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Full Length Research Paper

Studies on the optimization of D-erythorbic acid production by *Penicillium griseoroseum* FZ-13 in relevant fermented culture medium

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D-EA obtained by fermentation using micro-organism is a new biological food additive being widely used in the food industry. This work investigated the optimization of the fermented culture medium for maximization of D-erythorbic acid production produced by *Penicillium griseoroseum* FZ-13 using Response Surface Methodology (RSM). In the first optimization step, a Plackett–Burman design was used to evaluate the influence of 8 related factors. Glucose, urea, ammonium sulfate and zinc sulfate were found to be more significant factors. In the second step, the four significant factors aforementioned were further optimized using a Box–Behnken design. D-erythorbic acid production improved approximately 1.8-fold when the optimal medium was used compared with the original non-optimized medium, and its productivity reached a maximum of 7.88 g/L after a 120 h cultivation period. These results established a foundation for further investigating the direct fermentative production of D-erythorbic acid.

Key words: D-erythorbic acid, *Penicillium griseoroseum*, RSM, fermentation.

INTRODUCTION

D-Erythorbic acid (D-EA) is a C-5 epimer of L-ascorbic acid having only 1/20 of its vitamin activity. D-EA is not fortified with vitamin C and does not hinder the body's absorption of Vitamin C (Goldman et al., 1981). D-EA is a new biological food additive being widely used as an antioxidant in the food industry, whose safety is recognized by Food and Agriculture Organization and The World Health Organization (FAO/WHO Expert Committee on Food Additives, 1991). As a food antioxidant, D-EA has the same antioxidant property as ascorbic acid. D-EA and sodium erythorbate are widely used as a water-soluble antioxidant particularly in processed meat, fruit and vegetable products, drinking and canned food etc. D-EA and sodium erythorbate have many advantages in food industry. D-EA and sodium erythorbate can prevent the formation of carcinogenic nitrosamines in meat; D-EA and sodium erythorbate can help to reduce product spoilage and maintain the taste; D-EA and sodium erythorbate can extend fats and oils shelf life; D-EA and sodium erythorbate can increase the transparency of wine and inhibit enzymatic browning. Also, D-EA compounded with L-cysteine, sucrose and calcium chloride could inhibit polyphenol oxidase (PPO) activity effectively and improve quality and brittleness of banana slices (Zhuang et al., 2012).

D-EA has been found only in the filamentous fungus *Penicillium* (Takahashi et al., 1960; Yagi et al., 1967). Thereafter, it was identified in other *Penicillium* spp, including *Penicillium notatum*, *Penicillium chrysogenum* and *Penicillium griseofulvum* (Zhao and Li, 1988; Xu et al., 1995; Wang et al., 1991). At present, there are four kinds of technologies to produce D-EA, which are direct fermentation method (Takahashi et al., 1960), indirect fermentation method (Zhou et al., 2008), enzymatic method (Asakura et al., 2000; Hoshino et al., 2008) and genetic engineering method (Salusjarvi et al., 2004). Response surface methodology (RSM) is a common

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technique used in biotechnology to optimize microbial growth conditions in culture (Huang et al., 2010; Mamatha et al., 2008; Ratnam et al., 2005). In this study, the aim of this work was to optimize the medium in order to maximize D-EA production by *P. griseoroseum FZ13* and to reduce the cost of production.

This work was based on a statistics experimental design. The following optimization experiments were performed: (a) Plackett–Burman design to screen significant factors that affect the D-EA yield in the culture medium and (b) these significant factors were further optimized by Box–Behnken design (BB).

MATERIALS AND METHODS

Micro-organism

P. griseoroseum FZ13 was screened from local soil samples. The strain was maintained on PDA medium and stored in the microbiological laboratory of College of Food Science of Jiangxi Agriculture University.

Cultivation

Fermentation medium 50 ml in 250 ml conical flask was inoculated 2% spore suspension (10^6/ml), then shaking cultivation at 28°C and 180 r/min condition. Samples of each medium were taken after a 120 h fermentation period. The samples were filtered by filter paper, and then 1 ml filtrate was fetched and 100 ml diluted. The dilution was filtered by 0.45 um filter membrane, which was prepared for HLPC. All of these experiments were carried out in duplicate.

Screening of the significant factors using a Plackett–Burman design

Plackett–Burman design, a simple and efficient technique for fermented conditions optimization was used to pick factors that significantly influenced D-erythorbic acid production; then the insignificant ones were eliminated in order to obtain a smaller more manageable set of factors. The medium components were screened by a Plackett–Burman design for 8 variables at two levels. Each factor was tested at both high (+1) and low (–1) concentrations (Table 1).

Further optimization of the significant factors using a Box–Behnken design

Once critical factors were identified via screening, a Box–Behnken design for four independent variables, each at three levels with triplication, was employed to fit a polynomial model:

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_4D+\beta_{12}AB+\beta_{13}AC+\beta_{14}AD+\beta_{23}BC+\beta_{24}BD+\beta_{34}CD+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{44}D^2 \quad (1)$$

Where Y was the yield of D-EA; β0 was the intercept term; β1, β2, β3 and β4 were linear coefficients; β12, β13, β14, β23, β24, β34 were interactive coefficients; β11, β22, β33, β44 were quadratic coefficients and A, B, C, D were coded independent variables. Design-Expert 7.1.6 Trial (Stat-Ease Inc., Statistics Made Easy, Minneapolis, MN, USA) was used for the experimental design and analysis of variance of the data obtained.

HPLC-UV analysis

Erythorbic acid analysis was carried out by HPLC using phase column (Waters, 1525) and a gradient with acetonitrile in 0.1 mol/L monopotassium phosphate (a flow rate of 1 ml/min). The injection volume was 10 ul. The detection was carried out at the wave length of 254 nm. Detection and spectral characterization of peaks were accomplished with a dual λ UV absorbance detector (Waters 2478) and Breeze software (Waters).

RESULTS AND DISCUSSION

Screening of the significant factors using a Plackett–Burman design

According to the nutritional requirements of *P. griseoroseum* FZ-13, 8 factors were selected and their reference numbers were X1 to X8 (Table 1). A Plackett–Burman design was used for screening for the significant factors. In the experimental design, each row represented an experiment and each column represents an independent variable (Table 1). The yield of EA, determined for each experimental design was shown in Table 1. The analysis of variance for the experimental designs was calculated, and the significant levels of each medium variable were determined by analysis of variance (Table 1). The analysis showed that glucose, urea, ammonium sulfate and zinc sulfate had significant influence on the EA production. All the other insignificant variables were neglected, and optimum combinations of these four were further analyzed by a Box–Behnken design.

Further optimization of the significant factors using a Box–Behnken design

Through the experiments, an optimum combination of the four nutrients was reached using a Box–Behnken design. The variation levels were listed in Table 2 where these components were supplemented to the fermentation medium. The design and results of experiments carried out by the Box–Behnken design were presented in Table 2. The results obtained were submitted to an analysis of variance on Design-Expert 7.1.6 Trial, and the model was given as:

$$Y=7.026+0.828333333A+0.339166667B+0.159166667C-0.111666667D+0.2625AB+0.32AC-0.0075AD+0.0625BC+0.2625BD-0.015CD-0.3905A^2-0.39675B^2-0.40675C^2-0.138D^2$$

Where Y was the D-EA yield; A was the concentration of glucose; B was the concentration of urea; C was the concentration of ammonium sulfate and D was the concentration of zinc sulfate, respectively.

From Table 2, the model F-value of 17.32 implied the

Table 1. Design and results of Plackett-Burman response surface test and Variance analysis

Reference numbers	X_1(g/L)	X_2(g/L)	X_3(g/L	X_4(g/L)	X_5(g/L)	X_6(g/L)	X_7(g/L)	X_8(g/L)	D-EA (mg/ml)
	Glucose	Ammonium sulfate	(Urea)	Sodium nitrate	Dipotassium phosphate	Manganese sulfate	Magnesium sulfate	Zinc sulfate	
1	-1(20)	-1(1)	-1(2)	-1(1)	-1(3)	-1(0.3)	-1(0.4)	-1(0.01)	5.2
2	1(60)	1(3)	-1(2)	1(3)	-1(3)	1(0.5)	-1(0.4)	-1(0.01)	5.86
3	1(60)	-1(1)	-1(2)	1(3)	1(5)	1(0.5)	-1(0.4)	1(0.03)	5.81
4	-1(20)	-1(1)	1(4)	1(3)	-1(3)	1(0.5)	1(0.6)	-1(0.01)	5.26
5	1(60)	-1(1)	1(4)	-1(1)	1(5)	-1(0.3)	-1(0.4)	-1(0.01)	5.62
6	1(60)	-1(1)	1(4)	-1(1)	-1(3)	1(0.5)	1(0.6)	1(0.03)	5.28
7	-1(20)	1(3)	-1(2)	-1(1)	1(5)	1(0.5)	1(0.6)	-1(0.01)	5.76
8	-1(20)	-1(1)	-1(2)	1(3)	1(5)	-1(0.3)	1(0.6)	1(0.03)	5.6
9	1(60)	1(3)	1(4)	1(3)	1(5)	-1(0.3)	1(0.6)	-1(0.01)	5.88
10	-1(20)	1(3)	1(4)	1(3)	-1(3)	-1(0.3)	-1(0.4)	1(0.03)	5.5
11	-1(20)	1(3)	1(4)	-1(1)	1(5)	1(0.5)	-1(0.4)	1(0.03)	5.36
12	1(60)	1(3)	-1(2)	-1(1)	-1(3)	-1(0.3)	1(0.6)	1(0.03)	5.74

Variance analysis					
Source	Sum of squares	Df	Mean Square	F Value	p-value Prob > F
Model	3.7239	8	0.4654	69.505	0.002
X1	3.2552	1	3.2552	486.05	0.000
X2	0.0954	1	0.0954	14.245	0.032
X3	0.0720	1	0.0720	10.761	0.046
X4	0.0374	1	0.0374	5.5854	0.0991
X5	0.0044	1	0.0044	0.6582	0.476
X6	0.0014	1	0.0014	0.2102	0.677
X7	0.2380	1	0.2380	35.538	0.009
X8	0.0200	1	0.0200	2.9875	0.182
Residual	0.0200	3	0.0066		
Cor total	3.7440		11		

model is highly significant. There was only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BD, A^2, B^2, C^2 are significant model terms. The "Lack of Fit F-value" of 1.23 implies the Lack of Fit is not significant relative to the pure error. There is a 45.36% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good. The value of the determination coefficient (R^2) is 0.9454; and the "Pred R-Squared" of 0.7417 is in reasonable agreement with the "Adj R-Squared" of 0.8909. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 14.431 indicates an adequate signal and as such this model can be used to navigate the design space.

The 3D response surface curves were plotted to understand the interaction of medium components and their effect on D-EA production. Figure 1 showed the response surface plots as functions of glucose versus urea, ammonium sulfate and Zinc Sulfate, respectively. Figure 1a showed the mutual interaction between glucose and urea. From Figure 1a, it could be seen that irrespective of urea, increasing glucose concentration was paralleled with large increments in yield. The mutual interaction of glucose and ammonium sulfate was showed in Figure 1b. Predicted response surface for interaction of glucose and zinc sulfate for D-EA production was presented in Figure 1c. The combined effect of urea and ammonium sulfate on D-EA production was illustrated in Figure 1d. When ammonium sulfate concentration was at a certain value with increasing urea concentration, the yield was improved. But when ammonium sulfate concentration exceeded the fitted value, the downtrend was observed from Figure 1d. Figure 1e showed the mutual interaction between Zinc Sulfate and urea. At a fixed glucose concentration of 70.00 g/L and urea concentration of 4.00 g/L, some trends were observed in Figure 1f. When without regard to zinc sulfate, with enhancements of ammonium sulfate concentration, the yield was increased initially and then decreased.

The medium optimization of D-EA production by *P. griseoroseum* FZ-13 was predicted using the optimization function of the Design Expert 7.1.6 trial software. The optimal concentrations were 80 g/L glucose, 4.96 g/L urea, 4.65 g/L ammonium sulfate, and 0.04 g/L zinc sulfate. The relative deviation value was 2.16%. So under the given concentration, these models can be used to predict D-EA production within the experimental range.

These results indicated that the amount of glucose and nitrogen sources in the fermentation medium were important factors for D-EA production in *P. griseoroseum* FZ-13. Carbon source and nitrogen source are necessary nutrients for microbial growth, and zinc is a highly significant factor for the D-EA yield. D-EA synthesis pathway in *Penicillium* is comprised of

Table 2. Design and results of the Box–Behnken design and Analysis of variance for the experimental results.

Run	A(g/L) (Glucose)	B(g/L) (Urea)	C(g/L) (Ammonium sulfate)	D(g/L) (Zinc sulfate)	EA (mg/ml)
1	70	3	4	0.03	6.47
2	70	4	4	0.04	6.92
3	70	4	3	0.03	6.24
4	70	5	4	0.03	6.78
5	70	4	3	0.05	6.22
6	80	4	5	0.04	7.53
7	60	4	3	0.04	5.56
8	70	5	3	0.04	6.49
9	70	4	4	0.04	6.89
10	60	4	5	0.04	5.34
11	70	4	5	0.05	6.79
12	70	4	5	0.03	6.87
13	70	3	3	0.04	5.93
14	70	3	4	0.05	5.67
15	80	4	4	0.03	7.47
16	70	5	4	0.05	6.76
17	80	4	3	0.04	6.47
18	70	3	5	0.04	5.74
19	80	3	4	0.04	6.37
20	70	5	5	0.04	6.55
21	80	5	4	0.04	7.68
22	70	4	4	0.04	6.97
23	70	4	4	0.04	6.94
24	60	5	4	0.04	5.68
25	60	4	4	0.03	5.51
26	80	4	4	0.05	7.38
27	60	3	4	0.04	5.42
28	60	4	4	0.05	5.45
29	70	4	4	0.04	7.41

Analysis of variance

Source	Sum of squares	Df	Mean square	F value	p-value
Model	13.28736	14	0.949097	17.32468	<0.0001
A	8.233633	1	8.233633	150.2955	< 0.0001
B	1.380408	1	1.380408	25.19776	0.0002
C	0.304008	1	0.304008	5.549321	0.0336
D	0.149633	1	0.149633	2.731384	0.1206
AB	0.275625	1	0.275625	5.031216	0.0416
AC	0.4096	1	0.4096	7.476775	0.0161
AD	0.000225	1	0.000225	0.004107	0.9498
BC	0.015625	1	0.015625	0.285216	0.6017
BD	0.275625	1	0.275625	5.031216	0.0416
CD	0.0009	1	0.0009	0.016428	0.8998
A^2	0.989126	1	0.989126	18.05535	0.0008
B^2	1.021041	1	1.021041	18.63793	0.0007
C^2	1.07316	1	1.07316	19.5893	0.0006
D^2	0.123529	1	0.123529	2.254873	0.1554
Residual	0.766962	14	0.054783		
Lack of fit	0.579242	10	0.057924	1.234267	0.4536
Pure error	0.18772	4	0.04693		
Cor. total	14.05432	28			

two steps (Takahashi et al., 1976; Murakawa and Takahashi, 1977). During the synthesis, gluconolactonase had been observed to be secreted by the *Penicillium* (Takahashi et al., 1976), and gluconolactonase was a metalloprotein containing zinc (Carper et al., 1982). Zinc was presented at the active site of some enzymes involved in the association of regulatory and catalytic subunits (Kantrowitz and Lipscomb, 1990; Saire et al., 1990; Koshland, 1973). So an optimum amount of zinc in the fermentation medium is a critical factor for the D-EA production.

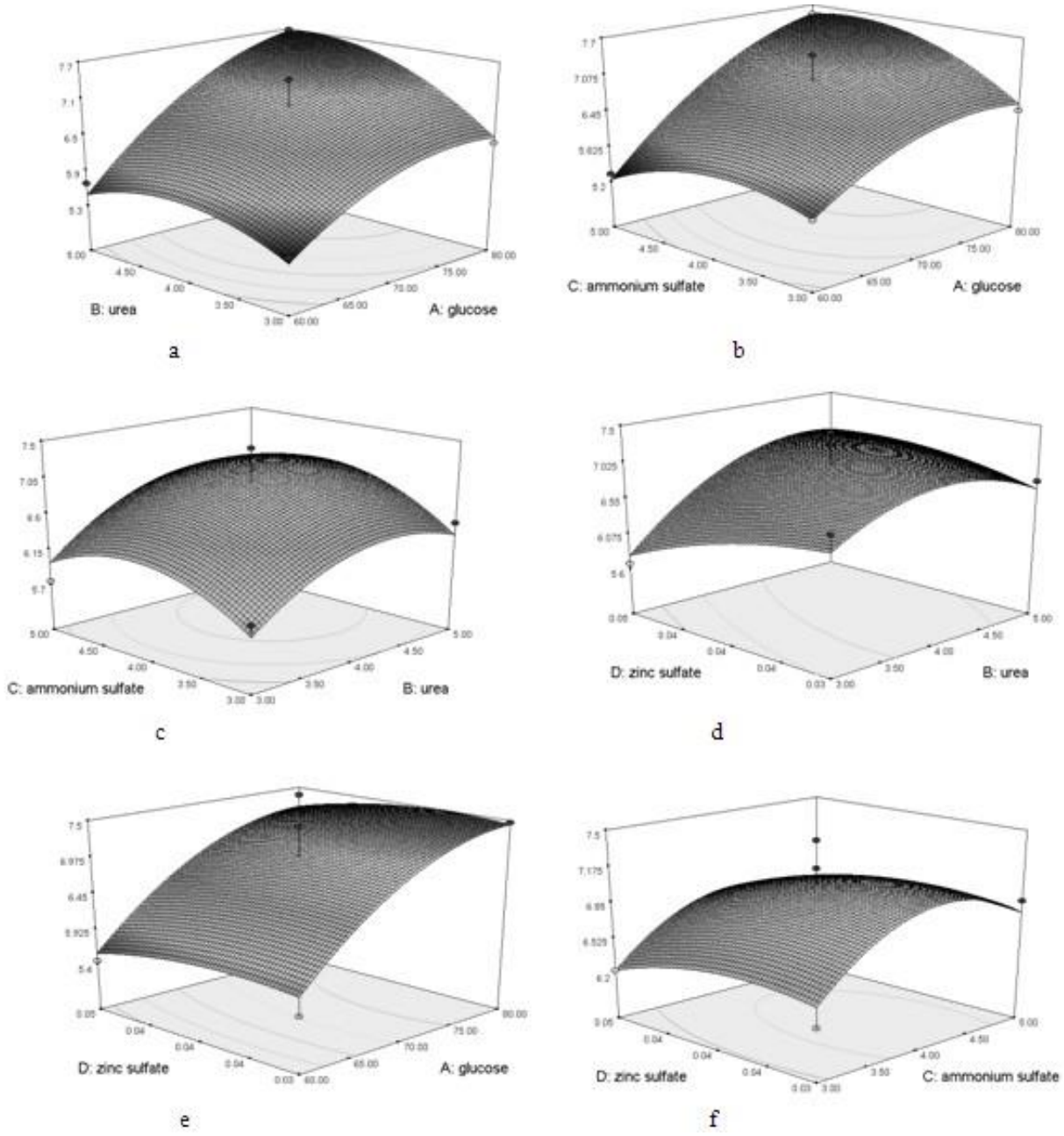

Figure 1. 3D Response Surface Plots for EA. a: glucose concentration versus urea concentration (AB), b: glucose concentration versus ammonium sulfate concentration (AC), c:glucose concentration versus zinc sulfate concentration (AD), d: urea concentration versus ammonium sulfate concentration (BC), e: urea concentration versus zinc sulfate concentration (BD), f: ammonium sulfate concentration versus zinc sulfate concentration (CD).

Conclusion

This work suggested RSM to be an effective way to optimize the medium for D-EA production in *P. griseoroseum* FZ-13. At 80g/L glucose, 4.96 g/L urea, 4.65 g/L ammonium sulfate and 0.04 g/L zinc sulfate, the maximum yield of D-EA was 7.88g/L predicted by the model. The actual maximum yield is 7.86 g/L, which was improved approximately 1.8-fold.

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