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Effect of physical parameters, carbon and nitrogen sources on the production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1

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Abstract - The effect of process physical parameters (initial pH of the medium, temperature and rpm of the shaking incubator) on the production of alkaline protease from a newly isolated *Bacillus pseudofirmus* SVB1 was studied using central composite design technique. The individual optimum levels of initial pH of the medium, temperature and rpm of the shaking incubator were found to be 9.2, 27.3 °C and 195, respectively for the production of alkaline protease (specific activity, U/mg of protein) and 11, 28.9 °C and 210, respectively for cell growth. By applying multiresponse analysis method of generalized distance approach for production and growth, the optimal levels were found to be 9.9, 28.1 °C and 202, for initial pH of the medium, temperature and rpm of the shaking incubator, respectively. After optimization, the production of alkaline protease and cell growth were enhanced by 36.23 and 44.29%, respectively. Also, the various nutritional parameters, which have significant effect on the production of alkaline protease, are delineated in the present study. Alkaline protease production was found to be influenced by media components, *viz*., types of C/N source and presence of metal ions. Finally an overall 6.2 fold increase in the production was achieved using casein as both carbon and nitrogen source.

Key words: alkaline protease; physical process parameters; statistical method for design of experiment.

INTRODUCTION

Alkaline proteases (EC.3.4.21-24, 99) are of immense interest due to their wide applications in detergent, tanning, textile and dairy industries, organic synthesis, peptide synthesis, instant recovery of silver from photographic plates and waste water treatment (Gupta *et al*., 2002). Alkaline proteases contribute greatly in the global market of industrial enzymes (Banerjee *et al*., 1999). In general, microbial proteases are extracellular and would simplify the downstream processing of the enzyme as compared to proteases obtained from plant and animal sources (ÖZTÜRK *et al*., 2009). Despite the long list of protease producing microorganisms, only a few are considered as appropriate producers for commercial exploitation, being 'generally regarded as safe' (GRAS), non-toxic and non-pathogenic. In view of this, search for new microorganisms, which are able to produce novel alkaline protease, is being carried out till date on the globe (Dipasquale *et al*., 2008).

The production of extracellular protease in microorganisms is influenced significantly by physical factors such as pH, temperature, dissolved oxygen, incubation time, inoculum level, aeration and agitation and medium constituents (Hanlon *et al*., 1982; McKeller and Cholette, 1984; Kole *et al*., 1988; Calik *et al*., 2002, 2003; Shafee *et al*., 2006; Potumarthi *et al*., 2007). Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum levels of parameters for desirable responses (Myers and Montgomery, 2002). This method has been successfully applied in many areas of biotechnology such as bioconversion of cheese whey to mycelia of *Ganoderma lucidum* (Lee *et al*., 2003), enzyme production (Bocchini *et al*., 2002), enzyme kinetics (Beg *et al*., 2002), bacteriocin production (Li *et al*., 2001) and protease production from *Bacillus* species (Puri *et al*., 2002, Beg *et al*., 2003).

In this study we report a novel strain, *Bacillus pseudofirmus* SVB1, which produces alkaline protease extracellularly in a wide range of pH and temperature. We have optimized the physical parameters *viz.,* initial pH of the medium, temperature and rpm of the shaking incubator for the production of alkaline protease and cell growth using central composite design (CCD). The effects of different carbon/nitrogen sources and different salts on alkaline protease production have been studied. This is the first comparative study between single responses and multiresponse analysis for alkaline protease production.

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MATERIALS AND METHODS

Organisms and culture conditions. The soil samples were collected in the premises of a tannery industry located at Park Circus, Kolkata, India and diluted in sterile saline solution (0.9% w/v). The diluted samples were plated onto nutrient agar plates (pH 10.0) containing (in g/l) beef extract 1.0, yeast extract 2.0, peptone 5.0, NaCl 5.0, agar 20.0 and were incubated at 30 °C. After 36 h, the isolates grown on the plates were streaked on gelatin agar plate (pH 10.0) containing (in g/l) gelatin 5.0, NaCl 5.0, and agar 20.0 to screen potential alkaline protease producers. Most efficient strain was selected based on its ability to form colony in very less time and large zone of clearance on gelatin agar plate. Based on its 16s rRNA sequence analysis, it was identified as *Bacillus pseudofirmus*. Cultures were regenerated every 2-3 weeks on a fresh Nutrient agar plates from the frozen stock culture. The cell densities were determined by reading the optical densities of the culture broth at 600 nm using UV-visible spectrophotometer (Perkin Elmer, Lamda 45). The cell-free supernatant was used to determine proteolytic activity and protein content.

Inoculum preparation and alkaline protease production. The inoculum was prepared by adding a loop full of pure culture into 50 ml of sterile Luria-Bertani broth medium (pH 10.0) containing (in g/l) casein enzymatic hydrolysate 10.0, yeast extract 5.0, and NaCl 5.0 in a 250 ml shake flask and incubated at 30 °C on a shaking incubator for 10 h. Inoculum (1%) from the seed culture ($A_{600} \approx 1.0$) was added to 100 ml of the medium in 500 ml shake flasks at different initial pH of the medium and incubated at different temperatures and rpm of the shaking incubator as per the central composite experimental design (Table 1). Samples were withdrawn at regular interval of time and growth of microorganism in each sample was measured at A_{600}. The cultures were centrifuged at 8000 rpm at 4 °C for 10 min. The cell free extract was used as crude preparation to measure alkaline protease activity.

Assay for proteolytic activity. Alkaline protease activity was measured by a modified method of Anson-Hagihara (Hagihara *et al*., 1958). The enzyme (35 µl) was added to 840 µl of casein solution (0.6%, w/v in 25 mM Borax-NaOH buffer, pH 10.0) and the reaction mixture was incubated at 30 °C for 10 min. The reaction was terminated by the addition of 896 µl of TCA mixture (0.11 M trichloro acetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). The terminated reaction mixture was incubated for 30 min at room temperature. The precipitates were removed by centrifugation at 13500 rpm for 15 min and absorbance of the supernatant was measured at 278.5 nm. One unit of alkaline protease activity (U/ml) was defined as the amount of enzyme required to liberate 1 µg of tyrosine/min under assay conditions. Enzyme unit was measured using tyrosine (0-150 µg) as standard. Specific enzyme activity was obtained by dividing the enzyme units by total protein content in mg per ml.

Protein concentration. The total protein contents of the samples were determined according to the method described by Lowry *et al*., (1951) using bovine serum albumin (Sigma) as standard.

Experimental design. The initial pH of the medium, temperature and rpm of the shaking incubator were considered in the present optimization process. Alkaline protease production (specific activity) and cell growth of *Bacillus pseudofirmus* SVB1 were taken as the responses. The physical process parameters were optimized using response surface methodology (Box and Hunter, 1957). The central composite design (CCD) with three variables at three levels was employed in this regard (Box and Wilson, 1951) using statistical software package MINITAB® Release 15.1, PA, USA. According to CCD, the total number of treatment

TABLE 1 - Experimental design of pH, temperature and rpm of the shaking incubator with coded and un-coded values for optimizing the two responses (specific activity and cell growth)

Run number	Variables		
	a (≡ A), pH	b (≡ B), ºC	c (≡ C), rpm
1	1.682 (≡ 9.78)	−1.682 (≡ 23.2)	1.682 (≡ 209)
2	1.682 (≡ 9.78)	1.682 (≡ 32.8)	1.682 (≡ 209)
3	0 (≡ 8)	0 (≡ 28)	1 (≡ 250)
4	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
5	−1.682 (≡ 6.22)	1.682 (≡ 32.8)	1.682 (≡ 209)
6	0 (≡ 8)	0 (≡ 28)	−1 (≡ 50)
7	0 (≡ 8)	−1 (≡ 20)	0 (≡ 150)
8	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
9	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
10	1.682(≡ 9.78)	1.682 (≡ 32.8)	−1.682 (≡ 91)
11	1 (≡ 11)	0 (≡ 28)	0 (≡ 150)
12	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
13	1.682(≡ 9.78)	−1.682 (≡ 23.2)	−1.682 (≡ 91)
14	−1.682 (≡ 6.22)	−1.682 (≡ 23.2)	1.682 (≡ 209)
15	−1.682 (≡ 6.22)	1.682 (≡ 32.8)	−1.682 (≡ 91)
16	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
17	0 (≡ 8)	1 (≡ 36)	0 (≡ 150)
18	0 (≡ 8)	0 (≡ 28)	0 (≡ 150)
19	−1.682 (≡ 6.22)	−1.682 (≡ 23.2)	−1.682 (≡ 91)
20	−1 (≡ 5)	0 (≡ 28)	0 (≡ 150)

A: pH, B: temperature (°C) and C: rpm of the shaking incubator. Values in the parenthesis indicate the un-coded values. The fermentation was carried out in 500 ml Erlenmeyer flasks with a working volume of 100 ml for 24 h on a shaking.

combinations was 20 (= 2^k + 2*k* + 6), where *k* is the number of independent variables (Araujo and Brereton, 1996). Fourteen experiments were augmented with six replications at the center points to evaluate the pure error (Table 1). The relationship among the variables, i.e. initial pH of the medium, temperature and rpm of the shaking incubator were expressed mathematically in the form of a quadratic model, which gave the response as a function of relevant variables (equation - 1).

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

Where *Y* is the response (enzyme production or cell growth), β_0 the constant coefficient, X_i (i = 1–3) are non-coded variables (initial pH of the medium: A, temperature: B and rpm of the shaking incubator: C), β_is are the linear, β_{ii}s are the quadratic, and β_{ij}s (*i* and *j* = 1–3) are the second-order interaction coefficients.

Multiple response optimization. An analysis in which a number of responses (output variables) are measured simultaneously for each setting of a group of parameters (input variables) is called multiresponse analysis. The difficulty raised is the fact that more than one response is studied simultaneously. The single response analysis has limitations in systems having a large number of input variables and responses. The meaning of optimum becomes unrealistic since the optimum conditions for one response may not be suitable or may be impractical for other responses. The optimal conditions evaluated by this analysis are sometimes called 'near' optimal for all responses. This is well understood fact that response is enhanced more in case of single response optimization as compared to multiple response optimization. The optimization was carried out based on the individual desirability using a desirability function (also called utility transfer function). Individual desirability evaluates how the settings optimize a single response; composite desirability evaluates how the settings optimize a set of responses overall. Optimal settings for input variables were determined by maximizing the composite desirability. These values are combined to determine the composite or overall desirability of the multiresponse system. An optimal solution occurs where composite desirability reaches its maximum. Desirability has a range of zero to one. One represents the ideal case; zero indicates that one or more responses are outside their acceptable limits.

Selection of most suitable carbon and nitrogen sources. Different carbon or nitrogen sources each at 1% w/v concentrations were used for each run. Samples were withdrawn after 24 h of incubation and growth of microorganism in each sample was measured at A_{600}.

RESULTS AND DISCUSSION

Isolation, screening and selection of microorganism

A total of 67 strains were isolated from the soil collected in the premises of tannery industry. Among them, 23 isolates were screened for the production of extracellular alkaline protease based on the diameter of clear zone due to gelatin hydrolysis on the agar plates. We have used gelatin as the sole source of carbon and nitrogen in the agar plates at pH 10.0 to screen alkaline protease producers. Hydrolysis of gelatin at pH 10.0 has been performed by alkaline protease, which solubilise gelatin by digesting peptide bonds. Hence, we confirmed that all the organisms grown on the gelatin agar plates were producing alkaline protease. The strain, which showed maximum zone of clearance was identified as *Bacillus pseudofirmus* SVB1 (GenBank accession no. EU533950). Based on 16s rRNA sequence analysis, this strain have shown 98 % similarity with its nearest neighbour in the phylogenetic tree. This microorganism has grown at a wide range of pH (6.2-11.0) and temperature (20-36 °C) and also ability to produce alkaline protease at similar conditions. The enzyme assay was carried out at pH 10.0 to inhibit neutral or acid proteases present in the culture filtrate.

Individual optimization of physical parameters

The production of the alkaline protease from *Bacillus pseudofirmus* SVB1 exhibits a characteristic relationship with growth of that organism (Fig. 1). Alkaline protease production reached its highest at the late log phase as it is evident from Fig. 1. Finally the enzyme production went down as stationary phase was reached. Hence, it seems from the plot that the alkaline protease may be one of the primary metabolite and might be growth associated and probably not a secondary metabolite. Similar profile for alkaline protease production has been reported earlier (Singh *et al*., 2004; Silva *et al*., 2007). The growth environment might have some influence on the alkaline protease synthesis. Hence, the effect of physical parameters on the production of alkaline protease (specific activity, U/mg of protein) and growth of *Bacillus pseudofirmus* SVB1 was studied.

Experiments were performed according to the central composite design (Table 1). Experimental and predicted responses (enzyme production (specific activity of alkaline protease, U/mg of protein), and growth of microorganism) are shown in Table 2. The second order regression equation provided the levels of the production and the cell growth as a function of initial pH of the medium, temperature and rpm of the shaking incubator, which can be predicted by the following equations:

$$Y_1 = -44.0018 + 6.4428A + 0.8256B + 0.0956C - 0.3965A^2 - 0.0128B^2 - 0.0003C^2 + 0.0080AB + 0.0033AC - 0.0010BC \quad (2)$$

$$Y_2 = -2.3210 + 0.2368A + 0.0742B + 0.0028C - 0.0104A^2 - 0.0013B^2 + 0.0008AB \quad (3)$$

Where Y_1 and Y_2 represent responses of production and cell growth, respectively. *A*, *B* and *C* are initial pH of the medium, temperature (°C), and rpm of the shaking incubator, respectively.

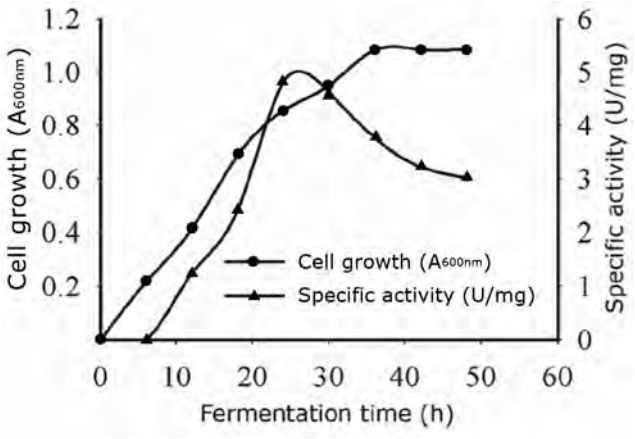

FIG. 1 - Cell growth and alkaline protease production profile under unoptimized conditions (for initial pH of the medium being 8, temperature 28 °C and rpm of the shaking incubator 250 rpm).

TABLE 2 - Experimental and predicted values of specific activity and cell growth obtained at various combinations of pH, temperature and RPM of shaking incubator as per the design given in Table 1

Run number	Enzyme production (specific activity, U/mg)		Cell Growth (A_{600nm})	
	Experimental	Predicted	Experimental	Predicted
1	6.002	6.024	0.4215	0.4126
2	6.2906	5.768	0.4304	0.4286
3	4.825	4.756	0.3414	0.3225
4	5.5235	5.425	0.3011	0.3075
5	1.829	2.000	0.0714	0.1001
6	0.925	1.075	0.2205	0.2125
7	4.878	4.451	0.2182	0.2066
8	5.3232	5.425	0.3013	0.3075
9	5.623	5.425	0.3051	0.3075
10	3.914	3.449	0.3909	0.3844
11	3.633	4.318	0.4545	0.4824
12	5.2237	5.425	0.3085	0.3075
13	2.781	2.557	0.3409	0.3345
14	2.116	2.528	0.0814	0.1110
15	1.165	1.090	0.0156	0.0474
16	5.3421	5.425	0.3089	0.3075
17	4.251	4.758	0.2542	0.2394
18	5.5234	5.425	0.309	0.3075
19	0	0.470	0.0003	0.0244
20	0	0.605	0.0001	0.0546

Experimental values are average of triplicates within ± 5% standard error.

TABLE 3 - ANOVA table for specific activity of alkaline protease: effect of physical process parameters

Source	Degrees of freedom	Sequential sum of squares	Adjusted sequential sum of squares	Adjusted mean of sum of squares	F	P
Regression	9	78.9278	78.9278	8.7698	36.79	0.000
Linear	3	45.7026	14.1149	4.7050	19.74	0.000
Square	3	31.5367	31.5367	10.5122	44.11	0.000
Interaction	3	1.6885	1.6885	0.5628	2.36	0.133
Residual error	10	2.3834	2.3834	0.2383	–	–
Lack-of-fit	5	2.2671	2.2671	0.4534	19.49	0.003
Pure error	5	0.1163	0.1163	0.0233	–	–
Total	19	81.3112	–	–	–	–

R^2 = 97.07% R^2 (predicted) = 78.52% R^2 (adjusted) = 94.43%

TABLE 4 - ANOVA table for SVB1 growth during alkaline protease production: effect of pH, temperature and RPM of shaking incubator

Source	Degrees of Freedom	Sequential Sum of Squares	Adjusted Sequential Sum of Squares	Adjusted Mean of Sum of squares	F	P
Regression	9	0.392529	0.392529	0.043614	54.16	0.000
Linear	3	0.364389	0.020420	0.006807	8.45	0.004
Square	3	0.027194	0.027194	0.009065	11.26	0.002
Interaction	3	0.000946	0.000946	0.000315	0.39	0.762
Residual error	10	0.008053	0.008053	0.000805	–	–
Lack-of-fit	5	0.007983	0.007983	0.001597	114.31	0.000
Pure error	5	0.000070	0.000070	0.000014	–	–
Total	19	0.400581	–	–	–	–

R^2 = 97.99% R^2 (predicted) = 84.85% R^2 (adjusted) = 96.18%

The analysis of variance (ANOVA) for production and growth demonstrates that the model is highly significant at $P < 0.0005$ (Table 3) and $P < 0.0005$ (Table 4) respectively. The *F* values corresponding to production of the alkaline protease and cell growth are 36.79 and 54.16, respectively. These values are much greater than the tabulated *F* value (25.27) at 0.001% level of significance and the null hypothesis is rejected for all the two responses. This shows that squared regression was significant at the level of 100% for the two responses. The value of R^2 (coefficient of determination) is the measure of the total variation of the observed values of responses about the mean explained by the fitted model, which is often expressed in percentage. In other words, R^2 describes the goodness of fit of the model. The values of R^2 corresponding to production of the alkaline protease and cell growth are 97.07 and 97.99%, respectively. This indicates the total variation of 97.07 and 97.99%, respectively for the production of alkaline protease and cell growth was explained by the model.

The optimum level of each variable and the effect of their interactions on the production of the alkaline protease and cell growth were studied by plotting three dimensional response surface curves against any two independent variables, while keeping the other variable at its respective middle level. According to the response surface plots, production increases sharply with initial pH of the medium and starts decreasing after some maximum value of initial pH of the medium and with temperature it increased and then decreased but the range of change being very small when rpm of the shaking incubator was kept constant at 150 rpm (Fig. 2A). When temperature was kept constant at 28 °C, production increased with initial pH of the medium up to a maximum value and then decreased so also with rpm of the shaking incubator (Fig. 2B). However, when initial pH of the medium was kept constant at 8, production increased very slowly with temperature and then decreased and with rpm it increased sharply and then started decreasing in the design space (Fig. 2C). This is very clear from the response surface plots that temperature has very little effect on the production, where as initial pH of the medium and rpm influences very strongly. The optimum levels of initial pH of the medium, temperature and rpm of the shaking incubator were determined by maximizing the regression (equation - 2) and were found to be 9.24, 27.3 °C and 195 rpm, respectively (Table 5). Experiment was performed at these optimal levels of parameters and the production (specific activity) was found to be 6.58 U/mg. When compared with levels before optimization (for initial pH of the medium being 8, temperature 28 °C and rpm of the shaking incubator 250 rpm, production was 4.83 U/mg), an enhancement of 36.23% in production (specific activity) was achieved.

According to Fig. 3A, cell growth increased in the design space with initial pH of the medium at a decreasing rate and finally reaches a plateau in the design space whereas, it varied very little with temperature when rpm of the shaking incubator was kept constant at 150 rpm. But, for both the variables, as the curvature shows, there is no point of maxima in the design space. Similarly, in Fig. 3B cell growth increased with initial pH of the medium and rpm of the shaking incubator when temperature was kept constant at 28 °C without passing through any point of maxima. But, Fig. 3C inferred that, maximum cell growth have been achieved at higher values of temperature and moderate values of rpm of the shaking incubator when the initial pH of the medium was kept constant at 8. Only from Fig. 3C it can be conferred that there is a maxima in the design space for temperature. Whereas, from the contour depicted on the surfaces; it can be seen that as the height in the z axis is increasing, the contours are converging in the design space. The elliptical nature of the contour indicates the presence of interactions among the variables. Actually, the surfaces are showing the interactions of two parameters at a time. Hence, to find the single response optima, all three figures are to be taken in to consideration and for getting multiresponse optima, all six surfaces in Fig. 2 and Fig. 3 are to be considered all together. Similar result has been reported previously (Reddy *et al*., 2008). By maximizing the regression (equation - 3), the optimum values of initial pH of the medium, temperature and rpm of the shaking incubator were found to be 11, 28.9 °C and 210 rpm, respectively, for cell growth. Experiment has been carried out under optimum levels of variables and the cell growth was found to be 0.4926 at 600nm. The cell growth under unoptimized condition was found to be 0.3414, and an enhancement of 44.29% was achieved. A similar type of response has been observed on the growth of some haloalkaliphilic archaea such as a *Natronoincula* (Zhilina *et al*., 1998) and *Natronorubrum bangense* (Xu *et al*., 1999), where the optimum pH for growth was 9-9.5. At low temperatures (up to optimal level), an increase in temperature would enhance the growth of the microorganism and therefore improvement in the enzyme activity was observed. At high temperatures (above optimal level), the volatilization of water in the medium restrains the growth and enzymatic production (Hongzhang *et al*., 2006).

Multiple response optimization

The optimum value for each of the variables predicted by multiresponse optimization for both the responses (enzyme production and cell growth) is shown in Table 5. The optimal levels of initial pH of the medium, temperature and rpm of the shaking incubator were found to be 9.85, 28.1 °C and 202 rpm, respectively, using multi response optimization. Experiment was performed at those optimum levels of parameters and the production of alkaline protease and cell growth obtained were 6.44 U/mg and 0.4623, respectively. Hence, an enhancement of 33.33 and 35.41% was achieved on the production of alkaline protease and cell growth, respectively. The main difficulty observed in multiresponse analysis is optimization of variables simultaneously to obtain maximum output for multiple responses (Muralidhar *et al*., 2003). Similarly, we observed that the final response value obtained in single response optimization is always higher than the value obtained in multiple response optimization (Table 5). This study revealed that the difference in values obtained for the responses in case of single and multiresponse optimization experiments are very small. Moreover with single response analysis we could not have an idea about the nature of the variation of other responses.

A high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of RSM to optimize the process for enzyme production. Similarly, an improved production was reported in other RSM experiments, most notably in the case of protease production using *Bacillus* sp. RGR-14 (Chauhan and Gupta, 2004). Response surface methodology has been used to optimization of protease production, particularly by *Bacillus* spp. (Puri *et al*., 2002; Beg *et al*., 2003). The production of protease from *Bacillus* sp. PE-11 was enhanced by 2.6 folds using response surface methodology (Oh *et al*., 1995). Similarly, an overall 4.2-fold increase in protease production from *Bacillus* sp. RGR-14 was achieved (Puri *et al*., 2002).

Validation of the experimental model for multi response analysis

The model was validated for all three variables set at the optimal values obtained from multiresponse analysis. The experimen-

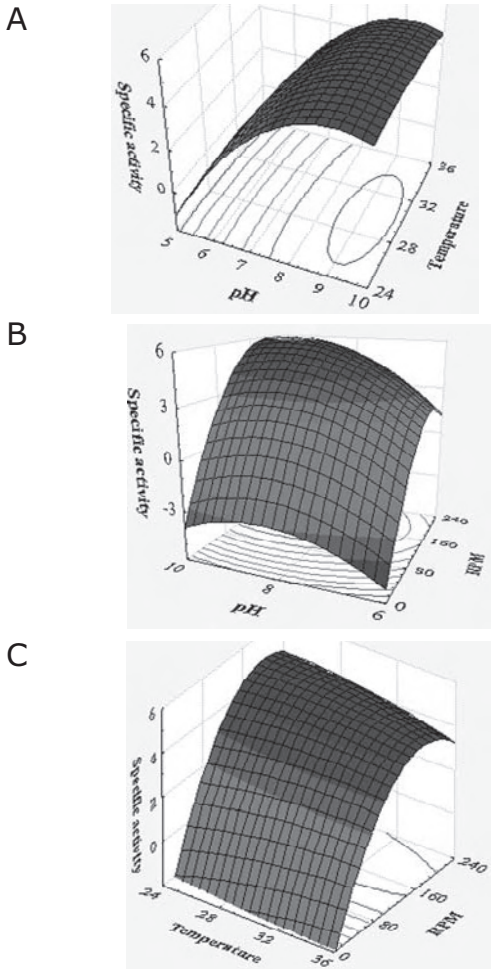

FIG. 2 - Surface plots for specific enzyme activity.

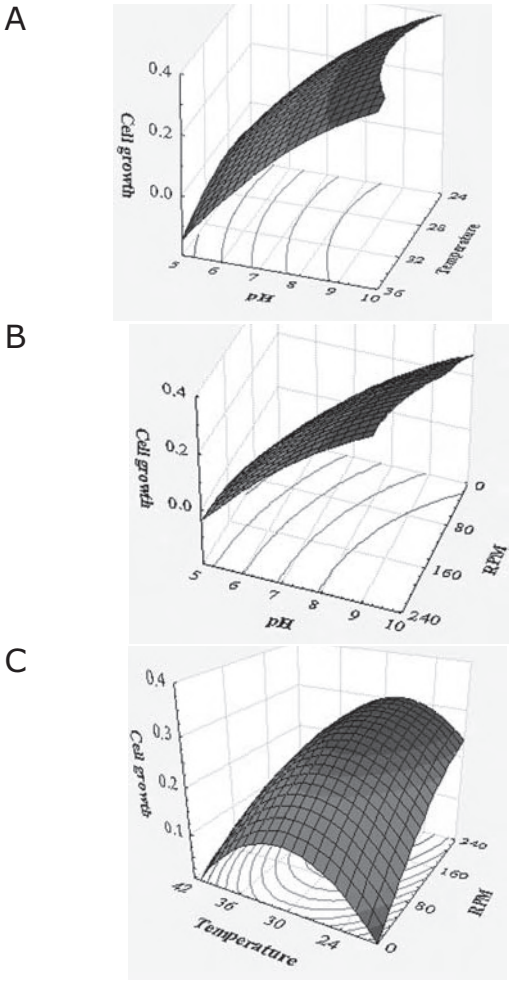

FIG. 3 - Surface plots for growth.

tally determined production values were in agreement with the statistically predicted ones (Table 5) confirming the model's authenticity. Production of alkaline protease and cell growth obtained in this run was higher than any of the other design experimental run.

Effect of carbon and nitrogen sources on alkaline protease production

Effect of different carbon and nitrogen sources on the production of alkaline protease (specific activity) is illustrated in Fig. 4. The maximum production of alkaline protease from SVB1 has been observed in the presence of casein (34.57 U/mg). The production of alkaline protease is 6.2 fold higher as compared to the basal value (4.83 U/mg). The production of alkaline protease was enhanced by complex organic nitrogen sources; whereas, the simple inorganic nitrogen sources did not support the growth of SBV1 and alkaline protease production (Fig. 4). It was observed that the supplementation of other carbon and nitrogen sources did not enhance the production of enzyme. It was observed that casein served as essential carbon and nitrogen source to maximize the production of enzyme by SVB1. Casein had been reported previously as an inducer for the production of alkaline protease (Saurabh *et al*., 2007). When Casein is the only C/N source, the microorganism is forced to produce extracellular alkaline protease in sufficient amount to hydrolyse casein as it cannot be taken in by the cell as a whole. But when ever any other C/N source as yeast extract or peptone or tryptone are present in the media, some smaller peptides are already present

TABLE 5 - The individual and simultaneous maxima, location of individual optima for optimization of physical parameters

Individual/Multiple response optimized		Optimal level of variables			Responses			
					Observed		Predicted	
		A	B	C	Y_1	Y_2	Y_1	Y_2
Individual response	Specific activity (Y_1)	9.24	27.3	195	6.5758	-	6.3437	-
	Cell growth (Y_2)	11	28.9	210	-	0.4926	-	0.4987
Multiple response	Specific activity and Cell growth	9.85	28.1	202	6.4428	0.4623	6.1836	0.4360

A: pH, B: temperature (°C), C: rpm (rpm) of the shaking incubator, Y_1: specific activity (U/mg) and Y_2: cell growth in terms of A_{600nm}. Variables coded are the same as mentioned in Table 1.

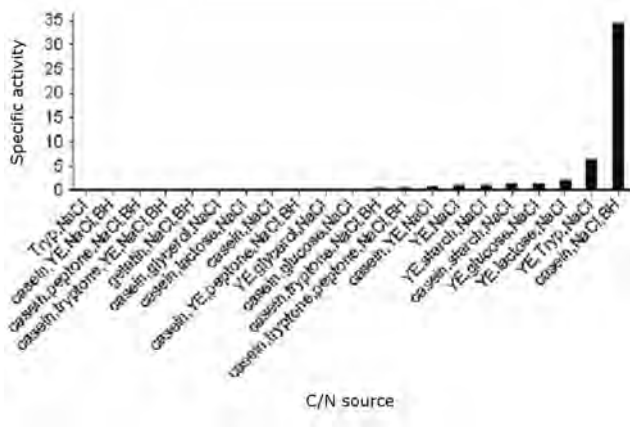

FIG. 4 - Surface plots for growth.

along with them which can be taken in by the bacterial cell without hydrolyzing even. Might be because of availability of easier substrates, the cell is not producing as much alkaline protease in the latter cases as in case of casein presented alone as C/N source. This type of reduction in protease production in presence of easier substrates like Yeast extract, peptone and tryptone has been reported previously (Ferrero *et al*., 1996; Manivannan and Karthiresan, 2007; Saurabh *et al*., 2007). In a similar study, soyacake was used as the sole source of carbon and nitrogen along with mineral solution at ambient temperature (Kanekar *et al*., 2002). Joshi and Ball (1993) reported that protease activity by alkaliphilic species of *Bacillus sphaericus*, *Bacillus brevis* and *Bacillus lactosporus* at 0.65, 1.55 and 1.85 U/ml, respectively, using nutrient broth medium containing 1% gelatin at pH 10 and 37 °C. Sinha and Satyanarayana (1991) have also reported the use of soya meal along with glucose, corn starch and wheat as carbon sources for the production of protease activity using thermophilic *Bacillus licheniformis*. The repression of alkaline protease synthesis from alkaliphilic actinomycete was studied by using glucose, peptone, yeast extract, KH_2PO_4, tyrosine, tryptophan, lysine and arginine. There was a critical limit of stimulation of enzyme production by these components. The low cost substrates such as molasses, wheat flour, and wheat bran were found to be effective for growth and enzyme production (Mehta *et al*., 2006). There are similar reports on enhanced alkaline protease production in the presence of complex carbon (Hubner *et al*., 1993; Gusek *et al*., 1988; Ferrero *et al*., 1996) and organic nitrogen sources (Hubner *et al*., 1993; Ferrero *et al*., 1996). This is in contrast to the report stating high protease yield in the presence of ammonium sulfate and potassium nitrate in *B. licheniformis* (Sinha and Satyanarayana, 1991).

CONCLUSION

Bacillus pseudofirmus SVB1 is found to be the promising alkaline protease producer among other isolates. Its ability to grow and produce alkaline protease in a wide range of pH may be of great use in different effluent treatment processes, where protein content is very high. Response surface methodology (RSM) was successfully applied to determine the optimum levels of physical parameters for enhanced production of this enzyme and cell growth. The optimum levels of parameters were found to be 9.85, 28.1 °C and 202 rpm for initial pH of the medium, temperature and rpm of the shaking incubator, respectively. Finally an overall 6.2 fold increase in specific activity was achieved with casein as a sole source of carbon and nitrogen in the medium. This is the first report on the comparison of individual and multiresponse optimization for the production of alkaline protease. This is also the first report to describe a new screening media for alkaline protease producers using gelatin as sole source of carbon and nitrogen.

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