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Genetic engineering and culture optimization of *Yarrowia lipolytica* for resveratrol production from p-coumaric acid

Yanlei Zhao, Meng Gao, Ruixue Wang, Feifei Xin, Haifang Xiao※, Huaiyuan Zhang※

(Colin Ratledge Center for Microbial Lipids, School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo 255000, Shandong, People's Republic of China)
※Corresponding Author: Haifang Xiao, Huaiyuan Zhang

Abstract: *Resveratrol is a plant based dietary polyphenol that has attracted huge attention, due to its various biological activities. Industrial production of resveratrol from microorganisms requires extensive genetic engineering. Therefore, the aim of this study was to construct the producer of resveratrol of Yarrowia lipolytica strains. In present study, 2 resveratrol-biosynthesis genes 4-coumaryl-CoA ligase gene (4cl) from Arabidopsis thaliana and the stilbene synthase gene (sts) from Vitis Vinifera，were cloned and transformed into Y. lipolytica. The results showed that expression of 4CL and STS genes in Y. lipolytica produced resveratrol through the tyrosine synthesis pathway. Furthermore, the final output of resveratrol of transformed strain was up to 1604.7 μg/L in presence of p-coumaric acid under response surface optimization experiments. This study suggests that Y. lipolytica is an attractive model for the production of resveratrol.*

Key words: *Resveratrol; 4-coumaryl-CoA ligase; Stilbene synthase; Yarrowia lipolytica*

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I. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) discovered in 1924 was first extracted and isolated from the root of *Veratrum grandiflorum* [1]. Resveratrol has several biological activities such as the antifungal activity[2, 3], anticancer[2, 3], anti-diabetes[4], prevention of cardiovascular diseases[5], antioxidant[6], anti-inflammatory[7], neuroprotective[8] and anti-aging effects[9-11]. One of the most significant biological activities of resveratrol is anti-cancer activity in the three stages of tumor development. Resveratrol has shown anti-tumor activity through mechanisms that regulates transcription factors and controls the expression of small RNAs[12]. In addition, resveratrol has a protective effect on diabetes[4] and neurodegenerative diseases[13] by inducing sirtuin-1 gene.

Two important aspects of the formation of natural resveratrol prevent it from being a widely used nutrient. First, even in the plants with the highest resveratrol content, the content of this compound is very low[14, 15]. Second, resveratrol can be extracted from plant tissues. However, the main disadvantage of this method is that it requires a large amount of plant materials and solvents, and involves a difficult purification process, resulting in a very low product yield[14, 16]. Although the production of genetically modified microbes has been achieved in commonly used plant varieties recently[17, 18], the titer is still very low, making resveratrol biosynthesis an ideal target for microbial fermentation[19, 20]. Microorganisms are able to produce resveratrol by introducing genes for the phenylpropanoid pathway. There are two main pathways for resveratrol production, one using tyrosine and the other phenylalanine as intermediates. For the tyrosine pathway, a tyrosine ammonia lyase (TAL) deaminates L-tyrosine to p-coumaric acid. Then a 4-Coumarate: CoA ligase (4CL) forms coumaroyl-CoA from coumaric acid. Finally, coumaroyl-CoA is condensed by three malonyl-CoA units to form resveratrol by a stilbene synthase (STS)[21]. For the phenylalanine pathway, cinnamic acid is generated by deamination of phenylalanine via a phenylalanine ammonia lyase (PAL). And then cinnamic acid is hydroxylated to p-coumaric acid by a cinnamic acid 4-hydroxylase (C4H). Finally, similarly as in the tyrosine pathway, cinnamic acid can be converted in resveratrol via 4CL and STS[22]. *Saccharomyces cerevisiae* has been used as host for resveratrol synthesis by expressing the genes encoding 4CL and STS[20]. The resveratrol produced by recombinant yeast through tyrosine fermentation reached 531 mg/L[20], while the resveratrol produced by phenylalanine in the batch fermentation of glucose supplement reached 812 mg/L[23]. The biosynthesis of resveratrol by microorganisms has become an important research field, and its research focuses mainly on synthetic biology, the interaction between microorganisms and plants, biocatalysis, and biological transformation[9].

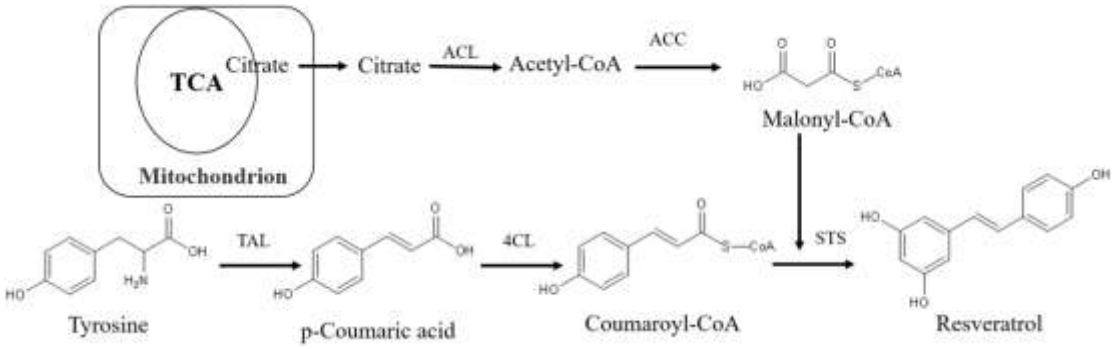

FIG. 1 Synthetic pathway of resveratrol from tyrosine. TCA, Tricarboxylic acid cycle; ACL, ATP：citrate lyase; ACC, Acetyl-CoA carboxylase; TAL, Tyrosine ammonia lyase; 4CL, 4-coumaroyl-CoA ligase; STS: Stilbene synthase.

In this study, *Yarrowia lipolytica* yeast was selected as model microorganism not only because it is safe in the pharmaceutical biotechnology and food industry, but also it could provide more malonyl-CoA for resveratrol biosynthesis (Fig. 1) due to its property of high lipid accumulation. To achieve non-de novo biosynthesis of resveratrol with the addition of aromatic precursors, the *4cl* gene from *Arabidopsis thaliana* and the *sts* gene from *Vitis vinifera* were cloned into *Y. lipolytica* (Fig.1) genome for resveratrol biosynthesis and culture conditions on recombinant strains were optimized to achieve the highest production of resveratrol.

II. Materials and Methods

2.1 Materials

All the materials used in this work were commercially available. The p-coumaric acid used in this experiment was purchased from Sangon Biological Engineering Co. LTD (Shanghai, China). Coumaric acid standard was purchased from Aladdin Biochemical Technology Co., LTD. (Shanghai, China). San Prep Column Plasmid DNA Mini Extraction Kit was purchased from Sangon Biological Engineering Co. LTD (Shanghai, China). San Prep Column PCR Product Purification Kit was purchased from Sangon Biological Engineering Co. LTD (Shanghai, China). Fast plant genome extraction kit was purchased from Tiangen Biochemical Technology Co., Ltd (Beijing，China). Gel recovery kit was purchased from Omega Bio-Tek Co. LTD (Guangzhou, China). Endonuclease, T4 DNA Ligase and DNA Marker, Taq DNA polymerase are purchased from TaKaRa Biomedical Technology Co., Ltd. (Beijing, China). Methanol, glucose, ammonium sulfate, and nitroprusside were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).YNB was purchased from BBI Life Science Co., Ltd (Shanghai, China). All reagents used in this work were analytical grade.

2.2 Strains and plasmids

Escherichia coli Top 10, plasmids pINA1312, *Y. lipolytica* were preserved in Colin Ratledge Center for Microbial Lipids of Shandong University of Technology.

2.3 Medium and culture conditions

Luria–Bertani (LB) medium: 0.5% yeast extract, 1% tryptone and 1% sodium chloride with or without 2% agar, sterilize at 121oC for 20 min. Yeast Peptone Dextrose (YPD) medium: 1% yeast extract, 2% peptone and 2% glucose. YNB-N5000 medium: 0.17% YNB without amino acids and ammonium sulfate, 1% glucose, 0.5% ammonium sulfate, 2% agar, pH 5.5-6.0, sterilize at 115 ^{o}C for 15 min. YEPD medium: 1% yeast extract, 2% peptone and 2% glucose supplemented with various concentrations of p-coumaric acid[24], solid medium add 1% agar powder, sterilize at 121 ^{o}C for 20 min.

The *E. coli* Top 10 strains were grown at 37 ^{o}C on Luria–Bertani (LB) agar plates or in liquid LB medium. The *E. coli* transformants were selected on LB solid medium containing 50 mg/mL Kanamycin. *Y. lipolytica* cells were cultured in YPD or YEPD medium at 28 ^{o}C. To produce resveratrol, the recombinant *Y. lipolytica* strains were grown at 28 ^{o}C and 200 rpm in baffle flask containing YEPD medium (1% yeast extract, 2% peptone and 2% glucose) supplemented with various concentrations of p-coumaric acid. A selective YNB-N5000 medium was used for the selection of the *Y. lipolytica* transformants.

2.4 Construction of recombinant strains

In order to construct a plasmid expression vector capable of expressing the dual genes of 4CL and STS, it is necessary to insert the promoter and terminator of the new cloning site behind the original cloning site of plasmid pINA1312 to obtain a new dual gene plasmid expression vector pCRCylm. The construction of

pCRCylm first cloned the promoter gene behind the original cloning site terminator of pINA1312 by BsiW Ⅰ/Hind Ⅲ, and then cloned the terminator gene behind the newly cloned promoter by Avr Ⅱ/EcoRI. The construction of plasmid pCRCylm-4cl-sts (Fig. 2) first clone *Arabidopsis thaliana* 4cl gene into the first cloning site of pCRCylm through BspE Ⅰ/Kpn Ⅰ, and then clone the *Vitis vinifera* sts gene into the second cloning site of pCRCylm through Hind Ⅲ/Avr Ⅱ. Transformation of *Y. lipolytica* was performed using the the Lithium Acetate Method[25], and the transformants were selected in YNB-N5000 plates.

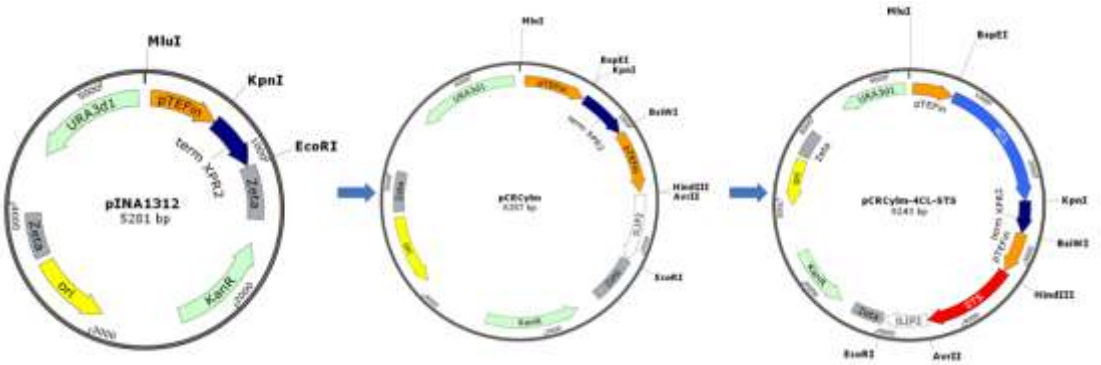

FIG. 2 Construction of plasmid for expressing *4cl* from *A. thaliana* and *sts* from *V. vinifera*.

2.5 Analytical methods

Extract resveratrol from the fermentation broth: Resveratrol was extracted twice from the culture medium and cell lysates with equal volumes of pure ethyl acetate，mix well and extract for 3 h, centrifuge at 4000 rpm for 10 minutes, take out of the upper ethyl acetate layer with a pipette into a clean centrifuge tube, blow dry with nitrogen, and then the residues were dissolved in chromatographic grade methanol, and filtered through a 0.02mm filter into a liquid phase flask.

High performance liquid chromatography analysis method: Resveratrol concentration was determined by HPLC (Agilent Technologies 1260 Infinity Ⅱ) with an InfinityLab Poroshell 120 EC-C18 column(4.6×150 mm)(Agilent, USA). The mobile phase A (acetonitrile + 0.1% (vol/vol) formic acid), mobile phase B (water + 0.1% (vol/vol) formic acid), flow rate Set to 0.8 mL/min, the mixed mobile phase composition is A mobile phase 40% and B mobile phase 60%[24]. The peak time of resveratrol is about 3 minutes (Fig.3). Extraction and analysis of resveratrol were carried out in triplicate.

The experimental design and statistically analysis of the results of Response Surface Methodology (RSM) were executed by Design Expert Version software (version 8.0.6.1, State-Ease,Inc., Minneapolis, MN, USA). All data were expressed as mean ± standard deviation (SD) and assessed using ANOVA. For all comparisons, a significant difference was judged to be statistically significant if $P < 0.05$.

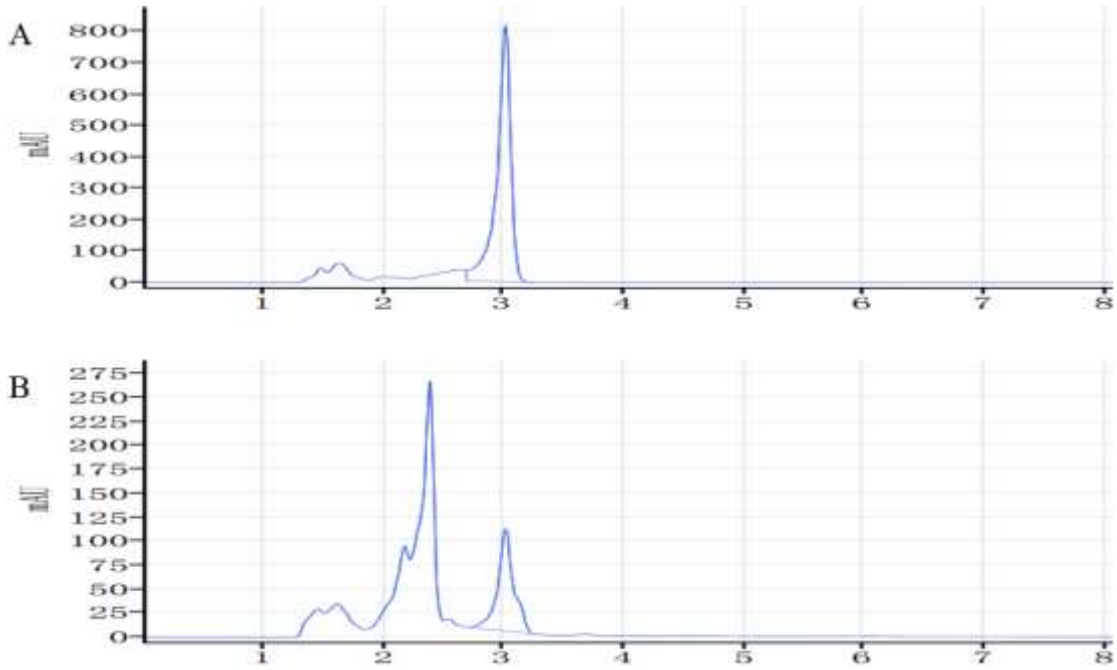

FIG.3 Resveratrol determination of culture samples by high performance liquid chromatogram (HPLC). A, resveratrol standard; B, resveratrol test sample. The peak of resveratrol was showed at 3 min.

III. Results and analysis

3.1 Construction of recombinant *Y. lipolytica*

The location and number of recombinant plasmids in the *Y. lipolytica* yeast genome are unclear, and high-yielding transformants need to be screened. Recombinant strains with high cell growth density and high-quality accumulation were selected for culture. The four transformants verified by PCR were chosen and fermented in a flask with 3 mg/L p-coumaric acid in YEPD (yeast extract peptone dextrose) medium, with continuous shaking. High-yielding transformants were selected based on the yield of resveratrol. The resveratrol yield of the recombinant strain was higher than that of the control strain, and the yields of the different transformants were significantly different. There may be two reasons for this phenomenon: firstly, the insertion position of the gene on the plasmid is unclear, which means that the plasmid may not be helpful to cell growth and the accumulation of metabolites after recombination. Secondly, activation, pre-germination, and repeated washing processes caused great damage to the cells. The recombinant strains TF3 and TF4 accumulated 624 μg/L and 674 μg/L of resveratrol, respectively, while the control strains did not generate any yield as expected (Fig. 4). Therefore, based on our data, TF4 recombinant strain transformant, referred to as Po1f-4CL-STS, was selected for subsequent experiments.

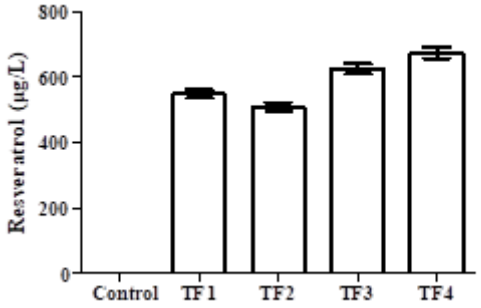

FIG.4 Resveratrol production by *Y. lipolytica* transformants. Control, *Y. lipolytica* Po1f with the control plasmid; TF 1-4, *Y. lipolytica* transformants 1-4.

3.2 Effects of culture conditions on resveratrol yield and *Y. lipolytica* biomass

To improve the resveratrol yield of recombinant *Y. lipolytica*, the culture conditions of yeast were explored. Firstly, the effect of p-coumaric acid amount supplemented in recombinant yeast was investigated. The final concentrations of p-coumaric acid added were 0, 4, 8, 16, and 32 mg/L, respectively. At 16 mg/L, the resveratrol produced by recombinant *Y. lipolytica* yeast reached the highest yield with 1467.2 μg/L. This may be

attributed to the fact that phenolic acids, such as levulinic acid, ferulic acid, and p-coumaric acid, interrupt the central metabolism of yeast cells; thus, high concentrations of p-coumarin acid have a toxic effect on yeast growth. The effect of p-coumaric acid on the biomass of recombinant yeast was more obvious. In fact, as the amount of p-coumaric acid increased, the biomass of recombinant *Y. lipolytica* decreased (Fig. 5 A). The growth inhibition of recombinant *Y. lipolytica* yeast may be another reason the addition of high concentrations of p-coumaric acid did not produce any high resveratrol yields.

With the delay in p-coumaric acid addition time, the biomass of the recombinant *Y. lipolytica* yeasts showed an upward trend (Fig. 5 B). The reason for this is still related to the toxicological effects of p-coumaric acid on yeast growth. In the early growth stage, the earlier the addition time, the more effective the growth inhibition on yeast was. The consumption of p-coumaric acid increased with an augmentation in yeast quantity, so the toxicity inhibition of p-coumaric acid decreased correspondingly. The yield of resveratrol was 1346.2 μg/L when p-coumaric acid was added at the beginning of the culture, 1600.4 μg/L after 12 h, and 1567.6 μg/L after 24 h. The addition of p-coumaric acid during the initial growth process inhibited the synthesis of resveratrol by recombinant *Y. lipolytica* yeasts. The early addition of p-coumaric acid inhibited the growth of yeast, leading to slow growth. The addition of p-coumaric acid at 24 h did not increase resveratrol production due to the total energy expended at the later stage of the medium, which reduced resveratrol synthesis. When p-coumaric acid was added at 12 h, the yeast was in the logarithmic growth phase and had a large amount of acetyl-CoA and malonyl CoA, leading to rapid accumulation of resveratrol. Although the biomass is reduced at a later stage, the rapid accumulation of resveratrol at this time leads to the highest final yield.

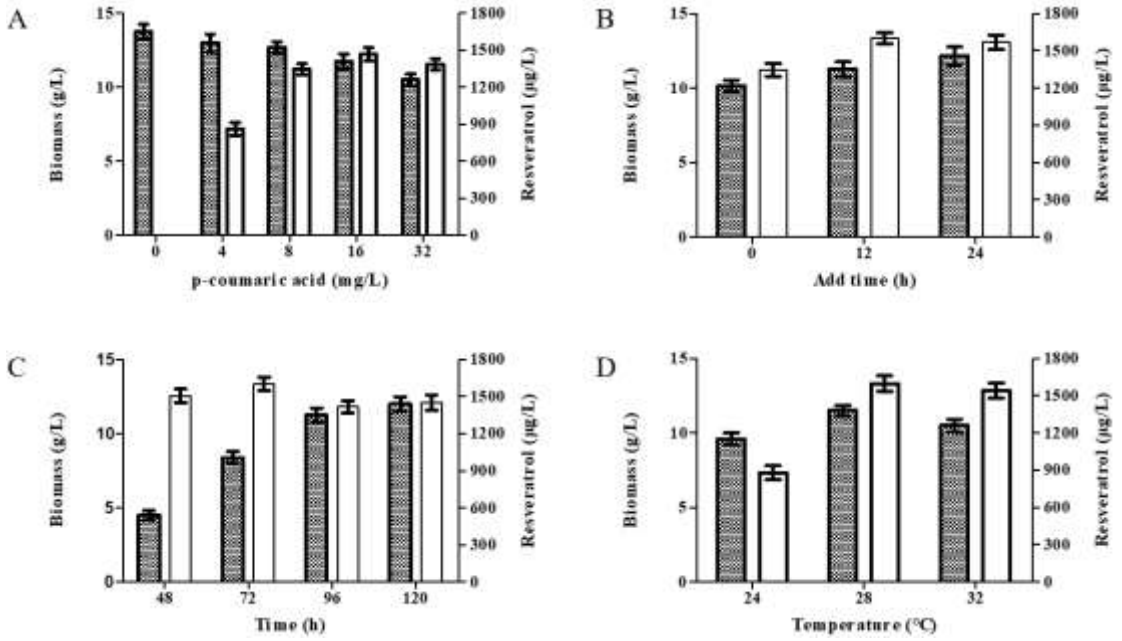

FIG.5 Effects of different culture conditions on the biomass (black columns) and resveratrol production (blank columns) of *Y. lipolytica* transformant TF4. A, Addition concentration of p-coumaric acid supplement; B, Addition time of p-coumaric acid; C, The culture time; D, The different culture temperatures.

The biomass of the recombinant lipolytic yeast was similar to that of the wild-type yeast (Fig. 5 C). The biomass of the final fermentation stage was still reduced, and the inhibition effect still existed. After 72 h of fermentation, the recombinant yeast strain produced the highest resveratrol content, reaching 1601.5 μg/ L, and at two subsequent test time points, increasing culture time did not increase resveratrol accumulation (Fig. 5 C). This is because resveratrol is oxidised at higher temperatures and when in contact with air; therefore, it is produced less.

The optimum fermentation temperature was determined to be 28^{o}C. When temperatures were decreased or increased, the biomass of recombinant yeast decreased (Fig. 5 D). The effect of temperature decrease on biomass was greater than that of temperature increase. The effect of culture temperature on the resveratrol yield of recombinant yeast was consistent with that of temperature on its biomass. The yield of resveratrol was 1597.8 μg/L at 28^{o}C and was the lowest with only 879.3 μg/L at 24 ^{o}C. At 32^{o}C, the yield was 1538.5 μg/L (Fig. 5 D). Therefore, the introduction of plant-derived genes is not very sensitive to temperature, and the yield of resveratrol varies with the growth status of the strain. If the strain grows well, the yield of resveratrol will be good. This also indicates that the genes of plant origin have no negative effect on yeast.

3.3 Response surface methodology (RSM) optimisation experiment

According to the results of single factor experiments, the amount of p-coumaric acid added, the time of p-coumaric acid addition, and the culture temperature play an important role in the production of resveratrol synthesized by recombinant *Y. lipolytica* yeast. Therefore, the three-level three-factor RSM of the Box-Behnken design method was used to further optimize the culture conditions for resveratrol production (Table 1). The design matrix and corresponding resveratrol production results are listed in Table 2. Various multiple regression analysis methods were used to process the experimental data. The relationship between the response value of resveratrol production and the test variables can be expressed by the following second-order polynomial equation (Equation 1):

$Y = 1568.64 + 5.00X_1 - 302.60X_2 + 1.50X_3 - 13.45X_1X_2 - 38.90X_1X_3 - 36.45X_2X_3 - 109.67X_1^2 - 354.92X_2^2 - 66.27X_3^2$.

Here, Y is the production of resveratrol. X_1, X_2, and X_3 are the amounts of p-coumaric acid added, culture temperature, and the time of p-coumaric acid addition, respectively. The quadratic model based on multivariate regression analysis can fully describe the data obtained by evaluating the experimental data. The significance of the parameters and the interaction of each coefficient were checked using the F-test and p-value. The precision and validity of the model were evaluated using the values of determination coefficient (R_2) and the adjusted determination coefficient (R_{2adj})[26]. Table 3 lists the analysis of variance (ANOVA) of the quadratic response surface model of resveratrol production. Model analysis, high F-value (15.66), and low p-value (< 0.0001) indicate that the model has extremely high statistical significance[27]. The lack of fit (F-values of 2.83 and p-value of 0.1684) of the model was insignificant ($p > 0.05$) relative to the pure error. In addition, the coefficient of determination ($R^2 = 0.9922$) is very close to the value of the adjusted coefficient of determination ($R^2_{adj} = 0.9821$), confirming the high correlation between the observations and illustrates the rationality and validity of the model[28]. Based on the above, the regression model was reasonable. As a tool to test the significance of each coefficient, the F- and p- values of each model item are listed in Table 3. The F-test larger F-value and smaller p-value indicated that the relevant coefficient was more notable. Hence, the linear coefficients (X_2) and quadratic coefficients (X_1^2, X_2^2, and X_3^2) revealed a significant influence ($p < 0.05$) on the yield of resveratrol. The culture temperature (X_2) was the main parameter that affected the resveratrol production while the other coefficients were not significant ($p > 0.05$).

Table 1 Factors and coding value of response surface experimental designment.

Levels	The amount of p-coumaric acid added (X_1)/mg/L	Culture temperature(X_2)/ ºC	The time of p-coumaric acid added(X_3)/h
-1	8	24	0
0	20	28	12
1	32	32	24

Table 2 Box-behnken experimental design and results of resveratrol production.

Runs	X_1 (mg/L)	X_2 (ºC)	X3 (h)	Y (μg/L)
1	0	0	0	1540.8
2	-1	0	-1	1362.5
3	-1	-1	0	1414.9
4	0	1	-1	877.4
5	1	0	1	1345.1
6	1	0	-1	1464.2
7	-1	0	1	1399
8	0	0	0	1551.3
9	-1	1	0	797.1
10	0	1	1	851.8
11	0	-1	-1	1370.2
12	0	0	0	1555.7
13	0	0	0	1581.3
14	1	1	0	766.3
15	0	0	0	1614.1
16	1	-1	0	1437.9
17	0	-1	1	1490.4

Table 3 Analysis of the variance (ANOVA) for the second-order polynomial model.

Source	Sum of squares	df	Mean square	F value	P value
Model	1.381E+006	9	1.534E+005	98.50	<0.0001[a]
X1	200	1	200.00	0.13	0.7306[c]
X2	7.325E+005	1	7.325E+005	470.35	<0.0001[a]
X3	18	1	18	0.012	0.9174[c]
X1X2	723.61	1	723.61	0.46	0.5174[c]

X1X3	6052.84	1	6052.84	3.89	0.0893[c]
X2X3	5314.41	1	5314.41	3.41	0.1072[c]
X12	50642.14	1	50642.14	32.52	0.0007[a]
X22	5.304E+005	1	5.304E+005	340.56	<0.0001[a]
X32	18491.42	1	18491.42	11.87	0.0108[b]
Residual	10901.97	7	1557.42		
Lack of fit	7431.90	3	2477.30	2.86	0.1684[c]
Pure error	3470.07	4	867.52		
Cor total	1.392E+006	16			

[a] $P < 0.01$.
[b] $p < 0.05$.
[c] Not significant.

To determine the interaction effect of various parameters on the response, a triaxial section of the model was generated, which was implemented by altering two parameters in the experimental scopes and maintaining the other variables at zero. In Fig. 6, a three-dimensional response surface and two-dimensional contour map was used to investigate the interaction of various factors on resveratrol production.

When the addition time of p-coumaric acid was fixed at 0, the production of resveratrol also increased first and then decreased with an increase in culture temperature (X2) (Fig. 6 A). The yield of resveratrol was greatly affected by culture temperature, but the amount of p-coumaric acid was only slightly affected. Fig. 6 B shows the interaction of p-coumaric acid addition amount (X_1) and addition time (X_3) on resveratrol production. Firstly, the yield of resveratrol increased with the addition of p-coumaric acid and the addition time. After resveratrol production peaked, there was no further increase in resveratrol production; Fig 6 C shows the effects of culture temperature (X_2) and addition time of p-coumaric acid (X_3) on resveratrol production. When the temperature was between 24°C and 28°C and the time of p-coumaric acid addition was from 0 h to 13 h, the resveratrol production increased at first but then decreased.

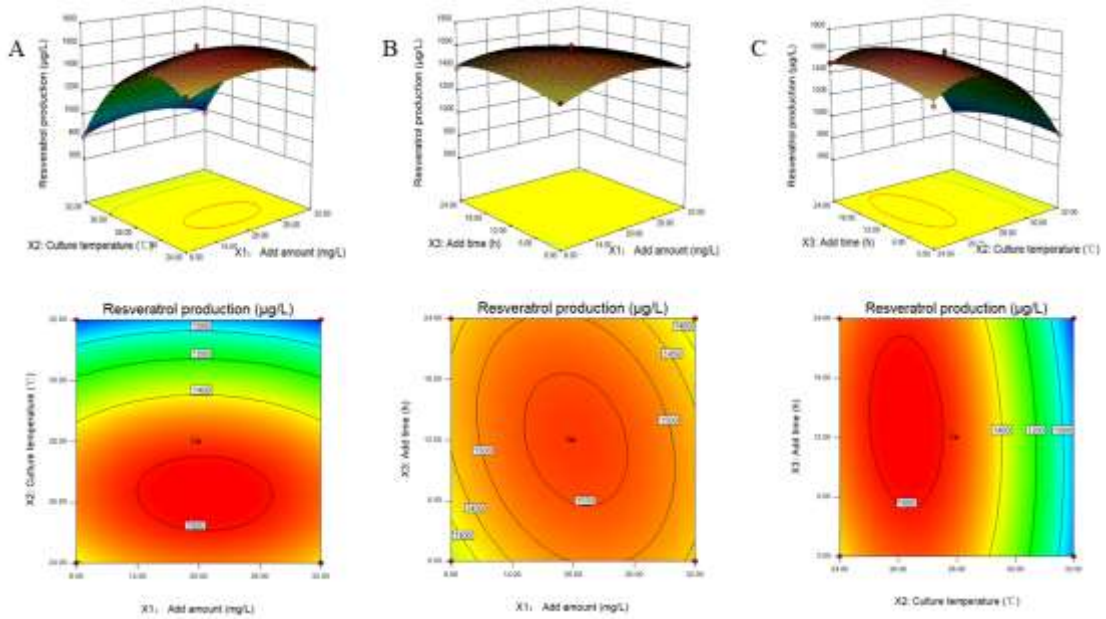

FIG.6 The effect of different variables on the resveratrol production showed by Response surface plots (2D) and contour plots (3D) (A-C). A, The effect of p-coumaric acid addition and culture temperature on the resveratrol production; B, The effect of p-coumaric acid addition concentration and time on the resveratrol production; C, The influence of culture temperature and time of adding p-coumaric acid on the resveratrol production.

Based on single-factor experiments and 3D response surface analysis, the optimal culture conditions for the resveratrol production by recombinant *Y. lipolytica* yeast were 19.407 mg/L of p-coumaric acid, added for 11.437 h, and 26.2°C for the culture temperature. The predicted yield of resveratrol was 1630.798 g/L under these conditions. When fermentation was performed and the experiments repeated three times, the average resveratrol yield was 1604.7 μg/ L, 1.59% lower than the predicted value, indicating that the model can be used to predict the yield of resveratrol synthesis by recombinant *Y. lipolytica* yeast.

IV. Conclusion

In this study, we explored the potential of *Y. lipolytica* to produce resveratrol. The direct addition of p-coumaric acid as a precursor through the tyrosine synthesis pathway can produce resveratrol, however, not directly. Furthermore, temperature had a significant effect on the yield of resveratrol, while the culture and addition time of p-coumaric acid had no significant effect. Through response surface optimization experiments, a high-yield resveratrol production synthesized by recombinant *Y. lipolytica* yeast reached 1604.7 μg/L. This study suggests that the tyrosine synthesis pathway can synthesise resveratrol in *Y. lipolytica* yeast.

Acknowledgments

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