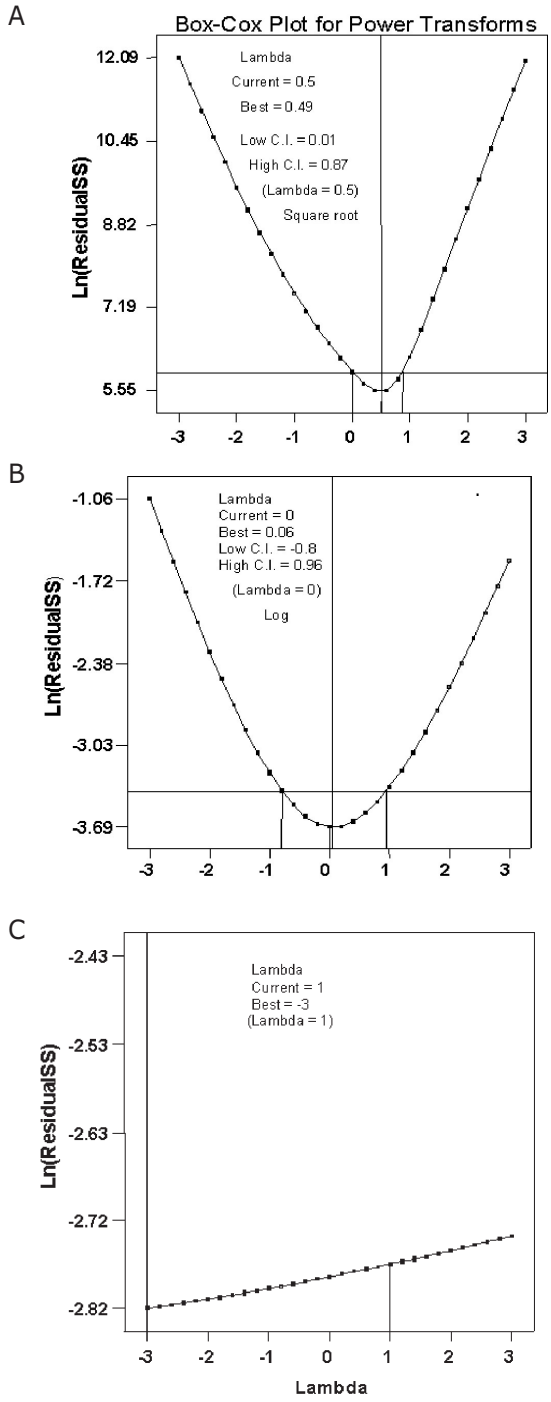

FIG. 4 - Box-Cox plot for power transformation in A) chitinase response, B) biomass response, and C) pH response.

Desirability analysis

The Design Expert software package was used to optimise the chitinase production with respect to minimum biomass and target pH, keeping all the four independent variables within the range, which were found by experimental analysis (Table 3). We set weight for this individual desirability equal to unity according to definition described in materials and methods. The numbers of different formulations were obtained with predicted chitinase with biomass and pH after adjusting such parameters (data not shown). However the best medium formation was selected on the basis of higher desirability with optimum chitinase value. It was observed that the medium consisting of (g/l): chitin 18, yeast extract 0.50, and $CaCl_2$ 0.08, was found to predict optimum chitinase of 82.93 U/ml with overall highest desirability of 0.842 as compared to other formulations. It was also found that in the chitinase production, pH and biomass were essential. Hence, the above medium composition was suggested for enhanced chitinase production.

Verification of the experiment

This statistically predicted optimised medium was further used for the verification for chitinase production. For that, *Paenibacillus sabina* strain JD2 was inoculated in this medium and chitinase production with respect to pH and biomass was checked after 96 h of incubation as described in Materials and Methods. It was found that 81.31 ± 7.15 U/ml chitinase was produced with biomass and pH as 0.248 ± 0.01 mg/ml and 7.36, respectively.

It was found that chitinase production was increased 2.74-fold through statistical optimised method as compared to unoptimised method (Monreal and Reese's medium) when acid swollen chitin was used as a substrate in chitinase assay system. By using statistical optimisation method, 4.33-fold chitinase production by *P. dispersa* (Gohel *et al.,* 2004c), 35% increase in riboflavin production was reported in U.V. mutant of *Eremothecium ashbyii* (Pujari and Chandra 2000), 35% higher recombinant hirudin production in *Saccharomyces cerevisiae* (Rao *et al.,* 2000), 141% increase in chitinase production in *Alcaligenes xylosoxydans* (Vaidya *et al.,* 2003).

In the present investigation, successful attempts were made to improve chitinase production using statistical approaches for optimising medium components. These findings prove that apart from cultural conditions like pH, temperature, incubation time and aeration, medium components also play a significant role in the production of chitinase. The results will facilitate large scale production of chitinase economically more viable. The behaviour of components in chitinase production with respect to growth and pH was efficiently understood through statistical methods and results also suggest that this method shows a much high level of chitinase production as compared to unoptimised or basal medium.

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