



Effects of aroma quality on the biotransformation of natural 2-phenylethanol produced using ascorbic acid



Xun Tian, Rui Ye, Jingwen Wang, Yifei Chen, Baoguo Cai, Shimin Guan, Shaofeng Rong *, Qianqian Li *

School of Perfume and Aroma Technology, Shanghai Institute of Technology, Shanghai, PR China

ARTICLE INFO

Article history:

Received 25 February 2015

Accepted 2 May 2015

Available online 28 May 2015

Keywords:

Aroma quality

Ascorbic acid

Biotransformation

By-products

2-Phenylethanol

ABSTRACT

Background: Natural 2-phenylethanol (2-PE) is an important flavoring that emits the aroma of roses. During biotransformation, the aroma quality of natural 2-PE is affected by its main by-products, which include butanol, isobutyric acid, butyric acid, and isovaleric acid. Thus, controlling undesirable by-product formation can reduce the effect of odor on 2-PE aroma quality.

Results: 2-PE was produced through biotransformation using L-phenylalanine as a substrate and glucose as a carbon source. Ascorbic acid was added to the system to improve the redox reaction and suppress the generation of by-products. Principal component analysis of the aroma quality of 2-PE was performed using an electronic nose. Similarity analysis revealed that the effects of four by-products on 2-PE aroma quality may be ranked in the following order: isovaleric acid > butyric acid > isobutyric acid > butanol. The sample that exhibited the best similarity to the standard 2-PE sample (99.19%) was the sample to which ascorbic acid had been added during glucose metabolism.

Conclusions: 2-PE produced through the addition of ascorbic acid exhibited the closest aroma similarity to the standard 2-PE sample.

© 2015 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. All rights reserved.

1. Introduction

2-Phenylethanol (2-PE), an aromatic alcohol, emits a fresh rose scent. Natural 2-PE, especially that in rose oil, is present in a number of plants [1,2,3]. As a result of its delicate aroma, 2-PE is the second most-often used flavor after vanillin. The compound and its derivatives can be used in food, tobacco, and cosmetics. The global annual output of 2-PE is nearly 10,000 t. Chemical synthesis is the basic method used to produce 2-PE. However, benzene or styrene, the raw materials used to synthesize 2-PE, are regulated chemicals [3, 4]. Methods to produce natural 2-PE include plant extraction and microbial transformation. Essential oils extracted from roses to produce 2-PE account for only a small proportion of the annual output of the compound [5,6,7]. As such, the production of 2-PE through biotransformation presents broad application prospects.

Aroma quality is an important consideration in the production of 2-PE through biotransformation. By-products such as butanol, isobutyric acid, butyric acid, and isovaleric acid are produced during biotransformation; these by-products are adsorbed, together with 2-PE, during post-processing, thereby affecting the aroma quality of natural 2-PE. High-temperature vacuum distillation is often used to

separate impurities from 2-PE [8]. However, high temperature and vacuum conditions can change the aroma quality of 2-PE. To address this problem, reduction of the concentration of by-products during biotransformation and separation of these compounds by atmospheric distillation has been proposed. Fusel alcohol (e.g., butanol) and miscellaneous acid (e.g., isobutyric acid, butyric acid, and isovaleric acid) generation during an alternative strategy of glucose metabolism and ethanol oxidation has been discussed [9]. However, little information on the control of impurities through addition of exogenous substances has been reported.

An electronic nose is an instrument that can discriminate and recognize various gases and odors [10]. Electronic noses consist of a headspace sampler, sensor array, and pattern recognition modules. These devices are capable of performing the following functions: generating volatile compounds of a sample, reacting on contact, and recording data as a fingerprint. Today, electronic noses are used to monitor food quality. These devices have also shown promising application potential in determination of aroma quality [11].

Taking our previous studies [12,13] as bases, a 2-PE biotransformation system was developed in the present work using L-phenylalanine (L-Phe) as the substrate. An alternative strategy of glucose metabolism and ethanol oxidation was applied to this system, and a post-processing procedure using macroporous resin was performed. Ascorbic acid was added during 2-PE biotransformation, and the effect of by-products on the aroma quality of the 2-PE obtained was investigated.

* Corresponding authors.

E-mail addresses: rong_sf@163.com (S. Rong), qqli@sit.edu.cn (Q. Li).

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

2. Materials and methods

2.1. Strains and reagents

Saccharomyces cerevisiae AS 2.1182 was used for 2-PE biotransformation. Macroporous adsorption resin (HZ816) was obtained from Shanghai Huazhen Science and Technology Co., Ltd. (Shanghai, China), and 99.5% L-Phe was acquired from Wuxi Jinghai Amino Acid Co., Ltd. (Wuxi, China). A standard 2-PE sample was provided by the Shanghai Research Institute of Fragrance and Flavor Industry (Shanghai, China). Analytical-grade ascorbic acid and other chemicals were purchased from Sigma (St. Louis, MO, USA) or Sinopharm Chemical Reagent Co., Ltd. (Beijing, China) unless otherwise indicated.

Two buffers, namely, buffers A and B, were used during high-performance liquid chromatography (HPLC) analysis. Buffer A contained water, while buffer B contained methanol.

2.2. Medium and biotransformation

The biotransformation medium consisted of 30 g/L glucose, 8 g/L L-Phe, 5 g/L MgSO₄, 5 g/L K₂HPO₄, and 1 g/L NaCl.

Biotransformation was conducted in a fermentor. Briefly, 3.5 L of 10 g/L *S. cerevisiae* AS 2.1182 was inoculated directly to a 5 L fermentor (BioFlo3000, New Brunswick Scientific Co., Inc., Edison, NJ, USA). The bioreactor was equipped with pH, temperature, and dissolved oxygen monitoring and control systems. The dissolved oxygen tension level was controlled under cascade mode with a constant air flow rate of 3 L/min. Biotransformation was conducted at normal pressure and 30°C with an agitation speed of 400 rpm. The concentrations of glucose, L-Phe, ethanol, and 2-PE were monitored during biotransformation. Glucose, L-Phe, and resin HZ816 were added batch wise according to the requirements of the reaction system.

Butanol, isobutyric acid, butyric acid, and isovaleric acid were added to the standard 2-PE sample at final concentrations of 10–50 mg/L. Samples were then analyzed by an electronic nose to determine the effects of the different by-products on the aroma quality of natural 2-PE.

Ascorbic acid (0.2 g/L) was added to the bioreactor at the start of glucose metabolism (Group II) and 4 h after ethanol oxidative degradation began (Group III). The sample without ascorbic acid (Group I) was designated as the control. This procedure was conducted to determine the effect of ascorbic acid on the produced by-products.

For post-processing, 30 g/L macroporous adsorption resin (HZ816) was added to the bioreactor after completion of fermentation. Adsorption was conducted for 0.5 h at 400 rpm with an aeration rate of 3 L/min.

2.3. Analytical methods

Samples obtained from the biotransformation solution were centrifuged for 10 min at 4000 rpm and 4°C. The supernatant was then used to determine the concentrations of 2-PE, ethanol, L-Phe, and glucose. After biotransformation, resin HZ816 was used to separate the products in the fermentation broth. The resin was loaded into a glass chromatographic column and washed with 95% ethanol. The resin was desorbed by ethanol flowing at a rate of 2 mL/min until the 2-PE concentration in the eluate was <10 mg/L. Macromolecules were removed through microfiltration membranes (0.22 μm), and ethanol was removed by distillation to obtain the pure 2-PE sample.

Residual sugar contents were determined using an SBA-90 Biosensor (Shandong Academy of Sciences, Shandong, China). A 6890A gas chromatograph (Agilent Technologies, Ltd., Santa Clara, CA, USA) was used to determine the concentrations of ethanol, 2-PE, butanol, butyric acid, isobutyric acid, and isovaleric

acid under the following conditions: chromatographic column, 19091J-413 HP-5 (30 m × 0.32 mm × 0.25 μm); injection amount, 1 μL; injection port temperature, 250°C; detector temperature, 250°C; and temperature gradient of 50°C for 3 min, 250°C for 10°C/min, and maintenance at 250°C for 5 min.

L-Phe contents were determined using an Agilent 1260 HPLC separation module fitted with a C18 column (250 mm × 4.5 mm; XDB-C18). Around 50 μL of the samples was injected into the column and eluted at a flow rate of 1 mL/min with 60% buffer B at the start of elution. The concentration of buffer B was gradually increased to 80% after 20 min, and the concentration of L-Phe was determined at 254 nm. Standard curves of the metabolites were obtained to achieve accurate quantification.

All of the 2-PE samples were analyzed using a FOX 4000 (Alpha MOS, Toulouse, France) electronic nose. This device included 18 metal oxide sensors that measure changes in electrical resistance in the presence of volatile compounds. The detection conditions were as follows: carrier gas, synthetic dry air; flow rate, 150 mL/min; 0.01 g of sample added to 1 mL of distilled water in a 10 mL vial; generation time, 600 s; temperature, 40°C; stirring speed, 500 rpm; injection volume, 500 μL; injection speed, 1000 μL/s; needle total volume, 2.5 mL; needle temperature, 60°C; acquisition time, 120 s; and lag time, 360 s [14]. The similarity (S, %) of the two perfume ingredients was calculated using the following formula:

$$S = \frac{1 - \sqrt{\sum_{i=1}^{18} (a_i - b_i)^2}}{\sqrt{\sum_{i=1}^{18} (a_i + b_i)^2}} \times 100\% \quad [\text{Equation 1}]$$

where *a* and *b* are the values of the corresponding fragrance sensor of the prepared 2-PE sample and the standard 2-PE sample, respectively.

2.4. Statistical analysis

One-way ANOVA was performed to estimate the overall significance of the data. A probability level of 5% (*p* < 0.05) was considered statistically significant. Correlation data analysis was conducted through principal component analysis (PCA) by using Xlstat. All experiments were performed in triplicate.

3. Results and discussion

3.1. Effects of the main by-products on the aroma quality of 2-PE

During 2-PE production by biotransformation, by-products, such as butanol, isobutyric acid, butyric acid, and isovaleric acid, produce a distinct and unpleasant odor that destroys the aroma quality of natural 2-PE. The present experiment was conducted to study the effect of different concentrations of by-products on 2-PE aroma quality. Different concentrations of the four by-products were added to the standard 2-PE sample, and the effects of these compounds on the aroma quality of 2-PE were analyzed. The effects of main by-products on the aroma quality of 2-PE showed the following order: isovaleric acid > butyric acid > isobutyric acid > butanol (Fig. 1). The similarity in aroma quality of the produced 2-PE sample and the standard 2-PE sample was apparent when the concentration of butanol was >40 mg/L. The aroma quality similarity between the 2-PE samples was <99.00% when the concentrations of isobutyric, butyric, and isovaleric acids were >30 mg/L. Moreover, the similarity of the products was >99.00% when equal concentrations of the four by-products were mixed provided that the concentrations of individual by-products were less than 20 mg/L. The similarity in aroma quality between 2-PE samples was <98.00% when the concentration of the four by-products exceeded 30 mg/L. Under this condition, a distinct smell that affected the aroma quality of 2-PE was detected.

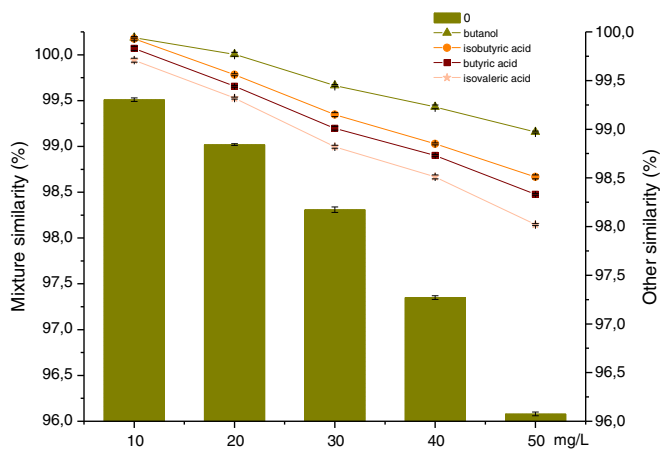


Fig. 1. Effects of the four main by-products on 2-PE aroma quality. The columns represent the similarities of mixtures of equal concentrations of butanol, isobutyric acid, butyric acid, and isovaleric acid as indicated in the abscissa (e.g. 10 mg/L in the abscissa represents 10 mg/L of butanol, isobutyric acid, butyric acid, and isovaleric acid in the mixture, and the total content of the four by-products is 40 mg/L). The curves represent the similarities of butanol (▲), isobutyric acid (●), butyric acid (■), and isovaleric acid (★) with concentrations shown in the abscissa adding standard 2-PE sample. Data reflect the means of the three experimental replicates. Bars indicate the standard errors. For all comparisons $p < 0.05$.

3.2. Effect of ascorbic acid on by-product concentration during 2-PE biotransformation

2-PE biotransformation was achieved through an alternative strategy of glucose metabolism and ethanol oxidation. By-products, such as ethanol, butanol, isobutyric acid, isovaleric acid, and other compounds, were produced when glucose metabolism entered the bypass pathway. As such, the concentration of by-products must be reduced at the beginning of the reaction to improve the final quality of 2-PE. Fig. 2 shows that by-product concentrations changed significantly when ascorbic acid was added to the reaction system during biotransformation. While the four main by-products were enriched during glucose metabolism, the concentrations of butanol, isobutyric acid, and butyric acid gradually decreased during ethanol oxidation. By contrast, the concentration of isovaleric acid in Group I gradually increased to a final concentration of 25.7 mg/L (Fig. 2). This result is significantly different from those observed in Groups II and III. The concentration of butanol in the three experimental groups was >60 mg/L. However, butanol contents in Groups II and III significantly decreased by about 41.27% and 58.10%, respectively, compared with that in Group I. The addition of ascorbic acid did not significantly affect the content of isobutyric acid during 2-PE biotransformation, and the peak butyric acid concentration was observed in the reaction

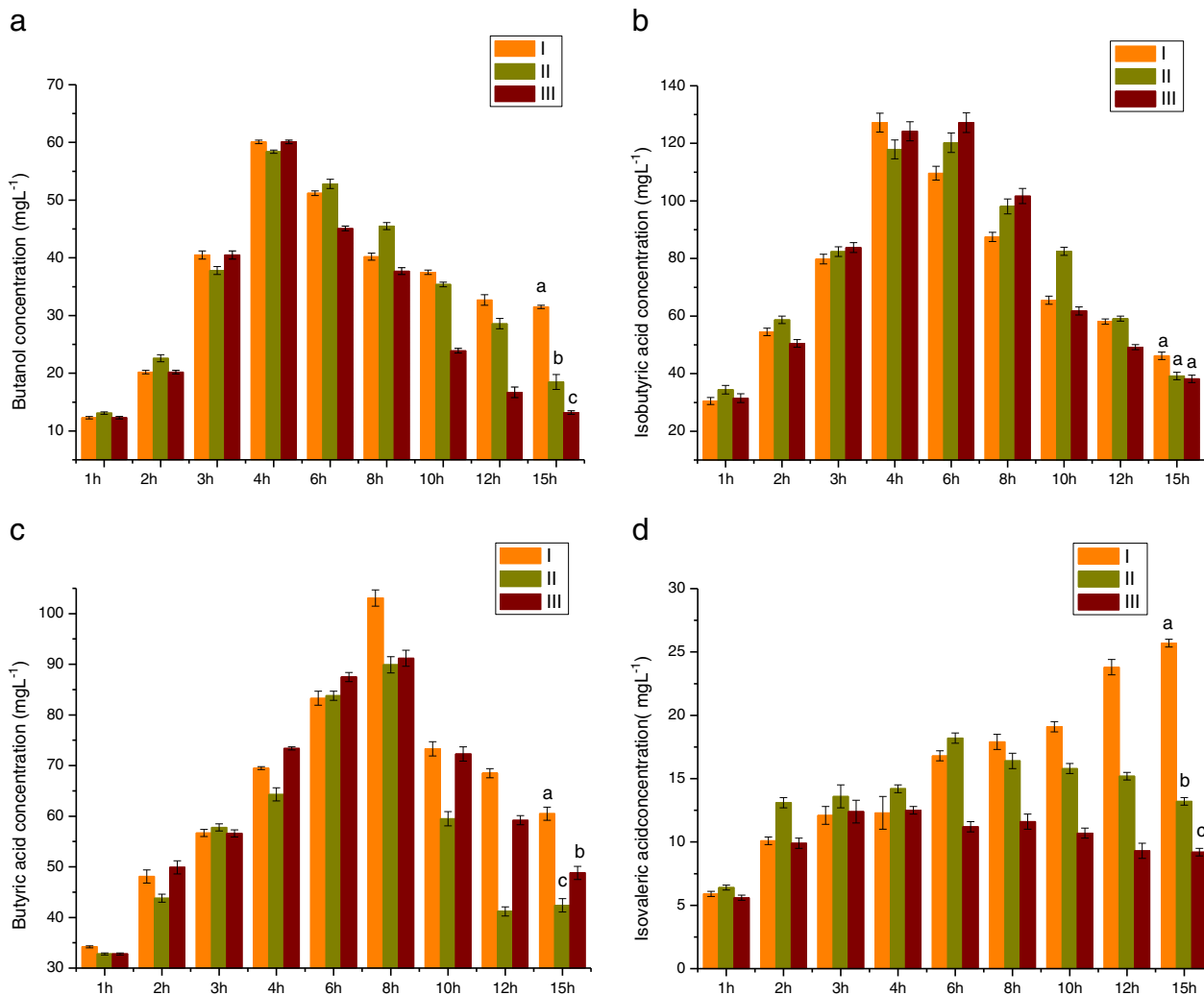


Fig. 2. Concentration changes in a) butanol, b) isobutyric acid, c) butyric acid, and d) isovaleric acid during application of the alternative strategy of glucose metabolism and ethanol oxidation: I, without ascorbic acid; II, with addition of ascorbic acid during glucose metabolism; and III, with addition of ascorbic acid during ethanol oxidation. During biotransformation, glucose metabolism occurred during the first 4 h, and ethanol oxidation began from the 4th to the 15th hour. Data represent the means of three experimental replicates. Bars indicate the standard errors. For all comparisons $p < 0.05$.

Table 1
Concentrations of the four main by-products during post-processing.

	Initial concentration ^a	Residual in the broth ^b	Elution ^c
<i>I</i>			
Butanol	31.25 ± 0.17	18.63 ± 0.47	13.14 ± 1.27
Isobutyric acid	41.63 ± 0.15	20.12 ± 0.27	21.35 ± 0.37
Butyric acid	60.51 ± 0.67	33.04 ± 0.59	27.01 ± 0.98
Isovaleric acid	25.70 ± 0.42	13.05 ± 0.79	12.12 ± 0.34
<i>II</i>			
Butanol	18.51 ± 0.33	10.14 ± 0.33	8.23 ± 0.12
Isobutyric acid	39.22 ± 0.25	26.52 ± 0.37	13.35 ± 0.14
Butyric acid	42.44 ± 0.56	29.18 ± 1.02	13.01 ± 0.09
Isovaleric acid	13.27 ± 0.29	7.44 ± 0.24	6.12 ± 0.22
<i>III</i>			
Butanol	13.21 ± 0.31	8.17 ± 0.35	5.14 ± 0.19
Isobutyric acid	38.20 ± 1.32	21.52 ± 0.21	16.35 ± 0.14
Butyric acid	48.81 ± .35	31.02 ± 1.32	17.01 ± 0.23
Isovaleric acid	9.22 ± 0.32	5.44 ± 0.27	4.12 ± 0.05

The concentration in the eluate was calculated and converted to the concentration in the fermentation broth: (I) without ascorbic acid, (II) with addition of ascorbic acid during glucose metabolism, and (III) with addition of ascorbic acid during ethanol oxidation. Data are presented as mean ± standard error ($n = 3$).

^a Initial concentration in the fermentation broth.

^b Residual concentration in the broth after post-processing.

^c Concentration in the eluate.

at the 8th h. The final 2-PE concentrations in Groups I, II, and III were 3.01, 3.24, and 3.18 g/L, respectively.

3.3. Effects of ascorbic acid on by-product generation during post-processing

The concentrations of the four main by-products during post-processing were analyzed by gas chromatography. Significant concentration changes among the four by-products were not observed during post-processing, but the by-product concentrations in Groups II and III were lower than that in Group I (Table 1). The final concentrations of the by-products in Groups II and III were <20 mg/L, and the total contents of these by-products in Groups II and III decreased to 44.70% and 42.11%, respectively, compared with that in Group I (Table 1).

3.4. Electronic nose analysis of 2-PE aroma quality

The total concentrations of the four main by-products in Groups I, II, and III after elution by ethanol and distillation were 125.43, 65.21, and 69.48 mg/L, respectively. The aroma quality of 2-PE was >99.00% similar among the samples when the total concentration of the four by-products was <80 mg/L. Electronic nose determination of 2-PE obtained through biotransformation and the standard sample showed that the aroma intensity in Group I is lower than that in Groups II and III. The respective aroma qualities of Groups I, II, and III were 94.76%, 99.19%, and 99.03% similar to that of the standard 2-PE sample.

PCA was used to analyze correlations with 2-PE aroma quality (Fig. 3). Samples with ascorbic acid (*i.e.*, Groups II and III) showed aroma qualities highly similar to that of the standard 2-PE, especially when ascorbic acid was added to the sample during glucose metabolism (Group II).

4. Conclusions

2-PE was synthesized through the Ehrlich pathway using *L*-Phe as the substrate and coenzyme NADH as the cofactor [12,15]. Biotransformation is the key step in the synthesis of 2-PE. NADH was mainly obtained through glucose metabolism and ethanol oxidation. The alternative strategy of using glucose and ethanol as a carbon source eliminated feedback inhibition of 2-PE and ethanol but produced by-products, including butanol, isobutyric acid, butyric acid, and isovaleric acid, during biotransformation. Post-processing adequately solved the problem of 2-PE inhibition. The addition of ascorbic acid showed good effects on by-product removal.

The ethanol utilization efficiency in Groups II and III during ethanol oxidation was higher than that in the control group (*i.e.*, Group I). Excess ethanol produced in Group I entered the metabolic pathway to produce butyric acid, butanol, and other compounds, thereby resulting in a high concentration of by-products in the final fermentation broth. The concentrations of by-products in Groups II and III after fermentation were lower than that in the control group. The total concentration of by-products in Groups II and III, especially after post-processing, was significantly lower than that in Group I. The aroma quality of the group to which ascorbic acid had been added was highly similar (>99.00%) to that of the standard 2-PE.

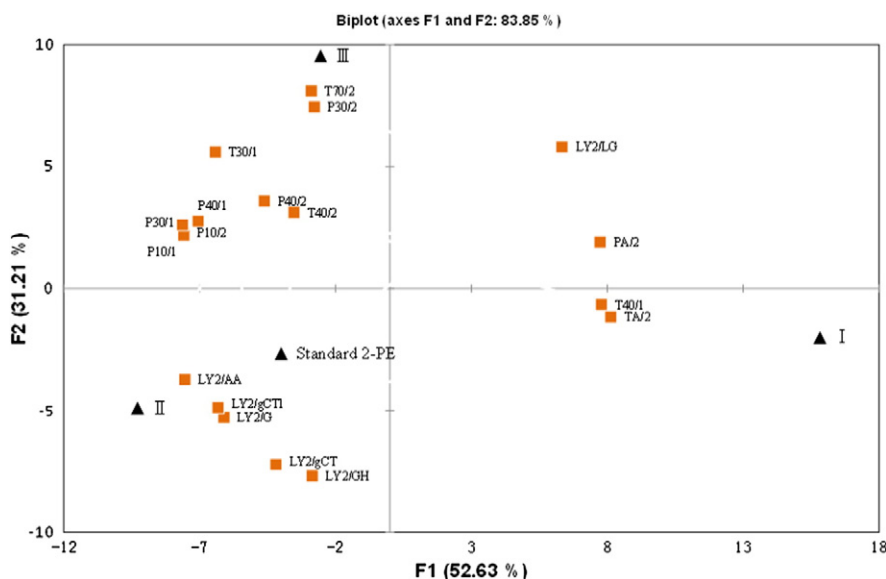


Fig. 3. PCA of 2-PE aroma quality. (I) Sample without ascorbic acid; (II) sample with ascorbic acid added during glucose metabolism, and (III) sample with ascorbic acid added during ethanol oxidation. ▲ Standard 2-PE; ■ Sensor array. Data reflect the means of three experimental replicates. For all comparisons, $p < 0.05$.

Financial support

This work was supported by grants from the Science and Technology Commission of Shanghai Municipality (Grant No. 14120503400), the National Natural Science Foundation of China (Grant No. 31301475) and from the Shanghai Institute of Technology (Grant No. YJ3013-23).

Conflict of interest

There is no conflict of interest.

Authors' contribution

Xun Tian and Rui Ye contributed equally to this work.

References

- [1] Garavaglia J, Flôres SH, Pizzolato TM, Peralba MC, Ayub MAZ. Bioconversion of L-phenylalanine into 2-phenylethanol by *Kluyveromyces marxianus* in grape must cultures. *World J Microbiol Biotechnol* 2007;23:1273–9. <http://dx.doi.org/10.1007/s11274-007-9361-3>.
- [2] Hua D, Xu P. Recent advances in biotechnological production of 2-phenylethanol. *Biotechnol Adv* 2011;29:654–60. <http://dx.doi.org/10.1016/j.biotechadv.2011.05.001>.
- [3] Etschmann M, Bluemke W, Sell D, Schrader J. Biotechnological production of 2-phenylethanol. *Appl Microbiol Biotechnol* 2002;59:1–8. <http://dx.doi.org/10.1007/s00253-002-0992-x>.
- [4] Chen XM, Kobayashi H, Sakai M, Hirata H, Asai T, Ohnishi T, et al. Functional characterization of rose phenylacetaldehyde reductase (PAR), an enzyme involved in the biosynthesis of the scent compound 2-phenylethanol. *J Plant Physiol* 2011;168:88–95. <http://dx.doi.org/10.1016/j.jplph.2010.06.011>.
- [5] Mihal M, Vereš R, Markoš J. Investigation of 2-phenylethanol production in fed-batch hybrid bioreactor: Membrane extraction and microfiltration. *Sep Purif Technol* 2012;95:126–35. <http://dx.doi.org/10.1016/j.seppur.2012.04.030>.
- [6] Wang H, Dong Q, Guan A, Meng C, Shi X, Guo Y. Synergistic inhibition effect of 2-phenylethanol and ethanol on bioproduction of natural 2-phenylethanol by *Saccharomyces cerevisiae* and process enhancement. *J Biosci Bioeng* 2011;112:26–31. <http://dx.doi.org/10.1016/j.jbiosc.2011.03.006>.
- [7] Etschmann MM, Sell D, Schrader J. Production of 2-phenylethanol and 2-phenylethylacetate from L-phenylalanine by coupling whole-cell biocatalysis with organophilic pervaporation. *Biotechnol Bioeng* 2005;92:624–34. <http://dx.doi.org/10.1002/bit.20655>.
- [8] Ding B, Fu Y, Zhang X, Rong S. Aroma quality analysis of natural 2-phenylethanol produced by fermentation. *Food Ferment Ind* 2011;11:55.
- [9] Tashiro Y, Takeda K, Kobayashi G, Sonomoto K, Ishizaki A, Yoshino S. High butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 in fed-batch culture with pH-stat continuous butyric acid and glucose feeding method. *J Biosci Bioeng* 2004;98:263–8. [http://dx.doi.org/10.1016/S1389-1723\(04\)00279-8](http://dx.doi.org/10.1016/S1389-1723(04)00279-8).
- [10] Cuk Supriyadi AN, Shimizu K, Suzuki M, Yoshida K, Muto T, Fujita A, et al. Maturity discrimination of snake fruit (*Salacca edulis* Reinw.) cv. Pondoh based on volatiles analysis using an electronic nose device equipped with a sensor array and fingerprint mass spectrometry. *Flavour Frag J* 2004;19:44–50. <http://dx.doi.org/10.1002/ffj.1272>.
- [11] Castel C, Fernandez X, Lizzani-Cuvelier L, Loiseau A, Perichet C, Delbecq C, et al. Volatile constituents of benzoin gums: Siam and Sumatra, part 2. Study of headspace sampling methods. *Flavour Frag J* 2006;21:59–67. <http://dx.doi.org/10.1002/ffj.1502>.
- [12] Rong S, Ding B, Zhang X, Zheng X, Wang Y. Enhanced biotransformation of 2-phenylethanol with ethanol oxidation in a solid-liquid two-phase system by active dry yeast. *Curr Microbiol* 2011;63:503–9. <http://dx.doi.org/10.1007/s00284-011-0008-0>.
- [13] Rong S, Fu Y, Wang T, He X. Research on the extraction of 2-phenylethanol in fermented broth by macroporous adsorption resin. *J Food Sci Biotechnol* 2010;29:693–7 [cited April 29, 2015. Available from Internet at: http://spyswjs.agrijournal.cn/ch/reader/create_pdf.aspx?file_no=201005010&year_id=2010&quarter_id=5&falq=1].
- [14] Li S, Li XR, Wang GL, Nie LX, Yang YJ, Wu HZ, et al. Rapid discrimination of Chinese red ginseng and Korean ginseng using an electronic nose coupled with chemometrics. *J Pharm Biomed Anal* 2012;70:605–8. <http://dx.doi.org/10.1016/j.jpba.2012.06.009>.
- [15] Hoelsch K, Sührer I, Heusel M, Weuster-Botz D. Engineering of formate dehydrogenase: Synergistic effect of mutations affecting cofactor specificity and chemical stability. *Appl Microbiol Biotechnol* 2013;97:2473–81. <http://dx.doi.org/10.1007/s00253-012-4142-9>.