J. Microbiol. Biotechnol. (2016), 26(8), 1348–1357
http://dx.doi.org/10.4014/jmb.1512.12084

jmb

A New Approach to Produce Resveratrol by Enzymatic Bioconversion

Jinxin Che[1], Junling Shi[2*], Zhenhong Gao[1], and Yan Zhang[1]

[1]*College of Food Science and Engineering, Northwest A & F University, Yangling, Shaanxi 712100, P.R. China*
[2]*Key laboratory for space biosciences and space biotechnology, School of life sciences, Northwestern polytechnical University, Xi'an, Shaanxi 710072, P.R. China*

Received: December 31, 2015
Revised: April 17, 2016
Accepted: April 25, 2016

First published online
April 27, 2016

**Corresponding author*
Phone: +86-29-88460541;
Fax: +86-29-88460541;
E-mail: sjlshi2004@nwpu.edu.cn

Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

An enzymatic reaction system was developed and optimized for bioconversion of resveratrol from glucose. Liquid enzyme extracts were prepared from *Alternaria* sp. MG1, an endophytic fungus from grape, and used directly or after immobilization with sodium alginate. When the enzyme solution was used, efficient production of resveratrol was found within 120 min in a manner that was pH-, reaction time-, enzyme amount-, substrate type-, and substrate concentration-dependent. After the optimization experiments using the response surface methodology, the highest value of resveratrol production (224.40 μg/l) was found under the conditions of pH 6.84, 0.35 g/l glucose, 0.02 mg/l coenzyme A, and 0.02 mg/l ATP. Immobilized enzyme extracts could keep high production of resveratrol during recycling use for two to five times. The developed system indicated a potential approach to resveratrol biosynthesis independent of plants and fungal cell growth, and provided a possible way to produce resveratrol within 2 h, the shortest period needed for biosynthesis of resveratrol so far.

Keywords: *Alternaria* sp., enzymatic synthesis, immobilized enzyme, resveratrol

Introduction

Resveratrol, a plant-derived stilbene, has been found to have many potential medicinal uses, including in extending lifespan; as an anticancer, anti-angiogenic, immunomodulatory, anti-inflammatory, antidiabetes, and cardioprotective drug; in lowering body weight; in reversing social deficits induced by pubertal stress; and as an antioxidant [7, 16, 18]. Resveratrol has been found in 70 types of plants, including grape [10, 11]. Extracting resveratrol from plants is the most widely used method of production, and technological development of production methods continues [1, 3]. However, potential yields are limited owing to the low resveratrol content in plant materials and the long time needed for plant growth. Great efforts have been made to explore new resveratrol-containing plants, construct genetically modified resveratrol-producing plants and microorganisms, and cultivate plant tissue or cells [4, 13, 20]. Comparatively, enzymatic biosynthesis should be a desirable approach because of its high efficiency, mild reaction conditions, and independence from cell growth [17, 24]. However, enzymatic biosynthesis of resveratrol has not yet been reported.

Enzymes contributing to the biosynthesis of resveratrol are found mainly in plants [22], but the resveratrol biosynthesis pathway has not been revealed in microorganisms until now. However, some endophytic fungi have been found to produce resveratrol during cultivation in vitro [19]. *Alternaria* sp. MG1, an endophytic fungus isolated from grapes, has a high capacity to produce resveratrol using glucose as the substrate [26]. However, resveratrol production varied greatly during the different stages of cell growth and decreased quickly after the peak.

To minimize the above adverse effects during the bioconversion of resveratrol, an enzymatic reaction system was developed in this study to produce stilbene, using enzymes extracted from *Alternaria* sp. MG1 cells. Bioconversion of resveratrol was successfully achieved in the enzymatic reaction system using either free enzyme solution or immobilized enzymes. The efficiency of resveratrol production was optimized by changing the pH, enzyme concentration, and substrate concentration, and by the addition of ATP

and coenzyme A (CoA). Ultimately, highly efficient bioproduction of resveratrol from glucose was achieved within 120 min, quicker than all previously reported methods and independent of cell growth.

Materials and Methods

Microorganism and Chemicals

Alternaria sp. MG1 (*Alternaria* sp. CCTCC M 2011348) was obtained from the China Center for Type Culture Collection. Methanol, acetonitrile, and *trans*-resveratrol were of chromatographic grade and purchased from Sigma Chemical Co., Ltd. Phenylalanine, CoA-SH (Sigma, USA), *p*-coumaric acid (Aladdin, China), and glucose-6-phosphate sodium salt (G-6-PNa2) and ATP (MP Biomedicals, USA) were used in the enzyme reactions to detect enzyme activity. All other chemicals were of analytical grade and purchased from Xilong Chemical Co., Ltd.

Preparation of Enzyme Extracts

Enzyme extracts were prepared from *Alternaria* sp. MG1 cells (4.0 g) using sodium phosphate buffer (8.0 ml, pH 7.0, 0.2 mol/l) containing 0.1 g/l $MgSO_4$, 0.1 g/l $CaSO_4$, and 0.6 mmol/l DTT [19, 26]. After centrifugation at 12,830 ×*g* for 10 min at 4°C, the liquid supernatant was obtained and then precipitated using solid ammonium sulfate at saturation of 75% at 4°C. The protein sediment was collected by 10 min centrifugation at 8,910 ×*g*, and then dissolved in 5 ml of the above-mentioned sodium phosphate buffer. The obtained enzyme solution was dialyzed against the same buffer using a MD25 dialysis tube (Sigma Chemical Co., Ltd.) to remove SO_4^{2-}, and then stored at 4°C until use.

Measurement of Enzyme Activities

According to that found in plants, activities of key enzymes for biosynthesis of resveratrol [7], phenylalanine ammonia lyase (PAL) or tyrosine ammonia lyase (TAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL) in the prepared enzyme extract, were tested according to the method reported by Zhang *et al*. [26]. One unit (U) of the enzyme was defined as an increase of 0.01 of OD value per hour, and the enzyme activity was expressed as the units of enzyme per milligram protein (U/mg). Protein concentration in the enzyme extracts was determined using a visible spectrophotometer, UVmini-1240 (Shimadzu, Japan) according to the Bradford method with bovine serum albumin as the standard. Determination of the enzyme activity was conducted in triplicates and the mean values were reported with their standard deviations.

Bioconversion of Resveratrol Using Enzyme Solution

The dialyzed enzyme solution was used for enzymatic conversion of resveratrol from glucose. All bioconversions were carried out at 37°C and 180 rpm in a reaction system consisting of 100 ml of buffer solution containing 0.1 g/l $MgSO_4$ and $CaSO_4$, and 0.6 mmol/l DTT. Unless specifically indicated, 0.36 g/l glucose, 5 ml of enzyme solution, and different substrates were separately or simultaneously added to this bioconversion system (pH 7.0, adjusted by 0.2 mol/l phosphate buffer) to start the biosynthesis as part of the experimental design. The amount of resveratrol accumulated in the system was determined after 120 min unless specifically indicated, using high-performance liquid chromatography (HPLC). The identity of the product resveratrol was confirmed using UPLC-QTOF-MS, as reported previously [26].

Subsequently, the effects of adding CoA, ATP, malonyl-CoA, *p*-coumaric acid, and phenylalanine were separately determined at the level of 0.02 mg/l. The CoA concentration effect was also tested at the levels of 0.01, 0.03, 0.04, and 0.05 mg/l.

Each treatment was performed in three separate replicates and the mean value is reported with standard errors.

Optimization of Bioconversion System by Response Surface Methodology

To enhance resveratrol production to the utmost extent, the conversion system was optimized in terms of pH value, and glucose, CoA, and ATP concentrations, using response surface methodology. Levels of the variable parameters are given in Table 1. A set of 30 experimental runs was carried out according to the central composite design with four variables (Table 2). All runs were carried out at 37°C and 180 rpm in 100 ml of enzymatic reaction system made of buffer containing 0.1 g/l $MgSO_4$, 0.1 g/l $CaSO_4$, and 0.6 mmol/l DTT, with glucose as the substrate and 5 ml of enzyme extract as the catalyst.

Results of the central composite design were analyzed using a quadratic equation (Eq. (1)):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 \quad (1)$$

where Y is resveratrol production; X_1, X_2, X_3, and X_4 are coded values representing pH, glucose, CoA, and ATP, respectively; b_0 is a constant for the model; and b_1, b_2, b_3, and b_4 are linear coefficients of X_1, X_2, X_3, and X_4. Design Expert (Version 8.0.5.0, Stat-Ease Inc., USA) was used to analyze the data by regression and graphical analyses.

Preparation and Application of Immobilized Enzyme

To simplify the bioconversion system and allow the reuse of the

Table 1. Levels and codes of variables tested by central composite design.

Factor	Code	Level				
		-2	-1	0	1	2
pH	X_1	6	6.5	7	7.5	8
Glucose (g/l)	X_2	0.09	0.27	0.36	0.45	0.54
Coenzyme A (mg/l)	X_3	0	0.01	0.02	0.03	0.04
ATP (mg/l)	X_4	0	0.01	0.02	0.03	0.04

Table 2. Results of central composite experimental design.

Run	pH	Glucose (g/l)	Coenzyme A (mg/l)	ATP (mg/l)	Resveratrol production (μg/l)	
					Experimental	Predicted
1	7.5	0.27	0.01	0.03	83.86	76.95
2	6.5	0.45	0.01	0.03	129.55	122.47
3	7.5	0.27	0.03	0.01	44.35	54.42
4	6.5	0.27	0.03	0.01	76.95	60.50
5	7.5	0.27	0.03	0.03	39.39	37.84
6	6.5	0.27	0.01	0.01	78.77	95.69
7	7.0	0.36	0.00	0.02	109.11	102.85
8	7.5	0.27	0.01	0.01	53.92	49.87
9	7.0	0.36	0.02	0.02	213.62	222.42
10	7.0	0.36	0.04	0.02	34.59	33.17
11	6.5	0.45	0.03	0.01	39.53	49.44
12	7.0	0.36	0.02	0.04	67.02	72.32
13	6.0	0.36	0.02	0.02	119.13	115.42
14	7.0	0.36	0.02	0.02	207.53	222.42
15	7.5	0.45	0.01	0.01	66.61	66.75
16	7.5	0.45	0.01	0.03	39.11	60.69
17	7.0	0.36	0.02	0.02	217.81	222.42
18	7.0	0.36	0.02	0.00	59.42	46.44
19	7.0	0.54	0.02	0.02	84.23	69.63
20	7.5	0.45	0.03	0.01	64.59	75.91
21	6.5	0.45	0.03	0.03	39.06	48.25
22	7.0	0.18	0.02	0.02	90.02	96.95
23	7.5	0.45	0.03	0.03	40.12	26.20
24	6.5	0.27	0.03	0.03	89.58	92.44
25	7.0	0.36	0.02	0.02	237.78	222.42
26	7.0	0.36	0.02	0.02	224.40	222.42
27	8.0	0.36	0.02	0.02	51.54	47.56
28	6.5	0.27	0.01	0.03	177.46	171.28
29	7.0	0.36	0.02	0.02	232.94	222.42
30	6.5	0.45	0.01	0.01	73.33	80.01

enzyme extracts, immobilization of the enzyme extract was carried out using either sodium alginate or chitosan-glutaraldehyde.

In the sodium alginate method, 2.0 g of sodium alginate was dissolved in 50 ml of distilled water, and then mixed with 10 ml of crude enzyme solution before being dribbled into a solution of 10 g/l calcium chloride by syringe. After 1 h at 4°C, the immobilized enzyme pellets (diameter 4 mm) were collected and washed several times using distilled water, and then stored in distilled water at 4°C until use.

In the chitosan-glutaraldehyde method, 2.26 g of chitosan was mixed with 15 ml of 50% (v/v) glutaraldehyde and kept at 25°C for 3 h to active the chitosan. Afterwards, the sediment of active chitosan was washed several times using distilled water to remove the glutaraldehyde. Ten milliliters of enzyme extract was added into the active chitosan sediments and kept at 4°C for 1 h to immobilize the enzyme. Finally, the sediment of immobilized enzyme was washed several times with distilled water and stored at 4°C until use.

The immobilized enzymes (made from 10 ml of enzyme extract) were used separately in 100 ml enzymatic reaction systems (pH 6.8, adjusted using Tris-HCl buffer solution because sodium alginate gel particles dissolved easily in sodium phosphate solution), containing 0.02 mg/l CoA, 0.02 mg/l ATP, and 0.35 g/l glucose. The accumulation of resveratrol was determined after reaction for 120 min at 37°C and 180 rpm.

The reusability of the sodium alginate immobilized enzyme

was also tested in the bioconversion system described above. In these tests, the same batch of immobilized enzyme was used many times, replacing the reaction solution after each 120-min reaction period. The accumulation of resveratrol was separately determined after each such test.

Extraction, Identification, and Determination of Resveratrol Produced in the Bioconversion System

Extraction, identification, and determination of resveratrol produced in the enzymatic bioconversion systems were carried out according to previously developed methods [25], with slight modifications. After each reaction, the bioconversion system was extracted twice with 100 ml of ethyl acetate for 10 h per extraction. The collected ethyl acetate phase was treated three times with 30 g/l $NaHCO_3$ water solution, 20 ml per treatment, and then subjected to vacuum concentration to dryness at 40°C. The residue obtained was dissolved in 2 ml of methanol and filtered through a Millex-HV filter membrane (0.45 μm; Millipore, USA) before the resveratrol concentration was measured.

It should be mentioned that a similar extraction operation was performed for the 100 ml resveratrol standard solution at different concentrations of 50, 25, 10, 5, and 1 μg/l and the recovery rate was calculated for each resveratrol concentration. The obtained standard curve for calculation of resveratrol production is shown in Fig. S1A, and an equation was obtained as Eq. (2):

$$\text{Recovery rate} = 9.4702 \times \ln(C) + 40.926 \quad (2)$$

where C is the original resveratrol concentration before extraction and determination operations.

The R^2 value of the equation was obtained as 0.9957, indicating the equation could be used to predict the recovery rate of resveratrol at different concentrations.

Quantitative measurement of resveratrol was conducted by using a Shimadzu Essentia LC-15C analytical HPLC system (Shimadzu). Resveratrol production of each sample was calculated according to the standard curve shown in Fig. S1B and adjusted according to the recovery rate corresponding to each concentration level. Verification of resveratrol was performed using a Waters UPLC-QTOF-MS system with a Waters Acquity-UPLC BEH C18 column (Waters, USA) and UV detector. The absorption value at 306 nm was recorded and used for calculation of resveratrol concentration according to the stability of resveratrol [6]. The liquid systems and testing conditions used for the measurements were the same as that previously reported [25]. The negative-ion mode selected multi-reaction monitoring mode was used, and molecular ions with $m/z = 226.5 - 227.5$ were the focus of analysis given the molecular weight of resveratrol (228.24).

Results

Activities of the Enzymes Related to Biosynthesis of Resveratrol

Experimental results showed the protein content was 0.549 ± 0.012 mg/ml in the enzyme extracts. The activity of PAL, TAL, C4H, and 4CL in the enzyme extract was 114.39 ± 0.36, 81.36 ± 1.69, 68.61 ± 0.82, and 308.50 ± 2.31 U/mg protein, respectively, which showed the presence of essential enzymes for biosynthesis of resveratrol in the enzyme extracts.

Verification of Resveratrol Production in the Enzymatic Reaction System

The resveratrol produced in the bioconversion systems and control check of crude enzyme are shown in Fig. S2. The compound having the same retention time (5.17 min) as standard resveratrol (5.14 min) at 306 nm was detected in the samples with enzyme extracts, but not in the enzyme extracts without substrates and blank bioconversion systems without enzyme extracts. In the ion chromatogram obtained from UPLC-QTOF-MS analysis, the suspected resveratrol detected in the samples (5.19 min) and *trans*-resveratrol standard (5.16 min) showed a similar retention time (Fig. 1), molecular ion of m/z = 227.1, and daughter ions of m/z = 185.1 and 143.1, verifying the production of resveratrol.

Moreover, it can be seen that the production of resveratrol was only determined in the reaction system with enzyme extracts, but not in the blank control.

Bioconversion of Resveratrol from Glucose under Different Conditions

As for all enzymatic reaction systems, the bioconversion of resveratrol was significantly influenced by pH (Fig. 2A), glucose concentration, reaction time (Fig. 2B), enzyme concentration (Fig. 2C), and the addition of malonyl-CoA, ATP, CoA, *p*-coumaric acid, and phenylalanine (Fig. 2D). Glucose and CoA showed the effect in a concentration-dependent manner (Fig. 2E).

Specifically, pH 6–7.5 yielded higher resveratrol production than other pH values (Fig. 2A), and phosphate buffer resulted in higher resveratrol production than citrate buffer at the same pH (pH 6). Adjustment to pH 7.0 with 0.2 mol/l phosphate buffer yielded the highest production of resveratrol (28.01 ± 0.87 μg/l), and indicated that pH 7.0 and phosphate buffer were more suitable for overall activities of the enzymes involved in the biosynthesis reactions.

Lower glucose concentrations (0.09 and 0.18 g/l) resulted in earlier peak production time (30 and 60 min, respectively) and lower maximum resveratrol production (15.89 ± 0.01 and 16.42 ± 1.18 μg/l) than higher glucose concentrations (0.36, 0.72, and 1.44 g/l), which had a later peak (120 min)

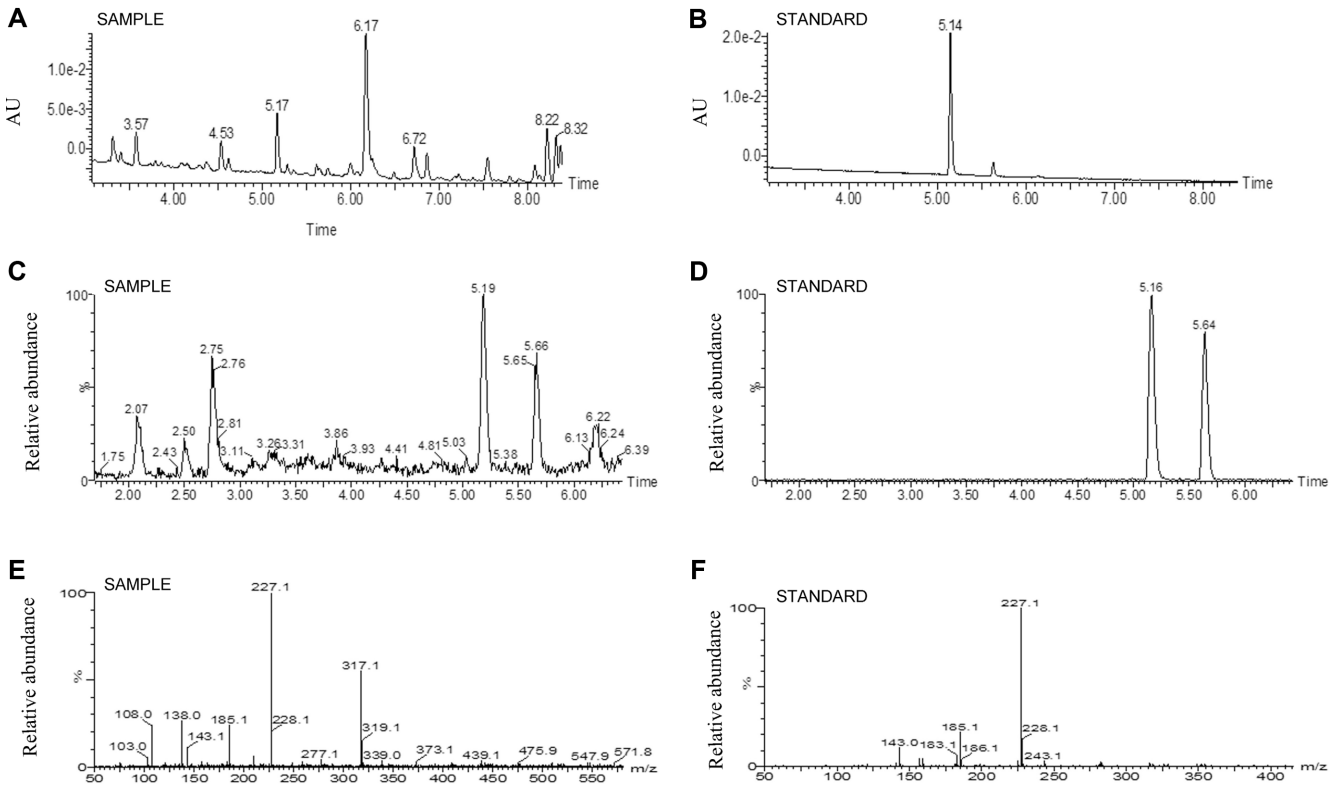

Fig. 1. UPLC-QTOF-MS analysis of resveratrol enzymatically produced from glucose.

UPLC chromatogram of a sample (**A**) and a *trans*-resveratrol standard (**B**). Extracted ion chromatogram of a sample (**C**) and *trans*-resveratrol standard (**D**) by TOF MS ES-227. Mass spectrum of a sample (**E**) and *trans*-resveratrol standard (**F**).

and higher production level (Fig. 2B). The highest resveratrol production (28.00 ± 2.10 μg/l resveratrol) was found after 120 min when 0.36 g/l glucose was used. The resveratrol production was undetectable at time zero. It should be noted that resveratrol production showed a sharp decrease after a certain time in all cases, indicating there are some kind of enzymes having capability to degrade or transform resveratrol to other substrates in the enzyme extracts. Therefore, it is necessary to stop the bioconversion in time to get high resveratrol production.

Increasing the enzyme concentration from 5 to 50 ml per 100 ml reaction solution (corresponding to concentration of 50 to 500 ml/l enzyme solution) enhanced the resveratrol production in an enzyme amount-dependent manner. The highest resveratrol production (79.91 ± 1.75 μg/l resveratrol) was obtained when enzyme solution was used at a concentration of 500 ml/l (Fig. 2C). However, it can be found that the increase of resveratrol production was very light when the enzyme concentration increased from 40 ml per 100 ml reaction solution. According to the enzymatic reaction kinetics, higher enzyme concentration could result in high product accumulation within a definite period when the amount of substrates was set at a definite value.

Addition of malonyl-CoA, ATP, CoA, *p*-coumaric acid, and phenylalanine was separately carried out in an attempt to improve resveratrol production because they are key compounds in the reported resveratrol biosynthetic pathway. The individual addition of 0.02 mg/l malonyl-CoA, 0.02 mg/l ATP, 0.02 mg/l CoA, and 0.02 mg/l *p*-coumaric acid significantly increased resveratrol production in the system (Fig. 2D). The addition of CoA yielded the most significant increase of resveratrol production (65.93 ± 10.59 μg/l) in a concentration-dependent manner (Fig. 2E). However, the addition of phenylalanine resulted in a small decrease in the level of resveratrol production. This differs from results in the resting cell system for bioconversion of resveratrol using whole cells of *Alternaria* sp. MG1, where the addition of phenylalanine increased resveratrol production [26]. This might be because of phenylalanine inhibiting the activity of some key enzymes involved in the biosynthesis

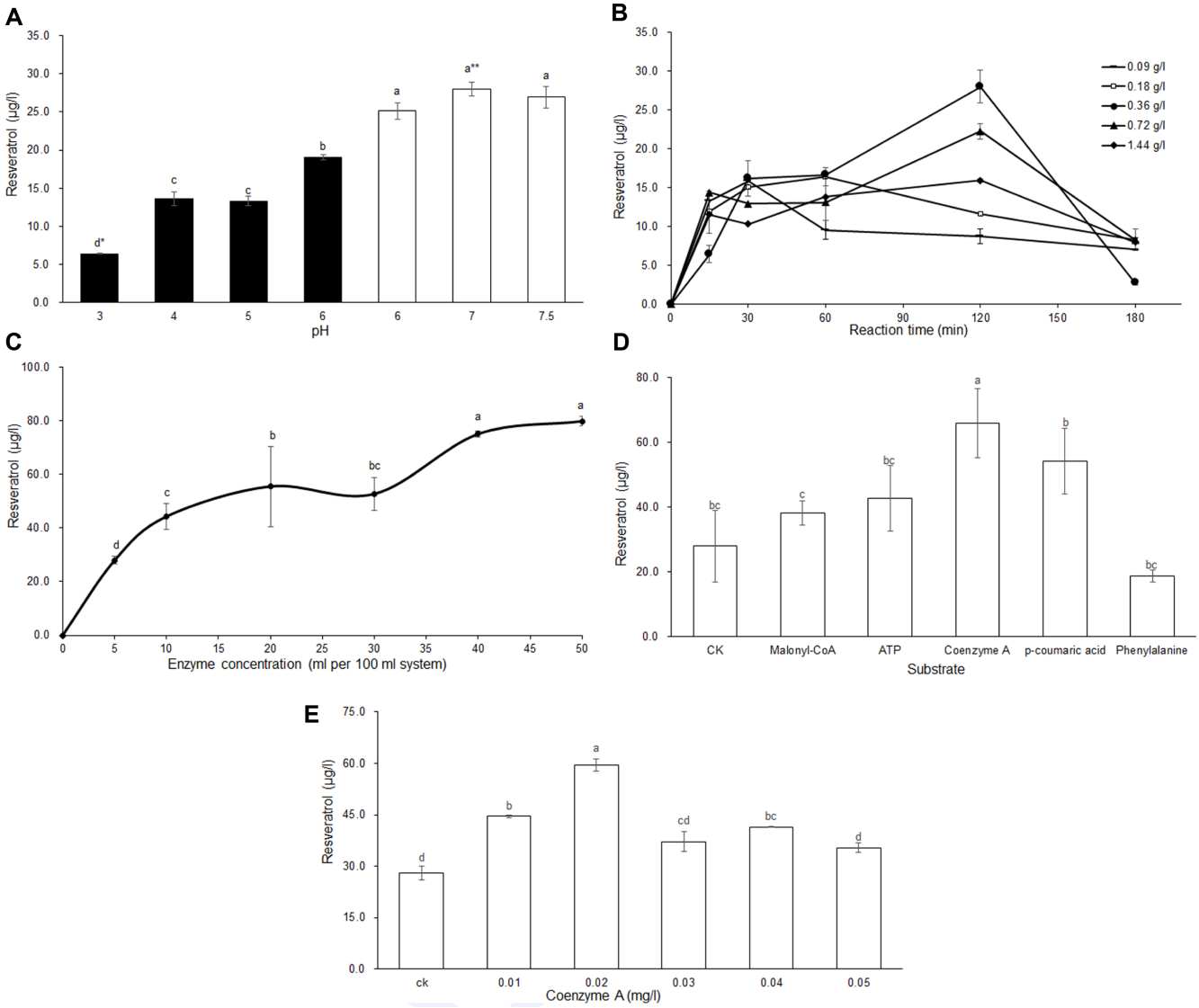

Fig. 2. Effects of different conditions on biosynthesis of resveratrol.

pH (**A**), Glucose concentration and reaction time (**B**), Enzyme dosage (**C**), Different substrates (0.02 mg/l) (**D**), and CoA concentration (**E**). *Black bars indicate pH values of the systems were adjusted using citrate buffer, and white bars indicate they were adjusted using phosphate buffer. **The same letters indicate the data are not significantly different from each other (Tukey's multiple comparison test, $p < 0.05$).

of resveratrol, or the enzyme activity corresponding to the bioconversion of resveratrol from phenylalanine being inhibited in the conditions used. The greatest improvement of resveratrol production caused by the additional supplement of *p*-coumaric acid might be due to the relatively lower activity of C4H (catalyzing the formation of *p*-coumaric acid from cinnamic acid) and higher 4CL activity (catalyzing the conversion of *p*-coumaric acid to *p*-coumaroly-CoA, the precursor of resveratrol) in the enzyme extracts, which caused low availability of *p*-coumaric acid during the reaction. Additional supplement of this *p*-coumaric acid could conquer this limitation and thus showed great improvement of resveratrol production.

Based on the above results, further optimization of the conditions for resveratrol production was carried out among pH, glucose concentration, CoA concentration, and ATP. *p*-Coumaric acid was not collected here because it is an intermediate of the secondary metabolic pathway in plants and has low content in nature, whereas ATP and CoA are widely available in microorganisms and thus are

Table 3. Analysis of variance for the response surface quadratic model.

Source	Sum of squares	DF	Mean square	F value	Prob> F
Model	1.32×10^5	14	9,444.75	46.67	<0.0001**
X_1	6,917.41	1	6,917.41	34.18	<0.0001**
X_2	1,120.30	1	1,120.3	5.54	0.0327*
X_3	7,282.54	1	7,282.54	35.99	<0.0001**
X_4	1,004.66	1	1,004.66	4.96	0.0416*
X_1X_2	1,059.53	1	1,059.53	5.24	0.0371*
X_1X_3	1,579.40	1	1,579.4	7.8	0.0136*
X_1X_4	2,353.69	1	2,353.69	11.63	0.0039**
X_2X_3	21.30	1	21.3	0.11	0.7501
X_2X_4	1,097.57	1	1,097.57	5.42	0.0343*
X_3X_4	1,905.28	1	1,905.28	9.42	0.0078**
X_1^2	34,048.56	1	34,048.56	168.26	<0.0001**
X_2^2	33,186.35	1	33,186.35	163.99	<0.0001**
X_3^2	40,873.56	1	40,873.56	201.98	<0.0001**
X_4^2	45,569.02	1	45,569.02	225.19	<0.0001**
Residual	3,035.43	15	202.36		
Lack of fit	2,364.48	10	236.45	1.76	0.2763
Pure error	670.95	5	134.19		
Cor total	1.35×10^5	29			

R^2= 0.9776; R_{Adj}^2= 0.9566

easily obtained from crushed microbial cells.

Optimum Conditions for the Enzymatic Bioconversion of Resveratrol

Based on the data in Table 2, a statistical model (Eq. (3)) was established to estimate the change of resveratrol production on variation of pH, glucose concentration, CoA concentration, and ATP concentration:

$$Y = -6{,}976.78 + 1{,}882.76X_1 + 1{,}908.55X_2 + 1{,}510.58X_3 + 39{,}426.60X_4 + 180.84X_1X_2 + 1{,}987.08X_1X_3 - 2{,}425.74X_1X_4 + 1{,}281.99X_2X_3 - 9{,}202.65X_2X_4 - 109{,}124X_3X_4 - 140.93X_1^2 - 4{,}294.31X_2^2 - 386{,}029X_3^2 - 407{,}599X_4^2 \quad (3)$$

where Y is the amount of resveratrol produced, X_1 is the pH, X_2 is the glucose concentration, X_3 is the CoA concentration, and X_4 is the ATP concentration.

According to the analysis of variance (Table 3), the model was significant, as evident from the Fisher F-test value (F = 46.67), low probability value ($p < 0.01$), high value for the determination coefficient ($R^2 = 0.9776$), and high adjusted determination coefficient (adjusted $R^2 = 0.9566$). The lack-of-fit F-value of 1.76 and the associated *p*-value of 0.2763 indicated that the lack of fit was not significant and the model fitted the data well. In addition, all the model terms were significant with values of "Prob> F" less than 0.05 (Table 3), except for X_2X_3 (interaction between glucose concentration and CoA concentration). pH and CoA showed lower *p* values ($p < 0.0001$) than glucose and ATP ($p = 0.0327$ and 0.0416 respectively), indicating a more significant influence on resveratrol production than glucose and ATP in the tested levels. Overall, pH and CoA were found as the comparatively more important factors influencing resveratrol production, although glucose and ATP concentrations also had significant influence on resveratrol production. Therefore, control of pH at the proper level would be important for resveratrol production because it would influence the enzyme actives contributing to resveratrol biosynthesis. The supplementation of CoA, glucose, and ATP was also important for the enhancement of resveratrol production because they are essential substrates or cofactors for resveratrol biosynthesis. Increasing the level of these substrates in a certain range was helpful to increase resveratrol production owing to the enzymatic reaction mechanism.

Finally, by solving the model, the optimum level of each factor corresponding to the highest resveratrol production was obtained. The results were pH 6.84, 0.35 g/l glucose, 0.02 mg/l CoA, and 0.02 mg/l ATP. In these conditions, resveratrol production was predicted to be 222.42 μg/l resveratrol. In practice, it was 224.40 μg/l, being 701.43% of that before optimization (28.00 μg/l resveratrol). Comparing the conditions used before and after optimization, it could be found that the simultaneous supplement of CoA and ATP was the major factor that caused a huge improvement of resveratrol production in the enzymatic reaction system. Under consideration of the influence of enzyme concentration, the resveratrol production would be further increased when higher enzyme concentration was used (here, 5 ml per 100 ml reaction system was used).

Table 4. Resveratrol content produced by differently immobilized enzymes (μg/l).

Material	CK[a]	Chitosan	Alginate
Resveratrol content	28.00 ± 0.72	7.08 ± 0.09	17.78 ± 1.06

[a]CK is the treatment with free enzyme solutions at the same enzymatic reaction conditions as that used for immobilized enzymes.

Enzymatic Bioconversion of Resveratrol Using Immobilized Enzymes

The process of immobilization decreased the efficiency

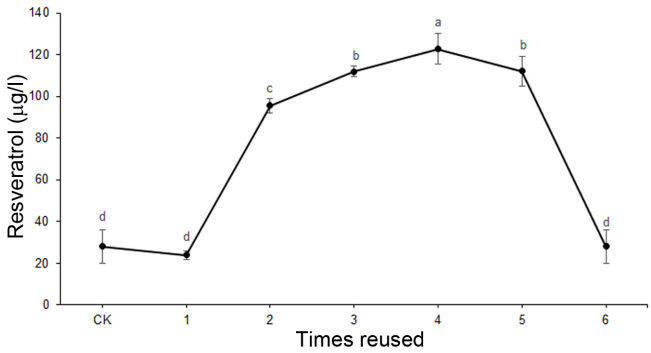

Fig. 3. Production of resveratrol in the biosynthesis system using sodium-alginate-immobilized enzyme.

of enzymatic bioconversion of resveratrol (Table 4). Comparatively, immobilization using sodium alginate retained higher enzyme activity than immobilization using chitosan. These differences may be because of the loss of some essential cofactors, or a decrease in enzyme activity during the process of immobilization. However, in the optimum conditions obtained from the above response surface analysis (pH 6.8, 0.05 mol/l Tris-HCl buffer, 0.1 g/l $MgSO_4$, 0.1 g/l $CaSO_4$, 0.6 mol/l DTT, 0.35 g/l glucose, 0.02 mg/l CoA, and 0.02 mg/l ATP), the sodium-alginate-immobilized enzymes retained high productivity in resveratrol production when they were reused for two to five times (Fig. 3). The average production value was 110.58 μg/l resveratrol and the highest was 122.79 ± 7.41 μg/l resveratrol on the fourth use. Reusability could not be achieved when liquid enzyme was used. Compared with liquid enzymes, the use of immobilized enzymes would simplify the operation and lower the cost for resveratrol production.

However, it should be mentioned that the highest resveratrol production in the immobilized enzyme systems was lower than that in liquid enzyme system. This might be because the immobilization materials formed a barrier between substrates and enzymes, and thus inhibited the reaction efficiency. Moreover, the barrier formed by the immobilized materials would retain and adsorb the formed resveratrol to the solid phase, and thus decrease the detected resveratrol production in the liquid phase of the system. This might also be the major reason for why the first time use of immobilized enzymes showed very low resveratrol production, whereas the resveratrol production was highly increased from the second time reuse when the maintenance dose and absorbance reached saturation level. The sharp decrease of resveratrol production after the fifth reuse of immobilized enzymes might be due to the inactive enzyme activity and the complete release of formed resveratrol from the immobilization materials.

Discussion

The enzymatic resveratrol bioconversion method developed in this study is advantageous relative to all earlier methods in terms of time scale and efficiency. Among the previously developed methods, extraction from plants normally needs months or years for plant growth plus 2 or 3 days for extraction operations [1, 3]. Methods using culture of plant cells, plant tissues, or genetically modified microorganisms shortened the growth period to 120 h (plant cell culture), 80 h (genetically modified yeast), and even 20 h (genetically modified *E. coli*) [4, 8, 23]. In the present study, the production period was decreased to 120 min.

The enzymatic biosynthesis system developed in this study provides a much simpler and cheaper method for production of resveratrol than all currently reported biosynthetic methods. In this work, especially when immobilized enzymes were used, biosynthesis of resveratrol could be quickly and easily restarted by changing the reaction solution, which could be easily operated in automation by pumping the liquid. What is even more important is that the accumulation of byproducts could be greatly inhibited in this work because only the substances that are essential for resveratrol biosynthesis would be supplied. This would greatly simplify the isolation and purification of resveratrol from the reaction system. Comparatively, the systems using plant cells and microbial cells need many nutrients to support cell growth and produce huge amounts of byproducts other than resveratrol, and thus cause complicated operation and high cost in isolation and purification of resveratrol from the system. Moreover, the addition of methyl jasmonate is normally needed to induce the biosynthesis of resveratrol in plant cultures, and cinnamic acid, *p*-coumaric acid, or phenylalanine are normally needed as starters to produce resveratrol using genetically modified microorganisms. Starting from glucose was previously achieved in biosynthesis of resveratrol by an endophytic fungus, but required 3–5 days [26]. In this work, biosynthesis of resveratrol was achieved within 2 h using glucose that is much cheaper than methyl jasmonate, cinnamic acid, *p*-coumaric acid, and phenylalanine. Therefore, we predict that this work might, at least possibly, provide a comparatively cheap and simple way to produce resveratrol in the future, especially when the additional supplement of CoA and ATP can be achieved by supplying crushed bacterial cells.

Resveratrol production in the developed enzymatic bioconversion systems using enzyme extracts from *Alternaria* sp. MG1 (224.40 μg/l) was much higher than that found when resting cells of *Alternaria* sp. MG1 were used (1.376 μg/l) [25] and a little lower than that when cell cultivation was used (422.04 μg/l) [19], indicating a potential of this method in resveratrol production. Cell cultivation had the highest resveratrol production in the above three cases because there is consistent production of enzymes, substrates, and cofactors for resveratrol biosynthesis in this case, but not in the other two cases. The system using resting cells showed the lowest production of resveratrol because the existence of the cell wall in this system would inhibit contact between the substrates outside of cells and the enzymes inside cells, as well as the absence of consistent supplement of enzymes and necessary cofactors, especially that are produced independent of the cell membrane, such as ATP. The developed enzymatic reaction system overcomes the disadvantages of cell cultivation in aspects of long term needed for cell growth and too much byproducts, and that of the resting cell system by removing cell walls and supplying cofactors.

However, further works are still required to improve resveratrol production in the developed enzymatic system because it is still much lower than that found in other biosynthesis systems. In previous reports, the production of resveratrol was 3.5–170 mg/l by extraction from plants [1, 2, 14], 11–35 g/l using cultivation of grape cells [9, 15], 8.2 mg/l using genetically modified *Saccharomyces cerevisiae* [21], 171 mg/l using genetically modified *Escherichia coli* [8, 23], and 200–315 mg/l using cultivation of genetically modified plant cells [5, 12]. These values are much higher than obtained in this study. The low resveratrol production of the enzymatic reaction system might be because the biosynthesis of resveratrol uses a series of enzymes that have different operating pHs, which could not be achieved in an uniform reaction system. Loss of enzyme activity and essential coenzyme factors caused by the disruption of cells might be another important factor that decreased the overall efficiency. For example, CoA and ATP are the essential substrates for biosynthesis of *p*-coumaric CoA, the direct precursor of resveratrol. In this study, the low resveratrol production under the conditions without additional supplement of CoA and ATP indicated low efficiency in biosynthesis of these two substrates when enzyme extracts were used. This might be due to the enzymes catalyzing the formation of ATP localized on cell membranes that tend to be destroyed by the enzyme extraction operation. ATP is one essential substrate for the formation of CoA. Low ATP biosynthesis resulted in low CoA and hence low resveratrol production. Expression of related enzymes on the microbial membrane might be a potential way to solve these problems.

Acknowledgments

This project was supported by the National Key Technology R&D Program (No. 2015BAD16B02), the National Natural Science Fund (No. 31471718), and the Northwestern Polytechnical University (No. 201410699086 and No. 3102014GEKY1010).

References

1. Ballard TS, Mallikarjunan P, Zhou KQ, O'Keefe S. 2010. Microwave-assisted extraction of phenolic antioxidant compounds from peanut skins. *Food Chem.* **120:** 1185-1192.
2. Benova B, Adam M, Pavlikova P, Fischer J. 2010. Supercritical fluid extraction of piceid, resveratrol and emodin from Japanese knotweed. *J. Supercrit. Fluids* **51:** 325-330.
3. Chainukool S, Goto M, Hannongbua S, Shotipruk A. 2014. Subcritical water extraction of resveratrol from barks of *Shorea roxburghii* G. Don. *Sep. Sci. Technol.* **49:** 2073-2078.
4. Donnez D, Jeandet P, Clement C, Courot E. 2009. Bioproduction of resveratrol and stilbene derivatives by plant cells and microorganisms. *Trends Biotechnol.* **27:** 706-713.
5. Dubrovina AS, Manyakhin AY, Zhuravlev YN, Kiselev KV. 2010. Resveratrol content and expression of phenylalanine ammonia-lyase and stilbene synthase genes in *rolC* transgenic cell cultures of *Vitis amurensis*. *Appl. Microbiol. Biotechnol.* **88:** 727-736.
6. Jensen JS, Wertz CF, O'Neill VA. 2010. Preformulation stability of trans-resveratrol and trans-resveratrol glucoside (piceid). *J. Agric. Food Chem.* **58:** 1685-1690.
7. Jun JH, Seu YB, Lee DG. 2007. Candicidal action of resveratrol isolated from grapes on human pathogenic yeast *C. albicans*. *J. Microbiol. Biotechnol.* **17:** 1324-1329.
8. Katsuyama Y, Funa N, Miyahisa I, Horinouchi S. 2007. Synthesis of unnatural flavonoids and stilbenes by exploiting the plant biosynthetic pathway in *Escherichia coli*. *Chem. Biol.* **14:** 613-621.
9. Kim JS, Lee SY, Park SU. 2008. Resveratrol production in hairy root culture of peanut, *Arachis hypogaea* L. transformed with different *Agrobacterium rhizogenes* strains. *Afr. J. Biotechnol.* **7:** 3785-3787.
10. Kim KS, Ghim SY, Seu YB, Song BH. 1999. High level of trans-resveratrol, a natural anti-cancer agent, found in Korean Noul red wine. *J. Microbiol. Biotechnol.* **9:** 691-693.
11. King RE, Bomser JA, Min DB. 2006. Bioactivity of

resveratrol. *Compr. Rev. Food Sci. Food Saf.* **5:** 65-70.
12. Kiselev KV, Dubrovina AS, Veselova MV, Bulgakov VP, Fedoreyev SA, Zhuravlev YN. 2007. The *rolB* gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells. *J. Biotechnol.* **128:** 681-692.
13. Li WL, Lu T, Yang YH, Yang YD. 2013. Chemoselectively synthesis of resveratrol oligomers. *Chin. J. Org. Chem.* **33:** 2443-2459.
14. Mantegna S, Binello A, Boffa L, Giorgis M, Cena C, Cravotto G. 2012. A one-pot ultrasound-assisted water extraction/cyclodextrin encapsulation of resveratrol from *Polygonum cuspidatum*. *Food Chem.* **130:** 746-750.
15. Medina-Bolivar F, Condori J, Rimando AM, Hubstenberger J, Shelton K, O'Keefe SF, *et al*. 2007. Production and secretion of resveratrol in hairy root cultures of peanut. *Phytochemistry* **68:** 1992-2003.
16. Pangeni R, Sahni JK, Ali J, Sharma S, Baboota S. 2014. Resveratrol: review on therapeutic potential and recent advances in drug delivery. *Expert Opin. Drug Deliv.* **11:** 1285-1298.
17. Park HJ, Uhm KN, Kim HK. 2010. Biotransformation of amides to acids using a co-cross-linked enzyme aggregate of *Rhodococcus erythropolis* amidase. *J. Microbiol. Biotechnol.* **20:** 325-331.
18. Poirier GL, Imamura N, Zanoletti O, Sandi C. 2014. Social deficits induced by peripubertal stress in rats are reversed by resveratrol. *J. Psychiatr. Res.* **57:** 157-164.
19. Shi JL, Zeng Q, Liu YL, Pan ZL. 2012. *Alternaria* sp. MG1, a resveratrol-producing fungus: isolation, identification, and optimal cultivation conditions for resveratrol production. *Appl. Microbiol. Biotechnol.* **95:** 369-379.
20. Shin SY, Han NS, Park YC, Kim MD, Seo JH. 2011. Production of resveratrol from p-coumaric acid in recombinant *Saccharomyces cerevisiae* expressing 4-coumarate:coenzyme A ligase and stilbene synthase genes. *Enzyme Microb. Technol.* **48:** 48-53.
21. Sun P, Liang JL, Kang LZ, Huang XY, Huang JJ, Ye ZW, *et al*. 2015. Increased resveratrol production in wines using engineered wine strains *Saccharomyces cerevisiae* EC1118 and relaxed antibiotic or auxotrophic selection. *Biotechnol. Prog.* **31:** 650-655.
22. Trantas E, Panopoulos N, Ververidis F. 2009. Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. *Metab. Eng.* **11:** 355-366.
23. Watts KT, Lee PC, Schmidt-Dannert C. 2006. Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*. *BMC Biotechnol.* **6:** 12.
24. Yeo HK, Hyun YJ, Jang SE, Han MJ, Lee YS, Kim DH. 2012. Development of fecal microbial enzyme mix for mutagenicity assay of natural products. *J. Microbiol. Biotechnol.* **22:** 838-848.
25. Zhang JH, Shi JL, Liu YL. 2013. Bioconversion of resveratrol using resting cells of nongenetically modified *Alternaria* sp. *Biotechnol. Appl. Biochem.* **60:** 236-243.
26. Zhang JH, Shi JL, Liu YL. 2013. Substrates and enzyme activities related to biotransformation of resveratrol from phenylalanine by *Alternaria* sp. MG1. *Appl. Microbiol. Biotechnol.* **97:** 9941-9954.