Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 10 (3): 83-99, 2015
ISSN 1816-4935 / DOI: 10.3923/jm.2015.83.99

Statistical Approach for the Production and Partial Characterization of Alkaline Stable Protease from a Newly Isolated *Bacillus* sp. IND6 for Silver Recovery

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ABSTRACT

A low-cost culture medium for the production of bacterial alkaline protease was developed after response surface methodological approach. Central composite design was applied to optimize the concentration of the three significant factors, namely pH, sucrose and yeast extract. A second-order model equation was obtained and then validated experimentally. The model adequacy was highly satisfactory as the coefficient of determination was 0.98. The maximum enzyme production was 2213 U mL^{-1} after 72 h of incubation, which showed about fivefold increase in enzyme production than unoptimized medium. The partially purified enzyme showed optimum activity at 50°C at pH 9.0. Crude enzyme exhibited a significant stability with most of the tested commercial laundry detergents. This enzyme removed the gelatin layer of used X-ray film effectively. Considering its promising properties, *Bacillus cereus* IND6 enzymatic preparations may be considered a potential candidate for detergent applications and silver recovery.

Key words: Agroresidues, solid-state fermentation, response surface methodology, alkaline proteases, detergent stable, silver recovery

INTRODUCTION

Proteases are the significant group of industrial enzymes that account for about 60% of the total worldwide sale of enzymes from biological sources (Adinarayana *et al*., 2003). Among proteases, alkaline proteases are widely used in leather, detergent, food and silk industries (Gessesse, 1997). These proteases, especially from the genus *Bacillus* having significant stability at high pH and temperature (Yang *et al*., 2000) and these dominate in leather processing and laundry detergent applications (Haddar *et al*., 2009; Jain *et al*., 2012; Nilegaonkar *et al*., 2007; Shrinivas and Naik, 2011; Joshi and Satyanarayana, 2013). Dehairing alkaline proteases have great applications in leather processing industry. These enzymatic dehairing processes have more advantages over the chemical dehairing process (Andersen, 1998). Alkaline proteases also find significant application in silver recovery. Used X-ray film contains 2.0% silver in its gelatin layers and the conventional method of silver recovery by burning used X-ray film causes environmental pollution. The alkaline proteases from *Bacillus* sp. B21-2, *Bacillus* sp. B189 and *B. coagulans* PB-77 (Fujiwara and Yamamoto, 1987; Fujiwara *et al*., 1991; Gajju *et al*., 1996) decomposed the gelatinous layer of the used X-ray films and the silver particle was recovered. Recently, protease has been produced from *Conidiobolus coronatus, Bacillus subtilis* NS and Thermoactinomyces (Shankar *et al*., 2010; Nisha and Divakaran, 2014; Verma *et al*., 2014) for silver recovery.

Submerged Fermentation (SmF) is generally used to produce alkaline proteases. Unlike SmF, Solid-State Fermentation (SSF) processes have been exploited to a lesser extent for the production of alkaline proteases (Malathi and Chakraborty, 1991; Chakraborty and Srinivasan, 1993; George *et al*., 1995). SSF has gained importance in the production of microbial proteases due to several economic advantages over SmF. The advantages of SSF include increased production, lower manufacturing costs, less preprocessing energy, effluent generation and better product recovery along with easy process management (Oliveira *et al*., 2006; Prakasham *et al*., 2006). In addition, it is eco-friendly, as mostly utilizes solid agro-industrial wastes (resides) as the substrate (source of carbon) (Thomas *et al*., 2013). Agroindustrial residues are widely used for the production of alkaline protease by many bacterial species including, *Bacillus* sp. (Prakasham *et al*., 2006; Mukherjee *et al*., 2008). The waste substances, such as corn steep liquor, feather meal (De Azeredo *et al*., 2006) and proteinaceous tannery solid waste, were also used as the substrate for the production of alkaline proteases (Ravindran *et al*., 2011). Agro-residues also used for the production of various enzymes, including lipases and cellulases (Chaturvedi *et al*., 2010; El-Metwally, 2014).

Reports on SSF of cow dung for the production of protease using *Bacillus* sp. are limited (Vijayaraghavan *et al*., 2012). There is no defined medium that has been proposed for the maximum production of alkaline proteases for any microbial species. Each organism required their own process conditions for maximum enzyme production (Kumar and Takagi, 1999). Response Surface Methodology (RSM) has been generally used for the optimized production of enzymes (Abdel-Fattah *et al*., 2013; Khalil *et al*., 2014). There are only few reports on statistical optimization of enzyme production using cheap substrates in SSF. The present investigation aimed to exploit cow dung for the production of a novel alkaline protease production. This article also reports some properties of the partially purified alkaline protease and its potential application in silver recovery, detergent and leather processing industries.

MATERIALS AND METHODS

Screening and identification of a protease producing *Bacillus cereus* IND6: About 1 g of fermented rice sample was serially diluted in double-distilled water to get a concentration range from 10^{-1}-10^{-7}. Isolation of protease producing organisms was carried out using nutrient agar medium containing (g L^{-1}): (w/v) peptone, 5; yeast extract, 5; $MgSO_4$, 0.2; KH_2PO_4, 0.1; skimmed milk, 10; NaCl, 5 and agar, 15. To screen gelatinase-positive isolates, the protease-secreting organisms were grown in the nutrient medium containing (g L^{-1}): peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5.0 and gelatin, 10 (pH 8.0). The isolates were streaked on the plates in a straight line and incubated at 37°C for 24 h. These plates were flooded with 5 mL of 15% (w/v) $HgCl_2$ solution containing 20% HCl. The enzyme activity was visualized as clear zones around the colony due to hydrolysis of gelatin.

Identification of the protease-secreting organism: The isolated strain IND6 with highest protease activity was identified on the basis of the phenotypical characteristics, biochemical properties and the 16S rRNA sequencing. The genomic DNA was extracted from the cells after 18 h of incubation at 37°C using a DNA purification kit (QIAGEN, Germany) according to the manufacturer's instructions. The 16S rRNA gene of the isolate IND6 was amplified by polymerase chain reaction. Amplification of DNA was carried out using the upstream primer P1: 5'-AGAGTTTGATCMTGGCTAG-3' and the downstream primer P2: 5'-ACGGGCGG TGTGTRC-3' and DNA polymerase (Sigma-Aldrich, USA) under the following conditions: denaturation at 95°C

for 3 min followed by 30 cycles at 95°C for 1 min, 55°C for 30 sec and 72°C for 1 min and 50 sec. The amplified product was sequenced and sequence comparison with databases was performed using BLAST through the NCBI server (Altschul *et al*., 1997). The isolate, IND6 was identified as *B. cereus* IND6. The 910 bp sequences were submitted to the GenBank database and an accession number was assigned.

Evaluation of cheap substrates for alkaline protease production: The substrates such as rice bran, banana peel, tapioca peel, green gram husk, wheat bran, orange peel and cow dung were collected and dried for 7 days and powdered. About 5.0 g of substrates was taken in an Erlenmeyer flask and the medium pH was adjusted as 8.0 using 0.1 M tris-HCl buffer. The moisture content was maintained as 100% (v/w) level. The medium was mixed thoroughly and sterilized at 121°C for 20 min. Then these flasks were cooled, inoculated with 0.5 mL (5%, v/w) of 18 h grown culture (OD 600 nm = 0.768) and incubated at 37°C for 72 h. After 72 h, ice-cold double-distilled water was added to the fermented medium and placed in an orbital shaker at 200 rpm for 20 min. The sample was further centrifuged at 10,000×g for 15 min and the supernatant was used as the crude enzyme.

Protease assay: The protease activity was determined by mixing 5.0 mL of casein (prepared in 0.1 M Tris buffer, pH 8.0) and aliquot of 0.05 mL of the crude enzyme. This mixture was incubated at 37°C for 30 min and the reaction was stopped on adding 5.0 mL of trichloroacetic acid (0.1 M). The mixture was filtered after 30 min. Two milliliter of the filtrate was added to 5.0 mL of 0.5 M sodium carbonate and 1.0 mL of Folin-Ciocalteu's phenol reagent. This mixture was incubated for 30 min at 37°C and the protease activity was read at 630 nm using an UV-Visible spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine/min under assay conditions (Chopra and Mathur, 1985).

Optimization of protease production by one-variable-at-a-time approach: The protease production of *Bacillus cereus* IND6 was optimized by varying the physical parameters and nutrient sources. The effect of fermentation period on enzyme production was studied by determining the protease activity for every 12 h of fermentation up to 96 h. The initial pH of the medium was adjusted from pH 6.0-11.0 to study the effect of pH. The buffer system (0.1 M) used were sodium phosphate (pH 6-7), Tris-HCl (pH 8) and glycine-NaOH (pH 9-11), respectively. The effect of moisture content on enzyme production was studied by adjusting the initial moisture content of the cow dung (60-140%, v/w) using glycine-NaOH buffer (pH 9.0). To determine the effect of inoculum size, the inoculum concentration was increased accordingly (3-15%, v/w). The effect of carbon sources (1%, w/w; glucose, sucrose, fructose, maltose and xylose), nitrogen sources (1%, w/w; casein, peptone, yeast extract, beef extract and oat meal) and ions (calcium chloride, sodium dihydrogen phosphate (NaH_2PO_4), ferrous sulphate, magnesium chloride and manganese chloride). The enzyme was extracted from the substrate as described previously and enzyme assay was carried out.

Screening of significant components with two-level full factorial design: A two-level full factorial design (2^5) was carried out to find the key ingredients for enhanced production of protease. The important physical parameters (pH and moisture) and nutrient factors (yeast extract, sucrose and sodium dihydrogen phosphate) were evaluated by statistical approach. The factors such as fermentation period (h) and inoculum (%) were kept at optimum level based on one-variable-at-a-time experimental results. In two-level full factorial design, each factor was examined at low

Table 1: Independent variables and their levels for the screening of significant factors by 2^5 factorial experimental design

		Coded levels	
Variables	Symbol	-1	+1
Moisture (%)	A	7	9
pH	B	80	100
Sucrose (%)	C	0.1	1
Yeast extract (%)	D	0.1	1
NaH_2PO_4 (%)	E	0.01	0.1

Table 2: Results of the 2^5 factorial design

Run	A-moisture (%)	pH	C. sucrose (%)	D. yeast extract	E-NaH_2PO_4 (%)	Response (Y)
1	80	9	0.1	1.0	0.01	275
2	80	9	1.0	1.0	0.1	540
3	100	9	1.0	1.0	0.01	850
4	80	7	1.0	1.0	0.01	385
5	80	7	0.1	1.0	0.1	230
6	100	7	1.0	0.1	0.1	460
7	100	7	1.0	1.0	0.01	875
8	100	9	1.0	0.1	0.01	455
9	100	9	1.0	0.1	0.1	1400
10	100	9	0.1	1.0	0.01	1000
11	100	7	0.1	0.1	0.1	1190
12	80	7	0.1	1.0	0.01	1590
13	80	9	0.1	0.1	0.1	495
14	80	7	1.0	1.0	0.1	700
15	80	9	0.1	0.1	0.01	2245
16	80	9	1.0	0.1	0.01	260
17	100	7	1.0	0.1	0.01	545
18	100	9	1.0	1.0	0.1	1220
19	100	7	0.1	1.0	0.01	1525
20	80	7	0.1	0.1	0.01	900
21	80	9	0.1	1.0	0.1	1325
22	100	7	1.0	1.0	0.1	120
23	80	7	1.0	0.1	0.01	375
24	80	7	0.1	0.1	0.1	475
25	100	9	0.1	0.1	0.1	790
26	80	9	1.0	0.1	0.1	780
27	100	7	0.1	1.0	0.1	925
28	100	9	0.1	0.1	0.01	505
29	80	9	1.0	1.0	0.01	510
30	80	7	1.0	0.1	0.1	670
31	100	7	0.1	0.1	0.01	325
32	100	9	0.1	1.0	0.1	680

and high level. The variables and the levels of factors were described in Table 1. Two-level full factorial designs were based on the following first-order polynomial model:

$$Y = \alpha_0 + \sum_i \alpha_i x_i + \sum_{ij} \alpha_{ij} x_i x_j + \sum_{ijk} \alpha_{ijk} x_i x_j x_k + \sum_{ijkl} \alpha_{ijkl} x_i x_j x_k x_l + \sum_{ijklm} \alpha_{ijklm} x_i x_j x_k x_l x_m$$

where, Y is the protease activity (response); α_{ij}, α_{ijk}, α_{ijkl} and α_{ijklm} were the ijth, ijkth, ijklth and ijklmth interaction coefficients, respectively; α_i was the ith linear coefficient and α_0 was an intercept.

Enzyme assay was carried out in duplicates and the average of these experimental values was taken as response Y (Table 2). The statistical software package "Design-Expert 8.0" (StatEase Inc., Minneapolis, USA) was used to analyze the experimental results. The factors that significantly affect protease production ($p<0.05$) were further selected for RSM.

Table 3: Independent variables and the level for CCD and RSM

		Coded values				
Variables	Symbol	-α	-1	0	+1	+α
pH	A	6.32	7.0	8.0	9.0	9.68
Sucrose (%)	B	-0.21	0.1	0.55	0.1	1.31
Yeast extract %)	C	-0.21	0.1	0.55	1.0	1.31

Table 4: Experimental design and results of the CCD

Run	A-pH	B-sucrose	C-yeast extract	Enzyme activity (U mL^{-1})
1	7.00	0.1	0.1	1372
2	9.00	0.1	0.1	873
3	7.00	1.0	0.1	841
4	9.00	1.0	0.1	2068
5	7.00	0.1	1.0	2213
6	9.00	0.1	1.0	1480
7	7.00	1.0	1.0	622
8	9.00	1.0	1.0	1590
9	6.32	0.55	0.55	810
10	9.68	0.55	0.55	1050
11	8.00	-0.21	0.55	2110
12	8.00	1.31	0.55	1500
13	8.00	0.55	-0.21	1200
14	8.00	0.55	1.31	1508
15	8.00	0.55	0.55	1290
16	8.00	0.55	0.55	1249
17	8.00	0.55	0.55	1308
18	8.00	0.55	0.55	1231
19	8.00	0.55	0.55	1269
20	8.00	0.55	0.55	1348

Statistical optimization of protease production by Central Composite Design (CCD) and RSM: RSM was used to elucidate the optimal concentration of the selected factors for experimental design. CCD was employed to optimize three significant factors, namely pH, sucrose and yeast extract at five levels (-α, -1, 0,+1 and +α) (Table 3). The CCD contains a total of 20 experimental runs (6 central, 6 axial points and 8 factorial). The experiments were conducted in duplicates and the mean value of protease activity was taken as the response (Y). The range of medium components is shown in Table 4. The statistical software package "Design-Expert 8.0" (StatEase) was used to analyze the experimental data. A second-order polynomial equation is as follows:

$$Y = \beta_0 + \sum_{I=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{ij=1}^{3} \beta_{ij} X_{ij}$$

where, Y is the response; β_0 is the offset term and β_i, β_{ii} and β_{ij} were the coefficients of linear terms, square terms and coefficients of interactive terms, respectively. X_is were A, B and C; X_{ij}s were AB, AC and BC (A-coded value of pH, B-coded value of sucrose and C-coded value of yeast extract). All trials were carried out in duplicate and the average protease yield was used as response Y. The optimum concentrations of the significant variables were obtained by analyzing three-dimensional plots.

Validation of the experimental model: To validate the model equation, experiments were conducted in triplicates for protease production under optimum conditions predicted by the model.

Partial purification and characterization of protease: The enzyme was purified by the combination of ammonium sulphate precipitation, dialysis and gel filtration chromatography. The crude enzyme was precipitated by applying solid ammonium sulphate (30-70% saturation) and the pellet was dissolved in minimal volume of double-distilled water. This sample was dialyzed against water (first exchange) and buffer (second and third exchange) for 4 h, respectively. This dialyzed sample was applied on a sephadex G-75 gel filtration column (2×25 cm) which was pre-equilibrated with sodium phosphate buffer (pH 7.5, 0.1 M). About 5.0 mL of dialyzed sample was applied on the column and eluted with the sample buffer. The flow rate was adjusted as 1.5 mL min^{-1} and 25 fractions were collected. All fractions were subjected to protein quantification and enzyme assay was carried out in all fractions. The active factions were pooled and used for characterization studies.

Effect of temperature and pH on protease activity and stability: The effect of temperature on protease activity was determined by holding the reactions at different temperatures (30-70°C) using the standard assay method. To evaluate the thermal stability of the enzyme, the sample was incubated at these temperatures and the enzyme activity was assayed. The effect of pH on the protease activity was studied by assaying the enzyme activity at various pH values ranging from 5.0-10.0 for 1 h. To evaluate the pH stability of the enzyme, the sample was incubated at these temperatures for 1 h and enzyme activity was assayed as described earlier.

Effect of ions on enzyme activity: To study the effect of ions on enzyme activity, the sample was preincubated with various divalent ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Fe^{2+}, Na^{+} and Zn^{2+}) at 37°C for 1 h and the activity was assayed.

Effect of detergent on enzyme activity: To examine the effect of detergents on enzyme activity, commercially available detergents, namely Ariel, Sun light, Mr. White, Henko, Ujala, Tide+and Surf excel, were added to the crude enzyme solution at 1% (v/v) level, allowed to stand for 1 h at room temperature and the enzyme activity was assayed.

Effect of enzyme on silver recovery: Used X-ray film was washed with double-distilled water and impregnated with ethanol. The washed X-ray film was dried in an oven at 40°C for 30 min. About 0.5 g of X-ray film (1.5×1.5 cm) was incubated in 5 mL of crude enzyme at room temperature (30°C), pH 8.0 in static condition.

RESULTS AND DISCUSSION

Screening of different agro-industrial and waste materials for alkaline protease production: Protease production by *B. cereus* IND6 varied with type of the substrate used in SSF (Fig. 1). Among the substrates used for enzyme production, cow dung supported more enzyme production (240 U mL^{-1}) than other substrates. All substrates used were supported the production of protease. This result was in agreement with the reports on the nature of the substrate. Protease production pattern varied with the types of residue used for enzyme bioprocess (Pandey *et al*., 2000). Based on these results, cow dung was further used as the substrate for optimized production of proteases.

Elucidation of process parameters by one-variable-at-a-time approach: The effect of fermentation period on protease production was carried out for a period of 96 h of incubation at 37°C. Enzyme production was found to be high after 72 h incubation (Fig. 2a) and the enzyme

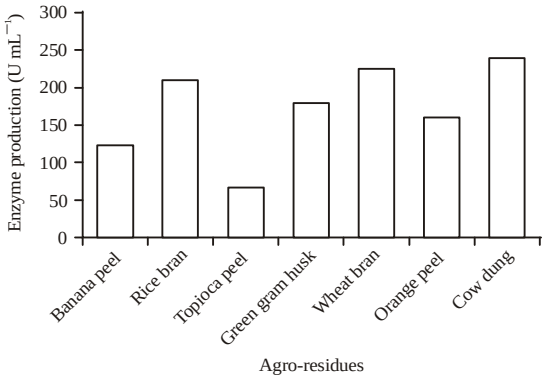

Fig. 1: Effect of agro-residues on enzyme production

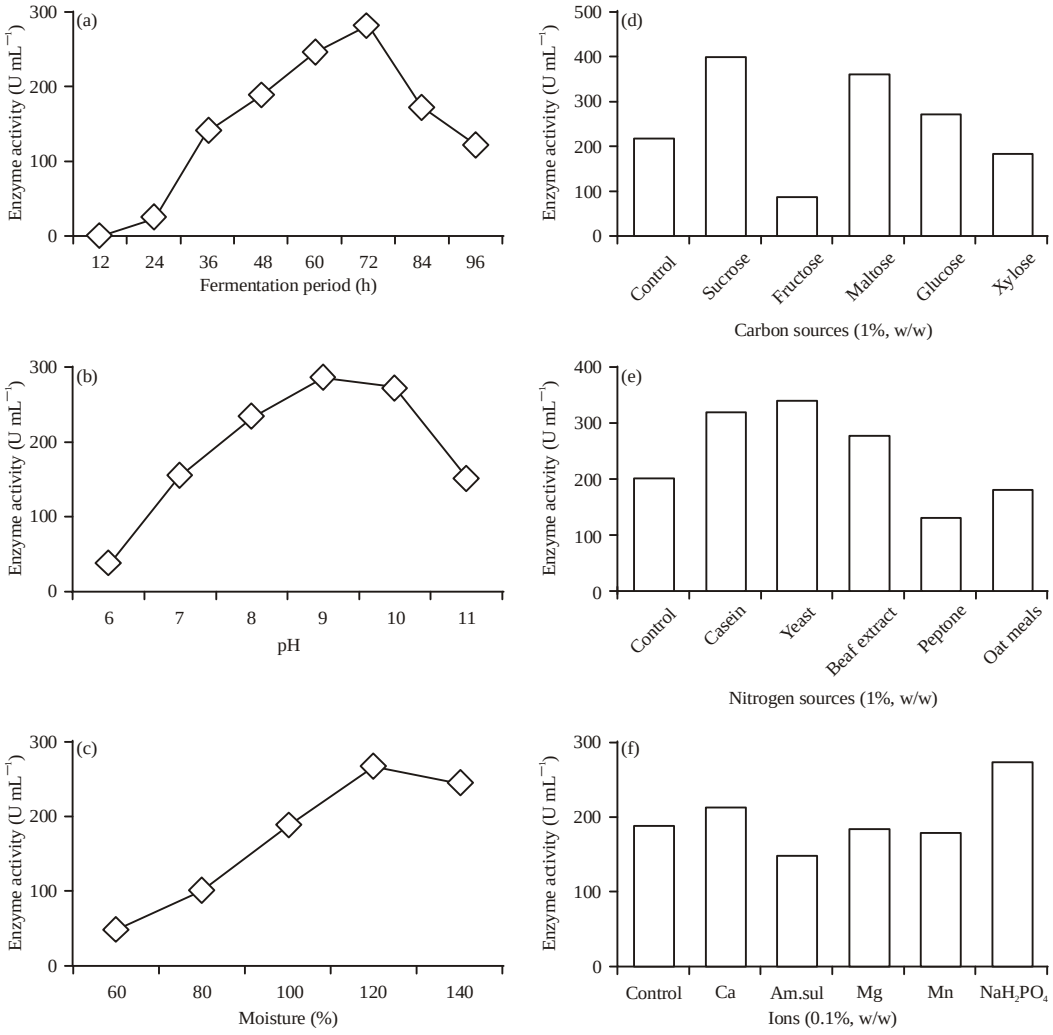

Fig. 2(a-f): Effect of fermentation (a) Period, (b) pH, (c) Moisture, (d) Carbon, (e) Nitrogen and (f) Ion on protease production

production decreased considerably. These results were in accordance with observations made by Kumar and Parrack (2003) with *Bacillus* sp. and George-Okafor and Mike-Anosike (2012) with *Bacillus* sp. SW2. This isolate was capable of producing protease in the pH range of 7-11. Enzyme production was maximum at pH 9 and decreased thereafter (Fig. 2b). At a higher pH, the metabolic action of the bacterium could have been suppressed, thus decreasing the enzyme production. These results were in accordance with observations made with *Bacillus* sp. (Prakasham *et al*., 2006; George-Okafor and Mike-Anosike, 2012). In the present study, enzyme production was high in a range of moisture content (100-140%) and the maximum enzyme production was observed at 120% moisture (Fig. 2c). Moisture content is one of the critical factors for the production of microbial growth and enzyme production in SSF (Pandey *et al*., 2000). The optimum moisture content varied based on the substrate used for the production of enzymes (Prakasham *et al*., 2006; Rajkumar *et al*., 2011).

The influence of carbon and nitrogen sources on protease production was studied. Among the carbon sources tested, sucrose supported maximum production of protease (396 U mL^{-1}) (Fig. 2d). The supplemented carbon and nitrogen sources had significant effect on the production of protease. The results showed that there was a significant increase in alkaline protease production with various tested carbon and nitrogen sources. Among the nitrogen sources, yeast extract showed the maximum production of protease (334 U mL^{-1}) than other nitrogen sources (Fig. 2e). These results were in accordance with observations made with *Bacillus* sp. (Prakasham *et al*., 2006). Protease production was found to be high in the medium containing NaH_2PO_4 (275 U mL^{-1}) (Fig. 2f) among the ionic sources.

Elucidation of significant factors by two-level full factorial design: Optimization of process parameters for protease production was carried out using the two-level full factorial design. In the present study, five significant factors (pH, moisture, sucrose, yeast extract and sodium dihydrogen phosphate) were selected, which contained total of 32 experimental runs. The matrix developed by the two-level full factorial design and the results were shown in Table 2. The proteolytic enzyme production varied from 120-2245 U mL^{-1}. According to the two-level full factorial design, the optimum medium compositions were as follows: pH 9.0, 80% moisture, 0.1% sucrose, 0.1% yeast extract and 0.1% sodium dihydrogen phosphate. The analysis of variance (ANOVA) was used to analyze the main effects and was shown in Table 5. The ANOVA of quadratic regression model is highly significant. The model F-value of 92.54 implies that the model is significant. There is only a 0.07% chance that a "Model F-value" this large could occur due to noise. The goodness of the model was checked using the determination of coefficient (R^2). In this model, R^2 value was 0.999. Values of "Prob>F" less than 0.05 indicate that model terms are significant. In this model, enzyme production was significantly affected by pH (<0.01), sucrose (<0.05) and sodium dihydrogen phosphate (<0.05). The coefficient estimate was negative to sucrose and sodium dihydrogen

Table 5: ANOVA table for 2^5 factorial experimental design

Source	Sum of squares	df	Mean square	F-value	p-value
Model	6.95E+06	29	2.40E+05	92.54	0.01
A-moisture	38503.13	1	38503.13	14.86	0.051
B-pH	1.30E+05	1	1.30E+05	50.20	0.019
C-sucrose	5.86E+05	1	5.86E+05	226.16	0.0044
D-yeast extract	2.42E+04	1	2.42E+04	9.34	0.05
E-NaH_2PO_4	1.20E+04	1	1.20E+04	4.64	0.016
Residual	5.18E+03	2	2.59E+03		
Cor total	6.96E+06	31			

phosphate. This states that the lower levels of sucrose and sodium dihydrogen phosphate concentrations would benefit proteolytic enzyme production. The coefficient estimate was positive to moisture, pH and yeast extract; these indicated that further increase of moisture content, pH and yeast extract of the cow dung medium will further enhance the production of enzyme. A significant factor in SSF that influences the microbial growth as well as production yield is the initial moisture content of the substrate. Each substrate has its own moisture holding capacity and anchorage of the microbial cells. Hence, depend upon the type of substrate, the enzyme production may vary. Previously, 30, 40, 100 and 140% initial moisture contents of wheat bran, lentil husk, *Imperata cylindrica* and potato peel, green gram husk and cow dung, were found to be optimum for maximum protease production by *Bacillus* sp. in SSF (Prakasham *et al*., 2006; Mukherjee *et al*., 2008; Uyar and Baysal, 2004; Vijayaraghavan *et al*., 2012). Adequate precision measures the signal-to-noise ratio with the ratio greater than 4 is desirable (Anderson and Whitcomb, 2005). The "adequate precision" ratio of 43.119 obtained in this result indicated an adequate signal. Neglecting the insignificant variables, the model equation for fibrinolytic enzyme production is as follows:

Final equation in terms of coded factors:

Enzyme activity = +769.38+34.69A+63.75B-135.31C+27.5D-19.38E+71.87AC+67.81AD+
63.44AE+54.06BC-60.63BD+90BE+121.56CE-60DE+128.12ABC+40.31ABD
+25.94ABE-58.13ACD-106.25ACE-147.19ADE+72.81BCD

Response surface methodology: The three independent variables (pH, sucrose and yeast extract) were chosen for optimized production of proteolytic enzymes using RSM design. The CCD model helps to study the interactions between the various variables and RSM helps to explore the optimum concentrations of each of the variables. Experiments were performed according to the given CCD experimental design. RSM is a sequential and effective procedure where the primary objective of the methodology is to run rapidly and efficiently along the path of enhancement toward the general vicinity of the optimum, identifying the optimal region for running the process (Mekala *et al*., 2008; Chennupati *et al*., 2009; Potumarthi *et al*., 2012). Twenty experimental runs with different combinations of three factors were carried out. The maximum protease production was observed at run 5 (Table 4). The results obtained from CCD were analyzed using ANOVA. The model F-value is 97.55. High F-value implies that the model is significant (Table 6). There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. ANOVA indicated that the model terms of A, B, AB, AC, BC, A^2, B^2 and C^2 were significant. The second-order polynomial model was used to correlate the independent variables with protease activity. The coefficient of determination (R^2) was calculated to be 0.988, indicating that the model could explain 98% of the variability. A value of >0.75 indicates appropriate for the model. The "lack-of-fit F-value" of 3.30 implies that the lack of fit is not significant relative to the pure error. There is only a 10.81% chance that a "lack-of-fit F-value" this large could occur due to noise. "Adequate precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. A ratio of 37.90 indicates the adequate signal. The data was fitted with a quadratic second-order polynomial equation. The model showed mean, standard deviation, c. v and Predicted Residual Sum of Square (PRESS) values of 1346, 62.04, 4.63 and 2.373E+0.05, respectively.

Table 6: Results of the regression analysis of the CCD

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	3.38E+06	9	3.38E+06	97.55	<0.0001	Significant
A-pH	1.37E+05	1	1.37E+05	35.54	<0.0001	
B-sucrose	2.49E+05	1	2.49E+05	64.62	<0.0001	
C-yeast extract	1.18E+05	1	1.18E+05	30.64	0.0002	
AB	1.47E+06	1	1.47E+06	381.46	<0.0001	
AC	3.04E+04	1	3.04E+04	7.89	1.85E-02	
BC	5.75E+05	1	5.75E+05	149.44	<0.0001	
A^2	2.13E+05	1	2.13E+05	55.34	<0.0001	
B^2	5.08E+05	1	5.08E+05	132.06	<0.0001	
C^2	1.16E+04	1	1.16E+04	3.01	1.14E-01	
Residual	3.85E+04	10	3.85E+03			
Lack of fit	2.95E+04	5	5.91E+03	3.3	0.1081	
Pure error	8.95E+03	5	1.79E+03			
Cor total	3.42E+06	19				

Final equation in terms of coded factors:

$$\text{Enzyme activity} = +1282.03+100.07A-134.94B+92.92C+428.38AB-61.62AC-268.12BC-121.57A^2+187.79B^2+28.34C^2$$

Response surface plots are generally used for identifying the optimal level of each factor for attaining maximum response (protease) production. Figure 3a-c shows the response surface obtained for the interaction effects of three selected variables. In each three dimensional graph, the effect of the two variables on protease production was shown when the other one variable was kept constant. Figure 3a shows the interaction relationship between the two independent variables, namely pH and sucrose. It was observed from Fig. 3a that protease production was significantly affected by pH and sucrose. It was previously reported that the increase of enzyme production in the culture medium containing sucrose (Phadatare *et al*., 1993; Kumar and Takagi, 1999). The choice of the carbon and nitrogen sources has a major influence on the production of protease. The present results as well as findings from previous studies suggested that different bacterial strains have different preferences for either inorganic or organic nitrogen for growth and protease production, although complex nitrogen sources are generally used for alkaline protease production (Pandey *et al*., 2000; Prakasham *et al*., 2006). Figure 3b shows the interaction between pH and yeast extract. Enzyme production was high at pH 9.0 and the enzyme production increased at increasing concentration of yeast extract. Figure 3c shows the interaction between sucrose and yeast extract. The combination of sucrose and yeast extract significantly increased the production of alkaline protease. Increased yields of alkaline proteases were reported by several workers who used different sugars such as sucrose (Phadatare *et al*., 1993), fructose (Sen and Satyanarayana, 1993), lactose (Malathi and Chakraborty, 1991) and maltose (Tsuchiya *et al*., 1991).

In *Bacillus clausii* and *Bacillus* sp. yeast extract stimulated the production of alkaline proteases (Prakasham *et al*., 2006; Oskouie *et al*., 2008). Among the variables used for RSM, yeast extract had a significant effect on protease production compared with other variables. Before optimization, the enzyme production was 402 U mL^{-1} and fivefold increased enzyme production was achieved after optimizing the medium by RSM. The obtained maximum protease production was estimated to be 2210 U mL^{-1}. There have been many studies were conducted on optimization of different physiochemical parameters for the production of proteases using RSM (Beg *et al*., 2003; Chauhan and Gupta, 2004; Rahman *et al*., 2004; Liu *et al*., 2004). Cost effective medium and optimization of process parameters by statistical methods for enzyme production is extremely

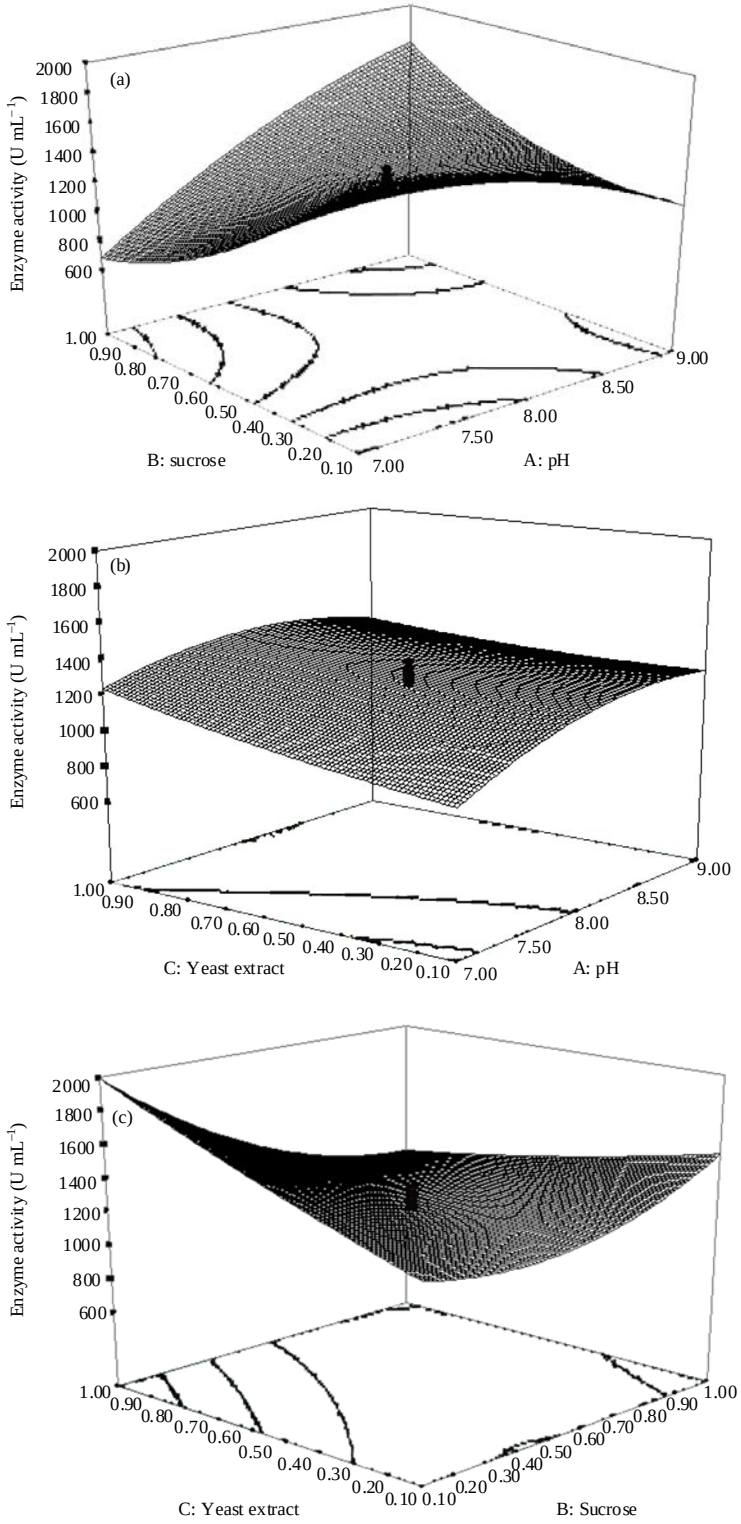

Fig. 3(a-c): Response surface plot for alkaline protease production by *Bacillus* sp. IND6 (a) Interactive effects of pH and sucrose, (b) pH and yeast extract and (c) Sucrose and yeast extract

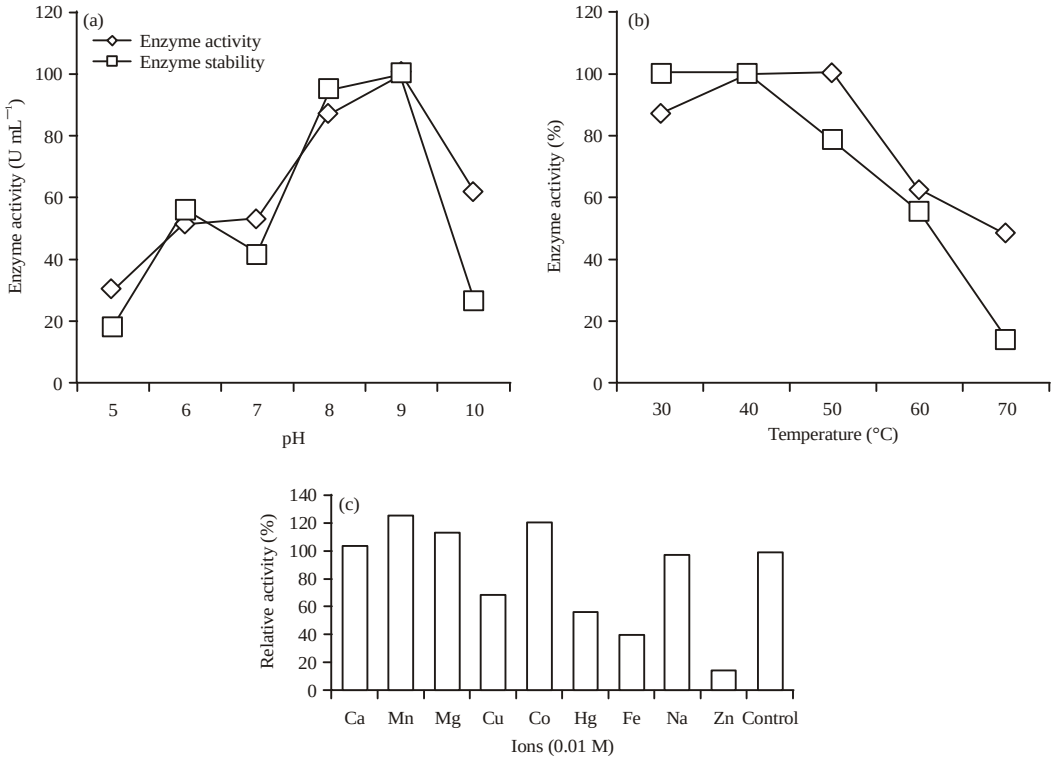

Fig. 4(a-c): Effect of (a) pH, (b) Temperature and (c) Ions on protease activity and stability

important in industrial scale for economic reason. Therefore, common low cost substrate, cow dung for optimizing the process using RSM will serve as a potential substrate for industrial microbial fermentation.

Optimization and validation: Validation of the predicted results was done under optimized conditions in three independent experiments. In this model, the experimental proteolytic activity of 2213 U mL^{-1} was obtained that correlated to the predicted activity (2318 U mL^{-1}) confirming the rationality of the model. This is fivefold higher than that obtained before optimization. Thus, overall fivefold increase in protease activity was observed after optimization.

Effect of temperature, pH and ions on enzyme activity and stability: Some of the biochemical properties of the partially purified enzyme were evaluated. Protease showed maximum activity at alkaline range of pH (8.0-10.0) and active in a broad range of temperature (30-50°C); however, optimum protease activity was observed at pH 9.0 (Fig. 4a) and 50°C (Fig. 4b). The optimum pH for protease activity of *B. cereus* IND6 is comparable with the pH optima of other microbial proteases. When the crude enzyme was incubated with different buffers for 1 h, the protease was very stable over a pH range of 8-9. Similar results were reported with *B. cereus* MCM B-326, *Bacillus* sp. RRM1 and *Bacillus* sp. (Nilegaonkar *et al*., 2007; Rajkumar *et al*., 2011; Haile and Gessesse, 2012). The enzyme activity was highly stable at pH 9 after 1 h incubation. These results were in accordance with the observations made with other *Bacillus* sp. (El Hadj-Ali *et al*., 2007). The commercial microbial proteases have the pH range between 8.0 and

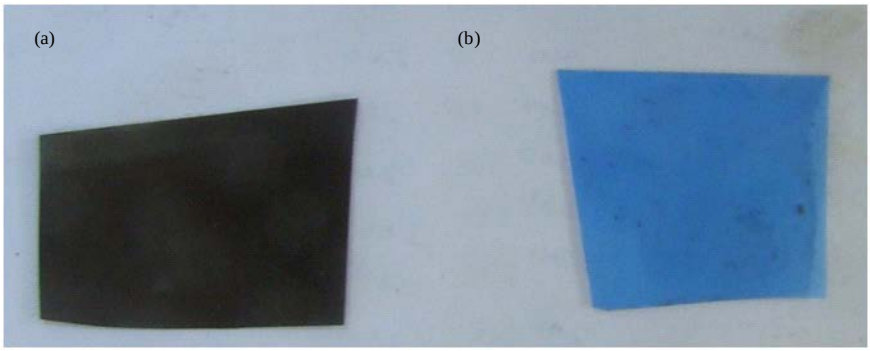

Fig. 5(a-b): Effect of crude protease on silver recovery (a) Control (used x ray film) and (b) Digestion of gelatine layer and removal of silver from the film

12.0 and *B. cereus* IND6 protease fell in these ranges. Enzyme activity was maximum between 40 and 50°C and the enzyme activity decreased rapidly above 50°C. This enzyme was stable up to 50°C for 1 h incubation and lost approximately 44% of its activity. A similar result was reported with *B. subtilis* RTSBA6.00 and *Bacillus* sp. (Toyokawa *et al*., 2010; Haile and Gessesse, 2012). Among the ions tested, Ca^{2+}, Mg^{2+}, $Mn^{2+}$and Co^{2+} positively regulated enzyme activity.

Detergent stability and silver recovery of alkaline protease: *B. cereus* IND6 alkaline protease was widely stable in the presence of various detergents (1%) after 1 h incubation. Enzyme stability was 41, 97, 99, 91, 93, 102 and 89%, respectively, for Ariel, Sun light, Mr. White, Henko, Ujala, Tide and Surf excel, respectively. This result was in accordance the observations made with *Bacillus subtilis* (Vijayaraghavan *et al*., 2012). The alkaline protease digested the gelatine layer of the used X-ray film and removed the silver ions from the X-ray film (Fig. 5a and b). It is also evident the secretion of gelatine degrading alkaline protease by *Pseudomonas aeruginosa* MCMI B-327 (Kumar *et al*., 2002). Successful recovery of silver from X-ray films have been reported by alkaline proteases derived from *Bacillus subtilis*, *Conidiobolus coronatus*, *Streptomyces avermectinus* and *Bacillus lehensis* (Nakiboglu *et al*., 2001; Shankar *et al*., 2010; Ahmad *et al*., 2008; Joshi and Satyanarayana, 2013).

CONCLUSION

From the present study it could be inferred that alkaline protease production by *Bacillus* sp. IND6 was influenced by physical parameters and nutritional factors such as pH, sucrose and yeast extract. The statistical optimized medium showed fivefold enzyme production than unoptimized medium. The strain *Bacillus* sp. IND6 may be successfully utilized for alkaline protease production for various industrial applications.

ACKNOWLEDGMENTS

The author P. Vijayaraghavan gratefully acknowledges the Council for Scientific and Industrial Research (CSIR), India for providing a Senior Research Fellowship (Ref: 09/652(0024)/2012 EMR-1).

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