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RESEARCH ARTICLE

# Optimization of Glucoamylase Production by *Colletotrichum* sp. KCP1 Using Statistical Methodology

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**Abstract** Glucoamylase is a key enzyme used in the food processing as well as in commercial production of glucose from starch. A natural fungal strain identified as *Colletotrichum* sp. KCP1 using 18S rDNA partial genome sequencing has been studied for optimization of glucoamylase production. Media components were screened and optimized through the statistical approach for the synthesis of glucoamylase in solid state fermentation using wheat bran as the substrate. The medium components influencing the enzyme production were identified using Plackett-Burman design. Among various variables screened along with wheat bran as major growth substrate, starch, whey, and casein acid hydrolyasate were found to be most significant. The optimum concentrations of these significant parameters were determined employing the response surface central composite design, revealing starch concentration (1.5 g), whey (0.1 mL), and casein acid hydrolysate (0.1 g) per 5 g of wheat bran for highest enzyme production.

**Keywords:** glucoamylase, *Colletotrichum* sp. KCP1, Plackett-Burman design, response surface methodology, solid state fermentation

## Introduction

Traditionally, glucoamylases have been produced by submerged fermentation (SmF). In recent years, however, solid-state fermentation (SSF) processes have been increasingly applied for the production of this enzyme (1). SSF compared to SmF is more simple, requires lower capital, has superior productivity, reduced energy requirement, simpler fermentation media and absence of rigorous control of fermentation parameters, uses less water and produces lower wastewater, has easier control of bacterial contamination and requires low cost for downstream processing (2,3). In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a result of evaporation and metabolic activities and thus optimum moisture level of the substrate is therefore most important (4). Amylase production and physicochemical parameter optimization using wheat bran has been studied by SmF and SSF (5). Tea waste and copra waste have been reported as substrates for glucoamylase production using *Aspergillus* sp. (6,7). Glucoamylase production by *Aspergillus* sp. using rice flakes manufacturing waste along with wheat bran and rice powder under SSF has been reported by Anto *et al*. (8). Glucoamylase is the major starch-degrading enzyme secreted by *Colletotrichum gloeosporioides* (9). Statistical experimental design has been employed for the production of intermediate temperature stable α amylase from *Aspergillus oryzae* (10).

Conventional approaches for increased microbial metabolite production usually employ manipulation of nutritional requirements, physical parameters, and genetic makeup of the producing strain (11). Development of economical medium requires selection of carbon, nitrogen, phosphorous, potassium, and trace element sources. Nutritional requirement can be manipulated by the conventional or statistical methods. Conventional method involves changing one independent variable at a time while keeping the others at fixed level. However, statistical method offers several advantages over conventional method being rapid and reliable, short lists significant

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nutrients, helps understanding the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously resulting in saving time, glassware, chemicals, and manpower (12). Initial screening of the ingredients is done to understand the significance of their effect on the product formation and then a few better ingredients are selected for further optimization (13). Statistical approach has also been used even for efficient recovery of amylase (14). Fungi from the genus *Colletotrichum* are ascomycetes found in environment, especially in association with the plant, either as pathogens, symbionts, or endophytic. *C. gloeosporioides* was reported to produce amylolytic enzymes through fermentation in semi solid medium composed of residues of the processing of rice (15). Assis *et al*. (16) have reported *C. gloeosporioides* having potential for producing α-amylase and glucoamylase enzymes but no detail statistical studies are available for producing starch hydrolysing enzymes using *Colletotrichum.*

In the present study a statistical approach has been employed in which a Plackett-Burman design is used for identifying various nutrients as significant variables influencing glucoamylase production by *Colletotrichum* sp. KCP1 a natural and novel isolate. The levels of the significant variables are further optimized using response surface methodology.

## Materials and Methods

**Strain isolation and identification** Fungal culture isolated from farm soil samples collected from Vallabh Vidyanagar, Anand, Gujarat, India on potato dextrose agar (PDA) (Himedia, Mumbai, India) was screened for amylase production on starch (Himedia) agar plate. Culture was maintained at 4ºC on Bushnell Hass agar (BHA) (Himedia) slants containing 1% starch (Himedia). Bushnell Hass mineral salt solution has the following composition (g/L): $MgSO_4$ 0.2; $CaCl_2$ 0.02; $KH_2PO_4$ 1.00; $K_2HPO_4$ 1.00; $NH_4NO_3$ 1.00; $FeCl_3$ 0.05 (pH 5.5). Genomic DNA of the isolate was extracted and the 18S rDNA gene was amplified using universal primers (F: 5'-CTG GTT GAT CCT GCC AGT AG-3', R: 5'-CCG CGG CTG CTG GCA CCA GA-3'). Amplification was carried out in a thermal cycler (2720; Applied Biosystems, Foster City, CA, USA) with reaction profile: initial denaturation at 95ºC for 1 min, followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 54ºC for 45 s, extension at 72ºC for 45 s, and finally extension at 72ºC for 5 min. The purified PCR product was sequenced and the phylogenic relationship of the isolate was determine by comparing the sequence data with the existing sequences available through the gene bank database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

**Identifying the significant variables using Plackett-Burman design** The present study was aimed at screening of the important medium components with respect to their main effects by Plackett-Burman design. This experimental design is a 2 factorial design, was used to identify the critical parameters required for prominent glucoamylase production by screening n variables in n+1 experiments (17). The variables chosen for the present study were glucose, starch, whey, sucrose, yeast extract, soyabean meal, and casein acid hydrolysate concentration while having wheat bran as a common substrate (Table 1). The experimental design for the screening of the variables is presented in Table 2. The Plakett-Burman design assumes that there are no interactions between the different media constituents. All the variables were denoted as numerical factors and investigated at 2 widely spaced intervals designated as -1 (low level) and +1 (high level). The effects of individual parameters on glucoamylase production were calculated by the following equation:

$$E(Xi)=2(M^{+}-M^{-})/N \quad (1)$$

where, $E$ is the effect of parameter under study; $M^{+}$ and $M^{-}$ are responses (glucoamylase activities) of trials at which the parameter was at its higher and lower levels, respectively; $N$ is the total number of trials.

Experimental error was estimated by calculating the variance among the dummy variables as

$$\text{Veff}=\Sigma(E\text{d})^2/\text{n} \quad (2)$$

where, Veff is the variance of the effect of level; $E$d is the effect of level for the dummy variables; and n is the number of dummy variables used in the experiment. The standard error (SE, $E$s) of concentration effect was the square root of variance of an effect, and the significance level ($p$-value) of each concentration effect was determined using the Student's $t$-test:

$$t(Xi)=E(Xi)/E\text{s} \quad (3)$$

where, $E(Xi)$ is the effect of variable $Xi$.

**Response surface methodology (RSM)** The levels of the significant parameters and the interaction effects between various medium constituents which may influence the glucoamylase production significantly were analysed and optimized by response surface central composite design (CCD). RSM is useful for small number of variables (up to 5) but is impractical for large number of variables, due to high number of experimental runs required. The concentrations of the 3 major components starch, casein acid hydrolysate, and whey (identified by Plackett-Burman design) were optimized, keeping temperature, pH, and inoculum size constant.

According to the design, the total number of treatment

**Table 1. Medium components and their variables used in Placket-Burman design for glucoamylase production using *Colletotrichum* sp. KCP1**

| Variable | Medium component | +Value | -Value |
|---|---|---|---|
| X1 | Glucose | 1.5 g | 0.5 g |
| X2 | Starch | 1.5 g | 0.5 g |
| X3 | Whey | 5.0 mL | 0.5 mL |
| X4 | Sucrose | 1.5 g | 0.5 g |
| X5 | Yeast extract | 2.0 g | 0.2 g |
| X6 | Soyabean meal | 2.0 g | 0.2 g |
| X7 | Casein acid hydrolysate | 2.0 g | 0.2 g |

combinations is $2^k$+2k+no, where k is the number of independent variables and no is the number of repetition of experiments at the central point. Each factor in the design was studied at 5 different levels (–α, –1, 0, +1, +α) as shown in Table 3. All variables were set at a central coded value of zero. The minimum and maximum ranges of variables were determined on the basis of our previous experiments. The full experimental plan with respect to their values in actual and coded form is listed in Table 4. Enzymatic activity was measured in triplicate in 20 different experimental runs. The glucoamylase production was analyzed by using a second order polynomial equation, and the data were fitted into the equation by multiple regression procedure. The model equation for analysis is given as:

$$Y = \beta_0 + \beta iXi + \beta iiXi^2 + \beta ijXiXj \quad (4)$$

where, $\beta_0$, $\beta i$, $\beta ii$, and $\beta ij$ represent the constant process effect in total, the linear, quadratic effect of *Xi,* and the interaction effect between *Xi* and *Xj*, respectively for the production of glucoamylase. Later, an experiment was run using the optimum values for variables given by response optimization to confirm the predicted value and amylase production was confirmed.

**Software and data analysis** The results of the experimental design were analyzed and interpreted using Design-Expert version 8.0 (Stat-Ease Inc., Minneapolis, MN, USA) statistical software.

**Enzyme extraction and assay** The experiments were performed according to the design matrix (Table 2, 4) in 250-mL Erlenmeyer flasks containing 5 g wheat bran as solid substrate. All experimental flasks were harvested after 5 days interval and amylase was extracted with 50 mL of 0.05 M sodium acetate buffer (pH 5.0) on a rotary shaker at 150 rpm for 30 min at 25ºC. The content was filtered through muslin cloth, centrifuged at 3,578×*g* for 25 min and the clear supernatant was used for determining amylase activity, which was expressed in Units/g dry substrate (U/gds). The reaction mixture consisted of 1.0 mL of 1% soluble starch, 0.9 mL 0.1 M acetate buffer (pH

**Table 2. Plackett-Bueman design generated by fractional rotation of full factorial design where X1 to X7 are independent variable and D1 to D4 are dummy variable**

| Run | Components | | | | | | | | | | | Glucoamylase (U/gds) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | X1 (w/w) | X2 (w/w) | X3 (v/w) | X4 (w/w) | X5 (w/w) | X6 (w/w) | X7 (w/w) | D1 | D2 | D3 | D4 | |
| 1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 51.75 |
| 2 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | 16.88 |
| 3 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 9.13 |
| 4 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | 43.14 |
| 5 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | -1 | 32.16 |
| 6 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 1 | 25.06 |
| 7 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 1 | 21.83 |
| 8 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | 58.43 |
| 9 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | -1 | 32.81 |
| 10 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | 38.83 |
| 11 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | -1 | 1 | -1 | 1 | 19.89 |
| 12 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 45.51 |

**Table 3. Experimental range and levels of the independent variables of selected components used for response surface central composite design**

| Variable | Components | Range | Levels of variable studied | | | | |
|---|---|---|---|---|---|---|---|
| | | | -α | -1 | 0 | +1 | +α |
| X1 | Starch (w/w) | 1.50-2.50 | -1.15 | 1.50 | 2.0 | 2.50 | 2.84 |
| X2 | Whey (v/w) | 0.10-0.20 | -0.06 | 0.10 | 0.15 | 0.20 | 0.23 |
| X3 | Casein acid hydrolysate (w/w) | 0.05-0.10 | -0.03 | 0.05 | 0.075 | 0.10 | 0.11 |

5.0), and 0.1 mL of enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) (Himedia) method of Miller (18). The color developed was read at 560 nm using a spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). Glucose (Himedia) was used as the standard. One unit (1 U) of glucoamylase is defined as the amount of enzyme releasing 1 μmol of glucose equivalent/min under the assay conditions. TLC analysis of reaction products of *Colletotrichum* sp. KCP1 amylolytic enzymes on starch indicated that it is glucoamylase since the major end product was glucose.

## Results and Discussion

**Identification of the fungal isolate** A 560 bp sized 18S rDNA sequence of the isolate was obtained through PCR amplification and sequencing. The sequence was subjected to a multiple sequence alignment using the BLAST programme of NCBI. The sequence showed a homology of 99% with *Colletotrichum* sp. The sequence was deposited in the gene bank of the NCBI (Accession no. GU353321). The phylogenetic tree as shown in Fig. 1 was drawn using bioinformatics software MEGA 4.0.

**Screening of parameters using Plackett-Burman design** A statistical approach has been used to screen the most effective supplement and select their concentrations to achieve highest possible glucoamylase production by *Colletotrichum* sp. KCP1 on wheat bran as a substrate. Usually, initial screening of the ingredients is done to understand the significance of their effect on the product formation and then a few better ingredients are selected for future optimization. A marked enhancement in production of amylase by *Bacillus amyloliquefaciens* in flask fermentation using statistical methods was reported by Zhao *et al*. (19). Use of Placket-Burman design to screen different nutrients affecting production of thermostable β-amylase and pullulanase by *Clostridium thermosulfurogenes* SV2 has been reported by Reddy *et al*. (20). Plackett-Burman design was used to screen 7 different medium components as carbon and nitrogen sources as 12 run experiment with 2 level of concentration of each variable. Studies were carried out as solid state fermentation using wheat bran as common solid substrate. The independent variables and their respective high and low concentrations used in the optimization study are represented in Table 1, whereas the Plackett-Burman experimental design for 12 trials with 2 level of concentration of each variable is given in Table 2, which was followed for the optimization of medium components for glucoamylase production. The variables X1-X7 represented the medium constituents and D1-D4 represented the dummy variables/unassigned variables. The results of Plackett-Burman experiment with respect to glucoamylase production, the effect, standard error, t (xi), *p*, and confidence level of each component are represented in Table 5. The components were screened at a confidence level of 95% on the basis of their effects. When components show significance at or above 95% confidence level and its effect is negative, it is considered effective for production but the amount required may be

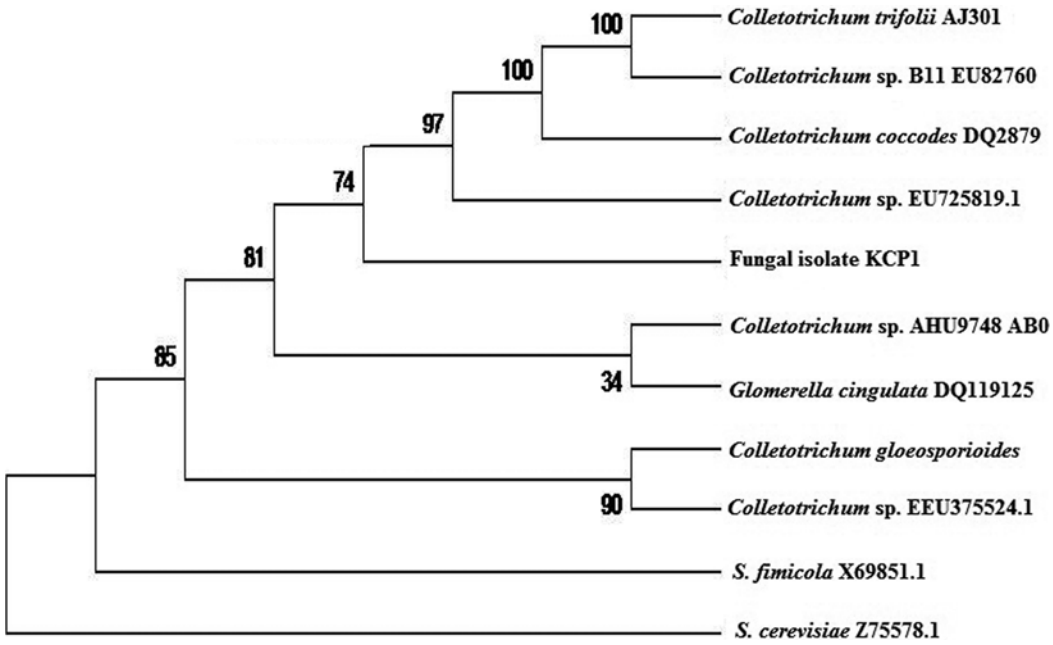


**Fig. 1. Phylogenetic relationship on the basis of homology index for a new fungal isolate *Colletotrichum* sp. KCP1.**

**Table 4. Full experimental central composite design with coded and actual level of variables and the response function**

| Run no. | A: Starch (w/w) | | B: Whey (v/w) | | C: Casein acid hydrolysate (w/w) | | Glucoamylase (U/gds) | |
|---|---|---|---|---|---|---|---|---|
| | Actual level | Coded level | Actual level | Coded level | Actual level | Coded level | Observed | Predicted |
| 1 | 1.5 | -1 | 0.1 | -1 | 0.05 | -1 | 29.062 | 27.803 |
| 2 | 2.5 | +1 | 0.1 | -1 | 0.05 | -1 | 20.451 | 21.483 |
| 3 | 1.5 | -1 | 0.2 | +1 | 0.05 | -1 | 37.458 | 41.461 |
| 4 | 2.5 | +1 | 0.2 | +1 | 0.05 | -1 | 28.416 | 33.526 |
| 5 | 1.5 | -1 | 0.1 | -1 | 0.1 | +1 | 65.659 | 59.560 |
| 6 | 2.5 | +1 | 0.1 | -1 | 0.1 | +1 | 29.277 | 24.286 |
| 7 | 1.5 | -1 | 0.2 | +1 | 0.1 | +1 | 54.25 | 52.229 |
| 8 | 2.5 | +1 | 0.2 | +1 | 0.1 | +1 | 15.069 | 15.340 |
| 9 | 1.1 | -α | 0.15 | 0 | 0.075 | 0 | 55.972 | 58.692 |
| 10 | 2.8 | +α | 0.15 | 0 | 0.075 | 0 | 23.680 | 22.358 |
| 11 | 2 | 0 | 0.06 | -α | 0.075 | 0 | 29.062 | 35.315 |
| 12 | 2 | 0 | 0.23 | +α | 0.075 | 0 | 44.131 | 39.277 |
| 13 | 2 | 0 | 0.15 | 0 | 0.032 | -α | 24.541 | 18.781 |
| 14 | 2 | 0 | 0.15 | 0 | 0.11 | +α | 23.034 | 30.193 |
| 15 | 2 | 0 | 0.15 | 0 | 0.075 | 0 | 24.756 | 28.197 |
| 16 | 2 | 0 | 0.15 | 0 | 0.075 | 0 | 17.868 | 28.197 |
| 17 | 2 | 0 | 0.15 | 0 | 0.075 | 0 | 31.861 | 28.197 |
| 18 | 2 | 0 | 0.15 | 0 | 0.075 | 0 | 36.166 | 28.197 |
| 19 | 2 | 0 | 0.15 | 0 | 0.075 | 0 | 15.930 | 28.197 |
| 20 | 2 | 0 | 0.15 | 0 | 0.075 | 0 | 42.840 | 28.197 |

**Table 5. Statistical analysis of components for glucoamylase production by *Colletotrichum* sp. KCP1**

| Component | Effect | Standard error | *t*-value | *p* | Confidence (%) |
|---|---|---|---|---|---|
| Glucose | -1.292 | 3.829 | -0.340 | 0.751 | 24.898 |
| Starch | 14.711 | 3.829 | 3.871 | 0.018 | 98.202 |
| Whey | -16.000 | 3.829 | -4.211 | 0.013 | 98.643 |
| Sucrose | -3.301 | 3.829 | -0.869 | 0.434 | 56.596 |
| Yeast extract | -4.019 | 3.829 | -1.057 | 0.350 | 65.008 |
| Soyabean meal | -1.435 | 3.829 | -0.378 | 0.725 | 27.515 |
| Casein acid hydrolyste | -16.289 | 3.829 | -4.287 | 0.013 | 98.722 |

lower than the indicated as low (–1) concentration in Plackette-Burman experiment. If the effect is found positive, a higher concentration than the indicated high value (+) concentration is required. In our experiment starch, whey and casein acid hydrolysate gave confidence level >95% and could be considered significant. Remaining components as glucose, sucrose, yeast extract, and soyabean meal showed confidence level <95% and were considered insignificant in the study. From the present study using PB design, starch, whey, and casein acid hydrolysate were short listed and studied further for optimization of their concentration requirement and to check their interaction effect. Methodology of Plackett-Burman was thus found to be very useful for determination of relevant variable for further optimization. Starch increases the glucoamylase production with a significant value. Thus presence of starch as an additional carbon source was found to have inductive effect, and also has remarkable efficiency in the production of enzyme, being an inexhaustible source of carbon compared to other carbon sources.

**RSM** The central composite design was employed to study the interaction among the significant factors and also determine their optimal levels. In the present work, experiments were planned to obtain a quadratic model consisting of $2^3$ triails. The plan includes 20 experiments and 2 levels of concentration for each factor. In order to study the combined effect of these variables, experiments were performed at different combinations. The central composite experimental plan along with the predicted and observed response for each individual experiment is summarized in Table 4. It shows the production of glucoamylase (U/gds) corresponding to combined effect of all 3 components in the specified ranges.

**Table 6. Analysis of variance of quadratic model for glucoamylase production using *Colletotrichum* sp. KCP1**

| Source | SS[1)] | df | MS | *F*-value | Prob > F | |
|---|---|---|---|---|---|---|
| Model | 2855.564 | 9 | 317.285 | 3.833 | 0.024 | Significant |
| A-Starch | 1593.565 | 1 | 1593.565 | 19.254 | 0.001 | Significant |
| B-Whey | 18.949 | 1 | 18.949 | 0.229 | 0.643 | |
| C-Casein H | 157.196 | 1 | 157.196 | 1.899 | 0.198 | |
| AB | 1.303 | 1 | 1.303 | 0.016 | 0.903 | |
| AC | 419.192 | 1 | 419.192 | 5.065 | 0.048 | Significant |
| BC | 220.281 | 1 | 220.281 | 2.661 | 0.134 | |
| $A^2$ | 273.790 | 1 | 273.790 | 3.308 | 0.099 | |
| $B^2$ | 149.145 | 1 | 149.145 | 1.802 | 0.209 | |
| $C^2$ | 24.794 | 1 | 24.794 | 0.299 | 0.596 | |
| Residual | 827.646 | 10 | 82.764 | | | |
| Lack-of-fit | 267.302 | 5 | 53.460 | 0.477 | 0.782 | Not significant |
| Pure error | 560.344 | 5 | 112.069 | | | |
| Correlation Total | 3683.210 | 19 | | | | |

[1)]SS, sum of squares; df, degrees of freedom; MS, mean square; $R^2$=0.87; CV=28.01%; Adequate precision=6.87

The optimum levels of the selected variables were obtained by solving the regression equation and by analyzing the response surface contour and surface plots (21). The regression equation obtained after the analysis of variance (ANOVA) provides an estimate of the level of glucoamylase production as a function of starch, whey, and casein acid hydrolysate concentration.

The production of glucoamylase may be best predicted by the following model:

$$\text{Glucoamylase}=(28.20)-(10.8021A)+(1.18B)+(3.39C)-(0.40AB)-(7.24AC)-(5.25BC)+(4.36A^2)+(3.22B^2)-(1.31C^2) \quad (5)$$

where, Y is glucoamylase production (U/gds); A is starch concentration (w/w); B is volume of whey (v/w); C is casein acid hydrolysate concentration (w/w).

The statistical significance of the second-order model equation was evaluated by *F*-test ANOVA which revealed that this regression is statistically highly significant for glucoamylase production. The model *F*-value of 3.83 implies that the model is significant. There is only a 2.39% chance that a large 'model *F*-value' could occur due to noise. Values of 'prob>*F*' less than 0.050 indicate that the model terms are significant. In this case A, AC are significant model terms (Table 6). The 'lack-of-fit *F*-value' of 0.48 implies the lack of fit is not significant relative to the pure error. Non-significant lack of fit is good for the model to fit. The $R^2$ value (multiple correlation coefficient) closer to 1 denotes better correlation between observed and predicted values. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case a low CV (28.01) denotes that the experiments performed are reliable. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In our case, the ratio is of 6.874, which indicates an adequate signal. This model can be used to navigate the design space.

The effect of interaction of variables on enzyme (glucoamylase production) yield was studied against any 2 independent variables while keeping the other independent variables at their constant level. These response surface plots or contour plots can be used to predict the optimal values for different test variables. Therefore, 3 response surfaces were obtained by considering all the possible combinations. Three-dimensional response plot shown in Fig. 2A describes the behaviour of glucoamylase production, main effect, interaction effect, and squared effect (nonlinear) of whey and starch at different concentrations. Lower and higher levels of both the whey and starch concentration did not result in higher enzyme yields. The shape of the response surface curves showed a moderate interaction between these tested variables. As observed in the contour plot, the middle level of whey and lower level of starch resulted in higher enzyme production. The 3-dimensional curve and contour plot of the calculated response surface from the interaction between starch and casein acid hydrolysate while keeping fixed concentration of whey are shown in Fig. 2B. Both components at their lower level did not result in the higher enzyme yield while starch at the lower level and casein acid hydrolysate at their higher level showed the maximum enzyme activity. The interaction plot of casein acid hydrolysate and whey is shown in Fig. 2C, where the shape of the response surface indicates positive interaction between these 2 factors. The enzyme yield was found to increase with simultaneous increase in both the factors.

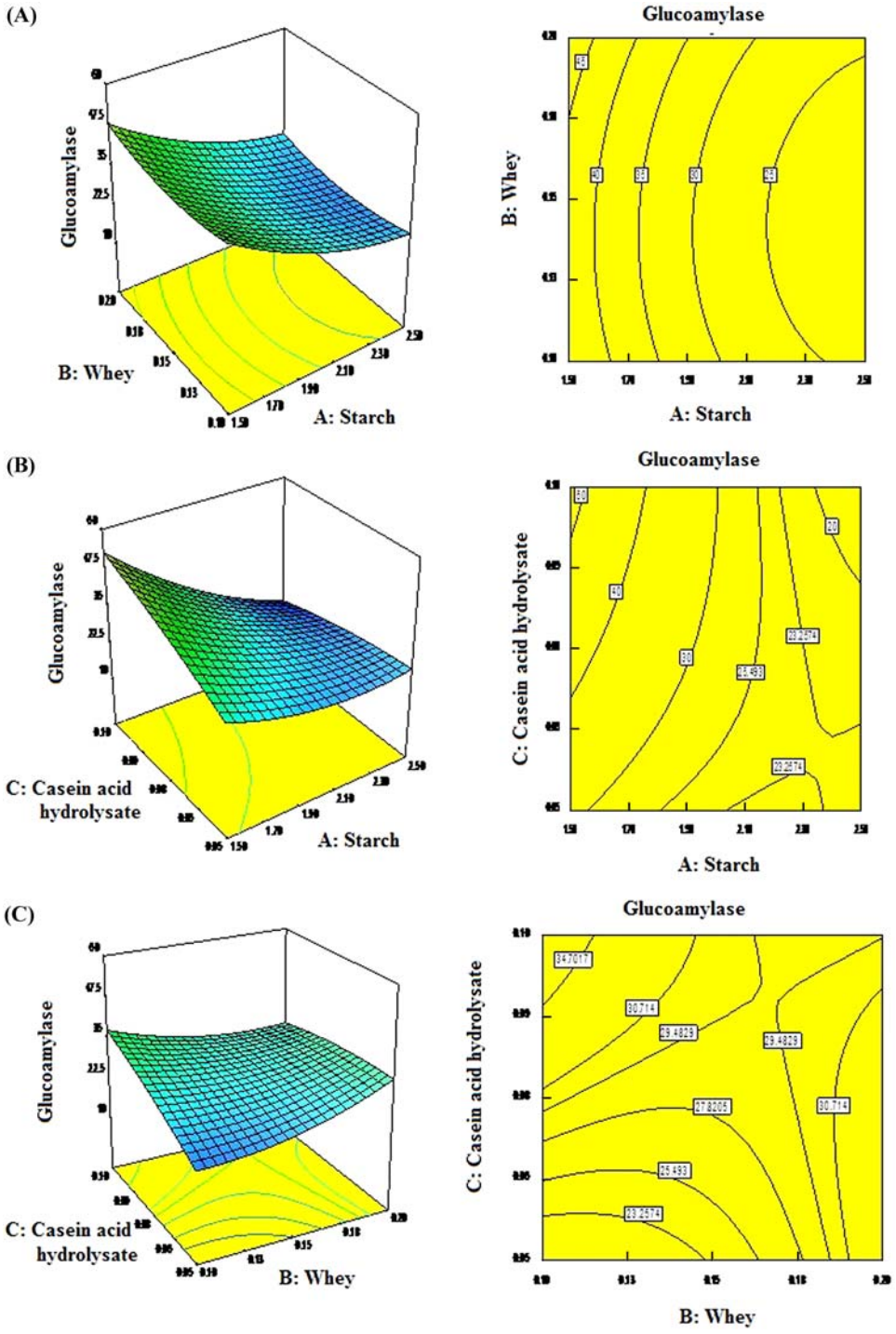


**Fig. 2. Response surface graph showing interaction effects between concentration of starch and whey (A), starch and casein acid hydrolysate (B), and casein acid hydrolysate and whey (C).**

**Validation of the model** Validation was carried out under conditions predicted by the model. The optimal concentrations estimated for each variable were 1.5 g starch, 0.1 mL whey, and 0.1 g casein acid hydrolysate per 5 g of wheat bran. The predicted amylase production obtained from the model using the above optimum concentration of medium components was 59.56 U/gds. To validate the prediction of the model, additional experiments in triplicate were performed with the optimized medium. These experiments yielded the maximum amylase activity of 60.38 U/gds. Agreements between the predicted and experimental results verified the validity of the model and the existence of the optimal points. The CCD helped in identifying significant levels that influenced the glucoamylase

production by *Colletotrichum* sp. The carbon and nitrogen sources are the most important factors affecting enzyme production.

It has also been applied for the production of various enzymes, such as cyclodextrin glucanotransferase (CGTase) (22,23), chitinase (13), α-amylase (24), pectinase (25), lipase (26), and vitamin riboflavin (27). In the present study, starch, whey, and casein acid hydrolysate were identified to be significant, and individual as well as interaction effects of these components were studied.

To the best of our knowledge, it is for the first time that statistical approach has been employed and showed significant results for screening and optimizing the medium components for maximal glucoamylase production under SSF using a *Colletotrichum* sp. This has also allowed rapid screening of a number of nutrients influencing glucoamylase production. The enzyme yield and the production were found to be significantly influenced by starch, whey, and casein acid hydrolysate concentration. The data obtained after optimization has resulted in 60.38 U/gds enzyme production.

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