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Response Surface Methodology for the Optimization of Alpha Amylase Production by *Serratia marcescens* SB08

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Abstract. In this work, central composite design combining with response surface methodology was successfully employed to optimize medium composition for the production of alpha amylase by *Serratia marcescens* SB08 in submerged fermentation. The process parameters that influence the enzyme production were identified using Plackett-Burman design. Among the various factors screened, inoculum concentration, pH, NaCl and $CaCl_2$ were found to be most significant. The optimum level of pH was 5.0, inoculum concentration 3%, NaCl 0.30 g/l and $CaCl_2$ 0.13 g/l. The actual enzyme yield before and after optimization was 56.43 U/ml and 87.23 U/ml, respectively. Thus, it is advisable to the microbial industry sponsors to apply such profitable bioprocess to maintain high yield for mass production of α amylase.

Keywords: alpha amylase; fermentation; *Serratia marcescens;* Plackett-Burman design; central composite design

Introduction

Alpha amylase catalyses hydrolysis of α-D-(1,4) glycosidic linkages in starch components and related carbohydrates. It is a key enzyme in the production of starch derivatives and also can be used in desizing fabrics, baking industry, pharmaceuticals and detergents. The growth and enzyme production of the organism are strongly influenced by medium composition thus optimization of media components and cultural parameters are the primary tasks in a biological process (Djekrif-Dakhmouche *et al*., 2006). The main strategy used is media engineering for which the operating conditions parameters are optimized by changing one parameter at a time and keeping the others at a constant level (Liu and Tzeng, 1998). Optimization studies do not take into consideration effects of interaction among different variables as any process can be influenced by several variables (Silva and Roberto, 2001). Limitations of single factor optimization can be eliminated by employing response surface methodology (RSM) which is used to explain the combined effects of all the factors in a fermentation process (Elibol, 2004). Single variable optimization methods are not only tedious, but also can lead to misinterpretation of results, especially because the effect of interaction between different factors are overlooked (Wenster-Botz, 2000). Response surface methodology may be summarized as a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables.

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Statistical methods are applied for optimization of alpha amylase (Kunamneni *et al*., 2005; Ahuja *et al*., 2004; Francis *et al*., 2002; Dey *et al*., 2001). No defined medium has been established for the optimum production of alpha amylase by different microbial sources. Each organism has its own special conditions for maximum enzyme production. A statistical approach has been employed in the present study for which a Plackett-Burman design is used for identifying significant variables influencing alpha amylase production by *Serratia marcescens* SB08 (GenBank Accession Number: AB061685). The levels of significant variables were further optimized using response surface methodology.

Materials and Methods

Microorganism and culture maintenance. Potent strains of bacteria were isolated from the gut of sulphur butterfly (*Kricogonia lyside).* The sulphur butterfly was washed with 70% ethanol and with sterile distilled water several times to eliminate surface bacteria. All dissections were performed under sterile conditions. After disrupting the walls, the contents of the stomach were collected in sterile eppendorf tube, containing phosphate-buffered saline; contents were serially diluted, spread onto the surface of nutrient agar plates and incubated for 48 h at 30 °C in order to record total colony forming units (CFU/ml). The *Serratia marcescens* SB08 isolated from the gut of sulphur butterfly was maintained at 4 °C on nutrient agar slants and subcultured every 2 to 4 weeks.

Amylase production. Five ml starch broth was inoculated with 1 ml of inoculum and was incubated at 30 °C for 18 h. This 5 ml

of 18 h old culture was then transferred to 95 ml of sterile starch broth medium and was incubated for 30 °C for 24 h. After incubation, the crude enzyme was obtained by centrifugation of the culture broth at 10,000 rpm for 10 min and this Cell Free Filtrate (CFF) was stored at -20 °C.

Alpha-amylase assay. Amylase production was assayed in terms of amylase activity exhibited by the culture supernatant. The reaction mixture containing 0.1 ml of crude enzyme and 1.0 ml (1.0%) solution of soluble starch in 50 mM phosphate buffer (pH 7.5) was incubated at 50 °C for 5 min. The reaction was stopped by addition of 1.0 ml of 1 N NaOH. The level of amylase activity was determined by measuring the reducing sugar released from soluble starch (Nelson, 1944). One unit of amylase activity was determined by measuring the amount of enzyme which liberates 1 µmol of reducing sugar as glucose per min under the conditions of assay. The experiment was performed in triplicate.

Optimization of process parameters. ***Screening of important nutrient components using Plackett-Burman design.*** This study was performed using Plackett-Burman design for screening medium components with respect to their main effects but not their interaction effects (Plackett and Burman, 1946) on enzyme production by *Serratia marcescens* SB08. The medium components were screened for eleven variables at two levels, maximum (+) and minimum (-). According to the Plackett-Burman design, the number of positive sign (+) is equal to (N+1)/2 and the number of negative sign (-) is equal to (N-1)/2 in a row. A column should contain equal number of positive and negative signs. The first row contains (N+1)/2 positive signs and (N-1)/2 negative signs and the choice of placing the signs is arbitrary. The next (N-1) rows are generated by shifting cyclically one place (N-1) times and the last row contains all negative signs. The experimental design and levels of each variable is shown in Table 1. The medium was formulated according to the design and the flask culture experiments for each enzyme were assayed as described earlier. Response was calculated as the rate of enzyme production, expressed as U/ml. All experiments were performed in triplicate and the average of the rates of enzyme production was considered as the response.

The effect of each variable was calculated using the following equation:

$$E = (\sum M_+ - \sum M-)/N$$

where:

E is the effect of tested variable, M_+ and M- are responses (enzyme activities) of trials at which the parameter was at its higher and lower levels, respectively, and N is the number of experiments carried out.

The standard error (SE) of the variables was the square root of variance and the significance level (P- value) of each variable was calculated using Student's t-test.

$$t = E_{xi} / SE$$

where:

E_{xi} is the effect of the tested variable. The variables with higher confidence level were considered to influence the response or output variable.

Optimization of concentrations of the selected medium components using response surface methodology. Response surface methodology is an empirical statistical modelling technique employed for multiple regression analysis using quantitative data obtained from factorial design to solve multivariable equations simultaneously (Rao *et al*., 2000). The screened medium components affecting enzyme production were optimized using central composite design (CCD) (Box and Hunter, 1957; Box and Wilson, 1951).

According to this design, total number of treatment combinations is $2^k + 2k + n0$, where 'k' is the number of independent variables and n0, the number of repetitions of the experiment at the centre point. For statistical calculation, the variable X_i has been coded as x_i according to the following transformation:

$$x_i = X_i - X_o / \delta X$$

where x_i is dimensionless coded value of the variable X_i, X_0 is the value of the X_i at the centre point, and δX is the step change. A 2^k-factorial design with eight axial points and six replicates at the center point with a total number of 30 experiments was employed for optimizing the medium components.

Behaviour of the system was explained by the following quadratic equation:

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where:

Y is the predicted response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} is the interaction effect. The regression equation was optimized for maximum value to obtain the optimum conditions using Design Expert Version 7.1.5 (State Ease, Minneapolis, MN).

Validation of the experimental model. The statistical model was validated with respect to alpha amylase under the conditions predicted by the model in shake flask conditions. Samples were withdrawn at the desired intervals and alpha amylase assay was determined as described above.

Results and Discussion

Plackett-Burman design. The influence of eleven medium factors namely pH, temperature, agitation, inoculum concentration, incubation time, sucrose, peptone, KH_2PO_4, yeast extract, NaCl and $CaCl_2$ in the production of alpha amylase was investigated in 12 runs using Plackett-Burman design. Table 1 represents the Plackett-Burman design for 11 selected variables and the corresponding response for alpha amylase production in 12 runs. Variations ranging from 24.35-79.07 U/ml in the production of alpha amylase in the 12 trials were observed by Plackett-Burman design.

Alpha amylases can be produced by submerged fermentation (Aguilar *et al*., 2000; Egas *et al*., 1998; Bose and Das, 1996). Alpha amylase production by *Serratia marcescens* SB08 was subjected to response surface methodology and pH, inoculum concentration, NaCl and $CaCl_2$ were found to be the positive factors. These factors have a positive effect on the production of alpha amylase. These findings support other investigations of the same enzyme belonging to other microbial species (whether fungal or bacterial) (Narang and Satyanarayana, 2001; Ilori *et al*., 1997).

Supplementation of metal ions has been reported to provide good growth and also influences higher alpha amylase production (Sivaramakrishnan *et al*., 2006). 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 playing an important role in enzyme stability. Both calcium and manganese are necessary for the alpha amylase biosynthesis (McTigue *et al*., 1994). Ca^{2+} ions are important for thermal stability of the enzyme activity, (Egas *et al*., 1998; Bose and Das, 1996).

Statistical analysis of the Plackett-Burman design demonstrates that the model F-value of 0.79 is significant. P-value < 0.05 indicates that model terms are significant. The model's goodness of fit was checked by determination of coefficient (R^2). In this case, the value of R^2 (0.85) closer to 1 denotes better correlation between the observed and the predicted responses. Coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. Lower reliability of the experiment is usually indicated by high value of CV. In the present case, a low CV (4.55) denotes that the experiments performed are highly reliable (Table 2).

Regression analysis was performed on the results and first order polynomial equation was derived representing alpha amylase production as a function of the independent variables:

Amylase = 47.00 + 5.10A + 8.58D + 1.92K + 1.33L

Magnitude of the effects indicates the level of significance of the variable on amylase production. Consequently, based on the results of the present experiment, statistically significant variables i.e., inoculum concentration, pH, NaCl and $CaCl_2$ with positive effects were further investigated with central composite design to find the optimal range of these variables. The optimized medium composition by Plackett-Burman design was as follows (g/l): soluble starch, 20.0; yeast extract, 4.0; peptone, 10.0; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $CaCl_2$, 0.2.

Table 1. Plackett - Burman experimental design for evaluating factors influencing alpha amylase production by *Serratia marcescens* SB08

Run	Factor A: pH	Factor B: Temp. (°C)	Factor C: Agit. (rpm)	Factor D: Inocul. conc. (%)	Factor E: Incub. time (h)	Factor F: Sucrose (g/l)	Factor G: Peptone (g/l)	Factor H: KH_2PO_4 extract (g/l)	Factor J:Yeast (g/l)	Factor K:NaCl (g/l)	Factor L:$CaCl_2$ (g/l)	α-amylase (U/ml)
1	4	40	200	1	96	20	6	2	2	0.1	0.2	24.35
2	4	40	200	5	6	5	3	6	2	0.5	0.2	67.32
3	4	40	0	5	96	5	6	6	4	0.1	0.05	27.97
4	4	20	0	1	6	5	3	2	2	0.1	0.05	63.11
5	6	20	200	5	96	5	3	2	4	0.1	0.2	79.07
6	6	20	0	1	96	5	6	6	2	0.5	0.2	45.99
7	4	20	200	1	96	20	3	6	4	0.5	0.05	27.49
8	6	40	0	1	6	20	3	6	4	0.1	0.2	32.08
9	4	20	0	5	6	20	6	2	4	0.5	0.2	41.18
10	6	40	200	1	6	5	6	2	4	0.5	0.05	37.54
11	6	40	0	5	96	20	3	2	2	0.5	0.05	74.04
12	6	20	200	5	6	20	6	6	2	0.1	0.05	43.91

Table 2. Analysis of variance for α-amylase production by *Serratia marcescens* SB08

Source	Sum of square	Degree of freedom	Mean square	F-value	P-value
Model	1260.603233	4	315.1508	0.792456383	0.0057
A-pH	312.2220083	1	312.222	0.785091826	0.005
D-Inoculum conc.	882.882075	1	882.8821	2.220034084	0.0098
K-NaCl	44.352075	1	44.35208	0.111524654	0.0482
L-$CaCl_2$	21.147075	1	21.14708	0.053174969	0.0242
Residual	2783.819658	7	397.6885		
Cor Total	4044.422892	11			

CV = 4.55; R^2 = 0.85

Central composite design. This is a very useful tool to determine the optimal level of medium factors and their interaction. Based on Plackett-Burman design, inoculum concentration, pH, NaCl and $CaCl_2$ were selected for further optimization using response surface methodology. To examine the combined effect of these factors on alpha amylase production, a central composite design (CCD) was employed within a range of -2 to +2 in relation to production of alpha amylase. The results obtained from CCD are given in Table 3. These were fitted to a second order polynomial equation to explain the dependence of alpha amylase production on the medium components:

$$Y = 66.50 + 15.83A + 4.00B + 2.67C + 0.33D - 3.38AB - 4.38AC + 3.63AD - 1.63BC + 4.13BD + 1.63CD - 4.48A^2 - 9.48B^2 - 3.48C^2 - 3.73D^2$$

where:

Y is the predicted response (alpha amylase production), A, B, C and D are the coded values of pH, inoculum concentration, NaCl and $CaCl_2$, respectively.

The analysis of variance of the quadratic regression model suggest that the model is very significant as is evident from the Fisher's F-test (Table 4). The model's goodness of fit was checked by determination coefficient (R^2). In this case, the value of R^2 (0.894) (multiple correlation coefficient) closer to 1 denotes better correlation between the observed and the predicted responses. The coefficient of variation (CV) indicates the degree of precision with which the experiments are comapared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case a low CV (5.45) denotes that the experiments performed are highly reliable. The P-value denotes the significance of the coefficients and is also important in understanding the pattern of the mutual interactions between the variables.

The fitted response and contour plotted for the above regression model is shown in Fig. 1. 3D response surface curves were plotted to understand the interactions of medium

Table 3. Experimental plan for optimization of alpha amylase production using central composite design

Run	pH	Inoc. conc. (%)	NaCl (g/l)	$CaCl_2$ (g/l)	Alpha amylase (U/ml) Experimental	Predicted
1	5	3	0.1	0.125	41.05	22.5
2	6	5	0.5	0.2	63.52	62.4167
3	5	3	0.3	0.125	34.02	32.25
4	6	5	0.1	0.05	54.14	58.6667
5	3	3	0.3	0.125	44.01	36.5833
6	4	1	0.1	0.2	63.14	59
7	6	5	0.5	0.05	39.84	39.8333
8	5	1	0.3	0.125	52.01	48.75
9	6	1	0.5	0.2	0	0
10	6	5	0.1	0.2	61.35	58.8333
11	5	3	0.7	0.125	38.03	30.6667
12	6	1	0.5	0.05	69.04	71.5833
13	5	7	0.3	0.125	24.54	25
14	6	1	0.1	0.2	59.54	61.9167
15	4	5	0.1	0.05	43.24	44.75
16	5	3	0.3	0.275	61.45	68.1667
17	5	3	0.3	0.125	0	0
18	5	3	0.3	0.125	87.23	80.25
19	4	1	0.1	0.05	0	0
20	5	3	0.3	0.125	47.52	36.5833
21	5	3	0.3	0.025	41.9	47.25
22	4	1	0.5	0.05	54.09	57.9167
23	4	5	0.5	0.05	41.44	50.9167
24	4	5	0.1	0.2	52.24	52.25
25	6	1	0.1	0.05	74.19	66.5
26	5	3	0.3	0.125	74.01	66.5
27	7	3	0.3	0.125	57.43	66.5
28	4	1	0.5	0.2	71.01	66.5
29	5	3	0.3	0.125	71.99	66.5
30	4	5	0.5	0.2	52.32	66.5

Table 4. ANOVA for the experimental results of the central composite design (quadratic model)

Source	Sum of square	Degree of freedom	Mean square	F-value	P-value
Model	10545.53	14	753.2524	4.459166894	0.0034
A-pH	6016.667	1	6016.667	35.61797014	< 0.0001
B-Inoculum conc.	384	1	384	2.273235546	0.1524
C-NaCl	170.6667	1	170.6667	1.010326909	0.3308
D-$CaCl_2$	2.666667	1	2.666667	0.015786358	0.9017
AB	182.25	1	182.25	1.078898902	0.3154
AC	306.25	1	306.25	1.812964546	0.1981
AD	210.25	1	210.25	1.24465566	0.2821
BC	42.25	1	42.25	0.250115109	0.6243
BD	272.25	1	272.25	1.611688483	0.2236
CD	42.25	1	42.25	0.250115109	0.6243
A^2	550.2976	1	550.2976	3.257698199	0.0912
B^2	2464.583	1	2464.583	14.59004802	0.0017
C^2	332.0119	1	332.0119	1.96547204	0.1813
D^2	381.4405	1	381.4405	2.258083461	0.1537
Residual	2533.833	15	168.9222		
Lack of fit	280.3333	10	28.03333	0.1934	0.0861

CV = 5.45; R^2 = 0.894

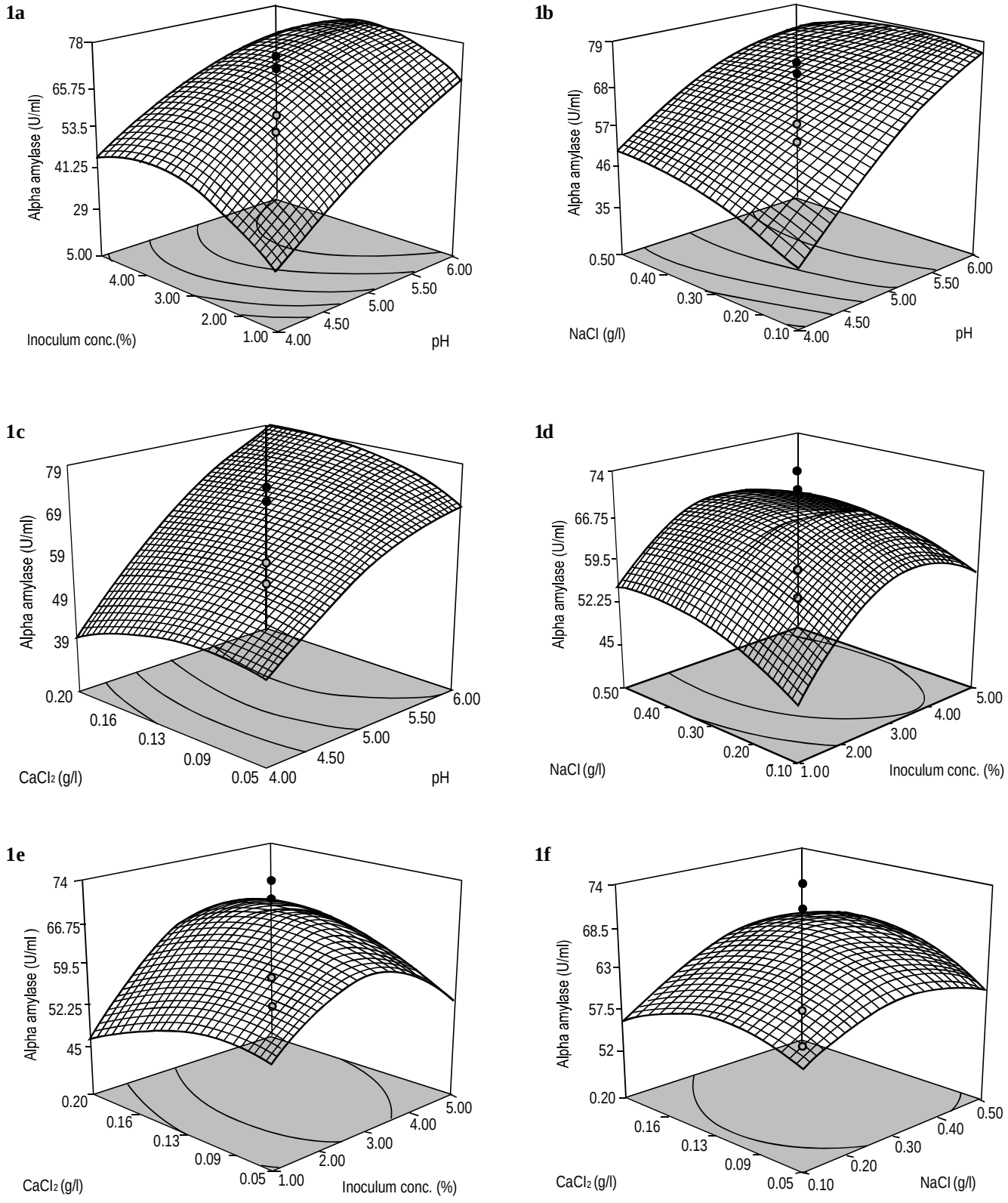

Fig. 1(a-f). Response surface and contour plot of the combined effect of factors: (a) inoculum concentration, pH; (b) NaCl, pH; (c) $CaCl_2$, pH; (d) NaCl, inoculum concentration; (e) $CaCl_2$, inoculum concentration; (f) $CaCl_2$, NaCl, on the production of alpha amylase by *Serratia marcescens* SB08.

components and their effect on enzyme production. Graphs highlight the roles played by various factors and also to emphasize the roles played by the physical constraints. From the central point of the contour plot the optimal process parameters were identified.

Fig. 1a shows the response surface plot obtained as function of pH *vs* inoculum concentration, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at pH 5.5 and inoculum concentration of 3%. Fig. 1b shows the response surface plot obtained as a function of pH *vs* NaCl, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at pH 5.35 and NaCl, 0.32 g/l.

Fig. 1c shows the response surface plot obtained as a function of pH *vs* $CaCl_2$, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at pH 5.23 and $CaCl_2$, 0.13 g/l.

Fig. 1d shows the response surface plot obtained as a function of inoculum concentration *vs* NaCl, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at inoculum concentration of 3.27% and NaCl, 0.43 g/l.

Fig. 1e shows the response surface plot obtained as a function of inoculum concentration *vs* $CaCl_2$, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at inoculum concentration of 3.83% and $CaCl_2$, 0.16 g/l.

Fig. 1f shows the response surface plot obtained as function of NaCl *vs* $CaCl_2$, while all other variables are maintained at zero level. An increase in alpha amylase yield was observed at NaCl, 0.38 g/l and $CaCl_2$, 0.15 g/l.

Validation of the model. The experiment was conducted for 12 runs, evaluated statistically and found significant. Optimum values of the tested variables are pH 5.0, inoculum concentration 3%, NaCl, 0.30 g/l and $CaCl_2$, 0.13 g/l. Maximum experimental response for alpha amylase production was 87.23 U/ml, whereas the predicted value was 80.25 U/ml indicating a strong agreement between experimental and predicted values, thus proving the validity of the model.

Alpha amylase production was sustainable (87.23 U/ml) in Ehrlenmeyer flask and in 2 litre fermentor, suggesting feasibility of scale up of alpha amylase production. There was a slight decline in enzyme production in 2 litre fermentor (86.92 U/ml), which could be due to the reduction in dissolved oxygen (Uma Maheshwar Rao and Satyanarayana, 2003).

Conclusion

It is important to discover new bacterial strains that produce enzymes with novel properties of industrial interest. In the present study, production of α amylase under submerged fermentation was obtained by optimizing medium components by RSM. Application of response surface methodology, represented by central composite design, to optimize the selected factors for maximum production, is an efficient method that tests the effect of factor interaction. Besides, it converts the bioprocess factor correlations into a mathematical model that predicts where the optimum is likely to be located. It is worthwhile to advise the microbial industry sponsors to apply such experimental design to maintain high efficiency and profit bioprocess. In the present investigation, the optimized medium succeeded to increase α amylase yield. The production level in basal medium was 56.43 U/ml and increased after applying RSM to 87.23 U/ml.

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