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Full Length Research Paper

Improved keratinase production for feather degradation by *Bacillus licheniformis* ZJUEL31410 in submerged cultivation

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Optimal medium was used to improve the production of keratinase by *Bacillus licheniformis* ZJUEL31410, which has a promising application in the transformation of feather into soluble protein. The results of single factor design revealed that the concentration of feather at 20 g/l and the initial pH at value 8 was the best for the production of keratinase and the degradation of feather. Ammonia salt and nitrate salt strongly restricted the production of keratinase and the degradation of feather. Result of Box-Behnken design (BBD) experiment which was used to optimize concentrations of glucose, corn steep flour and K_2HPO_4 for further improvement of keratinase productivity showed that the optimal medium was composed of glucose (20 g/l), corn steep flour (7.5 g/l), K_2HPO_4 (1 g/l) and feather (20 g/l). The result of submerged batch cultivation of *B. licheniformis* ZJUEL31410 in the 5 L fermentor indicated that the optimal medium had the highest keratinase and the degree of feather degradation (DFD) at 54.9 U/ml and 72.4%; both were 5 times more than the basal medium. The degradation of feather was verified by the analysis of scanning electron microscopy (SEM). This study provides a foundation for the production of keratinase and the conversion of feather to soluble protein through submerged fermentation process by *B. licheniformis* ZJUEL31410.

Key words: *Bacillus licheniformis* ZJUEL31410, keratinase, culture medium, optimization, Box-Behnken design, scanning electron microscopy, feather degradation.

INTRODUCTION

Feather waste which is generated in large quantities as a by-product of commercial poultry processing, contains a great deal of protein (keratin) and amino acids that could be beneficially utilized as animal feedstuff. However, the poor digestibility limited its application (Onifade et al., 1998). The digestibility of feather can be improved by thermal degradation (Wang and Parsons, 1997), which needs high cost for the thermo-energy and produces some poison by-products, restraining the practical use. A myriad of microorganisms, mostly fungi and bacteria, have been reported to utilize keratin (Santos et al., 1996; Simpanya and Baxter, 1996; Singh, 1997), suggesting the feasibility of biotechnological process for the utilization of feather as potential protein resources. In general, the biodegradation of feather can be achieved by the cultivation of keratinase-producing microorganisms on feather as well as the incubation of feather with crude or purified keratinase from some species of *Bacillus* (Williams et al., 1990; Takami et al., 1999), *Streptomyces* (Nickerson et al., 1963; Bockle et al., 1995), and some fungi (Mignon et al., 1998; Gradisar et al., 2000). In comparison, the enzymatic utilization of feather is regarded as the most promising method with the advent-

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ages of high specificity and efficiency and convenient operation in large-scale commercial production. In addition to feather degradation, the keratinase has important application in leather industry, in slow release nitrogen fertilizers, cosmetics and biodegradable films (Choi and Nelson, 1996; Raju et al., 1996; Riffel et al., 2003), which has being on rapid growth in the last two decades, greatly enlarging the commercial value up to $2,000,000,000/year which promoted the studies on the production and application of keratinase.

Keratinases produced by some species of *Bacillus* such as *Bacillus licheniformis* PWD-1 (Lin et al., 1992; Cheng et al., 1995), *B. licheniformis* Carlsberg NCINB (Evans et al., 2000), *Bacillus pumilus* FH9 (El-Refai et al., 2005) and *Bacillus subtilis* KD-N2 (Cai et al., 2008) have been characterized. However, there is no adequate information about the optimization of medium composition and cultivation conditions to promote the productivity and lower the cost of keratinase, which is the key factor for the industrial practices, especially for the degradation of feather to produce cheap feedstuff.

In this study, the medium composition for the submerged cultivation of *B. licheniformis* ZJUEL31410 which was isolated and verified safe (the maximum tolerated dose (MTD) of the feather hydrolysate was >10 mg/g in mice) to produce keratinase for food and feed use in our previous study was optimized to enhance the keratinase production which achieved the degradation of feather into soluble protein, promoting the utilization of insoluble feathers.

MATERIALS AND METHODS

Chemicals and microorganism

Folin-phenol reagent, standard proteins and tyrosine were purchased from Sigma Company. Chicken feather which was supplied by a local poultry processing factory was washed three times with distilled water and dried to constant weight at 60°C overnight and kept at 4°C until used. *B. licheniformis* ZJUEL31410 (CGMCC NO.1397) was isolated from animal feeding and processing factory, Hangzhou, China.

Preparation of the seed of *B. licheniformis* ZJUEL31410

The seed was prepared by the cultivation at 37°C for 24 h in the seed medium at initial pH 7.0 consisting of beef extract (4 g/l), peptone (4 g/l), yeast extract (2 g/l) and NaCl (5 g/l).

Optimization of the medium

For the optimization of the medium, 1 ml of fresh seed was inoculated into serial 250 ml flasks containing 30 ml medium followed by the incubation at 37°C in a shaker at 150 r/min for 30 h to analyze the keratinase activity, soluble protein content (SPC) and degree of feather degradation **(**DFD). The optimization was done with the aid of single factor experiment design and Box-Behnken design (BBD) design based on the basal feather medium consisting of glucose (20.0 g/l), chicken feather (10.0 g/l), corn steep flour (2 g/l), K_2HPO_4 (1.0 g/l) and $MgSO_4 \cdot 7H_2O$ (0.1 g/l) at an initial pH adjusted by sodium hydroxyl to 7.5 (Montgomery, 2008).

Optimization of the concentration of feather

B. licheniformis ZJUEL31410 was cultivated at different concentrations of feather, namely 5, 10, 15, 20 and 25 g/l in the basal medium to investigate the effects of the concentration of feather on the keratinase activity, SPC and DFD.

Optimization of the initial pH value of the culture medium

B. licheniformis ZJUEL31410 was cultivated at different initial pH (6.5, 7.5, 8.0 and 8.5) in the medium consisting of glucose (20.0 g/l), feather (20.0 g/l), corn steep flour (2 g/l), K_2HPO_4 (1.0 g/l), $MgSO_4 \cdot 7H_2O$ (0.1 g/l) to select the optimal initial pH by the comparison of the keratinase activity, SPC and DFD.

Investigation of the effects of ammonia salt and nitrate salt on the keratinase production and feather degradation

Based on our preliminary studies, the medium (glucose 20.0 g/l, feather 20.0 g/l, corn steep flour 2 g/l, K_2HPO_4 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l) with the initial pH at 8.0 containing serial concentration of sulfate ammonia and sodium nitrate which were the most common inorganic nitrogen source represented ammonium and nitrate salts which were considered having the highest efficiency among inorganic nitrogen sources, were used in the range of 0 to 15 g/l to culture *B. licheniformis* ZJUEL31410 to investigate the influence of inorganic nitrogen sources on the feather degradation and the keratinase production.

Investigation of the effects of amount of corn steep flour on the production of keratinase

B. licheniformis ZJUEL31410 was cultivated at different concentration of corn steep flour (0, 2, 7, 12 and 20 g/l) in the medium at initial pH 8.0 consisting of glucose (20.0 g/l), feather (20.0 g/l), K_2HPO_4 (1.0 g/l), $MgSO_4 \cdot 7H_2O$ (0.1 g/l) to compare the keratinase activity and SPC.

Optimization of the concentration of glucose, corn steep flour and K_2HPO_4 by BBD experiment for keratinase production

The Box-Behnken design (BBD) (Box et al., 1978) leading to 15 sets of experiments was adopted to optimize the concentration of glucose, corn steep flour and K_2HPO_4 to further increase the keratinase production. The variables were coded according to equation (1). The keratinase activity was considered as the dependent variable or response (Y_i). The second-order model which was obtained from BBD with four coded levels was performed according to equation 2 for the prediction of the optimal position based on the coded values. The proposed maximum point was verified in triplicate to show the real values of the optimization.

$$X_i = (x_i - x_0) / \sum x_i \quad (1)$$

Where, X_i is the coded value of an independent variable, x_i is the real value of an independent variable, x_0 is the real value of an independent variable at the center point, and $\square x_i$ is the step change value.

$$Y = b_0 + b_i X_i + b_{ii} X_i^2 + \sum b_{ij} X_j X_j \quad (2)$$

Where, Y was the response variable, b_0, b_i, b_{ii}, b_{ij} were the regression coefficients variables, for intercept, linear, quadratic and interaction terms, respectively and X_i and X_j were the coded value

of independent variables.

Investigation of the time course of keratinase production and feather degradation in 5 L fermentor

The optimal medium (glucose 20.0 g/l, feather 20.0 g/l, corn steep flour 7.5 g/l, K_2HPO_4 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.1 g/l) with initial pH value of 8.0 was applied for the culturing of *B. licheniformis* ZJUEL31410 in the 5 L pH-stat fermentor (Bio.Braun Biotech International Co.) containing 4 L fermentation medium for 120 h with the inoculants at 5 % (v/v), aeration at 1.0 L/min, temperature at 37°C, dissolved oxygen at 20 to 80% which was auto-controlled by changing the stir rate in the feedback model. Samples were fetched in every 24 h to monitor keratinase activity and other fermentative parameters.

Determination of keratinase activity

The assay of keratinase activity was based on the method of Goddard and Michaelis (1934) with little modification. Briefly, 2 ml of 0.05 mol/l Tris/HCl buffer (pH 7.5) and 1.0 ml of the enzyme solution were incubated with 10 mg feather powder for l h at 37°C, with constant agitation in a water bath. The enzyme reaction was stopped by the addition of 2.0 ml 10% trichloroacetic acid (TCA) and the samples were centrifuged for 15 min at 10,000 *g* and 4°C. The absorbance of the supernatant was measured at 280 nm on a UV-2401PC spectrophotometer (Shimadzu, Japan) to calculate the keratinase activity .One unit (U) of keratinase activity was defined as the amount of keratinase accounting for the increase of absorbance at 0.10 under specified measure conditions (at 37°C and pH 7.5 and in 1 h).

Determination of soluble protein content (SPC)

SPC was determined by Follin-hydroxybenzene method with the bovine serum albumin (BSA) as the standard reference protein (Bradford, 1976).

Determination of degree of feather degradation (DFD)

The residual feather was washed, dried and scaled to calculate DFD using equation 3.

$$DFD\ (\%) = (TF-RF) \times 100/TF \quad (3)$$

Where, TF is the total feather and RF is the residual feather.

Scanning electron microscopy (SEM)

The surface topography of chicken feather before/after fermentation was examined using SEM (XL-30-ESEM, Philips, Eindhoven and Netherlands) at the magnifications fold of 1, 000 and 2,000 times to verify the degradation. Before testing, the samples were fixed onto metallic sample holders with conducting silver glue and then sputtered with a layer of gold.

Statistical analysis

All the experiments were done in triplicate; the average of triplicate determinations was used to represent the result. Statistical analysis (Montgomery, 2008) such as analysis of variance (ANOVA) and the response surface regression (RSREG) were conducted with SAS software version 8.0 (SAS Institute Inc., Cary, NC, USA). Trends were considered as significant when mean values of compared sets were different at $P < 0.05$ by Tukey's pair wise comparison. However, both $P < 0.05$ and $P < 0.01$ were analyzed in the BBD experiment.

RESULTS AND DISCUSSION

Effect of feather concentrations on keratinase production

The amount of keratinase production was depended on feather concentrations. It was observed that 20 g/l feather had the highest keratinase production as well as the highest SPC and DFD (Figure 1). Keratinase produced at 5 g/l feather was seven times less than that of 20 g/l feather, while 25 g/l feather showed substrate repression on the keratinase production, which lead to the decrease in SPC and DFD. This result agreed with the study of Cheng et al. (1995) who reported that high feather concentration increased the viscosity of the medium which possibly resulted in oxygen limitation for bacterial growth, restricting the production of keratinase by *B. licheniformis* PWD-1.

Effect of initial pH of culture medium on keratinase production

The initial pH of the culture medium greatly affected the keratinase production, SPC and DFD; initial pH 8.0 was evidently optimal for keratinase production (Table 1). The results are in accordance with some previously reported keratinases which belonged to the family of alkaline protease (Altalo and Gashe, 1993; Cheng et al., 1995), indicating that the keratinase produced by *Bacillus* species might be most active in neutral or basic conditions. Therefore, it is reasonable to say that the highest keratinase production occur in the basic circumstance at pH 8.0 that helps the keratin dissolve into the culture medium. However, the keratinase started to decrease when the initial pH reached 8.5 which inhibit the growth of many of *Bacillus* family.

Effect of inorganic nitrogen sources on keratinase production

As the two fast nitrogen sources, ammonia and nitrate salts were added into the media. Keratinase activity, SPC and DFD were hardly detected and dramatically decreased in comparison with the control, suggesting that neither ammonia sulfate nor sodium nitrate at any concentrations exerted a positive effect on keratinase production (Table 2). On the contrary, the addition of fast nitrogen source (that is, ammonia salt and nitrate salt) restricted the induction of keratinase. Therefore, it is necessary to exclude any ammonia salt and nitrate salt in

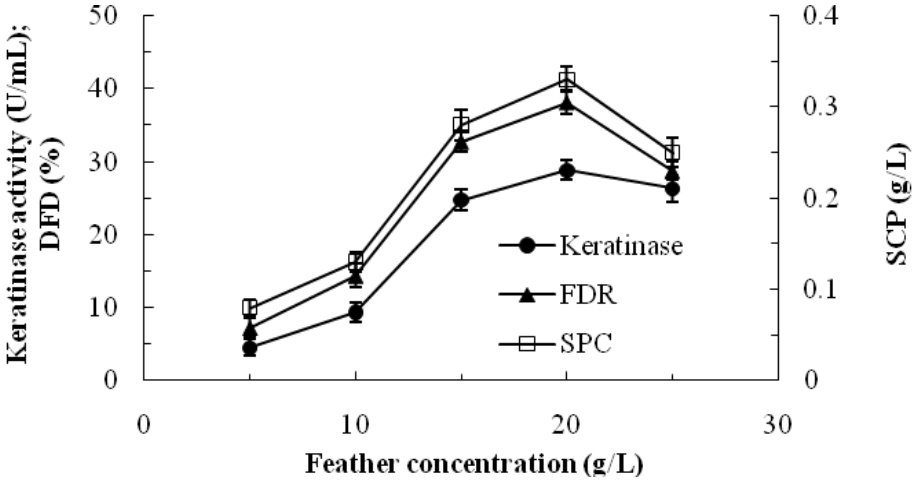

Figure 1. High keratinase production by *B. licheniformis* ZJUEL31410 at 20 g/l of feather.

Table 1. Effect of initial pH of culture medium on feather degradation and keratinase production*.

Original pH	Keratinase activity (U/ml)	DFD (%)	SPC (mg/ml)
6.5	8.3±0.52^{c}	7.9±0.42^{d}	0.2±0.16^{d}
7.5	26.1±0.56^{b}	35.4±0.52^{c}	0.3±0.22^{c}
8.0	35.7±0.68^{a}	42.3±0.66^{a}	0.7±0.18^{a}
8.5	23.7±0.71^{b}	29.6±0.67^{b}	0.6±0.20^{b}

*The number marked with different superscript letters in the same column show statistic difference at significant level 5%.

Table 2. Influence of different inorganic sources on feather degradation*.

Inorganic nitrogen source	Concentration (g/l)	SPC (g/l)	Keratinase activity (U/ml)
	5	0.4±0.11^{b}	17.4±0.49^{b}
$NaNO_3$	10	0.2±0.18^{b}	12.1±0.63^{c}
	15	0.1±0.04^{c}	9.3±0.72^{d}
	5	0.1±0.04^{c}	10.5±0.36^{d}
$(NH_4)_2SO_4$	10	0	8.2±0.51^{e}
	15	0	7.9±0.47^{e}
Control	0	0.7±0.17^{a}	35.1±0.68^{a}

*The number marked with different superscript letters in the same column **show** statistic difference at significant level 5%.

the medium for the production of keratinase by *B. licheniformis* ZJUEL31410. This result is in agreement with that of previous study (Cai and Zheng, 2009).

Effect of the concentration of corn steep flour on keratinase production

The results presented in Figure 2 demonstrated that 7 g/l of corn steep flour gave the highest keratinase activity. Additionally, the SPC displayed similar trend as the enzyme activity, confirming the presumption of Chen et al. (2002) that keratinase was induced to breakdown the feather into soluble protein to support cell growth.

Optimization of the medium using response surface methodology

The result of BBD experiment (Table 3) was regressively

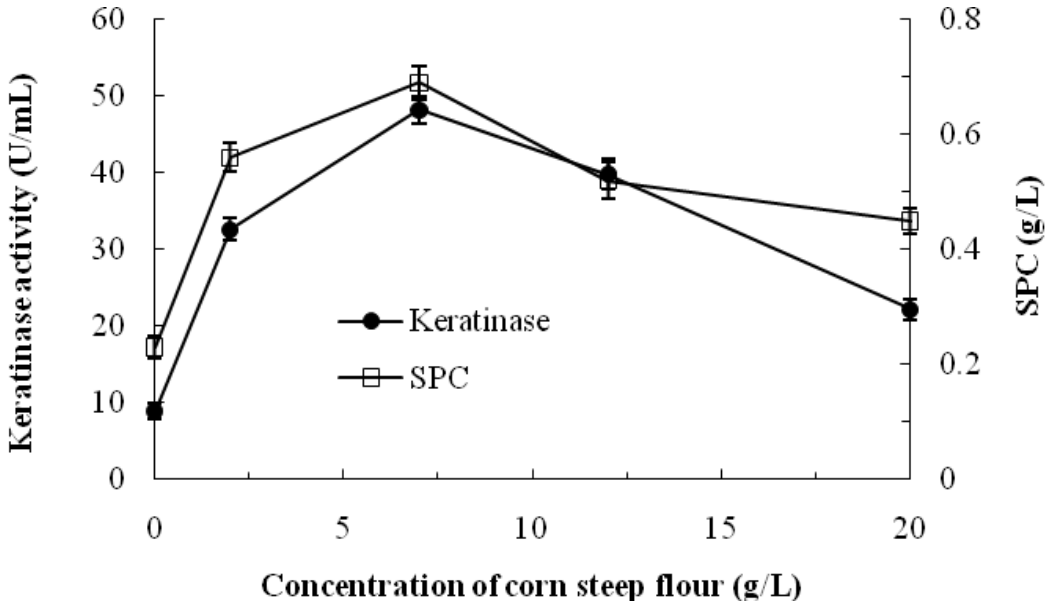

Figure 2. 7 g per liter of corn steep flour giving increased keratinase production.

Table 3. The experimental design and results of BBD for keratinase production*.

Runs	X_1	X_2	X_3	Y (U/mL)
1	-1	-1	0	13.0
2	-1	1	0	12.6
3	1	-1	0	6.2
4	1	1	0	19.6
5	0	-1	-1	19.1
6	0	-1	1	15.3
7	0	1	-1	18.3
8	0	1	1	24.4
9	-1	0	-1	14.5
10	1	0	-1	26.0
11	-1	0	1	24.0
12	1	0	1	20.0
13	0	0	0	49.5
14	0	0	0	48.8
15	0	0	0	50.1
16	0	0	0	54.4
17	0	0	0	48.9

*X_1 which was calculated by the formula (x_1-2)/1 and was the coded value of glucose (x_1 was the real concentration of glucose), X_2 which was calculated by the formula (x_2-0.7)/0.5 was the coded value of corn steep flour (x_2 was the real concentration of corn steep flour), X_3 which was calculated by the formula (x_3-0.1)/0.05 was the coded value of K_2HPO_4 (x_3 was the real concentration of K_2HPO_4), and Y refe to the keratinase activity.

analyzed to search the optimal medium composition based on surface response methodology. As shown by the regressive analysis (Table 4), the fit value, termed adjusted R^2 (Adj R-Squared, determinant coefficient) of the polynomial model was calculated to be 0.98, indicating that 98% of the variability in the response could be explained by the second-order polynomial prediction equation given below (Equation 4). The lack of fit was not significant which further indicated the possibility of model. It implied that the keratinase production by *B. licheniformis* ZJUEL31410 primarily depended on the cross product and quadratic terms of glucose and K_2HPO_4 and corn steep flour.

$$Y=48.8+0.85X_1+2.73X_2+0.68X_3-16.9X_1^2-18.9X_2^2-10.6X_3^2+3.4X_1X_2-3.8X_1X_3+2.5X_2X_3 \quad (4)$$

Where, X_1 was the coded value of glucose; X_2 was the coded value of corn steep flour; X_3 was the coded value of K_2HPO_4; Y was keratinase production.

The three-dimensional graphs (Figure 3) of the keratinase against the content of glucose, corn steep flour and K_2HPO_4 all showed maximum which indicated that the optimal composition for the keratinase production existed in the tested area. By forcing the derivative value of equation 4 at zero, the optimal value of X_1, X_2 and X_3 was calculated at 0, 0.1 and 0 to give the predicted highest keratinase productivity at 48.9 U/ml, very close to the real keratinase activity at 49.1±2.65 U/ml (N= 3), confirming the validity of the predictive model. Consequently, the optimal medium which had the keratinase activity (49.1 U/ml) 5 time greater than the basal medium (9.3 U/ml), was obtained in the composition of glucose (20.0 g/l), feather (20.0 g/l), corn steep flour (7.5 g/l),

Table 4. The ANOVA results of the second-order regression model for keratinase production.

Regression	DF	SS	R^2	*F*-value	*P*
Linear	3	68.33	0.1025	9.815	0.0155
Quadratic	3	454.66	0.6819	65.310	0.0002
Cross product	3	132.18	0.1982	18.987	0.0037
Total regression	9	655.16	0.9826	31.371	0.0007
Lack of fit	3	9.60		0.59	0.6533

Mean, 27.34; Standard Deviation, 2.11; C.V, 7.74; R-squared, 0.9924; adjusted R-squared, 0.9827; predicted R-squared, 0.9546; Adeq precision ,26.2940.

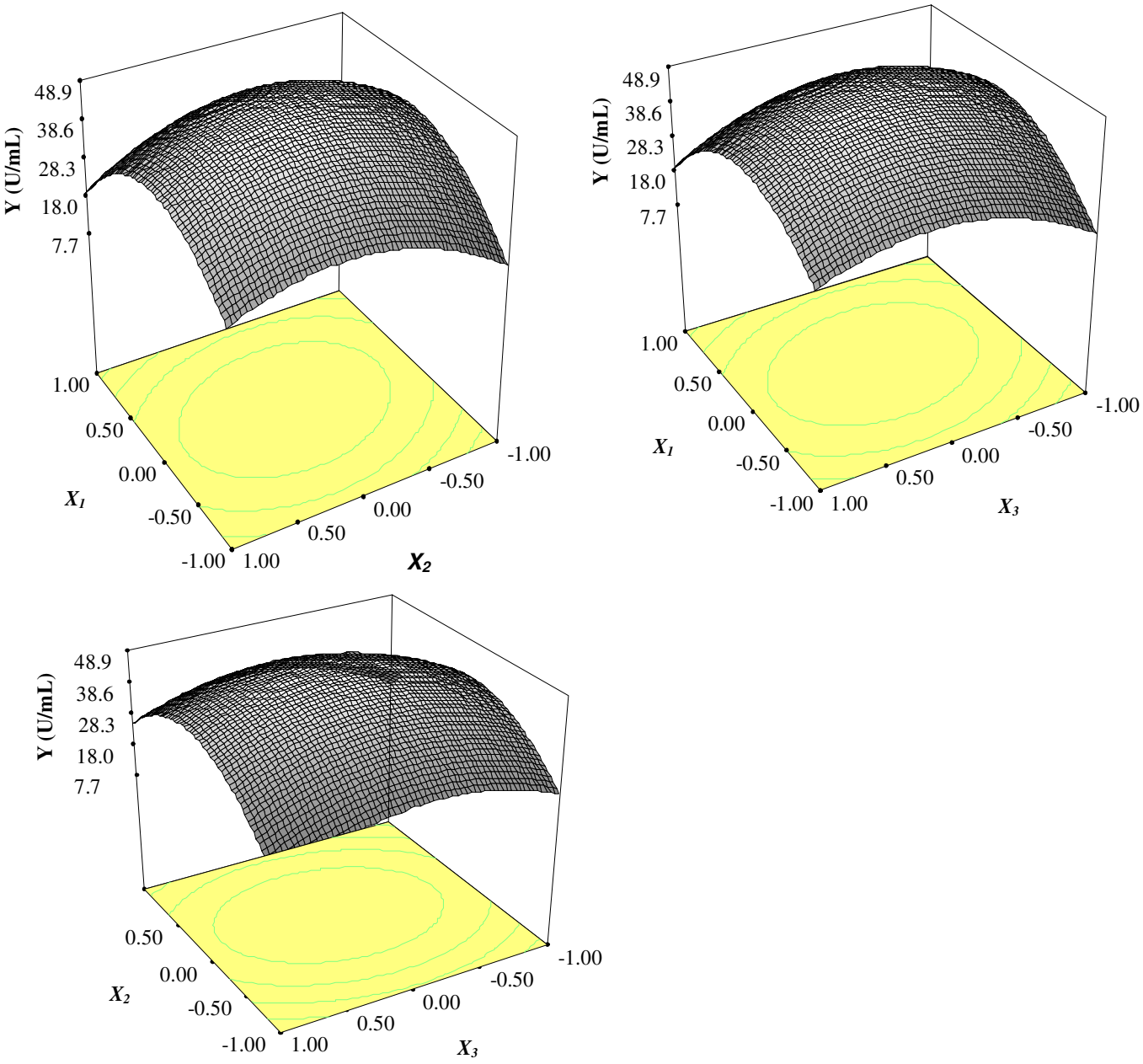

Figure 3. The response surface plots showing the influences of glucose (X_1), corn steep flour (X_2) and K_2HPO_4 (X_3) against keratinase production (Y) by *B. licheniformis* ZJUEL31410*.*X_1 was the coded value of glucose, X_2 was the coded value of corn steep flour, X_3 was the coded value of K_2HPO_4, and *Y* was keratinase production.

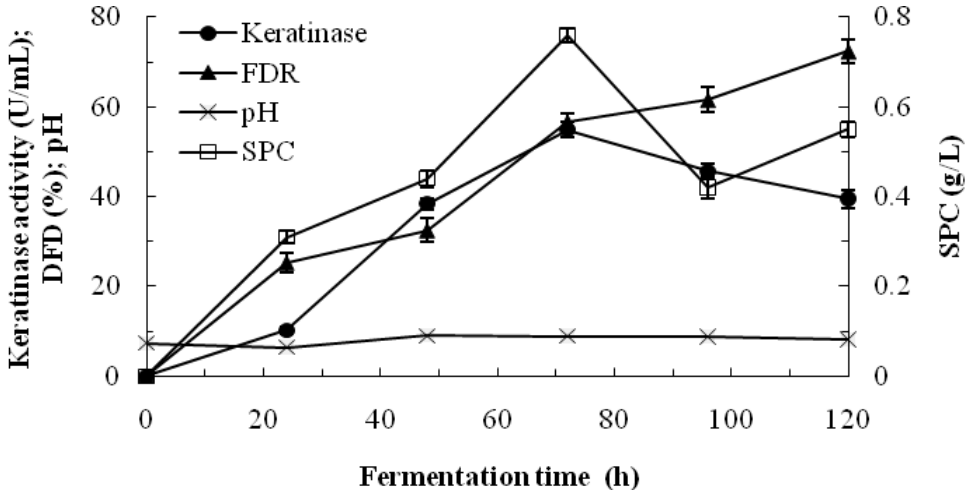

Figure 4. The time course of keratinase production and feather degradation.

K_2HPO_4 (1.0 g/l) and $MgSO_4 \cdot 7H_2O$(0.1 g/l) at initial pH 8.0 for the production of keratinase by *B. licheniformis* ZJUEL31410. This result indicated that nature keratin might be a good stuff for the keratinase production, which agreed with the study of Cai and Zheng (2009).

Time course of keratinase production and feather degradation

During the submerged cultivation in 5 L bioreactor, the keratinase activity reached the maximal point (54.9 U/ml) at the cultivation time of 72 h (Figure 4). SPC was observed to keep on increasing within 0 to 72 h followed by fluctuation in the rest of the cultivation, and the DFD kept increasing all over the cultivation (Figure 4), indicating the inner balance and regulation of SPC production and utilization: (1) the feather was broken down to produce SPC by the keratinase; (2) the SPC was utilized to support the growth of *B. licheniformis*; (3) the feather degradation was regulated by the concentration of SPC; (4) SPC reflected the balance of feather degradation and cell growth. Honeycomb-like structure which marked the deformation and disruption of feather, was observed after cultivation for 48 h by the SEM analysis (Figure 5), indicating the degradation of feather. The highest DFD measured at 72.4% in the optimized culture medium was five times higher than that of the basal medium (Figures 1 and 5), suggesting the optimized medium greatly improved the feather degradation. These comprehensive results indicate the optimal medium can greatly promote the keratinase production. Additionally, this culture medium demonstrated high efficiency in the degradation of non-soluble feather to soluble protein which has potential uses in feed industry through the simple biotechnological treatment by *B. licheniformis* ZJUEL31410. Compared with other keratin resource for production of keratinase (Cai et al., 2008; Cai and Zheng, 2009), feather causes serious environmental problem allover the world and is the richest keratin source, which makes it the most promising material for keratinase production. Moreover, *B. licheniformis* ZJUEL31410 is safe and efficient to converse feather into soluble protein which has important use in feed industry. Therefore, this study provided two useful approaches for the sustainable utilization of feather: keratinase production and feed protein. Thus, future study should focus on the purification of the keratinase and the nutritional evaluation of soluble protein after treatment with *B. licheniformis* ZJUEL31410.

Conclusions

Keratinases are extracellular proteolytic enzymes which have important use in pharmaceutical and cosmetic industry, as well as waste treatment, leather processing and feed processing, related to the ability to specifically degrade insoluble keratin. The medium for the cultivation of *B. licheniformis* ZJUEL31410 was optimized to produce keratinase. This strain displayed higher capability of keratinase production at the initial pH of 8 and feather concentration of 20 g/l. Ammonia salt and nitrate salt strongly restricted the production of keratinase and the degradation of feather. BBD experiment showed that the optimal medium for keratinase production was composed of glucose (20 g/l), corn steep flour (7.5 g/l), K_2HPO_4 (1 g/l) and feather (20 g/l). The highest keratinase, SPC and DFD was observed as 54.9 U/ml, 0.76 g/l and 72.4% respectively using the submerged batch culture in the 5 L fermentor. Significant structural change indicating the degradation of feather was observed by the aid of SEM. In summary, this improved medium provides a foundation for the fermentation of keratinase and the transformation

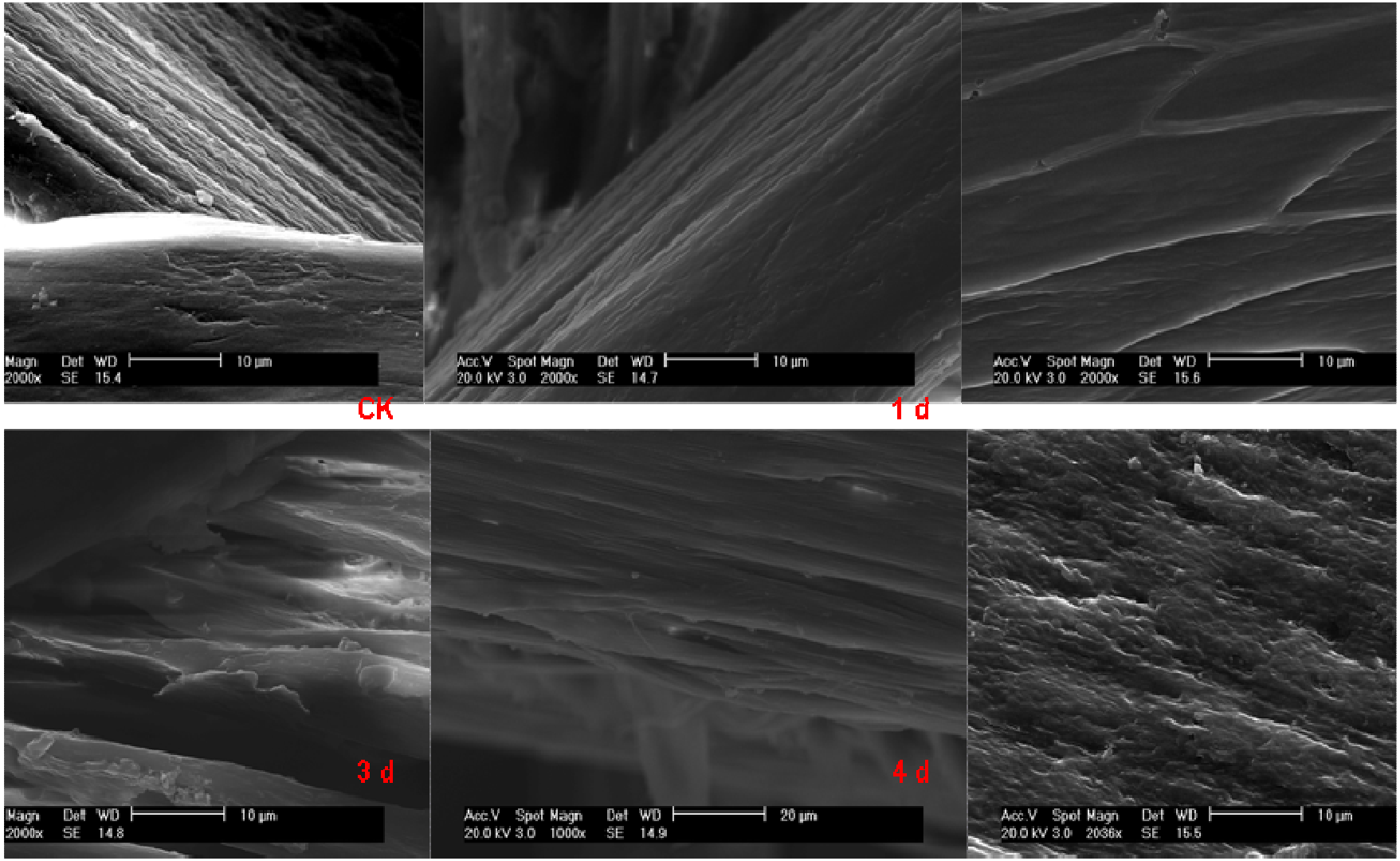

Figure 5. The observation of the degradation of chicken feather by scanning electron micrographs analysis*. d; day.

of feather to produce soluble protein by the bioprocess of *B. licheniformis* ZJUEL31410 under the submerged cultivation.

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