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Enhanced production of elastase by *Bacillus licheniformis* ZJUEL31410: optimization of cultivation conditions using response surface methodology

CHEN Qi-he[1], RUAN Hui[†1], ZHANG Hai-feng[1], NI Hui[2], HE Guo-qing[†‡1]

([1]*Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310029, China*)

([2]*School of Bioengineering, Jimei University, Xiamen 361021, China*)

[†]E-mail: ruanhui98@yahoo.com.cn; gqhe@zju.edu.cn

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Abstract: Sequential methodology based on the application of three types of experimental designs was used to optimize the fermentation conditions for elastase production from mutant strain ZJUEL31410 of *Bacillus licheniformis* in shaking flask cultures. The optimal cultivation conditions stimulating the maximal elastase production consist of 220 r/min shaking speed, 25 h fermentation time, 5% (v/v) inoculums volume, 25 ml medium volume in 250 ml Erlenmeyer flask and 18 h seed age. Under the optimized conditions, the predicted maximal elastase activity was 495 U/ml. The application of response surface methodology resulted in a significant enhancement in elastase production. The effects of other factors such as elastin and the growth factor (corn steep flour) on elastase production and cell growth were also investigated in the current study. The elastin had no significant effect on enzyme-improved production. It is still not clear whether the elastin plays a role as a nitrogen source or not. Corn steep flour was verified to be the best and required factor for elastase production and cell growth by *Bacillus licheniformis* ZJUEL31410.

Key words: Elastase, *Bacillus licheniformis* ZJUEL31410, Cultivation condition, Fractional factorial design (FFD), Response surface methodology

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INTRODUCTION

Elastase is the only enzyme that targets, solubilizes, and degrades elastin (Morihara, 1967). Clinically, elastase is used as a vascular dilator drug. In industries of food processing, elastase is used to tenderize meat and improve meat quality. In recent years, some researchers have found that although many Gram-negative and -positive bacteria secrete elastase, the bacterial forms of elastase either have a low activity or harmful effects so that few of them are of medical or industrial values (Tsuzuki and Oka, 1965; Ozaki and Shiio, 1975; Clark *et al*., 2000; Janda and Abbott, 1999). Others have exerted their efforts in isolating and screening microorganisms that produce a high level of elastase (Shibata *et al*., 1993; Tsai *et al*., 1988; Zins *et al*., 2001).

Although considerable efforts have been made to screen the elastase-producing bacterial strains in studying its pathogenic effects and characterizations (Tsai *et al*., 1988; Shibata *et al*., 1993; Zins *et al*., 2001), few studies focusing on bacterial fermentation, especially culture medium optimization and fermentation kinetics, have been reported. In the previous study (Chen *et al*., 2002) we have described an optimal cultivation medium. The main objective of the current work was to optimize the cultivation conditions of *Bacillus licheniformis* ZJUEL31410, a mutant strain that overproduces elastase, using three sequential experimental designs, and also to analyze the effect of growth factors and elastin on elastase production and cell growth.

‡ Corresponding author

In the previous study, we studied the optimization of culture medium with statistical method (Chen *et al*., 2002). The classical or empirical method has several adequacies towards complete optimization. The traditional one-factor at a time approach of 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 to account for the interactions. A combination of factors that generates a certain optimum response can be identified through the factorial design and the use of response surface methodology (Khuri and Cornell, 1987). Response surface methodology, described first by Box and Wilson (1951), an experimental strategy for seeking the optimum conditions for a multivariable system, is a much more efficient technique for optimization (Box and Hunter, 1978). This method had been successfully applied in the optimization of medium compositions (Roseiro, 1992; de O. Souza *et al*., 1999), conditions of enzymatic hydrolysis (Ma and Ooraikul, 1986), parameters of food preservation (King, 1983), and fermentation processes (Rosi *et al*., 1987; Kalil *et al*., 2000; Ramírez *et al*., 2001). A central composite experimental design was also used for the medium optimization reported here. An experimental design for fitting a second-order model involves at least three levels of each factor. The most widely used design for fitting a second-order model is the central composite design, which consists of a 2^k factorial design augmented by 2^k axial points and some center points (Box and Draper, 1987). In this approach, concentrations of medium components are the variables; each variable is referred to some base value and varies in a certain pattern.

MATERIALS AND METHODS

Elastin, elastin-Congo red and porcine pancreatic elastase were purchased from Sigma Chemical Company, USA. Casein was purchased from Biochemical Agent Company of Shanghai, China. All chemicals and reagents used were of analytical grade.

Microorganism and media

Bacillus licheniformis ZJUEL31410 (China General Microbiological Culture Collection Center, CGMCC No. 1397) was isolated and mutated from the soil of a meat-processing factory at Hangzhou, China. The method for screening microorganism described by Shiio *et al*.(1974) was modified and used in this study. We first identified *Bacillus* sp. as the elastase-producing strain, and then mutated it to have a high elastase activity. The seed strain was maintained in a 250 ml flask that contains 25 ml of culture media of LB (Luria-Bertani) slants (g/L): peptone 6, yeast extract 2, beef extract 4, NaCl 5, agar 2, at 37 °C, pH 7.0, and shaking at 180 r/min.

Culture conditions

All optimization experiments were carried out in unbaffled 250 ml Erlenmeyer flasks containing 25 ml of medium with optimized concentrations (g/L) of 74 glucose, 11.3 casein, 6.16 corn steep flour, 2.06 K_2HPO_4 and 0.34 $MgSO_4{\cdot}7H_2O$. The seed medium (g/L) contains peptone 6, yeast extract 2, beef extract 4, NaCl 5, pH 7.5. The media were sterilized at 121 °C for 20 min, and cooled to room temperature prior to use. Each flask (250 ml) containing fermentation medium was inoculated with the seed culture. The inoculated medium was incubated on the rotary shaker. The culture conditions were set up according to the experimental designs.

Analytical procedures

Elastolytic activity was assayed by the colorimetric method of Sacher (1955). Fermentation broth was recovered after batch fermentation, centrifuged at 1500×g for 15 min, and then the supernatant was suitably diluted. Enzyme preparation was incubated with 20 mg of elastin-Congo red in 2 ml of 0.2 mol/L boric acid buffer (pH 7.4) with shaking for 20 min at 37 °C. The reaction was stopped by adding 2 ml of 0.70 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 en-

zyme required to solubilize 20 mg elastin-Congo red under the tested conditions. Meanwhile, the standard curve was made by the method of Sacher (1955).

Biomass content was measured in terms of gravimetry (g/L). The culture samples (10 ml) were centrifuged (1500×g for 10 min), and the cell pellet was washed thoroughly with distilled water, dried to constant weight at 80 °C overnight, then cooled and weighed. Reduced reducing sugar (RRS) was measured with the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959), and the pH was measured with a pH meter.

Experimental design

Response surface methodology 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 (Montgomery, 1997); the objective is to optimize this response. To find the optimal cultivation conditions for an elastase production in submerged batch cultures, the key environmental factors affecting the elastase production must be determined. Based on the results of our preliminary experiments, the major factors were optimized using fractional factorial design (FFD) and response surface methodology (RSM) design. Table 1 shows the ranges of variables of fermentation time, shaking speed, inoculating volume, seed age and medium volume for FFD.

The correspondence between these values can be obtained by:

$$x_i=(X_i-X_{i0})/\delta_i,$$

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 1 unit of x_i.

A full second-order polynomial model obtained by a multiple regression technique using the SAS (statistical analysis software) package (SAS Institute, Cary, NC, USA) was adopted to describe the response surface. The response surface plot can be shown with Statistical software 6.4. For three factors, the model obtained is expressed as follows:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2,$$

where Y is the measured response (elastase activity), b_0 the intercept term, b_1, b_2 and b_3 linear coefficients, b_{12}, b_{13}, and b_{23} interactive coefficients, b_{11}, b_{22} and b_{33} quadratic coefficients, and x_1, x_2 and x_3 coded independent variables.

RESULTS AND DISCUSSION

Fractional factorial design (FFD)

The results of FFD and model regression are demonstrated in Tables 2 and 3, respectively.

Table 4 shows the analysis of variance (ANOVA) for elastase activity. Elastase production has a very high determinant of coefficient of 0.9985. It suggests that the model is reliable for explaining elastase production. According to the F-test results, it can be found that the important factors affecting responses are temperature, fermentation time, shaking speed and medium volume. The temperature, fermentation time and shaking speed all had an important positive effect on the enzyme production and cell growth, whereas both the medium and inoculating volumes had a negative effect. Therefore, considering the actual situation and medium evaporation, both medium and inoculating volumes were fixed at the zero level. The t-test for the FFD results was performed to check the experimental differences and showed no significant differences between the treatments (data not shown). The results of t-test for variance between averages of the observations of two-level experiment and center point show that the difference is significant ($P<0.01$). This indicates that the optimum point is within the domain of the experiment.

Table 1 Ranges of variables for fermentation time, shaking speed, inoculating volume, seed age and medium volume for fractional factorial design (FFD)

Code value	X_1 (°C)	X_2 (h)	X_3 (ml)	X_4 (%, v/v)	X_5 (h)	X_6 (r/min)
–1	30	18	20	3	10	180
0	35	24	25	5	18	200
1	40	30	30	7	26	220

X_1: Temperature; X_2: Fermentation time; X_3: Medium volume; X_4: Inoculating volume; X_5: Seed age; X_6: Shaking speed

Table 2 The experiments of factional factorial design (FFD) and the results

Runs	x_1	x_2	x_3	x_4	x_5	x_6	Y_{EA} (U/ml)	Y_{DCW} (g/L)
1	−1	1	1	1	−1	−1	294	7.10
2	1	−1	1	−1	−1	1	265	8.10
3	−1	−1	−1	1	−1	1	307	8.00
4	1	1	1	1	1	1	351	5.80
5	−1	1	−1	−1	1	1	311	8.45
6	1	1	−1	−1	−1	−1	355	6.05
7	−1	−1	1	−1	1	−1	110	4.25
8	1	−1	−1	1	1	−1	274	9.55
9	0	0	0	0	0	0	355	8.30
10	0	0	0	0	0	0	375	8.30
11	0	0	0	0	0	0	403	7.90

EA: Elastase activity (U/ml); DCW: Dry cell weight (g/L)

Table 3 Regression coefficient, standard error, and Student's *t*-test results for factional factorial design (FFD)

Parameter	*df*	*PE*	*SE*	*P*>*T*
Intercept	1	0.69850	0.006250	0.0057
x_1	1	0.06275	0.006250	0.0632
x_2	1	0.10050	0.010528	0.0395
x_6	1	0.05700	0.010528	0.0495
x_4	1	0.05275	0.010248	0.0751
x_5	1	−0.04950	0.013756	0.0800
x_3	1	−0.06475	0.010248	0.0713

df: Degree of freedom; *PE*: Parameter estimates; *SE*: Standard error

Table 4 Analysis of variance (ANOVA) for regression model of elastase production obtained from factional factorial design (FFD) experiment

Source	*df*	*SS*	*F*-ratio	*P*-value
Model	6	0.21370	113.972	0.0716
Error	1	0.00031		
CTR	7	0.21401		
R^2			0.9985	

df: Degree of freedom; *SS*: Sum of squares; CTR: Corrected total regression

Central composite design and response surface analysis for cultivation conditions

Once critical factors have been identified via screening and significant gross curvature has been detected in the design space, we proceeded to optimize the fermentation time and shaking speed. By determining the path of steepest ascent the vicinity of the optimum was reached. Thus, for cultivation conditions optimization, the levels of the two significant variables, fermentation time (x_2) and shaking speed (x_6), were further optimized with a central composite design. Table 5 shows the central composite design and the experimental results. The regression results in Tables 6 and 7 demonstrate that the most important factor for the elastase production is the shaking speed. The fit value, termed R^2, of the polynomial model was 0.7396, indicating that near 74% of the variability in the response could be explained by the second-order polynomial prediction equation given below (Eq.(1)). The ANOVA results show that this model is appropriate. Tables 6 and 7 also suggest that the elastase production is primarily determined by the linear term of x_2 and the quadratic term of x_1 of this model, with

Table 5 Experimental design and results of the central composite design (CCD)

Run	x_2	x_6	Y_{EA} (U/ml) observed	Y_{DCW} (g/L)
1	0	−1.414	319	8.45
2	1	1	341	9.25
3	1	−1	241	9.80
4	−1	−1	108	6.85
5	−1	1	437	8.10
6	−1.414	0	295	7.70
7	1.414	0	356	9.00
8	0	1.414	351	7.20
9	0	200	450	9.50
10	0	200	421	9.10
11	0	200	403	9.00
12	0	200	364	7.90
13	0	200	440	8.80

$x_2=(X_2-24)/4$; $x_6=(X_6-200)/30$. EA: Elastase activity (U/ml) observed; DCW: Dry cell weight (g/L)

Table 6 Results of parameter estimate for elastase production (Y_{EA})

Term	*df*	*PE*	*T* for H_0:	*P*>*T*
Intercept	1	415.46	14.642	0
x_2	1	15.37	0.688	0.5137
x_6	1	59.32	2.643	0.0377
$x_2 \times x_2$	1	−56.30	−2.367	0.0498
$x_2 \times x_6$	1	−57.25	−1.805	0.1141
$x_6 \times x_6$	1	−52.68	−2.187	0.0650

df: Degree of freedom; *PE*: Parameter estimates

Table 7 ANOVA results for elastase production obtained from central composite design (CCD) (Y_{EA})

Regression	*df*	Type I SS	R^2	*F*-ratio	*P*>*F*
Linear	2	30034	0.2775	3.730	0.0789
Quadratic	2	36895	0.3409	4.582	0.0534
Crossproduct	1	13110	0.1211	3.257	0.1141
TR	5	80039	0.7396	3.976	0.0499

TR: Total regression; *df*: Degree of freedom; *SS*: Sum of square

no significant interaction existing between the two factors (P<0.01). While the regression coefficients and P values suggest that both fermentation time and shaking speed have the positive effect on elastase production, the fermentation time, however, is not significant, indicating that oxygen is very important for cell growth and enzyme production. The poor oxygen supply, due to its poor solubility in the aqueous phase, to the growing cell population is the rate-limiting factor in many aerobic fermentation processes (Kapat *et al*., 2001).

Canonical analysis has been applied to validate linear models obtained from factorial and central composite designs (Khuri and Cornell, 1987). This is a mathematical procedure used to simplify a second-order polynomial model. In the present study, the results demonstrate that the response surface plot has a maximum point, and the optimal fermentation condition is determined to be the cultivation period of 25 h and the shaking speed at 220 r/min. The other conditions were fixed at zero levels, and the maximum response predicted from the model is 434 U/ml. The verified experiments were carried out based on the optimized cultivation conditions, of which the results [(420±15) U/ml] indicate that the predicted model is proper for explaining the elastase cultivation process. The effects of fermentation time and shaking speed on cell growth were also checked by the response surface plot (Fig.1).

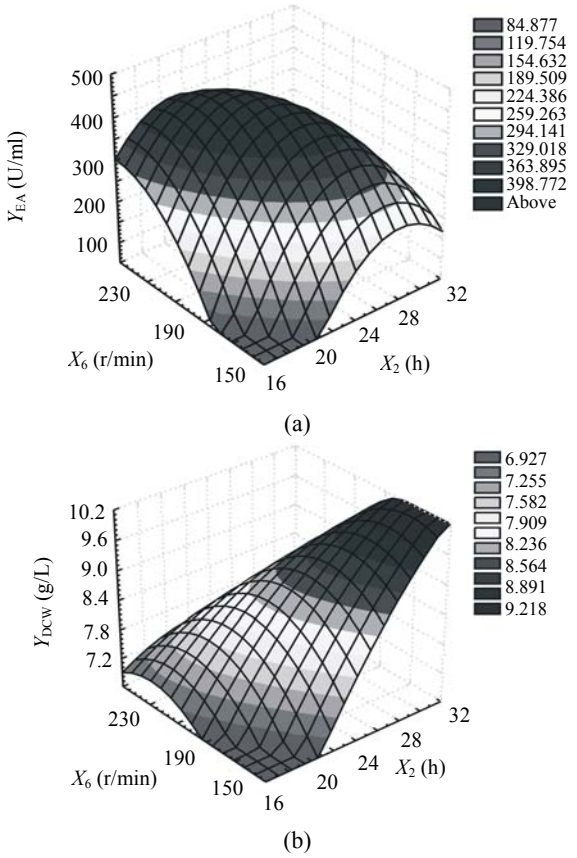

Fig.1 Response surface plots of fermentation time (X_2) and shaking speed (X_6) against (a) elastase production (Y_{EA}) and (b) dry cell weight (Y_{DCW}) by *Bacillus licheniformis* ZJUEL31410

The low activity of elastase produced by microorganisms has greatly hampered the research on the role of microbial elastases, and only a few attempts have been made to increase elastase productivity by manipulating physiological conditions and medium compositions (Tsai *et al*., 1988; Chen *et al*., 2002). Some recent studies in the field of the optimization of fermentation conditions have reported a significant increase in the yields of elastase (Chen *et al*., 2002; 2004; He *et al*., 2004). These developments include the use of fed-batch technique and statistical methodology. In the present study, we applied statistical optimization methods to increase the elastase production:

$$Y_{EA}=415.46+15.37x_2+59.32x_6-56.30x_2\times x_2 -57.25x_2\times x_6-52.68x_6\times x_6. \quad (1)$$

Effects of elastin addition on elastase production and cell growth by *Bacillus licheniformis* ZJUEL31410

The roles of elastin on elastase production and cell growth are illustrated in Fig.2. The experimental results demonstrate that elastin did not greatly induce elastase production compared to the control: the maximal elastase activity of both was not different, and, much more important, elastin added had no significant favorable effect on elastase secretion. Nevertheless, it is still necessary to further consider the effect of elastin on elastase production in view of its adding time. As shown in Figs.3 and 4, it is necessary to study the relation between fermentation time and elastase production. It was found that when adding elastin at 12 h, both the period of enzyme production and cell growth prolonged during the experimental time, and the enzyme activity also much increased, compared with that of 6 h. It suggests that the elastin could function as a part of nitrogen sources, while this remains under investigation. There was no report about the effect of elastin on elastase fermentation. Thus, a further instigation will be necessary to provide essential evidence for explaining elastase synthesis and secretion.

Effect of growth factor on elastase production and cell growth by *Bacillus licheniformis* ZJUEL31410

Corn steep flour greatly increases microbial elastase production and cell growth as illustrated in the previous study (Chen *et al*., 2002), and it has been further confirmed in the current study (Fig.5). The

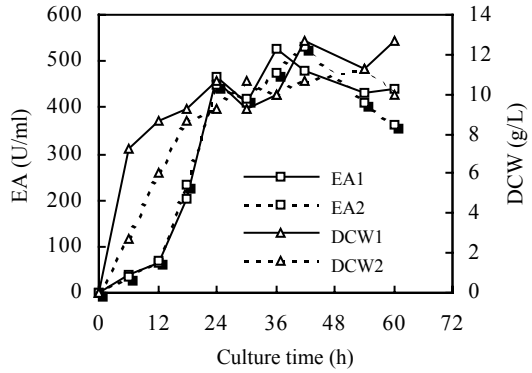

Fig.2 Effect of adding elastin into culture medium on elastase production and cell growth

1: Adding elastin, 2: No elastin; EA: Elastase activity; DCW: Dry cell weight. The cultivation conditions were referred to the above-optimized parameter. Addition amount of elastin is 0.8% (w/v)

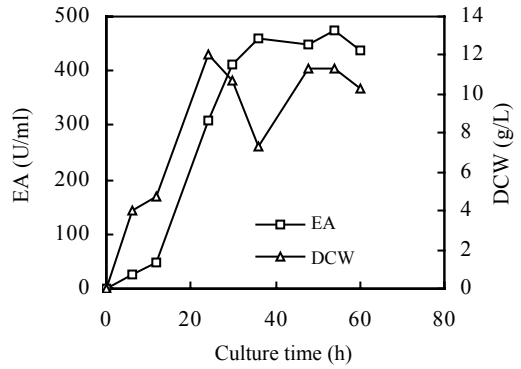

Fig.3 The elastase production and cell growth of *Bacillus licheniformis* ZJUEL31410 at 6 h culture time of elastin addition

EA: Elastase activity; DCW: Dry cell weight

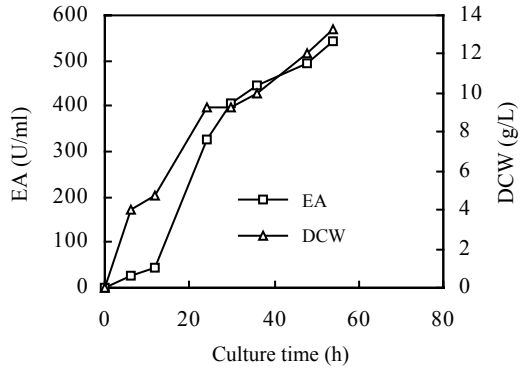

Fig.4 The elastase production and cell growth of *Bacillus licheniformis* ZJUEL31410 at 12 h culture time of elastin addition

EA: Elastase activity; DCW: Dry cell weight

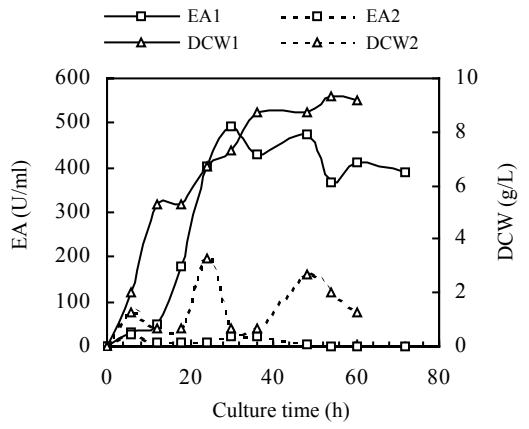

Fig.5 Effect of growth factor, corn steep flour, on cell growth and elastase production

1: Adding growth factor; 2: No growth factor. EA: Elastase activity; DCW: Dry cell weight

maximal elastase activity and production period were prolonged after 36 h cultivation as corn steep flour added; however, the elastase activity of the control was greatly lower than that of the medium added with corn steep flour. The cell growth of the experimental set was generally higher than that of the medium without corn steep flour. We postulate that corn steep flour contains abundant amino acids, vitamins and inorganic substances, most of which are the required nutrition elements for *Bacillus licheniformis* ZJUEL31410.

CONCLUSION

In the current study, we applied fractional factorial design (FFD) and response surface methodology (RSM) to optimize the fermentation conditions of elastase production by *Bacillus licheniformis* and the cell growth. We conclude that the culture time and oxygen supply are key factors that affect elastase production and cell growth, whereas other factors such as the seed age and inoculating volume are not as significant. We also conclude that corn steep flour increases the cell growth and elastase production, but the addition of elastin has no marked effect on the elastase production.

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