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APPLICATION OF PLACKETT-BURMAN EXPERIMENTAL DESIGN TO OPTIMIZE THE COLD-ACTIVE ALPHA AMYLASE BIOSYNTHESIS BY PSYCHROTROPHIC *STREPTOMYCES* 4 ALGA

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The aim of this work was to optimize the cultural and production parameters through the statistical approach for the synthesis of cold active alpha amylase by *Streptomyces* 4 Alga in submerged fermentation. The process parameters influencing the enzyme production were identified using Plackett-Burman design. Among the various variables screened, the yeast extract and $CaCl_2$ were the most significant. The optimum levels of these significant parameters were determined employing the Response Surface Methodology and the Central Composite Design. The most significant variables were determined as follows: yeast extract (5.00 g%) and $CaCl_2$ (0.25 g%). By using the optimal fermentation medium, the cold-active alpha amylase production was increased up to 23.96 AU, an approximate 4.4-fold improvement over the previous production (5.44 AU) with un-optimized medium. The cold-active alpha amylases could be used in bioethanol production at lower temperatures, waste-water treatment and bioremediation in cold climates.

Keywords*: Streptomyces* sp., alpha amylase, response surface methodology (RSM), submerged fermentation, Plackett-Burman design, cold-adapted alpha amylase production

Introduction

Alpha amylase (E.C. 3.2.1.1) which catalyses the hydrolysis of α-D-(1,4) glycosidic linkages in starch components and related carbohydrates constitutes a class of industrial enzymes having approximately 25% of the enzyme market (Gangadharan *et al*., 2008; Syed *et al*., 2009). The possibility of using actinomycetes, specifically *Streptomyces*, for enzyme production has recently been investigated. Starch hydrolyzing activity was widely distributed in species of *Streptomyces* and some of them can attack and hydrolyze raw starch granules with the release of maltose as the predominant product. Such enzymes are used for the industrial conversion of raw starch into sugar for fermentation (Syed *et al*., 2009). *Streptomyces* species served as an important source for numerous secondary

metabolites, enzymes and antibiotics mainly due to their shorter generation time, and the ease of genetic and environmental manipulation (De Azeredo *et al*., 2003; Ahmed *et a*l., 2008). Cold-active amylase has applications in different industries; *i.e.* baking, brewing, starch liquefaction and distillery and also in textile, detergents and bioremediation (Morita *et al*., 1997; Chessa *et al*., 1999; Gerday *et al*., 2000; Haki and Rakshit, 2003; Haq *et al*., 2003; Kar and Ray, 2007; Marx *et al*., 2007).

Designing an appropriate fermentation medium is of crucial importance because medium composition can significantly affect product yield (Gao *et al*., 2009).

The traditional 'one-factor at a time' technique used for optimizing a multivariable system is not only time consuming but also often easily misses the alternative effects between components (Kumar and Satyanarayana, 2007). Also, this method requires carrying out a number of experiments to determine the optimum levels. These drawbacks of the single factor optimizing process can be eliminated by optimizing all the affecting parameters collectively by Central Composite Design (CCD) using Response Surface Methodology (RSM) which is the one suitable for identifying the effect of individual variables and for seeking the optimum conditions for an efficient multivariable system (Bandaru *et al*., 2006; Kar and Ray, 2007). RSM may be summarized as a collection of experimental strategies, mathematical methods and statistical inferences for constructing and exploring an approximate functional relationship between a response variable and a set of design variables (Gangadharan *et al*., 2008). Response surface methodology has already been successfully applied for optimization of the media and culture conditions in many cultivation processes for the production of primary and secondary metabolites including amino acid, ethanol and enzymes such as α-amylase, xylanase, chitinase, protease, transglutaminase, and α-galactosidase (Morita *et al*., 1997; Bandaru *et al*., 2006; Kar and Ray, 2007; Khurana *et al*., 2007; Nawani and Kapadnis, 2007; Macedo *et al*., 2007; Anisha *et al*., 2008; Gangadharan *et al*., 2008; Abou-Elela *et al*., 2009; Tanyildizi *et al*., 2009).

A statistical approach has been employed in the present study for which a Plackett-Burman design is used for identifying significant variables influencing cold-active alpha amylase production under submerged fermentation (SmF) by psychrotropic *Streptomyces* 4 Alga. The levels of the significant variables were further optimized using response surface methodology and central composite design.

Materials and Methods

Microorganism

Psychrotrophic *Streptomyces* 4 Alga was isolated from Antarctic vegetation samples from Progress Lake 2 (East Antarctica) (Cotarlet et al., 2011). The strain was grown on Gause-agar slants (pH 7.0) containing (g%): starch 2.0; K_2HPO_4 0.5; $MgSO_4 \cdot 7H_2O$ 0.5; KNO_3 1.0; NaCl 0.5; $FeSO_4 \cdot 7H_2O$ 0.01; agar 25.0.

Optimization of process parameters

Identifying the significant variables using Plackett-Burman design

The present study was aimed at screening the important medium components with respect to their main effects by Plackett–Burman design. The Plackett–Burman experimental design is a two factorial design, which identifies the critical physico-chemical parameters required for elevated cold-active alpha amylase production by screening *n* variables in *n* + 1 experiments (Plackett and Burman, 1946). The variables chosen for the present study were starch, glycerol, maltose, malt extract, glucose, urea, sodium caseinate, yeast extract, peptone, $CaCl_2$ and KCl concentrations. The experimental design for the screening of the variables is described in Table 1. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level).

Table 1. Placket-Burman experimental design for screening significant process variables affecting cold-active alpha-amylase

Std	A*	B	C	D	E	F	G	H	I	J	K
1	2.00	1.25	0.50	1.00	1.00	0.20	0.50	2.00	2.00	3.00	1.00
2	0.75	1.25	1.00	0.50	1.00	0.20	2.00	2.00	2.00	1.00	3.00
3	2.00	0.50	1.00	1.00	0.50	0.20	2.00	5.00	2.00	1.00	1.00
4	0.75	1.25	0.50	1.00	1.00	0.10	2.00	5.00	5.00	1.00	1.00
5	0.75	0.50	1.00	0.50	1.00	0.20	0.50	5.00	5.00	3.00	1.00
6	0.75	0.50	0.50	1.00	0.50	0.20	2.00	2.00	5.00	3.00	3.00
7	2.00	0.50	0.50	0.50	1.00	0.10	2.00	5.00	2.00	3.00	3.00
8	2.00	1.25	0.50	0.50	0.50	0.20	0.50	5.00	5.00	1.00	3.00
9	2.00	1.25	1.00	0.50	0.50	0.10	2.00	2.00	5.00	3.00	1.00
10	0.75	1.25	1.00	1.00	0.50	0.10	0.50	5.00	2.00	3.00	3.00
11	2.00	0.50	1.00	1.00	1.00	0.10	0.50	2.00	5.00	1.00	3.00
12	0.75	0.50	0.50	0.50	0.50	0.10	0.50	2.00	2.00	1.00	1.00

**A-starch (g%), B-glycerol (g%), C-maltose (g%), D-malt extract (g%), E-glucose (g%), F-uree (g%), G-sodium caseinate (g%), H-yeast extract (%), I-peptone (g%), J-$CaCl_2$ (g%), K-KCl (g%)*

Response surface methodology

The levels of the significant parameters and the interaction effects between various medium constituents were analysed and optimized by Central Composite Design (CCD). In this study, the experimental plan consisted of 11 trials and the independent variables were studied at three different levels, low (-1), medium (0) and high (+1). The experimental design used for the study is shown in Table 2. All the experiments were done in triplicate and the average of cold-active alpha amylase production obtained was taken as the dependent variable or response (Y). The second order polynomial coefficients were calculated and analysed using the 'Design Expert' software (Version 8.0.2.0, Stat-Ease Inc., Minneapolis, USA) statistical package. The general form of the second degree polynomial Eq. (1) is

$$Y_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j, \quad (1)$$

where Y_i is the predicted response, $x_i x_j$ are input variables which influence the response variable Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient.

Table 2. Experimental design and results of CCD of response surface methodology for the optimization of cold-active alpha-amylase production

Run no.	Independent variables, g%	
	Yeast extract (H)	$CaCl_2$ (J)
1	5.00	0.25
2	10.00	0.25
3	5.00	1.00
4	10.00	1.00
5	3.96	0.63
6	11.04	0.63
7	7.50	0.09
8	7.50	1.16
9	7.50	0.63
10	7.50	0.63
11	7.50	0.63

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The contour plot and response surface curve were generated using 'Design Expert' software (Version 8.0.2.0, Stat-Ease Inc., Minneapolis, USA).

Enzyme production and assay

The experiments were performed according to the design matrix (Table 1) in 250 ml Erlenmeyer flask containing 100 ml medium. The samples were withdrawn at specific intervals, centrifuged at 9000 rpm for 15 min at 4°C and the supernatant was used for enzyme assay. Alpha-amylase assay was determined using a method based on the difference of the hydrolysis products in 0.1 N Lugol solution. One unit of α-amylase (AU) is the amount of enzyme which generates a 0.05 decrease of optical density (OD_{610} nm)/min, of the coloured iodine-starch complex from a 1 g% starch solution, at pH 7.0 and 20ºC (Cotarlet and Bahrim, 2011).

Results and discussion

Screening of parameters using Plackett-Burman design

The experiment was conducted in 12 runs to study the effect of the selected variables. Table 3 represents the results of the screening experiments using Plackett-Burman design. Statistical analyses of the responses were performed and they are represented in Table 4. The model F value of 17.81 implies that the model is significant. The values of Prob < 0.05 indicate model terms are significant. Regression analysis was performed on the results and a first order polynomial

equation was derived representing cold-active alpha amylase production as a function of the independent variables

$$Y = 11.78 + 3.23\ H - 3.23\ J \quad (2)$$

Table 3. Observed and predicted responses for the experiments performed using Plackett-Burman design

Run no.	Alpha-amylase activity	
	Observed (AU)	Predicted (AU)
1	6.33	4.71
2	14.91	15.49
3	15.05	14.49
4	30.04	26.46
5	14.93	15.51
6	7.58	5.96
7	7.44	8.00
8	15.03	14.55
9	7.49	9.12
10	7.56	8.04
11	7.57	8.05
12	7.47	8.03

The magnitude of the effects indicates the level of the significance of the variable on cold-active alpha amylase production. Among the variables screened yeast extract and $CaCl_2$ concentrations were identified as the most significant variables influencing cold-active alpha amylase production (Table 4).

Table 4. Statistical analysis of the model

Source	Sum of squares	Degree of freedom	Mean square	F value	Prob>F
Model	502.51	8	62.81	17.81	0.0187
H	124.81	1	124.81	35.39	0.0095
J	125.07	1	125.07	35.47	0.0095
Residual	10.58	3	3.53		
Corr.Total	512.09	11			

$R^2 = 0.97954$; Adj $R^2 = 0.9244$; Coefficient of variance = 15.94%.

IN literature, it was found that yeast extract is the preferred nitrogen source of *Streptomyces* strains (Suutari *et al*., 2002; Gill *et al*., 2003; Tuncer *et al.*, 2004; Lazim *et al*., 2009; Niladevi *et al*., 2009). In this study, as shown by PBD and RSM results, yeast extract as an important factor has a negative effect, and redundant yeast extract may block alpha-amylase synthesis. On the basis of data analysis, we appropriately decreased the amount of yeast extract and finally obtained a satisfactory result. Most of the alpha amylases are metalloenzymes and in most of the cases, Ca^{2+} ions are required for maintaining the spatial conformation of the

enzyme, thus they play an important role in enzyme stability (Gangadharan *et al.*, 2008). Thus the two variables yeast extract and $CaCl_2$ concentrations were selected and their optimal levels were identified using response surface methodology.

Response surface methodology

The Central Composite Design was employed to study the interactions among the significant factors and also determine their optimal levels (Table 5). The other variables in the study were maintained at a constant level which gave maximal yield in the Plackett–Burman experiments. Therefore, the media for submerged cultivation had the following composition, (g%): starch 0.75, glycerol 0.88, maltose 0.77, malt extract 0.78, glucose 1.00, urea 0.14, sodium caseinate 2.00, peptone 4.65 and KCl 1.00. The pH was adjusted to pH 7.0. The submerged cultivation was done at 20°C and 230 rpm.

Table 5. Observed and predicted responses obtained for Central Composite Design

Run no.	Alpha-amylase activity	
	Observed (AU)	Predicted (AU)
1	12.06	12.37
2	12.00	12.32
3	12.08	10.21
4	12.00	12.15
5	12.03	12.81
6	11.97	12.73
7	12.05	11.28
8	5.91	8.22
9	5.95	7.98
10	5.96	7.98
11	12.04	7.98

Multiple regression analysis was used to analyze the data and thus a polynomial equation was derived from regression analysis as follows:

$$Y = 7.98 - 0.021\ H - 2.17\ J - 5.000E\text{-}003\ HJ + 2.39\ H^2 + 0.88\ J^2 + 2.18\ I^2H - 0.014\ IH^2 \quad (3)$$

The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher's statistical analysis and the results are shown in Table 6. The model F value of 0.75 implies the model is not very significant and also shows that there is 66.27% chance that the model F value could occur due to noise. The R^2 value (multiple correlation coefficient) closer to 1 denotes better correlation between the observed and predicted values. In this case the value of R^2 (0.6358) indicates poor correlation between the experimental and predicted values. The coefficient of variation (CV) indicates the degree of precision to which the experiments are compared. The lower reliability of the experiment is usually indicated by a high value of CV. In the present case, a low CV (30.22) denotes that

the experiments performed are unwell reliable. The P values denote the significance of the coefficients and they are also important in understanding the pattern of the mutual interactions between the variables. The interaction effects and optimal levels of the variables were determined by plotting the response surface curve.

Table 6. Analysis of variance (AVOVA) for the cubic model

Source	Sum of squares	Degree of freedom	Mean square	F value	Prob>F
Model	51.44	7	7.35	0.75	0.6627
H_1	1.800E-003	1	1.800E-003	1.800E-004	0.9900
J_2	18.85	1	18.85	1.92	0.2599
HJ	1.000E-004	1	1.000E-004	1.018E-005	0.9977
H^2	32.38	1	32.38	3.30	0.1670
J^2	4.42	1	4.42	4.45	0.5504
H^2J	9.47	1	9.47	0.96	0.3985
HJ^2	3.802E-004	1	3.802E-004	3.871E-005	0.9954
H^3	0.000	0			
J^3	0.000	0			
Residual	29.46	3	9.82		
Lack of Fit	4.77	1	4.77	0.39	0.5974
Pure error	24.68	2	12.34		
Corr.Total	80.90	10			

R^2 = 0.6358; Coefficient of variance = 30.22%.

The response surface and contour curves are represented in Figures 1–2. Figure 1 represents the surface interaction between yeast extract and $CaCl_2$ concentrations. Higher levels of both variables did not result in higher enzyme yields.

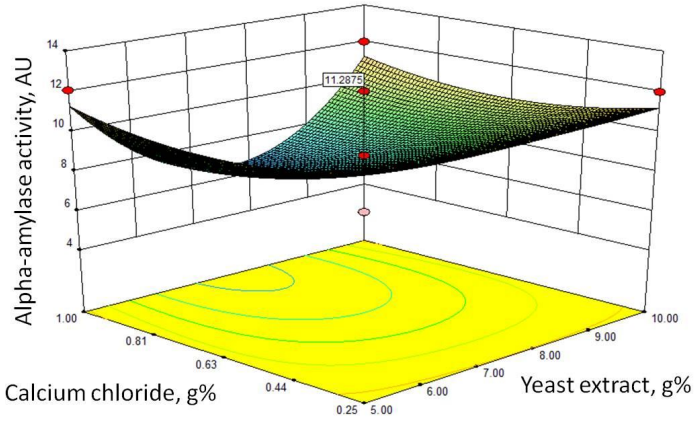

Figure 1. Response surface graph showing interaction between yeast extract and $CaCl_2$ concentrations

The shape of the contour surface curve showed a moderate interaction between these tested variables (Figure 2). Maximum enzyme production was recorded at minimum levels of both factors while further increase in the levels resulted in a gradual decrease in yield.

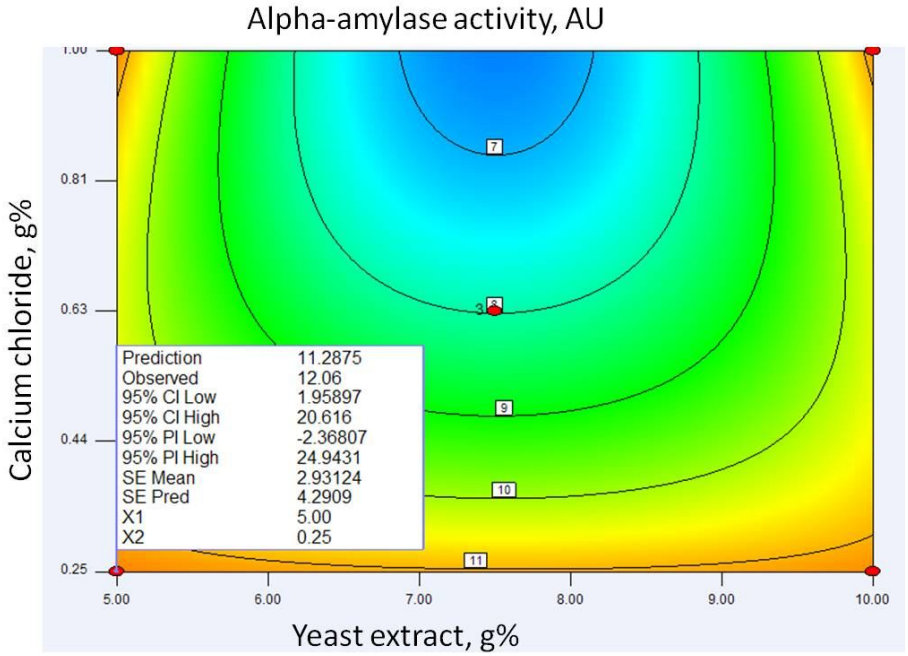

Figure 2. Contour graph showing interaction between yeast extract and $CaCl_2$ concentrations

Validation of the model

Validation was carried out under conditions predicted by the model. The model was successfully validated as the values predicted by the model were in good agreement with the results obtained. The time course of cold-active alpha amylase for both cases, before and after optimization, is also depicted in Figure 3. The optimal levels of the process variables for cold-active alpha amylase production under SmF by *Streptomyces* 4 Alga were 5.00 g% yeast extract and $CaCl_2$ 0.25 g%. A production of 23.96 AU, which was in agreement with the prediction, was observed in verification experiment. Compared to the production of original level (5.44 AU) a 4.4-fold increase in cold-active alpha-amylase production was obtained.

Conclusions

The statistical approach showed significant results for optimizing the process parameters for maximal cold-active alpha amylase production under SmF using psychrotrophic *Streptomyces* 4 Alga.

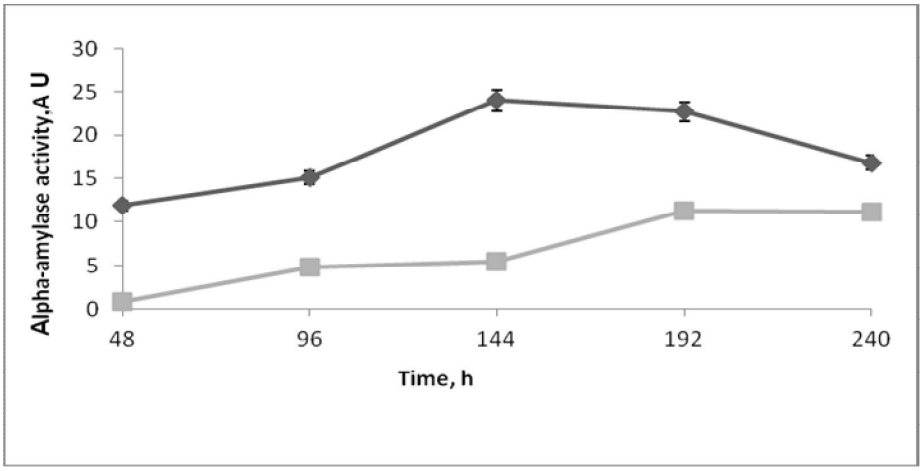

Figure 3. Time course of cold-active alpha amylase production in optimized (♦) and non-optimized (■) medium

The present study identified the effect of various process parameters on the enzyme yield and the production was found to be signficantly influenced by yeast extract and $CaCl_2$ concentrations. The results of this study have indicated RSM is an effective method for maximum production of cold-active alpha amylase with *Streptomyces* 4 Alga. The studied enzyme could be used in bioethanol production at lower temperatures, waste-water treatment and bioremediation in cold climates.

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