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Improved elastase production by *Bacillus* sp. EL31410 —further optimization and kinetics studies of culture medium for batch fermentation*

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Abstract: An efficient culture medium producing a bacterial elastase with high yields was developed further following preliminary studies by means of response surface method. Central composite design (CCD) and response surface methodology were applied to optimize the medium constituents. A central composite design was used to explain the combined effect of three medium constituents, viz, glucose, K_2HPO_4, $MgSO_4 \cdot 7H_2O$. The strain produced more elastase in the completely optimized medium, as compared with the partially optimized medium. The fitted model of the second model, as per RSM, showed that glucose was 7.4 g/100 ml, casein 1.13 g/100 ml, corn steep flour 0.616 g/100 ml, K_2HPO_4 0.206 g/100 ml and $MgSO_4 \cdot 7H_2O$ 0.034 g/100 ml. The fermentation kinetics of these two culture media in the flask experiments were analyzed. It was found that the highest elastase productivity occurred at 54 hours. Higher glucose concentration had inhibitory effect on elastase production. At the same time, we observed that the glucose consumption rate was slow in the completely optimized medium, which can explain the lag period of the highest elastase production. Some metal ions and surfactant additives also affected elastase production and cell growth.

Key words: Elastase, *Bacillus* sp. EL31410, Culture medium optimization, Central composite design, Response surface methodology, Batch fermentation, Fermentation kinetics studies

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INTRODUCTION

Elastase is an enzyme that attacks and solubilizes elastin. As elastase can degrade elastin (Morihara, 1967) that other proteases cannot; it has broad applications in medical therapy, food processing and daily use chemicals industry. Considerable efforts were made to screen the elastase-producing strains, to study its pathogen effect and its characterizations (Tsuzuki and Oka, 1965; Tsai *et al.*, 1988; Sharon *et al.*, 1997; Ozaki and Shiio, 1975). Shiio, 1975). Reported studies on the reaction mode between elastin and elastase were rare (Hall and Czerkawaki, 1961). The reaction was found to be a complex process of at least three steps (Heather *et al.*, 1999). The mechanism of the reaction is still not clear (Robert *et al.*, 1997).

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. Its three-dimensional structure was determined by Thayer (Thayer, 1991; Joelle *et al.*, 1989) after crystallization.

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So far, many authors have focused their efforts on isolating and screening of microorganisms for elastase production with higher activity and on purifying and characterizing newly found enzymes (Janda and Sharon, 1999; Tsuzuki and Oka, 1965; Ozaki and Shiio, 1975; Shibata *et al.,* 1993; Tsai *et al.*, 1988). To achieve high product yields, it is a prerequisite to design a proper production medium in an efficient fermentation process. Little information, however, is available in the scientific literature on complete optimization of culture media for elastase production. It is well known that medium optimization is approached either empirically or by statistical methods. But either the classical or empirical method has several problems for 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 interaction 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 for taking into account the interactions and it is efficient. A combination of factors that generate a certain optimum response can be identified through factorial design and the use of response surface methodology (Khuri and Cornell, 1987). Response surface methodology, described first by Box and Wilson (1951), is a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables (Box and Hunter, 1978). This method had been successfully applied in the optimization of medium compositions (Souza and Roberto, 1999; Sarra *et al.*, 1993; Roseiro, 1992), conditions of enzymatic hydrolysis (Ma and Ooraikul, 1986), parameters of food preservation (King, 1993), and fermentation processes (Rosi *et al.*, 1987; Ramírez and Anne, 2001). A central composite factorial experimental design was also used for the medium optimization reported here. In this approach, concentrations of medium components are the variables; each variable is referred to some base value and varied in a certain pattern.

Our preliminary studies showed that the most important factors in the culture medium are the casein and corn steep flour. And these two factors were also optimized with response surface methodology (Chen and He, 2002). This paper further reports an attempt to formulate a suitable production medium that can substantially reduce the medium cost and increase the elastase production from *Bacillus* sp. EL31410 by using statistical experimental design such as FFD (fractional factorial design) and RSM (response surface methodology), and also discusses the fermentation kinetics characteristics of completely optimized culture medium.

MATERIALS AND METHODS

Microbial strain

Bacillus sp. EL31410 was isolated from the soil at a meat-processing factory in Hangzhou, China. The modified method for screening microorganism (Shiio *et al.*, 1974) was used. A strain of *Bacillus* sp. that produced elastase was screened and identified as EL31410. Several ways such as violet and chemical agent were used to mutate the strain and obtain a positive mutant strain with high elastase activity. It was maintained on solid LB (g/L) slants (peptone 6, yeast extract 2, beef extract 4, NaCl 5, agar 2). The liquid seed medium contained the above components in addition to agar.

Shake-flask cultivations

All optimization experiments were carried out in 250-ml Erlenmeyer flasks containing 25 ml of medium with various concentrations of glucose, K_2HPO_4, and $MgSO_4 \cdot 7H_2O$ according to the experimental design. Each medium contained a fixed amount of casein (11.3 g/L) and corn steep flour (6.16 g/L), which were optimized in the previous study (Chen and He, 2002). Each flask (250 ml) containing 20 ml of fermentation medium was inoculated with 5% (v/v) seed culture, then cultivated for 24 hours at 37 ºC on the rotary shaker (200 rev/min). All media were sterilized at 121 ºC for 20

min, and cooled to room temperature prior to use.

Assays

Elastolytic activity was assayed by the colorimetric method of Sachar (1955). Fermentation broth was recovered after batch fermentation and centrifuged at 1 500 g 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 ºC. The reaction was stopped by adding 2 ml of 0.7 mol/L sodium phosphate buffer (pH 6.0), and immediately filtered. 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 (1955).

Biomass content was evaluated by dry cell weight (g/L). The culture samples (10 ml) were centrifuged (1 500 g for 10 min), and the cell pellet was washed thoroughly with distilled water, dried to a constant weight at 80 ºC overnight and then cooled and weighed. Reducing sugar was measured with the DNS method (Miller, 1959); pH was measured with pH meter.

Experimental designs

The experimental designs used were shown in the reference (Chen and He, 2002).

RESULTS AND DISCUSSIONS

The factorial design for medium development relies on three stages of experimentation: screening, optimization and verification. Screening experiments include many variables, but provide little information per variable. Screening aims at reducing a determination as to 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 experiment was used to validate the results under specific experimental conditions. In the previous studies (Chen and He, 2002), the optimized culture medium composition (g/L) was as follows: glucose 40, casein 11.3, corn steep flour 6.16, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.1, pH 7.5 with FFD and RSM experiment. In order to further increase the elastase production and decrease the medium cost, the following optimization was carried out.

Further factorial optimization and experimental results

In order to reduce the cost of medium sources, we attempted to improve the compositions of the medium by comparing different levels of several factors that were found to have lesser influence on the production of elastase by *Bacillus* sp. EL31410 in factorial design. The factors included different concentrations of the medium component glucose, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$. We used the central composition design to find the optimal concentrations of the three factors. The experimental design and results of the central composite design are given in Tables 1 and 2 (see the next page). The results of the experiment with CCD were fitted with a second-order polynomial function. Six repeats were included at the center of the design, and the total number of this design was 20. The results of the regression analysis are shown in Table 3.

The statistical significance of the second-order model equation was checked by an *F*-test. It was found that it adequately approximated to the experimental data at the 5% probability level. The coefficient of determination R^2, was calculated to be 0.9564, which showed that the regression model was very suitable for describing elastase production. The results demonstrated that the response surface had a maximum point at the coded level $-1.212894(x_1)$, $0.035835(x_2)$ and $0.823588(x_3)$. The optimal medium composition was calculated to be 7.4% glucose, 0.206% K_2HPO_4 and 0.034% $MgSO_4 \cdot 7H_2O$. As can be seen from the response surface plot (Figs.1a, 1b and 1c), the elastase production can be increased when the concentration of glucose decreased and both the magnesium sulfate and K_2HPO_4 decreased. This model was tested for adequacy by the analysis of variance (Table 4). The regression model for elastase production was highly significant ($P<0.001$).

Table 1 Definition and levels of independent variables in central composite design

Independent variables	Symbol	Coded levels				
		−1.682	−1	0	1	1.682
Glucose	X_1	8.2955	10	12.5	15	16.70450
K_2HPO_4	X_2	0.03182	0.10	0.20	0.30	0.368180
$MgSO_4{\cdot}7H_2O$	X_3	0.003182	0.01	0.02	0.03	0.036818

Table 2 Design and experimental results of the central composite design

Run	X_1	X_2	X_3	Y_1 (EA* U/ml)	Y_2 (Biomass* g/L)
1	−1	−1	1	365	11.6
2	0	0	−1	262	7.7
3	1	−1	1	200	7.2
4	−1	1	1	365	7.9
5	1	1	1	211	8.8
6	0	1.682	0	313	8.2
7	1	1	−1	238	7.2
8	−1	−1	−1	357	7.7
9	0	0	1	297	8.6
10	1	−1	−1	258	6.5
11	0	−1.682	0	312	6.6
12	−1.682	0	0	337	7.9
13	1.682	0	0	193	10.0
14	−1	1	−1	314	6.1
15	0	0	0	329	6.9
16	0	0	0	313	6.9
17	0	0	0	332	6.8
18	0	0	0	320	6.7
19	0	0	0	325	6.8
20	0	0	0	318	6.8

* EA: elastase activity; Biomass: dry cell weight

Table 3 Regression coefficient, standard error, and student's *t*-test results for the composite design (CCD-U) with six center points

Parameter	DF	Parameter estimates	Standard error	$P>T$
Intercept	1	0.8792430	0.015869	0
x_1	1	−0.1376440	0.010528	0
x_2	1	−0.0097610	0.010528	0.3757
x_3	1	0.0061780	0.010528	0.5703
x_1x_1	1	−0.0490640	0.010248	0.0007
x_2x_1	1	0.0108750	0.013756	0.4476
x_2x_2	1	−0.0064720	0.010248	0.5418
x_3x_1	1	−0.0456250	0.013756	0.0078
x_3x_2	1	0.0236250	0.013756	0.1167
x_3x_3	1	−0.0363400	0.010248	0.0053

Table 4 Analysis of variance (ANOVA) for regression model of elastase production obtained from the response surface experiments

Regression	DF	Sum of square	R^2	F-ratio	P
Linear	3	0.260590	0.7511	57.377	0.000
Quadratic	3	0.049142	0.1416	10.820	0.0018
Crossproduct	3	0.022064	0.0636	4.858	0.0245
Total regression	9	0.331796	0.9564	24.352	0.0263

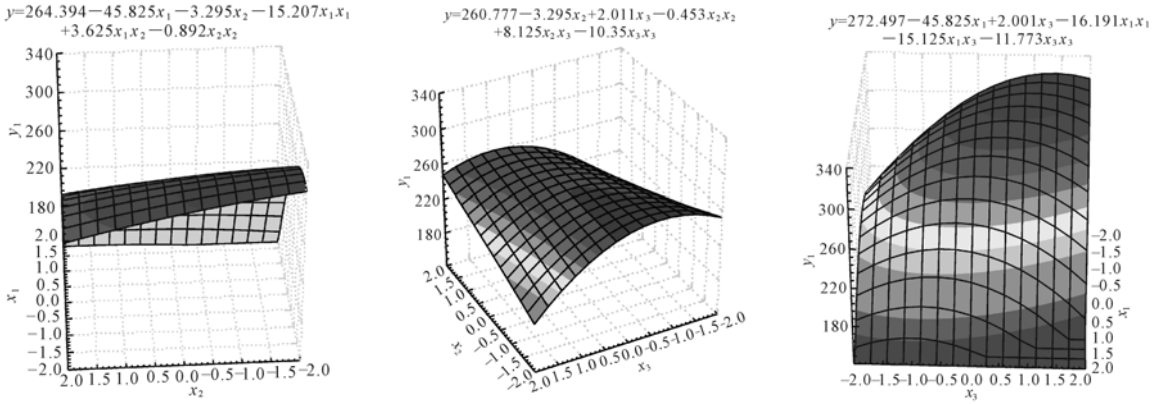

Fig.1 Response surface for the effects of (a) glucose (x_1) and K_2HPO_4 (x_2); (b) K_2HPO_4 (x_2) and magnesium sulfate (x_3); and (c) glucose (x_1) and magnesium sulfate (x_3) on elastase production (y_1)

Table 4 also suggests that elastase production was primarily determined by the linear terms and quadratic terms of the three factors. There existed interactions between the three factors. The results also showed that the response surface had a maximum point based on the response surface plot and the response model. The effects of the three variables on biomass were also seen according to the dry cell weight (data not shown). The effect was the same as that for elastase production. It was considered as the substrate inhibition during the elastase fermentation when the medium contained higher glucose. The K_2HPO_4 and $MgSO_4{\cdot}7H_2O$ all had positive effects on enzyme production, which may be due to the constant pH and the enzyme structure. It had been reported in other elastase-producing strains. The maximum response predicted by the model was 369 U/ml. The response equations obtained are as follows, and represent a suitable model for describing the effects of these three factors on elastase production.

To validate these predictions, flask cultivation using the completely optimized medium composition was done. The measured elastase activity (mean of three replicates) was 42% higher than that of the partially optimized medium.

Effects of various inorganic salts on enzyme production and cell growth

The effects of various inorganic salts are listed in Table 5. It was reported that phosphates are usually used as buffering reagents in order to keep the pH constant in culture medium, Ca^{2+}, Zn^{2+} and Fe^{2+} could increase the elastase production in the culture

Table 5 Effect of various inorganic salts on elastase production and cell growth

Metal ions	Concentration (g/100ml)	EA (U/ml)	Relative activity (%)	Biomass (g/L)
Ca^{2+}	0.01	295	112	7.35
	0.05	129	52	7.40
	0.10	46	22	7.50
Fe^{2+}	0.01	294	111	7.05
Zn^{2+}	0.01	264	101	7.35
	0.05	180	70	8.90
	0.10	153	60	7.60
Cu^{2+}	0.01	272	104	7.25
Control		277	100	7.40

medium, especially for gram-negative bacteria, as the Ca^{2+} and Zn^{2+} ions can keep the elastase stable. Mg^{2+} ion can increase the permeability of the cell wall, especially for the gram-positive bacteria. To find out the effects of Zn^{2+}, Ca^{2+} and Fe^{2+} ions on the cultivation of *Bacillus* sp. EL31410, a medium containing only the basic medium components that included casein/1.13% and corn steep flour/0.616% was tested. Results demonstrated that there was no significant difference in enzyme production and cell growth, but the morphology of bacterial growth changed greatly (data not shown). The enzyme production in the tested medium was in some ways increased, especially the effects of 0.01% Ca, Fe, Zn and Cu ions on the enzyme produced. When the concentration of several metal ions was above 0.01%, elastase production all decreased but the cell growth increased in some way. It was confirmed that Ca^{2+} and Zn^{2+} are required for catalysis and stabilization of elastase; reducing the availability of these ions should restrict autoprocessing of proelastase (Joan and Dennis, 1992). This had been supported in the *P.aeruginosa* strain. The low concentrations of Ca and Zn could induce elastase production and proelastase processing. Other metal ions increased the elastase activity in some way at the 0.01% concentration.

Effects of surfactants and polymeric additives

The effects of various polymer and surfactant additives on elastase production by *Bacillus* sp. EL31410 are shown in Table 6. It was found that 0.02% Tween80 had the maximal effect on elastase production. Both 0.1% SDS and 0.1% PEG had negative effect on elastase activity. It is possible that these substances can denature the elastase in the higher concentration. The cell growth also increased when the concentration of Tween80 increased. It may be thought that Tween80 can improve the gas availability. However, PEG and Gelatin showed different effects on cell growth. It was explained that the higher concentration of polymeric additives is not helpful for the bacteria growth, mainly owing to the high viscosity of polymeric compounds. The function of the surfactants and the polymeric additives in the fermentation broth is known to be very complicated. It was reported that addition of a polymer or surfactants could affect the rheological properties of the medium, availability of nutrients and gases, and physiological functions of the cell. The chemical structure, positioning of groups, degree of ionization in the medium, and charge density in the macromolecule would determine the effect of these changes (Chen and Li., 1996).

Table 6 Effect of surfactants and polymeric additives on elastase production and cell growth

Surfactants	Concentration (g/100ml)	EA (U/ml)	Relative activity (%)	Biomass (g/L)
Tween80	0.02	373	111	8.35
	0.04	351	104	9.65
	0.2	342	102	9.95
SDS	0.1	268	81	10.90
Polymeric additives				
PEG	0.1	291	87	8.10
	0.3	338	101	8.15
Gelatin	0.1	356	106	9.60
	0.3	252	77	6.75
Control		332	100	8.10

In order to verify its effect on cell growth and enzyme production, verifying experiment was performed. From the experimental results (data not shown), the elastase activity increased a little after the addition of these surfactants and polymeric additives. Consequently, in later studies, we did not add these substances to the culture medium.

Batch fermentation

Shake-flask fermentation was carried out using the completely optimized media and partially optimized medium to compare its cultivation kinetics. The time course of typical cultivation using the optimized medium is shown in Fig.2.

From Fig.2, it can be concluded that the completely optimized medium produced more elastase than the partially optimized culture medium during 72 hours fermentation.

Based on the kinetics analysis results (Table 7), it was obvious that the 18 hours cultivation was the best point for enzyme production with the two media. But the q_p of the later medium was higher than that of that the former, which means the completely optimized medium could increase the enzyme pro-

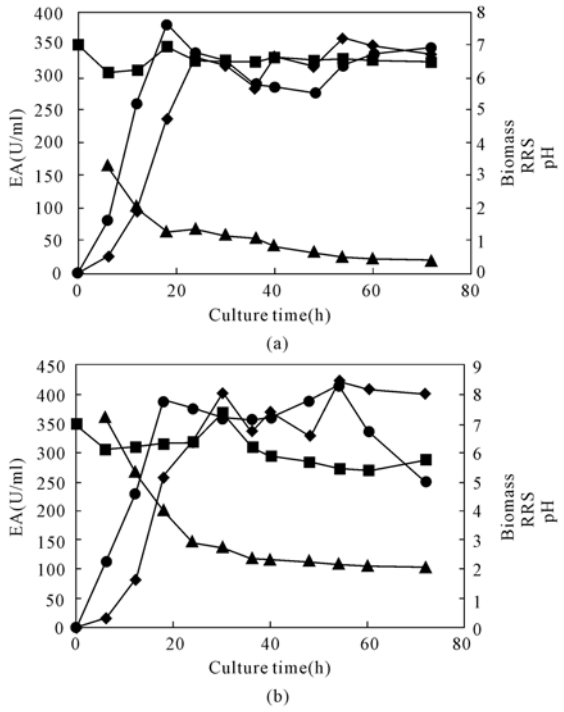

Fig.2 Enzyme productions by *Bacillus* sp. EL31410 on the partially optimized medium (a) and completely optimized medium (b) in the flask (EA: elastase activity; RRS: residual reduced sugar)

EA(U/ml) Biomass(g/L) RRS(g/100ml) pH

Table 7 Kinetics analysis of batch fermentation by *Bacillus* sp. EL31410 with optimized culture medium

Cultivation time (Hours)	Partially optimized medium		Completely optmized medium	
	μ_x	q_p	μ_x	q_p
0	0	0	0	0
6	0.17	2.34	0.170	1.20
12	0.12	2.31	0.083	2.39
18	0.05	3.09	0.068	3.75
24	−0.02	2.34	−0.007	1.30
30	−0.008	−0.32	−0.007	1.98
36	−0.02	−1.04	−0.002	−1.58
40	−0.008	2.10	0.004	1.15
48	−0.005	−0.35	0.010	−0.65
54	0.021	1.19	0.010	1.87
60	0.010	−0.33	0.040	−0.30
72	0.002	−0.12	0.030	−0.12

duction. However, cell growth per hour had the highest value at the 6 hours fermentation time in both kinds of media according to the μ_x value. It demonstrated that the bacterium at 6 hours cultivation time grew faster than at other fermentation time. At the same time, it was obvious that the reducing sugar in the partially optimized medium decreased more slowly than that in the completely optimized medium. And the elastase production was also prolonged in the completely optimized medium. That showed the glucose could provide the full carbon source and extend the cell growth and enzyme production. In contrast, the partially optimized medium was not really appropriate for enzyme production due to its low content of glucose and other inorganic salts that were verified to be beneficial for cell growth and elastase production.

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