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Full Length Research Paper

Optimizing culture medium for debittering constitutive enzyme naringinase production by *Aspergillus oryzae* JMU316

Dong-xiao Chen[1,2], Tian-gui Niu[1]* and Hui-nong Cai[2]*

[1]College of Food Science and Nutritional Engineering, China Agricultural University, P.O. Box 111, No. 17 Qinghua East Road, Beijing, P. R. China, 100083.
[2]Bioengineering College, Jimei University, No. 43 Yindou Road, Jimei District, Xiamen, Fujian, P. R. China, 361021.

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The objective of this study was to investigate nutrient requirements for extracellular constitutive naringinase production by *Aspergillus oryzae* JMU316. The one-factor-at-a-time method was used to determine the impact of different carbon and nitrogen sources on naringinase production. Naringin exhibited the highest naringinase activity compared to all other carbon sources and pomelo pericarp powder produced comparable naringinase activity. Pomelo pericarp powder was selected as carbon source because it is a waste of fruit process, which means that it is a cheap resource and has additional environmental benefits. Peptone proved to be the most suitable nitrogen source for naringinase production. Subsequently, the orthogonal matrix method was used to further optimize the concentration of pomelo pericarp powder, peptone, and minerals. The optimal concentration of the components were 15 g pomelo pericarp powder, 12 g peptone, 0.2 g $CaCl_2$, 0.4 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$ and 1 g K_2HPO_4 in 1 L distilled water for producing 408.28 IU/mL naringinase activity. The effects of medium components on naringinase were in the order of pomelo pericarp powder, K_2HPO_4, NaCl, peptone, $CaCl_2$, $MgSO_4 \cdot 7H_2O$. This two-step optimization strategy used in this study can be widely applied to other microbial fermentation processes.

Key words: Pomelo pericarp powder, orthogonal matrix method, naringinase, culture medium optimization, *Aspergillus oryzae* JMU316.

INTRODUCTION

Bitterness is the major limiting factor for commercial acceptance of processed citrus fruit such as juice, wine, and vinegar. Naringin is the major component in grapefruit with very bitter taste and a threshold of 20 mg/kg in water and detectable limit less than 1.5 mg/kg (Chandler and Nicol, 1975). All processed grapefruit juice contains more than 50 mg/kg naringin. Numerous techniques are used to reduce naringin, such as adsorptive debittering (Fayoux et al., 2007; Ribeiro, 2002), chemical methods (Berhow, 1992, 2000; Kimball, 1987), poly-styrene divinyl benzene styrene resin treatment (Puri, 1984), and β-cyclodextrin treatment (Fontananova et al., 2003; Shaw and Wilson, 1983; Wagner et al., 1988). These techniques have limitations in altering nutrient composition either through chemical reactions or removal of nutrients, flavor, and color etc.

Another suitable debittering procedure is the stepwise hydrolysis of naringin by naringinase (Habelt and Pittner, 1983; Ting, 1958). Naringinase is an enzyme complex containing both α-rhamnosidase and β-glucosidase. The former enzyme splits naringin into rhamnose and prunin and the latter further hydrolyses prunin to D-glucose and naringenin, a non-bitter component, which cannot be converted back to naringin (Ono et al., 1978; Thomas et al., 1958). Naringinase is also important in the production of sweetener precursors (Manzanares et al., 1997), preparation of prunin (Roitner et al., 1984), aroma enhancement in wine making (Caldini et al., 1994), biotransformation of antibiotics (Thirkettle, 2000), and manufacturing

*Corresponding author. E-mail: dxchen2000@163.com. Tel: +86-592-6182921; Fax: +86-592-6180470.

Table 1. Experimental factors and their levels for orthogonal layout $L_{18}(3^7)$.

Levels	Factors	Levels	Factors	Levels	Factors	Levels
	A * (g/L)	B (g/L)	C (g/L)	D (g/L)	E (g/L)	F (g/L)
1 ‡	5	5	0	0	0	0
2	15	10	0.2	0.2	1	1
3	25	15	0.4	0.4	2	2

* Symbols A, B, C, D, E and F represent factors of pomelo pericarp powder, peptone, $CaCl_2$, NaCl, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4, respectively; ‡ numbers 1–3 represent three concentrations for each factor.

of rhamnose, which is a chiral intermediate in organic synthesis and is used as a pharmaceutical and plant protective agent (Daniels et al., 1990). Immobilization of naringinase on different matrices has been studied by many researchers (Busto et al., 2007; Nunes et al., 2009; Puri et al., 2005b; Şekeroğlu et al., 2006). Yet limited information is available for commercial naringinase production even though industrial applications of naringinase are becoming more and more important.

The orthogonal matrix method is a useful technique for optimizing the culture conditions of fermentation processes. However, statistical methodology for medium optimization in naringinase production has been rarely used. The objective of this study is to optimize the culture medium for *Aspergillus oryzae* JMU316 in order to improve naringinase production using the one-factor-at-a-time and orthogonal matrix methods, and to provide practical guidance for industry.

MATERIALS AND METHODS

Chemicals

Naringin was obtained from Sigma (St. Louis. MO, USA). Acetonitrile and methanol (HPLC grade) were obtained from Tedia (Fairfield, OH, USA). Pomelo pericarp powder was made from dry Guanxi pomelos' pericarp, sieved through a 40 mesh. All other reagents were of analytical grade.

Microorganism and culture conditions

A. oryzae JMU316 was isolated from decayed 'Guanxi pomelo' flesh in Pinghe county, Zhangzhou, Fujian, P. R. China, and then cultured in our laboratory. The stock culture was maintained on potato dextrose agar (PDA) slants at 4℃ and subcultured every 4 weeks. Slants were incubated at 28℃ for 4 days and then stored at 4℃.

Inoculum preparation and fermentation

A. oryzae JMU316 was grown in petri dishes in an incubator at 28℃. Spore suspensions were prepared as inoculum by harvesting spores from 3-d old culture grown on PDA medium in 0.85% sufficiently sterile sodium chloride to reach the desired concentration of 10^8 spores/mL. The fermentation was inoculated with 3% (v/v) of the inocula and then cultivated in 300 mL flasks containing 30 mL medium at 28℃ on a rotary shaker incubator at 160 rpm for 5 days. The base medium contained K_2HPO_4 1.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, NaCl 0.2 g, $CaCl_2$ 0.2 g, $(NH_4)_2SO_4$ 0.2 g, yeast extract 1.0 g, naringin 5.0 g and distilled water up to 1 L (initial pH 6.0). All experiments were performed in triplicate.

Analyses of naringinase activity

The fermented culture solution was centrifuged at 15,000 × g for 15 min at 4℃ and supernatant was collected for analyses of naringinase activity. In brief, 0.05 mL supernatant was mixed with 0.5 mL 0.1% (w/v) naringin dissolved in 0.1 M sodium acetate buffer (pH 4.0). The mixture was stirred for 30 min at 40℃, and the reaction was terminated with 100 µL trichloroacetic acid (10%, w/v). Residual naringin was analyzed by HPLC (Waters 1525) with mobile phase A, a mixture of 3:1 methanol and acetonitrile, and mobile phase B, 0.01 M disodium hydrogen phosphate - citric acid buffer (pH 3.5). Flow rate was set at 0.8 mL/min with detection wavelength of 210 nm and column temperature at 35℃. One unit of naringinase activity (IU) was defined as 1 µg of naringin hydrolyzed per minute under the previously described conditions.

Optimization of medium

The one-factor-at–a-time method was used to select carbon and nitrogen sources and concentration for naringinase production. Various carbon sources (pomelo pericarp powder, lactose, rhamnose, sucrose, maltose, glucose, pomelo juice) were added individually at 10 g/L in the base medium compared to naringin control. Nitrogen compounds of organic nitrogen (peptone, beef extract, soybean meal, casein) and inorganic nitrogen (ammonium dihydrogen phosphate, ammonium nitrate, ammonium chloride) were also tested with pomelo pericarp powder as the carbon source. The orthogonal matrix method was used to optimize the concentrations of six nutrients (pomelo pericarp powder, peptone, $CaCl_2$, NaCl, $MgSO_4 \cdot 7H_2O$, K_2HPO_4) and determine the relationship between different variables of the medium components. The notation $L_a(b^c)$ is used to represent the orthogonal array where 'a' is the number of experimental runs, 'b' the number of levels for each factor or variable and 'c' the maximum number of factors investigated (Escamilla et al., 2000). For a problem with six design variables and three levels, the minimum orthogonal matrix method was selected as $L_{18}(3^7)$ with factors and level assignments noted in Table 1.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA). Tests of significant differences were determined by Duncan's multiple range tests at $P = 0.05$ using statistical package for the social sciences (SPSS) 11.5 (SPSS Inc., Chicago, IL, USA).

Table 2. Effects of carbon sources on naringinase production by *Aspergillus oryzae* JMU316 in shake flask culture*.

Carbon source	Naringinase specific activity (IU/mL) †	Relative activity (%)
Pomelo pericarp powder	336.22 ± 11.17 a	99.71
Lactose	184.73 ± 7.43 d	54.78
Rhamnose	286.37 ± 8.17 b	84.93
Sucrose	134.42 ± 5.24 e	39.86
Maltose	291.65 ± 13.22 b	86.49
Glucose	96.90 ± 6.48 f	28.74
Pomelo juice	224.49 ± 7.51 c	66.57
Naringin (control)	337.20 ± 7.77 a	100

* Fermentation experiments were carried out in shaking flask for 5 days at 28℃ with initial pH 6.0. † Values are mean ± S.D. of triple determinations. Values with the same letters are not significantly different by Duncan's multiple range test (P = 0.05).

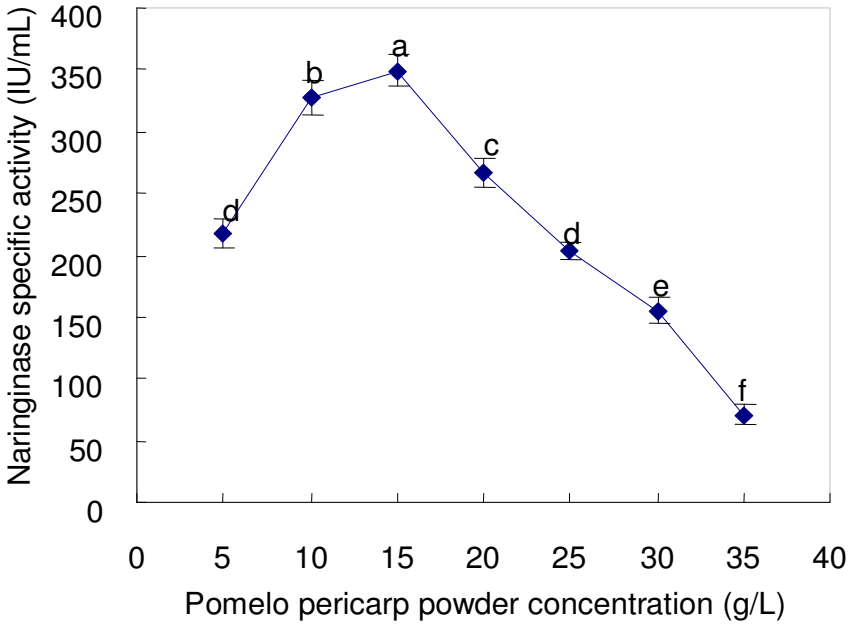

Figure 1. Effect of pomelo pericarp powder concentrations on naringinase activity produced by *Aspergillus oryzae* JMU316 in shaking flask culture *. * Fermentation experiments were carried out in shaking flask for 5 days at 28℃ with initial pH 6.0. Each value is expressed as mean ± standard deviation (n = 3). [a-f] means without the same superscripts differ by Duncan's multiple range test (P = 0.05).

RESULTS

One-factor-at-a-time method

After 5 days of fermentation, naringin exhibited the highest naringinase activity compared to all other carbon sources (P < 0.05, Table 2). Pomelo pericarp powder produced comparable naringinase activity and was not significantly different from naringin. Relative activities of other carbon sources to naringin were: pomelo pericarp powder, 99.71%; maltose, 86.49%; rhamnose, 84.93%; pomelo juice, 66.57%, lactose, 54.78%; sucrose, 39.86%; and glucose, 28.74%. Since pomelo pericarp powder is a cheap resource and has additional environmental benefits (pomelo pericarp powder is a waste of pomelo juice production), it was selected for medium formulation in subsequent experiments. Subsequently, a trial was conducted to determine optimum pomelo pericarp powder concentration (Figure 1). Naringinase activity was increased with increased pomelo pericarp powder from 5 to 15 g/L, then decreased sharply from 15 to 35 g/L with the highest activity at 15 g/L.

Table 3. Effects of nitrogen sources on naringinase production by *Aspergillus oryzae* JMU316 in shaking flask culture *.

Nitrogen source	Naringinase specific activity (IU/mL) †	Relative activity (%)
Control (basal medium)	340.16 ± 13.49 b	100.00
Organic nitrogen		
Peptone	361.94 ± 6.48 a	106.40
Beef extract	317.40 ± 15.14 c	93.31
Soybean meal	327.21 ± 7.48 bc	96.19
Casein	166.35 ± 13.14 f	48.90
Inorganic nitrogen		
$NH_4H_2PO_4$	209.54 ± 7.89 e	61.60
NH_4NO_3	108.02 ± 9.90 g	31.76
NH_4Cl	254.66 ± 6.48 d	74.86

* Fermentation experiments were carried out in shaking flask for 5 days at 28℃ with initial pH 6.0. † Values are mean ± S.D. of triple determinations. Values with the same letters are not significantly different by Duncan's multiple range test (P = 0.05).

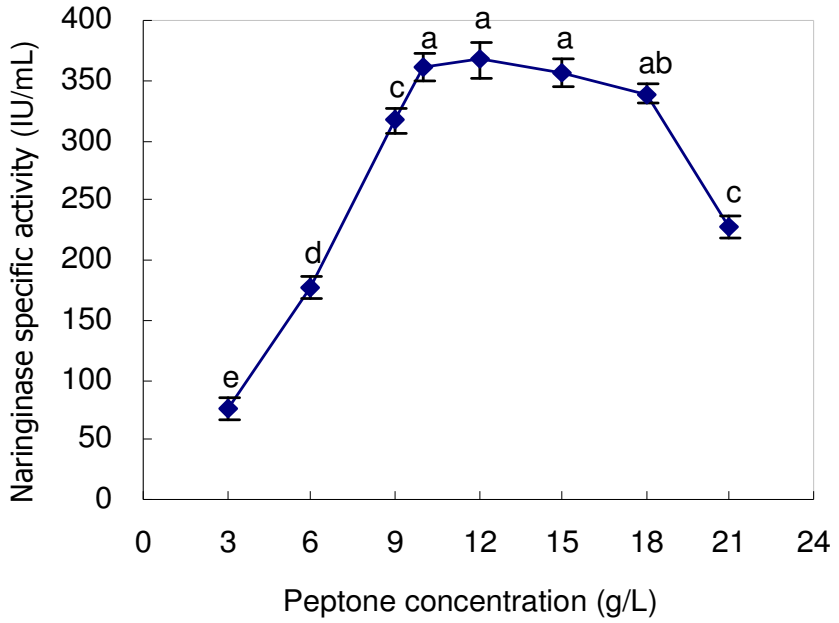

Figure 2. Effect of peptone concentrations on naringinase activity produced by *Aspergillus oryzae* JMU316 in shaking flask culture *. * Fermentation experiments were carried out in shaking flask for 5 days at 28℃ with initial pH 6.0. Each value is expressed as mean ± standard deviation (n = 3), [a-e] means without the same superscripts differ by Duncan's multiple range test (P = 0.05).

Different nitrogen sources were added at 10 g/L to replace potassium nitrate and ammonium sulphate in the base medium in a shaking flask culture of *A. oryzae* JMU316 (Table 3). Among all nitrogen sources, peptone was the most effective and was significantly better than the control (P < 0.05). In general, organic nitrogen sources yielded higher naringinase activity than inorganic nitrogen sources in liquid culture. Compared to the control, relative activities were: peptone, 106.40%; soybean meal, 96.19%; beef extract, 93.31%; NH_4Cl, 74.86%; $NH_4H_2PO_4$, 61.60%; casein, 48.90%; and NH_4NO_3, 31. 76%. Based on these results, peptone was selected as the nitrogen source in subsequent experiments. Naringinase activity was linearly increased with increased peptone concentration from 3 to 10 g/L, plateau from 10 to 15 g/L, and decreased dramatically at 18 g/L (Figure 2). Production was 361.10, 366.79 and 356.42 IU/mL at 10, 12 and 15 g/L peptone, respectively, with no significant

Table 4. Results of $L_{18}(3^7)$ orthogonal test of naringinase production by *Aspergillus oryzae* JMU316 in shaking flask culture*

Exp. group	A †	B	C	D	E	F	Naringinase specific activity (IU/mL) §
1 ‡	1 ∂	1	1	1	1	1	149.06 ± 17.15
2	1	2	2	2	2	2	332.67 ± 7.48
3	1	3	3	3	3	3	265.71 ± 23.37
4	2	1	1	2	2	3	304.59 ± 6.48
5	2	2	2	3	3	1	382.36 ± 6.48
6	2	3	3	1	1	2	274.35 ± 22.76
7	3	1	2	1	3	3	267.87 ± 16.31
8	3	2	3	2	1	1	140.41 ± 9.90
9	3	3	1	3	2	2	142.57 ± 23.37
10	1	1	3	3	2	2	319.71 ± 6.48
11	1	2	1	1	3	3	237.62 ± 13.49
12	1	3	2	2	1	1	179.30 ± 20.83
13	2	1	2	3	1	3	326.19 ± 22.76
14	2	2	3	1	2	1	244.11 ± 13.49
15	2	3	1	2	3	2	282.99 ± 9.90
16	3	1	3	2	3	1	110.17 ± 6.48
17	3	2	1	3	1	2	250.59 ± 13.49
18	3	3	2	1	2	3	138.25 ± 20.83

* Fermentation experiments were carried out in shaking flask for 5 days at 28°C with initial pH 6.0. † Symbols A, B, C, D, E and F represent factors of pomelo pericarp powder, peptone, $CaCl_2$, NaCl, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4 respectively. § Values are mean ± S.D. of triple determinations. ‡Each row of the experimental group number represents one experimental replicate, and every experimental group was replicated thrice. ∂ The arrangement of columns A–F were decided by orthogonal design for $L_{18}(3^7)$. Numbers 1–3 represent three concentrations for each factor as same as that in Table 1.

difference among the three levels. For economic reasons, 10 g/L peptone was used in the cultivation of *A. oryzae* JMU316 for the production of naringinase.

Orthogonal matrix method

The orthogonal matrix $L_{18}(3^7)$ method was used to investtigate the relationships among various medium components and to optimize their concentrations for naringinase production. The experimental conditions are listed in Table 4 and the results are included in the last column.

The effect of culture medium on naringinase production was calculated according to the orthogonal method (Ding et al., 2001; Lee et al., 1997). Based on the magnitude order of R value (maximum difference), the order of effects of factors was A, F, D, B, C, E, which corresponded to pomelo pericarp powder, K_2HPO_4, NaCl, peptone, $CaCl_2$, $MgSO_4 \cdot 7H_2O$ (Table 5). This result indicated that pomelo pericarp powder was the most important factor compared to other nutrients. To further test the effects of the six factors, analysis of variance was used and is shown in Table 6. All factors had significant effects on the naringinase production of *A. oryzae* JMU316 ($P = 0.05$). The results of ANOVA also demonstrated the order of significance of factors (Table5).

To obtain the optimal levels of each factor, the intuitive analysis was based on statistical calculation using the data in Table 4. In terms of the maximum K value of each column in Table 5, optimal levels of each medium ingredient for naringinase production by *A. oryzae* JMU316 were A2B2C2D3E3F2, corresponding to the optimal medium of 15 g pomelo pericarp powder, 12 g peptone, 0.2 g $CaCl_2$, 0.4 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4 in 1 L distilled water.

Verification test

To confirm optimal levels of each factor achieved by orthogonal design, experiments were carried out using optimal nutrient concentrations and the results are shown in Figure 3. The maximum naringinase activity of 408.28 IU/mL was achieved in the optimized medium by orthogonal design, which was 21.38 and 10.53% higher compared to the base medium and the medium optimized by the one-factor-at-a-time method, respectively ($P < 0.05$).

DISCUSSION

Naringinase is becoming more and more important in industrial applications. Production of naringinase by microorganisms is much cheaper and more efficient than chemical synthesis and extraction. Mateles et al. (1965)

Table 5. Analysis of media on naringinase production by *Aspergillus oryzae* JMU316 in shaking flask culture with $L_{18}(3^7)$ orthogonal test.

Parameter	Naringinase specific activity (IU/mL)					
	A †	B	C	D	E	F
K1 *	1484.07	1477.59	1367.42	1311.25	1179.48	1207.56
K2	1814.59	1913.96	1354.46	1350.14	1481.91	1728.18
K3	1049.87	1283.17	1626.65	1687.13	1546.72	1412.79
k1 §	247.35	246.27	227.90	218.54	219.98	201.26
k2	302.43	264.63	271.11	225.02	246.99	288.03
k3	174.98	213.86	225.74	281.19	257.79	235.46
R ‡	127.45	50.76	45.37	62.65	37.80	86.77
Optimal levels	2	2	2	3	3	2

† Symbols A, B, C, D, E and F represent factors of pomelo pericarp powder, peptone, $CaCl_2$, NaCl, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4, respectively. * $K_i^X = \sum$ naringinase activity at Xi, § $k_i^X = K_i^X / 3$, ‡ $R_i^X = \max(K_i^X) - \min(k_i^X)$. Symbol R means the maximum value of k_i^X minus the minimum value. Above symbol X represents A, B, C, D, E, and F respectively, and symbol i represents each level.

Table 6. The variance analysis of the results of $L_{18}(3^7)$ orthogonal test on the naringinase production by *Aspergillus oryzae* JMU316 in shaking flask culture.

Variance source	Sum of squares deviation	Degree of freedom	Mean square	F ratio	Significance level
A †	147095.08	2	73547.54	164.26	*
B	23783.70	2	11891.85	26.56	*
C	23575.65	2	11787.83	26.34	*
D	42727.28	2	21363.64	47.71	*
E	13649.15	2	6824.57	15.24	*
F	68771.07	2	34385.54	76.80	*
Error	18357.42	41	447.74		

$F_{0.05}(2, 41) = 3.23$, $F_{0.01}(2, 41) = 5.17$; * F ratio > $F_{0.01}$; † Symbols A, B, C, D, E and F represent factors of pomelo pericarp powder, peptone, $CaCl_2$, NaCl, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4, respectively.

reported that rhamnose or plant meal containing rhamnose glucoside increased naringinase production. The stimulation by naringin was much greater in shaking flasks than that by rhamnose (Bram and Solomons, 1965). In the present study, naringinase activity in the rhamnose medium was found to be lower than in the naringin or pomelo pericarp powder medium. Naringin exhibited the highest naringinase activity followed by pomelo pericarp powder, but the difference was not statistically significant. Pomelo pericarp powder produced high enzyme activity, presumably because it contains not only naringin and rhamnose but also other nutrients such as amino acid, protein, and vitamin etc. Glucose, sucrose, and lactose showed low naringinase activity. Repression of naringinase activity by glucose, sucrose and lactose was also reported by Puri et al. (2005a), although these carbon sources supported excellent growth. *A. oryza*e JMU316 produced naringinase without the need for an inducer in the medium. This clearly showed that the microorganism produces constitutive naringin-metabolizing emzyme. This is advantageous from a practical point of view because cells can produce naringinase conveniently and relatively cheaply by using inexpensive carbon sources. Although naringin was not essential for enzyme formation, its presence increased the naringinase yield in shaking flask experiments.

Pomelo pericarp powder is a good carbon source because it is readily available and inexpensive compared to other carbon sources and its use can solve environmental problems resulting from pomelo peel waste. Environmental protection has become a main concern worldwide and is an increasing concern in China. Waste produced by fruit processing industry has been increasing year by year and has become one of the main environmental pollutants. The amount of waste resulting from citrus fruit processing and consumption is enormous in China. Pomelo peel waste amounts to about 100 thousand metric tons annually. Moreover, most of this waste is dumped into the environment without any

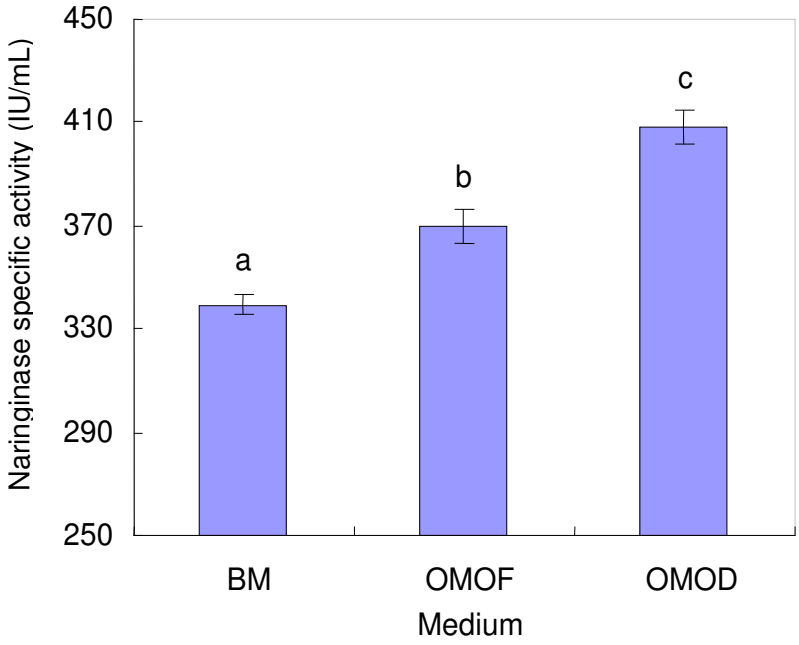

Figure 3. Effect of medium on naringinase production by *Aspergillus oryzae* JMU316 in verification test [*]. [*] Fermentation experiments were carried out in shaking flask for 5 days at 28°C with initial pH 6.0. Each value is expressed as mean ± standard deviation (n = 3). [a,b c]means without the same superscripts differ by Duncan's multiple range test (P = 0.05). BM represents base medium: K_2HPO_4 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 1.0 g/L, NaCl 0.2 g/L, $CaCl_2$ 0.2 g/L, $(NH_4)_2SO_4$ 0.2 g/L, yeast extract 1.0 g/L, naringin 5.0 g/L. OMOF represents optimal medium by one-factor-at-a-time: K_2HPO_4 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 1.0 g/L, NaCl 0.2 g/L, $CaCl_2$ 0.2 g/L, yeast extract 1.0 g/L, pomelo pericarp powder 15 g/L, peptone 10 g/L. OMOD represents optimal medium by orthogonal design: K_2HPO_4 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 2.0 g/L, NaCl 0.4 g/L, $CaCl_2$ 0.2 g/L, yeast extract 1.0 g/L, pomelo pericarp powder 15 g/L, peptone 12 g/L.

treatment. Although citrus fruit peel can be used for the extraction of essential oils or naringin, the cost of those applications is very high. This experiment provides an alternative way to recycle pomelo peel waste, which is not only beneficial to the pomelo processing industry but also to the environment. To the best of our knowledge, this is the first report that the pomelo peel can be used as a carbon source for the synthesis of naringinase in a fermentation process. The experiment suggests that other citrus fruit peel could be used as carbon source for naringinase production.

As far as nitrogen nutrition is concerned, *A. oryza*e JMU316 has a greater preference for organic nitrogen, which is typical for fungi. In general, in comparison with organic nitrogen sources, inorganic nitrogen sources apart from the control usually yielded relatively low naringinase production in shaking-flask cultures. These results are in agreement with those of submerged cultures of fungi reported by Norouzian et al. (2000). It has been suggested that inorganic nitrogen sources could only marginally synthesize certain essential amino acids in fermentation by fungi, and organic nitrogen sources were favorable for metabolite production (Hwang et al., 2003; Kim et al., 2003). Among all nitrogen sources used, peptone was the most effective in naringinase biosynthesis.

The orthogonal experimental design technique is a mathematical method that enables one to study the relationships among various factors. In comparison with full-factor experimental design, the orthogonal layout can reduce experimental difficulties, and it has been successfully applied to improve culture media for the production of primary and secondary metabolites in fermentation processes (Cai et al., 2009; Escamilla et al., 2000; Li et al., 2001; Xu et al., 2003). The conventional 'variation of one factor at a time' approach to optimization is not only time-consuming but often incapable of detecting interaction. It also becomes impractical when a large number of components have to be considered. In the present work, the one-factor-at-a-time method was used to observe effects of carbon and nitrogen sources on naringinase production at first. Subsequently, the concentration of each medium component including minerals was optimized using the orthogonal design. The optimal medium containing pomelo pericarp powder as the carbon source was developed by the one-factor-at-a-time method and orthogonal matrix method. It is noteworthy that the choice

of the fermentation system can have a significant impact on the industrial application because of the cost of medium ingredients. The result of the orthogonal layout indicated that all nutrients have significant effects on naringinase and the effect of pomelo pericarp powder was more important than that of the other five nutrients. Based on the L_{18} orthogonal array design, we carried out only 18 experiments in triplicate. In full-factorial experimental design, at least 2187 experiments would have been necessary to reach the same conclusion as those of the orthogonal array method with this number of factors.

Conclusions

Statistical methods were successfully applied to medium optimization of constitutive naringinase production from *A. oryzae* JMU316 in this study. The orthogonal matrix method of medium optimization was efficient, relatively simple, and time and material saving. The two-step optimization strategy used in this study may be worth attempting with other microorganism fermentation processes for enhancing production of naringinase, particularly those with industrial potential. Pomelo pericarp powder and peptone have been proved to be good carbon and nitrogen sources for naringinase production. Pomelo pericarp powder as carbon source could significantly lower the cost of naringinase production. Furthermore, it can reduce pomelo peel waste.

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