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Statistical optimization of chitinase production by Box–Behnken design in submerged fermentation using *Bacillus cereus* GS02

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ABSTRACT

Chitinase is a glycosyl hydrolase that cleaves chitin and has gained enormous attention due to its extensive biotechnological applications in various sectors including agricultural, industrial, medicinal, and environmental. It is important to have high yield of the enzyme with low cost of production for sustainable use. Culture medium is needed to be optimized to enhance the enzyme secretion and decreasing its cost. The present work describes optimization of the culture medium components and physical parameters for fermentation utilizing the statistical method to enhance chitinase production. A potent isolate *Bacillus cereus* GS02, which was locally isolated from soil earlier, was used for response surface methodology (RSM) mediated optimization of chitinase production. Four individual significant factors obtained after one factor at a time, namely, colloidal chitin, yeast extract, inoculum size, and pH were selected and utilized for fermentation process maximization. Box–Behnken design of RSM was applied for optimization of chitinase production for high yield. The optimum level was achieved at 15 g/l colloidal chitin, 0.72 g/l yeast extract, and 1.2% inoculum size and at pH 6.25 when incubated for 48 h in submerged fermentation, which resulted in 5.08-fold increase in chitinase production. Thus, *B. cereus* GS02 could be exploited for chitinase production at large scale for various biotechnological applications.

1. INTRODUCTION

Chitinase enzyme (EC. 3.2.1.14) is widely distributed in nature which catalyzes chitin hydrolysis into N-acetylglucosamine and its oligomers [1]. Due to the tremendous applications of chitinase in different industrial sectors, its economic production and enhancement are of crucial value [2]. The expenses in enzyme production act as essential aspect in determining the employment of chitinase for different applications. The culture medium and measures concerned in downstream processing of the enzyme carry large amount of the production cost [3]. In recent years, various advanced and novel biotechnological processes have been observed for production of chitinase which involves sub-merged fermentation (SmF) as a rising/favorable technology [4]. Further, various physical and chemical factors such as pH of the medium, incubation temperature, aeration, time of incubation, and inoculum size have influence on growth of the micro-organism. The utilization of statistical methods like design of experiment to optimize the medium of fermentation can minimize the drawbacks of ancient one-factor at a time (OFAT) concept [5]. On the contrary, with the help of statistical optimization, there is reduction in total number of the experiments allowing faster screening of enormous factors in lesser time [1,6,7]. The interaction among various parameters is also achieved by statistical optimization [7].

Response surface methodology (RSM) serves as a beneficiary mechanism to investigate the impact of various parameters on production of enzyme altering them concurrently in a defined experiments number, therefore sparing cost and time for optimization of production parameters. Every microbe is particular in itself with a specific necessity for multiplication and secretion of extracellular enzymes, thus the measure differs broadly to get the optimized production. RSM provides different tools such as central composite matrix design (CCD), Doehlert matrix (DM), full factorial design, and Box–Behnken design (BBD) to perform multiple regression studies [8]. RSM and Plackett–Burman design are analyzing tools, involving many variables to be tested concurrently. Due to this, lesser number of experiments is required which are more accurate and thus making these tools more effective in experimental biology [9].

Recently, various scientific investigations have been reported for accomplishing optimized production of chitinase using RSM [2,7]. Validation of the RSM results to increase chitinase secretion from *Stenotrophomonas maltophilia* was reported earlier by Ahmad *et al*. [10]. Subramanian *et al*. [11] reported optimization of chitinase from *Achromobacter xylosoxidans* using RSM and employed central composite design for maximum chitinase production. The

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present work focused on the enhancement of chitinase yield from *Bacillus cereus* GS02 using statistical approach. It is well reported that medium constituents extremely affect the extracellular chitinase production in microbes and various combination of them act as fundamental part in production of chitinase enzyme. Optimization of efficient bacterial strain for enhancement in production of chitinase enzyme (having industrial applications) by utilizing RSM was studied in a skilled way.

2. MATERIALS AND METHODS

2.1. Microbe Used

A bacterial strain *B. cereus* GS02 was isolated from soil enriched in chitinous waste collected from water sediments of Gambhir River, Mhow, Madhya Pradesh, India. The chitinolytic potential of the strain *B. cereus* GS02 (Accession number MN454860) has been described earlier [12]. The bacterial strain was sub-cultured periodically and maintained on nutrient agar slants.

2.2. Preparation of Colloidal Chitin

Colloidal chitin was prepared as per procedure given by Ferrari *et al.* [13] with few modifications as described here. Around 60 ml of HCl (12 N) was added to chitin flakes (5 g) and mixture was blended quickly for 10 min. Thereafter, it was incubated at low temperature (4–10°C) in a refrigerator for overnight. Further, addition of chilled water (2 l) was done to it and thereafter, incubation was done at 37°C for 24 h. Thereafter, precipitate was collected using centrifugation at 10,000 × *g* at 37°C for 20 min. The pellet was washed with water until its pH became 7.0. This neutral chitin pellet was autoclaved and kept at 4°C for further experiments.

2.3. SmF for Chitinase Production

Chitin flakes from shrimp shells were brought into colloidal form and used in the medium. SmF was carried out by growing the chitinase secreting bacterial isolate on autoclaved basal medium (sodium chloride; 0.5 g, KH_2PO_4; 3 g, ammonium chloride; 1 g, yeast extract; 0.05 g/l) containing 1% of neutral chitin pellet (substrate) [14]. Medium was inoculated by adding 1% of stationary phase culture (inoculum) aseptically and thereafter, it was incubated in an incubator shaker at 37°C temperature under shaking condition at the speed of 170 rpm for 48 h. After 48 h of incubation, centrifugation of the fermented medium was done at 10,000 × *g* for 15 min at 4°C temperature. The resulting supernatant was considered as crude chitinase extract and enzyme assay was performed.

2.4. Enzyme Assay

Chitinase activity was determined as explained by Dai *et al*. [15] with few alterations as written here. In the reaction mixture (2 ml), colloidal chitin (1 ml) was taken (prepared in 50 mM sodium phosphate buffer with 1.3% concentration) along with crude enzyme (0.1 ml) and distilled water (0.9 ml) and incubated at room temperature for 15 min. To stop the enzyme reaction, mixture was kept in boiling water and heated for about 3 min. Crude enzyme addition was done in the control tube just before putting it in the boiling water bath. Thereafter, centrifugation was performed at 10,000 × *g* (20 min) to separate the pellets. The concentration of reducing sugar was calculated using DNS method [16]. Chitinase activity was defined in a unit which was considered as the amount of the chitinase which produces one micromole of N-acetylglucosamine equivalent reducing power per minute under the experimental conditions [12]. All the analyses were carried out in triplicate.

2.5. Optimization for Production of Chitinase Using OFAT Approach

Earlier, nutritional, and physical conditions for the culture medium used for chitinase production by the GS02 isolate were optimized using OFAT conventional method in our laboratory. All the experiments were carried out in Erlenmeyer flasks (250 ml) consisting of basal medium (50 ml) augmented with colloidal chitin (1%) in a rotatory shaker with speed of 180 rpm. All the analyses were repeated 3 times and the mean (average) values were recorded. Analysis of variance (ANOVA) single factor was used for the analysis of the data and P value less than or equal to (≤) 0.05 was regarded as significant. Post hoc (Tukey) test was also carried out utilizing PRISM (5.0 version) of GraphPad software [12].

2.6. Enhancement of Chitinase Production by Statistical Optimization

Different physical and chemical parameters affecting chitinase production in SmF were investigated using OFAT approach. Out of these, four variables namely inoculum concentration, colloidal chitin concentration, pH, and concentration of yeast extract were recognized as critical parameters in fermentation methodology. The effect of interaction between these significant parameters and their optimum level was studied by response surface statistical analysis utilizing Box and Behnken design [17]. The experimental range was setup using Design expert software version 12 (State- Ease Inc., Minneapolis, Minnesota, United States), and levels of the four independent variables were used in Box–Behnken Design [Table 1].

Total experiments number (N) needed for the Box–Behnken design construction was interpreted by the equation given below:

$$N = 2k\,(k - 1) + C_0$$

Here, N represents the experiments number, k represents total number of variables, and C_0 represents total number of central points. The above equation was utilized for the development of numerical correlation among these four different variables on chitinase production by carrying out 29 experimental runs with five repeat points at central point. The response data were applied, using the quadratic equation to suit the polynomial equation

$$Y = \beta_0 + \Sigma\,\beta_i X_i + \Sigma\,\beta_{ii} X_i^2 + \Sigma\,\beta_{ij} X_{ij}$$

Here, the predicted response is symbolized by Y

X_i represents independent variables, the coefficient of fitted response is denoted by β_0; β_i and β_{ii} which are linear coefficient and quadratic coefficient, respectively, while β_{ij} is used for coefficient of interaction; X_{ij} denotes variables interacting with each other.

The experimental design developed using BBD of the four different variables in coded levels is given in Table 2. Temperature of 37°C for all the 29 experimental runs was used. Medium was analyzed for

Table 1: Levels of independent variables used in experimental design of BBD.

Variable	Name of factor	Unit	Range and levels		
			−1	0	+1
A	Colloidal chitin	g/l	5.00	10	15.00
B	Yeast extract	g/l	0.25	0.50	0.75
C	Inoculum size	%	0.5	1	1.50
D	pH	-	6.00	7	8.00

Table 2: Experimental design obtained by Box–Behnken used for optimization of four process parameters and results for chitinase production.

Std.	Run	Colloidal Chitin (g/l)		Yeast extract (g/l)		Inoculum size (%)		pH		Experimental value	Predicted value
		Coded	Decoded	Coded	Decoded	Coded	Decoded	Coded	Decoded		
23	1	0	10	−1	0.25	0	1	1	8	2.44	2.25
25	2	0	10	0	0.5	0	1	0	7	4.24	3.93
12	3	1	15	0	0.5	0	1	1	8	5.79	5.70
28	4	0	10	0	0.5	0	1	0	7	4.28	3.93
3	5	−1	5	1	0.75	0	1	0	7	3.25	2.92
21	6	0	10	−1	0.25	0	1	−1	6	4.05	4.16
20	7	1	15	0	0.5	1	1.5	0	7	6.87	6.92
11	8	−1	5	0	0.5	0	1	1	8	1.54	1.25
18	9	1	15	0	0.5	−1	0.5	0	7	5.69	5.85
15	10	0	10	−1	0.25	1	1.5	0	7	3.52	3.70
16	11	0	10	1	0.75	1	1.5	0	7	6.12	6.48
10	12	1	15	0	0.5	0	1	−1	6	6.57	6.94
19	13	−1	5	0	0.5	1	1.5	0	7	2.77	2.85
26	14	0	10	0	0.5	0	1	0	7	3.99	3.93
6	15	0	10	0	0.5	1	1.5	−1	6	6.34	5.62
29	16	0	10	0	0.5	0	1	0	7	3.87	3.93
7	17	0	10	0	0.5	−1	0.5	1	8	2.53	2.93
2	18	1	15	−1	0.25	0	1	0	7	5.02	5.03
5	19	0	10	0	0.5	−1	0.5	−1	6	4.42	4.05
1	20	−1	5	−1	0.25	0	1	0	7	1.22	1.40
22	21	0	10	1	0.75	0	1	−1	6	5.40	5.83
14	22	0	10	1	0.75	−1	0.5	0	7	4.68	4.58
27	23	0	10	0	0.5	0	1	0	7	3.27	3.93
4	24	1	15	1	0.75	0	1	0	7	8.54	8.04
24	25	0	10	1	0.75	0	1	1	8	4.98	5.11
9	26	−1	5	0	0.5	0	1	−1	6	2.46	2.63
13	27	0	10	−1	0.25	−1	0.5	0	7	3.10	2.82
8	28	0	10	0	0.5	1	1.5	1	8	4.08	4.13
17	29	−1	5	0	0.5	−1	0.5	0	7	0.97	1.16

chitinase activity after 48 h of incubation as programmed in Box–Behnken Design.

2.7. The RSM Model Validation

Validation of the RSM experimental model was done by achieving maximum production of chitinase using predicted parameters by the experimental design. The RSM model was compared with the experiment results obtained empirically with the predicted response [18].

3. RESULTS AND DISCUSSION

3.1. Identification of Significant Factors by OFAT

OFAT was selected to detect the influence of individual parameters affecting chitinase production by *B. cereus* GS02 using SmF. The secretion of extracellular chitinase by this bacterial strain was optimized with respect to fermentation time, inoculum size, pH, temperature, carbon and nitrogen sources, and substrate concentration. The highest chitinase yield by this isolate was attained after 48 h of incubation, at 37°C and pH 7.0. The highest chitinase was secreted when media consisted of colloidal chitin (1%) and 0.05% yeast extract which were in consideration with the results reported by Gueye *et al*. [19]. Factors with $P < 0.05$ were found to have positive influence on the chitinase production and were chosen for further statistical optimization analysis [12].

3.2. Statistical Optimization of Process Factors

The impact of various process factors on chitinase secretion was investigated by OFAT method and four variables, namely, colloidal chitin, yeast extract, pH, and inoculum level were identified to have positive influence in earlier study [12]. RSM using BBD was applied to optimize important variables to further improve chitinase secretion by *B. cereus* GS02. BBD was used to design experiments using 4 individual variables investigated at 3 levels and 5 central points (for estimation of error). Gonclaves *et al*. [20] also optimized the enzyme production in *Bacillus subtilis* using BBD. Experimental runs in total 29 were performed and the observed response has been reported [Table 2]. Recently, statistical optimization of different parameters for amylase production was done using central composite design, where five individual variables were investigated with 52 experimental runs [21].

ANOVA was used for determining the statistical significance of the polynomial model equation using the software, Design-Expert, version 12.0 [Table 3]. The produced response was fitted in the second-order polynomial equation given as follows:

Y = 3.93 + 2.19A + 1.13B + 0.6925C -0.6567D + 0.3725AB - 0.1550AC + 0.0350AD + 0.2550BC + 0.2975BD - 0.0925CD + 0.1067A2 + 0.3104B2 + 0.1567C2 + 0.0954D2

Where Y is the enzyme activity, A represents colloidal chitin, B represents the yeast extract, C represents the inoculum size, and D symbolizes the pH of the medium. The influence of experimental parameters with enzyme secretion was correlated using this equation. Coefficients of quadratic model were predicted by numerous linear regression and those with P value <0.05 were considered as significant. The linear effects of A, B, C, and D were observed to be significant, while the quadratic impacts of A, B, and D and interaction impacts of individual variables were found insignificant. The highest chitinase activity in the BB experimental design was found to be 8.54 unit/ml at 15 g/l colloidal chitin, 0.75 g/l yeast extract, inoculum level of 1% and pH 7.0 of fermentation medium as shown in Table 2. The actual experimental and predicted response obtained together with design matrix are also illustrated [Table 2] and analysis of the outcomes was done by ANOVA. The chitinase activity of the actual response concluded by regression quadratic model and those obtained after experiments was very close enough proving that the model is valid [Figure 1].

The F-value of model has been revealed to be 32.06 which implied that quadratic model is significant. The "Lack of fit F-value" of 1.22 revealed that the lack of fit is not significant relative to the pure error which is the requested aspect and conveys that experimental data fit the model. By determining the R^2 (coefficient), one can check the goodness of fit of the quadratic model produced by RSM. In the present analysis, R^2 value for this model was attained to be 0.969, while the predicted and adjusted R^2 values were as 0.939 and 0.857, respectively. It showed that this model cannot explain only 0.07% of total variations. Therefore, the current R^2 value reproduced a good fit between predicted and observed responses and suggested that the model is positive and steady for concluding chitinase secretion.

3.3. Response Surface 3-D Plot

The interactions between the individual variables, namely, colloidal chitin, yeast extract, inoculum concentration, and pH of the medium were described by the regression equation and are conferred as response surface 3-D graphs [Figure 2a-f]. The response surface plot showed the influence of two individual variables and kept the other variables at zero (0) levels.

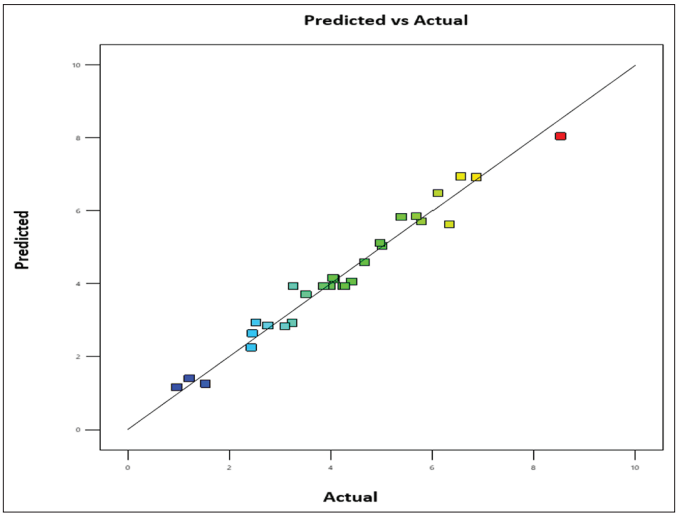

Figure 1: Experimental validation of the model for chitinase production showing the relation between predicted and actual values.

Table 3: Analysis of variance for the experimental results of Box–Behnken methodology for quadratic model (R^2=0.969, R^2 [pred]=0.857, R^2 [adj]=0.939).

Source	Sum of squares	df	Mean square	F-value	*P*-value	
Model	85.90	14	6.14	32.06	< 0.0001	Significant
A-colloidal chitin	57.51	1	57.51	300.54	<0.0001	
B-yeast extract	15.46	1	15.46	80.78	<0.0001	
C-inoculum size	5.75	1	5.75	30.07	<0.0001	
D-pH	5.17	1	5.17	27.04	0.0001	
AB	0.5550	1	0.5550	2.90	0.1106	
AC	0.0961	1	0.0961	0.5022	0.4902	
AD	0.0049	1	0.0049	0.0256	0.8752	
BC	0.2601	1	0.2601	1.36	0.2631	
BD	0.3540	1	0.3540	1.85	0.1953	
CD	0.0342	1	0.0342	0.1789	0.6788	
A^2	0.0738	1	0.0738	0.3857	0.5446	
B^2	0.6250	1	0.6250	3.27	0.0922	
C^2	0.1592	1	0.1592	0.8320	0.3771	
D^2	0.0591	1	0.0591	0.3086	0.5873	
Residual	2.68	14	0.1914			
Lack of fit	2.02	10	0.2018	1.22	0.4587	Not significant
Pure error	0.6614	4	0.1654			
Cor total	88.58	28				

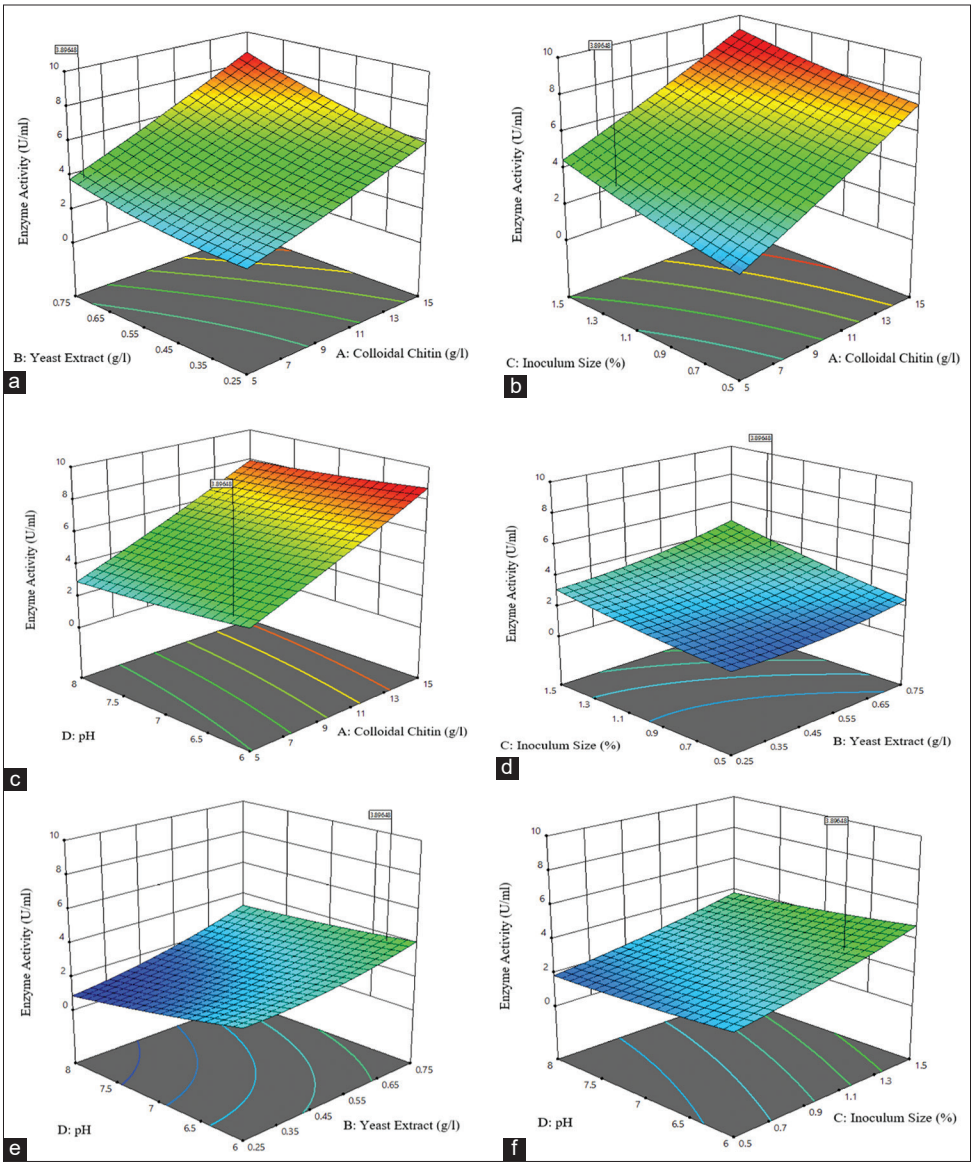

Figure 2: Response surface 3-D plots revealing correlation effects of (a) yeast extract and colloidal chitin, (b) inoculum size and colloidal chitin, (c) pH and colloidal chitin, (d) yeast extract and inoculum size, (e) yeast extract and pH, (f) pH and inoculum size.

A simultaneous interrelation was shown when enzyme activity was plotted against impact of colloidal chitin and yeast extract. It indicated that enhancement in yeast extract amount along with enhancement in colloidal chitin amount favored the optimum level [Figure 2a]. It was observed that there is enhancement in chitinase production by increasing the yeast extract concentration up to 0.75 g/l. Similar data indicated that chitinase secretion got stimulated by augmentation of yeast extract in the medium. Lee and Kim [5] reported requirement of yeast extract in the amount of 0.5 g/l for chitinase production, whereas Vaidya *et al*. [22] reported that 0.3 g/l of yeast extract favored maximum chitinase production. Low level of yeast extract (0.25 g/l) for enhanced chitinase production, using RSM has also been investigated [23].

Substrate concentration is a prime factor in fermentation of microbes. Chitinase production has been observed only in the presence of chitin (inducible in nature); hence, colloidal chitin influences the enzyme production [24]. However, the chitinase activity of different strains and species is associated with different concentrations of colloidal chitin. The response surface plot also showed that higher concentration of colloidal chitin (15 g/l) favored optimum chitinase secretion by *B. cereus* GS02. Optimization of chitinase by *Alcaligenes xylosoxydans* using BBD required colloidal chitin of 15 g/l for maximum secretion of chitinase [22]. Kuddus [7] reported optimum chitinase secretion by *B. cereus* GA6 with 20 g/l of colloidal chitin in medium. Warda *et al*. [25] observed maximum chitinase production in *Brevundimonas diminuta* when media was supplemented with 8 g/l of colloidal chitin. Optimum colloidal chitin concentration of 20 g/l was also reported for production of chitinase from *Streptomyces griseorubens* C9 using RSM [23].

Figure 2b, d, and f represented the response surface 3D graphs attained and revealed the interaction between inoculum size and colloidal chitin, yeast extract, and pH, respectively. It was observed that increasing the inoculum size favors increase in chitinase secretion and inoculum size of about 1.2% was noticed to be optimum for chitinase secretion. The correlation exhibited between the interaction of inoculum size

with colloidal chitin was revealed to be positive [Figure 2b]. High level of inoculum with increased level of yeast extract enhanced chitinase activity but at low pH level, it favors maximum enzyme activity. Earlier reports suggested that inoculum size had a large effect on secretion of chitinase enzyme. Sanjivkumar *et al*. achieved the maximum chitinase secretion from *Enterobacter* sp. NRG4, at an inoculum size of 2.6 ml for 4 g of solid substrate in solid-state fermentation employing RSM [18]. In another investigation, chitinase secretion by the *Streptomyces olivaceus* was optimized statistically by Box–Behnken design and was effectively secreted at the optimum level of 2.75% inoculum size [26].

Similarly, response surface plots showed the interaction between pH of the fermentation medium with colloidal chitin, inoculum size, and yeast extract [Figure 2c, e, and f]. The graph reflected minimal interaction of pH with variables such as yeast extract and inoculum size, while a positive interaction was observed between pH and colloidal chitin concentration [Figure 2c]. Thus, increasing the pH of the medium above 7.0 decreased the enzyme production and optimum level was predicted at pH 6.25. Hence, high concentration of colloidal chitin and low level of pH enhanced chitinase production. The outcome of the analyses is in close range with the earlier reported results. Rishad *et al*. [2] reported the optimum pH level at 6.5 for maximum secretion of chitinase from *Bacillus pumilus* MCB-7 employing response surface statistical analysis. However, the optimum level of the pH for a chitinase secretion in SmF from mutant *B*. *cereus* GA6 was found to be 9.0 [7].

3.4. RSM Model Validation

Fermentation of *B*. *cereus* GS02 under the calculated conditions was carried out to validate the RSM model. The optimized amount of four different factors used in statistical analysis were, colloidal chitin 15 g/l, yeast extract 0.73 g/l, inoculum size 1.2%, and pH 6.25. The predicted response for chitinase production was evaluated to be 8.04 (Unit/ml), while enhanced chitinase secretion of 8.54 (Unit/ml) was attained using optimum conditions. The data showed much similarity between actual experimental values and predicted response, therefore, proposed model is valid and very effective. This optimization of different culture variables by statistical method resulted in 5.08-fold enhancement in the chitinase secretion compared to results (1.68 U/ml) obtained in the basal medium, before any optimization.

Much work has been carried out with bacterial strains to maximize the physico-chemical parameters and medium components using RSM [27] since it is more efficient as compared to the traditional methods of optimization. Many studies as per literature indicated much low chitinase secretion even after optimization of various conditions. For instance, in *Humicola grisea* and *B. pumilus*, only 0.172 IU and 0.9 IU of chitinase activity, respectively, have been reported even after statistical optimization by RSM [28,29]. The variations found in chitinase yields of different species and strains, could be due to its inhabitant ecosystems. Kuddus [7] used central composite design of RSM to optimize medium and fermentation conditions for cold adaptive mutant *B*. *cereus* GA6 and achieved 4.4 folds enhancement in chitinase production. Rishad *et al*. [2] showed a 6.9-fold increase in enzyme production from *B*. *pumilus* MCB-7 using BBD for optimization of chitinase production. Jha and Modi [30] reported optimization of chitinase secretion from *Streptomyces rubiginosus* by statistical method using BBD and attained a 3-fold enhancement in enzyme yield after optimization. Sanjivkumar *et al*. [18] optimized medium for *S. olivaceus* using BBD and achieved up to 5.09-folds enhancement in chitinase secretion over the basal medium. The RSM mediated optimization of chitinase production in this work from *B*. *cereus* strain using BBD was revealed to be much compelling and could give a 5.08 folds enhanced production.

4. CONCLUSION

The extensive applications of chitinase in various industries require the more exploration of chitinases and support researchers for searching effective technologies which can minimize the prime expenses related with the enzyme production. Since statistical methods for optimization require lesser number of experiments and are time saving and more efficient, these can reduce the enzyme production cost. The present study was focused on statistical optimization of chitinase production using locally isolated bacteria, *B. cereus* GS02. Various production parameters were optimized earlier in our laboratory using OFAT approach including both medium components and physical parameters. Further, statistical optimization was performed using RSM to maximize the chitinase production. Various variables which were found to be significant in OFAT approach, namely, colloidal chitin concentration, yeast extract concentration, inoculum size, and pH were optimized by RSM. The experimental design was set up using BBD with 29 experimental runs, 4 factors, and 5 central points. The optimization approach resulted in 5.08-fold increase in chitinase production. The optimized conditions of inoculum size 1.2%, colloidal chitin 15 g/l, yeast extract concentration 0.73 g/l, and pH of 6.25 resulted in enhanced chitinase production in SmF. Due to cost efficient production and good yield of chitinase from *B*. *cereus* GS02, it could be exploited in enzyme production at the large scale for various biotechnological applications.

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6. CONFLICTS OF INTEREST

We declare “no conflicts of interest.”

7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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