Food Sci. Biotechnol. Vol. 16, No. 1, pp. 159 ~ 162 (2007)

RESEARCH NOTE

Food Science and Biotechnology

Optimization of Culture Conditions for Phenylethyl Alcohol Production by *Pichia anomala* SKM-T Using Response Surface Methodology

Eun Kyoung Mo and Chang Keun Sung[1]*

Research and Development Center, DBIO Incorporation, Daejeon 305-764, Korea

[1]Department of Food Science and Biotechnology, Chungnam National University, Daejeon 305-764, Korea

Abstract Optimization of the fermentation medium for phenylethyl alcohol (PEA) production by *Pichia anomala* SKM-T was performed. The carbon source (glucose), nitrogen source (L-phenylalanine), and initial pH value were independent variables of the optimized medium. The central composite rotatable design was used for the experimental design and the analysis of the results. The optimum medium composition for the maximal production (621.27 mg/L) of PEA was found to be an initial pH of 5.03, and concentrations of L-phenylalanine at 6.53 and glucose at 6.11 g/L (w/v). This experimental finding is in close agreement with the model prediction (702.79 mg/L; desirability 0.884) with an 11.6% difference.

Keywords: optimization, phenylethyl alcohol, *Pichia anomala* SKM-T, central composite rotatable design

Introduction

Phenylethyl alcohol (PEA) is a simple aromatic primary alcohol. Because of its rose-like aroma, it is used as a fragrance ingredient in a wide variety of cosmetic products and foods such as beer, wine, olive oil, grapes, tea, apple juice, and coffee (1). It has been estimated that approximately 700,000 kg of PEA is consumed annually as a component of foods (2). Biologically synthesized PEA is 250- to 300-fold more expensive than chemically synthesized products (3). Flavors and fragrances that can be labeled 'natural' both in the United States and Europe have to be produced from natural sources by physical, enzymatic, or microbiological processes (4, 5). From the chemist's point of view, there is no difference between a flavor compound synthesized in nature and the same molecule produced in the laboratory. Therefore, increasing demand for natural flavors has motivated research into the microbial production of PEA.

The most prominent microorganisms regarding the production of natural PEA are yeasts. *Saccharomyces cerevisiae* (6), *Pichia fermentans* L-5 (7), several *Kluyvermyces* strains (1, 8), and *Candida* sp. S-8 (9) are known to produce PEA. *Pichia anomala* can produce small volatile metabolites, e.g., ethyl acetate, ethyl propionate, phenylethyl acetate, and PEA (10, 11).

There are limited studies in the literature pertaining to PEA production in *P. anomala*, and in particular there has been no reported study on the optimization of PEA production in *P. anomala*. The application of statistical experimental design techniques in fermentation process development can lead to improved product yields and reduced overall costs. The conventional practice of single factor optimization by maintaining other factors involved at an unspecified constant level does not depict the combined effect of all the factors involved. This method is also a time consuming process and requires a number of experiments to determine optimum levels. Moreover, the determined levels are unreliable. These limitations of the single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by a statistical experimental design using response surface methodology (RSM, 12, 13). RSM can be used to evaluate the relative significance of several affecting factors even in the presence of complex interactions.

Earlier we identified PEA from *P. anomala* SKM-T using a batch extraction method and GC-MS analyses (11). In the present study, the effects of medium components on PEA production by *P. anomala* SKM-T were investigated using RSM.

Materials and Methods

Yeast strain and culture conditions *P. anomala* SKM-T was precultured in potato dextrose broth (Difco Laboratories, Detroit, MI, USA) in an Erlenmeyer flask for 24 hr. It was subsequently grown on semi-synthetic media containing 1.0 g/L KH_2PO_4 (Sigma Chemicals, St. Louis, MO, USA), 0.5 g/L $MgSO_4$ (Sigma), and various concentrations (g/L) of glucose (Sigma) and L-phenylalanine (Sigma). The pH of the semi-synthetic media was adjusted using 1 N HCl and/or 1 N NaOH. The batch culture (3 L-Erlenmeyer baffle flask) conditions were adjusted to various temperatures and/or agitation speeds and held at an initial biomass of 0.1 g/L (w/v).

PEA extraction and analyses The dry biomass of *P. anomala* SKM-T was measured after determining moisture content using the air-oven method (14). PEA was extracted with dichloromethane/pentane (2:1) three times and the PEA concentrations were analyzed using gas chromotography (6890N; Agilent Technologies, Santa Clara, CA, USA). Butyl benzene (Sigma) was used as an internal standard. The detailed procedures and analytical conditions have been described previously (11).

*Corresponding author: Tel: 82-42-821-6722; Fax: 82-42-822-2287
E-mail: kchsung@cnu.ac.kr
Received August 4, 2006; accepted November 7, 2006

Experimental design and statistical analyses RSM was used to investigate the influence of the concentrations of carbon and nitrogen sources, as well as the initial pH of the medium, on the production of PEA. A central composite rotatable design with three factors and 5 levels, including 6 replicates at the center point, was used for fitting a second order response surface. In the RSM experiment, 'Design Expert' software (Ver. 6.0.10., Stat-Ease, Minneapolis, MN, USA) was used for equation and graphical analysis of the obtained data.

Results and Discussion

Effect of carbon and nitrogen sources on PEA production Various carbon and nitrogen sources influence the quantity of the produced flavor compounds (1). In order to compare the efficiency of *P. anomala* SKM-T in terms of PEA production in the presence of different carbon substrates, the test strain was cultivated on a semi-synthetic media containing various carbon substrates for 24 hr. As shown in Table 1, the biomass of cultures metabolizing glucose, maltose, fructose, xylose, and sorbitol ranged between 0.89 and 1.98 g/L, and the PEA yields of these cultures were between 0.24 and 49.30 mg/g. Glucose appeared to be the most efficient carbon source with regard to the increase of biomass and PEA yields.

A critical step in PEA production is the phenylpyruvate decarboxylase reaction, which is the first specific step in phenylalanine catabolism. Phenylpyruvate decarboxylase activity was detected in an aerobic culture grown with phenylalanine as a sole nitrogen source (15). Therefore, the effect of L-phenylalanine concentrations on PEA production by *P. anomala* SKM-T was investigated. As shown in Table 2, PEA production yields increased with concentrations of L-phenylalanine up to 5 g/L. The highest levels of cell growth and PEA production were detected in medium supplemented with 9 g/L of L-phenylalanine. In media having a concentration of L-phenylalanine greater than 7 g/L, however, a delayed increase in PEA production was observed. Considering the price of L-phenylalanine and the cost of PEA production, 5 g/L was deemed an efficient concentration. Consequently, further experiments were performed with 5 g/L of glucose and 5 g/L of L-phenylalanine.

Table 1. Growth and phenylethyl alcohol production by *Pichia anomala* SKM-T in media with various carbon substrates[1)]

Substrates (5 g carbon /L)	Biomass (g/L)	PEA (mg/L)	Yield (PEA/Biomass, mg/g)
Glucose	1.98±0.06	97.61±1.06	49.30±0.11
Maltose	1.95±0.03	88.10±0.91	45.18±0.05
Fructose	1.75±0.05	41.09±0.06	23.48±1.23
Xylose	1.02±0.25	9.27±0.04	9.09±0.61
Sorbitol	0.89±0.01	0.21±0.01	0.24±0.03

[1)]Medium contained KH_2PO_4 1.0 g/L, $MgSO_4$ 0.5 g/L, L-phenylalanine 1.0 g/L and an initial biomass 0.1 g/L. Cultivation was conducted at 30°C and with an agitation speed of 200 rpm for 24 hr. The initial pH of media was 5.

Table 2. Growth and phenylethyl alcohol production by *Pichia anomala* SKM-T in media with various L-phenylalanine concentrations[1)]

Concentration (g/L)	Biomass (g/L)	PEA (mg/L)	Yield (PEA/Biomass, mg/g)
1	1.98±0.21	97.61±1.32	49.30±1.76
3	1.98±0.08	109.87±1.98	55.49±1.66
5	2.01±0.02	584.81±1.65	290.95±1.97
7	2.34±0.46	681.83±0.86	291.38±2.35
9	2.35±0.03	687.33±1.75	292.48±0.58

[1)]Medium contained KH_2PO_4 1.0 g/L, $MgSO_4$ 0.5 g/L, glucose 5.0 g/L and an initial biomass of 0.1 g/L. Cultivation was conducted at 30°C and with an agitation speed of 200 rpm for 24 hr. The initial pH of media was 5.

Effects of physical factors on PEA production The effects of cultivation temperature on PEA production by the test strain were investigated by varying the temperature from 15 to 40°C in 5°C increments. The highest PEA production was noted at 30°C with a yield of 290.95 mg/g (PEA/Biomass), which was significantly higher than the 213.43 mg/g (PEA/Biomass) produced at the second most effective temperature of 35°C. PEA production at different pH values ranging from 2 to 7 was also investigated. The yield (290.95 mg/g, PEA/Biomass) at pH 5 was found to be double that of the second highest yield at pH 4 (158.04 mg/g, PEA/Biomass). Alcohol production by microorganisms is affected by agitation speed and/or aeration rate. Accordingly, the effect of agitation speed (from 100 to 200 rpm with every 100 rpm) on PEA production by *P. anomala* SKM-T was examined. Although biomass increases with agitation speed, PEA production yield seem to be constant with no statistically significant differences in the range of speeds tested.

Optimization of PEA production On the basis of these results, experiments were performed to optimize PEA production. The first step in RSM is to find a suitable approximation for the true functional relationship between the dependent variable and the set of independent variables. From Table 3, a central composite rotatable design for three independent variables was used to obtain the optimum medium conditions. The optimal values of the experimental conditions were obtained by solving the regression equation and also by the response surface contour map.

In order to investigate the optimum medium conditions that affect PEA production utilizing RSM, the initial medium pH, L-phenylalanine concentration, and glucose concentration were selected as independent input variables. The response variable was PEA concentration (mg/L) and the center point of the design is the zero point. The range of the parameters, the central composite rotatable design matrix of five levels in coded values, and the resultant PEA concentrations are given in Table 3. Analysis of variance (ANOVA) for the quadratic model confirms the adequacy at a 95.85% confidence level, and the fit of the model was with a coefficient (R^2) of 0.8613. The final second order equation obtained (coded values) is shown below:

Table 3. Rotatable central composite design of optimum culture conditions for phenylethyl alcohol production by *Pichia anomala* SKM-T[1)]

No.	x_1		x_2		x_3		PEA (mg/L)
	C	A (g/L)	C	A (g/L)	C	A (g/L)	
1	-1	4.5	-1	5	-1	5	340.02±1.65
2	1	5.5	-1	5	-1	5	131.14±2.33
3	-1	4.5	1	7	-1	5	94.08±0.98
4	1	5.5	1	7	-1	5	313.57±0.25
5	-1	4.5	-1	5	1	7	79.23±1.23
6	1	5.5	-1	5	1	7	209.86±3.58
7	-1	4.5	1	7	1	7	362.12±3.69
8	1	5.5	1	7	1	7	250.19±2.74
9	-1.682	4.0	0	6	0	6	105.85±1.59
10	1.682	6.5	0	6	0	6	78.39±2.99
11	0	5.0	-1.682	4	0	6	94.33±1.98
12	0	5.0	1.682	8	0	6	567.51±0.06
13	0	5.0	0	6	-1.682	4	43.00±0.47
14	0	5.0	0	6	1.682	8	118.16±1.57
15	0	5.0	0	6	0	6	605.45±0.68
16	0	5.0	0	6	0	6	697.38±1.53
17	0	5.0	0	6	0	6	643.29±2.65
18	0	5.0	0	6	0	6	612.35±3.01
19	0	5.0	0	6	0	6	523.78±2.98
20	0	5.0	0	6	0	6	538.85±0.69

[1)] x_1, pH; x_2, L-phenylalanine; x_3, D-glucose; C, coded value; A, actual value.

$$y = 600.38 - 1.27x_1 + 77.29x_2 + 10.85x_3 - 163.7x_1^2 - 79.2x_2^2 - 167.60x_3^2 + 23.25x_1x_2 + 1.00x_1x_3 + 48.50x_2x_3$$

The results show that among the independent variables, L-phenylalanine has a significant effect (p=0.0358), whereas glucose (p=0.7407) and pH (p=0.9689) do not have significant effects. The circular contour of the response surfaces indicates that the interactions between the corresponding variables are negligible (17). Therefore, PEA production depended on the L-phenylalanine concentration. The optimum PEA production is obtained according to the above equation when the values of pH, L-phenylalanine and glucose are 5.03, 6.53 g/L (w/v), and 6.11 g/L (w/v), respectively. When *P. anomala* SKM-T was cultured at the optimum conditions, 621.27 mg/L of PEA was obtained. This experimental finding is in close agreement with the model prediction (702.79 mg/L; desirability 0.884) with an 11.6% difference.

In order to increase the final PEA concentration, *P. anomala* SKM-T, a Crabtree negative strain, was cultivated with various media and/or culture conditions. It has similarly been reported that the production of PEA is associated with cell growth in *P. anomala* SKM-T (7, 16). Although the glucose and initial pH of media affect the cell biomass, these two independent variables did not significantly affect PEA production. Brief observations during this study indicated that the PEA concentration depended significantly on the nature of the nitrogen source, L-phenylalanine. Considering the maximum theoretical yield of 0.75 g/L in *S. cerevisiae* (6), the PEA concentration from this study was 2.5 times lower than that of the reported strain (1). These results suggest that, under optimal conditions, complete molar conversion of the precursor and/or nutrients might be not achievable. Thereby, the main reason for the low PEA production yields in this study is insufficient salt concentration, which is essential for the bioconversion of L-phenylalanine to PEA. A second reason is the extraction power of the solvents used which were not optimal. The low PEA production yields in this study can be improved by more efficient *in situ* product recovery techniques than the batch extraction method. With regard to industrial processes and/or economic aspects, further studies are needed in order to establish the optimal salt concentration and an effective *in situ* product recovery technique. Unfortunately, this study did not determine these optimal conditions. Thus several of the above mentioned factors need to be addressed to produce a greater yield of PEA.

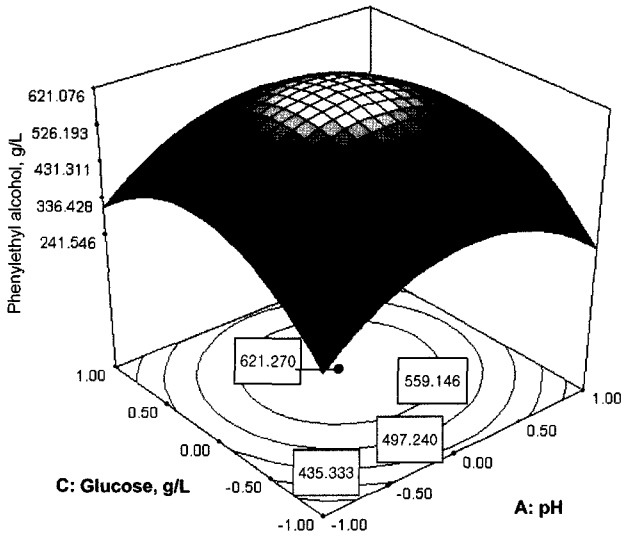

Fig. 1. Response surface contour map of phenylethyl alcohol production by *Pichia anomala* SKM-T. L-Phenylalanine was held at a level of 0.53 (coded value) and other conditions were held at 24 hr, 200 rpm, 30°C and an initial biomass of 0.1 g/L.

Acknowledgments

This study was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Korea.

References

1. Fabre CE, Blanc PJ, Goma G. Production of 2-phenylethyl alcohol by *Kluyvermyces marxianus*. Biotechnol. Progr. 14: 270-274 (1998)
2. Maarse H, Visscher CA, Willensens LC, Boelens MH. Volatile Components in Food-Qualitative and Quantitative Data. Central Institute Voor Voedingsondezioke. TNO Zeist, The Netherlands (2000)

3. Etschmann MMW, Bluemke W, Sell D, Schrader J. Biotechnological production of 2-phenylethanol. Appl. Microbiol. Biot. 59: 1-8 (2002)
4. US FDA. Code of Federal Regulations. 21CFR 101.22. Food and Drug Administration, Rockville, MD, USA (2001)
5. Jun MR, Jeong WS, Ho CT. Health promoting properties of natural flavor substances. Food Sci. Biotechnol. 15: 329-338 (2006)
6. Stark D, Munch BS, Marison IW, Stockar U. Extractive bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. Biotechnol. Progr. 18: 514-523 (2002)
7. Huang CJR, Lee SL, Chou CC. Production and molar yield of 2-phenylethanol by *Pichia fermentans* L-5 as affected by some medium components. J. Biosci. Bioeng. 90: 142-147 (2000)
8. Jiang J. Identification of flavor volatile compounds produced by *Kluyvermyces lactis*. Biotechnol. Tech. 8: 863-866(1993)
9. Seo WT, Ahn KC. Production of phenylethanol from L-phenylalanine by *Candida* sp. S-8. Food Sci. Biotechnol. 12: 644-648 (2003)
10. Westall S. Characteristics of yeast species by their production of volatile metabolites. J. Food Mycol. 1: 187-201 (1999)
11. Mo EK, Kang HJ, Lee CT, Xu BJ, Kim JH, Wang QJ, Kim JC, Sung CK. Identification of phenylethyl alcohol and other volatile flavor compounds from yeasts, *Pichia farinosa* SKM-1, *Pichia anomala* SKM-T, and *Galactomyces geotrichum* SJM-59. J. Microbiol. Biotechn. 15: 800-808 (2003)
12. Box GE, Hunter JS. Multiple experimental designs for exploring response surfaces. Ann. Math. Stat. 28: 195-241 (1957)
13. Lee SJ, Lee YB, Hong JH, Chung JH, Kim SS, Lee WJ, Yoon JR. Optimization of pine flavor microencapsulation by spray drying. Food Sci. Biotechnol. 14: 747-751 (2005)
14. AOAC. AOAC Official Method 925.10. Solid and Moisture in Foodstuff (air-oven method). Franklin, PA, USA (1995)
15. Vuralhan A, Morais MA, Tai SL, Piper MDW. Identification and characterization of phenylpyruvate decarbolyase genes in *Saccharomyces cerevisiae*. Appl. Environ. Microb. 69: 4534-4541 (2003)
16. Albertazzi E, Cardillo R, Servi S, Zucchi G. Biogeneration of 2-phenylehthanol and 2-phenylethylacetate important aroma components. Biotechnol. Lett. 16: 491-496 (1994)
17. Murthy MSRC, Swaminathan T, Rakshit SK, Kosugi Y. Statistical optimization of lipase catalyzed hydrolysis of methyloleate by response surface methodology. Bioprocess Eng. 22: 35-39 (2000)