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Medium optimization for the feather-degradation by *Streptomyces fradiae Var S-221* using the response surface methodology

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Abstract In order to accelerate biodegradation of feather into more amino acids, the fermentation medium of feather-biodegrading *Streptomyces fradiae Var S-221* was optimized in this paper. In the first optimization step, the effects of feather powder, beet molasses, $(NH_4)_2SO_4$ and KH_2PO_4 on amino acids formation were evaluated by using full factorial design. The results showed that feather powder and $(NH_4)_2SO_4$ had significant and positive effects on feather-biodegradation into amino acids. Then, the method of the steepest ascent was used to access the optimal region of the two significant factors. In the third step, the concentration of feather powder and $(NH_4)_2SO_4$ were further optimized with central composite design and response surface analysis. As a result, the composition of the optimal medium for *S. fradiae Var S-221* fermentation were as follows (g/100 ml): feather powder, 19.504; beet molasses, 4.0; $(NH_4)_2SO_4$, 1.467; KH_2PO_4, 0.3; $MgSO_4$, 0.15; $FeSO_4$, 0.001; $ZnSO_4$, 0.0001; and $MnSO_4$, 0.0001. Using this optimal fermentation medium, the amino acids concentration was increased from 4.61 to 6.13 g/100 ml.

Keywords Amino acid · Feather-biodegradation · Medium optimization · *Streptomyces fradiae Var S-221* · Response surface methodology

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Introduction

Millions of tons of feathers are produced annually as a waste byproduct at poultry processing plants. If these feathers are not disposed off in time, their accumulation will lead to a serious environmental pollution and feather protein wastage (Onifade et al. 1998; Gousterova et al. 2005). Feathers are composed of over 90% of beta-keratin, rich in stacked beta-pleated sheets, held together by hydrogen bonding, hydrophobic interactions and highly cross-linked by disulfide bonds (Lin et al. 1999). Therefore, feathers are mechanically stable and difficult to be degraded by common proteases. Most feathers generated in the industry are disposed off by incineration (Deydier et al. 2005).

The methods of feather-degradation include alkali hydrolysis, steam pressure cooking and biodegradation. However, due to feathers being almost pure keratin protein, alkali hydrolysis and steam pressure cooking will not only destroy the amino acids but also consume large amounts of energy (Cai et al. 2008). Therefore, the development of enzymatic and/or microbiological methods for the hydrolysis of feather into soluble proteins and amino acids is extremely attractive. Furthermore, feather can be biodegraded to feather meal for animals, slow-release nitrogen fertilizers, glues and films or used for the production of the rare amino acids like serine, cysteine and proline (Gupta and Ramnani 2006).

At present, keratinolytic microorganisms and their enzymes have become a subject of substantial

scientific interest (Worapot et al. 2005; Nadir et al. 2008; Matsui et al. 2009). Although many efforts have been focused on isolation and characterization of a feather-degrading microorganisms and enzyme, little information was available on the medium optimization for these microorganisms. We previously reported the isolation and characterization of a feather-degrading *Streptomyces fradiae Var S-221* strain, which was isolated from a poultry industry and produced a high keratinolytic activity (Tu and Ding 1994). In the present work, the fermentation medium of *S. fradiae Var S-221* was optimized to improve amino acids concentration by biodegrading feather.

Materials and methods

Preparation of feather powder

Poultry feathers (Yongxiu Sanjiao poultry processing company, PR China) were washed with water, and dried in oven at 45°C. To prepare feather powder, the feathers were cut into small fragments and milled in a ball mill, and then passed through a 100-mesh grid.

Microorganism and media

Feather-degradation in terms of amino acids concentration in the fermentation media was studied in cell cultures of *Streptomyces fradiae Var S-221* cells. *S. fradiae Var S-221* was maintained on agar slant containing (g/100 ml) soluble starch (Hua Rong Pharmaceutical Co. Ltd, PR China), 2.0; NaCl, 0.05; KNO_3, 0.10; K_2HPO_4, 0.05; $MgSO_4 \cdot 7H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.001; and agar, 2.0 (pH: 7.5 ± 0.1).

Preculture medium was composed of (g/100 ml) feather powder, 1.0; beet molasses (Hua Rong Pharmaceutical Co. Ltd, PR China), 2.0; $(NH_4)_2SO_4$, 0.25; KH_2PO_4, 0.15; $MgSO_4$, 0.1; $FeSO_4$, 0.001; $ZnSO_4$, 0.0001; $MnSO_4$, 0.0001.

Basal medium was composed of (g/100 ml) feather powder, 15.0; beet molasses, 4.0; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.3; $MgSO_4$, 0.15; $FeSO_4$, 0.001; $ZnSO_4$, 0.0001; $MnSO_4$, 0.0001.

The pH of the preculture medium and basal medium were both adjusted in the range of 7.3 ± 0.1 with NaOH prior to sterilization (autoclaved for 30 min at 121°C).

Culture conditions

Preculturing was carried out in a 250-ml Erlenmeyer flask containing 30 ml of preculture medium inoculated with approximately 1×10^9 spores from fresh slant, and incubated at 37°C on a rotary shaker at 180 rpm for 36 h. The seed culture was then transferred to a 250-ml Erlenmeyer flask containing 45 ml fermentation medium with 10% inoculum, and incubated at 37°C on a rotary shaker at 180 rpm for 96 h.

Experimental design and statistical analysis

Full factorial design was first used to identify the independent variables that significantly influence biodegradation of feather into amino acids by *S. fradiae Var S-221*. Based on the statistical model of the results of full factorial design, the significant factors used method of steepest ascent to arrive in the vicinity of the optimum rapidly. To efficiently explore the best condition obtained by the steepest method, the central composite design was performed, and the statistical analysis of results was accomplished by Data Processing System software (Tang and Feng 2007).

Quantification of amino acids in the fermentation broth

The concentration of amino acids in fermentation broth was determined by the ninhydrin-based analysis (Stanford 1968). Ninhydrin-hydrindantin was dissolved in 75% dimethyl sulfoxide-25% 4 mol/l lithium acetate buffer at pH 5.2, and then the amino acids concentration of fermentation broth was determined by the manual ninhydrin method.

Results and discussion

The results of the full factorial design

Firstly, the following four components of the fermentation medium were optimized for enhancing amino acids formation by feather-biodegrading *S. fradiae Var S-221*: feather powder, beet molasses, $(NH_4)_2SO_4$ and KH_2PO_4. The four components mentioned above were chosen on the basis of preliminary experiments. Table 1 shows the levels and actual values of the four factors tested in the full factorial design.

Table 1 Levels and actual values of factors for full fractional design

Factors (g/100 ml)	Levels		
	−1	0	+1
Feather powder, X_1	12.5	15.0	17.5
Beet molasses, X_2	2.0	4.0	6.0
$(NH_4)_2SO_4$, X_3	0.5	1.0	1.5
KH_2PO_4, X_4	0.15	0.3	0.45

The experimental design and results of full factorial design are listed in Table 2. A total number of 20 runs were required for the optimization of the four components, in which four replicates were conducted at the center point. Based on the levels of four variables and the experimental results (Table 2), the statistical analysis accomplished by Data Processing System software is summarized in Table 3.

From the results of regression analysis, the significant terms (significant at the 0.01 level) were as follows: X_1 (feather powder) and X_3 ($(NH_4)_2SO_4$). According to the regression analysis, the linear model obtained for amino acids concentration in the fermentation broth after 96 h of incubation was expressed as follows:

$$Y(\mathrm{g}/100\ \mathrm{ml}) = 4.4740 + 0.5275X_1 + 0.1125X_2 + 0.5000X_3 + 0.0525X_4 \quad (1)$$

In Eq. (1), Y was the amino acids concentration, and X_1, X_2, X_3 and X_4 were the coded levels of feather powder, beet molasses, $(NH_4)_2SO_4$ and KH_2PO_4, respectively. The statistical significance of the model was checked by Student t-test. As shown in Table 3, the model was highly significant ($P < 0.0001$) and $R^2 = 0.9125$.

The path of the steepest ascent

Coefficients of feather powder (X_1) and $(NH_4)_2SO_4$ (X_3) in the linear model (Eq. 1) were significant and positive, which demonstrated that increasing the concentrations of feather powder and $(NH_4)_2SO_4$

Table 2 Experimental design and results of full fractional design

Run	X_1 (feather powder)	X_2 (beet molasses)	X_3 ($(NH_4)_2SO_4$)	X_4 (KH_2PO_4)	Amino acids concentration (g/100 ml)
1	−1	−1	−1	−1	3.40
2	1	−1	−1	−1	4.41
3	−1	1	−1	−1	3.45
4	1	1	−1	−1	4.57
5	−1	−1	1	−1	4.20
6	1	−1	1	−1	5.44
7	−1	1	1	−1	4.20
8	1	1	1	−1	5.41
9	−1	−1	−1	1	2.90
10	1	−1	−1	1	4.48
11	−1	1	−1	1	4.02
12	1	1	−1	1	4.27
13	−1	−1	1	1	4.36
14	1	−1	1	1	5.41
15	−1	1	1	1	4.75
16	1	1	1	1	5.73
17	0	0	0	0	4.61
18	0	0	0	0	4.63
19	0	0	0	0	4.57
20	0	0	0	0	4.67

Table 3 Results of the regression analysis of the full fractional design

Term	Coefficient	*t*-value	Significant level
Intercept	4.4740	84.825	<0.0001**
X_1	0.5275	8.945	<0.0001**
X_2	0.1125	1.908	0.0758
X_3	0.5000	8.479	<0.0001**
X_4	0.0525	0.89	0.3874
$R^2 = 0.9125$, $F = 39.086$, Prob > $F = 0.0001$			

** Meant significant at the 0.01 level

would have positive effects on biodegradation of feather into amino acids. The path of the steepest ascent was determined to find the proper direction of changing variables, increasing the concentration of feather powder and $(NH_4)_2SO_4$ to improve the formation of amino acids, while the other components were fixed at zero level. One basal increment in the concentrations of feather powder and $(NH_4)_2SO_4$ was set at 2 and 0.2 g/100 ml each time, respectively. The experimental design and results of the steepest ascent are shown in Table 4. From Table 4, it was evident that the yield plateau was reached at the fifth step. Therefore, concentration of feather powder ($X_1 = 18$ g/100 ml) and $(NH_4)_2SO_4$ ($X_3 = 1.4$ g/100 ml) was chosen as the central point for the further experiments of central composite design.

Central composite design

The optimal concentration of media components was determined by using a central composite design with

Table 4 Experimental design and results of the steepest ascent

Run	X_1 (feather powder, g/100 ml)	X_3 ($(NH_4)_2SO_4$, g/100 ml)	Amino acids concentration (g/100 ml)
1	10	0.6	4.29
2	12	0.8	4.78
3	14	1.0	5.12
4	16	1.2	5.43
5	18	1.4	5.98
6	20	1.6	5.42
7	22	1.8	5.12
8	24	2.0	4.98

Table 5 Design and results of central composite design

Run	X_1 (feather powder)		X_3 ($(NH_4)_2SO_4$)		Amino acids concentration (g/100 ml)
	Level	Value (g/100 ml)	Level	Value (g/100 ml)	
1	+1	22	+1	1.7	5.65
2	+1	22	−1	1.1	5.37
3	−1	14	+1	1.7	5.19
4	−1	14	−1	1.1	4.89
5	−1.414	12.34	0	1.4	5.21
6	+1.414	23.66	0	1.4	5.57
7	0	18	−1.414	0.98	4.78
8	0	18	+1.414	1.82	5.41
9	0	18	0	1.4	5.98
10	0	18	0	1.4	5.92
11	0	18	0	1.4	5.87
12	0	18	0	1.4	5.96
13	0	18	0	1.4	5.93
14	0	18	0	1.4	5.89

the two variables, feather powder and $(NH_4)_2SO_4$. The levels of the two variable factors and the experimental results are presented in Table 5. Based on the levels of the two variables and the experimental results (Table 5), the regression analysis accomplished by Data Processing System software is summarized in Table 6.

From the results of statistical analysis, the significant terms (significant at the 0.01 level) were as follows: X_1, X_3, X_1^2 and X_3^2. Accordingly, the following second-order model for predicting amino acids concentration in fermentation broth after 96 h of incubation was obtained:

Table 6 Results of the regression analysis of central composite design

Term	Estimate	*t*-value	Pr (> |*t*|)
Intercept	−10.054951	−8.419	<0.0001**
X_1	0.629556	8.022	<0.0001**
X_3	13.536676	12.621	<0.0001**
$X_1 \times X_1$	−0.016068	−9.054	<0.0001**
$X_3 \times X_3$	−4.587984	−14.307	<0.0001**
$X_1 \times X_3$	−0.004167	−0.129	0.9
$R^2 = 0.9782$, $F = 71.72$, Prob > $F = 0.0001$			

** Meant significant at the 0.01 level

$$Y(\mathrm{g}/100\,\mathrm{ml}) = -10.054951 + 0.629556X_1 + 13.536676X_3 - 0.004167X_1 \times X_3 - 0.016068X_1^2 - 4.587984X_3^2 \quad (2)$$

In Eq. (2), Y was the amino acids concentration, and X_1 and X_3 were the actual concentrations of feather powder and $(NH_4)_2SO_4$ in fermentation medium, respectively. This regression model for amino acids concentration was highly significant ($P < 0.0001$) with a satisfactory value of determination coefficient ($R^2 = 0.9782$). Therefore, the Student t-test of regression demonstrated that the second-order model was adequate for the results obtained in experiments. The resulting response surface showed the effect of feather powder and $(NH_4)_2SO_4$ on the formation of amino acids, as shown in Fig. 1. These results demonstrated the response surface had a maximum point.

From equations derived by differentiation of the second-order model, the optimal values of the model could be obtained as follows: $X_1 = 19.504$, $X_3 = 1.467$. Thereby, the theoretical maximum of amino acid was 6.107 g/100 ml, when the feather powder and $(NH_4)_2SO_4$ were 19.504 and 1.467 g/100 ml, respectively. To validate the second-order model, four replicated experiments were performed at optimal point of feather powder and $(NH_4)_2SO_4$, and the average amino acids concentration obtained in the experiments with optimal medium was 6.13 g/100 ml, which was close to the optimum value predicted by the model. The good correlation between the experimental and predicted results demonstrated that the second-order model was a valid model for predicting amino acids concentration obtained in feather biodegradation.

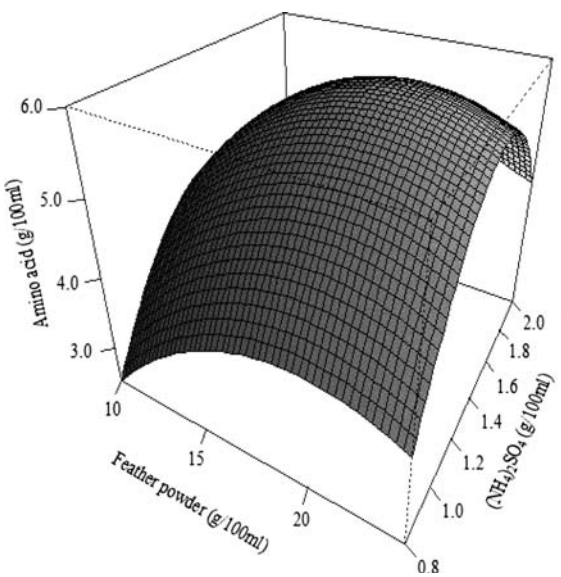

Fig. 1 The three-dimensional presentation of the response surface for the concentrations of feather powder and $(NH_4)_2SO_4$ on amino acids concentration

Statistically based experimental design is a more efficient approach to deal with a large number of variables compared with the traditional "one-factor at a time" technique (Zhuang et al. 2006). As a useful statistical technique, response surface methodology has widely and successfully been applied to the optimization of the medium components (Hao et al. 2006; Wu et al. 2007). At present, large research was aimed to isolate the feather-degrading microorganisms and investigated the characterization of feather-degrading enzyme (Matsui et al. 2009; Kojima et al. 2006). However, medium optimization to obtain amino acids by feather-biodegradation was little reported. In the present work, the fermentation medium of *S. fradiae Var S-221* was optimized for feather-biodegradation by response surface methodology. As a result, the amino acids concentration obtained under the optimal medium was 32.97% higher than that obtained with the original medium recipe.

Conclusions

The present study using the full fractional design and central composite design proved to be valuable tools in optimizing medium for amino acids concentration by feather-biodegrading *Streptomyces fradiae Var S-221*. The final composition of the medium for *S. fradiae Var S-221* fermentation was as follows (g/100 ml): feather powder, 19.504; beet molasses, 4.0; $(NH_4)_2SO_4$, 1.467; KH_2PO_4, 0.3; $MgSO_4$, 0.15; $FeSO_4$, 0.001; $ZnSO_4$, 0.0001; and $MnSO_4$, 0.0001. Using this optimization strategy, the amino acids concentration obtained by feather-biodegradation was increased from 4.61 to 6.13 g/100 ml.

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