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Influence of medium components on elastase production using crude sources by *Bacillus* sp. EL31410*

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Abstract: A newly isolated strain EL31410, producing elastase (E.C3.4.4.7) with high elastolytic activity was identified as *Bacillus* sp. In the medium optimization, it was found that wheat bran and soybean flour hydrosate were the best crude carbon and nitrogen source for enzyme production, respectively. Addition of corn steep flour can affect the bacterium growth and elastase production. A fractional factorial design was applied to study the main factors that affect the enzyme production, and central composite experimental design and response surface methodology were adopted to derive a statistical model for the effect of wheat bran and soybean flour hydrosate on elastase production. The experimental results showed that wheat bran had positive effect but soybean flour hydrosate had negative effect, on enzyme production. An initial concentration of 3.4%(w/v) wheat bran and 9.4%(v/v) soybean flour hydrosate were found to be optimal for enzyme production in batch culture. The time course of elastase production in the optimized medium composition was also described.

Key words: Elastase, *Bacillus* sp. EL31410, Crude sources, Medium optimization, Fractional factorial design (FFD), Central composite design (CCD), Response surface methodology (RSM), Batch cultivation

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INTRODUCTION

An increasing demand from consumers for healthy and low cost medicals has led to the development of alternative medical therapy agent such as elastase. At present elastase is mainly derived from the pig and other animal organs. The shortage of enzyme source limited its applications to medicine and food processing; and so, necessated study of microbial elastase as an alternative.

Elastase can degrade elastin (Morihara, 1967), so it has broad applications in medical therapy, food processing and daily use chemicals industry. Study of the reaction between elastin and elastase had been primarily directed to purified pancreatic elastase. The reaction was found to be a complex process of at least three steps. Among the microbial elastases, the elastase produced by *P. aeruginosa* had been thoroughly studied. *P. aeruginosa* elastase shows considerable amino acid sequence homology with other microbial neutral metalloproteinases, especially thermolysin(Saulnier et al., 1989). Its three-dimensional structure after crystallization was determined by Thayer (1991). In recent years, many researchers found that some gram-negative and gram-positive bacteria can secrete elastase, especially gram-negative species (Janda and Abbott, 1999; Tsuzuki and Oka, 1965; Ozaki and Shiio, 1975). However, few of them have potency for industrial applications due to their low elastase activity.

So far, many authors have focused their efforts on the isolation and screening of microorganisms for enzyme production with high elastase activity, and on purifying and characterizing newly found enzymes (Shibata et al., 1993; Tsai

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et al., 1988; Zins et al., 2001). In contrast, there are few reports on culture medium optimization in order to increase elastase production and improve application effects. We screened the elastase-producing strains and studied the nutritional conditions of cultivation.

Carbon and nitrogen sources, inorganic salts, and growth factors are important variables affecting the growth and products of microbe. It is difficult to find the most important factors and to optimize. It is well known that medium optimization is approached by either empirical or statistical methods. But the classical or empirical methods have several limitations towards complete optimization. The traditional one-factor at a time approach to optimization is time-consuming and incapable of reaching the true optimum due especially to interactions among factors. Moreover, it assumes that the various fermentation parameters do not interact and that the process response is a direct function of the single varied parameter. In contrast, the observed behavior of fermentation results from the interactive influences of the various variables. Unlike conventional optimization, statistical optimization methods can take into account the interaction of variables in generating the process response (Haaland, 1989). Factorial design of optimization experiments is especially suitable to account for the interactions. A combination of factors generating a certain optimum response can be identified through factorial design and the use of response surface methodology (Khuri and Cornell, 1987). Response surface methodology, an experimental strategy for seeking the optimum conditions for a multivariable system, described first by Box and Wilson (1951), is a much more efficient technique for optimization (Box and Hunter, 1978). This method had been successfully applied to the optimization of medium compositions (Roseiro, 1992; Souza and Roberto, 1999), conditions of enzymatic hydrolysis (Ma and Ooraikul, 1986), parameters of food preservation (King, 1993) and fermentation processes (Rosi et al., 1987; Kalil et al., 2000) and wastewater process (Hwang et al., 2001). This pattern was designed by using statistical methods to yield the most information from a minimum number of experiments.

The aim of this work was to study the possibility of using crude material as culture medium. Fractional factorial design (FFD) was applied to identify the most important components in the media makeup, then focused on the critical subset of media components; and finally used central composite design to examine and optimize the culture media for elastase production by *Bacillus* sp. EL31410 in shaking flask experiments.

MATERIALS AND METHODS

1. Microorganism and culture media

The method for screening microorganism described by Shiio et al. (1974) was modified and used in this study. We screened and identified this elastase-producing strain *Bacillus* sp., then used several ways to mutate the strain, and obtained a positive mutant strain with high elastase activity.

All optimization experiments were carried out in unbaffled 250-ml Erlenmeyer flasks. The seed medium contained: peptone 6, yeast extract 2, beef extract 4, NaCl 5, pH 7.5. The culture conditions in the experiments were designed as follows. The basic culture medium (g/L) contained wheat bran flour 40, soybean flour 30, corn steep flour 1, K_2HPO_4 1 and $MgSO_4 \cdot 7H_2O$ 0.1 (initial pH 7.5). Cultivation medium was changed according to experimental design. Erlenmeyer flasks (250 ml) containing 25 ml of culture medium were inoculated with 5% (v/v) seed culture, then incubated for 24 hours at 37℃ on rotary shaker (200 r/min). All media were sterilized at 121℃ for 20 minutes, and cooled to room temperature prior to use.

2. Assays

Elastolytic activity was assayed by the colorimetric method of Sachar (1955). Fermentation broth was recovered after batch cultivation and centrifuged at 6000 r/min for 15 minutes. Then the supernatant was suitably diluted. Enzyme preparation was incubated with 20 mg of congo-red elastin in 2 ml of 0.2 mol/L boric acid buffer (pH 7.4) with shaking for 20 minutes at 37℃. The reaction was stopped by adding 2 ml of 0.7 mol/L sodium phosphate buffer (pH 6.0), and immediately filtrated. Absorbency of the filtrate was read at 495 nm against a control (no enzyme). One unit of elastase activity was defined

as the amount of enzyme required to solubilize 20 mg elastin-congo red under the tested conditions. The standard curve was made by the method of Sachar.

The protease activity was assayed as the reduction of casein at 40℃ at pH 9.0 for 10 minutes according to the method described previously. Reducing sugar was measured with the DNS method of Miller (1959), pH was measured with pH meter.

3. Experimental design

A series of statistically designed studies were conducted to investigate the effect of various media components on elastase activity to optimize the media. The optimization process firstly entails identifying the most important components in the media ingredients using fractional factorial design, and then focuses on the critical subset of media components. The steepest ascent design was to use to determine the direction toward predicted higher responses. Finally a central composite design was derived to optimize the critical components and maximize the elastase activity (Cockshott and Sullivan, 2001).

A 2^{6-2} fractional factorial design leading to 16 sets of experiments, performed in duplicate, was used to verify the most significant factors affecting the elastase activity. The variables were coded according to the following equation (Montgomery, 1991):

$$\chi_i = (X_i - X_0)/\Delta\chi_i$$

Where χ_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 $\Delta\chi_i$ is the step change value.

The range and level of the variables investigated in this study are given in Table 4. The elastase activity was considered as the dependent variable or response, Y_i. Based on the first-order model equation obtained by the FFD used, a new series of trials were performed in the direction of the steepest ascent.

In order to fit the empirical second-order polynomial model, a central composition design with five coded levels was performed. The quadratic model for predicting the optimal point was expressed according as:

$$y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_j x_j \quad (1)$$

Where y is the response variable, b the regression coefficient, and x the coded levels of the independent variable.

A full second-order polynomial model obtained by a multiple regression technique for two factors using the SAS Package (SAS Institute, Inc, Cary, NC, USA) was adopted to describe the response surface.

RESULTS AND DISCUSSION

1. Effect of carbon sources on enzyme production

The effects of various crude carbon sources on the production of elastase by *Bacillus* sp. EL31410 are illustrated in Table 1. Wheat bran

Table 1 Effect of various carbon sources on enzyme production[a]

Carbon source (%, w/v)	Fermentation time (hours)	EA[c] (U/ml)	PA[d] (U/ml)	pH
Wheat bran	24	31	385	
	48	78	1632	8.73
Straw flour	24	65	1194	
	48	57	1397	8.86
Corn flour	24	6	78	
	48	45	403	7.22
Soybean flour	24	31	515	
	48	51	751	9.01
Glucose	24	43	636	
	48	113	2295	6.28

[a] The medium consisted of soybean flour 3%(w/v), corn steep flour 0.1%(w/v), K_2HPO_4 0.1% (w/v) and $MgSO_4 \cdot 7H_2O$ 0.01% (w/v) (initial pH 7.5); [c] EA means elastase activity, [d]PA means protease activity.

was found to be the best substitute carbon for glucose. Alkaline protease activity changed similarly as that of elastase production. It had been reported that protease could induce elastase production and elastase processing. Corn flour is very poor carbon source for cell growth and elastase production. This had been reported in other studies. The experimental results showed that 48 hours cultivation time is better than 24 hours fermentation in terms of resulting elastase and protease activity. Among the carbon sources tested, only wheat bran has growth factors very beneficial for cell growth and elastase production by *Bacillus* sp. EL31410. Soybean flour is not good carbon source for elastase production, probably because soybean flour contains too much nitrogen composition than required of carbon source; and at the same time contains the inhibition factors of protease.

2. Effects of glucose as part of carbon source on elastase production

The carbon source choosen was 4% wheat bran. And the glucose added resulted in enzyme production listed in Table 2 showing that elastase activity increases with increasing glucose addition; and that the protease changes similarly as elastase production. It was thought that much more carbon can increase the cell growth. Considering the medium cost and fermentation time, there was no need to add glucose. However, it is possible that glucose can be added when carring out fed-batch cultivation.

Table 2 The effect of adding glucose into the medium on enzyme production

Medium	EA (U/ml)	PA (U/ml)
2% glucose	106	1627
3% glucose	137	1892
4% glucose	173	2508
control	67	1497

3. Effects of various nitrogen sources on enzyme production

The nitrogen source and carbon source are two important factors affecting cell growth and enzyme production of microorganisms. The effects of various nitrogen sources on elastase production by *Bacillus* sp. EL31410 are listed in Table 3 showing that hydrolysate of soybean flour is the best crude nitrogen source. This had been reported in another study on alkanine elastase production (Takagi et al., 1995). Soybean flour hydrosate has been proved useful for elastase production; contains abundant protein and amino acid after its hydrolysis; and also contains some Ca ions verified to be beneficial for elastase production and stabilization (Chen and He, 2002).

Table 3 Effects of nitrogen sources on enzyme production[b]

Nitrogen source	Fermentation time(h)	EA (U/ml)	PA (U/ml)
Soybean flour	24	59	430
	48	68	889
Feather Flour	24	61	649
	48	92	1897
Elastin	24	84	1108
	48	33	1459
Hydrosate of soybean flour (%, v/v)	24	76	1924
	48	132	3608
Casein	24	139	ND[c]
	48	196	ND[c]

[b] The medium consisted of wheat bran 4% (w/v), corn steep flour 0.1% (w/v), K_2HPO_4 0.1% (w/v) and $MgSO_4\ 7H_2O$ 0.01% (w/v) (initial pH 7.5); [c] ND: not determined

4. The optimization of cultivation medium for enzyme production

The factorial design approach to medium development relies on three stages of experimentation: screening, optimization, and verification. Screening aims at reducing the problem to determination of which few process variables have the greatest impact on performance. Optimization experiments are designed to provide in-depth information about a few variables identified during screening as having the greatest impact on performance. Finally, verification experiments were used to validate the results under specific experimental conditions. According to previous study, 4% (w/v) wheat bran and 8% (v/v) soybean flour hydrosate were chosen as carbon source and nitrogen source, respectively.

(1) Screening experiments: the fractional factorial design and analysis

The screening experiments were designed to evaluate the impact of five factors, concentrations of the carbon source, the nitrogen source, corn steep flour, potassium dihydrogen phos-

phate, and magnesium sulfate. A two-level fractional factorial design was employed; the results of the fractional factorial design are shown in Table 4 and Table 5. These results suggest that these variables significantly affect elastase activity. As can be seen from Table 6, the factors wheat bran (x_1) and corn flour hydrosate (x_2) were found to be significant at probability level of 95% and 80% for elastase production, respectively. The main effects of these variables were negative (x_1) and positive (x_2), respectively. There is no evidence of any interactions.

Table 4 Range of values for the FFD

Independent variables	Variable name	−1	0	+1
X_1(g/100ml)	Wheat bran	2	4	6
X_2(ml/100ml)	Hydrosate liquid of corn flour	4	8	12
X_3(g/100ml)	Corn steep flour	0.05	0.1	0.15
X_4(g/100ml)	K_2HPO_4	0.1	0.2	0.3
X_5(g/100ml)	$MgSO_47H_2O$	0.01	0.02	0.03

Table 5 Experimental designs and the results of the FFD[a]

Run	χ_1	χ_2	χ_3	χ_4	χ_5	EA (U/ml)	PA (U/ml)
1	6	4	0.15	0.1	0.01	35	381
2	2	12	0.15	0.1	0.03	167	2834
3	6	4	0.05	0.3	0.03	35	264
4	6	12	0.15	0.3	0.03	57	503
5	2	4	0.15	0.3	0.01	114	1086
6	2	12	0.05	0.3	0.01	151	2161
7	2	4	0.05	0.1	0.03	105	1492
8	6	12	0.05	0.1	0.01	20	134
9	4	8	0.1	0.2	0.02	191	1241
10	4	8	0.1	0.2	0.02	181	1496
11	4	8	0.1	0.2	0.02	185	1512

[a] Results are from three replicates.

The effects of wheat bran, soybean flour hydrosate liquid, corn steep flour, potassium dihydrogen phosphate and magnesium sulfate concentrations on elastase yield were also analyzed by multiple regression techniques. The values of the regression coefficients were calculated and an equation of the first order was attained with a very high coefficient of determination, $R^2 = 0.9538$.

Table 6 Results of parameter estimates for elastase production (Y_1)

Term	Parameter estimate	T for H0:	$P > T$
intercept	0.272	3.18	0.0057
x_1	−0.125	−6.07	0.0261
x_2	0.035	1.696	0.20
x_3	0.021	1.006	0.4205
x_4	0.009	0.436	0.7053
x_5	0.0131	0.642	0.5866

Table 7 ANOVA results for elastase production obtained from FFD(Y_1)

Surce	DF	Sum of square	F-Ratio	$P > F$	R-square
Model	5	0.1408	8.267	0.10	0.9538
Error	2	0.0068			
C total	7	0.1476			

The results of t-test for variance between average of observation of two-level experiment and center point showed that the difference was not significant (Table 8). This result indicated the optimum point was not in the domain of our experiment. Experimentation of steepest ascent path is necessary to reach optimum domain.

Table 8 T-Test for elastase production obtained from FFD

X	N	Mean	Std dev	Std error	Minimum	Maximum
1	8	0.27200000	0.14521413	0.05134095	0.10500000	0.48100000
2	4	0.42275000	0.21077219	0.10538610	0.10700000	0.54200000
Variances		T	DF	$P > \|T\|$		
Unequal		-1.2860	4.5	0.2615		
Equal		-1.4689	10.0	0.1726		
For H0: Variances are equal,		$F' = 2.11$			$P > F' = 0.3756$	

(2) Steepest ascent experiment and analysis

The results in Table 5 and Table 8 clearly showed that the optimal region is outside the current design space. In this situation, a directional search method, like steepest ascent, can be used to determine the next set of experiments. Steepest ascent is a method using the magnitude and sign of the linear effects to determine the direction toward predictive higher responses. The path begins at the center of the current design space and stretches well outside the design space. A sequence of equally spaced locations along the path is then selected which form a set of experiments. Thus the path of steepest ascent was aimed to increase hydrosate of soybean flour and decrease wheat bran in order to improve elastase production. The other factors were fixed at zero level. The values of elastase activity obtained in these experiments are summarized in Table 9 clearly showing that elastase activity increased when the soybean flour hydrosate concentration increased and wheat bran concentration decreased. After the second step on the path, further experimentation could not increase elastase activity. The highest enzyme activity was achieved in the second step at 48 hours cultivation. And protease production also changed similarly as elastase production. This further verifies that protease production can affect elastase production during the fermentation period in some way. According to the pH change, it is clearly demonstrated that the final culture pH increases as soybean flour hydrosate increases. However, it was found that too high pH is not beneficial for elastase production and elastase stability in my preliminary studies. These results indicated that the experimentation was approximating the neighborhood of the optimum elastase activity.

Table 9 Experimental designs of the steepest ascent and corresponding responses

Run	X_2 (wheat bran)	X_3 (hydrosate of soybean flour)	Elastase activity (U/ml) 48 hours	Protease activity (U/ml) 48 hours	pH
1	4.0%	8%	194	2870	5.25
2	3.5%	9%	216	3089	8.30
3	3.0%	10%	163	2586	8.45
4	2.5%	11%	100	1131	8.76
5	2.0%	12%	80	965	8.90
6	1.0%	13%	47	474	9.10

(3) Response surface methodology

A response surface design is appropriate when the optimal region for running the process has been identified. A Box-Wilson central composite design with four-star points and five replicates at center point was used for optimizing elastase production for each of the two factors. Table 5 shows the design of this experiment and the results. Regression analysis was conducted to fit the response function with the experimental data. An F-test (ANOVA) checked the statistical significance of the second-order model equation and data are shown in Table 10. The fit value, termed R^2, of the polynomial model was calculated to be 0.73, indicating that 73% of the variability in the response could be explained by the second-order polynomial prediction equation given below (Fig. 1a). ANOVA results

showed that this model was appropriate. It was also suggested that the crossproduct and linear terms of wheat bran and soybean flour hydrosate of the model primarily determined elastase production by *Bacillus* sp. EL31410. And wheat bran (x_1) was next to $x_1 * x_2$. The equation for elastase production showed positive linear effect and negative quadratic effect. And both these factors also changed protease production and pH in the same way as that of elastase production. This can be seen in Fig. 1b and Fig. 1c.

Table 10 Experimental design and results of the central composite design (CCD)[b]

Run	Factors*		Elastase activity (Y_1) (U/ml)	Protease activity (Y_2) (U/ml)	pH (Y_3)
	χ_1	χ_2			
1	-1	-1	113	2303	8.40
2	1	-1	254	4270	8.40
3	-1	1	158	2389	8.64
4	1	1	44	470	5.85
5	-1.414	0	127	1627	8.44
6	1.414	0	255	5454	5.43
7	0	-1.414	188	3654	8.01
8	0	1.414	232	4092	8.35
9	0	0	172	3897	8.42
10	0	0	204	4130	8.60
11	0	0	166	3838	8.53
12	0	0	209	5076	8.50
13	0	0	202	4093	8.35

* $\chi_1 = (X_1 - 3.5)/1.0$; $\chi_2 = (X_2 - 9.00)/1.50$ [b] Results are from three replicates.

Table 11 Results of parameter estimate for elastase production (Y_1)

Term	DF	T for H0:	$P > T$
Intercept	1	10.034	0
x_1	1	2.008	0.085
x_2	1	-0.552	0.598
$x_1 * x_1$	1	-0.545	0.602
$x_1 * x_2$	1	-3.734	0.007
$x_2 * x_2$	1	-0.048	0.963

Table 12 ANOVA results for elastase production obtained from CCD (Y_1)

Regression	DF	Type of sum of square	R-square	F-ratio	$P > F$
Linear	2	0.063	0.1696	2.169	0.1849
Quadratic	2	0.004	0.0117	0.149	0.8642
Crossproduct	1	0.203	0.5451	13.942	0.0073
Total Regression	5	0.270	0.7263	3.716	0.0582

The three-dimensional graph obtained from the calculated contour plots is shown in Fig. 1. The three-dimensional contour plots of wheat bran and soybean flour hydrosate concentrations against elastase activity, protease activity and pH can further explain the results of the statistical and mathematical analyses. It is evident from the plot that elastase activity reached maximum at a combination of uncoded level 3.42% (x_1) and 9.4% (x_2). Both factors had different effects on protease production. Increasing wheat bran and decreasing soybean flour hydrosate, it can increase elastase production. Fig. 1 shows the effect of x_1 and x_2 on pH. With increasing concentration of soybean flour hydrosate, the final culture pH also increased. This can be explained by the fact that soybean flour hydrosate

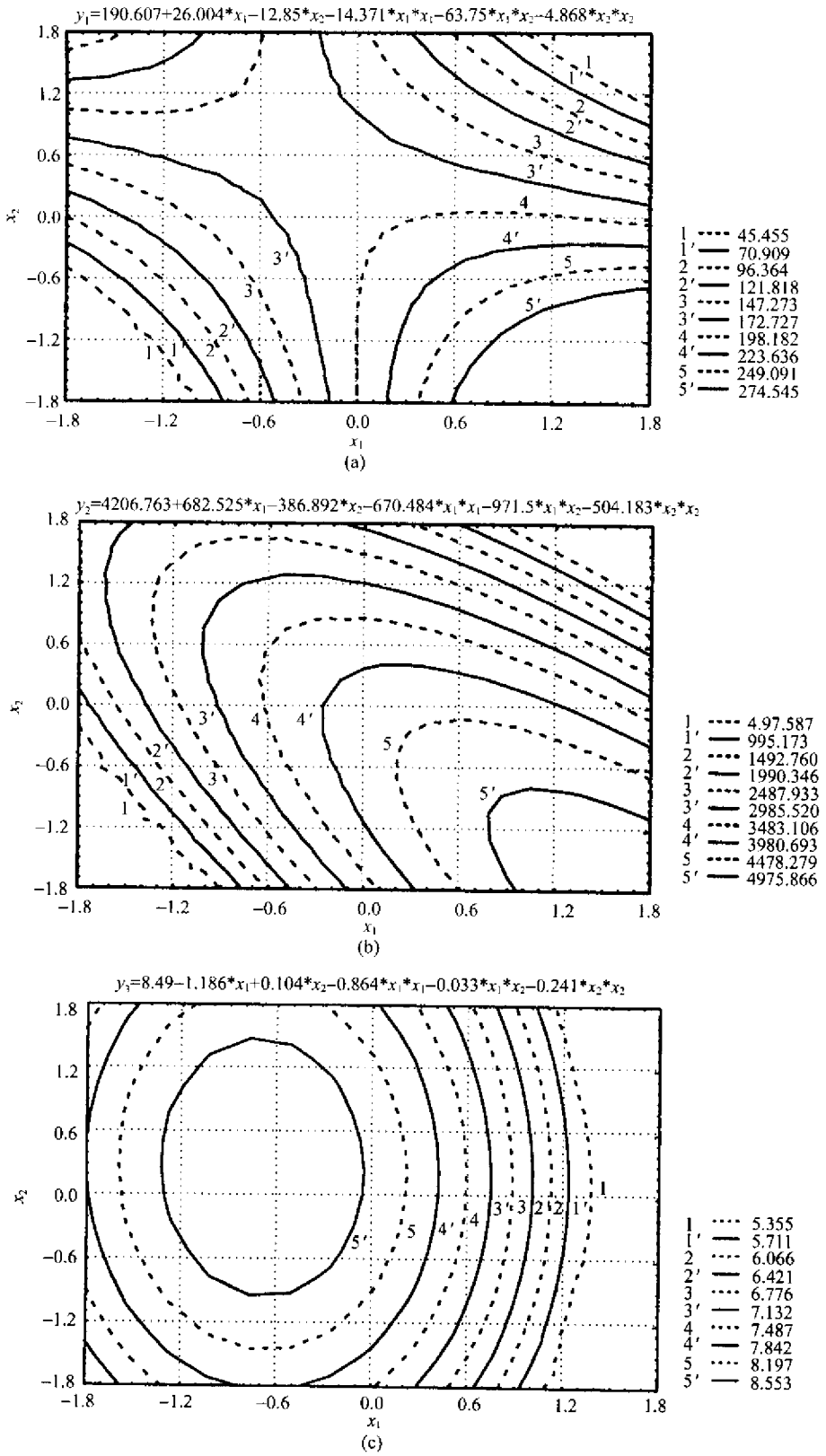

Fig.1 Contour plots for the effects of wheat bran (x_1) and soybean flour hydrosate (x_2) on (a) elastase activity (U/ml); (b) protease activity (pH9, U/ml); (c) pH

contains many peptides and amino acids or other alkaline substances. These substances can be degraded by this strain and produce large quantities of alkaline metabolic substances.

5. The time course of elastase production by *Bacillus* sp. EL31410 cultivated in medium with optimized compositions

The time course of elastase production by *Bacillus* sp. EL31410 cultivated in optimized medium is shown in Fig. 2. Elastase production reached maximum after 24 hours cultivation. While protease production reached maximum after 36 hours cultivation. At 54 hours cultivation, both elastase production and protease production decreased, and then increased somewhat. This may be considered as due to the complex compositions of the medium, especially when it contains various carbon sources. This was reported in my preliminary studies (Chen and He, 2002). The culture pH determined during the cultivation ranged from 5-8. It could also be seen that the alkaline cultivation condition was favorable for enzyme production. Both elastase and alkaline protease production appeared to have the same change direction. However, still under investigation is whether the protease production had positive effects on the elastase. The reduced sugar consumption rate was a strange phenomenon still not understood. The interaction between elastase production and strain growth is not clear, and requires research by culture kinetics experiments.

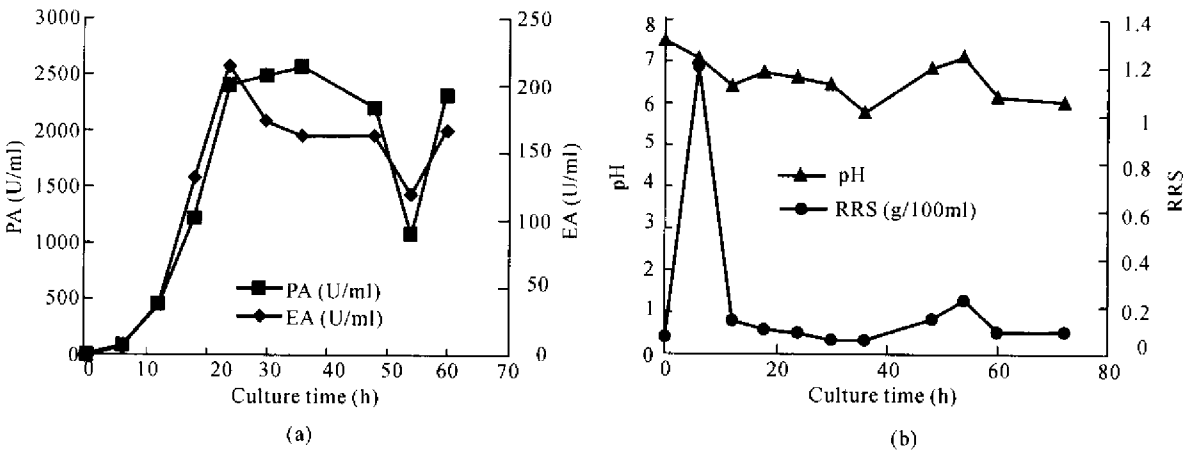

Fig. 2 The curve of *Bacillus* sp. EL31410 cultivation with optimized culture medium: (a) enzyme production; (b) pH and residual reduced sugar (RRS) change

CONCLUSIONS

Batch cultures of *Bacillus* sp. EL31410 were investigated and optimized for the production of microbial elastase. The types of nitrogen source and carbon source affect elastase and protease production. Wheat bran is a suitable carbon source; soybean flour hydrosate is the best nitrogen source; corn steep flour as the best growth factor can induce elastase production. Through statistically designed optimization, elastase production could be increased from an average of 66 U/ml in screening experiments to an average of 171 U/ml in the optimization experiments. The experiment results also revealed that protease production could affect the elastase production, both changed in the same way. Fig. 2 shows that the maximum elastase production was at 24 hours cultivation.

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