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Production of chitin deacetylase by *Aspergillus flavus* in submerged conditions

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**ABSTRACT**

Chitosan is a biopolymer obtained by deacetylation of chitin and has been proven to have various applications in industry and biomedicine. Deacetylation of chitin using the enzyme chitin deacetylase (CDA) is favorable in comparison to the hazardous chemical method involving strong alkali and high temperature. A fungal strain producing CDA was isolated from environmental samples collected from coastal regions of South Kerala, India. It was identified as *Aspergillus flavus* by morphological characteristics and ITS DNA analysis. Nutritional requirement for maximum production of CDA under submerged condition was optimized using statistical methods including Plackett–Burman and response surface methodology central composite design. A 5.98-fold enhancement in CDA production was attained in shake flasks when the fermentation process parameters were used at their optimum levels. The highest CDA activity was 57.69 ± 1.68 U under optimized bioprocess conditions that included 30 g L⁻¹ glucose, 40 g L⁻¹ yeast extract, 15 g L⁻¹ peptone, and 7 g L⁻¹ MgCl₂ at initial media pH of 7 and incubation temperature of 32°C after 48 hr of incubation, while the unoptimized basal medium yielded 9.64 ± 2.04 U.

**KEYWORDS**

*Aspergillus flavus*; bioprocess optimization; central composite design; chitin deacetylase; microbial enzyme production; Plackett–Burman design

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**Introduction**

Chitosan is a random heteropolymer of *N*-acetylglucosamine and its deacetylated counterpart, *N*-glucosamine. It is present in limited amounts in the exoskeletons of many crustaceans and insects and also in the fungal cell wall. It has gained immense importance due to its physicochemical properties like biodegradability, biocompatibility, solubility, and nontoxicity.¹ Chitosan is utilized in various areas, including biomedicine (burn and wound dressings), food and feed additives (antioxidant and hypocholesterolemic agent), cosmetics (ingredient in hair and skin care products), pharmaceuticals (encapsulating agent for drug delivery), agriculture (antifungal agent and biopesticide), and water treatment (removal/recovery of metal ions and dyes from wastewaters).²⁻³ At present, chitosan is mainly produced by deacetylation of chitin using a harsh thermochemical process, which involves the use of a large amount of concentrated alkali (40–50% NaOH or KOH) and high temperatures (100°C or higher), which in turn cause environmental concern. This process is non-eco-friendly, tedious to control, and results in reduced product quality with generation of a heterogeneous range of deacetylated products.⁴⁻⁵ Bioconversion of chitin to chitosan using the enzyme chitin deacetylase (CDA; EC 3.5.1.41) is a competent alternative to the chemical method as it overcomes the various disadvantages of the chemical process.⁶ CDA catalyzes the deacetylation of monomeric acetyl glucosamine residues of chitin by multiple attack mechanisms.⁷ Chitosan produced by the enzymatic method offers the possibility of a controlled, nondegradable deacetylation process that results in a more regular pattern of deacetylation.⁸ In spite of its industrial importance and increasing demand for chitosan in various commercial fields, the high cost of production of CDA has hindered its industrial application in bioconversion of chitin to chitosan. CDA has been reported in several microorganisms like fungi, yeast, and bacteria and is also present in some insect species.⁹ Fungal CDA has been studied more widely than those from bacteria and insects. On the basis of localization of CDA in fungi, it has been divided broadly into two subgroups: intracellular CDA and extracellular CDA. Intracellular CDA is secreted into the periplasm, while extracellular chitin deacetylase is secreted into the external medium. Numerous fungi displaying both intracellular and extracellular CDA activity have been identified in the past few years.¹⁰⁻¹⁵ CDA-assisted enzymatic conversion of chitin to chitosan needs intensive screening of novel CDA hyperproducers. For industrial application, exploration of novel CDA hyperproducers with an inherent capability to secrete the CDA, with further optimization of the production process, is necessary. However, most of the research carried out so far has been related to the purification and characterization of CDA. Medium components and culture conditions have significant influence on extracellular enzyme production and are different for each microorganism. Bioprocess parameter optimization plays a crucial role in the development of any fermentation process since it significantly influences the economy and efficiency of the process. The conventional approach of optimizing one factor at a time, though, gives us an initial understanding of the effect of individual bioprocess parameters on enzyme production; it cannot provide...
the information on mutual interactions between the variables on the desired outcome. On the other hand, statistical experimental designs offer an organized plan for optimization of variables by experimentation and can eliminate the limitations of one-factor-at-a-time approach. Statistical data analysis involves a limited number of experiments where the interactions among several experimental variables can be visualized simultaneously. Further computational analysis leads to the prediction of data in the areas not directly covered by experimentation. In the current study, preliminary analysis was carried out using one-factor-at-a-time studies for CDA production under submerged fermentation by an isolated culture of *Aspergillus flavus*. Further Plackett–Burman design was used to determine the influential media components for CDA production by *Aspergillus flavus*, followed by response surface methodology to optimize the concentration of these components for CDA production.

**Materials and methods**

**CDA assay**

Extracellular CDA activity was determined by incubating 0.5 mL of crude enzyme with 1.0 mL of 0.1 M citrate-phosphate buffer, pH 6.5, and 0.5 mL of 1% colloidal chitin in the same buffer. The mixture was kept in a water bath at 50°C for 1 hr along with appropriate substrate and enzyme blanks. After this, the reaction mixture was heated in a boiling water bath to inactivate the enzyme and centrifuged at 10,000 × g for 5 min, and the aqueous supernatant was removed into a new tube. The supernatant was then analyzed for its acetate content using high-performance liquid chromatography (HPLC; Shimadzu). The conditions maintained in the HPLC were as follows: ROA-Organic acid H + (Rezex, 300 × 7.8 mm, Phenomenex) column, refractive index detector (Shimadzu), 0.01 N H2SO4 as mobile phase, flow rate at 0.6 mL min⁻¹, and temperature maintained at 55°C. One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 µmole mL⁻¹ min⁻¹ of acetate under the assay conditions.

**Identification of isolate by ITS sequencing and phylogenetic analysis**

For the identification of fungal isolate I8, the genomic DNA was isolated using standard protocol, and further, the internal transcribed spacer (ITS) region was amplified using universal forward and reverse primers. This ITS amplicon was further analyzed for its sequence data. The identification of the phylogenetic neighbors was initially carried out by the NCBI-BLAST and megaBLAST programs against the database of type strains with validly published prokaryotic names. The 20 sequences with the highest scores were then selected for the calculation of pairwise similarity using global alignment algorithm, which was implemented using MEGA 6 software. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model.

**Preparation of colloidal chitin**

The colloidal chitin was prepared by the method of Hsu and Lockwood with minor modifications: 40 g of flaked chitin was dissolved in 400 mL of concentrated HCl by stirring for 1 hr. The chitin was precipitated as a colloidal suspension by adding it slowly to 4 L of water at 4°C. The suspension was repeatedly washed by centrifugation until the pH of the suspension was about 4.0. The colloidal chitin thus prepared was stored at 4°C for subsequent use.

**Single-parameter optimization for CDA production**

The initial production medium consisted of (g L⁻¹) glucose, 10.0; yeast extract, 3.0; peptone, 5.0; KH2PO4, 1.0; K2HPO4, 1.0; and NaCl, 0.5 (pH 6.0). Spore suspension (0.5 mL, 10⁷ spores mL⁻¹) of the fungal strain was inoculated in 50 mL CDA production medium in a 250-mL Erlenmeyer flask. It was then incubated at 30°C and 200 rpm shaking with appropriate modifications as demanded by the specific conditions of the experiment. For every 24-hr intervals up to 96 hr, a culture sample was aseptically collected, centrifugation was done at 10,000 rpm for 10 min at 4°C, and the supernatant was used for the CDA assay as mentioned earlier.

**Effect of incubation time**

The culture was inoculated in the production medium and incubated at 30°C up to 96 hr in a rotary shaker at 200 rpm. Samples were withdrawn at periodic intervals of 12 hr.

**Effect of inoculum size**

The spore suspension was made from a week-old mature PDA slant using saline supplemented with 0.1% Tween 80. The production medium was inoculated with 1 mL of 10³, 10⁴, 10⁵, ..., 10⁸ spores mL⁻¹. After optimizing the spore concentration, the fermentation medium was inoculated with various volumes (0.5, 1.0, 1.5, ..., 5 mL) of the optimized spore concentration inoculum.
Effect of initial pH and incubation temperature

The effect of the initial pH value on the CDA production was investigated by varying the initial pH of the culture medium from 3 to 12 with a difference of 1 pH unit. The effect of temperature on enzyme production was determined by incubating the inoculated medium at different temperatures (26, 28, 30, 32, 34, 37, and 40°C) at 200 rpm.

Effect of glucose concentration

Glucose was the only carbon source provided for CDA production. To determine the effect of glucose concentration on the induction and production of CDA enzyme, it was added at increasing concentrations of 0, 2, 4, 6, 8, 10, 12, and 14 g L⁻¹ to the CDA production medium.

Effect of variation of nitrogen source

The effect of different sources of inorganic and organic nitrogen on CDA production was determined by individually supplementing the production medium with various nitrogen sources, such as urea, ammonium nitrate, ammonium chloride, ammonium sulfate, ammonium citrate, sodium nitrite, sodium nitrate, potassium nitrate, diammonium hydrogen phosphate, dihydrogen ammonium phosphate, peptone, yeast extract, beef extract, casein enzyme hydrolysate, and corn steep liquor. The nitrogen sources were supplemented such that the final nitrogen content in the medium would be 2 g L⁻¹. A control flask containing no added source of nitrogen was also maintained.

Effect of addition of metal ions

Influence of various metal ions on CDA production was determined by individually supplementing the medium with different divalent metal cations such as Co²⁺, Ca²⁺, Hg²⁺, Mg²⁺, Mn²⁺, Sn²⁺, and Zn²⁺ in their chloride form; Fe²⁺, Ni²⁺, and Cu²⁺ in their sulfate form; and Ag⁺ in its nitrate form. The metal ions were added at final concentrations of 5 mM and 10 mM to the production medium. A control flask containing no added metal ions was also maintained.

Effect of addition of surfactants

For studying the effect of addition of surfactants on the production of CDA, the production medium was supplemented with 0.2% (w/v) of sodium dodecyl sulfate (SDS), cetyl trimethylammonium brmide (CTAB), and 0.2% (v/v) of Triton X-100, Tween 20, Tween 80, Tween 60, and Tween 80. A control flask containing no added surfactants was also maintained.

Statistical optimization

The statistical software package Minitab 17.1.0, Minitab, Inc., State College, PA, was used to create suitable experimental designs and analyze the results of the experiments.

Screening of parameters affecting CDA production by Plackett–Burman statistical design

Identification of essential medium constituents to improve CDA production by Aspergillus flavus was carried out using Plackett–Burman design. This experimental design is a two-factor design, which identifies the critical nutritional parameters required for CDA production by screening n variables in n + 1 experiments. The relevant nutritional parameters affecting the enzyme production were screened using a design with six variables at two levels (Table 1) in a total of 28 experimental runs as shown in Table 2. The parameters tested were concentrations of glucose, yeast extract, peptone, MgCl₂, CuSO₄, and ZnCl₂ in the production medium. The variables were represented at two levels: a higher level designated as +1 and a lower level designated as −1. The actual and coded values tested for each parameter are given in Table 1. The number of positive and negative signs per trial was (k + 1)/2 and (k − 2)/2, respectively. Each row represents a trial, and each column represents an independent (assigned) variable. The effect of each variable was determined by the following equation:

\[ E(X_i) = \frac{\sum (M^+ + M^-)}{n} \]

where E(Xᵢ) is the concentration effect of the tested variable, M⁺ and M⁻ represent CDA production from the higher and lower levels, respectively.

<table>
<thead>
<tr>
<th>Code</th>
<th>Parameter name</th>
<th>Low level (−1)</th>
<th>High level (+1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁</td>
<td>Glucose</td>
<td>20 g L⁻¹</td>
<td>30 g L⁻¹</td>
</tr>
<tr>
<td>X₂</td>
<td>Yeast extract</td>
<td>10 g L⁻¹</td>
<td>20 g L⁻¹</td>
</tr>
<tr>
<td>X₃</td>
<td>Peptone</td>
<td>10 g L⁻¹</td>
<td>20 g L⁻¹</td>
</tr>
<tr>
<td>X₄</td>
<td>MgCl₂</td>
<td>1.0 g L⁻¹</td>
<td>2.0 g L⁻¹</td>
</tr>
<tr>
<td>X₅</td>
<td>CuSO₄</td>
<td>1.25 g L⁻¹</td>
<td>2.5 g L⁻¹</td>
</tr>
<tr>
<td>X₆</td>
<td>ZnCl₂</td>
<td>0.68 g L⁻¹</td>
<td>1.36 g L⁻¹</td>
</tr>
</tbody>
</table>

Table 1. Range of experimental values for the Plackett–Burman design.

Table 2. Experimental design matrix for CDA production using Plackett–Burman design.
lower levels respectively for each parameter, and \( n \) is the total number of trials. All experiments were conducted in duplicate, and the mean value of CDA activity was taken as the response. CDA assay was performed as per the protocol mentioned earlier. Analysis of variance (ANOVA) was performed on the data to determine the significance of the fitted model and to test the significance of the effect of individual parameters on the CDA production. The most significant parameters affecting the CDA production were identified.

**Optimization of significant parameters affecting CDA production by response surface methodology (RSM)**

Response surface methodology (RSM) central composite design (CCD) was used in this investigation to optimize the concentration of the significant parameters identified by the Placket–Burman design for further fine-tuning of CDA production. These included glucose, yeast extract, peptone, and \( \text{MgCl}_2 \), for enhanced CDA production. This has a few steps, including initial determination of the optimum region for the variables, behavior of the response in the optimum region, and estimation of the optimal condition and verification \([21,22]\).

Each factor in the design was considered at five levels (\(-2, -1, 0, +1, +2\)) (Table 3) in a set of 30 experiments including 8 axial points, 16 factorial points, and 6 center points (Table 4). All experiments were performed in duplicate, and the mean value of CDA activity was taken as the response. CDA assay was performed as per the protocol mentioned earlier. A second-order polynomial equation with interaction terms was then fitted to the data by multiple regression analyses. This resulted in an empirical model that related the response measured to the independent variables of the experiment. The optimum levels of media components including glucose, yeast extract, peptone, and \( \text{MgCl}_2 \) to achieve maximum CDA production were obtained by solving the regression equation, as well as by analyzing the response surface contour plots. Validation of the model was performed under the conditions predicted by the model in triplicates.

**Results and discussion**

The isolate P6B2 was identified as an *Aspergillus flavus* by ITS DNA analysis using NCBI-BLAST and the MEGA6 software. Figure 1 displays the phylogenetic tree generated based on ITS sequences of the fungal strains, which were similar to our isolate as obtained from the BLAST results. The *Penicillium chrysogenum* strain (ATCC10106) was used as the outgroup. Phenotypically the strain produced velvet-textured, floccose, olive-colored colonies with a white border and a creamy-colored underside on PDA plates.

<p>| Table 3. Range of experimental values for the central composite design. |</p>
<table>
<thead>
<tr>
<th>Code</th>
<th>Parameter name</th>
<th>Levels (Values in g L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1</td>
<td>Glucose</td>
<td>–2 30 35 40 45</td>
</tr>
<tr>
<td>X2</td>
<td>Yeast extract</td>
<td>25 30 35 40 45</td>
</tr>
<tr>
<td>X3</td>
<td>Peptone</td>
<td>10 15 20 25 30</td>
</tr>
<tr>
<td>X4</td>
<td>( \text{MgCl}_2 )</td>
<td>1 2 3 4 5</td>
</tr>
</tbody>
</table>

The study of effect of incubation time on CDA production by *Aspergillus flavus* showed that the highest level of CDA was produced at 48 h of incubation as observed in (Figure 2). After this, the level of CDA in the fermentation broth decreased significantly with time. This may be due to either the depletion of nutrients in the fermentation medium or production of inhibitory products in the medium resulting in the inactivation of secretory machinery of the enzymes, or the CDA enzyme playing a significant role only during the growth phase of the fungus. A significant increase in the CDA production is observed only after 24 hr of incubation. *Absidia corymbifera*, *Penicillium oxalicum*, *Rhizopus oryzae*, and *Scopulariopsis brevicaulis* were reported to produce maximum CDA after 3, 4, 5, and 6 days of incubation, respectively.

The size of the inoculum determines total biomass production during fermentation. An increase in the spore concentration in the inoculum ensures a rapid proliferation and biomass synthesis. But after a certain limit with increased spore concentration, the competition for the nutrients results in decreased growth and metabolic activity, which results in decreased production of CDA. When an optimum inoculum size is used for enzyme production, the availability of nutrients is balanced between that which is required for biomass proliferation and that which sustains the production of the enzyme. The inoculum size of 2.0 mL of \( 10^7 \) spores mL\(^{-1}\) resulted in the highest production of CDA by *Aspergillus flavus*. An inoculum size of \( 10^8 \) spores mL\(^{-1}\) and \( 3.6 \times 10^6 \) spores mL\(^{-1}\) was used for production of CDA by *Mortierella sp.* and *Penicillium oxalicum*, respectively.

Among the tested pH levels from 3 to 11, maximum CDA production by the *Aspergillus flavus* strain was observed at an
initial pH of 7. The level of enzyme production decreases rapidly at pH values above and below pH 7 (Figure 3). Initial pH values of 5, 6, 7, 8, and 9 were found to be optimum for CDA production by Mortierella sp.,[12] Bacillus amyloliquefaciens,[17] Scopulariopsis brevicaulis,[15] Penicillium oxalicum,[14] and Absida corymbifera,[13] respectively.

To evaluate the optimum growth temperature for CDA production, the Aspergillus flavus strain was grown at 25–40°C. CDA production was highest at 32°C. The enzyme production progressively decreased at temperatures above and below 32°C (Figure 3). Highest CDA production was observed at incubation temperatures of 28°C for Mortierella sp.[12] and Absida corymbifera,[13] 29°C for Scopulariopsis brevicaulis,[15] 30°C for Rhizopus oryzae,[23] and Penicillium oxalicum[14] and 37°C for Bacillus amyloliquefaciens.[17]

Highest CDA production was observed at a glucose concentration of 10 g L⁻¹. The level of CDA production decreased gradually at glucose concentrations greater than 10 g L⁻¹.

Among the various nitrogen sources amended in the production medium individually, the organic nitrogen sources were comparatively more effective in inducing the CDA production than the inorganic nitrogen sources, as observed in Figure 4. Yeast extract and peptone were the most effective of the organic nitrogen sources, while among the inorganic nitrogen sources sodium nitrate was the best for production.

![Figure 1](image-url) Phylogenetic tree based on ITS sequences of the fungal strains. Numbers at the internodes represent the bootstrap values. The Penicillium chrysogenum strain (ATCC10106) was used as the outgroup.

![Figure 2](image-url) Effect of incubation period on CDA production.

![Figure 3](image-url) Effect of initial pH and incubation temperature on CDA production (filled squares ■, initial pH; filled circles ●, incubation temperature).
of CDA by the Aspergillus flavus strain. However, dihydrogen ammonium phosphate entirely suppressed the CDA production. From earlier reports also it became evident that organic nitrogen sources were more effective in inducing CDA production as compared to inorganic nitrogen sources. Beef extract, yeast extract, and peptone were determined to be best for CDA production by Bacillus amyloliquefaciens, Paenibacillus sp., and Scopulariopsis brevicaulis. Among inorganic nitrogen sources, ammonium sulfate was best at inducing CDA production, albeit lower than organic nitrogen sources.

The results showed that CDA production was enhanced by the addition of Mg$^{2+}$ in the production medium of Aspergillus flavus strain as given in Figure 5. When the concentration of Mg$^{2+}$ was increased from 5 mM to 10 mM, there was increased production of CDA, almost double as compared to that of the control. There was total inhibition of CDA production when Ag$^{+}$, Co$^{2+}$, Hg$^{2+}$, Mn$^{2+}$, and Ni$^{2+}$ were added to the medium even at 5 mM concentration. As reported by Cai et al., Mn$^{2+}$, Mg$^{2+}$, Co$^{2+}$, and Zn$^{2+}$ enhanced the production of CDA while Fe$^{2+}$ and Cu$^{2+}$ inhibited the production of CDA by Scopulariopsis brevicaulis. CDA production was increased by the addition of Ca$^{2+}$ while it was decreased by the addition of Cu$^{2+}$ for Paenibacillus sp.

Nonionic surfactants like Tween 40, Tween 60, and Tween 80 were found to increase CDA production, while Tween 20 and Triton X-100 reduced the CDA production to one-third in comparison to that of the control, as shown in Figure 6. The addition of ionic surfactants like SDS and CTAB to the production medium entirely inhibited the growth of the Aspergillus flavus strain and production of CDA.

The concentration of the media components was optimized using statistical methods. Plackett–Burman screening experimental design was employed to determine the combined influence of media components on the production of CDA by Aspergillus flavus. The components were screened at the confidence level of 95% on the basis of their effects (either positive or negative). If the component shows at or above 95% confidence level and its effect is positive, this indicates that the component is effective in CDA production. But the amount required is higher than the stated high concentration in the Plackett–Burman experiment. As observed in Figure 7, the Pareto chart illustrates the order of significance of the variables affecting the CDA production. Here the factors showing significant positive effect on CDA production are glucose, yeast extract, MgCl$_2$, and peptone, in the order of significance indicated by the Pareto chart. The effect of CuSO$_4$ and ZnCl$_2$ is not significant. The regression equation obtained from the Plackett–Burman design for CDA production is

$$\text{CDA(U)} = 34.797 + 3.882X_1 + 3.696X_2 + 2.200X_3 + 2.658X_4 + 0.432X_5 - 0.765X_6$$

(1)

The four significant factors selected based on the results of Plackett–Burman design, namely, glucose, yeast extract, peptone, and MgCl$_2$, were optimized using CCD. The results generated from the thirty experimental runs were analyzed by standard analysis of variance (ANOVA) as indicated in Table 5. Figure 8 depicts the contour plots of CDA activity, showing interactions between different variables in the CCD.
The quadratic regression equation obtained was

$$\text{CDA (U)} = 50.79 - 1.275X_1 + 1.896X_2 - 1.977X_3 + 1.477X_4$$
$$- 3.093X_1^2 + 2.195X_2^2 - 1.411X_3^2 + 0.029X_4^2$$
$$- 0.976X_1X_2 + 0.150X_1X_3 - 1.634X_1X_4$$
$$+ 0.753X_2X_3 + 1.926X_2X_4 + 0.289X_3X_4$$

The appropriateness of the model fitting was examined by coefficient of determination ($R^2$), which for CDA production was calculated to be 0.9073, which can explain up to 90.73% of the variability of the response. Model terms having values of $p$ less than 0.05 were considered significant, whereas those greater than 0.10 are insignificant. As suggested by the present model, CDA production is significantly affected by the linear and squared terms of glucose, yeast extract, peptone, and MgCl$_2$ and interaction effect of glucose–MgCl$_2$ and yeast extract–MgCl$_2$. However, other interactions including glucose–yeast extract, glucose–peptone, yeast extract–peptone, and peptone–MgCl$_2$, were observed to be insignificant.

Verification of the calculated maximum was carried out with experiments that were performed using the production medium representing the optimum combination found using statistical analysis, as well as in the unoptimized basal medium, under submerged condition. The results predicted by the equation showed that a combination of 30 g L$^{-1}$ glucose, 40 g L$^{-1}$ yeast extract, 15 g L$^{-1}$ peptone, and 7 g L$^{-1}$ MgCl$_2$ would favor CDA production, giving 55.90 U. Under the predicted optimal conditions, CDA production experimentally reached 57.69 ± 1.68 U, while the unoptimized basal medium yielded 9.64 ± 2.04 U. Thus, the CDA production increased 5.98-fold in comparison to that in the basal level medium. The predicted response for CDA production was observed to be very near to the actual response. The excellent correlation between predicted and experimental values justifies the validity of the response model.

A 2.0-fold enhancement in CDA titers was achieved in shake flasks, when the media components were used at their optimum levels by Pareek et al.\cite{25} for a mutant Penicillium oxalicum strain. The optimization was carried out using statistical methods including Plackett–Burman design and RSM central composite design. Similarly, optimization of the physical parameters affecting CDA production by the same Penicillium oxalicum strain was carried out by formulating an RSM central composite design.\cite{26} Similarly, by Plackett–Burman and RSM central composite design, CDA production from Rhodococcus erythropolis HG05 was increased 4.12-fold, as reported by Sun et al.\cite{27}

**Conclusions**

Statistical optimization was carried out to study the combined effects of various media components on CDA production by Aspergillus flavus. The CDA production determined experimentally at optimal conditions predicted by the model equation obtained by statistical analysis was found to be 57.69 U. This was in good agreement with the values predicted by the quadratic model (55.90 U), confirming the validity of the model.
model. A 5.98-fold increase in the CDA production was observed in comparison to the production using unoptimized basal level medium. The results obtained from the current study concluded that *Aspergillus flavus* isolate is a mesophilic fungal strain that can produce a significant amount of CDA enzyme in a short time. In addition to this, by optimization of fermentation conditions under submerged system, significant enhancement in CDA production by *Aspergillus flavus* could be achieved.

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**References**
