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Computational approach for the optimization of keratinase enzyme production from *Bacillus cereus*

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Abstract

Feathers made up of keratin protein are resistant to physical, chemical and environmental factors. Keratins are classified as heterogeneous proteins due to the structure and composition of amino acid. Keratinase enzyme can easily degrade keratin waste and converts into feed stuffs and fertilizers. Keratinase enzyme is secreted by various microorganisms which mostly include fungi, actinomycetes and *Bacillus*. In the present study, *Bacillus cereus* KM209198, which was isolated from feather dumped soil for production of keratinase enzyme was selected. Statistical tools Minitab pro 16.1.0.0 and Design expert 7 were used for optimization of keratinase production. Plackett-Burman method was used for identifying variables influencing the enzyme production and further optimized by RSM. Placket-Burman design, four out of seven variables such as glucose, casein, magnesium sulphate and pH were identified. These significant variables were further optimized through hybrid system of response surface methodology. Optimized concentration was identified as glucose 2.5 g l^{-1}, casein 7.5 g l^{-1}, $MgSO_4^{-2}$ g^{-1} and pH 10. Initially enzyme activity without optimization was 2.2 U ml^{-1} and after optimization, the enzyme activity was increased upto 3.5 U ml^{-1}.

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Introduction

Keratins are valuable animal proteins found abundantly in slaughter houses, meat and poultry plants containing skin remains, bristle, animal hair, horns and hooves, feathers etc. Keratin waste was classified as animal by-products, not intended for human consumption in regulation (EC) 1774/2002 of the European Parliament and Council of 3rd October 2002. Keratins have high number of disulfide bonds of α-keratin (Fraser *et al.,* 1972; Filipello Marchisio, 2000) which makes them insoluble and resistant to chemical and environmental factors. Keratins are found in skin and hair of animals, human hair, horns, or claws rich in keratin, which can be easily degraded by keratinolytic microorganisms such as prokaryotic or keratinophilic fungi and some bacterial strains. They use native keratin as the source of carbon, nitrogen for energy (Noval and Nickerson, 1959; Korniłłowicz-Kowalska, 1997a). Disposal of bulk poultry waste is a major global environmental problem accounting to pollution of land and underground water resources.

Microbial keratinase is a type of protease enzyme, and accounts 60% of total industrial enzyme market. These enzymes are used in detergents, leathers for dehairing and bating of skins and hides. Keratinase enzyme is produced by various bacteria, actinomycetes and keratinophilic fungi. Keratinolytic bacteria, particularly from genus *Bacillus*, are mostly used. The requirement of media and condition varies largely with species and cannot be generalized (Burtt and Ichida, 1999). The enzyme yield also varies between strains and should be optimized, and cost requirement for enzyme

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production is a major challenge in industries. Thus, process optimization is necessary for enzyme production.

A popular approach for media optimization among researchers is the one-factor-at-a-time approach. But, since it is a time-consuming process, requires more experimental runs and interaction between factors cannot examine are major limitations. Statistical approaches like Placket-Burman and Response Surface Methodology are highly successful and preferred for optimization, increasing enzyme production and reduction of cost. Using these approaches, a study was undertaken for the production of inexpensive keratinase enzyme from *Bacillus cereus* KM209198 isolated from feather dumped soil. Keratinase enzymes are important in biotechnological processes involving keratin-containing wastes from poultry and leather industries.

Materials and Methods

Processing of chicken feathers : Chicken feathers collected from poultry farm were washed thoroughly with tap water and distilled water, and air-dried. Then, it was soaked in diethyl ether for 24 hr and washed thoroughly with distilled water and air-dried. The feathers were then cut into small pieces without the midportion; autoclaved and stored at room temperature.

Enzyme production in batch cultivation : Enzyme was produced by inoculating 1ml of bacterial culture into 500 ml Erlenmeyer flask containing 100 ml MR-VP liquid medium. The media consisted of 3 g l^{-1} dextrose, 7 g l^{-1} peptone and 5 g l^{-1} di-potassium phosphate. The flask was incubated at 37 ºC and 150 rpm for 3 days in a shaker incubator.

Extraction of keratinase enzyme : After completion of 3 days, the culture medium was filtered through Whatman No.4 filter paper to remove unwanted residues. Filtrate was then centrifuged at 10,000 rpm for 10 min to remove bacterial residue and the supernatant containing crude enzyme was used for enzyme assay.

Keratinase assay : Keratinase activity was determined by the modified method of Letourneau *et al*. (1998) using keratin Azure (Sigma Chemicals, USA) as substrate. The keratin azure was suspended in carbonate buffer (10 mM, pH 10) at a concentration of 4 mg ml^{-1}. The reaction mixture contained 1 ml of culture supernatant and 1 ml of keratin azure suspension. The sample was incubated at 37 ºC, 300 rpm for 1hr. After incubation, the mixture was kept in ice for 15 min followed by centrifugation at 5000g for 15 min to remove unutilized substrate. The supernatant was spectrophotometrically measured for the release of azo dye at 595 nm. A control was kept with enzyme and buffer without substrate. One unit (1 U ml^{-1}) of keratinase is defined as the amount of enzyme required to increase 0.1 absorbance between sample and control at 595 nm.

Effect of carbon source on keratinase production : To study the influence of different carbon sources, glucose, fructose and sucrose were used for the production of keratinase enzyme. The flasks were incubated at 37^{0}C with 150 rpm for 3 days in a shaker incubator.

Effect of nitrogen source on keratinase production : Different nitrogen sources such as ammonium sulphate, urea, casein and peptone were used along with the production medium to study the influence on keratinase production. The flasks were incubated at 37 ºC and 150 rpm for 3 days in a shaker incubator.

Effect of pH on keratinase activity : To study the influence of various pH such as 4, 7 and 10 in the production medium, the flasks were incubated at 37^{0}C with the agitation of 150 rpm for 3 days in a shaker incubator.

Optimization of medium using Plackett-Burman design : Plackett–Burman design is an effective way to screen the major influencing parameters among a large number of process variables that are required for enhanced keratinase production. In this study, seven variables-which included four components glucose, casein, K_2HPO_4, $MgSO_4$, and three cultivation parameters, pH, rpm and inoculum size-were studied using PB design to identify the major variables that influence keratinase production. It identifies the main physio-chemical parameters required for maximal keratinase production by screening n variables in n+1 experiments, each variables was examined at two levels. 'Minitab pro 16.1.0.0' was used to analyze the experimental Plackett-Burman design (Sudhir *et al.,* 2010). The design matrix consisted of experimental runs, and the corresponding response of twelve keratinase activity is shown in Table 2. All the twelve experimental runs were incubated at 37 ºC and 150 rpm for 3 days in a shaker incubator. Major influencing parameters were further optimized using Response Surface Methodology.

Optimization of medium using Response Surface Methodology : Further optimization using face-centered central composite design (FCCCD) was employed to estimate the main effects, interaction effects, and quadratic effects of the variables on the response. Based on the PB design, four major influencing variables (Glucose, Casein, pH and $MgSO_4$) were selected for further statistical optimization. The response selected was maximum keratinase production (Y).To detect the effect of major key factors responsible for keratinase production, each factor in the design was considered at five different levels (-α, -1, 0, +1, +α) in CCD. The minimum and maximum range of

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variables were investigated.

All the thirty runs of experiments were conducted in duplicate, and production of keratinase enzyme (Y) was recorded for all the experiments. A second order polynomial equation was then fitted to the data by using multiple regressions. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

Results and Discussion

Effect of carbon source on keratinase production : Maximum production of keratinase was obtained when glucose was used as carbon source, as shown in Fig. 1 and minimum production was observed in sucrose, making all other variables constant.

Effect of nitrogen source on keratinase production : Maximum keratinase production was obtained when casein was used as nitrogen source as shown in Fig. 2 and minimum production was observed in ammonium sulphate, making all other variables constant.

Effect of pH on enzyme activity : Maximum keratinolytic activity was obtained at pH 10 as shown in table 1,which indicates that alkaline condition was suitable for enzyme production. The leather and detergent industries mostly use enzyme which has maximum enzyme activity at alkaline condition. Thus, keritinase enzyme is used for serval purposes in these industries (Anapaula *et al.*, 2010)

Optimization of medium using Plackett-Burman method: The effect of each parameter on keratinase production was calculated as difference between the average measurement calculated at higher (+) and lower (-1) levels of parameters. The results showed that glucose, casein, pH and $MgSO_4$ had a positive effect on keratinase production as shown in Table 2. On the other hand K_2HPO_4, inoculum size had negative effect on the activity of keratinase.

Table 1 : Effect of pH on enzyme activity

pH	Enzyme activity ($U\ ml^{-1}$)
4	No Activity
7	No Activity
10	0.85

Optimization of medium using Response surface Methodology : Using FCCCD, thirty experiments were carried out with various combinations of four independent variables and corresponding responses of keratinase activity as shown in Table 3. The three-dimensional response of surface graphs are shown in Fig. 3. These graphs were plotted to determine the interaction effects among the variables. The main effect is present when different levels of a factor affect the characteristic differently. Minitab creates the main effects plot by plotting the characteristic average for each factor level (Prafulla *et a*l., 2012). Maximum keratinase activity was found to be 326 Uml^{-1}.

The data were fitted with the second-order non-linear polynomial equation represented by equation in terms of actual values of independent variables:

$$R1=126.50000+ 6.20833*GLUCOSE-30.45833*CASEIN+8.87500*MgSO_4+7.37500*pH+30.06250*GLUCOSE*CASEIN-12.43750*GLUCOSE*MgSO_4+3.18750*GLUCOSE*pH-27.56250*CASEIN*MgSO_4+15.06250*CASEIN*pH-20.93750*MgSO_4*pH+9.32292*GLUCOSE^2+10.44792*CASEIN^2+10.94792*MgSO_4^2+33.82292*pH^2$$

In Table 4, the model F- value of 8059.59 implied that the model was significant. Through F-test and probability p-value shown in analysis of variance (ANOVA) table, significance of each variable, interaction and quadratic term

Table 2 : Optimization of medium using Placket-Burman method

Run order	Glucose	Casein	K_2HPO_4	$MgSO_4$	pH	Rpm	Innoculum	Yield
1	1	1	-1	1	1	-1	1	398
2	-1	1	-1	-1	-1	1	1	224
3	1	-1	1	1	-1	1	-1	213
4	1	-1	-1	-1	1	1	1	101
5	-1	-1	-1	1	1	1	-1	78
6	-1	1	1	1	-1	1	1	282
7	-1	-1	-1	-1	-1	-1	-1	43
8	1	-1	1	-1	-1	-1	1	170
9	-1	1	1	-1	1	-1	-1	16
10	1	1	-1	1	-1	-1	-1	431
11	-1	-1	1	1	1	-1	1	6
12	1	1	1	-1	1	1	-1	65

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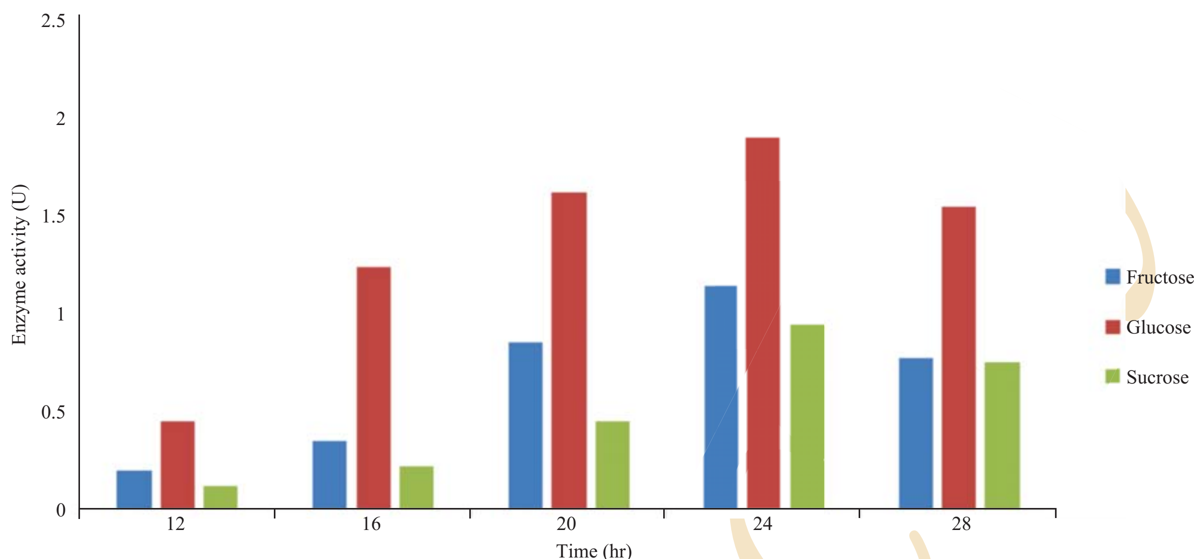

Fig. 1 : Effect of carbon source on keratinase production

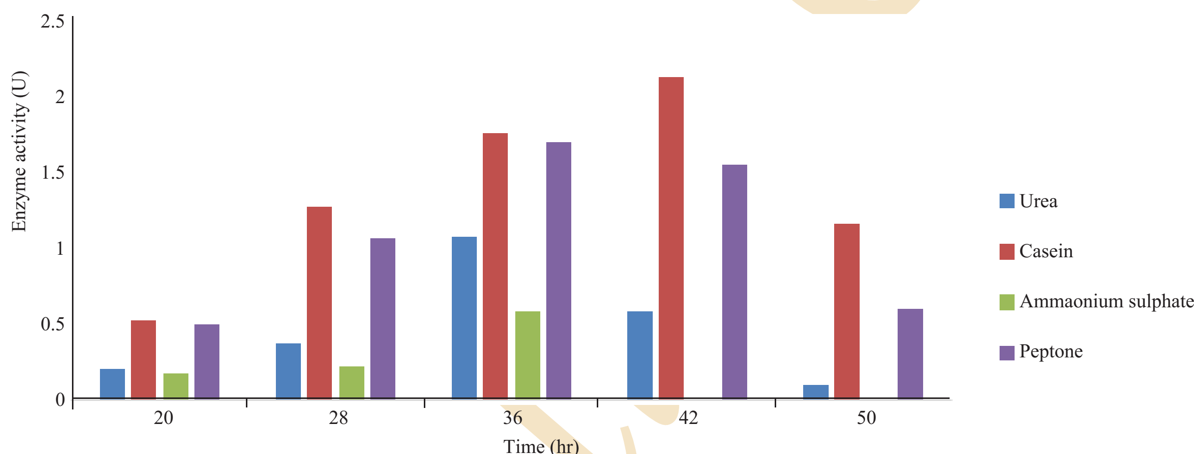

Fig. 2 : Effect of nitrogen source on keratinase production

was determined. Response surface plot is a graphical representation of the regression equation from which the response i.e., keratinase enzyme production was plotted agaist any two variable while other variables were fixed at their middle level.(Nahed *et al*., 2010). Model F-value was large due to noise. Value of Prob> F less than 0.0500 indicated that model terms were significant and was good fit. In the present study, from ANOVA table A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2, D^2 were found to be significant model terms from p-value. If the values were greater than 0.1, it indicated that the model terms were not significant. If there were many insignificant model terms (not counting those required to support hierarchy), model reduction might improve the model. The "Lack of Fit F-value" of 0.70 implied that the Lack of Fit was not significant relative to pure error. There was 70.25% chance that a "Lack of Fit F-Value" could occur due to noise. Non-significant lack of fit was good. Std. Dev-0.94, R-Squared-0.9999, Mean-178.13, Adj R-Squared-0.9997, C.V. %-0.53, Pred R-Squared-0.9995, PRESS-52.56, Adeq Precision-354.610. The "Pred R-Squared" of 0.9995 is reasonable agreement with the "Adj R-Squared" of 0.9997. "Adeq Precision" measures the signal to noise ratio and was found to be greater than 4, which was desirable which indicated an adequate signal (Singh *et al.,* 2015). From this study, this model could be used to navigate the design space. The relationship between keratinase activity and independent variables was described by second order polynomial model, based on

Table 3 : Optimization of medium using Response Surface Methodology

Run	A:GLUCOSE	B:CASEIN	C:$MgSO_4$	D:pH	Response
1	1.00	1.00	1.00	1.00	171
2	-2.00	0.00	0.00	0.00	152
3	0.00	0.00	0.00	0.00	128
4	1.00	1.00	-1.00	-1.00	182
5	0.00	0.00	0.00	2.00	277
6	1.00	1.00	-1.00	1.00	275
7	-1.00	-1.00	1.00	1.00	262
8	-1.00	-1.00	-1.00	-1.00	186
9	0.00	0.00	0.00	-2.00	246
10	0.00	0.00	-2.00	0.00	152
11	2.00	0.00	0.00	0.00	175
12	0.00	0.00	2.00	0.00	188
13	0.00	0.00	0.00	0.00	125
14	1.00	-1.00	-1.00	-1.00	158
15	-1.00	1.00	1.00	1.00	116
16-	1.00	-1.00	-1.00	1.00	190
17	0.00	0.00	0.00	0.00	127
18	1.00	-1.00	1.00	-1.00	248
19	1.00	1.00	1.00	-1.00	161
20	-1.00	-1.00	1.00	-1.00	326
21	0.00	2.00	0.00	0.00	107
22	-1.00	1.00	-1.00	-1.00	91
23	-1.00	1.00	1.00	-1.00	120
24	0.00	-2.00	0.00	0.00	229
25	0.00	0.00	0.00	0.00	126
26	1.00	-1.00	1.00	1.00	196
27	0.00	0.00	0.00	0.00	127
28	-1.00	1.00	-1.00	1.00	170
29	-1.00	-1.00	-1.00	1.00	207
30	0.00	0.00	0.00	0.00	126

Table 4 : Analysis of variance

Source	Sum of squares	df square	Mean value	F Prob>F	p value
Model	99670.22	14	7119.30	8059.59	<0.0001 s
A-GLUCOSE	925.04	1	925.04	1047.22	<0.0001
B-CASEIN	22265.04	1	22265.04	25205.71	<0.0001
C-MgSO4	1890.38	1	1890.38	2140.05	<0.0001
D-pH	1305.38	1	1305.38	1477.78	<0.0001
AB	14460.06	1	14460.06	16369.88	<0.0001
AC	2475.06	1	2475.06	2801.96	<0.0001
AD	162.56	1	162.56	184.03	<0.0001
BC	12155.06	1	12155.06	13760.45	<0.0001
BD	3630.06	1	3630.06	4109.50	<0.0001
CD	7014.06	1	7014.06	7940.45	<0.0001
A2	2384.00	1	2384.00	2698.87	<0.0001
B2	2994.07	1	2994.07	3389.52	<0.0001
C2	3287.50	1	3287.50	3721.70	<0.0001
D2	31378.00	1	31378.00	35522.27	<0.0001
Residual	13.25	15	0.88		
Lack of Fit	7.75	10	0.78	0.700.7025	ns
Pure Error	5.50	5	1.10		
Cor Total	99683.47	29			

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regression analysis (Kaushlesh *et al.*, 2015).

Bacillus cereus KM209198 isolated from feather dumped soil exhibited complete degradation of feather after 3 days of incubation at 150 rpm. The maximum keratinase was produced when glucose and casein were used as carbon and nitrogen sources. Maximum activity was obtained at pH 10 at 40° C. Glucose, casein, $MgSO_4$ and pH was screened as significant variables by Plackett-Burman. The optimized media concentration was identified as follows: glucose 2.5 g l^{-1}, casein 7.5 g l^{-1}, $MgSO_4$ 2 g l^{-1} and pH 10. Initially, enzyme activity without optimization was 2.2 U ml^{-1} and after optimization the enzyme activity increased upto 3.5 U ml^{-1}. Response Surface Methodology is an efficient tool to handle a large number of media and process parameters to maximize the keratinase yield. To conclude, the modest attempt was made and these statistical tools might be used for media optimization.

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