

Original Article

Optimization of Medium Ingredients for Keratinolytic Protease Production by *Bacillus licheniformis* MZK-03 using Statistical Experimental Designs

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A culture medium was optimized for the production of keratinolytic protease by a newly isolated strain of *Bacillus licheniformis* MZK-03 in shake-flask culture. Based on the results of preliminary experiments, feather mill, molasses and trace elements were found to be major variables in keratinolytic protease production. The concentrations of these ingredients were optimized by using two statistical approaches, namely Box-Wilson method and central composite design. The optimized culture medium, finally determined by using the statistical approaches, composed of 0.95% feather mill, 0.12% molasses and 1.44% trace elements. The keratinolytic protease production was increased by approximately 2-fold when the strain was grown in the optimized medium (95.2 U/ml) compared to the un-optimized medium (56.05 U/ml).

Keywords: Keratinolytic protease, Optimization, *Bacillus licheniformis* MZK-03, Statistical designs

Introduction

Keratins are the most abundant proteins in epithelial cells of vertebrates and represent the major constituents of skin and its appendages such as nail, hair, feather, and wool¹. Keratins are fibrous and insoluble structural proteins extensively cross-linked with disulphide, hydrophobic, and hydrogen bonds²⁻³. Their high degree of cross-linking by disulfide bonds, hydrophobic interactions, and hydrogen bonds stabilizes keratin filament structure¹. Therefore, keratinous material is water insoluble and extremely resistant to degradation by proteolytic enzymes such as trypsin, pepsin and papain¹⁻².

A group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases are called keratinases². They are produced by some insects and mostly by microorganisms. The best studied are keratinases from the dermatophytic genera *Microsporum*⁴ and *Trichophyton*⁵ as well as from bacteria of the genera *Bacillus*⁶⁻¹¹ and *Streptomyces*¹². Bacterial keratinases are of particular interest because of they act on insoluble keratins with a broad range of protein substrates¹³. These enzymes have been studied for dehairing processes in the leather industry¹⁴ and hydrolysis of feather keratin¹³, which is a by-product generated in huge amounts by the poultry industry. Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed^{1,15-16}. In addition, keratin hydrolysates have potential use as organic fertilizers, production of edible films and rare amino acids¹⁷⁻¹⁸.

Some of the important strains *Bacillus licheniformis* are used as a tool in industrial biotechnology to manufacture enzymes, antibiotics, biochemicals and consumer products^{6-7,13}. All of them

exhibit keratinolytic activities by extracellular enzymes. A strain of *Bacillus licheniformis* MZK-03 has been previously isolated from Bangladesh¹⁹. It has been found to secrete a substantial amount of keratinolytic protease in the medium¹⁹, which can degrade the feather consisting mainly of β -keratin. Therefore, it has a promising future as source of keratinolytic proteases for use in poultry and leather industry of Bangladesh.

Cultivation conditions are essential in successful production of an enzyme, and optimization of parameters such as pH, temperature and media composition are important in developing the cultivation process. Despite all the work that has been done on production of proteolytic enzymes, relatively little information is available on keratinases²⁰. Optimization of medium by the classical method involves changing one independent variable while maintaining all others at a fixed level is extremely time consuming and expensive when a large number of variables are evaluated. To overcome this difficulty, experimental factorial design and response surface methodology can be employed to optimize the medium components. The statistical approaches like Box-Wilson method and central composite design have been previously used by several researchers for media ingredient optimization²¹⁻²². This paper reports on the optimization of some medium ingredient using the statistical techniques for production of keratinase by *Bacillus licheniformis* MZK-03.

Materials and Methods

Microorganisms and maintenances

The microorganism used was *Bacillus licheniformis* MZK-03. This organism was previously isolated from feather-decomposed soil collected from a local poultry farm in Dhaka, Bangladesh and

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identified using 16S rRNA typing by the International Centre for Biotechnology, Osaka University, Japan¹⁹. Stock culture of the organism was maintained at -70°C in nutrient broth containing 10% glycerol.

Enzyme production

A single colony from the agar plate was aseptically transferred to 50 ml nutrient broth in 250-ml Erlenmeyer flask. The flask was incubated for 7-8 h at 37°C and 200 rpm in an orbital shaker until it reached to an absorbance of 0.5-0.8 at 600 nm. To induce enzyme production 5 ml of the seed culture was transferred to 95 ml of feather degrading enzyme-producing broth in a 500-ml Erlenmeyer flask. The feather degrading enzyme-producing broth contained 0.75 g/l NaCl, 0.09 g/l CaCl₂, 1.50 g/l MgCl₂·6H₂O, 1.05 g/l KH₂PO₄ and 2.10 g/l K₂HPO₄ supplemented with 1% feather mill corresponding to 10 g/l (initial pH 7.5). The inoculated flasks were incubated at 37°C at 150 rpm in an orbital shaker unless otherwise stated. Crude enzyme was collected after 42 h of incubation by centrifuging at 5,000 rpm for 10 min. The supernatant was preserved at 4°C and assayed for enzymes and protein (in duplicate).

Protein and enzyme assay

Soluble protein in the culture supernatant was estimated according to the Bradford method using bovine serum albumin (BSA) as standard²³. Keratinolytic protease activity was determined by azocasein (Sigma, USA) hydrolysis method²⁴ with some modifications and the method has been described previously²⁵. One unit of enzyme activity was defined as an increase of 0.01 absorbance unit per min at 440 nm under the assay conditions.

Experimental design

Based on the results obtained in preliminary experiments, feather mill, molasses and trace element solution were found to be major variables in the keratinolytic protease production. Hence, these variables were selected to find the optimized conditions for higher keratinolytic protease production using two well known statistical methods namely, Box-Wilson Method²⁶ and central composite design²⁷⁻²⁹.

According to the Box-Wilson Experimental method if the proportions of three ingredients x_1 , x_2 and x_3 are to be optimized and y is the yield, then y can be related to the three ingredients as follows:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 \dots \dots$$

b represents the regression coefficients. If yield is taken to be the surface of a mountain, the method explores a small part of the yield surface, in which the linear function equation, $y = b_0 + b_1x_1 + b_2x_2 + b_3x_3$, is calculated. Computer software Statistica edition 99 (StatSoft Inc) was used to map contours of the whole yield surface.

In the first experiment of the Box-Wilson Method, which begins at some distance from the yield summit, the regression coefficient b was determined or the direction of the steepest slope to the summit was established. The goal was to optimize a medium containing feather mill, molasses and trace elements. In the first step a factorial experiment is designed in which two levels of feather mill, molasses

and trace elements are used. Both levels of each were run in combination with all the others. The levels chosen varied the same amount above and below a chosen mean. The results from this experiment indicated how much the concentrations of each ingredient should be increased or decreased proportionally in the next experiment. In the second experiment, by changing the levels of the medium ingredients in accordance with the obtained regression coefficient values the 'ascent' was actually made. Finally, the medium was optimized by using a central composite design, which is basically a two factor experimental design where each factor is studied at three levels. After completion of the experiment, the optimum concentration of each nutrient was calculated using defined statistical equations.

Results and Discussion

Keratinolytic proteases are found frequently in fungi (*Trychophyton mentagrophytes*, *Trychophyton rubrum*)³⁰, actinomycetes (*Streptomyces* spp.) and bacteria (*Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus*, *Brevibacillus borstelensis*)³¹. Keratinolytic protease production by *Bacillus licheniformis* was carried out in a feather mill medium containing feather mill, molasses and trace elements (NaCl, CaCl₂, MgCl₂·6H₂O, K₂HPO₄, and KH₂PO₄). Preliminary experiments demonstrated the best effects at 1% feather mill, 1.5% trace element, and 0.10% molasses. This was the base level ingredient concentration used for our experiment.

The concentrations of the ingredients like feather mill, trace element and molasses were then optimized using Box-Wilson method³². This method is a factorial design which consists of two sets of experiments. The first step (Table 1) consists of running two steps of each factor in combination with all others. Total eight combinations were possible. The levels chosen were varied in same amount above or below the base levels. The upper limit has an assigned value of +1 and lower limit has a value of -1.

Table 1. Experimental design for optimization of ingredient concentrations for keratinolytic protease production by *Bacillus licheniformis* MZK-03 using Box-Wilson method and the results obtained

Ingredient	Experimental design			Results obtained	
	Feather mill (%)	Trace element (%)	Molasses (%)	Keratinolytic protease (U/ml)	Final pH
Lower level	0.75	1	0.05		
Upper level	1.25	2	0.15		
Base level	1	1.5	0.10		
Coding unit (±)	0.25	0.50	0.05		
Medium No. 1	0.75 (-)	1 (-)	0.05 (-)	57.50	8.75
2	0.75 (-)	2 (+)	0.05 (-)	48.20	8.22
3	0.75 (-)	1 (-)	0.15 (+)	45.00	8.72
4	0.75 (-)	2 (+)	0.15 (+)	49.50	8.27
5	1.25 (+)	1 (-)	0.05 (-)	47.00	9.00
6	1.25 (+)	2 (+)	0.05 (-)	46.50	8.53
7	1.25 (+)	1 (-)	0.15 (+)	54.70	8.79
8	1.25 (+)	2 (+)	0.15 (+)	50.00	8.58

The base levels and coding units were selected based on the results obtained in preliminary experiments carried out before. Only keratinolytic protease yields were considered for the determination of slope *b* or regression coefficient. The calculated values gave an indication of increasing molasses concentration by 0.02%, and decreasing feather mill and trace element by 0.1 % and 0.3 % respectively from the corresponding base level.

The second experiment to obtain the ‘ascent’ yielded results in accordance with Table 2. The yield plateau with respect to keratinolytic protease was reached at medium No. 2 containing 0.9% feather mill, 0.12% molasses and 1.2% trace element. The enzyme activity in this medium was 58.55 U/ml. The results obtained in this experiment indicated a gradual decrease of keratinolytic protease production with progressively decreasing feather mill and trace element, and increasing molasses concentration. This could be due to low mineral element concentration and increasing reducing sugar level²⁷ from molasses.

Table 2. Second experimental design of Box-Wilson method to obtain the ‘ascent’ and the results obtained

Parameter	Experimental design			Results obtained		
	Feather mill (%)	Trace element (%)	Molasses (%)	Keratinolytic protease (U/ml)	Final pH	
Medium No.	1	1.0	1.5	0.10	57.85	7.65
	2	0.9	1.2	0.12	58.55	7.61
	3	0.8	0.9	0.14	56.75	7.73
	4	0.7	0.6	0.16	53.65	7.77
	5	0.6	0.3	0.18	47.15	7.63
	6	0.5	0.0	0.20	32.75	7.63
	7	0.4	0.0	0.22	25.60	7.47
	8	0.3	0.0	0.24	20.80	7.23

It was evident that feather mill and trace element were the most important factors for keratinolytic protease production. So the optimal concentrations of these two major components were optimized using a 3² central composite design²⁷⁻²⁹. The results are given in Table 3. The optimum concentration of each nutrient was calculated using the following second order equations:

$$x_1 = \frac{b_2b_{12} - 2b_1b_{22}}{4b_{11}b_{22} - b_{12}^2}$$

and

$$x_2 = \frac{b_2b_{12} - 2b_2b_{11}}{4b_{11}b_{22} - b_{12}^2}$$

where, x_1 and x_2 are variables, namely feather mill and trace element solution, respectively, b_0 = constant, b_1 and b_2 = linear coefficients, b_{12} = cross product coefficient, b_{11} and b_{22} = quadric coefficients. The response surface diagram (Figure 1) generated from these statistical analyses was used to calculate the most appropriate concentrations of feather mill and trace element.

Table 3. Experimental design for optimization of feather mill and trace element concentration for keratinolytic protease production using 3² central composite design and the results obtained

Parameter obtained	Variable (%)		Results	
	Feather mill (x_1)	Trace element (x_2)	Keratinolytic protease (U/ml)	Final pH
Lower level	0.8	0.9		
Upper level	1	1.5		
Central level	0.9	1.2		
Medium No. 1	1 (+)	1.5 (+)	56.05	8.05
2	1 (+)	1.2 (0)	56.10	8.18
3	1 (+)	0.9 (-)	53.10	8.26
4	0.9 (0)	1.5 (+)	54.80	8.00
5	0.9 (0)	1.2 (0)	56.00	8.02
6	0.9 (0)	0.9 (-)	54.35	8.31
7	0.8 (-)	1.5 (+)	59.10	7.99
8	0.8 (-)	1.2 (0)	56.80	8.18
9	0.8 (-)	0.9 (-)	56.45	8.19

The x_1 and x_2 values were determined according to following equations:

$$x_1 = \frac{b_2b_{12} - 2b_1b_{22}}{4b_{11}b_{22} - b_{12}^2} = \frac{(1.008)(0.075) - 2(-1.183)(-0.643)}{4(1.2335)(-0.643) - 0.075} = 0.445 \text{ (approximately)}$$

Variable: Feather mill (x_1)

The calculated feather mill concentration:

Base level + variation from base level $\times x_1$ value = $0.9 + 0.1 \times 0.445 = 0.9445$ H” 0.95 % (approximately)

$$x_2 = \frac{b_2b_{12} - 2b_2b_{11}}{4b_{11}b_{22} - b_{12}^2} = \frac{(-1.183)(0.075) - 2(-1.008)(1.2335)}{4(1.2335)(-0.643) - 0.075} = 0.79 \text{ (approximately)}$$

Variable: Trace element (x_2)

The calculated trace element concentration: Base level + variation from base level $\times x_2$ value = $1.2 + 0.3 \times 0.79 = 1.4379$ H” 1.44 % (approximately).

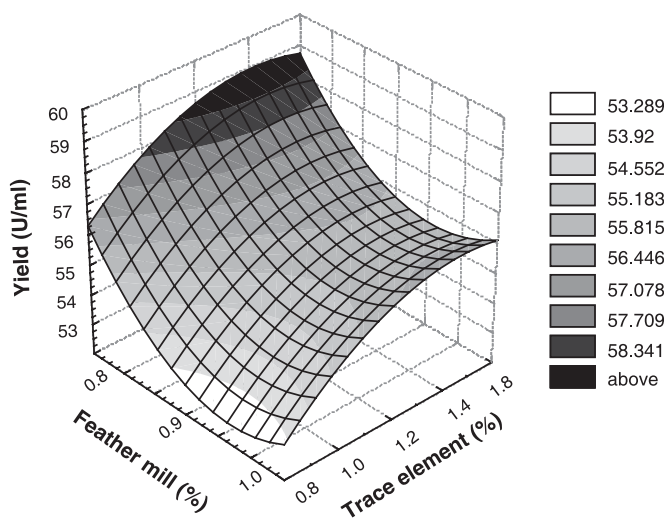


Figure 1. 3-D graphical representation of the response surface for enzyme yield generated by the central composite design.

The optimum response analyzed by response surface statistical analysis was identified as 0.95% feather mill and 1.44% trace element, which corresponded to keratinolytic protease activity of 95.2 U/ml (average value obtained from five separate cultures). The base level medium showed 56.05 U/ml of enzyme activity. So the enzyme activity in the optimized medium was remarkably higher than that in base level medium. The optimized medium thus developed contained only few cheap components as feather mill (0.95%), molasses (0.12%), NaCl (0.072%), CaCl₂ (0.086%), MgCl₂·6H₂O (0.0144%) and KH₂PO₄ + K₂HPO₄ (1:2 ratio, 0.10%). Thus, the medium seems economically feasible for large-scale production of keratinolytic protease by *Bacillus licheniformis* MZK-03.

Keratinolytic protease is a promising enzyme in several industrial sectors. At poultry processing plants, feathers are produced in huge amounts reaching millions of tons per year worldwide³³. Feathers, mainly constituted of pure keratin are utilized on a limited extent as dietary protein supplement for animal feed because feather mill production is an expensive chemical process requiring significant amount of energy. It also results in a product of poor digestibility and variable nutrient quality³⁴. Microorganism-derived keratinolytic protease can be a suitable alternative for carrying out feather mill production. On the other hand, keratinolytic protease produced by microorganisms can reduce the cost and complexity of poultry waste and effluent treatment, amino acid production, bating and dehairing in leather industry.

It is apparent from this study that *Bacillus licheniformis* MZK-03 is a potent producer of keratinolytic enzyme while grown on readily available and cheap substrate in submerged cultivations. Optimization of the medium by two sophisticated statistical approaches certainly increases the efficiency of enzyme production. This experiment finely identified the optimum requirements and fulfilled the requirements of stringent optimization of keratinolytic production medium. The findings of

this experiment could significantly contribute to biotechnological application of microorganism derived keratinolytic protease as a widely used enzyme.

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