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Research paper

Optimization of Alkaline Protease Production by Alkaliphilic *Bacillus lehensis* G1 using Statistical Design

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Abstract

A few parameters were optimized to maximize the production of *Bacillus lehensis* G1's extracellular protease. The *B. lehensis* G1 was cultivate using the statistical method with ten different variables. These ten variables were initially screened using the Plackett-Burman design and subsequently, the significant variables were further optimized via response surface methodology. A statistical model for the effect of the four variables which includes casein, corn flour, temperature and NaCl was generated using the central composite design (CCD). The result showed that the production rate of the extracellular protease was twenty-fold higher when compared to the reference medium. These experimental data showed that casein and temperature give a positive and negative effect on protease production, respectively.

Keywords: *Bacillus lehensis, enzyme, extracellular protease, culture medium optimization, response surface methodology*

1. Introduction

Alkaline protease is a universal class of enzymes used extensively in pharmaceutical, detergent, leather, food and agriculture industries. It constitutes 60–65% of the global industrial enzyme market [1, 2] with an estimated global market worth $2,767 million by 2019 (http://www.marketsandmarkets.com/PressReleases/protein-hydrolysis-enzymes.asp). Alkaline proteases are suitable as detergent additive because this enzyme could eliminate the proteinaceous stains such as keratin, blood, gravy and milk. Furthermore, proteases could retain their activity in high pH environment and compatible with detergent's formula. The first reported of commercial protease used in detergent is Subtilisin Carlsberg from *B. licheniformis* in the 1960s [3].

Bacillus species, such as *B. subtilis*, *B. koriensis* and *B. circulans*, is the major microbial source of proteases due to their capacity to secrete high-level of active enzyme, good high pH stability and temperature stability [4, 5]. However, only a few *Bacillus* species are identified as commercial producers of proteases [6]. An ideal enzyme and host cell that could withstand harsh industrial conditions and produced cheaply has not been found. This warrant continued the search for new microbial sources of alkaline proteases. Cost of the enzyme is the main obstacle for large-scale application of proteases in industry. An effective approach to reduce enzyme cost is to maximize production yield and reduce fermentation-associated cost. In this aspect, approximately 40% of the production cost of industrially important enzymes is estimated to derive from the cost of growth medium [7, 8]. Hence, optimization of fermentation conditions can effectively reduce the cost of enzyme production.

The traditional 'one-factor-at-a-time' (OFAT) approach is probably the oldest and most frequently used methods to improve biological systems to obtain high-level cell growth and desired metabolic products. This approach is time-consuming, labour intensive and also ignores potential interactions between physiological parameters [9]. This often leads to suboptimal conditions for bioproducts production. A better optimization strategy is by statistical design such as Plackett-Burman and Response Surface Methodology (RSM) [10]. Major advantages of statistical design over traditional OFAT approach are the ability to reveal significant interactions between two or more parameters and to perform a more exhaustive search for the optimum desired outcome. Therefore, the statistical design is a widely accepted for modelling and analysis of biological systems and problems in which a response (i.e., level of extracellular enzyme production) is influenced by several variables (i.e., temperature, pH and media components). The present study aims to improve the protease production by alkaliphile *B. lehensis* G1, a strain locally isolated from rubber tree plantation site [11], in a Horikoshi medium through optimization of fermentation conditions, medium components and their concentrations using statistical approaches.

2. Materials and methods

2.1. Microorganism and culture condition

B. lehensis G1 was used to produce an extracellular protease. The seed culture was grown in Horikoshi medium at 37°C/250 rpm. This was prepared for inoculation in the production flasks.

2.2. Protease Assay

The protease was assayed at 37°C using casein (Sigma, USA) as a substrate, and the method as previously described in Joshi & Satyanarayana [12]. One unit of the protease was equivalent to the

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amount of enzyme required to release 1 µg of tyrosine/min under standard assay conditions.

2.3 Screening of significant variables

Design-Expert®v10 (STATEASE Inc., Minneapolis, MN, USA) software was used to screen and optimized the nutritional and physical factors. Various carbon sources such as glucose, wheat bran and corn flour and organic nitrogen sources such as casein were tested in this study for protease production from *B. lehensis* G1. Both the carbon and nitrogen sources were added at 0.5-1% (w/v) of final concentration. Furthermore, inorganic and standard salts (%): FeSO4.7 H2O, NaCl, and CaCl2 were added and tested to the medium as well. These salts were supplement in the medium with a final concentration of 0.01- 0.05% (w/v), 0.1-0.5% (w/v), and 0.002-0.02 g/l, for CaCl2, NaCl, and FeSO4.7 H2O, respectively. Among physical variables that were screen included production time (24-72 hr), temperature (30-37°C) and inoculum size (1-5% w/v).
All variables were screened to optimize the carbon, nitrogen and physical effect at two levels as shown in Table 1. The protease yield was determined after 24 h of incubation at 37°C. The Plackett-Burman was used to find the positive effect of nutritional and physical factors in the production of protease and the factor values as shown in Table 2. The table showed the design of twelve Plackett-Burman runs along with levels and response of each of the ten factors. This statistical design does not involve the interactions between the selected nutrient variables and follows a linear approach for screening of factors [13]. A first-order polynomial equation explained the model variables are:

$$Y= \beta o+ \Sigma \beta i\ xi$$

Whereas Y is the response, βo is the interception coefficient, βi is the linear coefficient. From the ANOVA as given in Table 3, variables ($P< 0.05$) were considered significant response which will be further optimized by response surface methodology (RSM). (Table 2 and 3 were shown on result and discussion section).

Table 1: Ten experimental variables that were screened to produce protease from *B. lehensis* G1 using Plackett-Burman design.

		Range of levels		
Variables	Symbol codes	Units	-1	+1
Casein	A	% (w/v)	0.5	1
Wheat flour	B	% (w/v)	0.5	1
Corn flour	C	% (w/v)	0.5	1
Glucose	D	% (w/v)	0.5	1
Inoculum size	E	% (w/v)	1	5
Time	F	Hours	24	72
Temperature	G	°C	30	37
$FeSO_4.7\ H_2O$	H	g/l	0.002	0.02
$CaCl_2$	I	% (w/v)	0.01	0.05
NaCl	J	% (w/v)	0.1	0.5

2.4 Experimental design and protease production

In the medium optimization for protease production, four variables which includes casein, corn flour, temperature, and NaCl was screened to find their optimal concentration at five different levels (-2, -1, 0, +1, +2). The lowest and the highest level was labelled as -2 and +2, respectively, with six central value labelled as zero coded. By using the Central Composite design (CCD) from Design-Expert® v10 software (Stat-Ease Inc., Minneapolis, MN, USA), a set of 30 experiments suggested and was carried out. The predicted and actual values were presented in Table 4.
CCD was used to obtain the quadratic model to estimate quadratic effects and central points for the estimation of protease production. CCD approached was used to fit a quadratic surface, to optimize the variables with a minimum number of experiments and to analyse the interaction between the variables. The collected data were analysed in a statistical manner to determine the relationship between the factors and response variables. The relationship was explained using the second order polynomial equation:

$$Y= \beta o+ \Sigma \beta ixi + \Sigma \beta iixi^2i + \Sigma \beta ijxixij$$

Where Y: predicted response, β: regression coefficient, and x: coded levels of the independent variable.
The second order polynomial equation was shown as the three-dimensional response surface curve. The four variables used were coded according to the following equation:

$$Z= \frac{(X-Xo)}{\Delta X}$$

Where "Z" is the coded value of the independent variable, "X" is the corresponding real value, "Xo" is at the real value of an independent variable at the centre point and "ΔX" is the step change of the real value at the variable for the value "Z".

Table 4: Experimental variables, symbol codes in bracket, units, ranges of levels of the independent variables for response surface methodological experiments.

		Range of levels				
Variables	Units	-2	-1	0	+1	+2
Casein (A)	% (w/v)	0.25	0.5	1	1.5	2
Corn flour (C)	% (w/v)	0.25	0.5	1	1.5	2
Temperature (G)	°C	25	27	30	37	40
NaCl (J)	% (w/v)	0.05	0.07	0.1	0.25	0.5

3. Results and discussion

3.1 Variables Screening by Plackett-Burman design

The effect of variables on the production of protease from *B. lehensis* G1 was determined by using Plackett-Burman screening design. Ten variables were screened as indicated in Table 2. The values for protease activity were recorded between 1.97 U/ml and 17.30 U/ml. High values of protease were obtained when maximal level casein and corn flour were used thus suggested that these variables have significant impact on the protease activity. The P-value of the model is statistically computed with the value lesser than 0.05 indicates the significance of the model [13]. The determination on the effect of significant variables was computed by using analysis of variance (ANOVA) and the values are as indicated in Table 3.
As showed in Table 3, casein, corn flour, temperature, and NaCl were found to be significant while proving other variables as insignificant.
In the experimental variables tested, the factor such as casein and corn flour were found to be significant with P-values of 0.042 and 0.0136, respectively. The analysis showed that other two glucose sources; wheat flour and glucose were not significant as well as the physical effects tested, time and inoculum size. It is also apparent from ANOVA that this model is highly significant with P-value of 0.0083. By using Design Expert, the equation obtained for Plackett-Burman design was as follows:

$$Y= +0.3834 + 0.1904A + 0.1361C-0.0137G – 0.1177J$$

Where A= casein, C= corn flour, G= temperature, J= NaCl
According to the equation, these four significant variables were further optimized to maximize the production of protease by using central composite design of response surface methodology while non-significant variables were retained at their central values in this study to produce a high yield of the enzyme.

Table 2: Plackett-Burman design used for screening of ten variables with twelve runs.

Experimental Values											
Run no	A	B	C	D	E	F	G	H	I	J	Response (U/ml)
1	1	1	1	1	-1	-1	1	-1	1	1	10.86
2	-1	1	1	1	1	1	1	-1	-1	-1	7.41
3	-1	-1	1	1	1	1	-1	1	1	1	10.44
4	1	1	-1	1	-1	1	-1	1	1	-1	12.52
5	1	1	-1	-1	1	1	-1	-1	-1	1	16.56
6	-1	1	-1	-1	1	-1	1	1	1	-1	5.50
7	-1	1	1	-1	-1	-1	-1	1	-1	1	8.85
8	1	-1	1	-1	-1	1	1	1	-1	-1	14.91
9	1	-1	-1	1	1	-1	1	1	-1	1	2.10
10	1	-1	1	-1	1	-1	-1	-1	1	-1	17.30
11	-1	-1	-1	-1	-1	1	1	-1	1	1	1.97
12	-1	-1	-1	1	-1	-1	-1	-1	-1	-1	8.68

Table 3: ANOVA (analysis of variance) for the experimental parameters of Plackett-Burman design affecting protease production.

Variables	Coefficients	Standard error	F-value	p-value
Model	-	-	16.53	0.0083
Casein	0.048	8.088E-003	34.64	0.0042
Corn flour	0.020	8.088E-003	17.71	0.0136
Temperature	-0.048	8.088E-003	35.07	0.041
NaCl	0.024	8.088E-003	8.48	0.0436

R^2 = 0.9666, Adj. R^2= 0.9081, Root MSE=0.028, C.V.%=16.42
* Non-significant values at p< 0.05 in the result is not shown.

3.2 Optimization by Central Composite Design (CCD)

The screening process was later followed by CCD with 30 experiments conducted in determining the optimal levels for the factors proven to be significant (casein, corn flour, temperature and NaCl) that affected the production of protease (Table 4 and 5). Design Expert was utilized to compute the values for coefficient and regression as indicated in the following equation:

$$Y= +263.15 +43.29A + 22.24C + 0.055G – 10.63J – 15.14AC + 26.48AG – 0.60AJ + 2.27CG – 7.20\ CJ – 2.26GJ – 1.06\ A^2 + 9.01C^2 – 4.29G^2 – 5.39J^2$$

According to the regression analysis of the RSM, (Table 6) the model terms *A*, *C*, *G*, *J*, *AC*, *AG*, *AJ*, *CG*, *CJ*, *GJ*, A^2, C^2, G^2, and J^2 were significant ($p < 0.05$). The linear effects of *A* and *C* the interactive effects of *AC* were most significant with all have *P*-value <0.0001. These results indicated that casein and corn flour exhibited a direct relationship to the production of protease. The ANOVA for the second-order polynomial model showed that R^2 was 0.9444 which indicates that the model could explain 94.44% of the variation in response to protease production. The square of the linear correlation is termed as the coefficient of determination (R^2), which is the measure of the strength of the linear relationship between the experimental and the predicted values [13]. The model F-value 18.22 implies that protease production exhibit a good fit with the model whilst non-significant lack-of-fit (0.4896) showed that the model was significant. The coefficient of variation % (CV%) is a reliability of the model by measure the residual variation of the data relative to the size of the mean. Generally, the higher the value of CV, the lower is the reliability of the experiment [14]. A low coefficient variation (CV=6.75) showed the reliability of the experiments conducted. Adequate precision 17.158 obtained in this analysis explained significantly the good fit of the model as this value should be above "4" explaining the signal to noise to navigate the design space excellently [15].

Table 5: Central Composite Design for the response of protease along with its predicted and observed values

	Experimental Values						
Run no	A	C	G	J	Response (U/ml)	Predicted (U/mL)	Residual value
1	0	2	0	0	335.09	334.90	0.19
2	1	-1	-1	-1	278.45	275.87	2.58
3	1	-1	1	1	329.68	316.02	13.66
4	-1	-1	-1	-1	237.43	210.45	26.98
5	-1	1	1	-1	266.95	251.26	15.69
6	-1	-1	-1	1	213.46	209.31	4.15
7	1	1	1	1	306.98	320.35	-13.37
8	0	0	0	0	280.78	278.20	2.58
9	0	0	0	0	275.86	275.14	0.72
10	1	1	1	-1	364.23	361.34	2.89
11	0	0	0	-2	244.69	255.16	-10.47
12	2	0	0	0	337.54	345.92	-8.38
13	0	-2	0	0	245.90	244.07	1.83
14	0	0	0	0	281.08	264.58	16.5
15	0	0	-2	0	225.75	246.33	-20.58
16	0	0	0	0	239.57	263.58	-24.01
17	1	-1	1	-1	333.86	328.62	5.24
18	-1	1	-1	1	273.49	265.13	8.36
19	1	1	-1	1	278.55	267.26	11.29
20	-2	0	0	0	161.07	172.75	-11.68
21	-1	-1	1	-1	153.07	154.18	-1.11
22	-1	-1	1	1	143.26	147.39	-4.13
23	0	0	0	2	220.96	220.57	0.39
24	1	1	-1	-1	317.37	299.62	17.75
25	-1	1	-1	-1	287.84	295.07	-7.23
26	-1	1	1	1	216.70	212.28	4.42
27	0	0	0	0	252.66	263.58	-10.92
28	0	0	2	0	247.98	244.33	3.65
29	1	-1	-1	1	271.05	269.61	1.44
30	0	0	0	0	254.01	249.80	4.21

Table 6: ANOVA for the experimental parameters of Central Composite design affecting protease production.

Variables	Sum of Squares	df	Mean Square	F-value	p-value
Model	79600.1	14	5685.78	18.22	< 0.0001
Residual	4682.21	15	312.15	-	-
Lack of fit	3215.36	10	321.54	1.10	0.4896
Pure error	1466.85	5	293.37	-	-
Total	84283.1	29	-	-	-

Std Dev = 321.37, Mean = 4762.50, C.V.% = 6.75, R^2 = 0.9444, Adj R^2 =0.8926, Pre R^2 = 0.7552, Adeq Precision = 17.158

Figure 1 showed the response surface curves thus providing information on the interaction between the variables and their response. The response curves were generated in paired combination of the variables while keeping the other two at their centre point levels. The 3D response curves as depicted in Figure 1A further explained the computed response from the interaction between corn flour and casein while retaining the level of other two variables (temperature and NaCl) at zero. The result indicated that the increase of corn flour and casein at 2% (w/v) increased the production of enzyme production to 337 U/mL. Figure 1B displayed the effect of casein and temperature on the production of protease while keeping other two variables (corn flour and NaCl) at zero. The curves indicated that the least production of protease took place at 0.25% (w/v) casein and 40°C which fit the model. The interaction between casein and NaCl on the production of the protease was observed by retaining the central level of corn flour and temperature as indicated in Figure 1C. The curves signified that the peak of protease production took place with casein at 2% (w/v) with irrespective of NaCl concentration, which coincided with the model. The reaction of corn flour at 2% (w/v) resulted in maximum protease production with the temperature at 25-40°C as indicated in Figure 1D. Interaction between NaCl (0.05% w/v) and corn flour 2% (w/v) as presented in Figure 1E indicated that the maximum enzyme production occurred at 291U/mL. Figure 1F presented the response curves of the interaction between temperature and NaCl, and the lack of curvature indicated that the response did not deviate when NaCl concentration was increased or when the temperature changed.

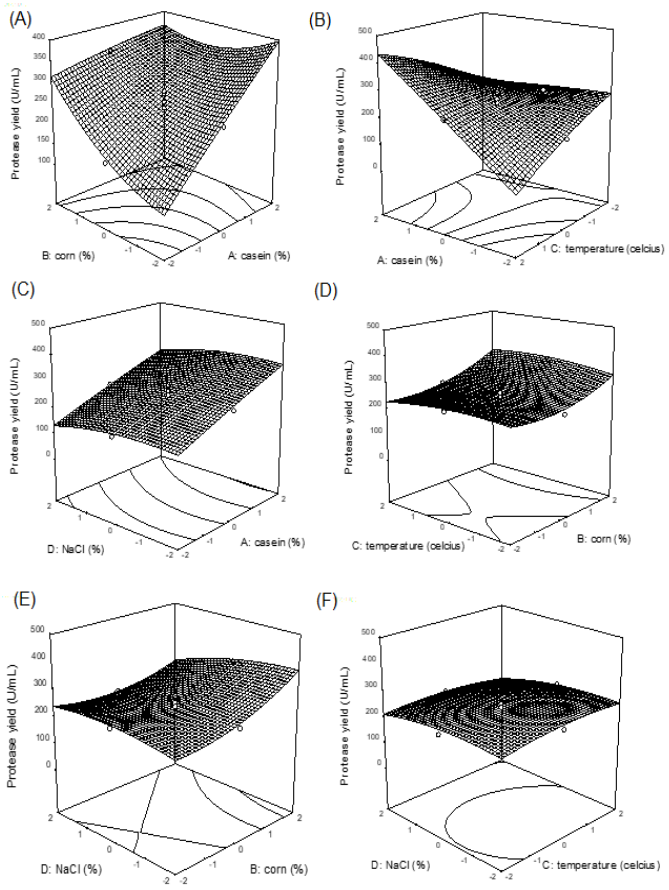


Figure 1: Response surface curve from *B. lehensis* G1 showing the interaction between (A) corn flour and casein, (B) temperature and casein, (C) NaCl and casein, (D) temperature and corn flour, (E) NaCl and corn flour and (F) NaCl and temperature for production of extracellular protease.

4. Conclusion

In this work, the statistical method, Plackett–Burman and CCD proved to be efficient for the production of protease. Plackett–Burman was used to find the variables that were important in the medium components. Among the ten variables tested, casein, corn flour, temperature and salt were found to be the most significant variables. Further experimentation optimization using CCD, the highest protease activity reached 364.23 U/mL with a residual value of 2.89 U/mL between the predicted and response activity. The optimized medium established in this work might benefit in a cost reduction for protease production from B. *lehensis* G1.

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