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Statistical Approach for the Production of Protease and Cellulase from *Bacillus cereus* KA3

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Abstract: A cheap agro-industrial waste was used as the substrate for the production of protease and cellulase from *Bacillus cereus* KA3. The process parameters were optimized by a two level full factorial design and response surface methodology. Two level full factorial designs revealed that the factors namely, pH, peptone and NaH_2PO_4 were significantly influenced on the production of protease and cellulase. These three significant factors were selected for central composite design and response surface methodology. The maximum protease and cellulase production was 3127 U/g, and 482 U/g, respectively, after statistical approach, which showed over fourfold increase in enzyme production than unoptimized medium.

Keywords: Solid State Fermentation, Agro-residues, Response Surface Methodology, Optimization

1. Introduction

Microorganisms are known to play a vital role for the production of intracellular and extracellular proteases on an industrial scale. Most of the proteases produced commercially are of microbial source. The alkaline proteases produced by the genus *Bacillus* are the important group of enzymes produced commercially [1] and these enzymes constitute 60–65% of the global enzyme market [2]. Alkaline proteases are very widely used in various industries, including, leather, detergents, and pharmaceutical industries. Cellulolytic enzymes have various applications in laundry, textile, paper, animal feed and fruit juice extraction [3]. Cellulase also finds potential application in saccharification of lignocellulosic agroresidues to fermentable sugars which can be used for production of lactic acid, bioethanol, and single-cell protein [4]. Bacteria have been explored for cellulase production owing to their high growth rate, stability at extreme temperature, expression of multienzyme complexes, pH and ability to withstand variety of environmental stress [3]. Among the bacteria, the genus *Bacillus* continues to be dominant bacterial workhorse due to the capacity to secrete large quantities of enzymes [5, 6].

Solid-state fermentation (SSF) has gained significance in the production of enzymes over submerged fermentation. The advantages of SSF include increase production with less manufacturing costs, less pre-processing energy and less effluent generation, better product recovery and easy process management [7, 8]. The traditional one-at-a-time-approach is widely used to optimize the process parameters to enhance the production of enzymes. This traditional method consumes more time in addition ignores the mutual interactions among various selected variables. Statistical optimization techniques such as Placket and Burman [9, 10], two level full factorial design [11] and Response Surface Methodology (RSM) helps in evaluating the significant factors and building models to study the interaction and to select optimum conditions of variables for a desirable response [12]. Statistical experimental designs for the optimization process have been successfully employed, demonstrating that these statistical methods are useful and powerful tools [13].

2. Materials and Methods

2.1. Screening and Isolation of Cellulase and Protease Producing Bacteria

The soil sediment samples were collected from the estuary at Rajakkamangalam, Kanyakumari district, Tamil Nadu, India and transferred to the laboratory for further studies. Standard method was followed for the screening of both protease and cellulase producing bacterial isolate. Skimmed milk agar plate was used for the screening of proteolytic enzyme producing organism and this medium composed of: 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4$, 1% (w/v) skimmed milk, 0.5% (w/v) NaCl, and 1.5% (w/v) agar. The protease producing bacterial isolates were further selected for cellulase screening. Cellulase producing ability of the bacterial isolate was screened using the medium composed of (g/l): carboxymethylcellulose (CMC), 10.0; tryptone, 2.0; KH_2PO_4, 4.0; Na_2HPO_4, 4.0; $MgSO_4.7H_2O$, 0.20; $CaCl_2.2H_2O$, 0.001; $FeSO_4.7H_2O$, 0.004; agar, 15 and pH was adjusted to 7.0 [14]. CMC agar plates were incubated at 37°C for 48 h. The plates were flooded with 1% Congo red and 1M NaCl to visualize the cellulolytic activity of isolated bacterial strain.

2.2. Identification of Protease and Cellulase Producing Bacterial Isolates

The biochemical and morphological tests, the strain was identified according to the methods described in Bergey's Manual of Systematic Bacteriology [15]. 16S rDNA was amplified using a Polymerase Chain Reaction machine with oligonucleotide primers designed to anneal conserved positions in the 3′ and 5′regions of the 16S rRNA genes [16].

2.3. Optimization of Protease and Cellulase Production by One-Variable-at-a-Time Approach

Protease and cellulase production of *B. cereus* KA3 was optimized by varying the physical parameters and nutrient sources. The effect of fermentation period on enzyme production was studied by determining the protease and cellulase activity 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 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 inoculums size, the inoculums concentration was increased (3-15%, v/w). The effect of carbon sources (1%, w/w; glucose, sucrose, fructose, maltose and xylose), nitrogen sources (1%, w/w; ammonium sulphate, peptone, yeast extract, beef extract and oat meal) and ions (calcium chloride, sodium dihydrogen phosphate, ammonium sulphate, magnesium chloride and manganese chloride) were studied.

2.3.1. Protease Assay

The protease activity was determined by mixing 1.0 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). It 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 [17].

2.3.2. Cellulase Assay

Cellulase activity was measured following the method of Irfan et al. [18]. Briefly, a reaction mixture composed of 0.2 ml of crude enzyme solution and 1.0 ml of 0.5% carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 7.0). It was incubated at 37 ˚C in a shaking water bath for 30 min. The reaction was terminated by adding 3 ml of DNS reagent. The colour was then developed by boiling the mixture for 5 min and OD of the samples was measured at 540 nm against reagent blank.

2.4. Screening of Significant Components with Two-Level Full Factorial Design

A two-level full factorial design was carried out to find the key ingredients for enhanced production of protease and cellulase. The important physical parameters (pH and moisture) and nutrient factors (Oat meal, Glucose, $MgCl_2$), 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 and high level (Table 1). Two-level full factorial designs were based on the following first-order polynomial model:

$$Y = \alpha_0 + \sum_i \alpha_i x_i + \sum_{ij} \alpha_{ij} x_i x_j + \sum_{ijk} \alpha_{ijk} x_i x_j x_k + \sum_{ijkl} \alpha_{ijkl} x_i x_j x_k x_l + \sum_{ijklm} \alpha_{ijklm} x_i x_j x_k x_l x_m \quad (1)$$

where, Y is the protease and cellulase activity (response); αij, αijk, αijkl and αijklm were the ijth, ijkth, ijklth and ijklmth interaction coefficients, respectively; αi was the ith linear coefficient and α0 was an intercept. Enzyme assay was carried out in duplicates and the average of these experimental values was taken as response Y (Table 2). Analysis of variance was used to evaluate the significance of protease and cellulase production.

The statistical software "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.

2.5. Statistical Optimization of Protease Production by Central Composite Design (CCD) and RSM

In the present study, CCD was employed to optimize three significant factors, namely pH, sucrose and yeast extract at

five levels (-α, -1, 0, +1 and +α) (Table 5). The CCD design consists of 20 experimental runs (6 central, 6 axial points and 8 factorial). The experiments were conducted in duplicates and the mean value of protease and cellulase activity was taken as the response (Y) (Table 6). A second-order polynomial equation is as follows:

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

where, Y is the response; β0 is the offset term and βi, βii and βij were the coefficients of linear terms, square terms and coefficients of interactive terms, respectively. Xis were A, B and C; Xijs were AB, AC and BC (A-pH, B- sucrose and C-yeast extract). All trials were carried out in duplicate and the average protease and cellulase yield was used as response Y. ANOVA was used to elucidate the significance of protease and cellulase production. The optimum concentrations of the variables were obtained by analyzing three-dimensional plots. To validate the model equation, experiments were conducted in triplicates for protease and cellulase production under optimum conditions predicted by the model.

3. Result and Discussion

3.1. Screening, Isolation and Identification of Protease and Cellulase Producing B. cereus KA3

The bacterial isolate was screened from estuarine sediment using skimmed milk- and CMC-agar plates. Initially the selected organism was screened for the production of protease and further subjected for the production of cellulases. It showed 15 mm and 4 mm zone on skimmed milk and CMC agar plates. The 16S rDNA sequence was deposited in Genbank (Accession number KR108282).

3.2. Elucidation of Process Parameters by One-Variable-at-a-Time Approach

The process parameters were initially screened by one-variable-at-a-time approach. Among the carbon sources tested, sucrose enhanced more protease production (878.4 U/g), followed by maltose (647.8 U/g) and fructose (271.7 U/g). This result was in accordance the observations made with other *Bacillus* sp. [19]. In the present study, *B. cereus* KA3 utilized the carbon sources such as, glucose, fructose, maltose, xylose, sucrose (1%, w/w) for its growth and the production of cellulase. Among the carbon sources, sucrose enhanced more cellulase production (366 U/g). Similar results on maximum yields of cellulase production were obtained with *T. viride* [20]. Yeast extract supported more protease production (558.1 U/g) than the tested nitrogen sources. Beef extract significantly enhanced the production than other tested nitrogen sources (986.3 U/g) followed by peptone (795.1 U/g). This result was in accordance the observations made previously with other bacterial species [21, 22]. Among the nitrogen sources tested, beef extract significantly influenced on cellulase production (527.9 U/g). Similar results were reported by various research groups on *Pseudomonas flourescens*, *Monascus purpureus* and *Streptomyces* sp. BRC2 [23-25]. The effect of different ions on protease production was studied by supplementing the sources such as, $CaCl_2$, ammonium sulphate, $MgSO_4$, $MgCl_2$, NaH_2PO_4, at 1% concentration. Among the different sources $MgSO_4$ significantly influenced the cellulase production (414.4 U/g). It was previously reported that the addition of $Ca^{2+,}$ Co^{2+} and Mg^{2+} ions stimulated protease production by 135%, 118% and 110% [26]. The present investigation showed that *Bacillus* sp. produced maximum protease at pH 8.0. Similar result was reported with other bacterial species [27]. In the present study, the maximum enzyme production was observed with 100% moisture content (346.7 U/g) and enzyme production decreased thereafter. Among the several factors that are important for microbial growth and enzyme production under SSF, moisture content is a critical factor [28].

3.3. Elucidation of Significant Factors by Two-Level Full Factorial Design

In *B. cereus* KA3 proteolytic enzyme production varied from 950 to 3470 U/g material. According to the two –level full factorial design, the optimum medium compositions were as follows: pH 9.0, 80% moisture, 0.1% peptone, 0.1%NaH_2PO_4 and 0.1% sucrose. The analysis of variance (ANOVA) was used to analyze the main effects for the production of protease was shown in Table 3. The ANOVA of quadratic regression model is highly significant. The model F-Values of 11.54 implies the model is significant. There is only a 0.03%chance that an F-Value this large could occur due to noise. Values of "Prob>F" less than 0.050 indicate model terms are significant. In this model enzyme production was significantly affected by pH (< 0.01), peptone (< 0.05) and NaH_2PO_4 (< 0.05). The coefficient estimate was negative to peptone and NaH_2PO_4. The "Pred R-Squard" of 0.9361 is reasonable agreement with the "Adj R-Squared"of 0.9657. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. In this model the signal to noise ratio is 28.223 indicates an adequate signal. The ANOVA of quadratic regression model is highly significant.

In this study cellulase production varied considerably. The model F-Value of 4.13 implies the model is significant. There is only a 1.87% chance that an F-value this large could occur due to noise. In this model, enzyme production was significantly affected by pH (< 0.01), peptone (< 0.05) and NaH_2PO_4 (< 0.05) (Table 4). The coefficient estimate was negative to peptone and NaH_2PO_4. The "Pred R-Squared" of 0.6880 was in reasonable agreement with the "Adj R-Square" of 0.9012. "Adeq Precision" measures the signal to noise ratio. The ratio of 13.620 indicates an adequate signal and this model can be used to navigate the design space.

Table 1. *Independent variables and their levels for the screening of significant factors by 2^5 factorial experimental designs for protease and cellulase.*

Symbol	Variable	Units	Coded levels	
	Name		-1	+1
A	Moisture	%	90	120
B	pH		7	9
C	Sucrose	%	0.1	1
D	Peptone	%	0.1	1
E	NaH_2PO_4	%	0.01	0.1

Table 2. *2^5 factorial designs for the production of protease and cellulase.*

Std	Sucrose%	B: Peptone%	C: NaH_2PO_4%	D: pH	E: Moisture	Protease activity (U/g)	Cellulase activity (U/g)
1	0.1	0.1	0.01	6	90	2068	84
2	1	0.1	0.01	6	90	1144	88
3	0.1	1	0.01	6	90	1546	130
4	1	1	0.01	6	90	933	41
5	0.1	0.1	0.1	6	90	924	344
6	1	0.1	0.1	6	90	2031	135
7	0.1	1	0.1	6	90	2086	90
8	1	1	0.1	6	90	1876	146
9	0.1	0.1	0.01	8	90	1153	130
10	1	0.1	0.01	8	90	915	103
11	0.1	1	0.01	8	90	1144	498
12	1	1	0.01	8	90	2773	254
13	0.1	0.1	0.1	8	90	1153	66
14	1	0.1	0.1	8	90	924	78
15	0.1	1	0.1	8	90	4374	441
16	1	1	0.1	8	90	2754	373
17	0.1	0.1	0.01	6	110	1400	125
18	1	0.1	0.01	6	110	1171	125
19	0.1	1	0.01	6	110	924	225
20	1	1	0.01	6	110	1381	297
21	0.1	0.1	0.1	6	110	3642	314
22	1	0.1	0.1	6	110	1107	100
23	0.1	1	0.1	6	110	924	402
24	1	1	0.1	6	110	1208	134
25	0.1	0.1	0.01	8	110	1830	145
26	1	0.1	0.01	8	110	1153	132
27	0.1	1	0.01	8	110	1162	213
28	1	1	0.01	8	110	2983	400
29	0.1	0.1	0.1	8	110	1848	171
30	1	0.1	0.1	8	110	1848	150
31	0.1	1	0.1	8	110	1134	109
32	1	1	0.1	8	110	4786	262

Table 3. *ANOVA table for 2^5 factorial experimental design of protease production.*

Soucre	Squares	Df	Square	Value	Prob>F
Model	3.15E+07	24	1.31E+06	104.12	<0.0001
A-Sucrose	87675.78	1	87675.78	6.96	0.0335
B-Peptone	1.84E+06	1	1.84E+06	146.27	<0.0001
C-NaH_2PO_4	2.50E+06	1	2.50E+06	198.31	<0.0001
D-pH	1.79E+06	1	1.79E+06	142.18	<0.0001
AB	2.60E+06	1	2.60E+06	206.64	<0.0001
AD	1.53E+06	1	1.53E+06	121.64	<0.0001
AE	4.68E+05	1	4.68E+05	37.19	5.00E-04
BC	4.17E+05	1	4.17E+05	33.12	7.00E-04
BD	5.20E+06	1	5.20E+06	412.67	<0.0001
BE	1.39E+06	1	1.39E+06	110.44	<0.0001
CD	1.92E+05	1	1.92E+05	15.23	5.90E-03
DE	1.81E+05	1	1.81E+05	14.35	6.80E-03
ABC	7.99E+04	1	7.99E+04	6.35	3.99E-02
ABD	5.32E+05	1	5.32E+05	42.27	3.00E-04
ABE	3.24E+06	1	3.24E+06	257.44	<0.0001
ADE	1.38E+06	1	1.38E+06	109.32	<0.0001
BCD	7.43E+05	1	7.43E+05	58.98	1.00E-04
BCE	1.21E+06	1	1.21E+06	96.35	<0.0001
CDE	7.13E+04	1	7.13E+04	5.67	4.89E-02
ABCD	2.13E+05	1	2.13E+05	16.89	4.50E-03
ABCE	2.09E+06	1	2.09E+06	165.78	<0.0001
ABDE	4.23E+04	1	4.23E+04	3.36	1.10E-01
ACDE	3.55E+06	1	3.55E+06	282.07	<0.0001
BCDE	1.19E+05	1	1.19E+05	9.45	1.80E-02
Residual	8.81E+04	7	1.26E+04		
Cor Total	3.16E+07	31			

Table 4. *ANOVA table for 2^5 factorial experimental design of cellulase production.*

Source	Sum of Squares	Df	Mean Square	F Value	p-Value Prob>F
Model	4.75E+05	28	16950.21	58.24	0.0031
A-Sucrose	13986.28	1	13986.28	48.06	0.0062
B-Peptone	92988.28	1	92988.28	319.51	0.0004
C-NaH2PO4	3300.78	1	3300.78	11.34	0.0435
D-pH	17344.53	1	17344.53	59.6	0.0045
E-Moisture	2869.03	1	2869.03	9.86	0.0517
AB	2227.78	1	2227.78	7.65	0.0698
AC	6300.03	1	6300.03	21.65	0.0187
AD	12285.28	1	12285.28	42.21	0.0074
AE	6641.28	1	6641.28	22.82	0.0174
BC	8679.03	1	8679.03	29.82	0.0121
BD	63457.03	1	63457.03	218.04	0.0007
BE	850.78	1	850.78	2.92	0.1858
CD	18769.53	1	18769.53	64.49	0.004
CE	4163.28	1	4163.28	14.31	0.0324
DE	32832.03	1	32832.03	112.81	0.0018
ABC	3676.53	1	3676.53	12.63	0.038
ABE	8352.78	1	8352.78	28.7	0.0127
ACD	19750.78	1	19750.78	67.86	0.0037
ACE	17251.53	1	17251.53	59.28	0.0046
ADE	20250.78	1	20250.78	69.58	0.0036
BCD	2064.03	1	2064.03	7.09	0.0761
BCE	3719.53	1	3719.53	12.78	0.0374
BDE	64890.03	1	64890.03	222.97	0.0007
ABCE	13081.53	1	13081.53	44.95	0.0068
ABDE	21269.53	1	21269.53	73.08	0.0034
ACDE	1638.78	1	1638.78	5.63	0.0982
BCDE	8745.03	1	8745.03	30.05	0.0119
ABCDE	3220.03	1	3220.03	11.06	0.0448
Residual	873.09	3	291.03		
Cor Total	4.76E+05	31			

Final equation in term of coded factors:

Protease activity= +1759.34+52.34*A+239.91*B+279.34*C+236.53*D+285.16*AB+ 218.78*AD+120.97*AE+114.16*BC+402.97*BD- 208.47*BE+77.41*CD+75.16*DE-9.97*ABC+128.97*ABD 318.28*ABE+207.41*ADE+152.34*BCD-194.72*BCE- 47.22*CDE- 81.53*ABCD+255.41*ABDE+333.16*ACDE+60.97*BCDE (3)

Cellulase activity= +197.03- 20.91*A+53.91*B+10.16*cC+23.28*D+9.47*E+8.34*AB- 14.03*AC+19.59*AD+14.41*AE-16.47*BC+44.53*BD- 5.16*BE-24.22*CD-11.41*CE- 32.03*DE+10.72*ABC+16.16*ABE+24.84*ACD- 23.22*ACE=25.16*ADE+8.03*BCD-10.78*BCE- 45.03*BDE-20.22*ABCE+25.78*ABDE+7.16*ACDE- 16.53*BCDE+10.03*ABCDE (4)

3.4. CCD and Response Surface Methodology

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 [29]. RSM was employed for the production of various enzymes, including protease [30], amylase [31], cellulase [32] and fibrinolytic enzyme [33]. In this study twenty experimental runs with different combinations of three factors were carried out. The maximum protease production was observed at run 5.

The Model F-value of 60.36 implies the model is significant for protease production. There is only a 0.01% chance that an F-value this large could be due to noise. Values of "Prob > F"less than 0.0500 indicate model terms are significant. In this case B, C, AB, AC, BC, A^2, B^2 were significant model terms (Table 7). Values greater than 0.100 indicate the model terms are not significant. The "Lack of Fit F-value" of 0.47 implies the Lack of Fit is not significant relative to the pure error. There is a 78.46% chance that a "Lack of Fit F-value "this large could occur due to noise. Non-significant lack of fit is good. The"Pred R-Squared"of 0.9361 is in reasonable agreement with the "Adj R-Squared" of 0.9657. "Adequate precision" measures the signal to noise ratio. The ratio was 28.223 indicated an adequate signal.

The Model F-Value of 20.25 implies the model is significant for cellulase production (Table 8). There is only a 0.01% chance that an F-Value this large could occur due to noise. Values of "Prob> F" less than 0.050 indicate model terms are significant. In this case A, B, C, AB, A^2, B^2, C^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-

value" of 2.39 implied the lack of fit is not significant relative to the pure error. There is 18.01% chance that a "Lack of Fit F-value "this large could occur due to noise. The "Pred R-Squared" of 0.8880 is close to the "Adj R-Squared" of 0.9012.

Table 5. *Independent variables and the level for CCD and RSM.*

Factors	Units	- α	-1	0	+1	+ α
Peptone	%	-0.21	0.1	0.55	1.0	1.31
NaH_2PO_4	%	-0.21	0.1	0.55	1.0	1.31
pH		6.32	7.0	8.0	9.0	9.68

Table 6. *Experimental design and results of the CCD model on protease and cellulase for B. cereus* KA3.

Run	A: pH	B: Peptone%	C: $NaH_2PO_{4\%}$	Protease activity (U/g)	Cellulase activity (U/g)
1	8	0.55	0.55	3600	472
2	7	1	0.1	1964	281
3	8	0.55	0.55	4098	451
4	8	0.55	1.31	3790	361
5	9.68	0.55	0.55	1657	381
6	9	1	1	4072	421
7	9	0.1	0.1	3985	301
8	8	0.55	0.55	4290	447
9	7	0.1	1	1987	172
10	8	0.55	0.55	3793	471
11	7	1	1	4590	372
12	9	1	0.1	3602	301
13	9	0.1	1	401	292
14	8	1.31	0.55	4012	451
15	8	0.55	-0.21	4620	161
16	8	0.55	0.55	4209	412
17	6.32	0.55	0.55	1598	38
18	7	0.1	0.1	3982	67
19	8	0.55	0.55	3982	511
20	8	-0.21	0.55	2193	268

Table 7. *ANOVA for the production of protease using CCD.*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob>F	
Model	2.72E+07	9	3.03E+06	60.36	<0.0001	Significant
A-pH	9689.76	1	9689.76	0.19	0.6696	
B-Peptone	3.52E+06	1	3.52E+06	70.17	<0.0001	
C-NaH2PO4	1.10E+06	1	1.10E+06	21.97	9.00E-04	
AB	9.13E+05	1	9.13E+05	18.21	1.60E-03	
AC	1.75E+06	1	1.75E+06	34.96	1.00E-03	
BC	9.41E+06	1	9.41E+06	187.59	<0.0001	
A2	9.32E+06	1	9.32E+06	185.86	<0.0001	
B2	1.15E+06		1.15E+06	22.97	7.00E-04	
C2	1.65E+05	1	1.65E+05	3.3	9.95E-02	
Residual	5.02E+05	10	5.01E+04			
Lack of Fit	1.61E+05	5	3.22E+04	0.47	7.85E-01	Not Significant
Pure Error	3.40E+05	5	6.81E+04			
Cor Total	2.77E+07	19				

Table 8. *ANOVA for the production of cellulase using CCD.*

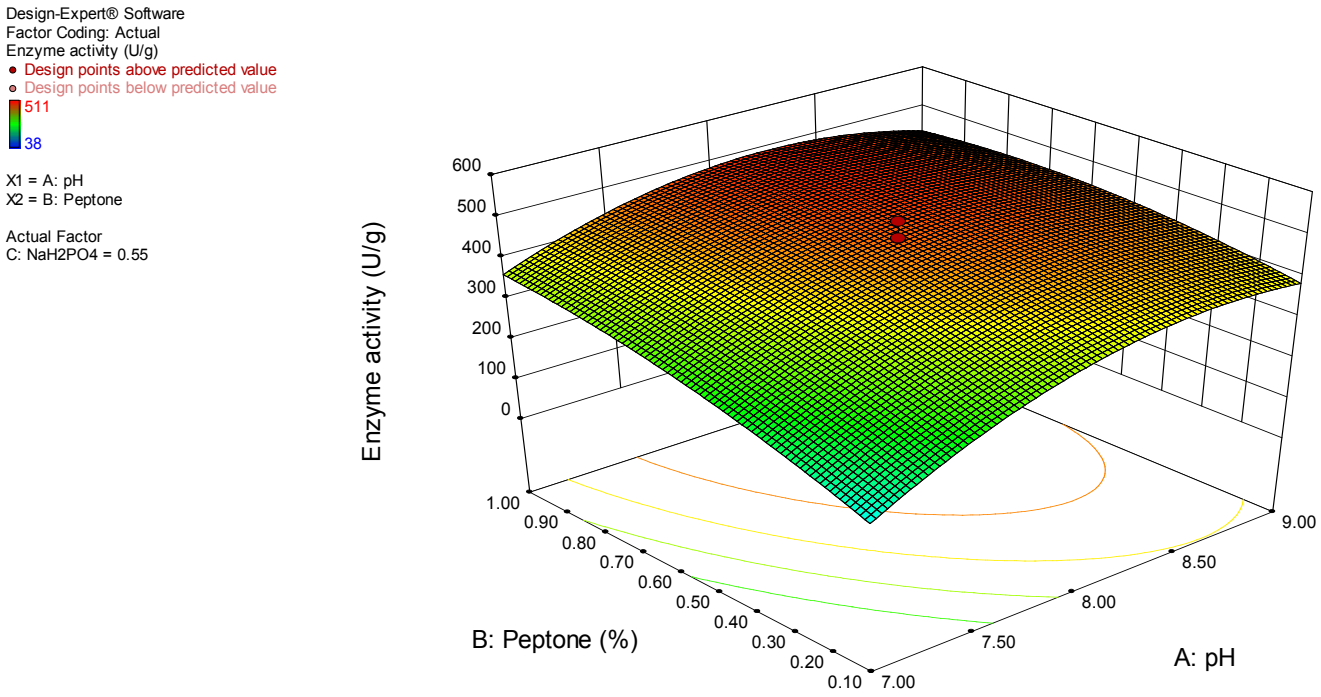
Source	Sum of Squares	df	Mean Square	F Value	p-value	
Model	3.35E+05	9	37223.24	20.25	<0.0001	Significant
A-pH	73202.06	1	73202.06	39.83	<0.0001	
B-Peptone	52999.49	1	52999.49	28.84	0.0003	
C-NaH_2PO_4	30307.88	1	30307.88	16.49	0.0023	
AB	10153.12	1	10153.12	5.52	0.0406	
AC	903.13	1	903.13	0.49	0.4993	
BC	1653.13	1	1653.13	0.9	0.3653	
A2	1.08E+05	1	1.08E+05	58.82	<0.0001	
B2	16249.96	1	16249.96	8.84	0.014	
C2	67433.06	1	67433.06	36.69	0.0001	
Residual	18379.83	10	1837.98			
Lack of Fit	12962.5	5	2592.5	2.39	0.1801	Not Significant
Pure Error	5417.33	5	1083.47			
Cor Total	3.53E+05	19				

Final equation in terms of coded factors

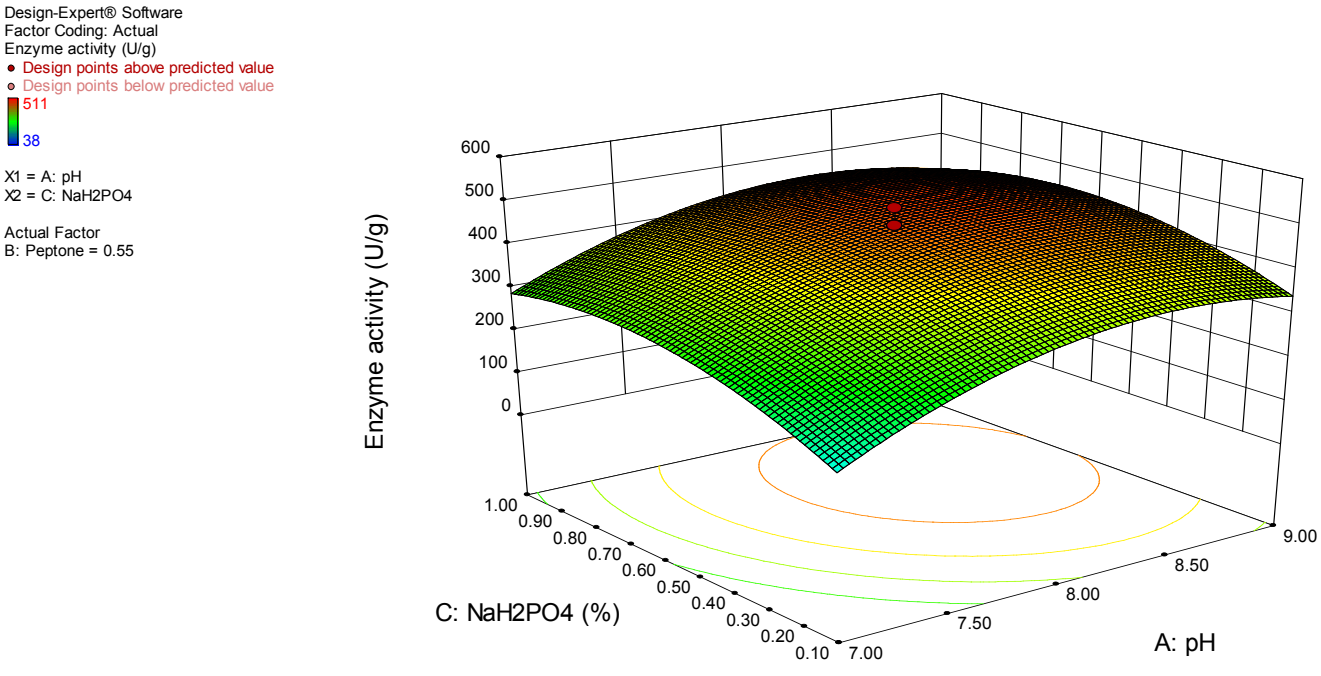
Protease activity= +3990.29-26.64*A+507.60*B-284.03*C+337.88*AB-468.12*AC+1084.38*BC-804.19*A^2-282.70*B^2+107.10*C^2 (5)

Cellulase activity= +460.33+73.21*A+62.30*B+47.11*C-35.62*AB-10.62*AC+14.38*BC-86.61*A^2-33.58*B^2-68.40*C^2 (6)

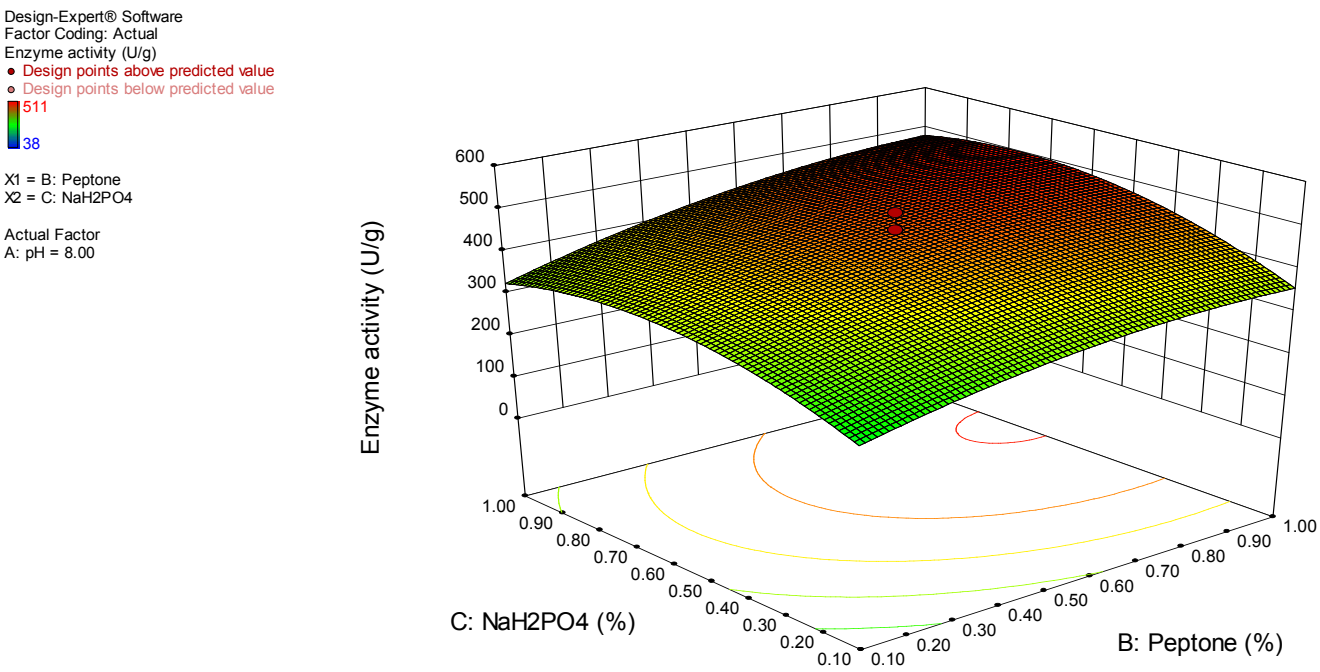
Figure 1 shows the interaction relationship between the two independent variables, namely pH and peptone and NaH_2PO_4. It was observed from Figure 1a that protease production was significantly affected by altering pH and peptone. Figure 1b shows the interaction between pH and NaH_2PO_4. Figure 1c shows the interaction between peptone and NaH_2PO_4. The combination of peptone and NaH_2PO_4 significantly increased the production of alkaline protease.



(a)



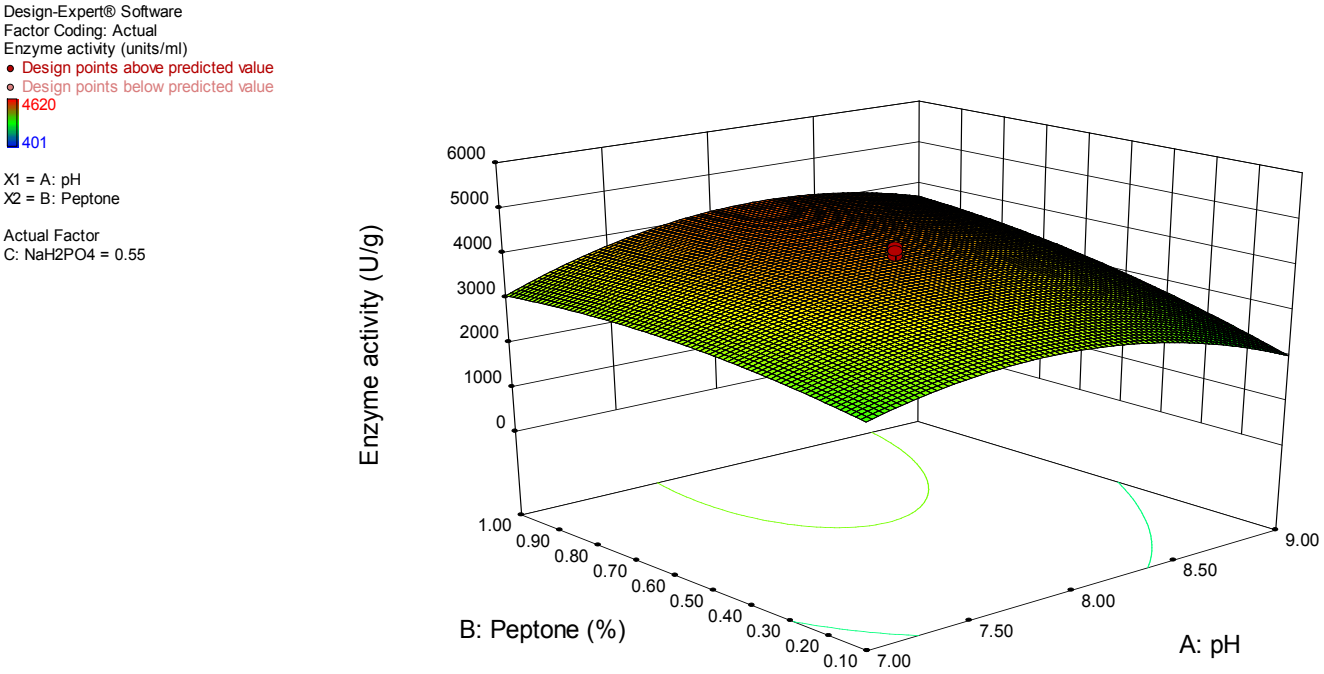
(b)



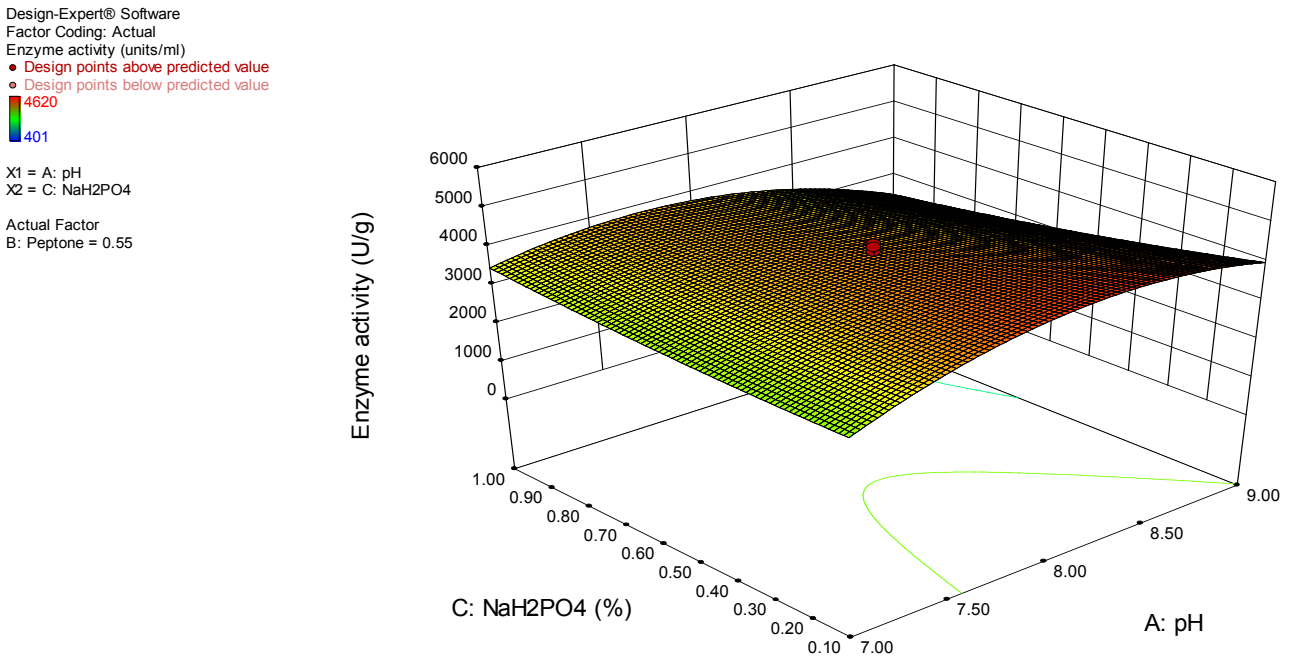
(c)

(a) Interactive effects of NaH_2PO_4 and pH (b) Peptone and pH (c) NaH_2PO_4 and Peptone

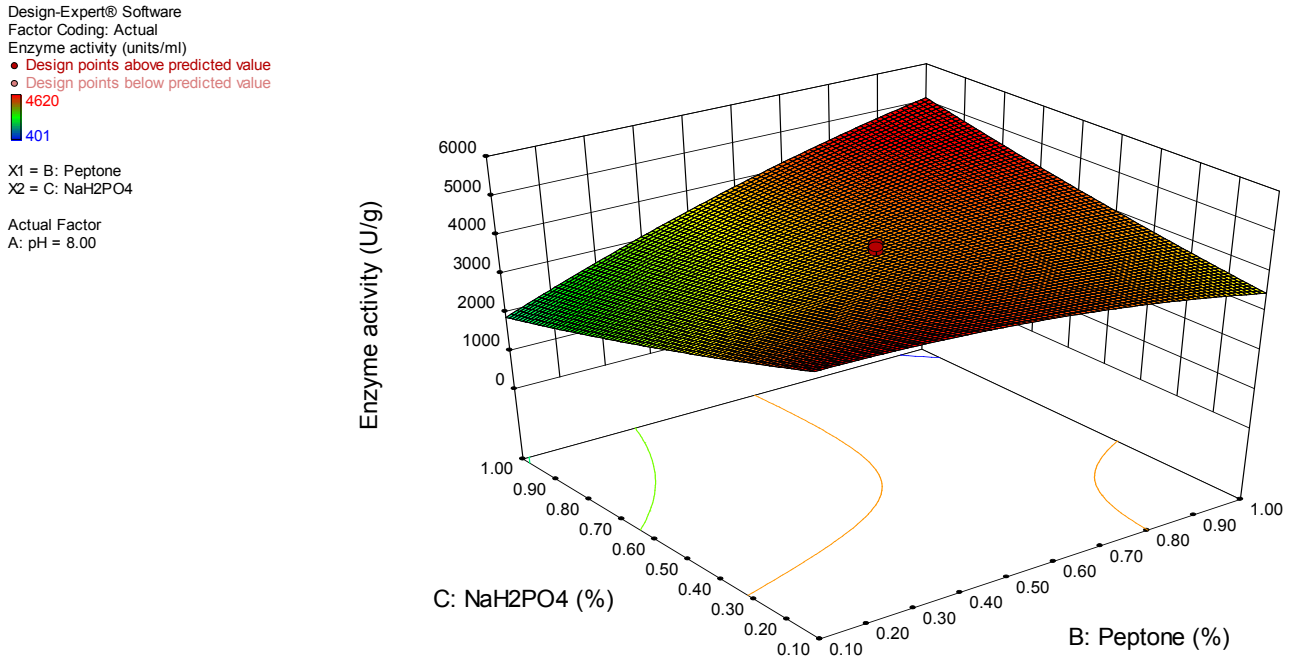
Figure 1. *Response surface plot for alkaline protease production by B. cereus* KA3.



(a)



(b)



(c)

(a) Interactive effects of NaH_2PO_4 and pH (b) Peptone and pH (c) NaH_2PO_4 and Peptone

Figure 2. *Response surface plot for cellulase production by B. cereus* KA3.

Figure 2 shows the interaction between the independent variables, namely pH and peptone and NaH_2PO_4. Cellulase production was significantly affected by altering pH and peptone (Figure 2a). Figure 2b shows the interaction between pH and NaH_2PO_4. Enzyme production increased at increasing concentration of Na_2HPO_4. Figure 2c shows the interaction between peptone and NaH_2PO_4. The combination of peptone and NaH_2PO_4 significantly increased the production of cellulase.

3.5. 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 3127 U/g which was close to the predicted response 3082 U/g. Cellulase production was 482 U/g, which was highly correlated with predicted activity (501 U/g) confirming the rationality of the model. The overall fourfold increase in

protease-, and cellulase activity was registered after statistical optimization.

4. Conclusion

It could be inferred that protease and cellulase production by *B. cereus* KA3 was influenced by physical parameters and nutritional factors. The statistical optimized medium showed fourfold enzyme production than unoptimized medium. The strain *B. cereus* KA3 may be successfully utilized for protease and cellulase production for various industrial applications.

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