

Enhanced 2-phenylethanol production by newly isolated *Meyerozyma* sp. strain YLG18 and characterization of its synthetic pathways

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Research

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Abstract

Background

2-Phenylethanol (2-PE) is an aromatic alcohol which has been widely used in cosmetics, perfume and food industries owing to its delicate rose scent. The newly isolated yeast *Meyerozyma guilliermondii* YLG18 was able to tolerate high exogenous 2-PE and produce 2-PE with two different pathways.

Results

A unique *Meyerozyma* sp. strain YLG18 was obtained in this study, which was capable of tolerating 4.0 g/L exogenous 2-PE. Response surface methodology (RSM) was implemented to improve the maximum 2-PE production. At optimized conditions: temperature, 24.7°C; initial glucose, 63.54 g/L; initial L-phe, 10.70 g/L, 2-PE production was increased to 2.55 g/L, leading to 104% increase compared to the pre-optimized one. In situ product recovery (ISPR) could further help improve the final 2-PE production to 3.20 g/L with fatty acid methyl ester as the extractant, representing the highest 2-PE production by using *Meyerozyma* sp.. Furthermore, genes involved in 2-PE synthesis were identified and their expression levels between Shikimate pathway and Ehrlich pathway were compared. Based on the genomic and transcriptional analysis, a penta-functional enzyme AroM in Shikimate pathway and an aspartate aminotransferase (AAT) with the potential to convert phenylalanine into phenylpyruvate in Ehrlich pathway were identified.

Conclusions

These findings would help broaden our knowledge and add to the pool of known 2-PE generating microbes and genes. Moreover, this study describes a potential, new 2-PE producer that lays foundation for the industrial-scale production of 2-PE and its derivatives in the future.

Key words: 2-phenylethanol; *Meyerozyma guilliermondii*; RSM; ISPR; gene analysis

Background

2-Phenylethanol (2-PE) is an aromatic alcohol, which can be naturally found in essential oils of many plants, such as hyacinths, jasmine and lilies. 2-PE has been widely used in cosmetics, perfume and food industries owing to its delicate rose scent [1]. Furthermore, 2-PE is also an important raw material for the derivatives synthesis, among which phenylethyl acetate is a valuable fragrance compound, and p-hydroxyphenylethanol is widely used in pharmaceutical and fine chemicals industries [2, 3]. Although 2-PE can be extracted from flowers and plants, the extremely low concentration hinders its application [4]. Alternatively, 2-PE can be chemically synthesized, however, its quality is greatly affected because of the harsh condition and toxic reagents used [5]. Currently, the price of 2-PE produced through natural routes, such as extraction from rose petals or bio-converted from renewable resources is approximately USD 1,000/kg. However, it is only USD 5.0/kg for chemically synthesized 2-PE from benzene and styrene [6].

Therefore, bio-synthesis of 2-PE has become an appealing option owing to its environmentally friendly property and mild conditions.

In nature, many wild-type microorganisms have been identified and characterized to be capable of producing 2-PE, most of which are from eukaryotes, including *Saccharomyces* sp., *Kluyveromyces marxianus*, *Yarrowia lipolytica*, *Aspergillus oryzae*, and *Pichia* sp., etc [2, 7]. In addition, some prokaryotes have also been reported to produce 2-PE, such as *Microbacterium foliorum*, *Proteus vulgaris*, and *Psychrobacter* sp.. 2-PE can be either converted from L-phenylalanine (L-phe) through three steps catalysis of Ehrlich pathway or produced from glucose through Shikimate pathway. As multiple steps are needed for 2-PE production through Shikimate pathway, lower concentration usually occurred when 2-PE was directly synthesized from glucose. Accordingly, Ehrlich pathway is thought to be more promising for 2-PE production. It should be noticed that the lipophilic 2-PE could make the lipid membrane structure a preferential binding target, resulting in the collapse of transmembrane gradients and consequently the loss of cell viability [8], hence, strain development including mutation, selection, or genetic modification has been comprehensively adopted to improve the final 2-PE production. For instance, the newly isolated *K. marxianus* CCT 7735 could generate 3.44 g/L of 2-PE through Ehrlich pathway under optimized conditions [9]. *S. cerevisiae* BY4741, which overexpressed ARO10 and contained an *aro8Δ* deletion could produce 96 mg/L of 2-PE from glucose [10]. *S. cerevisiae* SPO810, in which ARO8 and ARO10 were co-expressed could finally produce 2.61 g/L of 2-PE with fed batch fermentation [3].

In the present study, novel 2-PE-generating microbes were first isolated and characterized. To further improve 2-PE production, statistical design of experimental strategy and in situ extraction strategy were used to improve final 2-PE titer. Genes involved in 2-PE synthesis were also identified and characterized by transcriptome analysis.

Results And Discussion

Isolation and phylogenetic analysis of *Meyerozyma* sp. strain YLG18

In this study, L-phe was used as the sole nitrogen source for the isolation of 2-PE generating strains (Fig. 1A). After more than 5 consecutive transfers in the medium spiked with 5 g/L of L-phe and 40 g/L of glucose, one colony named YLG18 gave the highest 2-PE production and molar conversion. Furthermore, YLG18 can even produce 100 mg/L of 2-PE in synthetic medium without supplementation of L-phe via the de novo pathway (Fig. 1B), which surpassed that using metabolically engineered *S. cerevisiae* (96 mg/L) via de novo pathway [10]. The 18S rDNA genes amplified from the genomic DNA of culture YLG18 showed 99% identity to *Meyerozyma guilliermondii* when blasted with the microbio sequences in the GenBank database. Thus, this culture is designated as *Meyerozyma* sp. strain YLG18 (Table 1). *M. guilliermondii* is known to be an ascomycetous yeast, which is broadly used for the production of riboflavin, xylitol and industrial enzymes [11]. Actually, *M. guilliermondii* has been reported to produce 2-PE through biological conversion of L-phe, and the highest 2-PE production could reach 1.61 g/L [12], however, the underlying mechanism for 2-PE production has not been clearly elaborated. Therefore, this

newly isolated wild-type 2-PE-producing *Meyerozyma* sp. strain YLG18 may further broaden our knowledge and add to the pool of known 2-PE generating microbes.

Table 1
Top matches of 18S rRNA gene sequence of strain YLG18 against known strains.

Microorganisms	Identity (%)
<i>Meyerozyma guilliermondii</i> culture CBS:2030 internal transcribed spacer 1, partial sequence	99
<i>Pichia guilliermondii</i> strain ATCC6260; ATCC46036 internal transcribed spacer 1, partial sequence	99
<i>Candida athensensis</i> strain BG02-5-23-003I-4 18S ribosomal RNA gene, partial sequence	95
<i>Carpophila</i> culture CBS:5256 internal transcribed spacer 1, partial sequence	98
<i>Meyerozyma caribbica</i> CBS 9966 ITS region; from TYPE material	98
<i>Meyerozyma amylolytica</i> strain DSM 27310 small subunit ribosomal RNA gene, partial sequence	93

2-PE can increase the cell membrane fluidity and reduce the uptake of amino acids and glucose, leading to the low microbial growth and 2-PE production. The increased permeability of membrane would accelerate the transmembrane diffusion of ions and small molecular metabolites, and disrupt the transmembrane proton potential [13]. Studies have reported that 2.0 g/L of 2-PE could completely inhibit the growth of *S. cerevisiae* W303-1A and *S. cerevisiae* Giv 2009 [14]. Therefore, to further determine the 2-PE tolerance potential of strain YLG18, the 2-PE tolerance of strain YLG18 was investigated. Different concentrations of exogenous 2-PE ranging from 1.5 g/L to 4.0 g/L were added into the fermentation medium initially. As seen from Fig. 1C, with the increase of exogenously added 2-PE, the inhibition effect on strain growth was obviously observed. For instance, with the supplementation of 3.5 g/L of 2-PE, OD₆₀₀ reached 10, which is only half of that in the presence of 1.5 g/L 2-PE. When the concentration of exogenous 2-PE reached 4.0 g/L, strain growth was almost completely inhibited. Nevertheless, the higher 2-PE tolerance level of strain YLG18 compared to current reported 2-PE producers indicated that it may be promising candidate for high 2-PE production [15].

Determination of influencing factors for 2-PE production by *Meyerozyma* sp. strain YLG18

To further improve the final 2-PE titer, various strategies including fermentation condition optimization and process integration have been developed. During fermentation process, carbon source has been proved as an important factor influencing cell growth, L-phe consumption and 2-PE molar conversion. Therefore, different carbon sources including glycerol, glucose, xylose, rapeseed oil and NaAc were chosen for 2-PE production (Fig. 2A). After 96 h fermentation in mineral salts medium, the highest 1.25 g/L of 2-PE with glucose as the carbon source occurred with 51.3% molar conversion, which is

38.89%, 56.25%, 316.67% and 150% higher than that using xylose, glycerol, rapeseed oil and NaAc as substrate, respectively. It should be noticed that although glycerol is more reduced than glucose and provide more NADH, which is critical for the last reduction step in Ehrlich pathway, it only gave 0.8 g/L of 2-PE. Also, previous studies have shown that lower pH is relatively favorable for cell growth, but unfavorable for 2-PE production [16]. However, when sodium acetate was used as carbon source to increase pH, 2-PE production was only 0.5 g/L.

The effect of temperature ranging from 25 to 37°C was also evaluated in synthetic medium containing 30.0 g/L of glucose (Fig. 2B). The highest 1.55 g/L of 2-PE was obtained at 30°C with molar conversion of 70.86%. Studies have shown *M. guilliermondii* preferred 37°C for ethanol production, however, slightly lower 2-PE production of 1.3 g/L occurred, and an OD₆₀₀ of 14.32 was achieved at 37°C, indicating that elevated temperatures unfavored 2-PE production and microbial growth for strain YLG 18. Similar results were also obtained when other *M. guilliermondii* strain, such as *M. guilliermondii* WUT22 was used for bioconversion of L-phe to 2-PE [17, 18].

During the bioconversion process, 30 g/L of glucose can be rapidly consumed during 48 h, however, 3.65 g/L of L-phe was still left over with initial concentration of 5 g/L (data not shown). Hence, different initial glucose concentrations were assessed for their effects on 2-PE production. As seen in Fig. 2C, when initial glucose concentration was 40.0 g/L, the highest 2.17 g/L of 2-PE and 77.6% of molar conversion were achieved, respectively. The molar conversion would decrease with the increase of glucose concentration. When glucose concentration was above 40 g/L, OD₆₀₀ dropped from 38.15 to 31.96 with the increase of glucose concentration. Especially, 2-PE production kinetics is paralleled to that of microbial growth, suggesting that 2-PE production by strain YLG18 was closely related to cell growth.

As L-phe was used as the nitrogen source for both microbial growth and 2-PE production, different amounts of L-phe were also evaluated for the improvement of 2-PE production. It can be seen from Fig. 2D that 2-PE production kinetics was basically consistent with the yeast growth. When L-phe concentration was 7 g/L, 2.22 g/L of 2-PE and a maximum OD₆₀₀ of 35.72 were achieved, which was higher than that using *S. cerevisiae* Ye9- 612 E (0.85 g/L) [19, 20]. However, the molar conversion with 7.0 g/L of 2-PE was only 79%, which could be because the molar conversion was negatively correlated with the initial L-phe concentration. This finding proved that high L-phe concentration in a certain range is beneficial for 2-PE production, but would reduce the conversion yield, which was also verified by other studies, in which L-phe concentrations above 4.0 g/L did not lead to the increase of 2-PE titer by using *K. marxianus* CCT 7735 [9].

Optimization of fermentation conditions for enhanced 2-PE production from L-phe

Based on the above results using the method of “one time one factor”, further statistical experimental design methodology was applied to evaluate the interaction and determine the optimal level of three influencing factors including temperature, initial glucose and initial L-phe levels. For the response surface analysis, 17 experiments with triplicates were conducted according to the RSM design, where Y is 2-PE

production (g/L), X_1 denotes temperature ($^{\circ}\text{C}$), X_2 denotes initial glucose level (g/L) and X_3 denotes initial L-phe level (g/L) (Table 2). According to the response values obtained from the experimental results, a second order regression equation was generated for the response surface, as follows:

Table 2
RSM experimental design for 2-
PE production.

Run	X_1	X_2	X_3	Y
1	30	60	5	1.56
2	25	30	15	1.54
3	20	60	15	2.06
4	25	60	10	2.35
5	25	90	5	1.60
6	30	30	10	1.29
7	30	90	10	1.99
8	25	60	10	2.35
9	20	90	10	1.55
10	25	60	10	2.35
11	25	90	15	1.66
12	20	60	5	1.60
13	30	60	15	1.66
14	25	30	5	1.40
15	25	60	10	2.35
16	25	60	10	2.35
17	20	30	10	1.61

$Y = 2.28 + 0.013X_1 - 0.12X_2 + 0.072X_3 - 0.12 \times 1 \times 2 + 0.032 \times 1 \times 3 + 0.0016 \times 2 \times 3 - 0.34X_1^2 - 0.87X_2^2 - 0.50X_3^2$ (Eq. 2, [$R^2 = 0.9710$; $R^2(\text{Adj}) = 0.9337$; $R^2(\text{Pred}) = 0.5362$]). The regression model (Eq. (1)) gave a high R^2 value of 0.9710 (Table 3), which indicated aptness of the model [21]. The R^2 value is between 0 and 1. When R^2 is close to 1.0, the model will better predict the response [22, 23]. Notably, the model F value of 26.06 and values of probability (P) > F (0.0001) indicated that the model terms were significant [24]. The three-dimensional response surface plot is generally used to demonstrate relationships between the response and experimental levels of each variable (Fig. 3). The highest point on the contour profiles in

Fig. 3 indicates the optimal parameter values of the highest 2-PE production. As shown, there was obvious interaction between each pair of variables, and the interaction between the selected three variables was significant. The optimal 2-PE production value predicted from the response surface model was 2.37 g/L, when the temperature was controlled at about 24.7°C and initial glucose and L-phe concentrations were 63.5 and 10.7 g/L, respectively.

Table 3
Analysis of variance (ANOVA) for the quadratic model^a.

Source	Sum of Squares	df	Mean Square	F Value	Prob>F	Significance
Model	2.28	9	0.25	26.06	0.0001	significant
Residual	0.068	7	0.009743			
Lack of Fit	0.068	3	0.023			
Pure Error	0.000	4	0.000			
Cor Total	2.35	16				
^a Coefficient of determination (R^2) = 0.9710; Adjusted R^2 = 0.9337;						

The following validation experiment in 3.0 L bioreactor was carried out under the optimized conditions with 63.54 g/L of initial glucose, 10.70 of L-phe g/L at 24.7°C. The 2-PE production was finally improved up to 2.55 g/L after 96 h, which is 7.8% higher than the predicted level, representing the highest 2-PE production from L-phe by using *M. guilliermondii* [12]. Hence, the models developed were considered to be accurate and reliable for predicting 2-PE production from L-phe by using strain YLG18.

Biosynthesis of 2-PE by using *Meyerozyma* sp. strain YLG18 with ISPR technology

As mentioned, 2-PE is toxic to microbial cells, as it can make the lipid membrane structure a preferential binding target, resulting in the collapse of transmembrane gradients and the loss of cell viability [8]. To further increase the final 2-PE production and productivity, more robust strains or novel extraction technology should be developed. In situ product recovery (ISPR) techniques can simultaneously remove 2-PE from the fermentation broth while it is produced. Thereby, 2-PE concentration could be maintained below the inhibitory level, and microbes are able to continuously produce 2-PE. Suitable extractants including fatty acid methyl ester (FAME), oleic acid and ethyl acetate were first identified. As seen from Fig. 4A, the partition coefficients of these organic solvents were similar for 2-PE. However, different organic solvents had different impacts on the dissolved oxygen level, which would affect microbial growth and lead to significant difference in the final 2-PE titer [25]. Among these organic solvents, FAME gave the highest 2.53 g/L of 2-PE. The volume ratio of medium and extractant phases will also affect the distribution and mass transfer of products. Low ratio of FAME could not extract 2-PE effectively, affecting the bioconversion rate. High ratio of FAME may increase toxicity to cells. Results showed that the highest 2.48 g/L of 2-PE was obtained when FAME : water ratio was maintained at 1:1 (Fig. 4B). It should be

noticed that when the ratio of FAME to water was 1:2, the production of 2-PE was 2.45 g/L, which is close to the highest one. Accordingly, the optimal ratio of 1:2 was chosen for following experiments.

Generally, microbes would show a log phase after inoculated into the fresh medium. The presence of FAME in the initial stage may prolong this period and affect the transformation activity of yeasts. Therefore, a proper delay of FAME addition may help to improve the metabolic activity of yeasts and ultimately increase 2-PE production. From Fig. 4C, it can be seen that the earlier extractants were added, the more obvious the strain growth inhibition was. Compared with the experimental results of groups 4, 5 and 6, when FAME was supplemented when strain growth reached exponential phase, the death rate would be decreased significantly. In addition, it can be seen from Fig. 4D that when the strain death rate decreases, the 2-PE production in the group 4 was also the highest, and it can reach 3.20 g/L, which was improved by 25.49% compared to single-phase biotransformation.

Characterization of related genes and biosynthetic pathway of 2-PE by *Meyerozyma* sp. strain YLG18 through transcriptome analysis

At present, only limited literatures have reported 2-PE production by using *M. guilliermondii*. To further elaborate the 2-PE production mechanism by strain YLG18, genes involved in substrate consumption, L-phe metabolism and 2-PE production were identified and characterized, which could help to speculate the biosynthesis pathway of 2-PE and provided clues on the divergence of gene expression level between Shikimate pathway and Ehrlich pathway (Fig. 5). After integrating the annotations of strain genome sequences in GO, COG and KEGG, total 10 main unigenes (*aroF*, *aroM*, *CM*, *aroC*, *pheA*, *AAT*, *ARO8*, *HisC*, *PDC*, *ADH*) with functions assigned to 2-PE biosynthesis were identified. According to KEGG annotation, the first five genes are key ones in the Shikimate pathway, which were mainly responsible for conversion of glucose into phenylpyruvate; while the last five genes are key ones in the Ehrlich pathway, which were mainly responsible for conversion of L-phe into 2-PE. As for the Shikimate pathway in strain YLG18, a penta-functional enzyme of *AroM* was identified, which is different from some prokaryotes, whose Shikimate pathway reactions are mainly catalyzed by enzymes encoded by individual genes (*aroB*, *aroD*, *aroE*, *aroK*, *aroA*). To further confirm the function of this penta-functional enzyme *AroM*, the protein sequence was blasted in NCBI website of non-redundant protein sequences (nr) database. Only 59.33% similarity was shown between *AroM* from strain YLG18 and the penta-functional protein *ARO1p* from *S. cerevisiae* S288C (NP_010412.1). Previous studies have reported that the penta-functional protein *ARO1* in *S. cerevisiae* is essential for aromatic amino acid production (Gold et al., 2015; Hassing et al., 2019). Although *AroM* in *M. guilliermondii* complicates tuning gene expression at individual activity level, it can convert 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) to 5-enolpyruvyl-shikimate-3-phosphate (EPSP), which is important in Shikimate pathway. This newly identified penta-functional enzyme *AroM* from strain YLG18 may broaden our knowledge of 2-PE production through Shikimate de novo pathway.

To confirm the reproducibility and accuracy of differential gene expression identified through the Illumina analysis, genes related to phenylalanine biosynthesis and phenylalanine metabolism were selected for qRT-PCR analysis to determine the FPKM value. As can be seen from Fig. 5, the expression levels of

genes in Ehrlich pathway were higher than those in Shikimate pathway except for HisC. The overall change in the expression pattern for Ehrlich pathway vs. Shikimate pathway ranged from 0.95-fold to 62.68-fold. This result can effectively explain that the 2-PE production synthesized through Ehrlich pathway was generally higher than that synthesized through Shikimate pathway. It is important to point out that the expression of aspartate aminotransferase (AAT) shows nearly 62 times difference in these two pathways. Although substrates were different, the expression of ARO8, which is also an amino acid aminotransferase was far lower than that of AAT. This means that AAT plays an important role in the Ehrlich pathway of strain YLG18. Different from the existing results on 2-PE production by other yeasts such as *S. cerevisiae*, various amino acid transaminases (Aro8, Aro9, Bat1, and Bat2) have shown to catalyze the first step of Ehrlich pathway in the catabolism of aromatic amino acids [3, 26]. However, AAT has rarely been reported for 2-PE production. Shrawder et al. [27] have proved that AAT from porcine heart had phenylalanine transaminase activity, and Cárdenas-Fernández et al. [28] have successfully synthesized L-phenylalanine with immobilized AAT. Therefore, AAT may be a potential enzyme for 2-PE synthesis, and *Meyerozyma* sp. strain YLG18 may also serve as a potential candidate for industrial 2-PE production from L-phe. Future studies are needed to elaborate AroM and AAT function, and the tolerance mechanism of strain YLG18.

Conclusion

This study presents how a newly identified *M. guilliermondii* strain YLG18 can be used as a potential candidate for high 2-PE production from L-phe. After determination of influencing factors, process optimization using RSM and reducing toxicity of 2-PE with ISPR techniques, 3.20 g/L of 2-PE can be produced from 63.54 g/L of glucose and 10.70 g/L of L-phe. The high 2-PE production by using strain YLG18 indicates that it may show great potential for 2-PE production. Candidate genes related to 2-PE biosynthesis and metabolism can be used as target genes for marker-assisted selection through genetic engineering to further improve final 2-PE titer in future studies.

Materials And Methods

Isolation and molecular identification of strain YLG18

Soil samples from Xuanwu Lake, Nanjing, China were used as inocula to screen microorganism capable of producing 2-PE. The obtained samples were added into flasks containing 50 mL of defined mineral salts medium (MMT) spiked with 40 g/L of glucose as the carbon source. After 96 h fermentation at 30 °C and a shaking speed of 200 rpm, 0.2 mL culture was spread on a Petri dish which contained MMT medium. Then different colonies were selected and added into MMT using L-phe as the sole nitrogen source for detecting production capabilities of 2-PE. Eventually, a 2-PE-producing strain named YLG18 was obtained. Unless stated otherwise, the strain was grown in MMT with L-phe as the sole nitrogen source at 30 °C.

The defined MMT contained (per liter of distilled water): glucose 40 g, L-phe 5 g, NaCl 1.0 g, MgCl₂·6H₂O 0.5 g, KH₂PO₄ 0.2 g, KCl 0.3 g, CaCl₂·2H₂O 0.015 g, TES 2.292 g, uracil 0.1 g, thiamine 3 mg. In addition, 1 mL of trace element solution and 10 mL of salts solution were added to 1 L medium. Then the medium was dispensed into triangle shaker, autoclaved for 20 minutes and cooled down to room temperature.

DNeasy tissue kit (Zomanbio, Beijing) was used to extract and purify cultures' genomic DNA according to the instructions of manufacturer. The 18S rRNA gene was then PCR amplified using genomic DNA as a template with a pair of common fungi primer ITS1 (5'-CCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The obtained PCR products were purified with a PCR purification kit (Zomanbio, Beijing) and sequenced using an ABI DNA sequencer (Applied Biosystems, USA). The 18S rRNA gene sequence was aligned using the BLAST algorithm and was deposited in the GenBank database with an accession number of MN461543.

Optimization of fermentation conditions

All batch fermentation studies were carried out in 250 mL triangle shakers containing 50 mL of mineral salts medium with initial pH of 5.8. The medium-filled triangle shakers were wrapped with gauze and aluminum foil before being autoclaved. L-phe was first filter-sterilized and then added into the medium as the sole nitrogen source. Inoculation was carried out by adding 2% of seed culture (strain YLG18) to the medium. The bottles were incubated at 30 °C and 200 rpm in a shaking incubator for 96 h. Detailed optimization studies regarding the production of 2-PE were carried out by using the response surface methodology (RSM). Three factors (temperature, initial glucose concentration, and initial L-phe concentration) were chosen as independent variables, while 2-PE concentration was the dependent variable. An experimental strategy based on central composite design (CCD) was obtained using Design Expert version 7.0 (Stat-Ease, Minneapolis, USA). The values of the response variable obtained were fitted to a second-order polynomial equation:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

where Y_i is the predicted response, x_i , x_j are independent variables, which influence the dependent variable Y ; β_0 is the offset term; β_i is the i^{th} linear coefficient; β_{ii} is the i^{th} quadratic coefficient and β_{ij} is the ij^{th} interaction coefficient. Statistical analysis of the model was performed by using analysis of variance (ANOVA) in a statistical software package.

Subsequently, the optimized condition obtained from RSM was applied to batch fermentation in a 3.0-L bioreactor (BIOSTAT® B plus, Sartorius, Germany). The bioreactor was filled with 2.0 L of mineral salts medium and operated at 30 °C with an agitation rate of 200 rpm.

Analytical procedures

Fermentation broth samples were analyzed for biomass growth, glucose and L-phe utilization, and 2-PE production. Biomass was determined by measuring optical density at 600 nm with appropriate dilution using a UV-visible spectrophotometer (Lambda-25, Perkin-Elmer, USA). Glucose was analyzed by a 1260

Series HPLC system (Agilent Technologies Inc.) equipped with an Aminex HPX-87H column (BioRad, Richmond, CA, USA) and a Refractive Index Detector (RID). Samples were run at 75 °C with 0.6 mL/min eluent of 5 mM sulfuric acid. Substrate (L-phe) and product (2-PE) were measured by a 1260 Series HPLC system (Agilent Technologies Inc.) equipped with an Acclaim™ 120-C18 column (Thermo Scientific, China) and UV-detector at 210 nm. 50% sterile water and 50% (v/v) methanol were pumped isocratically through Agilent 1260 quat pump at 0.6 mL/min. The column was kept at temperature of 30°C. Five-point standard curves were obtained by running standard solutions containing L-phe and 2-PE.

In situ product removal (ISPR) for 2-PE production

Extractive bioconversion was carried out with the addition of different organic solvents as extractants. These extractants were added directly with an aqueous/organic ration of 1:1 (v/v) when the bioconversion started. Controls were conducted without addition of any solvent. The bioconversion was carried out at 180 rpm, 30 °C in 500-mL flask. At the end of the bioconversion, samples were collected and 2-PE concentration in both organic and aqueous phases was measured [29].

qRT-PCR-based validation and analysis

qRT-PCR was conducted to verify the expression of genes potentially involved in the biosynthesis pathway. Total RNA was extracted from strain YLG18 cultured after 48 h using the FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, Nanjing, China) and reverse-transcribed into cDNA using the HiScript II Q RT SuperMix for qPCR (+ gDNA wiper) (Vazyme, Nanjing, China). PCR primers were designed with SnapGene 1.1.3 based on the nucleotide sequence of reference and antioxidant-related genes from the RNA-Seq data (Table 4). qRT-PCR was performed using ChamMq™ SYBR qPCR Master Mix (High ROX Premixed) (Vazyme, Nanjing, China) and an ABI StepOnePlus (Applied Biosystems). qRT-PCR was performed with the follow thermocycling parameters: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 55 °C for 30 s. Triplicate analyses of each cDNA sample were performed, and the relative expression levels of genes in each group were normalized to 18SrRNA expression. The $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method was used to evaluate the relative expression levels of target genes [30]. The values reported represent the average of 3 biological replicates [31].

Table 4
Primers used for qRT-PCR analysis.

KO ID	Predicted function	Gene name	Primers used for qPCR	
K13830	pentafunctional AROM polypeptide	aroM	F R	GCGGAATCGAACATTTGTTCGAGCA TTGAGGGTTTTTCAGCGGCCAAATA
K01736	chorismate synthase	aroC	F R	ATTCAGAGTGACCACCTATGGTGAG CACAAGCATACCAATTGGAGATCCC
K01626	3-deoxy-7-phosphoheptulonate synthase	aroF	F R	ATACGATGACACACGTATCTTGGCA CGTACTCTATAGCAGACGTGGGGT
K03786	3-dehydroquinate dehydratase	aroQ	F R	CAACCTTTTTGGGTACCAGAGAGCC TGGCCACAGAAGTATGAGTATATGCC
K01850	chorismate mutase	CM	F R	AACCAGAAACTGTTCTTGATCTCGG GCCTCGTATCTACGGATCTGCGAG
K00817	histidinol-phosphate aminotransferase	HisC	F R	GTGAACCTGCGGAAGGATCATTACA TCATCGATGCGAGAACCAAGAGAT
K04518	prephenate dehydratase	pheA	F R	TCCAGGCACATATACTCATCAGGCG TGTTCTCCCACAATTCTAAACAGCG
K14454	aspartate aminotransferase	AAT	F R	CGTGTCTGTGGATCCAGTTATGG TTCTAGCTCCTTCAATGTA CT CAGG
K00838	aromatic amino acid aminotransferase	ARO8	F R	CTTCTATCTGAACAAGCCAAAGCGA GTCGGTTGAATCCTGCGAAACTGTA
K12732	phenylpyruvate decarboxylase	PDC	F R	GGTGAAAGCAGTAGCCAAGATTCTC TTGTCTCGATTGACGAGGGTGGTAT
K00002	alcohol dehydrogenase	ADH	F R	CAAGGTTTTGGAGTCGACGATAAGG AGCGTAACTTTGTCACCAACGGAGA

Abbreviations

2-PE

2-phenylethanol

L-phe

L- phenylalanine

RSM

Response surface methodology

ISPR

In situ product recovery

MMT

mineral salts medium

FAME
fatty acid methyl ester
AAT
aspartate aminotransferase

Declarations

Authors' contributions

YW conceived, designed the experiments, performed the laboratory work, analyzed, and interpreted the data and drafted the paper. QXJ performed the sample determination. ZWM, ZJ and XB analyzed the metabolic pathway and enzyme function, and critically revised the manuscript. DWL, JM and XFX contributed to experimental design and data interpretation and also critically revised the manuscript. All authors read and approved the final version.

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Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

Not applicable.

Availability of data and materials

The information about accession numbers is given in the manuscript.

Consent for publication

All authors consent to publication.

Ethics approval and consent to participate

Not applicable.

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All of the information of the authors has been listed on the title page.

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Figures

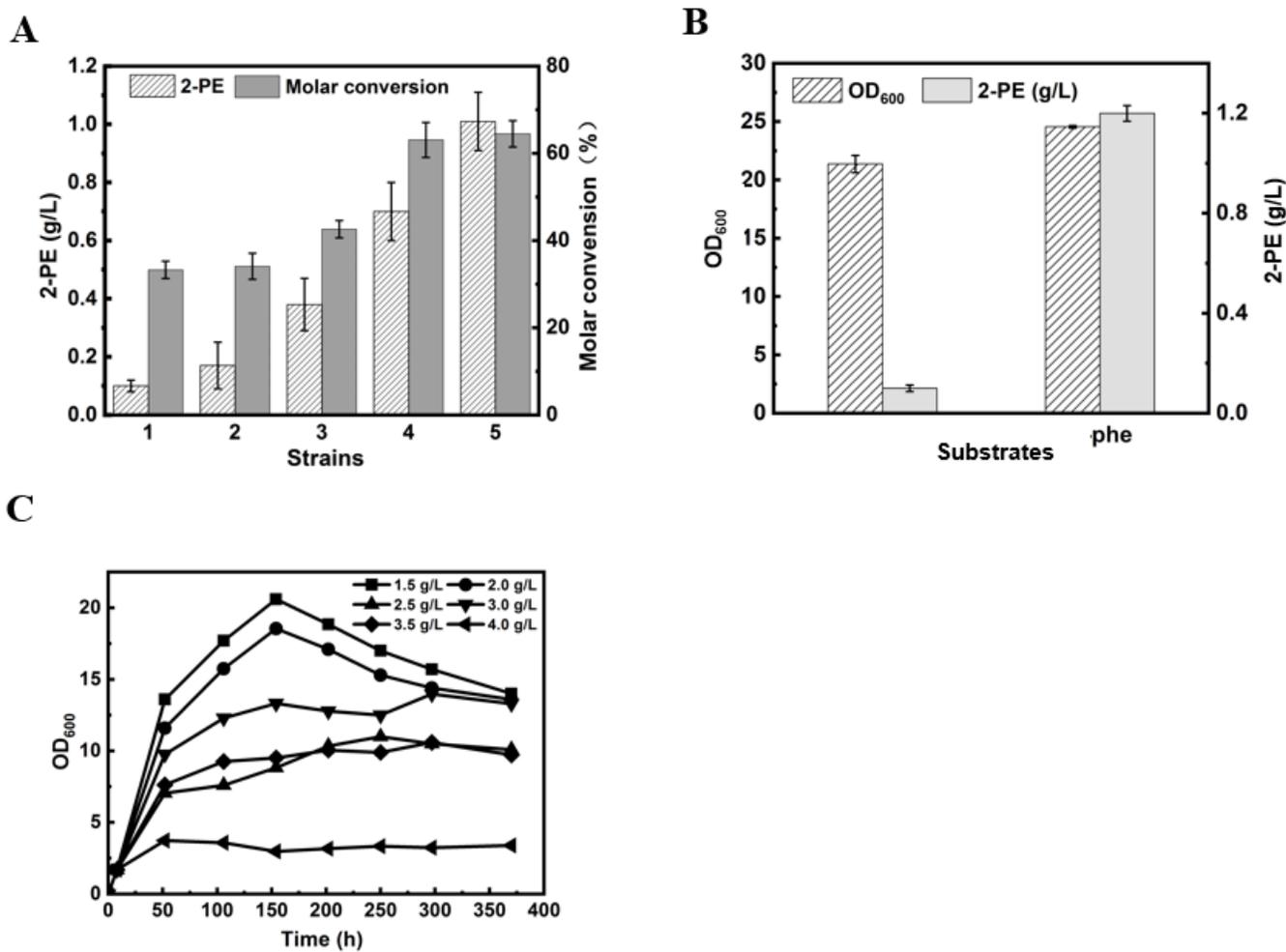


Figure 1

2-PE production and conversion yield by using different screened strains (A), Growth and 2-PE production of strain YLG18 under two synthetic pathways (B), Tolerance of strain YLG18 to exogenous 2-PE (C).

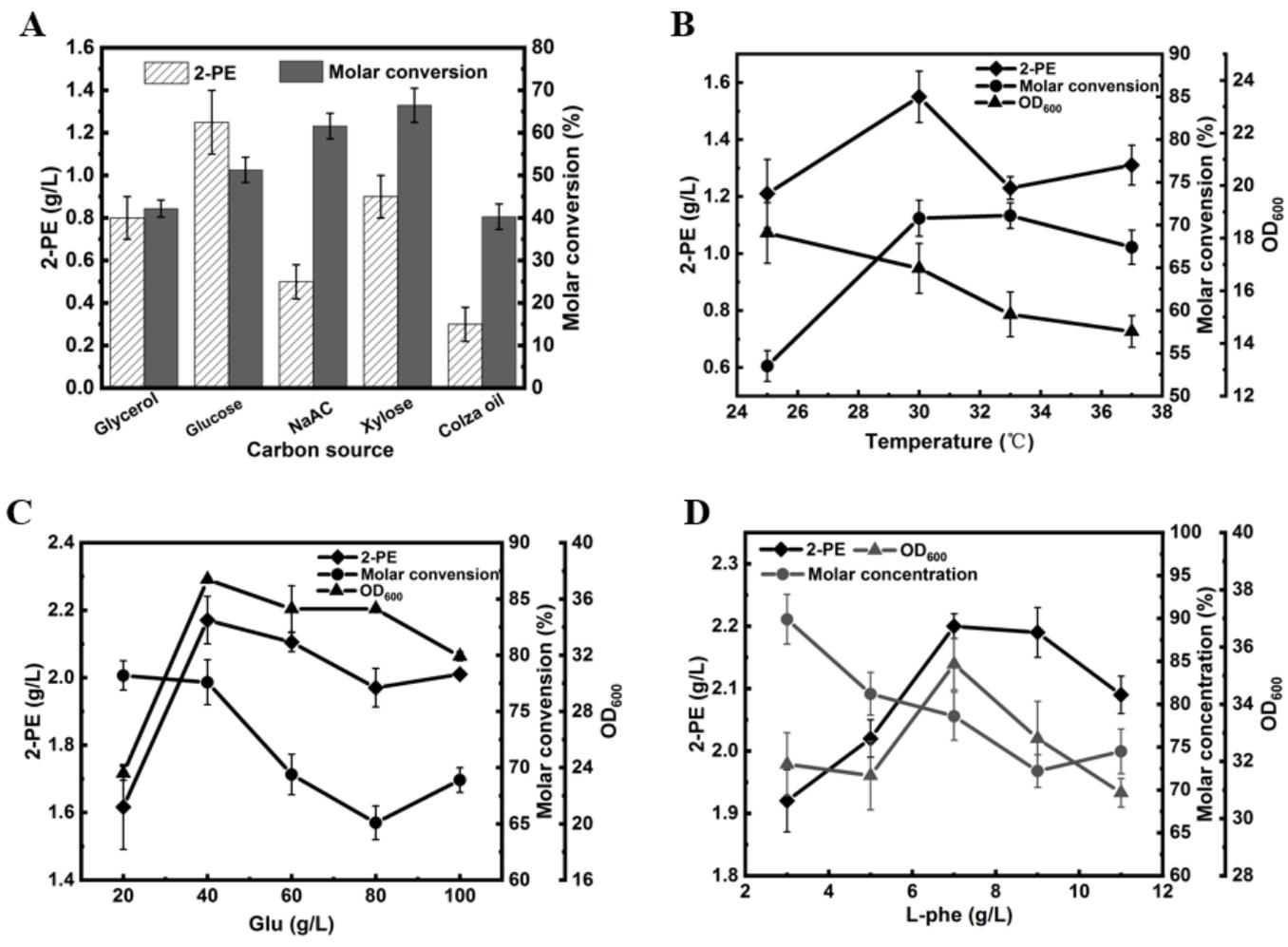


Figure 2

Optimization of single factor fermentation conditions: carbon sources (A), fermentation temperatures (B), initial glucose concentrations (C), initial L-phe concentrations (D).

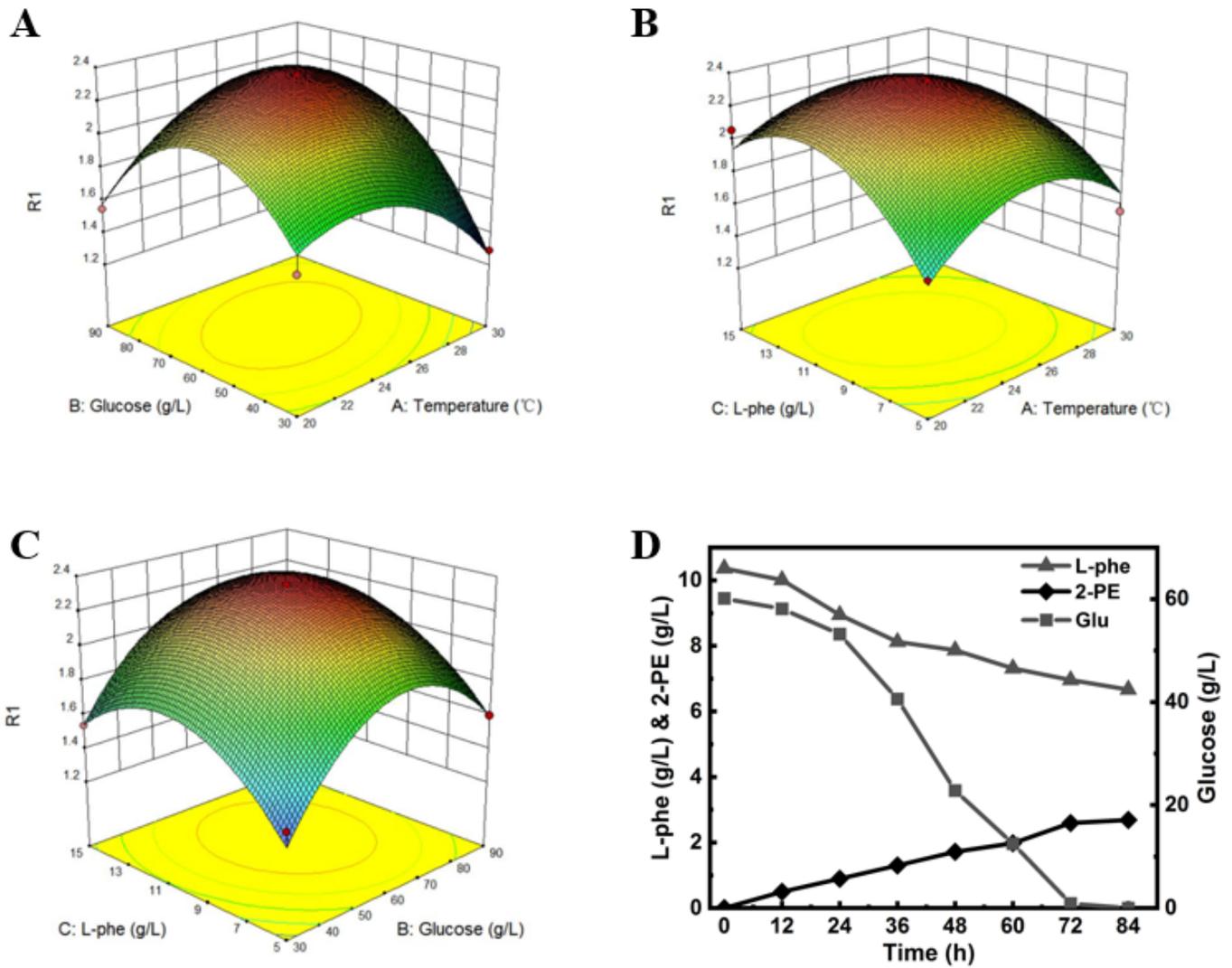


Figure 3

Three dimensional response surface and contour plot showing the effect of temperature, initial glucose concentration and initial L-phe concentration on 2-PE production (g/L): the effect of temperature and initial glucose concentration on 2-PE production (g/L) (A), the effect of temperature and initial L-phe concentration on 2-PE production (g/L) (B), the effect of initial glucose concentration and initial L-phe concentration on 2-PE production (g/L) (C), growth and fermentation profiles of strain YLG18 in mineral salts medium amended with 63.54 g/L of glucose and 10.70 g/L of L-phe with temperature controlled at 24.7°C (D).

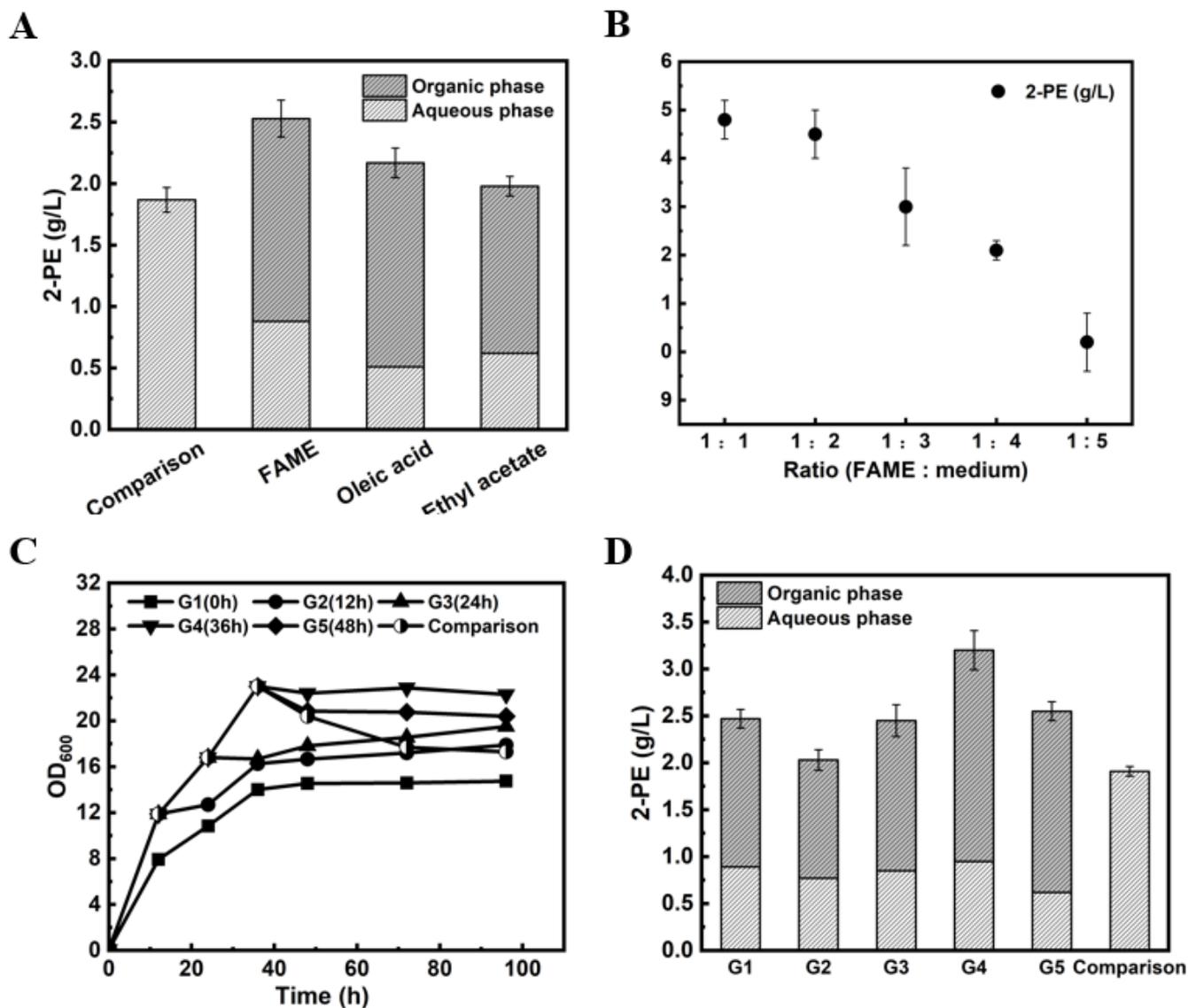


Figure 4

Biosynthesis of 2-PE with ISPR technology: 2-PE yield using different extractants (A); 2-PE production with different phase-volume ratios (B); Effects of adding time of FAME on the growth of cells (C); Effects of FAME adding time on 2-PE production(D).

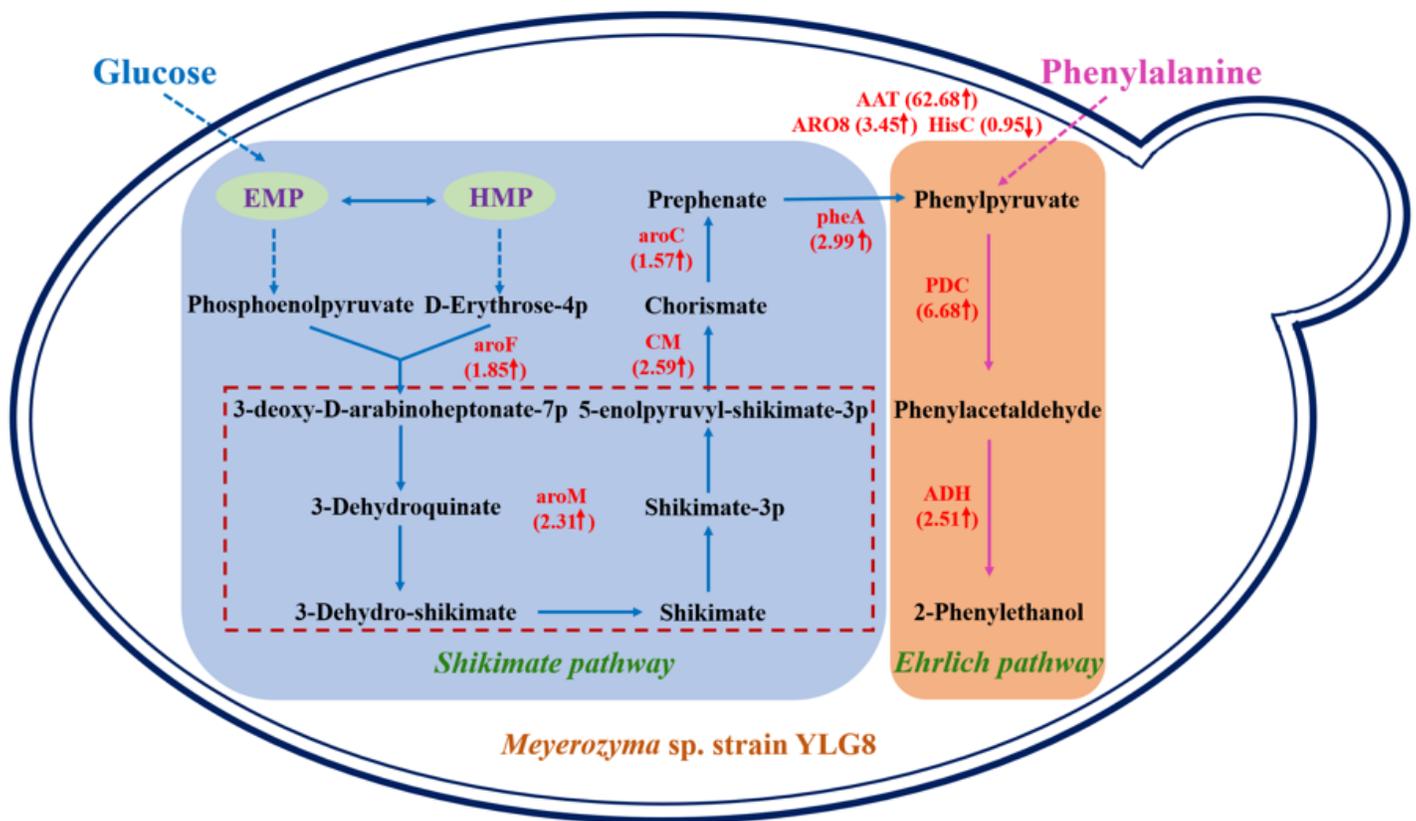


Figure 5

Proposed metabolic pathway for 2-PE production within strain YLG18 and comparison of expression levels of related genes in two different 2-PE synthesis pathways.