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ORIGINAL PAPER

Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology

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Abstract Culture medium for keratinase production from hair substrate by a new *Bacillus subtilis* strain, KD-N2, was optimized. Effects of culture conditions on keratinase production were tested, and optimal results were obtained with 10% inocula (v/v), 16 g/L hair substrate, an initial pH value of 6.5 and a culture volume of 20 mL. Several carbon sources (sucrose, cornflour) and nitrogen sources (yeast extract, tryptone and peptone) had positive effects on keratinase production, with sucrose giving optimal results. To improve keratinase yield, statistically based experimental designs were applied to optimize the culture medium. Fractional factorial design (FFD) experiments showed that $MgSO_4$ and K_2HPO_4 were the most significant factors affecting keratinase production. Further central composite design (CCD) experiments indicated that the optimal $MgSO_4$ and K_2HPO_4 concentrations were 0.91 and 2.38 g/L, respectively. Using an optimized fermentation medium (g/L: NaCl 1.0, $CaCl_2$ 0.05, KH_2PO_4 0.7, sucrose 3, $MgSO_4$ 0.91, K_2HPO_4 2.38), keratinase activity increased to 125 U/mL, an approximate 1.7-fold increase over the previous activity (75 U/mL). Human hair was degraded during the submerged cultivation.

Keywords Human hair · Keratinase · Optimization · Response surface methodology

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Introduction

Keratins cannot be degraded solely by common proteolytic enzymes such as pepsin, trypsin and papain. Feathers, which consist of over 90% keratin, do not accumulate in nature because keratins can be degraded by keratinases [EC 3.4.21/24/99.11] produced by some microorganisms [1] including bacilli [2–4], fungi [5–7] and actinomycetes [8, 9]. On the other hand, feathers have not been utilized economically and can lead to environmental pollution and feather protein wastes [1, 10]. Traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking not only destroy the amino acids but also consume large amounts of energy. Biodegradation of feathers by keratinase from microorganisms may provide a viable alternative. The ability of microbes to degrade keratin, and keratinase production levels, vary according to species, keratin substrates and culture conditions. Substrates utilized in keratinase activity determination including white guinea pig hair [11], stratum corneum [12], azo-keratin [13] and keratin azure [14], etc., and the ways in which enzyme activity is documented varies based on the substrate selected. Thus it is difficult to compare keratinase activities reported in the literature. In addition, microbes degrade keratin substrates to different degrees during cultivation. For example, *Bacillus licheniformis* RG1 isolated from compost degrades chicken feathers completely in 24 h [15]; *Fervidobacterium islandicum* AW-1 [16] and *Fervidobacterium pennavorans* [17] degraded feathers in 48 h; *Bacillus licheniformis* PWD-1 in 10 days [2]; and *Bacillus subtilis* KD-N2 in the present study required 36 h [18]. The time required for a bacterium to degrade keratins completely depending on the culture conditions may be an indicator of the utilization potential of that keratinase.

Keratinases from microorganisms have many applications in the feed, fertilizer, detergent, leather and

pharmaceutical industries [19]. For example, feather hydrolysates of *B. licheniformis* PWD-1 and *Vibrio* sp. strain kr2 [20, 21] can be used as animal food, keratinase from *B. licheniformis* PWD-1 can degrade the infectious form of prion, PrP^{sc}, in the presence of detergents and heat treatment [22]. In the leather industry, keratinase from *B. subtilis* S14 exhibits remarkable dehairing capabilities [23] without the degradation of collagen; this ecofriendly dehairing approach shows great utilization potential. Thus it is important and necessary to improve keratinase yields for such applications.

Several methods, including optimization of culture conditions and medium composition, have been applied to improve enzyme yield [15, 24]. Optimization of culture medium by response surface methodology is an effective method widely utilized in microbial enzyme production [25–27]. Almost all keratinases are inducible, and different keratin-containing materials such as feathers, hair and wool can be used as substrates for keratinase production [19]. Among keratin-containing materials, feathers have been most often utilized; human hair, although abundant, has been used only rarely, except for the utilization of keratin from human hair in keratinase production by *Stenotrophomonas* sp. D-1 [28].

Our laboratory have screened a new keratin-degrading *Bacillus subtilis*, KD-N2. This isolate is able to degrade feathers, human hair and other keratin-containing materials [18]. The crude keratinase produced using feathers and human hair as substrate showed dehairing capability and application potential [29]. The aim of this study was to optimize the culture medium for keratinase production by the new isolate with human hair as substrate.

Materials and methods

Bacteria

Strain KD-N2 was a *Bacillus subtilis* screened from a local poultry plant and kept in our laboratory [18].

Culture media and growth conditions

Luria–Bertani (LB) medium [peptone 1% (w/v), yeast extract 0.3% (w/v), NaCl 0.5% (w/v), pH 7.2] was used for inoculum preparation, with the addition of 20 g agar for isolate maintenance.

The basal medium used for keratinase production contained the following constituents (g/L): NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), $MgSO_4$ (0.1), $CaCl_2$ (0.02) and human hair (10), pH 7.0. Cultivation was performed in 250 mL Erlenmeyer flasks containing 50 mL medium for 36 h at 28°C with constant shaking (200 rpm). As inocula, 5% bacteria grown in LB broth for 16 h was used. Culture supernatants obtained after centrifugation (8,000 *g* for 20 min) were used to assay keratinase activity.

Human hair was obtained from a local barbershop, washed with tap water twice and distilled water once, then dried at 60°C to constant weight and cut into 0.5–1 cm lengths.

Effect of substrate concentration on keratinase production

Concentrations of 2, 6, 10 and 16 g/L human hair were selected as sole sources of carbon and nitrogen for keratinase production. Cultivation was performed at 200 rpm and 28°C for 36 h.

Effect of culture conditions on keratinase production

For optimization, keratinase production by KD-N2 was studied using 10 g/L human hair substrate under the following conditions: initial pH value 6.0–7.5 with increments of 0.5 units, inoculum size 2–10% (v/v) of cells cultivated for 20 h, culture volumes of 20, 30, 40, 50 mL with 10 g/L substrate. Erlenmeyer flasks (250 mL) containing 50 mL culture medium were incubated at 28°C and 200 rpm for 36 h.

Effects of extra sources of carbon and nitrogen on keratinase production

Addition of extra carbon and nitrogen sources may have a stimulatory effect on product formation. How much of these should be supplemented depends on the availability of carbon and nitrogen from the substrate and the requirements of the organism. In this study, the effects of extra-addition of 0.5 g carbon (maltose, corn flour, glucose, sucrose) or nitrogen (urea, tryptone, ammonium chloride, beef extract, yeast extract, peptone) sources were studied in the basic medium defined above. All chemicals used in this investigation were of analytical reagent grade and were purchased from local chemical suppliers in China.

Keratinase activity determination

Keratinase activity was measured using soluble keratin (0.5%, w/v) as substrate. Soluble keratin was prepared from white chicken feathers by the method of Wawrzkiewicz et al. [30]. Native chicken feathers (10 g) in 500 mL dimethyl sulfoxide were heated in a reflux condenser at 100°C for 2 h. Soluble keratin was then precipitated by addition of cold acetone (1 L) at −70°C for 2 h, followed by centrifugation at 10,000 *g* for 10 min. The resulting precipitate was washed twice with distilled water

and dried at 40°C in a vacuum dryer. Quantified precipitate (1 g) was dissolved in 20 mL 0.05 mol/L NaOH. The pH was adjusted to 8.0 with 0.1 mol/L Tris and 0.1 mol/L HCl and the solution was diluted to 200 mL with 0.05 mol/L Tris–HCl buffer (pH 8.0).

Keratinase activity was assayed as follows: 1.0 mL crude enzyme properly diluted in Tris–HCl buffer (0.05 mol/L, pH 8.0) was incubated with 1 mL keratin solution at 50°C in a water bath for 10 min, and the reaction was stopped by adding 2.0 mL 0.4 mol/L trichloroacetic acid (TCA). After centrifugation at 1,450 *g* for 30 min, the absorbance of the supernatant was determined at 280 nm (UV-2102, UNICO Shanghai, China) against a control. The control was prepared by incubating the enzyme solution with 2.0 mL trichloroacetic acid (TCA) at 50°C for 10 min before the addition of keratin solution. One unit (U/mL) of keratinase activity was defined as an increase of corrected absorbance at 280 nm (A_{280}) [31] of 0.01 per minute under the conditions described [18].

Experimental design and statistical analysis

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems, in which a response of interest is influenced by several variables [32] and the objective is to optimize this response. To find the optimal cultivation conditions for keratinase production in submerged cultures, the key medium factors affecting keratinase production should first be determined using fractional factorial design (FFD), and then optimized by RSM design. Table 1 shows the ranges of variables of the medium for FFD during keratinase production. The correspondence between these values can be obtained from Eq. 1

$$x_i = \frac{X_i - X_{i0}}{\delta_i} \quad (1)$$

where x_i is the coded value, X_i the corresponding natural value, X_{i0} the natural value in the center of the domain, and δ_i the increment of X_i corresponding to one unit of x_i.

Table 1 Factors and levels (g/L) in the culture medium for fractional factorial design (FFD)

Code value	Factor	−1	0	1
X_1	NaCl	0.2	1.0	1.8
X_2	$MgSO_4$	0.1	0.3	0.5
X_3	$CaCl_2$	0	0.05	0.1
X_4	KH_2PO_4	0.1	0.4	0.7
X_5	K_2HPO_4	0.2	0.8	1.4
X_6	Sucrose	1	3	5

Maximal keratinase production was investigated using a central composite design (CCD) with two variables [24, 33]. Each factor in the design was studied at five different levels ($-\alpha$, −1, 0, +1, $+\alpha$) (Table 2). Based on the FFD design, the processing variables of $MgSO_4$ (X_1) and K_2HPO_4 (X_2) were chosen for the CCD experiments.

As shown in Table 3, a set of 13 experiments was carried out. All variables were taken at a central coded value set at zero. The minimum and maximum ranges of the variables and the full experimental plan with respect to their values in coded forms are also listed in Table 3. Upon completion of the experiments, keratinase activity was taken as the response (Y). A second-order polynomial equation was then fitted to the data by a multiple regression procedure. The equation resulted in an empirical model that relates the measured response to the independent variables of the experiments. When several factors are involved, the model is expressed as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2 \quad (2)$$

where Y is the predicted response (keratinase activity); X_1 and X_2 the coded forms of the input variables ($MgSO_4$ and K_2HPO_4, respectively); b_0 a constant; b_1 and b_2 the linear coefficients; b_{12} a cross-product coefficient; b_{11} and b_{22} the quadratic coefficients.

Keratinase production data were subjected to analysis of variance (ANOVA) using Expert-Design software to estimate t values, P values and confidence levels. Optimal values of keratinase were estimated using the solver function of Expert-Design software.

Results and discussion

Effects of substrates on keratinase production

Different keratin-containing materials, e.g., feather, human hair and wool, can be used as substrates for keratinase production by *Bacillus subtilis* KD-N2. The optimal concentration of feather has been determined, but that of hair has not [18]. In this experiment, human hair at 2, 6, 10, 16 g/L was tested and the results showed that, with the increase in hair concentration, the production of keratinase increased with maximum production at 16 g/L among the concentrations tested. There was no significant difference

Table 2 Factors and levels in central composite design (CCD)

Factor	Description	Level (g/L)				
		−1.4142	−1	0	1	1.4142
X_1	$MgSO_4$	0.16	0.4	1	1.6	1.84
X_2	K_2HPO_4	0.09	0.5	1.5	2.5	2.91

Table 3 Experimental and predicted results of central composite design (CCD) for keratinase production

Experiment	X_1	X_2	Experimental results	Predicted results
1	1.41	0.00	111.4	104.07
2	0.00	0.00	122.5	126.3
3	0.00	1.41	132.7	123.17
4	0.00	0.00	110.8	116.62
5	1.00	−1.00	68	68.68
6	−1.00	1.00	122.5	126.3
7	1.00	1.00	105.7	116.7
8	−1.00	−1.00	60.2	53.68
9	0.00	0.00	124.1	116.62
10	0.00	0.00	125.1	116.62
11	0.00	−1.41	32.8	37.86
12	0.00	0.00	100.6	116.62
13	−1.41	0.00	97.4	100.25

in the results between concentrations of 6 and 10 g/L (Fig. 1). Using feather as substrate, a concentration of 10 g/L was optimum for keratinase production [18]. Considering the ratio of medium to substrate, and the fact that little human hair was degraded during cultivation, 10 g/L human hair was selected as the substrate for further studies.

Effect of culture conditions on keratinase production

The optimal temperature and age of inoculum were found to be 28°C and 16 h, respectively, in previous studies using feather substrate [18], and were the same for the human hair substrate used here (data not shown), which may be due to the growth characteristics of the isolate. The effects of initial pH, inoculum size and medium volume on keratinase production were further investigated in the context of using human hair substrate.

The optimal initial pH value for keratinase production was 6.5 and the medium pH increased to a relatively steady level of about 7.0 during cultivation; less keratinase was produced when the initial pH value was less than 6.0 or greater than 7.5 (Fig. 2). The production of keratinase may be affected by the growth of seed in the medium during cultivation. For feather substrate, the optimum initial pH value was 7.5 and the medium pH increased to a relatively stable level of about 8.5 [18]; more ammonia and alkaline compounds may be produced in the feather substrate.

Inoculum size is an important factor affecting cell growth and product formation. An inoculum size of 10% (v/v) was optimal for keratinase production in hair substrate, followed by 8% (v/v), but the difference between the two concentrations was not significant. Concentrations of 2% and 5% gave lower yields (Fig. 3). The amount of keratinase produced increased with inoculum size with human hair substrate. In contrast, for feather substrate, keratinase yield decreased with inoculum size, with the optimum value being 2% [18].

Medium volume in cultivation also affected keratinase production. Volumes of 20, 30, 40 or 50 mL medium in 250 mL Erlenmeyer flasks with 10 g/L human hair were tested and the results showed that production of keratinase decreased as culture medium volume increased (Fig. 4); the optimal volume was found to be 20 mL, which may be

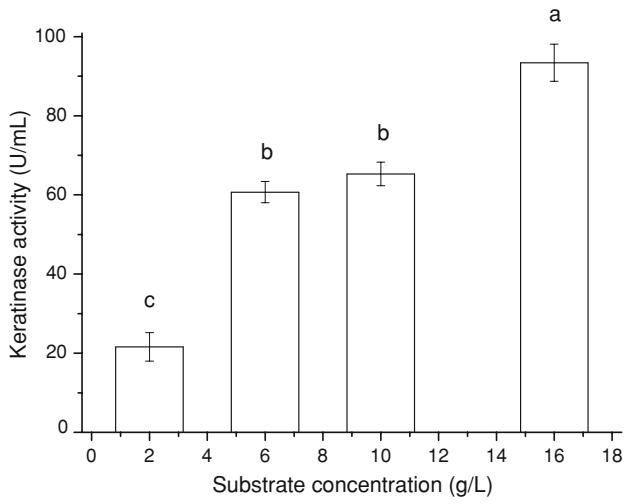

Fig. 1 Effect of human hair concentration on keratinase production. Cultivation was performed at 200 rpm and 28°C for 36 h with 5% inoculum. *Bars* standard error of the mean. Means with the different letters are significantly different according to Duncan's multiple range test at $P = 0.05$ using SAS (SAS Institute, version 6.12, Cary, NC)

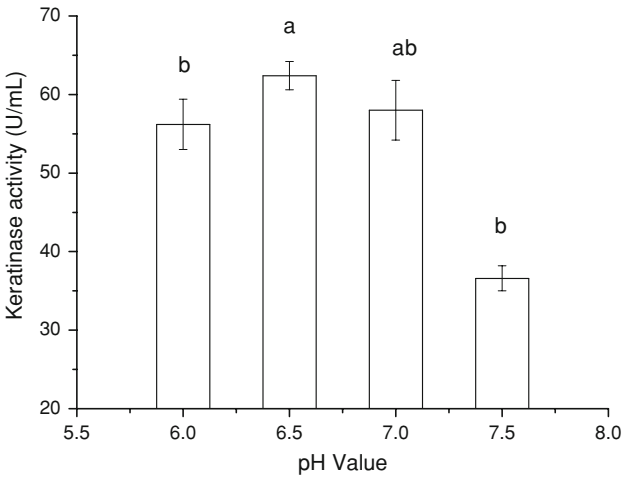

Fig. 2 Effect of initial pH value on keratinase production. Cultivations were performed at 28°C and 200 rpm for 36 h with 5% inoculum in 10 g/L hair substrate. *Bars* standard error of the mean. Means with the different letters are significantly different according to Duncan's multiple range test at $P = 0.05$ using SAS

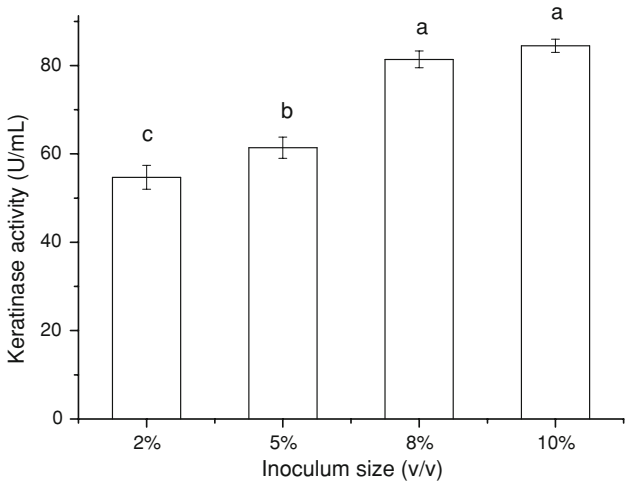

Fig. 3 Effect of inoculum size on keratinase production. Cultivations were performed at an initial pH of 6.5, 28°C and 200 rpm for 36 h in 10 g/L hair substrate. *Bars* standard error of the mean. Means with different letters are significantly different according to Duncan's multiple range test at $P = 0.05$ using SAS

due to the high dissolved oxygen and keratinase concentration in the medium.

Effects of extra sources of carbon and nitrogen on keratinase production

Extra carbon (sucrose, cornflour) and nitrogen (urea, yeast extract, tryptone, peptone) sources had positive effects on keratinase production, while glucose, maltose and ammonium chloride had negative effects (Fig. 5). Sucrose stimulated keratinase production most and was selected as an extra carbon source in further optimization.

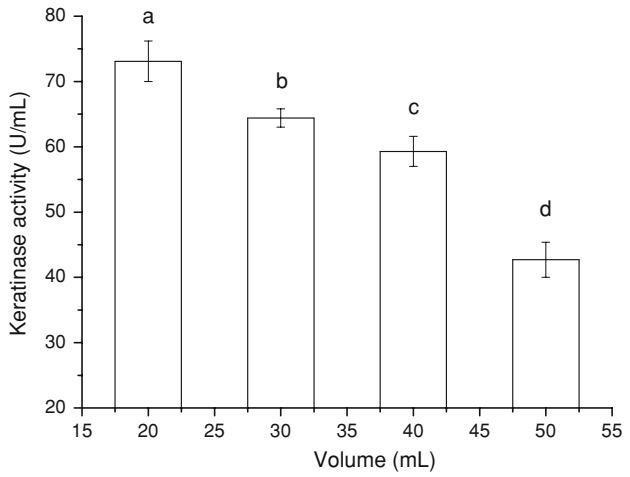

Fig. 4 Effect of culture volume on keratinase production. Cultivations were performed at an initial pH of 6.5, 28°C and 200 rpm for 36 h with 5% inoculum in 10 g/L hair substrate. *Bars* standard error of the mean. Means with different letters are significantly different according to Duncan's multiple range test at $P = 0.05$ using SAS

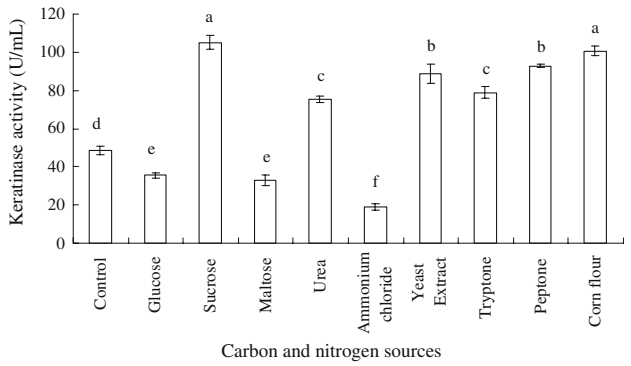

Fig. 5 Effects of extra carbon and nitrogen sources on keratinase production. Cultivations were performed at an initial pH of 6.5, 28°C and 200 rpm for 36 h with 5% inoculum in 10 g/L hair substrate. *Bars* standard error of the mean. Means with different letters are significantly different according to Duncan's multiple range test at $P = 0.05$ using SAS

Any effects of extra carbon and nitrogen sources on product formation are normally optimized further. In the case of keratinase, addition of extra sources had been discussed widely. In a study designed to optimize medium for keratinase production by *Bacillus subtilis* RG1, glucose, glutathione and peptone were found to have positive effects [15]. The effect of glucose on keratinase production was different in this study; glucose partially inhibited keratinase production by KD-N2 (Fig. 5). Usually, glucose has negative effects on microbial proteinase (keratinase included) production. For example, the keratinases produced by strain *Aspergillus fumigatus Fresenius* [34], *Thermoactinomyces candidus* [8] and *Stenotrophomonas* sp. D-1 [28] are partially inhibited by glucose. As for other carbon sources, Antarctic actinomycete strains of *Streptomyces flavis* 2BG and *Microbispora aerata* IMBAS-11A produced much more keratinase in wool substrate upon the addition of starch [9]. The effects of nitrogen sources on keratinase production also vary. Supplementation of yeast extract resulted in maximal keratinase production by *Stenotrophomonas* sp. D-1 [28]. As with *A. fumigatus*, nitrates decreased keratinase production [34]. Usually, the effects of extra carbon and nitrogen sources on keratinase production vary according to the species, substrate and carbon or nitrogen concentration. Thus it is necessary to optimize culture composition on a case-by-case basis to improve keratinase production.

Optimization of culture medium

Fractional factorial design

The results of fractional factorial design (FFD) and analysis of variance (ANOVA) for keratinase activity are shown in Tables 4 and 5, respectively. Keratinase

Table 4 Results of fractional factorial design (FFD) experiments

Run	X_1	X_2	X_3	X_4	X_5	X_6	Y (U/ml)
1	−1	−1	−1	1	−1	1	47.7
2	1	−1	1	−1	−1	1	71.6
3	1	1	1	−1	1	−1	86
4	−1	−1	1	1	1	−1	92.8
5	1	1	−1	1	−1	−1	49.1
6	0	0	0	0	0	0	57.4
7	0	0	0	0	0	0	80.7
8	1	−1	1	1	−1	−1	11.1
9	−1	1	−1	1	1	−1	90.7
10	−1	1	−1	−1	1	1	116.5
11	−1	−1	−1	−1	−1	−1	60.5
12	1	−1	−1	−1	1	−1	80.4
13	−1	−1	1	−1	1	1	34.8
14	−1	1	1	1	−1	1	88.1
15	1	1	−1	−1	−1	1	51.6
16	1	1	1	1	1	1	111.3
17	0	0	0	0	0	0	61
18	−1	1	1	−1	−1	−1	77.6
19	1	−1	−1	1	1	1	87.3
20	0	0	0	0	0	0	73.5

production has a determinant of coefficient of 0.7454, and the model was very significant ($P = 0.0047$). Compared with other factors, the concentrations of $MgSO_4$ (X_2) and K_2HPO_4 (X_5) were considered significant for keratinase production. However, the interactions between $MgSO_4$ and K_2HPO_4 were not examined. Thus a linear regression equation was obtained by applying the following fractional factorial equation:

$$Y = 72.94 + 10.92X_2 + 17.03X_5 \quad (3)$$

Considering the actual situation, other factors were fixed at the zero level. The t test for the FFD results was performed to check the experimental differences; the treatments showed no significant differences. This indicated that the optimum point is within the domain of the experiment.

Optimization of important medium components by response surface methodology

A CCD was used to screen combinations of process variables that would lead to high enzyme yield. The results of CCD and ANOVA for keratinase activity are shown in Table 6. Based on a regression analysis of the data from CCD experiments, the effects of two independent variables ($MgSO_4$ and K_2HPO_4) on keratinase production were predicted by a second-order polynomial function:

$$Y = 120.62 + 1.59X_1 + 24.62X_2 - 11.22X_1^2 - 14.55X_2^2 - 5.67X_1X_2 \quad (4)$$

where X_1, X_2 are the concentrations of $MgSO_4$ and K_2HPO_4, respectively. The corrective measures for estimating the regression equation were the multiple correlation coefficients R and the determination coefficient R^2. The closer the R value is to 1, the better the correlation between the observed and the predicted values. The results from Eq. 4 were considered significant ($P = 0.0006$). The value of the determination coefficient ($R^2 = 0.9301$) indicated a high degree of correlation between the observed and the predicted values.

The effects of $MgSO_4$ and K_2HPO_4 on keratinase production are shown in Fig. 6. Maximum keratinase activity was obtained when the initial concentrations of $MgSO_4$ and K_2HPO_4 were 0.91 and 2.38 g/L, respectively. The maximum keratinase activity (131.2 U/mL) was predicted by using CCD. The corresponding optimal medium composition for efficient keratinase production was finalized as follows (g/L): NaCl (1.0), $CaCl_2$ (0.05), KH_2PO_4 (0.7), sucrose (3), $MgSO_4$ (0.91), K_2HPO_4 (2.38).

The formulated optimal medium from RSM experiments was verified experimentally and compared with the predicted data from the model. The average keratinase activity in the broth was 125.0 U/mL from triple-duplicated

Table 5 Analysis of variance (ANOVA) for selected factorial model

Source	Sum of square	*df*	Mean square	*F* value	Prob > *F*	
Model	6,971.00	6	1,161.83	5.86	0.0047	Significant
X_1	403.01	1	403.01	2.03	0.1796	
X_2	2,132.13	1	2,132.13	10.75	0.0066	
X_3	6.89	1	6.89	0.035	0.8553	
X_4	95.55	1	95.55	0.48	0.5009	
X_5	4,306.64	1	4,306.64	21.71	0.0006	
X_6	26.78	1	26.78	0.13	0.7197	
$R^2 = 0.7454$						

Table 6 Analysis of variance (ANOVA) of central composite design (CCD)

Source	Sum of squares	*df*	Mean square	*F* value	Prob > *F*	
Model	7,085.4	5	1,417.08	18.62	0.0006	Significant
X_1	20.16	1	20.16	0.26	0.6226	
X_2	4,848.84	1	4,848.84	63.72	<0.0001	
X_1^2	876.14	1	876.14	11.51	0.0115	
X_2^2	1,472.21	1	1,472.21	19.35	0.0032	
X_1X_2	128.82	1	128.82	1.69	0.2904	
Residual	532.67	7	76.1	4.04	0.1054	
Lack of fit	400.52	3	133.51			
R^2	0.9301					
Adjusted R^2	0.8801					

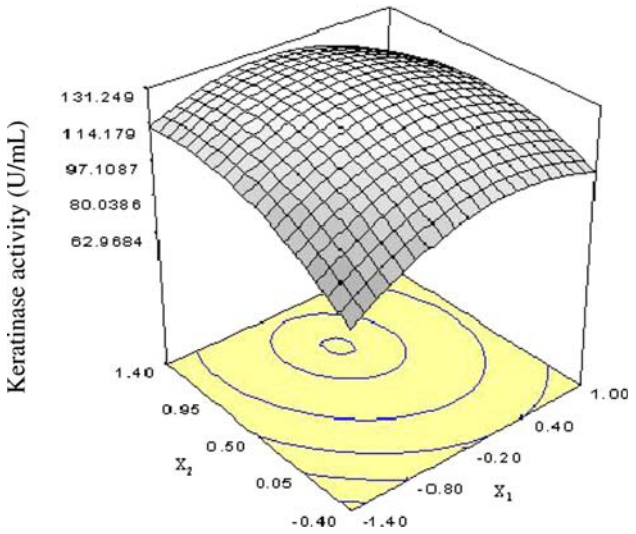

Fig. 6 Response surface methodology (RSM) plot for the combinatory effects of $MgSO_4$ (X_1) and K_2HPO_4 (X_2) for keratinase production

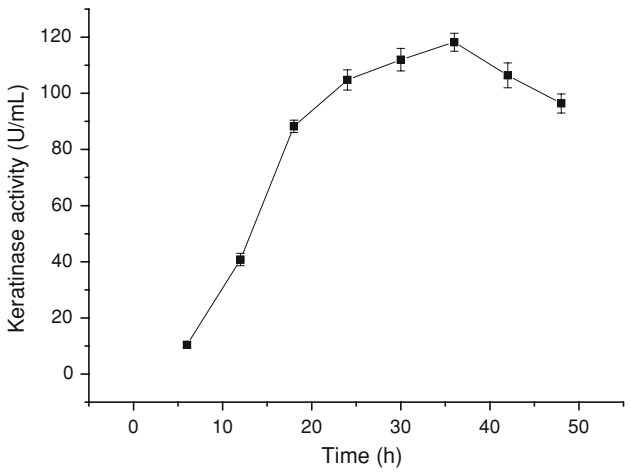

Fig. 7 Time course of production of keratinase in hair substrate. *Bars* standard error of the mean

experiments, suggesting that the accuracy of the model was over 95%.

Keratinase production and hair degradation

Keratinase was produced in submerged cultivation for 50 h with an inoculum size of 5% at 28°C, 200 rpm. Keratinase activity peaked at about 125 U/mL at 36 h and then decreased (Fig. 7). During keratinase production in submerged cultivation, hair was degraded gradually (Fig. 8).

Several keratin-containing substrates have been used in keratinase production—especially feathers. Statistical methods involving RSM have been performed to optimize keratinase production in feather substrate by *Bacillus licheniformis* RG1 [15]. We previously optimized substrate concentration and culture conditions, including initial pH value, inoculum size etc., for keratinase production by KD-N2 in feather substrate. In this study, culture medium for keratinase production using hair substrate were optimized using single factors and CCD design. Culture conditions for human hair content, the effect of initial pH, inoculum size and culture volume etc., were optimized.

Conclusion

Optimal culture conditions for keratinase production using human hair as substrate were 10% inocula (v/v), 16 g/L hair substrate, an initial pH value of 6.5 and a culture volume of 20 mL. Carbon (sucrose, cornflour) and nitrogen (yeast extract, tryptone and peptone) sources could stimulate the production of keratinase, with sucrose being optimal. FFD experiments indicated that $MgSO_4$ and K_2HPO_4 were significant factors affecting keratinase production, and their optimal concentrations of 0.91 and

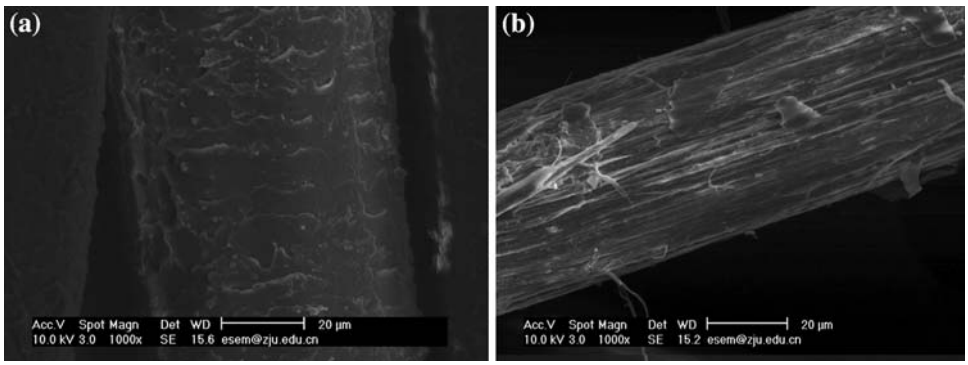

Fig. 8a,b Degradation of hair by KD-N2 in submerged cultivation. **a** Hair control, **b** hair cultivated for 50 h. Hair was filtered, washed with distilled water, dried with a Hitachi HCP-2 critical point dryer, and then coated using an Eiko IB-5 ion coater. Specimens were examined with an XL30-ESEM environment scanning electron microscope

2.38 g/L were determined by CCD experiments. Keratinase activity increased to 125 U/mL after 36 h fermentation in the optimal medium (g/L) of NaCl 1.0, $CaCl_2$ 0.05, KH_2PO_4 0.7, sucrose 3, $MgSO_4$ 0.91, K_2HPO_4 2.38. Human hair as the substrate was degraded by the isolate during fermentation. Utilization of human hair as a substrate for the optimization of keratinase production by CCD design by *Bacillus subtilis* KD-N2 is feasible, and further purification and characterization of the keratinase should be performed.

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