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Statistical optimization of alkaline protease from *Bacillus amyloliquefaciens* SP1

Shiwani Guleria Sharma[1]*[#], Abhishek Walia[3], Anjali Chauhan[2] & CK Shirkot[1]*

[1]Department of Basic Sciences; [2]Department of Soil Science and Water Management, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan - 173 230. Himachal Pradesh, India
[3]Department of Microbiology, D.A.V. University, Jalandhar - 144 001, Punjab, India

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Alkaline protease has tremendous applications in different industries, and to fulfil industrial and commercial enzyme requirements, overproduction and optimization of production medium are prerequisites. Protease production by *Bacillus amyloliquefaciens* SP1, isolated from apple tree rhizosphere was studied. Plackett Burmann design and response surface methodology was used to optimize medium for alkaline protease production. Higher levels of protease activity were observed in presence of disaccharides owing to diauxic growth and inorganic nitrogen sources proved to be less favourable. After screening through one-factor-at-a time experiments and Plackett Burmann design, casein, yeast extract, maltose and KH_2PO_4 were identified as the most significant variables. Statistical design based three-dimensional (3-D) and contour plots were generated to understand the relationship between enzyme activity and medium variables. The protease production was found to increase from 1730 to 3630 µg/ml/min in optimized medium i.e. approx. 2.1 fold increased compared with original medium. The study assumes significance in the ability of bacterium to survive in wide variety of salts and agricultural wastes and yield optimum levels of extracellular protease.

Keywords: Apple rhizosphere, Central composite design, Placket Burmann design, Protease, Response surface methodology (RSM)

Proteases (EC 3.4) are one of the largest and most diverse families of enzymes known. These are also termed as proteinases and peptidases. These hydrolyze proteins into short peptides or free amino acids. These are basically classified under Group III (Hydrolases) and sub-group IV according to the nomenclature committee of International Union of Biochemistry and Molecular Biology (IUBMB). Proteases do not refer to a single enzyme but a mixture of enzymes including proteinases and amidases[1]. These enzymes have versatile applications in various industries, such as detergents, food, pharmaceutical, leather, silk, silver recovery and production of protein hydrolysates[2]. Among different types (acidic, neutral and alkaline) of proteases, alkaline proteases are the most commonly used industrial enzymes in view of their activity and stability at high pH[3,4]. Currently, a large proportion of the commercially available alkaline proteases are derived from *Bacillus* species because of their high pH and temperature stability[5].

Protease production by microorganisms is greatly influenced by media components, especially carbon and nitrogen sources and physical factors such as temperature, pH, incubation time, agitation and inoculum density[6,7]. Newer statistical experimental designs such as experimental factorial design and response surface methodology have been used for bioprocess optimization considering the interactive effects among the control factor for hyper production of enzymes[8]. Optimization of media components by classical methods which involves the change of one variable at a time, is extremely time consuming and expensive when a large number of variables are considered. In order to overcome this difficulty and determine the interaction between the studied variables, an experimental factorial design and response surface method was employed for optimization process[5,9]. This study attempted to optimize medium components for protease production by *Bacillus amyloliquefaciens* SP1, newly isolated strain from apple rhizosphere that could be a potential candidate for industrial use.

*Correspondence:
Phone: +91 9418608991 (Mob.)
E-Mail: shg1988@gmail.com, shiwani@pau.edu (SGS);
profckshirkot@gmail.com (CKS)
[#]*Present add.*: Department of Microbiology, College of Basic Sciences & Humanities, Punjab Agricultural University, Ludhiana, Punjab, India

Material and Methods

Microorganism and Culture conditions

B. amyloliquefaciens SP1 was isolated previously from apple tree rhizosphere soil[10] of District Chamba, Himachal Pradesh, India from an altitude of 2880 m from sea level and maintained at 4ºC on the nutrient agar medium and preserved at −20°C in glycerol as cryoprotectant.

The skimmed milk medium used for production of protease contained 0.25% yeast extract, 0.5% casein, 0.1% glucose and 0.7% skimmed milk, (pH 8.0). The strain SP1 was inoculated at 1% inoculum level into the skimmed milk medium and mixed thoroughly by keeping the flasks on a rotary shaker at 120 rpm for 48 h at 35ºC. The liquid culture was then centrifuged at 12000 rpm for 20 min at 4ºC, followed by collecting the supernatant which was further used in enzyme assay[10].

Determination of proteolytic activity

The cell free supernatant was used for protease assay and enzyme activity was measured according to the method described by Sigma Aldrich with slight modifications[11]. The reaction mixture containing 1.0 mL of enzyme was added to 5 mL of 0.5% (w/v) casein solution in 0.2 M Tris-HCl buffer of pH 8.0 and the mixture was incubated for 5 min at 60ºC. The reaction was stopped by adding 5 mL of trichloro-acetic acid (110 mM) and kept there for 30 min at room temperature (30ºC) and then centrifuged for 5 min at 10000 rpm. Then 2 mL of filtrate was mixed with 5 mL of 500 mM Na_2CO_3 solution followed by 1.0 mL Folins-Ciocalteu's reagent. The amount of tyrosine released was determined spectrophoto-metrically at 660 nm against the enzyme blank. The control was treated in the same way, except TCA was added before addition of enzyme solution. One unit of protease activity was equivalent to the amount of enzyme required for releasing 1.0 µg of tyrosine/mL/min under standard assay conditions.

Protein determination

The concentration of protein in culture supernatant was estimated using the Lowry's method[12] of protein estimation with bovine serum albumin as a standard.

Medium optimization using one-factor-at-a-time

The production of protease was optimized based on varying carbon sources, nitrogen sources, metal ions, salts, surfactants as part of culture conditions. Six carbon sources i.e. starch, lactose, maltose, wheat bran, rice bran and apple pomace at 1% concentration with or without glucose (0.1%) were used to determine their effect on protease production by *B. amyloliquefaciens* SP1 after 48 h. Five nitrogen sources i.e. tryptone, peptone, urea, ammonium chloride and ammonium nitrate at 1% concentration with or without yeast extract (0.25%) were used to determine their effect on protease production by *B. amyloliquefaciens* SP1 after 48 h. Different metal ions ($MgSO_4$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, $CaCl_2$, $CuSO_4$ K_2HPO_4, and KH_2PO_4), salts **(**NaCl, KCl, $MnCl_2$ and $FeCl_3$) and Surfactants (Tween 20, Tween 80 and Triton X-100) were added in liquid medium at 0.05% (w/v) concentration to determine their effect on protease production[4].

Effect of casein

To elucidate the effect of casein on protease production, above experiment was repeated with selected carbon and nitrogen source in production medium with or without casein.

Plackett Burmann Design

Based on 'one-factor-at-a-time' experiments, eleven independent variables were chosen for further optimization by Plackett Burmann design[13]. Eleven independent medium components were evaluated at two levels (high and low) and designated as level + 1 and level –1, respectively with one centre point (Table 1). The significant variables were screened in 13 combinations in accordance with the design matrix, and the responses were measured according to the protease activity. For the selection of these factors, Design Expert 8.0.5 (Stat-Ease, Inc., Minneapolis, USA) was used to generate and analyze the experimental design of Plackett–Burmann.

Central Composite Design and Response Surface Methodology

The independent variables selected from Plackett Burmann design for this study were: casein (A), yeast

Table 1 — Coded values of variables at different levels used in Plackett–Burman (PB) design

Variables	Factors	Concentration (%)	
		-1 Levels	+1 Levels
A	Caesin	0.1	1.0
B	Yeast Extract	0.1	0.5
C	Maltose	0.1	2.0
D	Gelatin	0.1	2.0
E	KH_2PO_4	0.01	0.1
F	K_2HPO_4	0.01	0.1
G	$MgSO_4$	0.01	0.1
H	KCl	0.01	0.1
I	NaCl	0.01	0.1
J	$FeSO_4$	0.01	0.1
K	Tween-80	0.01	0.1

extract (B), maltose (C) and KH_2PO_4 (D). Each variable was studied at three levels (−1, 0, +1) and set of 30 experiments was performed in triplicate. The range and the levels of these variables are given in Table 2. The experimental design included 30 flasks with three replicates at their central coded values[14]. The mathematical relationship of response (enzyme production) and variable A, B, C, and D was approximated by a quadratic model equation.

The data obtained from the Response Surface Methodology (RSM) on protease production were subjected to analysis of variance (ANOVA). Protease production was analysed by multiple regression through the least squares method to fit the following equation:

$$Y = A_0 + \_AiXi + \_AiiXi + \_AijXiXj$$

where Y is the predicted response variable; $A0$, Ai, Aii, Aij are constant regression coefficients of the model, and Xi, Xj (i=1,3; j=1,3, $i \neq j$) represent the independent variables (medium composition) in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2. Each experimental design was carried out in triplicate, and the mean values were given.

Statistical analysis

The statistical software package Design-Expert 8.0.5 (StatEase, Minneapolis, MN) was used for regression analysis of experimental data to obtain working parameters and to generate response surface graphs. ANOVA was used to estimate statistical parameters.

Results

Effect of different carbon sources on alkaline protease production by *B. amyloliquefaciens* SP1 in skimmed milk medium with or without glucose

To elucidate the influence of different carbon sources at 1% (w/v) concentration, on the growth, alkaline protease production and specific activity of supernatant was estimated by adding one carbon source at a time in skimmed milk medium with and without glucose. The results (Fig. 1 A and B) revealed that, maximum alkaline protease production and specific activity was found in maltose (1990 µg/mL/min; 76.69 U/mg protein) in skimmed milk medium without glucose at 35°C and pH 8.0. Minimum protease activity (1630 µg/mL/min) and specific activity (63.30 U/mg protein) was found in skimmed milk broth (SMB) without glucose, but having apple pomace as sole carbon source.

Table 2 — Coded values of independent variables at different levels used in Central Composite Design

Independent variables	Symbol	Levels (%)		
		-1	0	+1
Casein (%)	A	0.50	1.50	2.50
Yeast Extract (%)	B	0.10	0.30	0.50
Maltose (%)	C	0.50	1.50	2.50
KH_2PO_4 (%)	D	0.05	0.15	0.25

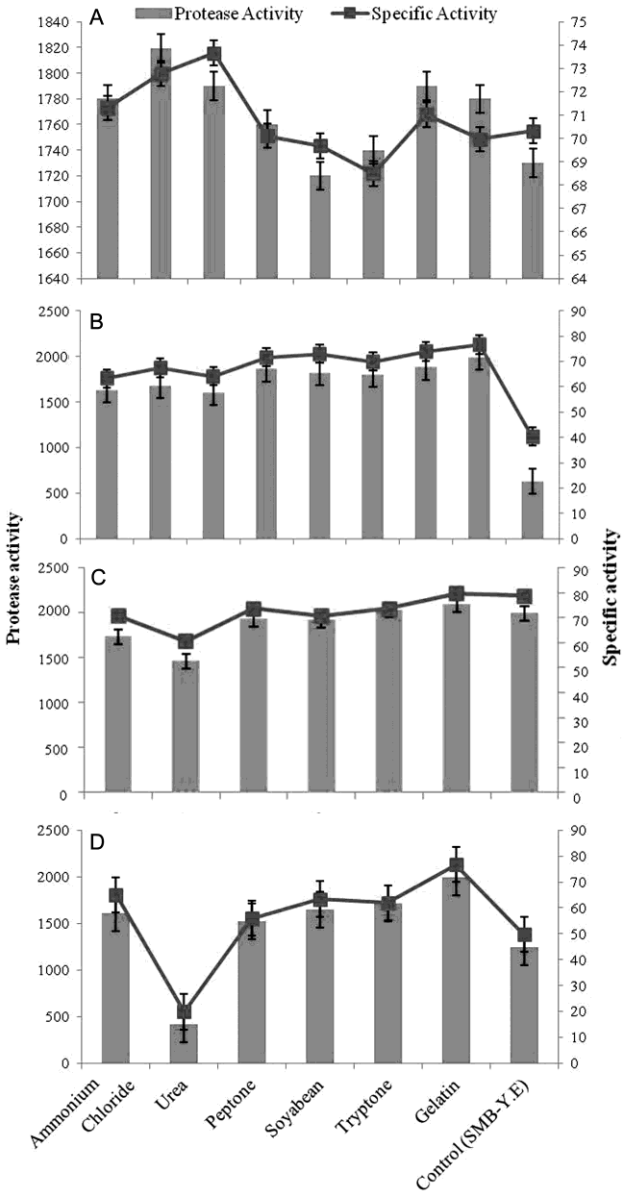

Fig. — 1 Effect of different (A, &B) carbon; and (C & D) nitrogen sources on alkaline protease production in skimmed milk medium by *B. amyloliquefaciens* SP1 with or without yeast extract at pH 8.0 after 48h (Y.E: Yeast Extract)

The addition of cellulose and sucrose in skimmed milk broth with glucose had non significant effect on alkaline protease production. In case of skimmed milk medium containing glucose, maximum alkaline protease production was observed after supplementing it with wheat bran (1819.29 µg/mL/min) followed by rice bran (1790 µg/mL/min) which was found statistically at par with lactose, maltose and apple pomace. However, addition of agricultural wastes i.e. wheat bran, rice bran and apple pomace in skimmed milk medium without glucose, decreased the alkaline protease production ability of *B. amyloliquefaciens* SP1 by about 3-8%. Overall, maximum alkaline protease production was found in maltose (1990 µg/ml/min) followed by lactose (1880 µg/mL/min) and starch (1860 µg/mL/min) as carbon sources in skimmed milk broth without glucose.

Viable count varied between maximum 9.39 log cfu/mL in skimmed milk broth without glucose having maltose as carbon source to minimum 8.0 log cfu/mL in control skimmed milk broth with rice bran. The difference in viable count between skimmed milk broth having sucrose and lactose; starch and cellulose as sole carbon sources was statistically insignificant (data not shown).

Effect of different nitrogen sources on alkaline protease production by *B. amyloliquefaciens* SP1 with or without yeast extract

The results revealed that (Fig. 1 C and D), maximum alkaline protease production (2090 µg/mL/min) and specific activity (79.68 U/mg protein) was found in gelatin followed by tryptone (2030 µg/mL/min; 73.6 U/mg protein) as nitrogen source in skimmed milk medium with yeast extract. Overall, minimum alkaline protease production and specific activity was found in urea (415 µg/mL/min; 19.85 U/mg protein) followed by peptone (1520 µg/mL/min; 55.9 U/mg protein) as nitrogen sources in skimmed milk broth without yeast extract.

The difference in alkaline protease production between peptone and soyabean in presence of yeast extract was found to be insignificant statistically. However, in control skimmed milk medium without yeast extract, addition of various nitrogen sources tested in present study decreased the alkaline protease production of *B. amyloliquefaciens* SP1 by about 14.07 to 79.15% except for gelatin (100.25%).

Viable count varied between maximum 9.39 log cfu/mL in control skimmed milk broth which was found statistically equivalent with soyabean, gelatin and tryptone to minimum 7.51 log cfu/mL in skimmed milk broth having urea as sole nitrogen source. The difference in viable count between skimmed milk broth having only soyabean and gelatin as sole nitrogen sources was insignificant statistically (data not shown).

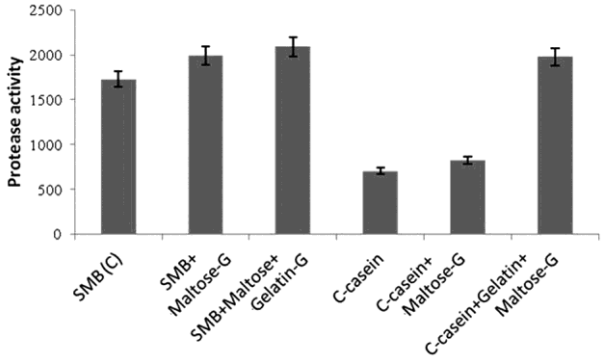

Fig. 2 — Effect of casein on alkaline protease production by *B. amyloliquefaciens* SP1 at pH 8.0 after 48h [C, Control; and G, Glucose]

Effect of casein on alkaline protease production by *B. amyloliquefaciens* SP1 at pH 8.0 after 48 h

To determine the effect of casein on enzyme production, the following treatments: Control + maltose – glucose and control + maltose-glucose + gelatin were further tested for alkaline protease production in the absence of casein (Fig. 2). The deletion of casein from control medium showed significant decrease in protease production in all treatments except for treatment: Control – casein + maltose – glucose + gelatin. The results revealed that ability of protease production of *B. amyloliquefaciens* SP1 decreased by about 59.25, 58.85 and 5.27% in the absence of casein in control skimmed milk broth, SMB + maltose-glucose and SMB + maltose – glucose + gelatin, respectively (Fig. 2). The difference in alkaline protease activity between treatments: SMB – casein + gelatine + maltose and SMB + maltose-glucose were found statistically at par with each other.

Effect of various ions and salts on alkaline protease production by *B. amyloliquefaciens* SP1

The effect of different metal ions and salts on the growth of *B. amyloliquefaciens* SP1 and alkaline protease production is shown in Fig. 3. The isolate was capable of producing alkaline protease in a wider range of chemical conditions including $CuSO_4$, $MgSO_4$, KH_2PO_4, K_2HPO_4, $FeSO_4$, NaCl, $FeCl_3$, $CaCl_2$, KCl, $MnCl_2$ and $ZnSO_4$ at 0.05% (w/v) concentration than in

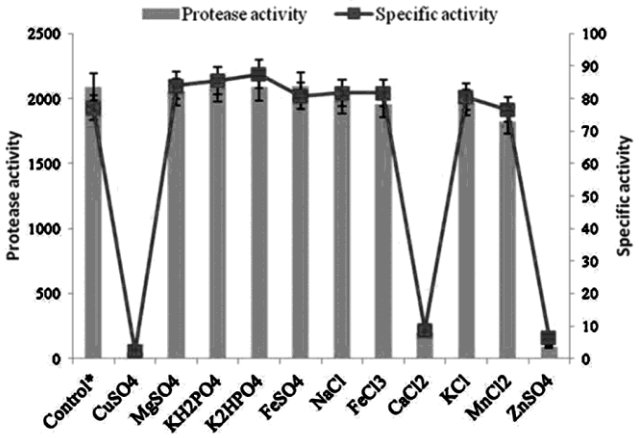

Fig. 3 — Effect of different metal ions and salts on alkaline protease production after 48 h of incubation

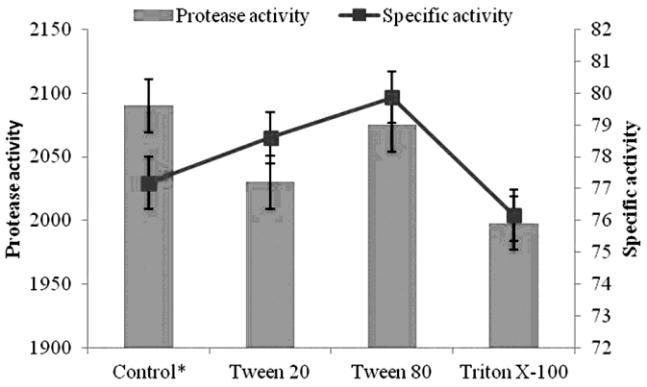

Fig. 4 — Effect of surfactants on alkaline protease production by *B. amyloliquefaciens* SP1 after 48 h of incubation [*Control: skimmed milk medium contained l^{-1}: casein (5g), yeast extract (2.5 g), maltose (10 g), gelatin (10 g) and skimmed milk (7.00%)]

control which did not contain any ions. Among all, $CuSO_4$ (45 µg/mL/min), $CaCl_2$ (205 µg/mL/min), and $ZnSO_4$ (90 µg/ml/min) resulted in very low yield of alkaline protease along with significantly lower count of viable cell number. However, $FeSO_4$ (2095 µg/mL/min), KH_2PO_4 (2080 µg/mL/min), and K_2HPO_4 (2085 µg/mL/min) favoured maximum growth and alkaline protease production which was found statistically at par with control. A significant level of alkaline protease production was also observed with $MgSO_4$ (2050 µg/mL/min) followed by NaCl (1984 µg/mL/min), $FeCl_3$ (1955 µg/mL/min), and KCl (1970 µg/mL/min) which were statistically at par with each other. However, specific activity obtained in KH_2PO_4 (85.53 U/mg protein) was significantly higher than control and other ions. Minimum specific activity was found with $CuSO_4$ i.e. 2.21 U/mg protein. The difference in specific activity between NaCl, $FeCl_3$, and KCl was insignificant statistically.

Maximum viable count (9.39 log cfu/mL) was observed in $FeSO_4$ which was statistically equivalent with control, KH_2PO_4, NaCl and $MgSO_4$ and minimum i.e. 7.06 log cfu/mL in $ZnSO_4$ which was equivalent with $CuSO_4$. The final pH varied from 5.83 to 8.40 (data not shown).

Effect of surfactants on alkaline protease production by *B. amyloliquefaciens* SP1

Surfactants are known to increase extracellular enzyme production. Therefore, an experiment was designed to study their effect on alkaline protease production by *B. amyloliquefaciens* SP1 in submerged fermentation (SmF). The data presented in Fig. 4 revealed that the medium containing 0.05% (v/v) Tween 80 resulted in maximum enzyme activity (2075 µg/mL/min) at the end of 48 h which was significantly higher than medium containing Tween 20 (2030 µg/ml/min) and Triton X-100 (1998 µg/mL/min) at same level of concentration but was at par with control.

Similar pattern was observed with respect to protein content and specific activity. However, final pH and viable count of Tween-20, Tween-80 and Triton X-100 was found to be statistically at par with each other.

Plackett Burmann Design

In this method, prior knowledge obtained from previous experiments i.e. one factor at a time approach (understanding of the cultivation condition variables under investigation) was necessary for achieving a more realistic model. The matrix of 13 experimental runs for the optimization of independent variables of alkaline protease production was developed by the Plackett Burmann design and the results are presented in Table 3. The variables used for the factorial analysis were casein (A), yeast extract (B), maltose (C), gelatin (D), KH_2PO_4 (E), K_2HPO_4 (F), $MgSO_4$ (G), KCl (H), NaCl (J), $FeSO_4$ (K) and Tween-80 (L).

Data appended in Table 3 revealed that maximum alkaline protease production obtained was 2100 µg/mL/min in Run No. 1, which consisted of 1.00 g casein (%),

Table 3 — Actual and predicted values of alkaline protease activity recorded in experimental set up of Placket Burman Design

Std	Run	Casein	Yeast extract	Maltose	Gelatin	KH_2PO_4	K_2HPO_4	$MgSO_4$	KCl	NaCl	$FeSO_4$	Tween -80	Alkaline protease activity (µg/mL/min) Actual	Alkaline protease activity (µg/mL/min) Predicted
11	1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	2100.00	2100.71
6	2	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	300.00	301.00
3	3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	1700.00	1700.71
7	4	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	900.00	901.00
8	5	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	1500.00	1500.71
2	6	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	1950.00	1950.71
10	7	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	1150.00	1151.00
13	8	0	0	0	0	0	0	0	0	0	0	0	1230.00	1231.00
5	9	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	600.00	600.71
9	10	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	1250.00	1250.71
1	11	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	1050.00	1051.00
4	12	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	1100.00	1100.71
12	13	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1050.00	1050.71

Table 4 — ANOVA for Plakett Burman Factorial model

Source	Sum of square	df	Mean square	F-value	p-value
Model	3.027×10^6	11	2.752×10^5	3548.14	0.0131 significant
A-Caesin	4.602×10^5	1	4.602×10^5	5933.26	0.0083
B-Yeast Extract	1.519×10^5	1	1.519×10^5	1958.06	0.0144
C-Maltose	6.769×10^5	1	6.769×10^5	8726.65	0.0068
D-Gelatin	1875.00	1	1875.00	24.17	0.1277
E- KH_2PO_4	46875.00	1	46875.00	604.34	0.0259
F- K_2HPO_4	16875.00	1	16875.00	217.56	0.0431
G- $MgSO_4$	5208.33	1	5208.33	67.15	0.0773
H-KCl	46875.00	1	46875.00	604.34	0.0259
J-NaCl	75208.33	1	75208.33	969.63	0.0204
K- $FeSO_4$	1.435×10^6	1	1.435×10^6	18503.51	0.0047
L-Tween-80	1.102×10^5	1	1.102×10^5	1420.87	0.0169
Residual	77.56	1	77.56		
Cor Total	3.027×10^6	12			

[The Model F-value of 3548.14 implies the model is significant. There is only a 1.31% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, E, F, H, J, K, L are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve this model.
Std. Dev., 8.81; Mean, 1221.54; C.V. %, 0.72; PRESS, 1.453E+005; R-Squared, 1.0000; Adj R-Squared, 0.9997; Pred R-Squared, 0.9520; and Adeq Precision, 212.727. The "Pred R-Squared" of 0.9520 is in reasonable agreement with the "Adj R Squared" of 0.9997. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. This model ratio of 212.727 indicates an adequate signal. This model can be used to navigate the design space]

0.10 g yeast extract (%), 2.00 g maltose (%), 2.00 g gelatin (%), 0.10 g KH_2PO_4 (%), 0.01g K_2HPO_4 (%), 0.01g $MgSO_4$ (%), 0.01 g KCl (%), 0.10 g NaCl (%), 0.01 g $FeSO_4$ (%) and 0.10 g Tween-80 (%). However, minimum alkaline protease production was 300 µg/mL/min obtained in Run No. 2 which consisted of 0.10 g casein (%), 0.10 g yeast extract (%), 0.10 g Maltose (%), 2.00 g Gelatin (%), 0.01g KH_2PO_4 (%), 0.10 g K_2HPO_4 (%), 0.10 g $MgSO_4$ (%), 0.01 g KCl (%), 0.10 g NaCl (%), 0.10 g $FeSO_4$ (%) and 0.10 g Tween-80 (%). These results suggested that the data was deviated and the flask experiments were accurate. In general, the maximum alkaline protease activity was obtained in medium supplemented with high level of casein, maltose, gelatin, KH_2PO_4, NaCl and Tween-80.

By applying multiple regression analysis on the experimental data, the following model was generated for the response of alkaline protease activity. The significant model terms were evaluated by ANOVA in the optimization study (Table 4) (P <0.05) and were identified as A, B, C, E, F, H, J, K and L. The model was reconstructed by removing the insignificant terms and is present in Eq. 1 as coded factors:

Protease activity = 1221.54 +195.83 A + 112.50 B + 237.50 C + 62.50 E – 37.50 F-62.50H – 79.17J – 345.83 K + 95.83 L (Eq. 1)

The statistical significance of Eq. (1) was checked by *F*-test, and the analysis of variance (ANOVA) for the Plackett Burmann factorial model is shown in Table 4. It is evident that the model was highly significant, as suggested by the model *F* value and a low probability value [(*P* model >*F*] = 0.0131). A lower value of CV (0.72) indicated a better precision and reliability of the experiments. The determination coefficient (R^2) implies that the sample variation of 99.97% for alkaline protease production was attributed to the independent variables. Here, the value of *R* (1.00) for Eq. (1) indicated a close agreement between the experimental results and the theoretical values predicted by the model equation.

The estimated parameter and the corresponding *P*-values (Table 4) suggested that, among the independent variables, casein (A), yeast extract (B), maltose (C), KH_2PO_4 (E), K_2HPO_4 (F), KCl (H), NaCl (J), $FeSO_4$ (K), and Tween-80 (L) have a significant effect on alkaline protease production. Among them, A, B, C, E and L were the positive coefficients having linear effect on increase in alkaline protease production, while F, H, J and K were negative coefficient having decreased linear effect on alkaline protease production.

Response Surface Methodology

Regression model of response

Response surface methodology (RSM) was employed to optimize the four most significant factors, i.e. casein, yeast extract, maltose and KH_2PO_4 for enhancing the alkaline protease production, which was selected from prior knowledge obtained from Plakett Burmann design. For RSM based on the central composite design (CCD), used for the optimization of independent variables for the alkaline protease production, 30 experimental runs with different combinations of four factors i.e. casein (A), yeast extract (B), maltose (C) and KH_2PO_4 (D) were carried out. The levels of these variables and their responses are given in Table 5. The experimental responses for the 30 runs were presented in Table 5, which showed considerable variation in the amount of alkaline protease production depending on the four independent variables in the medium.

Table 5 — Actual and predicted values of alkaline protease activity recorded in experimental set up of Response surface methodology

Std	Run	Block	Casein	Yeast extract	Maltose	KH_2PO_4	Alkaline protease activity (μg/ml/min) Actual	Alkaline protease activity (μg/ml/min) Predicted
9	1	Block 1	-1	-1	-1	+1	1200.00	1161.67
15	2	Block 1	-1	+1	+1	+1	1360.00	776.67
5	3	Block 1	-1	-1	+1	-1	1950.00	1686.67
14	4	Block 1	+1	-1	+1	+1	1850.00	1753.33
2	5	Block 1	+1	-1	-1	-1	2400.00	2406.67
17	6	Block 1	0	0	0	0	1800.00	2100.00
8	7	Block 1	+1	+1	+1	-1	3630.00	2811.67
3	8	Block 1	-1	+1	-1	-1	2650.00	2170.00
12	9	Block 1	+1	+1	-1	+1	1600.00	1286.67
18	10	Block 1	0	0	0	0	2400.00	2100.00
11	11	Block 2	-1	+1	-1	+1	1300.00	1572.08
19	12	Block 2	0	0	+1	0	1450.00	2100.00
6	13	Block 2	+1	-1	+1	-1	3030.00	3203.75
10	14	Block 2	+1	-1	-1	+1	1400.00	1403.75
20	15	Block 2	0	-1	0	0	2050.00	2100.00
13	16	Block 2	-1	-1	+1	+1	600.00	638.75
1	17	Block 2	-1	-1	-1	-1	1650.00	1762.08
16	18	Block 2	+1	+1	+1	+1	1050.00	1363.75
4	19	Block 2	+1	+1	-1	-1	1900.00	2287.08
7	20	Block 2	-1	+1	+1	-1	1400.00	1822.08
29	21	Block 3	0	0	0	-1	2500.00	2100.00
24	22	Block 3	0	+2	0	0	1550.00	1734.58
21	23	Block 3	-2	0	0	0	1350.00	1534.58
22	24	Block 3	+2	0	0	0	2800.00	2766.25
25	25	Block 3	0	0	-2	0	900.00	849.58
28	26	Block 3	0	0	0	+2	1150.00	1276.25
27	27	Block 3	0	0	0	-2	3300.00	3324.58
26	28	Block 3	0	0	+2	0	650.00	851.25
23	29	Block 3	0	-2	0	0	1750.00	1716.25
30	30	Block 3	0	0	0	-1	2400.00	2100.00

Data appended in Table 5 revealed that maximum and minimum alkaline protease production was 3630 and 600 µg/mL/min in 30 runs, respectively. The highest alkaline protease activity was obtained from Run No. 7, which consisted of: 2.00 g casein (%), 0.40 g yeast extract (%), 2.00 g maltose (%) and 0.10 g KH_2PO_4 (%); while the lowest activity was obtained in Run No. 16, which consisted of: 1.00g casein (%), 0.20 g yeast extract (%), 2.00 g maltose (%) and 0.20 g KH_2PO_4 (%). Treatment runs were repeated three times for estimation of error. In general, the highest alkaline protease activity was obtained in medium supplemented with high level of casein, yeast extract and maltose.

By applying multiple regression analysis on the experimental data, the following quadratic model was generated for the response of alkaline protease activity. The significant model terms were evaluated by ANOVA in the optimization study (Table 6) (P <0.05) and were identified as A, D, AC, C^2. From the analysis, only the linear effect of B, C and interaction effect of AB, AD, BC, BD, CD and quadratic effect of A^2, B^2, D^2 found to be insignificant terms in the quadratic model. The model was reconstructed by removing the insignificant terms and is present in Eq. 2 as coded factors:

$$\text{Protease activity} = 2100.00 + 307.92\,A - 512.08\,D + 218.13\,AC - 312.40\,C^2 \quad \text{Eq (2)}$$

It is evident that the model was highly significant, as suggested by the model F value and a low probability value [(P model >F] = 0.0002) (Table 6). The analysis of variance (F-test) showed that the second model was well adjusted to the experimental data. A lower value of CV (18.31) indicated a better precision and reliability of the experiments. The determination coefficient (R^2) indicated that the sample variation of 79.36% for alkaline protease production was attributed to the independent variables. Here, the value of R (0.9006) for Eq. (2) indicates a close agreement between the experimental results and the theoretical values predicted by the model equation. Linear and quadratic terms were both significant at the 1% level. Therefore, the quadratic model was selected in this optimization study. Smaller P-values indicated higher

Table 6 — ANOVA for Response surface quadratic model

Source	Sum of Squares	df	Mean Square	F value	p-value (Prob > F)
Block	1.111×10^6	2	5.553×10^5		
Model	1.316×10^7	14	9.398×10^5	8.42	0.0002 significant
A-Casein	2.276×10^6	1	2.276×10^6	20.38	0.0006
B-Yeast Extract	504.17	1	504.17	4.516×10^{-3}	0.9474
C-Maltose	4.17	1	4.17	3.732×10^{-5}	0.9952
D-KH_2PO_4	6.294×10^6	1	6.294×10^6	56.37	< 0.0001
AB	2.783×10^5	1	2.783×10^5	2.49	0.1384
AC	7.613×10^5	1	7.613×10^5	6.82	0.0215
AD	1.620×10^5	1	1.620×10^5	1.45	0.2498
BC	74256.25	1	74256.25	0.67	0.4295
BD	6.25	1	6.25	5.598×10^{-5}	0.9941
CD	2.003×10^5	1	2.003×10^5	1.79	0.2034
A^2	4357.44	1	4357.44	0.039	0.8464
B^2	2.405×10^5	1	2.405×10^5	2.15	0.1659
C^2	2.677×10^5	1	2.677×10^6	23.98	0.0003
D^2	68857.44	1	68857.44	0.62	0.4463
Residual	1.451×10^5	13	1.116×10^5		
Lack of Fit	1.086×10^6	10	1.086×10^5	0.89	0.6133 non significant
Pure Error	3.650×10^5	3	1.217×10^5		
Cor Total	1.572×10^7	29			

[The Model F-value of 8.42 implies the model is significant. There is only a 0.02% chance that a "Model F-Value" this high could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, D, AC, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve this model.

The "Lack of Fit F-value" of 0.89 implies the Lack of Fit is not significant relative to the pure error. There is a 61.33% chance that a "Lack of Fit F-value" this high could occur due to noise. Non-significant lack of fit is appropraite.

Std. Dev., 334.14; Mean, 1825.33; C.V. %, 18.31; PRESS, 7.150E+006; R-Squared, 0.9006; Adj R-Squared0.7936; Pred R-Squared, 0.5106; and Adeq Precision, 11.672. The "Pred R-Squared" of 0.5106 is not as close to the "Adj R-Squared" of 0.7936 as one might normally expect. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction response transformation, outliers, etc. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 11.672 indicates an adequate signal. This model can be used to navigate the design space]

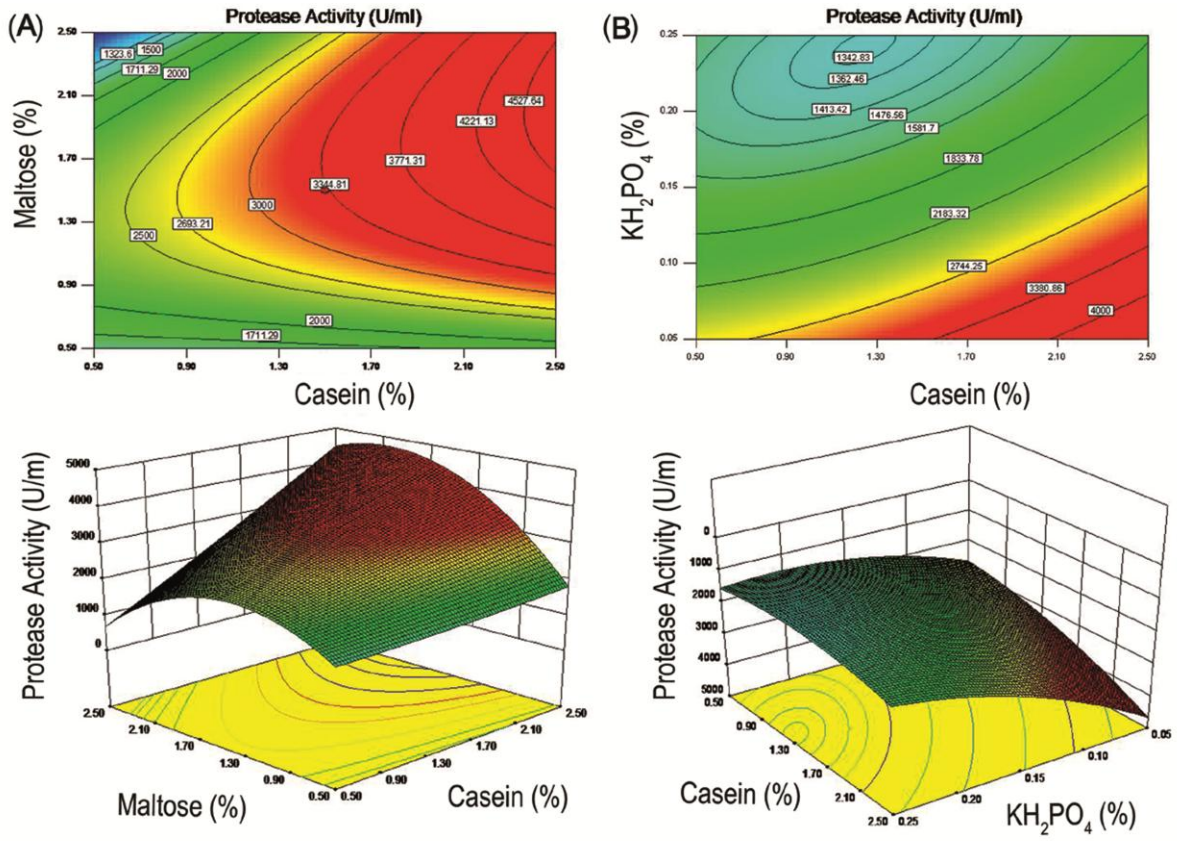

Fig. 5 — Response surface plot and contour plot of the combined effects of (A) casein and KH_2PO_4; and (B) casein and maltose on the alkaline protease production of *B. amyloliquefaciens* SP1

the significance of the corresponding coefficient (Table 6). The estimated parameter and the corresponding *P*-values (Table 6) suggested that, among the independent variables, A, D, AC and C^2 have a significant effect on alkaline protease production. Variable A was the positive coefficient having linear effect on increase in alkaline protease production, while negative coefficient i.e. D was showing decreased linear effect on alkaline protease production. The quadric term of variable C^2 also had a significant effect. However, no interactions between the AB, AD, BC, BD, CD variables were found to contribute to the response at a significant level except AC.

Localization of optimum conditions

Fig. 5A depicted the 3D plot and contour plot, showing the effects of concentration of KH_2P

O_4 and casein on the enzyme production at fixed level of other two factors. Fig. 5A indicated that alkaline protease production decreased gradually as the KH_2PO_4 concentration increased at low concentration of casein. With the increase in the concentration of casein, alkaline protease production significantly increased from 1342.83 to 4000.00 µg/mL/min at low initial KH_2PO_4 concentration. This suggested that increasing casein concentration within the tested range was beneficial for alkaline protease production. The results also showed that by increasing the concentration of KH_2PO_4 beyond 0.15 g (%) decrease in alkaline protease production was observed.

Fig. 5B presents 3D plot and its corresponding contour plot showing the effects of maltose and casein on the alkaline protease production, while other two factors were fixed at their middle level. It is evident that alkaline protease production increased simultaneously when both maltose and casein concentration increased.

Discussion

Fermentation profile of an organism is affected by nutritional and physiological factors, such as incubation time, pH, inoculum size, incubation

temperature and carbon and nitrogen sources. Hence, optimization of these factors has to be carried out in order to maintain a balance among various medium components, thus minimizing the amount of non utilized components at the end of fermentation.

In order to screen different carbon sources, apple pomace, wheat bran and rice bran (complex carbon sources), starch and cellulose (polysaccharides) and sucrose, lactose and maltose (disaccharides) were added at 1% (w/v) concentration in presence or absence of glucose in reference skimmed milk media (Fig. 1 A and B). This is evident that *B. amylo-liquefaciens* SP1 chose to utilize disaccharide over monosaccharide and complex carbon sources for its growth and alkaline protease production. Similar results were obtained in the study Tari *et al*.[5] in the determination of the effect of various carbon sources on proteolytic activity of *Bacillus* sp. L21. They had also reported that maximum alkaline protease production obtained when maltose was used as carbon source instead of whey, potato starch, glucose and fructose. This choice of carbon source of strain SP1 was further confirmed by adding all tested carbon sources under study one by one in presence of glucose (monosaccharide) in media. This phenomenon refers to the diauxic growth generated by an organism when grown on growth media containing two types of sugars, one of which is easier to metabolize than the other (for example glucose and lactose). In present work, it was observed that growth as well as alkaline protease production was higher when glucose is present in media along with wheat bran, apple pomace and rice bran (complex carbon sources) as compared to the media having them without glucose. However, alkaline protease production was lower in media having glucose along with maltose, sucrose and lactose, as compared to media having them without glucose, although, significant growth was recorded in both cases. The effect of casein was also studied in presence of maltose and it was evident that significant decrease in growth as well as in alkaline protease production was observed in absence of casein in medium (Fig. 2). This is in accordance with the earlier report of Suleiman *et al*.[15].

Ammonium chloride, urea, soyabean, tryptone and gelatin were screened at 1% (w/v) concentration as main nitrogen sources, by supplementing and replacing the yeast extract at a concentration 2.5 g/L as indicated in the reference skimmed milk medium. According to the results, the lowest protease activity and specific activity in the amounts of 415 µg/mL/min and 19.85 U/mg protein was obtained with urea (Fig. 1 C and D). The negative effect of inorganic nitrogen sources on protease production by *Bacillus* sp. has been observed in earlier investigations[16,17]. The repression of protease biosynthesis may be attributed to the release of ammonia from these inorganic nitrogen sources. Our results revealed with the repression of the enzyme production by urea were consistent with previously published other studies[9]. Better performance was seen for gelatin, followed by tryptophan and soyabean which showed protease activities of 1995, 1710 and 1650 µg/mL/min, respectively. Even though gelatin had the similar alkaline protease production as control medium when used alone, present investigation conducted experiments to determine how its interaction with yeast extract influence enzyme activity, if presented together in same media fermentation. According to results (Fig. 1), gelatin and yeast extract could be used together as nitrogen sources, since it resulted in slightly higher activity than control. In the study of Tari *et al*.[5], different nitrogen sources were screened for protease production from *Bacillus* sp. The best result was attained when soyabean meal together with corn steep liquor was used as the main nitrogen sources. Peptone was used in addition to these components which resulted slight increase in activity. The effect of casein on alkaline protease activity was also studied in medium having gelatin and yeast extract and it was evident that significant decrease in alkaline protease activity was observed in absence of casein (Fig. 2).

Surfactants were used in production medium experiments in order to determine if it had a promoting or inhibiting effect on the enzyme synthesis (Fig. 3). Although, strain SP1 retained its activity and growth in presence of three surfactants used in present study, but maximum protease activity along with maximum specific activity was observed with Tween-80. Tween-80 is a well known industrial surfactant whose presence in media would affect the homogeneity of the broth and facilitate the nutrient and oxygen transfer to the microorganisms[5]. The present investigation is in accordance with the study of B.K.M Lakshmi *et al*.[18] that the protease from *Bacillus clausii* and *Bacillus* sp. retain their activity with different surfactants such as Triton X 100, Tween-20 and Tween-80.

The strain SP1 was capable of producing protease in a wider range of chemical conditions including copper sulphate, magnesium sulphate, potassium dihydrogen phosphate, potassium hydrogen phosphate, ferrous sulphate, sodium chloride, ferric chloride, calcium chloride, potassium chloride, manganese chloride and zinc sulphate at 0.05% (w/v) concentration than the control medium which did not contain any salt (Fig. 4). While, calcium chloride and manganese chloride resulted in very low yields of protease, zinc sulphate and copper sulphate did not support bacterial growth. However, rest of them favored optimum growth and protease secretion. A similar study was conducted on the microbial production of extracellular protease[15,16] where protease production was shown in different concentrations of sodium and potassium salts and the maximum protease activity was found in the presence of 7.5-10% (w/v) sodium sulphate and 3% (w/v) sodium acetate (4.6 U/mL). These results indicate the ability of *B. amyloliquefaciens* SP1 to utilize various salts for primary metabolism and subsequently produce optimum yields of protease, thereby increasing its commercial viability.

The effect of medium components on alkaline protease production plays an important role in the expression and repression of the enzyme and each organism has its own specific requirements for maximum production of enzyme. The greatest difficulty in optimization is the presence of interactive effects of medium components. The "one-factor-at-a-time" method, in which one independent variable is studied while maintaining all the other factors at a fixed level, is laborious and cannot provide the information about mutual interactions of the parameters on the desired outcome[19]. Statistical experimental design methods, such as Plackett Burmann design and response surface methodology, can provide systematic and efficient plan for experimentation to achieve certain goals, so that several control factors are simultaneously studied[20,21].

The optimization of medium conditions was carried out by a combination of non-statistical and statistical based experimental designs. The selection of medium compositions was carried out through the one-factor-at-a-time experiments and 2090 µg/mL/min of the alkaline protease activity was observed. The obvious significant factors (casein, yeast extract, maltose and KH_2PO_4) that influenced the production of alkaline protease were obtained using Plackett Burmann design, and then optimal concentrations of these factors were sequentially investigated using the response surface methodology with a central composite design. The final optimal medium components included: 2.00 g casein (%), 0.40g yeast extract (%), 2.00 g maltose (%) and 0.10 g KH_2PO_4 (%) (Table 5). When using this medium, the yield of alkaline protease was increased 2.10 fold (3630 µg/mL/min) compared with the protease production in an unoptimized medium (1730 µg/mL/min). There have been reports on optimization of culture media using statistical approaches for a few bacterial proteases[5,20,21]. The results (Table 6) of CCD indicate the significance of casein (A), KH_2PO_4 (D), and some interaction i.e. casein*maltose (AC) on production of alkaline protease by *B. amylo-liquefaciens* SP1. Higher level of maltose significantly increased the alkaline protease production of strain SP1 is in agreement with the findings reported earlier by Tari *et al*.[5].

Conclusion

Enhanced production of protease has been achieved with the help of Plackett-Burmann screening of media components, statistically designed experiments such as central composite design (CCD) and subsequent optimization analysis by RSM have also been successful for yield optimization. Statistical experimental approaches proved useful for optimization, which resulted in about 2.1 time higher enzyme activity by *B. amyloliquefaciens* SP1 than that using the original medium in a shake-flask culture. This study introduces a basal medium for further optimization studies of this and similar strains. Moreover, the growth of the microorganism and the protease produced were favorable over a wide variety of salts and agricultural wastes, which proved strain SP1 as a potential candidate for industrial alkaline protease production.

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Conflict of interest

Authors declare no competing interests.

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