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Full Length Research Paper

Purification and medium optimization of α-amylase from *Bacillus subtilis* 168

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α-Amylase was first time isolated and purified from *Bacillus subtilis* 168 (1A1). Purified α-amylase fraction showed a single protein band with a molecular weight of 55 kD. Chemical characterization of the purified α-amylase revealed optimum amylolytic activity at 37°C and pH 7.0 using starch as substrate. It was stable at pH 5.0 to 9.0 and at temperatures 25–70°C. Culture conditions were optimized by using statistics-based experimental designs to enhanced α-amylase (EC.3.2.1.1) production. A two level fractional factorial Plackett-Burman design was used for the preliminary screening significant media components and conditions. Response surface methodology (RSM) involving a 2^4 full-factorial central composite design (CCD) and a second-order polynomial equation was then employed to identify the relationship between the α-amylase yield and the four significant variables. Optimal levels of the significant variables for the maximum α-amylase yield were starch 2.55 g/l, yeast extract 8.4 g/l, sodium chloride 8.1% and 48 h of incubation. Mean value of α-amylase yield was 639.7 IU/ml, which was in excellent agreement with the predicted value (633.5 IU/ml).

Key words: *Bacillus,* α-amylase*,* optimization, Plackett-Burman design, response surface methodology.

INTRODUCTION

Microbial enzymes find increasing industrial application and among them amylases occupy a large share of the commodity enzyme market. The genus *Bacillus* produces a large variety of extracellular enzymes of which amylases are of significant industrial importance. α-Amylase is most important commercial enzyme, having wide applications in starch-processing, brewing, alcohol production and finds applications in food, textile, pharma-ceuticals and detergent industries (Prescott and Dunn, 1987). α-Amylase is an endo-acting enzyme that hydrolyzes the 1,4 α-linkages between the glucose residues in the polysaccharide chains (Godfrey and Reichelt, 1983). Highly active α-amylase from *Bacillus* is essential to meet the demand of the above-mentioned industries (Pedersen and Nielsen, 2000).

Media engineering and genetic manipulations are use to enhance enzyme production, but leads to the over-production of enzymes by medium handling considered as advanced approach (Li et al., 2001). Modifications of nutrients and culture conditions with the help of statistical experimental design techniques are useful tools for screening of nutrients with significant impact on growth rate as they can provide statistical models, which aid in understanding the interactions among the process parameters at different levels. Several authors have described the modification of nutrients and culture conditions for α-amylase production by various *Bacillus* strains using flask cultures and batch fermentation (Sun et al., 2009). A well-acknowledged conventional method "one variable at a time (OVAT)" frequently used but time consuming, does not account for the interactions of variables among the medium components (Korbhati et al., 2007). Therefore, an alternative tactics involving statistical approach, for example, Plackett-Burman factorial experimental design and response surface methodology (RSM) could be

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Abbreviations: RSM, Response surface methodology; **CCD,** central composite design; **DNS,** dinitrosalicylic acids; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 1. Fermentation conditions for α-amylase production used in screening of variables using a Placket-Burmen design.

Variables	Symbol code	Units	Experimental Values		
			Lower (-1.000)	Centre (0.000)	Higher (1.000)
Starch	X1	g/l	0.5	1.75	3
Glucose	X2	g/l	1	2	3
Yeast Extract	X3	g/l	1	5.50	10
Peptone	X4	g/l	1	8	15
Amm. Nitrate	X5	g/l	0.1	0.3	0.5
S. Chl	X6	%	2	6	10
P. Chl	X7	%	2	6	10
Inoculum Size	X8	%	0.5	1.75	3
Incubation time	X9	hrs	24	48	72
Temp	X10	C	30	37	44
Rpm	X11	rpm	150	200	250

employed to overcome these difficulties and to determine the interactions between the variables (Sharma et al., 2009). RSM is a collection of mathematical and statistical methods that are useful for modeling problems in which response is influenced by several variables and the objective is to optimize the response. This optimization process involves three major steps: Performing the statistically designed experiments, fitting experimentally determined response data into a quadratic model and estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model (Vohra and Satyanarayana, 2002). The major advantage of RSM is the fewer number of experimental trials needed for identification and quantification of significant interactions between the variables (Karacan et al., 2007).

In this study, significant nutrients and culture conditions affecting α-amylase production were screened by Plackett-Burman factorial design and then response surface methodology was applied to optimize the growth medium composition for obtaining high yield of α-amylase. A 2^4 full-factorial central composite design (CCD) was used to identify the optimum levels of the significant variables to generate the maximum α-amylase yield.

MATERIALS AND METHODS

Microorganism and inoculum preparation

Bacillus subtilis 168 (1A1) was a generous gift from the *Bacillus* Genetic Stock center (BGSC, Columbus, OH, USA; 6th Edn., 1th July, 1994) for research purpose. The culture was preserved on nutrient agar slant overlaid with mineral oil and 70% glycerol stock, and then stored at −70°C. Twenty five (25) ml of medium, whose composition was varied based on the experimental design contained in 250 ml conical flask, was sterilized at 121°C in an autoclave for 15 min. The inoculated flasks were incubated in a temperature controlled shaker incubator at 200 rpm and 37°C for 24 h. All the experiments were carry out in parallel as triplicates.

Identification of significant nutrients and culture conditions by Plackett-Burman design

To identify the significant nutrients and culture conditions, Plackett-Burman design was used (Majumder and Goyal, 2008). Eleven variables were selected for this study according to their effect on α-amylase production. Each variable was studied at 3 levels, high (+1), centre point (0) and low (−1) as given in Table 1. Seventeen trials including five center points were conducted to evaluate the linear and curvature effects of the variables. Table 2 shows the Plackett-Burman experimental design with eleven variables including level of each variable, experimental α-amylase response and predicted response by software. Each run was carried out in triplicate and mean averages was taken as response. Plackett-burman design is based on the first-order polynomial equation

$$Y = ß_o + \sum ß_i Xi \quad (1)$$

Where, Y, is the α-amylase yield; $ß_o$, is the model intercept; ßi, is the linear coefficient; Xi, is the level of the independent variable. It is important to note that Plackett-burman design was only used to screen and evaluate the significant variables that can influence yield because this model does not describe the interaction among various variables (Purama and Goyal, 2008).

Optimization of selected significant nutrients and culture conditions

The effect of four significant variables starch (X1), yeast extract (X3), sodium chloride (X6) and incubation time (X9) on production of α-amylase was studied by response surface methodology. A central composite design (CCD) was used to study the interactions among significant variables and for optimizing the maximum α-amylase yield. The respective low and high levels with the coded levels of the four significant factors are given in Table 3. The concentrations of the other factors were fixed at zero level as shown in Table 1. Total 21 experiments with five center points were run in triplicates covering maximum range of combinations of variables as shown in Table 4. To determine a relationship among variables, a second order polynomial equation was employed to obtained data from 21 runs. The equation could be written as:

$$Y = ß_o + \sum ß_i xi + \sum ß_{ii} xi^2 + \sum ß_{ij} x_i x_j, \quad i = 1, 2, 3... k \quad (2)$$

Table 2. The experimental design using the Placket Burmen method for screening of α-amylase fermentation conditions along with observed and predicted α-amylase activity.

Run No.	Experimental values											α-Amylase (IU/ml)	
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Observed	Predicted
1	3.00	3.00	10.00	1.00	0.10	2.00	10.00	0.50	72.00	44.00	150.00	203.3	189.3
2	0.50	3.00	10.00	1.00	0.50	10.00	10.00	0.50	24.00	30.00	250.00	172.8	172.8
3	1.75	2.00	5.50	8.00	0.30	6.00	6.00	1.75	48.00	37.00	200.00	240.5	167.9
4	1.75	2.00	5.50	8.00	0.30	6.00	6.00	1.75	48.00	37.00	200.00	242	167.9
5	3.00	1.00	1.00	1.00	0.50	2.00	10.00	3.00	24.00	44.00	250.00	167.5	167.5
6	0.50	1.00	1.00	15.00	0.10	10.00	10.00	0.50	72.00	44.00	250.00	162.8	162.8
7	1.75	2.00	5.50	8.00	0.30	6.00	6.00	1.75	48.00	37.00	200.00	245	167.9
8	3.00	1.00	10.00	15.00	0.10	10.00	10.00	3.00	24.00	30.00	150.00	173.4	173.4
9	3.00	3.00	1.00	15.00	0.50	10.00	2.00	0.50	24.00	44.00	150.00	139.4	139.4
10	3.00	3.00	1.00	1.00	0.10	10.00	2.00	3.00	72.00	30.00	250.00	207.6	207.6
11	1.75	2.00	5.50	8.00	0.30	6.00	6.00	1.75	48.00	37.00	200.00	242.5	167.9
12	0.50	3.00	1.00	15.00	0.50	2.00	10.00	3.00	72.00	30.00	150.00	137.4	137.4
13	3.00	1.00	10.00	15.00	0.50	2.00	2.00	0.50	72.00	30.00	250.00	160.5	160.5
14	0.50	1.00	1.00	1.00	0.10	2.00	2.00	0.50	24.00	30.00	150.00	159.7	159.7
15	0.50	3.00	10.00	15.00	0.10	2.00	2.00	3.00	24.00	44.00	250.00	149.7	149.7
16	1.75	2.00	5.50	8.00	0.30	6.00	6.00	1.75	48.00	37.00	200.00	235.5	167.9
17	0.50	1.00	10.00	1.00	0.50	10.00	2.00	3.00	72.00	44.00	150.00	180.8	180.8

Table 3. Experimental variables, codes, units, ranges and levels of the independent variables for response surface methodological experiments.

Variable	Symbol code	Units	Levels				
			-1.682	-1	0	+1	+1.682
Starch	X1	g/l	-0.35	0.5	1.75	3	3.85
Yeast Extract	X3	g/l	-2.07	1	5.5	10	13.07
Sodium Chloride	X6	%	-0.73	2	6	10	12.73
Incubation time	X9	hrs	7.64	24	48	72	88.36

Where, Y, is the predicted response; k, is the number of factor variables; $ß_o$, is the model constant; $ß_i$, is the linear coefficient; $ß_{ii}$, is the quadratic coefficient; $ß_{ij}$, is the interaction coefficient. The relationship between the coded forms of the input variables and the actual values of a variable are described in below as

$$xi = Xi - Xo / \Delta Xi \quad 3$$

Where, xi, is the coded value of the ith independent variable; Xi, is the actual value; Xo, is the value of Xi at centre point; ΔXi, is the step change.

Validation of model

The optimized conditions generated during response surface methodology implementation were validated by conducting experiment on given optimal medium setting. Three runs were carried out in triplicate to confirm the results.

Fermentation process

Starch was used as substrate in the production of α-amylase. The inoculum (1.75%) was incubated at 37°C in Sakaguchi flasks (50ml/flask) for 48 h in rotary shaker (200rpm). After 48 h, the culture broth was centrifuged at 10,000 rpm for 15 min at 4°C. The crude enzyme extract was collected and immediately assayed for amylolytic activity.

Enzyme assay and purification of crude enzyme extract

α-Amylase activity was determined (Fischer and Stein, 1961) by adding 1 ml of the enzyme solution to 1 ml of starch solution and incubated at 37°C for 3 min. After incubation, 2 ml of 3, 5 dinitro-salicylic acids (DNS), was added to stop the reaction. Color due to reducing sugar was developed by heating the reactants in boiling water bath for 5 min then rapidly cool to room temperature. The extinction value (absorbance) was determined at 550 nm against standard maltose curve. One unit of enzyme activity is the amount of enzyme that liberates reducing sugar equivalent to 1.0 mg maltose hydrate under specific assay conditions. The crude enzyme was precipitated with ammonium sulfate (80% saturation) and allowed to stand overnight at 4°C with stirring. The mixture was centrifuged at 10,000 rpm, 4°C for 15min. The precipitate was collected and re-dissolved in a minimum quantity of distilled water and dialyzed against 10 mM phosphate buffer (pH 6.9) for 24 h at

Table 4. Central composite experimental design data for α-amylase production with predicted and experimental values.

Run No.	Experimental Values				α-amylase (IU/ml)	
	X1	X3	X6	X9	Observed	Predicted
1	-0.35	5.50	6.00	48.00	34.1	32.3
2	1.75	5.50	6.00	48.00	549	561.1
3	0.50	10.00	10.00	72.00	168.3	173.2
4	1.75	5.50	6.00	48.00	563.2	561.5
5	1.75	5.50	12.73	48.00	517.4	506.1
6	1.75	5.50	-0.73	48.00	452.1	459.2
7	0.50	10.00	2.00	72.00	179.1	177.2
8	3.00	10.00	10.00	24.00	334.5	339.3
9	1.75	-2.07	6.00	48.00	242.2	240.1
10	1.75	13.07	6.00	48.00	458.6	456.5
11	3.00	1.00	2.00	72.00	213.4	211.5
12	1.75	5.50	6.00	88.36	243.3	241.5
13	0.50	1.00	10.00	24.00	131.7	136.6
14	0.50	1.00	2.00	24.00	147.3	145.1
15	1.75	5.50	6.00	7.64	114.5	112.1
16	1.75	5.50	6.00	48.00	557.3	561.1
17	1.75	5.50	6.00	48.00	572.2	561.3
18	3.85	5.50	6.00	48.00	457.1	455.2
19	3.00	10.00	2.00	24.00	254.6	252.2
20	1.75	5.50	6.00	48.00	559.4	561.4
21	3.00	1.00	10.00	72.00	240.2	245.5

4°C. The dialyzed mixture was centrifuged at 10,000 rpm, 4°C for further purification. The partially purified α-amylase was further purified at a temperature below 4°C. Precipitates formed, after ammonium sulfate precipitation, were dissolved in a small volume of 10 mM phosphate buffer of pH 7.4 (Sephadex) over the course of 16 h against 50 volume of the same buffer. The column (G-75-120 {30Kda-80Kda}) was washed with the equilibration buffer, and the crude protein was concentrated with 10 Kda filter in medium ultra filtration cell. The concentrate was put on a column of Sephadex (G-75-120) that had been equilibrated with 10 mM Sephadex buffer; proteins were eluted as active fractions at a flow rate of 25 mlh^{-1} and checked on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after enzyme assay.

To confirm the above purification, partially purified α-amylase was again purified by cation exchange chromatography using phosphate cellulose column that had been equilibrated with 100 mM P-11 buffer, pH 7.6. The same buffer with a linear salt gradient of KCl (0.0-1M) was used for the elution of different proteins. Fractions of 5.6 ml per tube were collected and the absorbance of each fraction was measured at 550 nm. The amylolytic activities of the fractions obtained at each purification step were measured.

Homogeneity and molecular weight determination of the enzyme

Electrophoresis was performed according to the method described previously (Davis, 1964). One-half of the gel was stained with coomassie brilliant blue for about 15 min and destained with a mixture of 7.5% acetic acid and 5% methanol. While the second half of the gel containing the pure protein was checked through activity staining method as zymography (Hatada et al., 1996). The molecular weight of the purified α-amylase was determined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described earlier (Laemmli, 1970). Proteins with known molecular weight such as bovine serum albumin (BSA) and broad range marker were used as standards for estimating the molecular weight of the purified α-amylase in 10% separating gel and 5% stacking gel. The purified enzyme and standards were dissolved in sample buffer (0.05Mtris-HCl, pH6.8, 40% glycerol, 2% SDS, 0.4% β-mercapto ethanol, 0.1% bromophenol blue) and incubated at 100°C for 5 min to ensure complete dissociation of proteins.

Statistical analysis

The statistical analysis of the data was performed using design Expert 7.1.3 (Stat-ease, Minneapolis, USA) to evaluate the Fisher's F-test for analysis of variance (ANOVA) to determine the significance of each term in equations and statistical significance of the model. Student t-test was used to evaluate the statistical significance of regression coefficients. The adequacy of the model can be expressed by the coefficient of multiple determinations (R^2) and lack-of fit value. A difference was considered statistically significant when $P< 0.05$. Response surfaces (3D and counter plots) were drawn for experimental results obtained from the interactive effects of the independent variables on the α-amylase production. Optimized conditions were predicted by using response surface plots and point prediction method.

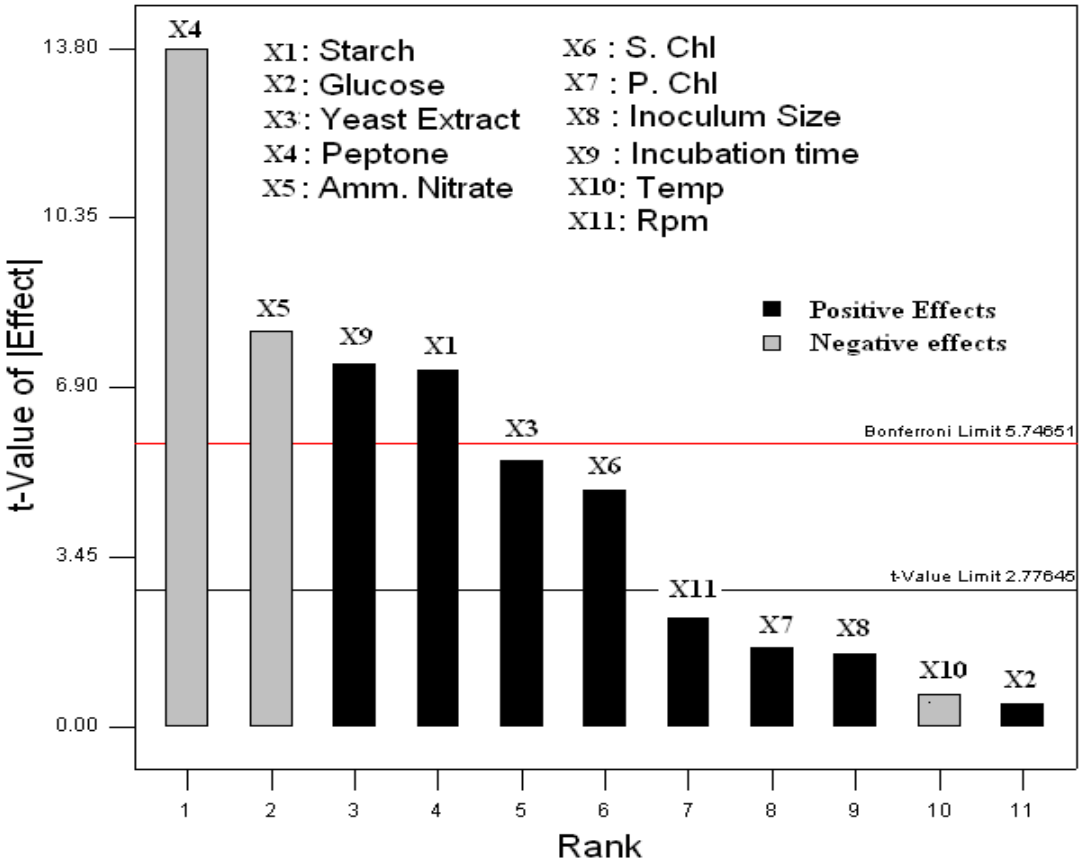

Figure 1. Pareto chart of nutrients and conditions for the selection of significant variables for α-amylase produced from *Bacillus subtilis* 168.

RESULTS

Identification of significant variables by using Plackett-Burman design

α-Amylase yield was determined in *B. subtilis* 168. The data in Table 2 showed wide variation of α-amylase production from 137.4 to 242.5 due to significant effect of variables on α-amylase production. Six variables out of eleven have significant effect on α-amylase production. Out of six significant variables, four variables, that is, starch, yeast extract, sodium chloride and incubation time were selected for further study. Yeast extract was preferred on peptone and ammonium sulphate as nitrogen source because peptone and ammonium sulphate showed a negative effect on production of α-amylase as shown in Pareto chart (Figure 1). The model (Equation 1) for α-amylase production can be written as

Y= (α-amylase predicted response) = +167.91+7.37X1+0.46X2+5.51X3−14.04X4−8.18X5+4.89X6+1.63X7+1.49X8+7.49X9−0.66X10+2.24X11

Where, X1, Starch; X2, glucose; X3, yeast extract; X4, peptone; X5, ammonium sulphate; X6, sodium chloride; X7, potassium chloride; X8 inoculum's size; X9, incubation time; X10, temperature; X1, RPM.

Optimization of significant variables using response surface methodology

Starch, yeast extract, sodium chloride and incubation time were found to be significant variables using Plackett-Burman design. To examine the combined effects of these independent variables, 21 runs with five center points were performed. The central composite design and respected high and low levels of each variable with the coded values are given in Table 4. The 3D response surface graphs with counter plots (Figure 2) were used for the graphical representation of the regression equation.

Validation of statistical model

To confirm the validity of the response surface methodology, three additional experiments were conducted under the predicted optimal conditions by counter plot

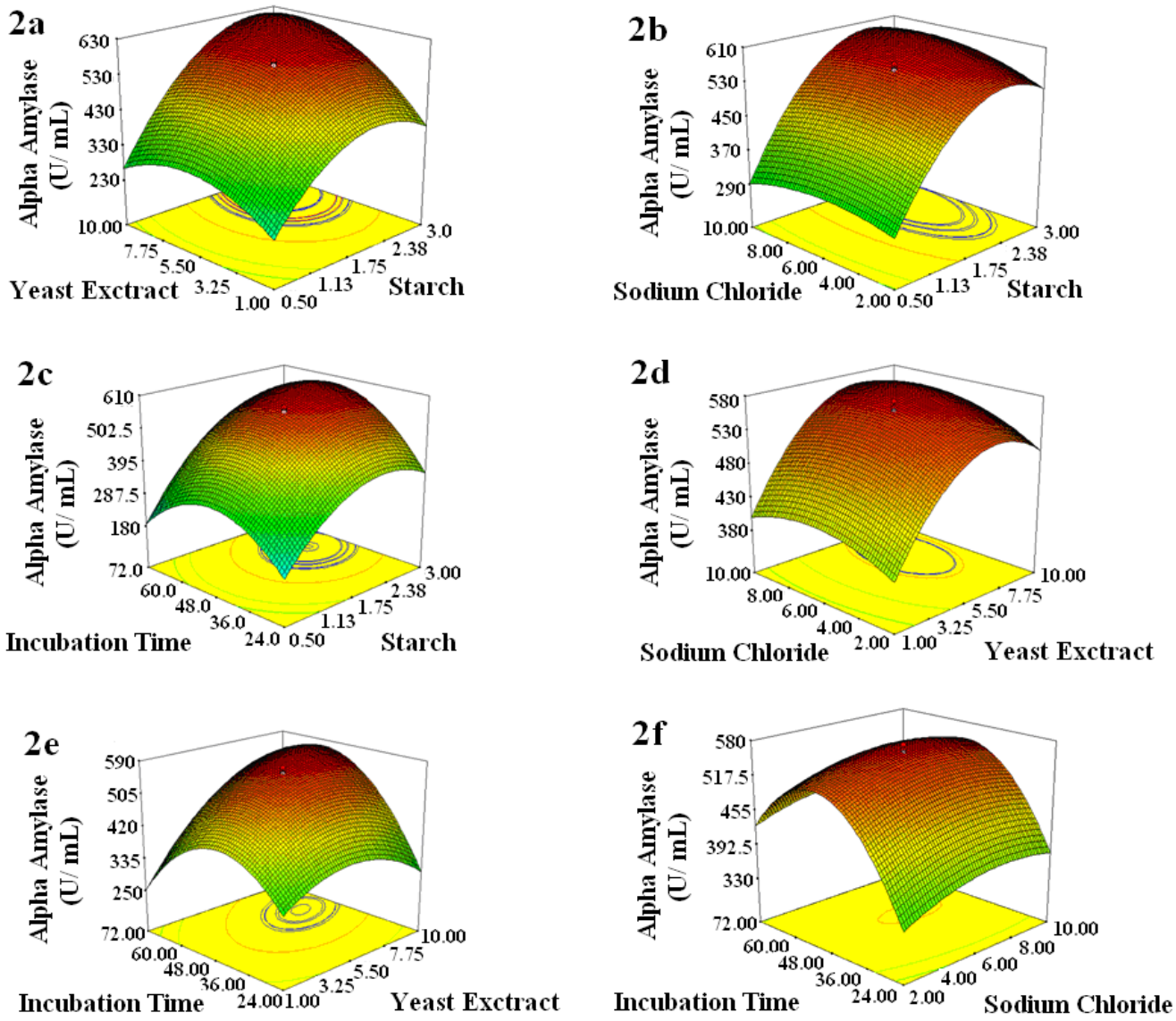

Figure 2. 3D response surface graph along with counter plot showing effect of starch, yeast extract, sodium chloride and incubation time at fixed concentration of two variables and different concentrations of the other two variables on α-amylase yield. (2a) Starch and yeast extract, fixed level: sodium chloride and incubation time; (2b) starch and sodium chloride, fixed level: yeast extract and incubation time; (2c) starch and incubation time, fixed level: yeast extract and sodium chloride; (2d) yeast extract and sodium chloride, fixed level: starch and incubation time; (2e) yeast extract and incubation time, fixed level: starch and sodium chloride; (2f) sodium chloride and incubation time, fixed level: starch and yeast.

results. These experiments showed the mean value of observed (639.7 IU/ml) and predicted (633.5 IU/ml) response (α-amylase yield).

Purification, homogenization and molecular weight determination of α-amylase

Crude enzyme of *B. subtilis* 168 was assayed using 1% starch as substrate. Results of each purification step for α-amylase are shown in Figures 3, 4 and 5. The crude enzyme was precipitated with ammonium sulphate at 80% saturation. The ammonium sulphate precipitated enzyme obtained from the previous step was de-salted by dialysis. Subsequently, two-step column chromatography that is, gel filtration and cation exchange was done as previously described (Bolton et al., 1997). Keeping the view above, α-amylase was purified to homogeneity through Rotofor.

DISCUSSION

Plackett-Burman design has shown (Table 5) a good fit

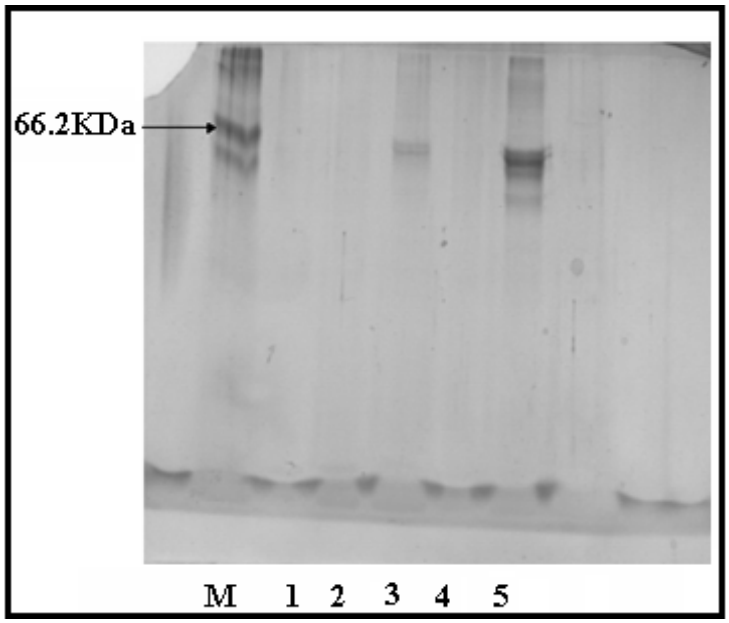

Figure 3. SDS-PAGE of gel filtration: Lane M; shows BSA. Lanes 1,2,3,4 and 5: show protein purification in the form of 5 different peaks, using sephadex.

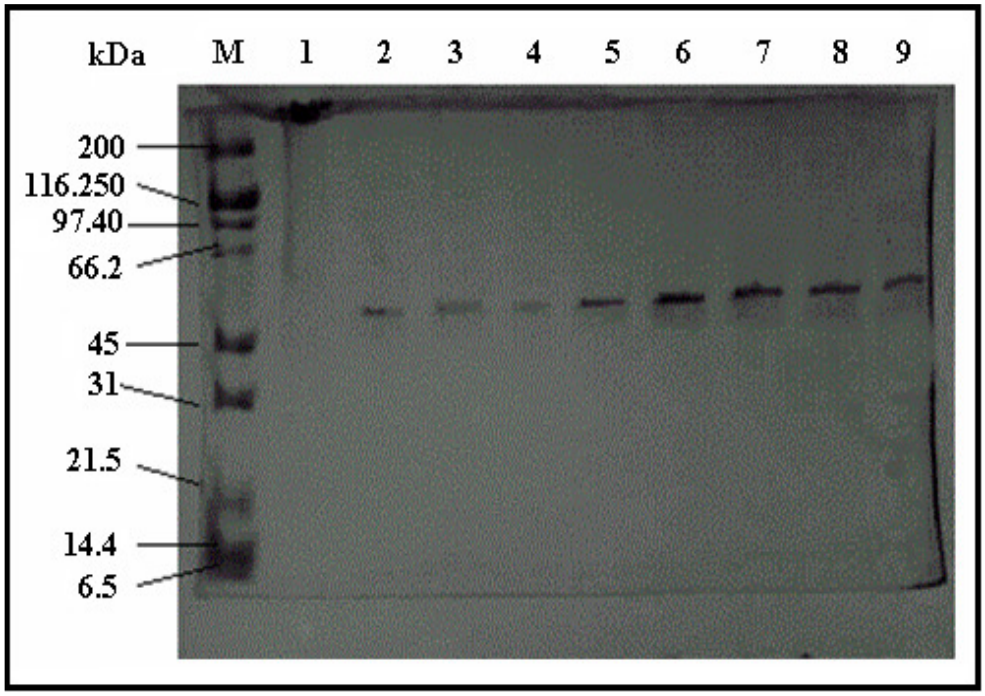

Figure 4. SDS-PAGE of Ion-exchanger: Lane M; shows broad range marker. Lane1; shows (negative) control. Lanes 2-9, shows active fractions having α-amylase purified through phosphate cellulose resin.

with the experimental data, since the coefficient of determination R^2 had a value of 0.9907. This means that the fitted model could explain 99.07% of the total variability within the range of values studied. Significance of each variable was determined by p and F-values. Smaller p-value and larger magnitude of F-value showed high significance of the corresponding variables (Karthikeyan et al., 1996). The variables with p-value less

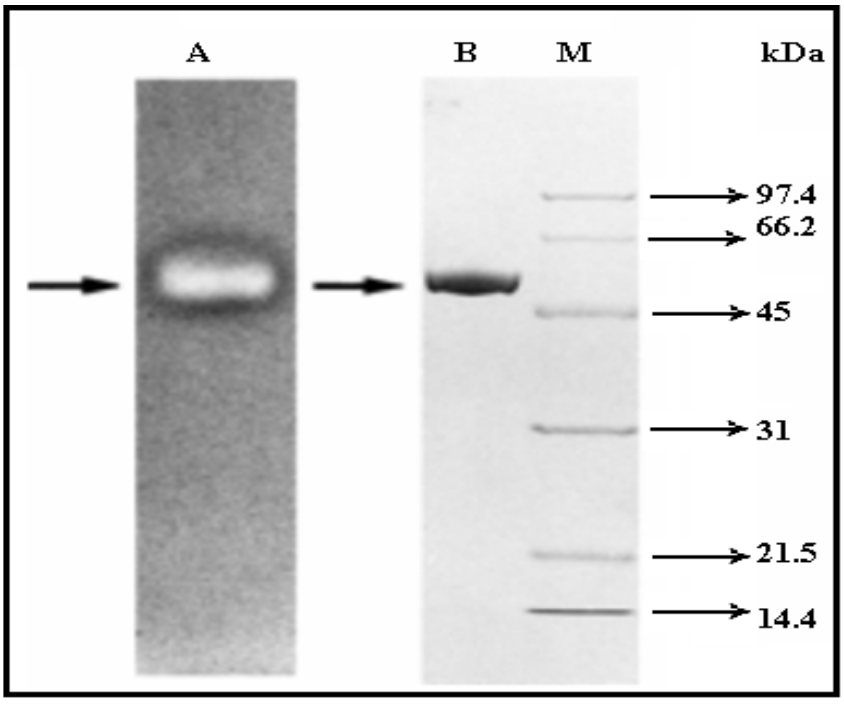

Figure 5. SDS-PAGE of purified α-amylase from *Bacillus* sp. The purified enzyme was visualized by activity staining (lane A) and coomassie brilliant blue staining for protein (lane B). Lane M, molecular mass marker (calibration in kilo Daltons). The arrows indicate the positions of the purified enzyme.

Table 5. Regression co-efficient and corresponding F and p values for α-amylase activity in eleven variables Placket Burman design experiment.

Variable	Sum of Squares	Standard df	Coefficient Estimate	F- value	p-value
Model	5271.83	11	-	38.57	0.0015*
Curvature	18907.13	1	-	1521.70	<0.0001*
X1	652.69	1	7.37	52.53	0.0019*
X2	2.52	1	0.46	0.20	0.6757**
X3	364.10	1	5.51	29.30	0.0056*
X4	2366.02	1	-14.04	190.42	0.0002*
X5	801.97	1	-8.18	64.54	0.0013*
X6	287.14	1	4.89	23.11	0.0086*
X7	31.69	1	1.63	2.55	0.1855**
X8	26.70	1	1.49	2.15	0.2165**
X9	673.50	1	7.49	54.21	0.0018*
X10	5.20	1	-0.66	0.42	0.5529**
X11	60.30	1	2.24	4.85	0.0923**

Std Dev = 3.52; Mean = 189.4; C.V% = 1.86; R^2 = 0.9907; Adj R^2 = 0.9650; Adeq Precision = 33.642; *significant values, **non-significant at p< 0.0.

than 0.05 (<0.05) were considered as significant. ANOVA of the model showed high F- (38.57) and p-value (0.0015), which explained that the model itself is significant (Tanyildizi et al., 2006). Table 6 represents the experimental design and results obtained for α-amylase production. The analysis of variance (ANOVA) performed for these significant variables showed that X1, X3, X6, X9, X1X3, X1X6, X1X9, X3X9, X12,X32,X62 and X92 are significant model terms (P = <0.05). Data was best fitted by following a two level quadratic polynomial equation

Table 6. Regression co-efficient and corresponding F, t and p values for α-amylase activity in four selected variables using RSM.

Variable	Sum of Squares	df	Coefficient Estimate	Standard Error	t- value	F- value	p-value
Model	6.457E+005	14	-	-	-	441.38	<0.0001
Lack of Fit	342.95	2	-	-	-	2.42	0.2052
X1	89464.50	1	125.76	4.30	29.24	856.19	<0.0001
X3	23328.0	1	64.22	4.30	14.93	223.25	<0.0001
X6	2624.38	1	13.86	2.77	5.003	25.12	0.0024
X9	8320.50	1	38.35	4.30	8.91	79.63	0.0001
X1X3	196.51	1	46.60	5.62	8.29	68.87	0.0002
X1X6	2244.50	1	16.75	3.61	4.63	21.48	0.0036
X1X9	4967.32	1	38.72	5.62	6.88	47.54	0.0005
X3X6	420.50	1	7.25	3.61	2.008	4.02	0.0917
X3X9	18027.70	1	73.76	5.62	13.12	172.53	<0.0001
X6X9	288.00	1	-6.00	3.61	-1.66	2.76	0.1479
$X1^2$	1.883E+005	1	-112.25	2.64	-42.51	1802.18	<0.0001
$X3^2$	84753.57	1	-75.31	2.64	-28.52	811.10	<0.0001
$X6^2$	11512.55	1	-27.76	2.64	-10.51	110.18	<0.0001
$X9^2$	2.762E+005	1	-135.94	2.64	-51.49	2643.02	<0.0001

Std Dev = 10.22, Mean = 332.52, C.V% = 3.07, R^2 = 0.9990, Adj R^2 = 0.9968, Pre R^2 = 0.9646, Adeq Precision = 61.233, PRESS = 22860.5.

(Equation 2):

$$Y= +560.96+125.76\ X1+64.22\ X3+13.86\ X6+38.35\ X9+46.60\ X1X3+16.75\ X1X6+ 38.72\ X1X9+7.25\ X3X6+73.76\ X3X9-6.00\ X6X9-112.25\ X1^2-75.31\ X3^2-27.76\ X6^2-135.94\ X9^2$$

Where, Y, is predicted response; X1, X3, X6 and X9 are the coded values for starch, yeast extract, sodium chloride and incubation time, respectively. Starch, yeast extract, sodium chloride and incubation time were found to be significant using Plackett-Burman design. To validate the fit of the equation-3 of RSM, the regression based determination coefficient R^2 was evaluated (Manikandan et al., 2009). As reported earlier, the value of R^2 of the model nearer to 1.0 would explain better variability of experimental values to predict response (Sayyad et al., 2006). This model presented a high R^2 value (0.9990) explaining 99.9% of the variability in the response. The statistical significance of the above equation was further checked by the results of ANOVA and F-test (Table 6). Adjusted R^2 value (0.9968) is in reasonable agreement with predicted R^2 value (0.9646) which showed an excellent correlation among the predicted and experimental values (Khuri and Cornell, 1987). A significant value of model (p = <0.0001), a non-significant value of lack of fit (p = 0.2052) and low value of coefficient of variation (CV = 3.07%) indicates that model is significant (Box et al., 1978; Li et al., 2007).

The responses taken from Table 6 reveal that all the four significant variables have remarkable effects on α-amylase production. Starch as carbon source for α-amylase medium showed the highest level of significance. As described by Gangadharan et al. (2006), starch can be a preferable carbon source over glucose due to feedback inhibition caused by reducing sugars for α-amylase production in *Bacillus* sp. F-value of 52.53 and a very low p-value (0.0019) of starch demonstrated its significance and larger effect on α-amylase production. These results confirmed the suggestion of Tanyildizi et al. (2006) that concentration of starch has a direct effect on α-amylase production. Organic nitrogen sources produce high α-amylase yield as compared to inorganic nitrogen sources from several *Bacillus* sp. (Babu and Satyanaryana, 1993; Narang and Satyanarayana, 2001; Haq et al., 2002; Aiyer, 2004; Swain et al., 2006). In our study, we observed significant effect of yeast extract on α-amylase production. High F-value (29.30) and significant p-value (0.0056) of yeast extract revealed that it could act as limiting variable as starch in α-amylase production. A small variation in starch and yeast extract concentration can alter α-amylase yield as cleared from observed values in Table 4. Sodium chloride acts as Na^+ source and required for usually thermostable α-amylase. Significant effect of sodium chloride on α-amylase production in our study suggests that α-amylase produced from this *Bacillus* sp. is thermostable (Stefanova and Emanuilova, 1992). Incubation time with highest F-value (54.21) and p-value (0.0018) among culture conditions explained its importance on α-amylase yield.

The 3D response surface graphs with counter plots (Figure 2) are the graphical representation of the regression equation generally used to visualize the relationship between the response and experimental levels of each variable and the type of interaction between the variables to deduce the optimum conditions (Chen et al., 2009).

The counter plots predict the observed response at any certain point between two variables while keeping the other two at hold (center point) value. Effect of starch and yeast extract on α-amylase yield as illustrated in Figure 2A revealed that by increasing the amount of starch, α-amylase yield increased but when it crosses the 2.75 value, it gradually decreased. Yeast extract showed the maximum α-amylase yield in the range of 6-9. Figure 2B explains the quadratic effect of starch and sodium chloride on α-amylase yield while Figure 2C represents the quadratic effect between incubation time and starch. Maximum yield of α-amylase achieved between 45-50 h of incubation. Figure 2D showed quadratic effect of both variables, sodium chloride and yeast extract on production of α-amylase. Figure 2E represents the quadratic effect of incubation time and yeast extract while Figure 2F showed the quadratic effect between sodium chloride and incubation time. All these graphs revealed that the optimum conditions for α-amylase production from *B. subtilis* 168 are in the model range.

The pI value of the isolated α-amylase was around pH 9-11. α-Amylase was homogenous with molecular mass approximately 55 Kda as judged by SDS-PAGE (Figure 3 & 4). Purified enzyme was checked through activity staining method on SDS-PAGE, a clear band on a dark background after I_2-KI staining, represented the amylolytic activity (Figure 5) confirmed the size and homogeneity of the protein.

Optimum levels of the variables were obtained by solving the regression equation and analyzing the counter plots as well as point prediction method using design expert software. The statistical analysis based on central composite design showed significant effects of starch, yeast extract, sodium chloride and incubation time for improvement of α-amylase production. The mean value of α-amylase yield was 639.7 IU/ml, which was in excellent agreement with the predicted value (633.5 IU/ml). The final media composition and optimized cultural conditions were, starch 2.55 gl^{-1}, yeast extract 8.4gl^{-1}, sodium chloride 8.1%, inoculum size 1.75%, 37°C incubation temperature, 200 rpm agitation speed and 48 h of incubation, which resulted in an overall 3-fold increase of α-amylase yield as compared with original medium used. These optimized medium and cultural conditions will be helpful for further study using large-scale batch fermentation for α-amylase production from isolated *B. subtilis* 168.

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