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ARTICLE

# Cow dung is an ideal fermentation medium for amylase production in solid-state fermentation by *Bacillus cereus*

**Ponnuswamy Vijayaraghavan** *, **M. Kalaiyarasi, Samuel Gnana Prakash Vincent**

*International Centre for Nanobiotechnology, Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam 629 502, Kanyakumari District, Tamil Nadu, India*

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Cow dung

**Abstract** Amylase production by *Bacillus cereus* IND4 was investigated by solid state fermentation (SSF) using cow dung substrate. The SSF conditions were optimized by using one-variable-at-a-time approach and two level full factorial design. Two level full factorial design demonstrated that moisture, pH, fructose, yeast extract and ammonium sulphate have significantly influenced enzyme production ($p < 0.05$). A central composite design was employed to investigate the optimum concentration of these variables affecting amylase production. Maximal amylase production of 464 units/ml of enzyme was observed in the presence of 100% moisture, 0.1% fructose and 0.01% ammonium sulphate. The enzyme production increased three fold compared to the original medium. The optimum pH and temperature for the activity of amylase were found to be 8.0 and 50 °C, respectively. This enzyme was highly stable at wide pH range (7.0–9.0) and showed 32% enzyme activity after initial denaturation at 50 °C for 1 h. This is the first detailed report on the production of amylase by microorganisms using cow dung as the low cost medium.



## 1. Introduction

Amylases are the most important industrial enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25–30% of the world enzyme market [1]. These enzymes have a great commercial value in biotechnological applications ranging from food, fermentation, textile to paper industries [8]. Submerged fermentation (SmF) is generally used for the production of enzymes including amylases. However, Solid-state fermentation (SSF) replaces SmF as it mimics the natural habitat of microorganisms. SSF is a better choice over SmF due to its simplicity, low capital investment, lower energy requirement, less water output, and lack of foam built up [4,12].

Agrowastes like wheat bran, rice bran, and coconut oil bran have replaced the high cost media generally used in submerged fermentation for amylase preparation because of their simplicity, low cost, easy availability, and lesser water output. Additionally it solves the pollution problem occurring due to their disposal in the surrounding [19]. Recently, various agrowastes

* Corresponding author. Tel.: +91 09486007351.
E-mail address: venzymes@gmail.com (P. Vijayaraghavan).
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were used as the substrates for the production of amylases in SSF. The organisms namely, *Bacillus amyloliquefaciens* (MTCC 1270) [18], *Anoxybacillus flavithermus* sp. [11], *Bacillus licheniformis* ZB-05 [7], *B. amyloliquefaciens* P-001 [5], *Bacillus subtilis* (MTCC 121) [17] and *B. licheniformis* RT7PE1 [21] were used for the production of amylases and enzyme properties were studied. Recently, cow dung was used as the substrate for the production of proteolytic enzymes [23,24]. However, reports on the utilization of cow dung for the production of amylase may be little or perhaps nil. For the maximum enzyme production, medium optimization is a first step for its commercial usage. The present work describes the effects of culture conditions on amylase production in SSF using cow dung substrate and the properties of enzyme by *Bacillus cereus* IND4. Optimal culture conditions and fermentation parameters were assessed by using one variable at a time method followed by $2^5$ full factorial design and CCD. The amylase enzyme was partially characterized for various industrial applications.

## 2. Materials and methods

### 2.1. Microorganism

Around 0.1 g of fermented rice was transferred to an Erlenmeyer flask (100 ml) with 50 mL of sterile double-distilled water, shaken for 20 min, and 1 ml of this solution was resuspended in sterile double-distilled water and aliquots were then spread on nutrient agar plates composed of the following (g/L): peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5 and sodium chloride, 5.0 (pH 7.0). Twelve organisms were isolated and the isolated organisms were screened for amylase activity. The organisms were grown on nutrient agar plate containing 1% soluble starch. After 24 h incubation at 37 °C, 1% iodine was poured on the starch agar plates. A clear zone of hydrolysis around the colony indicates a positive result.

### 2.2. Identification of the amylase enzyme-secreting organism

The isolated strain IND4 with highest activity was identified on the basis of the biochemical properties, the phenotypical characteristics, and the 16S rRNA gene sequencing. The genomic DNA was extracted from the cells of an 18-h cultured IND4 strain by using a QIAGEN DNA purification kit (Germany) according to the manufacturer's instructions. The 16S rRNA gene of the isolate was amplified by polymerase chain reaction (PCR) using the upstream primer P1: 5′-AGA GTTTGATCMTGGCTAG-3′ and the downstream primer P2: 5′-ACGGGCGG TGTGTRC-3′ (Sigma–Aldrich). Amplification of DNA was carried out using the research gradient Peltier Thermal cycler machine PTC-225 and a DNA polymerase (Sigma) under the following conditions: denaturation at 95 °C for 3 min followed by 30 cycles at 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min and 50 s. The amplified product was sequenced at Scigenome Laboratories, India. Sequence comparison with databases was performed using BLAST through the NCBI server. The isolate IND4 was identified as *B. cereus* IND4. The sequence was submitted to the GenBank database, and an accession number was assigned. The GenBank accession number of the sequence reported in this article is KF250420.

### 2.3. Solid state fermentation

Cow dung was obtained from the farm house. It was dried for 7 days, powdered and used as the substrate. Fermentation was carried out in Erlenmeyer flasks (100 ml) with 2.0 gm cow dung substrate, supplemented with carbon source (1%), nitrogen source (1%) and inorganic ion (0.1%). The pH of the medium was adjusted using 0.1 M buffer at various pH range (6.0 to 10.0). Moisture of the medium was adjusted to 100% and autoclaved for 121 °C for 20 min. During the preliminary screening process, the experiments were carried out for 96 h and it was found that after 72 h, maximum enzyme production occurs. Hence, all experiments were carried out for 72 h.

### 2.4. Enzyme extraction and assay

The fermented substrate was mixed thoroughly with 20 ml of sterile distilled water and placed in an orbital shaker at 150 rpm for 30 min. After this, it was centrifuged at $10{,}000\times g$ for 10 min, and the supernatant was used as the crude enzyme. The amylase enzyme was assayed accordingly to the method described by Miller [9] using the UV–visible spectrophotometer (Eltek, India). One unit of amylase activity was defined as the amount of enzyme that releases 1 μg of reducing sugar as glucose per ml per min under the assay conditions.

### 2.5. Statistical optimization of amylase production by *B. cereus IND4*

In this study, maximum amylase production by *B. cereus* IND4 was attained by response surface statistical optimization methods employing different process parameters under SSF. Significance of various medium constituents towards amylase production was tested initially by a full factorial experimental design (FFD). The factors and ranges were selected by one-factor experiments (data not shown). The $2^5$ full factorial design consisted of a set of 32 experimental runs in which the selected five factors (moisture, pH, fructose, yeast extract and ammonium sulphate) were kept either at their high (+) or low (−) levels to find out the most significant factors on amylase production. Table 1a lists the variables and levels in detail. All these experiments were carried out in 100 ml Erlenmeyer flasks containing 2.0 gm of production medium (cow dung) with appropriate media components.

The $2^5$ factorial design was based on the following first-order polynomial model:

**Table 1a** Independent variables and their levels for the $2^5$ factorial experimental design.

| Symbol | Variable name | Units | Coded levels | |
|---|---|---|---|---|
| | | | −1 | +1 |
| *A* | Moisture | % | 80 | 100 |
| *B* | pH | | 7 | 8 |
| *C* | Fructose | % | 0.1 | 1 |
| *D* | Y. extract | % | 0.1 | 1 |
| *E* | A. sulphate | % | 0.1 | 0.5 |

**Table 1b** Results of the $2^5$ factorial design.

| Run | *A*-moisture | *B*-pH | *C*-fructose | *D*-yeast extract | *F*-A. sulphate | Enzyme activity (units/ml) |
|---|---|---|---|---|---|---|
| 1 | 80 | 7 | 0.1 | 0.1 | 0.1 | 218 |
| 2 | 100 | 7 | 0.1 | 0.1 | 0.1 | 85 |
| 3 | 80 | 8 | 0.1 | 0.1 | 0.1 | 192 |
| 4 | 100 | 8 | 0.1 | 0.1 | 0.1 | 34 |
| 5 | 80 | 7 | 1 | 0.1 | 0.1 | 4 |
| 6 | 100 | 7 | 1 | 0.1 | 0.1 | 93 |
| 7 | 80 | 8 | 1 | 0.1 | 0.1 | 77 |
| 8 | 100 | 8 | 1 | 0.1 | 0.1 | 102 |
| 9 | 80 | 7 | 0.1 | 1 | 0.1 | 75 |
| 10 | 100 | 7 | 0.1 | 1 | 0.1 | 31 |
| 11 | 80 | 8 | 0.1 | 1 | 0.1 | 67 |
| 12 | 100 | 8 | 0.1 | 1 | 0.1 | 47 |
| 13 | 80 | 7 | 1 | 1 | 0.1 | 7 |
| 14 | 100 | 7 | 1 | 1 | 0.1 | 105 |
| 15 | 80 | 8 | 1 | 1 | 0.1 | 173 |
| 16 | 100 | 8 | 1 | 1 | 0.1 | 72 |
| 17 | 80 | 7 | 0.1 | 0.1 | 0.5 | 71 |
| 18 | 100 | 7 | 0.1 | 0.1 | 0.5 | 101 |
| 19 | 80 | 8 | 0.1 | 0.1 | 0.5 | 87 |
| 20 | 100 | 8 | 0.1 | 0.1 | 0.5 | 293 |
| 21 | 80 | 7 | 1 | 0.1 | 0.5 | 215 |
| 22 | 100 | 7 | 1 | 0.1 | 0.5 | 260 |
| 23 | 80 | 8 | 1 | 0.1 | 0.5 | 99 |
| 24 | 100 | 8 | 1 | 0.1 | 0.5 | 138 |
| 25 | 80 | 7 | 0.1 | 1 | 0.5 | 94 |
| 26 | 100 | 7 | 0.1 | 1 | 0.5 | 208 |
| 27 | 80 | 8 | 0.1 | 1 | 0.5 | 171 |
| 28 | 100 | 8 | 0.1 | 1 | 0.5 | 175 |
| 29 | 80 | 7 | 1 | 1 | 0.5 | 93 |
| 30 | 100 | 7 | 1 | 1 | 0.5 | 28 |
| 31 | 80 | 8 | 1 | 1 | 0.5 | 27 |
| 32 | 100 | 8 | 1 | 1 | 0.5 | 142 |

**Table 2** ANOVA table for $2^5$ factorial experimental design.

| Source | Sum of squares | df | Mean square | *F* value | *P*-value |
|---|---|---|---|---|---|
| Model | 1.69E+05 | 24 | 26.49 | 60.27 | <0.0001 |
| *A*-moisture | 1860.5 | 1 | 1860.5 | 15.96 | 0.0052 |
| *B*-pH | 1352 | 1 | 1352 | 11.6 | 0.0114 |
| *C*-fructose | 3081.13 | 1 | 3081.1 | 26.43 | 0.0013 |
| *D*-yeast extract | 9591.13 | 1 | 9591.1 | 82.26 | <0.0001 |
| *E*–A. sulphate | 21012.5 | 1 | 21,013 | 180.23 | <0.0001 |
| Residual | 816.12 | 7 | 116.59 | | |
| Cor total | 1.68E+08 | | | | |

$$Y = \alpha_0 + \sum_i \alpha_i x_i + \sum_{ij} \alpha_{ij} x_i x_j + \sum_{ijk} \alpha_{ijk} x_i x_j x_k + \sum_{ijkl} \alpha_{ijkl} x_i x_j x_k x_l + \sum_{ijklm} \alpha_{ijklm} x_i x_j x_k x_l x_m \quad (1)$$

where $Y$ is the response (enzyme activity); $x_i$, $x_j$, $x_k$, $x_l$ and $x_m$ are variables of moisture, pH, fructose, yeast extract and ammonium sulphate, $\alpha_{ij}$, $\alpha_{ijk}$, $\alpha_{ijkl}$, and $\alpha_{ijklm}$ are the *ij*th, *ijk*th, *ijkl*th, and *ijklm*th interaction coefficients, respectively; $\alpha_i$ is the *i*th linear coefficient; and $\alpha_0$ is an intercept.

The cow dung substrate was supplemented with carbon, nitrogen and metal ions at appropriate concentrations predicted by FFD were used for statistical optimization. These statistically designed media were inoculated with the seed culture at 10% (v/w) and incubated at 37 °C for 72 h. At the end of the fermentation, amylase activity in the cell-free medium was estimated. All experiments were performed in duplicate and at two different occasions and the responses considered for analysis represent mean of these responses (Table 1b). Analysis of variance (ANOVA) was used to estimate the significant parameters and the values of "Prob > *F*" < 0.05 indicate that the model terms are significant (Table 2).

The three significant factors (moisture, fructose and ammonium sulphate) that affect amylase enzyme production significantly ($p < 0.05$) were further optimized by CCD. Each of the variables used was analysed at five coded levels ($-\alpha$, $-1$, 0,

+1, +α) (Table 3a). Central point of the CCD is the actual level of variables designed on the basis of initial experiments. A $2^3$ full factorial CCD for three test variables each at five levels with six replicates at the centre points was employed to fit a quadratic model, indicating that 20 experiments were required for the procedure.

The second-order polynomial equation was employed to fit the experimental data. For a three-factor system, the second-order polynomial equation is as follows (2):

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{ij=1}^{3}\beta_{ij} X_{ij} \quad (2)$$

where $Y$ is the response; $\beta_0$ is the offset term; and $\beta_i$, $\beta_{ii}$, and $\beta_{ij}$ are the coefficients of linear terms, square terms, and coefficients of interactive terms, respectively. $X_i$s are $A$, $B$, and $C$; $X_{ij}$s are $AB$, $AC$, and $BC$ ($A$ = moisture; $B$ = fructose; $C$ = ammonium sulphate). The enzyme assay was carried out in duplicates, and the average of these experimental values was taken as response $Y$ (Table 3b). Values of "Prob > $F$" < 0.05 indicate that the model terms are significant. In this model, the $P$ value was < 0.05; hence this model was significant (Table 4).

The model determined an analysis of variance (ANOVA) statistically from the regression model developed from the responses, and the $F$ and $R^2$ (correlation coefficient) values were evaluated for generating three dimensional response surface graphs. The 3D plot was used to understand the interaction of different factors and to predict optimum medium composition for amylase production. The optimal values of the experimental conditions were obtained by solving the regression equation and analysing the response surface plot. All statistical analyses were carried out by Design-Expert software package (version 8.0.0.7, Stat-Ease, Inc., USA).

**Table 3a** Independent variables selected for CCD and RSM.

| Variables | Symbol | Coded values | | | | |
|---|---|---|---|---|---|---|
| | | −α | −1 | 0 | +1 | +α |
| Moisture (%) | *A* | 73.18 | 80 | 90 | 100 | 106.82 |
| Fructose | *B* | −0.21 | 0.10 | 0.55 | 1.0 | 1.31 |
| Sulphate | *C* | −0.02 | 0.01 | 0.06 | 0.1 | 0.13 |

**Table 3b** Experimental design and results of the CCD.

| Run | Moisture (*A*) | Fructose (*B*) | A. sulphate (*C*) | Enzyme activity (units/ml) |
|---|---|---|---|---|
| 1 | 90 | 0.55 | −0.02 | 387 |
| 2 | 80 | 1 | 0.1 | 148 |
| 3 | 90 | 0.55 | 0.06 | 342 |
| 4 | 100 | 1 | 0.01 | 306 |
| 5 | 90 | 0.55 | 0.13 | 174 |
| 6 | 90 | 0.55 | 0.06 | 354 |
| 7 | 106.82 | 0.55 | 0.06 | 331 |
| 8 | 100 | 1 | 0.1 | 138 |
| 9 | 90 | 1.31 | 0.06 | 148 |
| 10 | 80 | 0.1 | 0.1 | 295 |
| 11 | 90 | 0.55 | 0.06 | 346 |
| 12 | 100 | 0.1 | 0.01 | 464 |
| 13 | 90 | 0.55 | 0.06 | 329 |
| 14 | 90 | −0.2 | 0.06 | 451 |
| 15 | 90 | 0.55 | 0.06 | 367 |
| 16 | 73.18 | 0.55 | 0.06 | 266 |
| 17 | 80 | 1 | 0.01 | 264 |
| 18 | 90 | 0.55 | 0.06 | 352 |
| 19 | 100 | 0.1 | 0.1 | 300 |
| 20 | 80 | 0.1 | 0.01 | 396 |

### 2.6. Partial characterization of amylase

The crude amylase sample was used for characterization studies. The optimum temperature for amylase activity was determined at selected temperatures ranging from 30 °C to 70 °C. The temperature stability of the enzyme was evaluated by incubating the enzyme at these temperatures for 1 h at pH 8.0 and the relative enzyme activity was determined. The enzyme activity was also tested for pH optima ranging from 5.0 to 10.0 using 0.1 M buffer systems (succinate buffer, pH 5.0; phosphate buffer, pH 6.0–7.0; *tris*–HCl buffer, pH 8.0 and glycine–NaOH buffer, pH 9.0–10.0). In order to determine the enzyme pH stability, the amylase was incubated in buffer at 37 °C for 1 h after which the remaining enzyme activity was determined. The impact of various metal ions (0.010 M) ($Ca^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Hg^{2+}$, $Fe^{2+}$, $Na^{+}$ and $Zn^{2+}$) was evaluated.

## 3. Results and discussion

### 3.1. Screening of *B. cereus* IND4 for amylase activity

The bacterium *B. cereus* IND4 showed more activity on starch agar plate. It produced approximately an 17-mm zone on the starch agar plate, which was higher than the other isolates. The isolated strain was Gram positive, oxidase positive, rod shaped and catalase positive and had tested negative for citrate utilization, indole formation, and the hydrolysis of urea. It was able to hydrolyse starch and casein and also tested negative for gelatin hydrolysis. A clear zone of hydrolysis observed on the

**Table 4** Results of the regression analysis of the CCD.

| Source | Sum of squares | df | Mean square | *F* value | *P*-value |
|---|---|---|---|---|---|
| Model | 1.70E+05 | 1 | 18894.38 | 104.02 | < 0.0001 |
| *A*-moisture | 3363.26 | 1 | 3363.26 | 18.52 | 0.0016 |
| *B*-fructose | 89988.28 | 1 | 89988.28 | 495.43 | < 0.0001 |
| *C*-A. S | 60266.55 | | 60266.55 | 331.8 | < 0.0001 |
| *AB* | 210.12 | 1 | 210.12 | 1.16 | 0.3074 |
| *AC* | 1653.12 | 1 | 1653.12 | 9.1 | 0.013 |
| *BC* | 45.13 | 1 | 45.13 | 0.25 | 0.629 |
| $A^2$ | 4537.19 | 1 | 4537.19 | 24.98 | 0.0005 |
| $B^2$ | 4358.17 | 1 | 4358.17 | 23.99 | 0.0005 |
| $C^2$ | 8375.48 | 1 | 8375.48 | 46.11 | < 0.0001 |
| Residual | 1816.36 | 10 | 181.64 | | |
| Lack of fit | 1003.03 | 5 | 200.61 | 1.23 | 0.4118 |
| Pure error | 813.33 | 5 | 162.67 | | |
| Cor total | 1.72E+05 | 19 | | | |

starch agar plate showed amylase activity of *B. cereus* IND4. Several reports have described amylase production from this particular genus isolated from various sources [14,3,22].

### 3.2. Cow dung: an ideal substrate for the production of amylase

Cow dung contains high amount of essential nutrients. It consists of nitrogen (1.2–1.6%), cellulose (35.4%), hemicelluloses (32.6%), ash (13.3–13.4%) and growth factors [10]. The selection of an ideal agrobiotech waste for enzyme production depends upon several factors, mainly related to the cost and availability of the substrate material [13]. In the present study, maximum enzyme production (160 units/ml) was observed with cow dung than wheat bran (125 units/ml). Cow dung is cheaper than other agroresidues and the availability is higher than other solid substrates. The results described here is highly significant because no reports so far evidenced on the use of cow dung as the substrate for the production of amylases. Based on this fact, cow dung can be effectively utilized for the production of amylases.

### 3.3. Screening of nutrients and physical factors by FFD

Experiments were carried out based on two level full factorial design and the results obtained were given in Table 1b. From the Table 1b, it was observed that the variation in amylase activity was 4–293 units/ml. The variable factors such as moisture, pH, fructose, yeast extract and ammonium sulphate showed significant effect on amylase production as evident by the correlation coefficient values (ANOVA) obtained after $2^5$ full factorial optimization (Table 2). The equation in terms of the coded factors is given below:

$$\begin{aligned}\text{Enzyme activity} = {} & +112 + 7.63A + 6.50B - 9.81C - 17.31D \\ & + 25.63E + 7.69AC + 22.88AE - 4.94BC \\ & + 8.06BD - 4.00CD - 4.81ABC \\ & - 5.81ABD + 15.75ABE - 8.12ACD \\ & - 21.44ACE - 8.19ADE + 13.00BCD \\ & - 22.69BCE - 28.38CDE + 9.00ABCD \\ & + 11.56ABCE + 13.37ACDE \\ & + 15.13BCDE + 20.50ABCDE\end{aligned}$$

where $A$ is moisture, $B$ is pH, $C$ is Fructose, $D$ is Yeast extract and $E$ is Ammonium sulphate.

The physical factors such as moisture and medium pH strongly influenced amylase production. Among the several factors that are important for microbial growth and enzyme production under SSF using particular substrates, moisture content is one of the most critical factors [13]. In this study, results show that enzyme synthesis is affected by carbon and nitrogen sources and maximal activity is attained with fructose and yeast extract. In *B. subtilis*, yeast extract enhanced amylase production [20]. Rao and Sathyanarayana [16] reported that different carbon sources have a varied influence on the extracellular enzymes especially amylase strains. In the present study, ammonium sulphate was found to be the most suitable inorganic nitrogen source for *B. cereus* IND4. Ramachandran et al. [15] reported that ammonium salts enhanced the production of amylases.

Based on ANOVA, the "*F*-value" for the overall regression model (60.27) is significant at the 5% level. Based on the full factorial design, the optimum levels of following parameters such as 100% moisture, 0.1% fructose, 0.1% yeast extract,

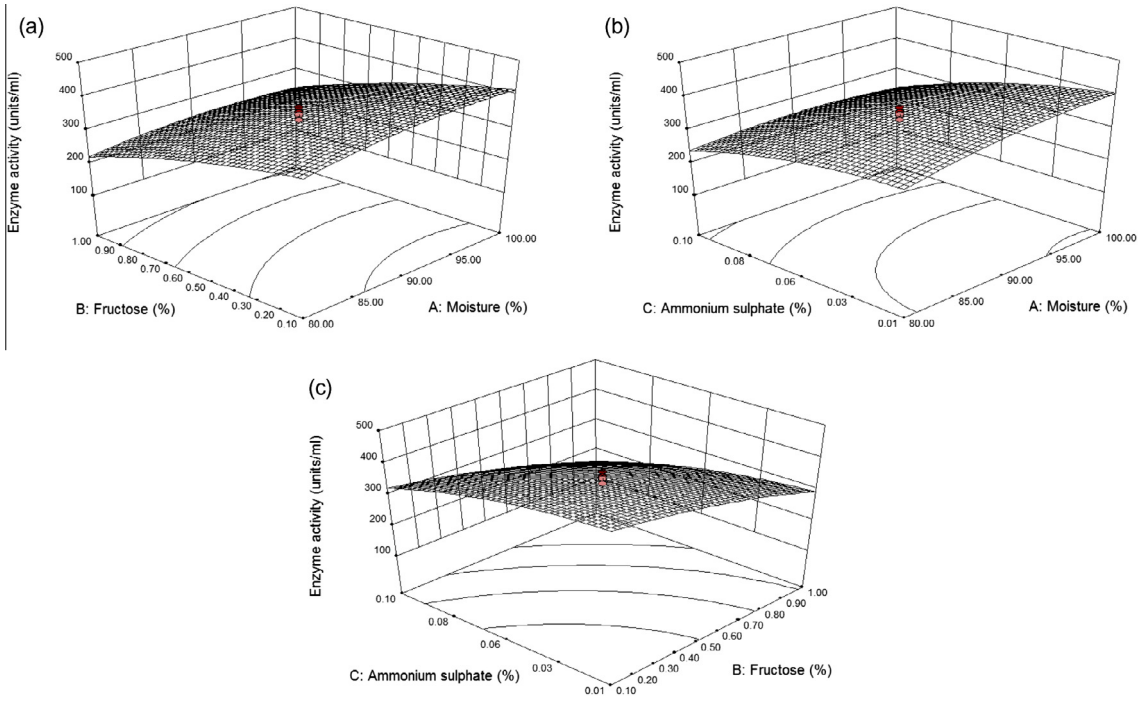


**Figure 1** Response surface plot for amylase production by *B. cereus* IND4. (a) The interactive effects of moisture and fructose; (b) the interactive effects of moisture and ammonium sulphate; (c) the interactive effects of fructose and ammonium sulphate.

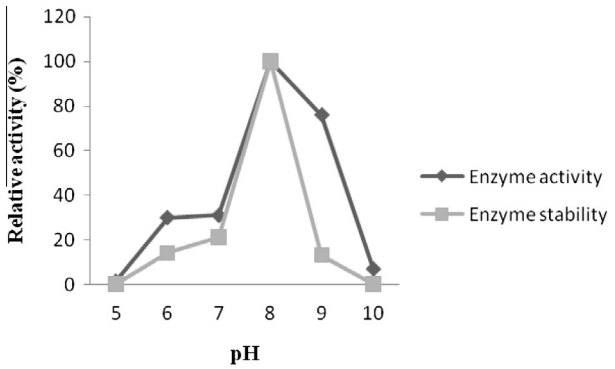


**Figure 2** Effect of pH on enzyme activity and stability.

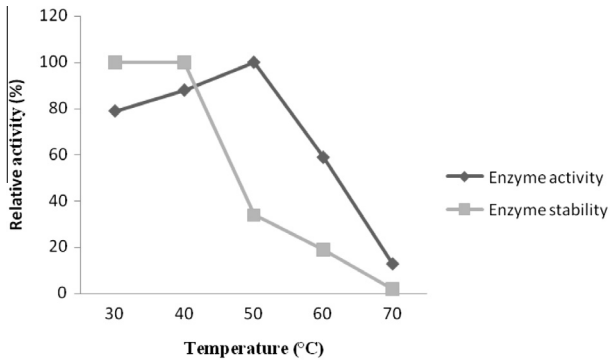


**Figure 3** Effect of temperature on enzyme activity and stability.

0.5% ammonium sulphate and pH 8.0 increased the amylase yield, and hence these parameter levels were maintained for further optimization with CCD. A CCD was developed to understand the interactions among most significant independent variables (moisture, fructose and ammonium sulphate) and their effect on amylase production. The generated CCD experimental design with their response values is listed in Table 3b. The "*F*" test for an ANOVA was developed to understand the statistical significance and reliability of the regression model (Table 4). ANOVA result suggested that, all the model terms except *AB* and *BC*, in the examined range were found to be significant ($p = < 0.05$). The fit value, termed $R^2$ (determinant coefficient), was calculated to be 0.9894 for amylase production by *B. cereus* IND4 suggesting that 98.94% of the variability in the response could be explained by the polynomial model and hence the final equation in terms of coded factor may be written as follows:

$$\text{Amylase activity}(Y) = +348.35 + 15.69A - 81.17B - 66.43C - 5.12AB - 14.37AC - 2.37BC - 17.74A^2 - 17.39B^2 - 24.11C^2$$

where, $Y$ is the response of amylase yield and $A$, $B$, and $C$ are the coded terms for the independent variables of moisture, fructose and ammonium sulphate, respectively.

The three dimensional response surface of significant factors (*AB*, *AC*, *AD* and *BD*) interaction on amylase production generated by the CCD model is shown in Fig. 1a–c, whereas the other two factors were kept constant. The predicted $R^2$ of 0.9487 was in reasonable agreement with the adjusted $R^2$ of 0.9799. An adequate precision value greater than 4 is desirable. The adequate precision value of 35.07 indicates an adequate signal and suggests that the model can be used to navigate the design space. The optimum conditions for the maximum production of amylase were determined by the response surface analysis and also from the regression equation. The CCD model predicted a maximum amylase concentration of 351.7 units/ml, with optimal concentration values of 94.05% for moisture, 0.55% for fructose and 0.055% for ammonium sulphate. Fig. 1a–c shows the response surfaces obtained for the interaction effects of tested variables. Fig. 1a shows the interaction relationship between the two independent variables, namely, moisture/fructose and their effects on amylase production. It was observed from Fig. 1a that amylase production was significantly affected by moisture content. Amylase production was increased with an increase in moisture content up to 95% and further increase in moisture content decreased amylase production.

Validation of the experimental model was tested by carrying out the experiment under optimal experimental conditions. Three repeated experiments were performed and the results were compared with the predicted value. The amylase activity obtained from experiments (345 units/ml) was very close to the predicted response (351.73 units/ml) which proved the validity of the model.

### 3.4. Properties of amylase

The results illustrated in Fig. 2 indicate that the optimum temperature for amylase activity was 50 °C, maintained about 59% of the maximal enzyme activity at 60 °C. The optimum temperature for amylase activity from *B. cereus* IND4 was similar or quite higher to that of optimum temperature of amylase from *Bacillus* sp. AB68 and *B. subtilis* BS5 reported in the previous study [2,6]. The enzyme was highly active at pH 8.0 and it lost its activity considerably at pH 9.0 (Fig. 3). This pH optimum was higher than the pH optimum (6.0) of *B. subtilis* BS5 [6]. The activities of the amylase were stimulated by $Ca^{2+}$, $Mg^{2+}$ and $Na^{+}$ and the enzyme activities were 128%, 103% and 103.5%, respectively. Enzyme activity was inhibited by $Cu^{2+}$, $Co^{2+}$ and $Zn^{2+}$ ions and the relative enzyme activity was 0%, 9%, and 0%, respectively. These results are in accordance with observations made by Femi-Ola and Olowe [6] with *B. subtilis* BS5.

## 4. Conclusion

Cow dung is an ideal substrate for the production of amylases. The response surface methodology was effectively applied for the production of amylases from *B. cereus* IND4 using cow dung substrate in SSF. The statistical design of experiment offers efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments for amylase production by *B. cereus* IND4. Due to its availability and low cost, cow dung may be a key substrate in enzyme bioprocesses.

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