



# STATISTICAL OPTIMIZATION OF EXPERIMENTAL VARIABLES ASSOCIATED WITH PRODUCTION OF ALPHA AMYLASES BY *BACILLUS SUBTILIS* USING BANANA AGRO-RESIDUAL WASTES IN SOLID-STATE FERMENTATION

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## ABSTRACT

The synthesis of  $\alpha$ -amylase using *Bacillus subtilis*, NCIM 2439, was studied using substrate banana waste in the solid-state fermentation medium. The effect of incubation time, inoculum size, initial moisture content, temperature, pH and additional nutrients on the production of alpha-amylase was characterized. The pH, temperature and moisture content in the solid-state fermentation medium were statistically optimized using response surface methodology. A maximum yield of 53.3 U/g was recorded from banana waste with 70% initial moisture content, 8.0 pH, 35°C temperature and additional nutrients (2.0%, ammonium sulphate, 4.0% beef extract, 1.0% glucose and 1.0% sodium chloride) in the medium an inoculum to substrate ratio of 20% (v/w) for 24h incubation. The study provides information on the appropriate temperature, pH and moisture content in the solid-state fermentation, yielding the highest enzyme levels.

**Key words:** Banana waste, solid-state fermentation, optimization, response surface methodology, central composite design.

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## INTRODUCTION

Banana (*musa sapientum*) is grown extensively in tropical and subtropical countries and 37% of the world's production is shared by India. From the banana produce, in addition to the fruit waste, the stem, leaves and pseudostem are also accumulated as waste in the environment posing serious environmental problems. Several attempts have been made to utilize these wastes through ensilaging and to eliminate or reduce the negative nutritional effects. In recent years the new potential of using microorganisms as biotechnological sources of industrially relevant enzyme has stimulated renewed interest in the exploration of extra cellular enzymatic activity in several microorganisms<sup>1-3</sup>. Amylases are important enzymes employed in starch processing industries for the hydrolysis of polysaccharides and also find application in food, baking, brewing, detergent, textile, paper and distilling industry. Alpha amylases (endo-1, 4- $\alpha$ -d-glucanglycohydrolase EC 3.2.1.1) constitute the family of; endo amylases that randomly cleave the 1,4- $\alpha$ -d-glycosidic linkages between adjacent glucose unit in the linear amylose chain with retention of alpha – anomeric configuration in the products.

Production of alpha amylases has been investigated through submerged (SMF) and solid-state fermentation (SSF)<sup>4,5,10</sup>. However, it has been reported that SSF is the most appropriate process in developing countries due to the advantages it offers. In our experiment the production of  $\alpha$ -amylase was investigated through solid-state fermentation using banana agro residual waste (banana peel). The optimization of the process parameters is done to enhance the yield of  $\alpha$ -amylase using response surface methodology. The results achieved in this work gives complete information for utilization of banana agro residual wastes (banana peel) for the production of  $\alpha$ -amylase.

## EXPERIMENTAL

### Microorganism

The lyophilized culture of *Bacillus subtilis* NCIM 2439 was procured from the National Collection of Industrial Microorganism, Pune, India. The organism was maintained on nutrient agar slants and subcultured every 2-3 weeks.

### Preparation of substrate

The banana peel were sliced, spread on trays and oven dried at 50°C for 48 hrs. The dried slices of banana peel were grounded and sieved through standard-mesh sieves to obtain particles of various sizes ranging from 0.4 mm to 3.2 mm that were stored in polyethylene bags at room temperature ( $30 \pm 2^\circ\text{C}$ ) until use.

### Cultivation medium

The mineral salt medium recommended by <sup>12</sup> for alpha amylase production under solid-state fermentation using wheat bran was used in the present study. The composition of the medium used included 1.1 g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 0.61 g of  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 0.3 g of KCl, 0.01 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 100ml distilled water, pH of 8.0 and Banana agro-residual wastes as substrate, added to the medium. This medium was used for optimizing various process parameters. Necessary changes were made in the composition of the medium to provide optimal requirements for maximal enzyme production after standardization.

### Alpha-amylase production using banana agro-residual waste (banana peel) under solid-state fermentation

Procedures used for  $\alpha$ -amylase production by *Bacillus* sp. using wheat bran during SSF reported by <sup>12</sup> are adopted for SSF studies using banana agro-residual wastes as substrate. The following procedure was used, banana agro-residual waste (banana peel of particle sizes ranging from 600-1200  $\mu\text{m}$ ) was weighed 10 g, moisturized in 250ml conical flask with the prepared medium to an initial moisture content of 70%, autoclaved at 121°C for 60 min, cooled to about 30°C and inoculum with 20% (v/w) cell suspension. The content of the flask were mixed thoroughly to ensure uniform distribution of the inoculum and left for incubation in a slanting position at 35°C for 24 h samples were removed after 6 h for analysis. The content were extracted and assayed for alpha amylase.

### Optimization of process parameter

The various process parameters that influence the enzyme yield during SSF were optimized over a wide range. The strategy followed was to optimize each parameter independently of the other, and subsequently optimal conditions were employed in all experiment. The sequential order the various process parameters were optimized for maximal enzyme production: initial moisture content, substrate concentration, pH and incubation temperature. An additional nutrient,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{NaNO}_3$ , peptone, beef extract, yeast extract, glucose, sucrose and lactose, NaCl and KCl, each at concentrations of 1% - 4%, w/w on the basis of the weight of the dry banana fruit peel, inoculum size (10% - 40%) and incubation period (12 - 48 h). The optimization of the values obtained by the above procedure, initial moisture content, substrate concentration, and initial pH of the medium, is conducted using response surface methodology. After optimization of all the process parameters,  $\alpha$ -amylase production under SSF using banana agro-residual wastes, was carried out under optimal conditions as described above.

### Enzyme assays

Alpha amylase was determined according to the method of <sup>8</sup>. The reaction mixture consisted of 1.25 ml 1% (w/v) soluble starch solution, 0.25 ml, 0.1M acetate buffer, pH 6.0, 0.25 ml of distilled water, and 0.25 ml of properly diluted crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars were estimated by the dinitrosalicylic acid (DNS) method <sup>6</sup>. Biochemical analyses of total reducing sugars were also determined by DNS method.

### Central composite experimental design

A  $2^3$ -factorial central composite experimental design with six star- ( $\alpha$ )-points ( $\alpha = 1.682$ ), six replicates at the center point and eight cube points, all in duplicate, leading to 20 sets of experiments, was used to optimize the synthesis of pectinase <sup>7</sup>.

The variables were coded according to the equation

$$x_i = (X_i - \bar{X}_i) / \Delta X_i$$

Where  $x_i$  = Dimensionless value of an independent variable,  $X_i$  = real value of an independent variable,  $\bar{X}_i$  = real value of an independent variable at the center point,  $\Delta X_i$  = step change.

The levels of the independent variables, viz., moisture content, pH and temperature chosen for this study are given in Table 1. The average maximum enzyme activity was taken as the dependent variable or response,  $Y_i$ . Regression analysis was performed on the data obtained. The results of a central composite design are usually used to fit a second-order polynomial equation as it represents the behavior of such systems more appropriately than first-order designs <sup>7</sup>. A second order polynomial of the following form was fitted:

$$\begin{aligned} \hat{Y}_i = & \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 \\ & + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{23} x_2 x_3 + \beta_{31} x_3 x_1 \end{aligned}$$

Where  $\hat{Y}_i$  = predicted response (pectinase),  $\beta_0$  = offset term,  $\beta_1, \beta_2, \beta_3$  = linear effect,  $\beta_{11}, \beta_{22}, \beta_{33}$  = squared effect,  $\beta_{12}, \beta_{23}, \beta_{31}$  = interaction effect.

The proportion of variance explained by the polynomial models obtained, was given by the multiple coefficient of determination,  $R^2$ . Statistica software (Version 6.0, by StatSoft Inc., Tulsa, USA) was used for the study.

#### Abbreviations

SSF: Solid State Fermentation; RSM: Response surface methodology; CCD: Central Composite Design.

### RESULTS AND DISCUSSION

#### Optimization of process parameters for $\alpha$ -amylases production using banana agro-residual wastes under solid-state fermentation

Optimal conditions that favored maximal production of  $\alpha$ -amylase by *B. subtilis* on banana agro-residual wastes (banana peel) were determined as 70% moisture content, pH 8.0. The incubation temperature and substrate concentration of the agro-residual waste (banana peel) was at 35°C and 10g (Table 2). Additions of ammonium sulphate or sodium nitrate at 2.0% (w/w), beef extract, peptone at 4 % (w/w), yeast extract at 2 % (w/w), sugars (glucose, maltose, sucrose) at 1% (w/w) and salts (sodium chloride or potassium chloride) at 1% (w/w), to the production medium containing all the banana agro-residual waste (banana peel) resulted in maximum productivity (Table 1). The optimization of the process parameters that is the initial moisture content 70% and pH 8.0 and substrate concentration of banana peel 10 grams were conducted using response surface methodology (central composite design).

Results obtained for the optimization of process parameters for SSF production of  $\alpha$ -amylase with banana agro residuals as substrate demonstrated clearly the impact of the process parameters on the gross yield of enzymes as well as their independent nature in influencing the organism's ability to synthesize the enzyme.

#### Optimization of screened medium constituents for $\alpha$ -amylase production

Based on the results of the preliminary studies, the selected variables are moisture content, pH and temperature, for the production of  $\alpha$ -amylase. Central composite design (Table 3) was employed to optimize their individual concentrations. Table 4 shows the predicted values and observed values of the enzymatic activities obtained by central composite experimental design. On regression analysis of the experimental data, the following second order polynomial equation was shown to account for  $\alpha$ -amylase production.

$$Y = 52.81 + 4.0354X_1 + 2.3034X_2 - 0.2385X_3 - 6.1415X_1^2 - 10.0871X_2^2 - 10.2657X_3^2 - 0.1275X_1X_2 - 0.6225X_1X_3 - 0.1250X_2X_3$$

Where Y represents the response variable and  $X_1$ ,  $X_2$  and  $X_3$  represent the coded values of moisture content, pH and temperature respectively. From the regression equation the optimized values are calculated by partial differentiating the above equation with respect to  $X_1$ ,  $X_2$  and  $X_3$  and equating to zero. Solving the three equations obtained from partial differentiation gives the optimal values of moisture content, pH and substrate concentration.

The contour plot (Fig.-1) shows the relative effects of moisture content, pH and temperature. The optimal values obtained from the contour plot were almost equal to the results obtained by optimizing the regression equation. To test the goodness of fit of the regression equation, the multiple coefficient of correlation R and the determination coefficient  $R^2$  were evaluated. The coefficient of determination,  $R^2 = 0.89373$ , which indicates a good agreement between the experimental and predicted values <sup>4</sup>. The experiment with three replicates is conducted with the optimal values of moisture content, pH and substrate concentration. The enzyme activity obtained was 53.3 U/g, which is near to the theoretical value 55.36 U/g conforming the fitness and adequacy of the experiment. The results were in well agreement with the enzymatic activities of rice bran and wheat bran as the method adopted for substrate and media preparation, inoculation, incubation and enzyme extraction were similar to those reported by <sup>11-12-13</sup>.

### CONCLUSION

Unlike the classical method of optimizing medium components, statistical techniques were performed, where the levels of variables were changed simultaneously to study their collective effect on  $\alpha$ -amylase production and efficient utilization of banana agro residual wastes. Enzyme synthesis was about 25 % more using the optimized medium (53.3 U/g) than using the unoptimized medium (42.64 U/g). In the present study, the central composite design was proved to be the potent tools in optimizing medium composition for  $\alpha$ -amylase production by *Bacillus subtilis* NCIM 2439 using banana agro residual waste (banana peel).

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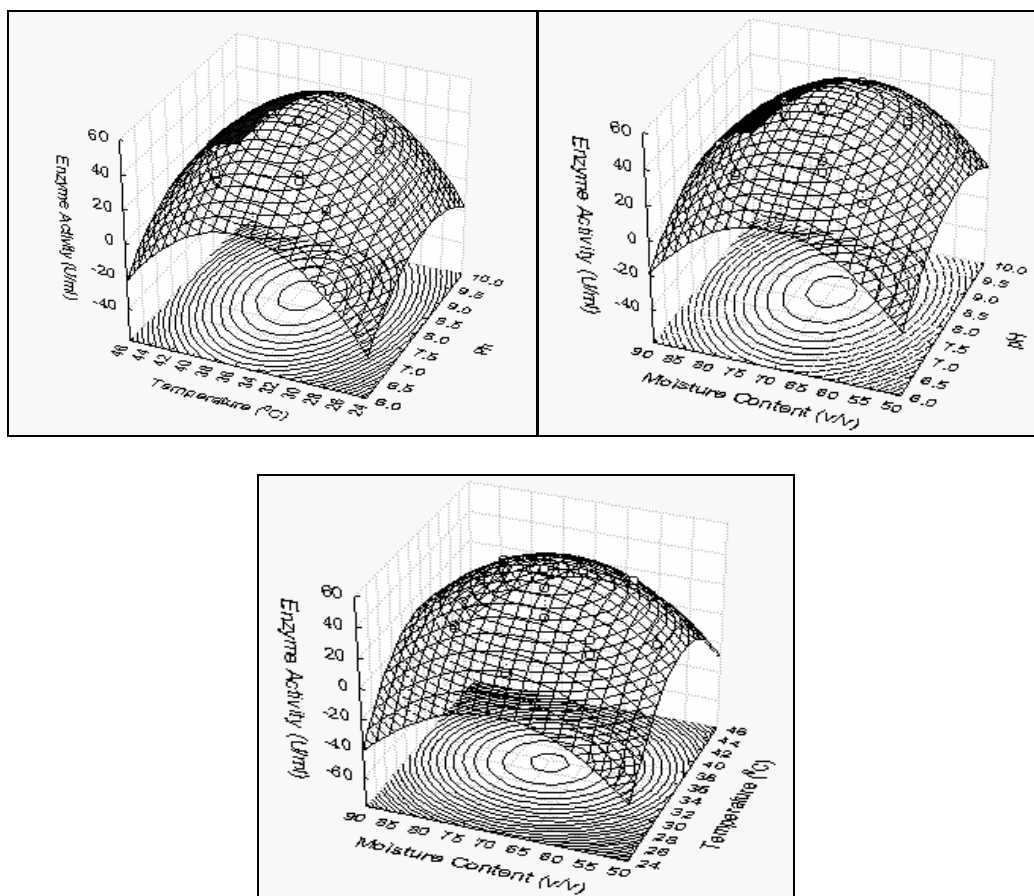


Fig.-1: Effect of pH, temperature and moisture content on the production of  $\alpha$ -amylase using *Bacillus subtilis* NCIM 2439

Table-1: Independent variables (pH, temperature and moisture content) and the levels studied

| Variables | Components                         | Range Studied | Levels of Variables |    |    |    |          |
|-----------|------------------------------------|---------------|---------------------|----|----|----|----------|
|           |                                    |               | $-\alpha$           | -1 | 0  | 1  | $\alpha$ |
| $X_1$     | pH                                 | 6-10          | 6                   | 7  | 8  | 9  | 10       |
| $X_2$     | Temperature ( $^{\circ}\text{C}$ ) | 25-45         | 25                  | 30 | 35 | 40 | 45       |
| $X_3$     | Moisture content (v/v)             | 50-90         | 50                  | 60 | 70 | 80 | 90       |

$X_{i(i=1-3)}$ ; real value of components of the independent variable,  $+\alpha, -\alpha$ : lowest and highest values in the range studied for each variable,  $+1, -1$ : Intermediate values between the central and extreme levels of each variable, 0: Central value in the range studied for each variable.

Table-2: Experimental plan of the optimization design (coded values)

| Runs | pH        | Temperature | Moisture content |
|------|-----------|-------------|------------------|
| 1    | -1        | -1          | -1               |
| 2    | -1        | -1          | 1                |
| 3    | -1        | 1           | -1               |
| 4    | -1        | 1           | 1                |
| 5    | 1         | -1          | -1               |
| 6    | 1         | -1          | 1                |
| 7    | 1         | 1           | -1               |
| 8    | 1         | 1           | 1                |
| 9    | $-\alpha$ | 0           | 0                |
| 10   | $+\alpha$ | 0           | 0                |
| 11   | 0         | $-\alpha$   | 0                |
| 12   | 0         | $+\alpha$   | 0                |
| 13   | 0         | 0           | $-\alpha$        |
| 14   | 0         | 0           | $+\alpha$        |
| 15   | 0         | 0           | 0                |
| 16   | 0         | 0           | 0                |
| 17   | 0         | 0           | 0                |
| 18   | 0         | 0           | 0                |

Table-3: Experimental plan of the optimization design with the experimental and predicted values of  $\alpha$ -amylase activity

| Runs | pH       | Temperature (°C) | Moisture content (v/v) | Observed Response (U/g) | Predicted Response ( $\hat{Y}_i$ ) |
|------|----------|------------------|------------------------|-------------------------|------------------------------------|
| 1    | 7.000000 | 30.00000         | 60.00000               | 23.26                   | 19.34636                           |
| 2    | 7.000000 | 30.00000         | 80.00000               | 25.23                   | 20.36446                           |
| 3    | 7.000000 | 40.00000         | 60.00000               | 32.25                   | 24.45822                           |
| 4    | 7.000000 | 40.00000         | 80.00000               | 27.24                   | 24.97632                           |
| 5    | 9.000000 | 30.00000         | 60.00000               | 33.23                   | 28.91716                           |
| 6    | 9.000000 | 30.00000         | 80.00000               | 26.23                   | 27.44526                           |
| 7    | 9.000000 | 40.00000         | 60.00000               | 35.23                   | 33.51901                           |
| 8    | 9.000000 | 40.00000         | 80.00000               | 34.21                   | 31.54711                           |
| 9    | 6.000000 | 35.00000         | 70.00000               | 20.63                   | 28.65860                           |
| 10   | 10.00000 | 35.00000         | 70.00000               | 40.96                   | 42.23201                           |
| 11   | 8.000000 | 25.00000         | 70.00000               | 16.52                   | 20.41142                           |

|    |          |          |          |       |          |
|----|----------|----------|----------|-------|----------|
| 12 | 8.000000 | 45.00000 | 70.00000 | 22.75 | 28.15919 |
| 13 | 8.000000 | 35.00000 | 50.00000 | 16.81 | 24.18133 |
| 14 | 8.000000 | 35.00000 | 90.00000 | 21.45 | 23.37928 |
| 15 | 8.000000 | 35.00000 | 70.00000 | 53.26 | 52.81607 |
| 16 | 8.000000 | 35.00000 | 70.00000 | 53.45 | 52.81607 |
| 17 | 8.000000 | 35.00000 | 70.00000 | 53.26 | 52.81607 |
| 18 | 8.000000 | 35.00000 | 70.00000 | 52.89 | 52.81607 |

Table-4: Effect of various process parameters for the production of  $\alpha$ -amylase using banana agro-residual waste by *Bacillus subtilis* NCIM 2439

|                             |                         | pH                                                       |      |      |      |                            | Temperature (°C) |      |      |                        | Substrate concentration (g) |      |      |                         | Moisture content (%) |      |      |      |
|-----------------------------|-------------------------|----------------------------------------------------------|------|------|------|----------------------------|------------------|------|------|------------------------|-----------------------------|------|------|-------------------------|----------------------|------|------|------|
| Banana agro-residual wastes | Process Parameter       | 5                                                        | 6    | 7    | 8    | 9                          | 25               | 30   | 35   | 40                     | 5                           | 10   | 15   | 20                      | 60                   | 70   | 80   | 90   |
| Banana peel                 | Reducing Sugars (g/Lt)  | 2.8                                                      | 2.7  | 2.76 | 2.6  | 2.6                        | 2.7              | 2.78 | 2.74 | 2.8                    | 2.6                         | 2.4  | 2.7  | 2.8                     | 2.7                  | 2.46 | 2.84 | 2.82 |
|                             | α-amylase activity(U/g) | 8.8                                                      | 12   | 16   | 28   | 21                         | 8.8              | 16   | 29   | 21                     | 24                          | 34   | 28   | 16                      | 17                   | 34   | 16   | 10.2 |
| Banana peel                 | Process Parameter Conc. | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (%, w/w) |      |      |      | NaNO <sub>3</sub> (%, w/w) |                  |      |      | Peptone (%, w/w)       |                             |      |      | Beef extract (%, w/w)   |                      |      |      |      |
|                             |                         | 1                                                        | 2    | 3    | 4    | 1                          | 2                | 3    | 4    | 1                      | 2                           | 3    | 4    | 2                       | 3                    | 4    | 5    |      |
|                             | Reducing sugars (g/Lt)  | 2.32                                                     | 2.16 | 2.34 | 2.6  | 2.4                        | 2.2              | 2.3  | 2.46 | 2.56                   | 2.64                        | 2.73 | 2.52 | 2.62                    | 2.6                  | 2.46 | 2.7  |      |
|                             | α-amylase activity(U/g) | 34.6                                                     | 53.3 | 27.5 | 26.0 | 24.6                       | 40               | 29   | 21.3 | 9.68                   | 34.6                        | 35.5 | 40.2 | 35.5                    | 39.5                 | 49.7 | 36   |      |
| Banana peel                 | Process Parameter Conc. | Yeast Extract (%, w/w)                                   |      |      |      | Glucose (%, w/w)           |                  |      |      | Sucrose (%, w/w)       |                             |      |      | Lactose (%, w/w)        |                      |      |      |      |
|                             |                         | 1                                                        | 2    | 3    | 4    | 1                          | 2                | 3    | 4    | 1                      | 2                           | 3    | 4    | 1                       | 2                    | 3    | 4    |      |
|                             | Reducing sugars (g/Lt)  | 2.72                                                     | 2.54 | 2.62 | 2.48 | 2.4                        | 2.6              | 2.6  | 2.7  | 2.13                   | 2.4                         | 2.56 | 2.6  | 2.5                     | 2.63                 | 2.7  | 2.82 |      |
|                             | α-amylase activity(U/g) | 8.52                                                     | 28.4 | 27.5 | 26.0 | 34                         | 17               | 14.2 | 10.2 | 32                     | 27                          | 25   | 26   | 23                      | 18.6                 | 18.8 | 19.2 |      |
| Banana peel                 | Process Parameter Conc. | NaCl (%, w/w)                                            |      |      |      | KCl (%, w/w)               |                  |      |      | Inoculum size (%, v/w) |                             |      |      | Incubation Period (hrs) |                      |      |      |      |
|                             |                         | 0.5                                                      | 1    | 1.5  | 2    | 0.5                        | 1                | 1.5  | 2    | 10                     | 20                          | 30   | 40   | 12                      | 24                   | 36   | 48   |      |
|                             | Reducing sugars (g/Lt)  | 2.6                                                      | 2.42 | 2.32 | 2.46 | 2.5                        | 2.44             | 2.3  | 2.5  | 2.7                    | 2.6                         | 2.8  | 2.82 | 2.48                    | 2.46                 | 2.78 | 2.82 |      |
|                             | α-amylase activity(U/g) | 24.6                                                     | 34.6 | 17.7 | 17   | 21                         | 24               | 18   | 16   | 24                     | 35.5                        | 21.3 | 16   | 24.4                    | 32                   | 30   | 29.3 |      |