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Optimization of fermenting medium by statistical method for production of alkaline protease by *Bacillus licheniformis* MZK05M9

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ABSTRACT

To optimize the fermentation medium for the production of alkaline protease by *Bacillus licheniformis* MZK05M9 (*Bl*M9) molasses as a carbon source, soybean meal as a nitrogen source, and the salts NaCl, $MgSO_4.7H_2O$, and K_2HPO_4 were selected by Plackett–Burman approach. The response surface methodology based on central composite design revealed that the optimum values for the tested variables were found as (% w/v) molasses (0.92%), soybean meal (0.79%), NaCl (0.125%), $MgSO_4$ (0.125%), and K_2HPO_4 (0.59%) with the protease activity 761 U/ml predicted by statistical software Minitab Version 17. The experimental value was found as 765 U/ml. The granular size of soybean meal 4.7 mm supported the enzyme production 5% higher than that of the mixed sizes between 6 and 4 mm. Fermentation in 7 l bioreactor exhibited the enzyme activity 1020 U/ml after 28 h. The statistically optimized medium based on cost-effective agro-industrial C and N sources yielded a high productivity 36,428 U/l h of protease by the mutant strain of *B. licheniformis*.

1. INTRODUCTION

Extracellular microbial alkaline proteases are commercially important enzymes used extensively in several industries such as in the detergent, food, pharmaceutical, chemical, leather, silk, and waste treatment [1]. A large proportion of commercially important alkaline proteases is derived from the strains of *Bacillus* [2-4]. In fermentation technology, improvement in the productivity of the microbial enzyme can be achieved by optimization of physicochemical parameters and genetic manipulation such as mutation or gene cloning [5]. In our laboratory, previously, a wild strain *Bacillus licheniformis* MZK05 was improved to *B. licheniformis* MZK05M9 (*Bl*M9) by genetic manipulation through classical mutation. The mutant *Bl*M9 produces an alkaline protease with novel properties of high quality bating potential in leather processing [6]. As a consequence of the research, the further optimization of the production medium was required for overproduction of the alkaline protease to make it industrially as well as commercially viable. For optimization of the medium, the "one-at-a-time-approach" is frequently used to obtain the maximum level of enzyme in a fermentation system. However, this technique is time-consuming and also ignores the combined interactions among the variables [7]. On the other hand, the statistical methodologies are generally preferred due to their recognized advantages of their use [8,9]. The statistical Plackett–Burman designs (PBD) are used for selecting main variables from a large number of process variables. Therefore, these designs are very useful in primary studies in which the main target is to find the main factors from a large number of variables that can be fixed or eliminated in further optimization processes. On the other hand, response surface methodology (RSM) is a competent tactical experimental method which can determine the optimal conditions of a multivariable system. Many researchers used statistical methods to select carbon and nitrogen sources for the production of enzymes [2]. The complex carbon and nitrogen sources facilitate the large-scale production of protease by providing some trace elements and vitamins [10]. The main objective of the present study was to select significant carbon and nitrogen sources and further to optimize the concentration level of the screened ingredients using statistical methodologies for the production of alkaline protease by employing the mutant *Bl*M9.

2. MATERIALS AND METHODS

2.1. Microorganism

B. licheniformis MZK05M9 (*Bl*M9), a mutant developed through classical mutation [11] was used in this experiment. This organism was preserved in the Enzyme and Fermentation Biotechnology laboratory, the Department of Microbiology, University of Dhaka. The organism

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was maintained on Tryptic Soy Agar medium at 4°C for routine use and 15% glycerol broth at −70°C for long-term preservation.

2.2. Preparation of Seed Culture

Sterilized 50 ml of Tryptic Soy Broth in a 100 ml Erlenmeyer flask was inoculated with one loop-full *Bl*M9 culture and was incubated for 16 h at 37°C with 150 rpm.

2.3. Fermentation and Separation of the Enzyme

5 ml of seed culture was transferred to 100 ml of fermentation medium (pH 7.5) containing (% w/v): Soybean meal (1%), molasses (0.5%), K_2HPO_4 (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.05%), and $CaCl_2.2H_2O$ (0.05%) in a 250 ml Erlenmeyer flask and incubated at 37°C and 150 rpm in an orbital shaker for 48 h. After fermentation, the samples were collected and centrifuged at 6000 rpm for 10 min. The cell-free supernatant was used for enzyme assay.

2.4. Determination of Enzyme Activity

Protease activity was determined according to the modified method of Kreger and Lockwood [12]. In brief, 400 µl of appropriately diluted cell-free enzyme solution was incubated with 400 µl of 1% azocasein (Sigma Co. St. Louis. Mo.) solution in 0.05 M Tris-HCI buffer at pH 8.5 for 1 h at 37°C in a water bath. The reaction was terminated by addition of 135 µl of 35% trichloroacetic acid (TCA) and keeping the mixture at 4°C for 10 min. The reaction mixture was then centrifuged at 13,000 rpm for 10 min. Then, 0.75 ml supernatant was mixed with 0.75 ml of 1 M NaOH, and the absorbance was read at 440 nm against the control. The control was treated in the same way except TCA was added to the enzyme before mixing with azocasein solution. One unit of proteolytic activity was determined as the amount of enzyme that produces an increase in absorbance of 0.01 under the assay conditions.

2.5. Selection of Ingredients by Statistical Plackett-Burman Design (PBD)

Locally available cost-effective complex carbon and nitrogen sources such as rice bran (A), wheat bran (B), mustard seed meal (C), molasses (D), and soybean meal (E), and the salts such as $CaCl_2$ (F), $MgSO_4.7H_2O$ (G), K_2HPO_4 (H), and NaCl (J) were studied to find out their effects on protease production. In the PBD factorial design, each variable was examined on two levels: For a low level (−1) and for a high level (+1) (Table 1).

In the first-order model, the factors were screened linearly using the approach: $Y = \beta_o + \sum \beta_i x$ (I = l-k). In this equation, Y is the target function, β_o and β_i are the intercept and regression coefficient, respectively.

Table 1: Low level and high-level concentration of the variables for Plackett–Burman factorial design.

Indication	Ingredients	(1) High (%)	(−1) Low (%)
A	Rice bran	2	0.1
B	Wheat bran	2	0.1
C	Oil cake	2	0.1
D	Molasses	2	0.1
E	Soybean meal	2	0.1
F	$CaCl_2 2H_2O$	0.2	0.01
G	$MgSO_4.7H_2O$	0.2	0.01
H	K_2HPO_4	0.5	0.03
J	NaCl	0.2	0.01

2.6. Optimization of the Concentration of the Selected Ingredients by Response Surface Methodology (RSM)

The independent variables selected by PBD such as soybean meal as a nitrogen source, molasses as a carbon source, K_2HPO_4, $MgSO_4.7H_2O$, and NaCl were indicated by A, B, C, D, and E, respectively. Then, the second-order polynomial equation was found as: $Y = \beta_o+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_5E+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{44}D^2+\beta_{55}E^2+\beta_{12}AB+\beta_{13}AC+\beta_{14}AD+\beta_{15}AE+\beta_{23}BC+\beta_{24}BD+\beta_{25}BE+\beta_{34}CD+\beta_{35}CE+\beta_{45}DE$.

The experimental design and analysis of data were performed using the software Minitab version 17.

2.7. Fermentation in Bioreactor

The fermentation experiments for the production of extracellular alkaline protease by *Bl*M9 were carried out in a bench-top bioreactor (7.5 l) (Bioflo 110, New Brunswick Scientific, USA) with a working volume of 3.5 l. The fermentation medium was inoculated with *Bl*M9 inoculum at a level of 5% (v/v) based on total working volume. The fermentation was carried out at a temperature of 37°C and initial pH 7.5. The dissolved oxygen levels in the culture were controlled by cascading mode maintained by both agitation and aeration where high and low limits of agitation were 300 rpm and 150 rpm, respectively, and high and low limits of aeration were 3.5 SLPM and 1 SLPM, respectively.

3. RESULTS AND DISCUSSION

3.1. Screening of Significant Nutrients by PBD

PBD was used to select the nutrients with most positive effects on the production of the *Bl*M9 enzyme. The *P* value is used to check the significance of each of the parameters. A low *P*-value implies significant effect. In our study, the *P* value for the model was 0.000 indicating that the model was significant. From the 24 set of fermentation run designed by Minitab version 17, the Pareto chart showed that the soybean meal demonstrated the most positive effect on protease production (Fig. 1). The rank order of the effect of the ingredients on the production of the protease was Soybean meal > molasses > NaCl > mustard seed meal > K_2HPO_4 > $MgSO_4$. On the other hand $CaCl_2$, rice bran and wheat bran had a diminutive effect on the enzyme production. Therefore, the soybean meal as a nitrogen source, molasses as a carbon source, and other salts NaCl, K_2HPO_4, and $MgSO_4$ were selected for further optimization of the medium to determine the optimum concentration of the ingredients using RSM based on central composite design (CCD).

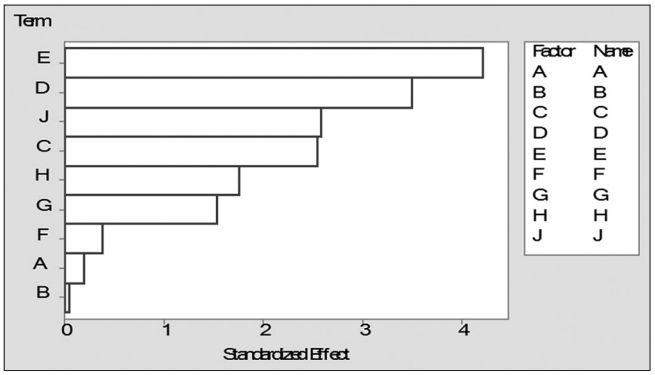

Fig. 1: Pareto chart showing the rank order of the effect of the ingredients on enzyme production.

3.2. Optimization of the Concentration of the Selected Ingredients by RSM

CCD was designed to study the effects of five selected independent variables, namely, soybean meal (A), molasses (B), K_2HPO_4 (C), $MgSO_4$ (D), and NaCl (E). The designed CCD has been described in Table 2. After 32 set of fermentation experimental run, the enzyme yields were found which are given in Table 3.

Polynomial regression equation was found-

Y = 684.28+8.29A+10.21B-1.87C+7.46D+5.79E-41.78A*A-40.28B*B-17.78C*C-2.53D*D+1.22E*E-15.94A*B-5.31A*C-0.31A*D-15.31A*E+6.56B*C+19.06B*D-0.94B*E+3.44C*-D+0.94C*E+8.44D*E.

The *P* value of the model was found as 0.000 which indicates that the model is significant. If the values of "*P* > *F*" are found as <0.0500 then model terms would be said significant. The correlation coefficient (R^2) of the polynomial equation was found 0.9570 which implies that 95.70% of the variability in the response (production of alkaline protease) can be explained by this model (Table 4).

The response surface plots of the RSM generated by the software showed the interactions among the ingredients. The three-dimensional surface of the relative effects of soybean meal and molasses showed strong degree of curvature where the optimum level can be determined (Fig. 2).

The contour plot of the same interaction exhibited maximum protease production 685 U/ml when the concentration levels of soybean meal and molasses were at their nearly central value of 1% and 0.75%, respectively (Fig. 3).

When protease production was observed as a response to the interaction of soybean meal and molasses keeping other ingredients' concentration at central points, an enhancement in protease production was observed at the central concentration of soybean meal and molasses. Thus, maximal enzyme production could be obtained at midvalue of soybean meal and molasses. The experimental value showed a high degree of similarity with the predicted value, and thus, this indicated the accuracy and applicability of the RSM to optimize the medium for the production of the *Bl*M9 enzyme. Previously, other researchers found improved production by optimizing media using statistical RSM methods such as in the case of α-amylase from *Bacillus circulans* GRS313 and in the case of protease production using *Bacillus* sp. RGR-14 [13,14]. The result of the present study harmonizes the earlier reports in case of protease production in which better production has been observed in the media formulated with complex carbon and nitrogen sources [2].

3.3. Validation of the Prediction of Software at Shake Flask Level (Validation of the Model)

The software Minitab version 17 predicted that the highest enzyme activity 761 U/ml would be found in the optimized medium comprising (% w/v): Molasses (0.92%), soybean meal (0.79%), NaCl (0.125%), $MgSO_4.7H_2O$ (0.125%), and K_2HPO_4 (0.59%). However, the protease yield was found 765 U/ml in experimental fermentation which validated the prediction of the software.

3.4. Effect of Mesh Size of Soybean Meal on Enzyme Production

The soybean meal was differentiated in different mesh size of 6.3 mm, 4.7 mm, and 4 mm (Fig. 4). The mesh size 4.7 mm of soybean meal in the optimized medium supported the enzyme production most than those of other mesh sizes and original mixed sizes of soybean meal

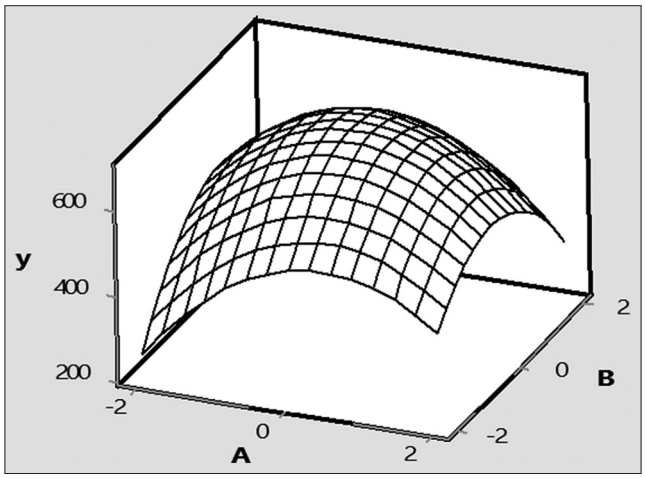

Fig. 2: Response surface plot of protease production by *Bl*M9 showing the interaction between soybean meal and molasses. A – soybean meal, B – molasses, Y – enzyme activity (U/ml).

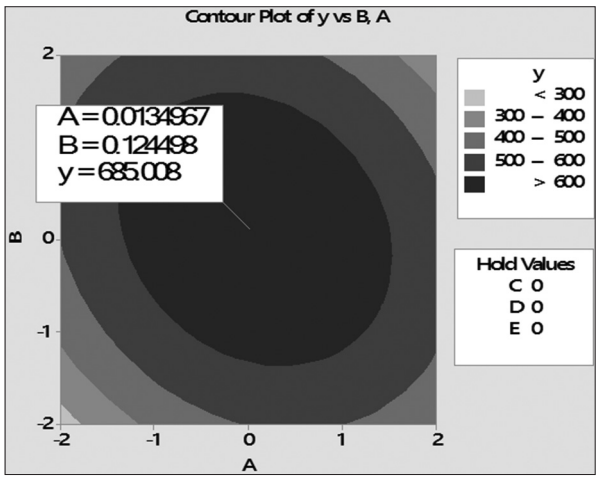

Fig. 3: Contour plot of protease production by *Bl*M9 showing the interaction between soybean meal and molasses. A – soybean meal, B – molasses.

Table 2: Experimental range and level of the independent variables.

Independent variables		High (+1) (% w/v)	Low (-1) (% w/v)	Mean (0) (% w/v)	+α (% w/v)	-α (% w/v)
Soybean meal	A	1.5	0.5	1	2	0
Molasses	B	1	0.5	0.75	1.25	0.25
K_2HPO_4	C	0.75	0.25	0.5	1	0
$MgSO_4.7H_2O$	D	0.1	0.05	0.075	0.125	0.025
NaCl	E	0.1	0.05	0.075	0.125	0.025

For five variables, α=2

(Fig. 5). The smaller granular size makes the medium condition more viscous which subsequently may prevent the transfer of the oxygen to the medium. On the other hand, the larger size of the granule may interfere with the microbe in breaking down the granule and taking the nutrition.

Table 3: 32 set of fermentation experimental run and their enzyme yields.

Run order	Variables					Enzyme activity (U/ml)
	A	B	C	D	E	
1	0	0	0	2	0	677
2	0	0	0	0	0	688
3	−1	1	1	−1	1	600
4	1	1	1	1	1	625
5	1	1	−1	1	−1	630
6	−1	1	−1	−1	−1	570
7	−1	−1	−1	−1	1	530
8	−2	0	0	0	0	570
9	0	0	−2	0	0	665
10	0	0	0	0	−2	655
11	1	−1	−1	1	1	600
12	1	−1	1	1	−1	580
13	1	1	1	−1	−1	590
14	1	−1	1	−1	1	580
15	0	0	0	−2	0	640
16	0	−2	0	0	0	505
17	0	0	0	0	0	690
18	0	0	0	0	0	691
19	−1	−1	1	1	1	565
20	−1	1	1	1	−1	620
21	−1	−1	−1	1	−1	510
22	−1	1	−1	1	1	650
23	0	0	0	0	2	692
24	0	2	0	0	0	510
25	1	−1	−1	−1	−1	655
26	0	0	2	0	0	645
27	2	0	0	0	0	575
28	0	0	0	0	0	691
29	0	0	0	0	0	692
30	−1	−1	1	−1	−1	540
31	0	0	0	0	0	685
32	1	1	−1	−1	1	560

Table 4: ANOVA for the experiments.

DF	20
Adj SS	120205
Adj MS	6010.3
F-value	12.24
P-value	0.000
S	22.1573
R^2	95.70%
R^2 (adj)	87.88%
R^2 (pred)	0.00%

3.5. Bioreactor Cultivation of the *Bl*M9 for Production of the Protease in Optimized Medium

A drastic fall of dissolved oxygen level was observed from the start to 12 h of the fermentation suggesting that the mutant *Bl*M9 has high growth rate and ability to reach exponential phase immediately under the fermentation conditions with the optimized medium (Fig. 6a). It was found that the highest protease activity 1020 U/ml with 36428 U/l h and extracellular protein concentration 0.87 mg/ml were achieved after 28 h at stationary phase (Fig. 6b). In the bioreactor, the optimized medium supported the enzyme production 1.3-fold higher than that in shake flask suggesting that the optimized medium is suitable for large-scale enzyme production by *Bl*M9.

To produce alkaline protease, many researchers used inexpensive substrates coupled with expensive one as a carbon and nitrogen source such as glucose and soybean meal [15-17], soybean meal and trypton [18], wheat bran and soybean meal [19], starch and soybean meal [20], molasses and potassium nitrate [21], molasses and urea [22], starch, wheat bran and soya flour [23], wheat bran and beef extract [24], and glucose and groundnut meal [25]. However, there are fewer attempts reported to induce protease production using inexpensive both carbon and nitrogen sources [2,26].

4. CONCLUSION

In the present study, the established optimized medium containing locally available cheap agro-industrial residues, molasses, and soybean meal as carbon and nitrogen source, respectively, resulted in a significantly enhanced production of protease and reduction in the cost of medium constituents. Therefore, the present study will facilitate the effective economization of overproduction of the alkaline protease by *Bl*M9.

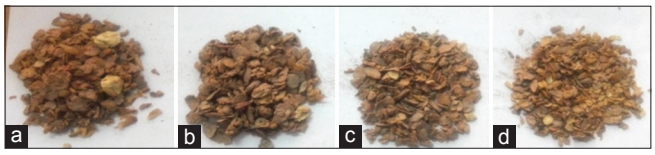

Fig. 4: Different mesh size of soybean meal. a – Mixed, b – 6.3 mm, c – 4.7 mm, d – 4.0 mm.

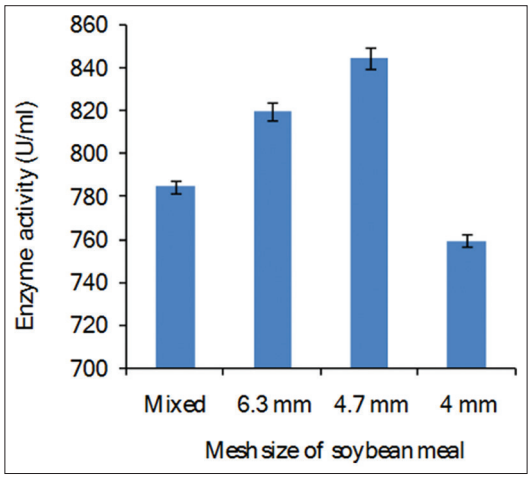

Fig. 5: Effect of mesh size of soybean meal on protease production by *Bl*M9 in shake culture.

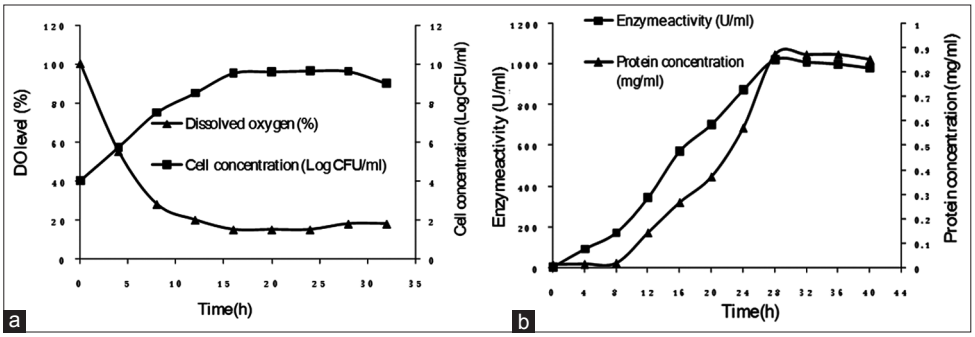

Fig. 6: (a) Time course for cell growth of *Bl*M9 in the optimized medium in 7 l bioreactor, (b) time course for protease production by *Bl*M9 in the optimized medium in 7 l bioreactor.

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