



Original Research Article

Metabolic reprogramming and computation-aided protein engineering for high-level de novo biosynthesis for 2-phenylethanol in *Pichia pastoris*

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ABSTRACT

2-Phenylethanol (2-PE), an aromatic compound with a characteristic rose fragrance, is extensively used in the food and cosmetic industries as a flavoring and fragrance agent. Due to limitations in obtaining 2-PE from natural plant sources, microbial cell factories offer a promising alternative for sustainable biosynthesis. In this study, *Pichia pastoris* was engineered to efficiently synthesize 2-PE. Using computer-assisted predictions of interactions between the key phenylpyruvate decarboxylase KDC2 and its substrates or products, an optimal enzyme variant was rationally designed to boost 2-PE production. Additionally, the shikimic acid pathway was enhanced, and a dynamic regulation promoter was employed to reduce competition from alternative pathways. These strategies significantly increased metabolic flux toward 2-PE production, achieving a titer of 2.81 g/L and 45.8-fold improvement over the non-engineered strain. By integrating controlled carbon feeding and in situ extraction to alleviate acetic acid inhibition and product toxicity, the recombinant strain achieved a final 2-PE titer of 7.10 g/L and a yield of 0.14 g/g glucose, the highest reported microbial production to date. This study highlights the significant potential of *P. pastoris* as a versatile cell factory for the green biosynthesis of 2-PE and other natural products.

1. Introduction

2-Phenylethanol (2-PE), an aromatic alcohol with a pleasant floral scent, is a key flavor and fragrance widely used in the cosmetic and food industries. Compared to chemical synthesis and phytoextraction, fermentative production of 2-PE provides a more sustainable, safer, and cost-effective alternative [1]. Natural products like α -santalene [2], catharanthine [3], and chondroitin sulfate [4] have been synthesized using *Pichia pastoris*, highlighting its potential for efficient natural product synthesis. Additionally, *Pichia* species role in 2-PE production during Baijiu brewing [5] suggests they could be promising for 2-PE biosynthesis. However, there are few studies on the ability of *Pichia* to synthesize 2-PE [6]. To date, various host strains, including

Saccharomyces cerevisiae, *Yarrowia lipolytica*, and *Bacillus licheniformis* [7–9], have been engineered to enhance 2-PE production. For instance, promoter engineering in *B. licheniformis* enabled the highest reported 2-PE titer of 6.95 g/L [10], while *Y. lipolytica* produced 2.4 g/L [11]. However, fermentation complexity and low production titers limit industrial viability.

Efficient microbial synthesis of 2-PE faces several challenges, such as uneven metabolic flux distribution, balancing cell growth with product synthesis, by-product inhibition, and product toxicity. To elevate 2-PE production in yeast, several strategies have been applied, as shown in Table 1, such as breeding high-performance strains [12], fermentation optimization [13–15], and synthetic microbial consortium [16,17]. Moreover, the implementation of pathway metabolic engineering to

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Table 1
Comparison of the green advances of different production strategies of 2-PE.

Strains	Strategies	Titer	Yield	Ref.
<i>Saccharomyces cerevisiae</i>	metabolic engineering	1.59 g/L	0.08 g/g glu	[9]
<i>Yarrowia lipolytica</i>	metabolic engineering	2.4 g/L	0.06 g/g glu	[11, 23]
<i>Kluyveromyces marxianus</i>	UV mutagenesis	0.25 g/L	0.01 g/g glu	[24]
<i>Escherichia coli</i>	enzymatic engineering	2.5 g/L	0.16 g/g glu	[25]
<i>Bacillus licheniformis</i>	metabolic engineering	6.95 g/L	0.15 g/g glu	[10]
<i>Escherichia coli</i> and <i>Meyerozyma guilliermondii</i>	microbial consortium	3.8 g/L	0.05 g/g glu	[16]

increase 2-PE production has been demonstrated to be an effective strategy. The construction of a “push-pull” strategy in *Y. lipolytica* by co-expressing exogenous feedback-resistant genes (*EcAROG^{fbr}*, *EcPheA^{fbr}*) and endogenous genes (*ylARO10*, *ylPAR4*) significantly enhanced shikimate pathway metabolic flux, achieving a 2-PE titer of 2.4 g/L. This approach effectively alleviated metabolic bottlenecks in the 2-PE pathway [11]. By introducing *Bifidobacterium breve* phospho-ketolase (Bbxfpk) or overexpressing pyruvate kinase variants to rewire central carbon metabolism for optimized erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) supply to the shikimate pathway, and the key enzymes were systematically strengthened to construct a 2-PE production strain [9,18].

Recent advances in computer-aided semi-rational design combined with directed evolution have streamlined screening efforts while boosting enzyme catalytic activity [19,20]. This approach has been validated through multiple studies. Cui et al. predicted the mutation site by computer-aided $\Delta\Delta G$ value calculation and B-factor analysis. Two mutants Q246V and K249V with enhanced thermal stability obtained by site-directed mutagenesis were enhanced to 2.41- and 2.96-fold of alginate lyase AlyMc, respectively [21]. The γ -glutamylmethylamide synthetase was modified by bioinformatics analysis and computer-aided design. The obtained mutant E179K/N105R had higher catalytic capacity and better thermal stability, and the yield of L-theanine was increased by 36.61 % [22]. Combining metabolic engineering and protein engineering has proven highly effective, demonstrating the feasibility of applying this strategy to microbial production of high-value compounds.

The production efficiency of microbial cell factories depends on the growth performance, product synthesis ability, and stress resistance of the strains. In 2-PE production, the tolerance of the chassis strain to 2-PE largely determines its maximum production capacity. This is due to the inherent cytotoxicity of 2-PE, which damages microbial cells and restricts their ability to produce it at high concentrations [26,27]. To mitigate product toxicity, strategies like tolerance engineering and adaptive laboratory evolution are commonly employed. A previous study improved the robustness of *P. pastoris* to 2-PE using transcriptomics and tolerance engineering, which enhanced strain productivity [28]. However, achieving high 2-PE production also requires mitigating product toxicity during fermentation. In-situ product recovery (ISPR) technology can address this challenge by separating 2-PE from the aqueous phase, maintaining low product concentrations to minimize toxicity and feedback inhibition, and ultimately improving 2-PE titers. Liquid-liquid extraction methods within ISPR provide key advantages, such as improved process efficiency through partial product purification, reduced waste and solvent use in downstream processing, and support for continuous fermentation at scale [29]. Notably, studies on two-phase systems using organic solvents like oleyl alcohol, oleic acid, and polypropylene glycol have shown that incorporating an organic phase during microbial fermentation can boost 2-PE production by 2–4 times [30,31].

In this study, we developed a *P. pastoris* cell factory for efficient 2-PE

synthesis using multiple strategies (Fig. 1). First, the shikimic acid pathway was enhanced by alleviating feedback inhibition and increasing precursor supplies of E4P and PEP. Next, the catalytic activity of the key phenylpyruvate decarboxylase KDC2 was improved through rational design, streamlining the downstream pathway. Dynamic regulation promoter was then employed to minimize by-product accumulation, and optimize carbon utilization. Finally, fermentation conditions were also optimized in a two-phase culture, enabling the engineered strain (*P. pastoris* Pp18) to achieve the highest reported microbial 2-PE titer from glucose to date [29].

2. Material and methods

2.1. Strains, plasmids and culture conditions

The strains and plasmids used in this study are listed in [Supplementary Table 1](#). All plasmids were constructed in *Escherichia coli* DH5 α . The wild-type (WT) *Pichia pastoris* strain GS115 and its derivative strains were activated and cultured on YPD medium at 30 °C. Minimal dextrose (MD) medium consisted of 20 g/L glucose, 0.4 mg/L biotin, and 13.4 g/L yeast nitrogen base. All engineered yeast strains were constructed and cultured in MD medium at 30 °C. The engineered strains were verified using 2-PE fermentation in a fermentation medium (YPD) containing 50 g/L glucose, 20 g/L peptone, 10 g/L yeast extract, 0.5 g/L Mg₂SO₄, and 5 g/L K₂HPO₄. Genetic modifications were performed using the CRISPR/Cas9 method developed by Cai et al., with gRNA expression plasmids constructed as previously described [28]. The N20 sequences used in this study are listed in [Supplementary Table 2](#). The construction method of engineering strains was described in the materials and methods of [Supplementary File](#).

2.2. Modeling, docking, and virtual saturation mutation

The crystal structure of the decarboxylase from *Kluyveromyces lactis* (PDB ID: 2VK4, 1.95 Å) was utilized as a template to generate a three-dimensional (3D) homology model of KDC2 using Swiss-Model software. Visualization and analysis of the generated model structures were performed using the PyMOL Molecular Graphics System ([Supplementary Figs. 1 and 2](#)). The ligands phenylpyruvate and phenylacetaldehyde were obtained from PubChem for energy minimization. Molecular docking and binding energy calculations for the WT KDC2 were performed using Discovery Studio (DS) 2019. Virtual saturation mutagenesis was performed on residues within 5 Å of the binding sites of phenylpyruvate and phenylacetaldehyde in KDC2.

2.3. Construction of the mutant library and screening

The plasmid pET28(a)-KDC2 was used as a template, and mutant primers were used to construct a mutant library of KDC2 ([Supplementary Table 2](#)). The PCR products were transformed into *E. coli* DH5 α to obtain mutant transformants. Plasmids from all transformants were extracted and transformed into the expression host *E. coli* BL21 to establish the mutant library. Target colonies from the mutant library were screened using a 96-well plate. Single colonies were inoculated into 96-well plates for culture, and expression was induced by adding 0.05 mM IPTG at 16 °C. After 16–18 h, the plates were centrifuged at 5000 rpm, washed twice with PBS buffer, and resuspended in 0.2 mL cell lysate. The supernatant, containing crude enzyme, was obtained by centrifugation. The reaction mixture (0.2 mL) consisted of 70 mM potassium phosphate buffer (pH 7.0), 2 mM NADH, 0.2 mM thiamine diphosphate, 0.35 U yeast alcohol dehydrogenase (dissolved in 1 mM DTT), and 2 mM phenylpyruvate. The reaction was initiated by adding crude enzyme, incubated at 30 °C for 15 min, and the absorbance at 340 nm was measured.

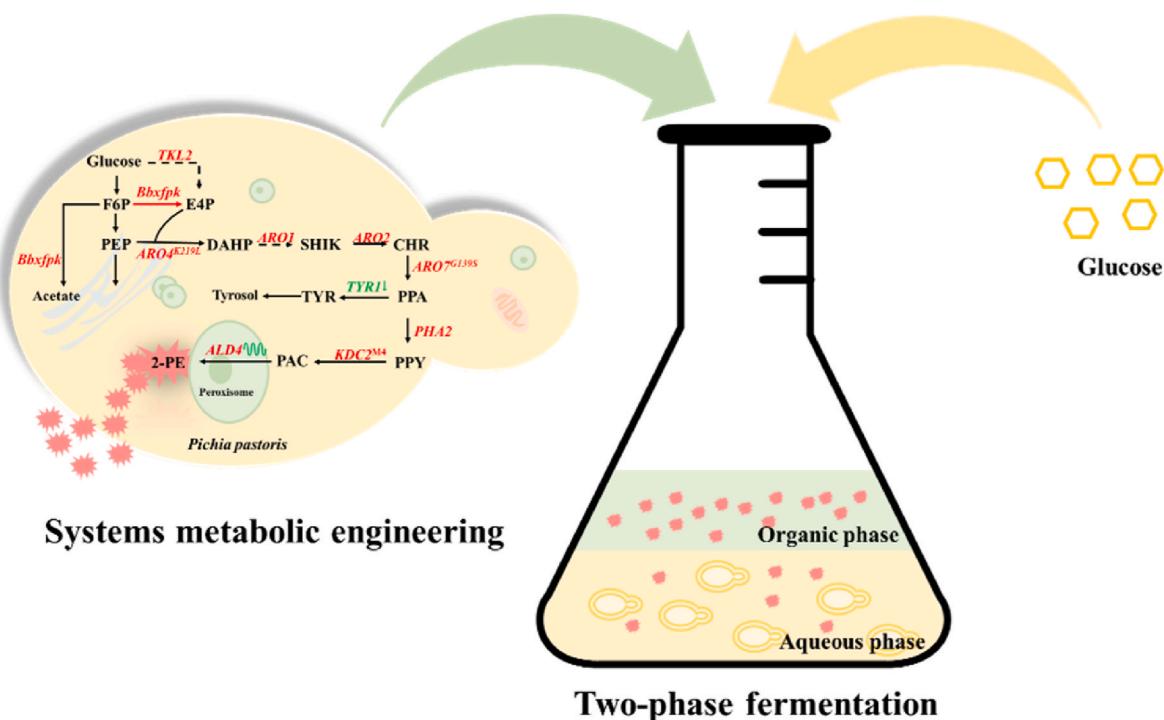


Fig. 1. Schematic diagram of multiple strategies for 2-PE overproduction in *P. pastoris*. The overexpressed gene is shown in red, the down-regulated gene is shown in green. *ALD4* gene was fused with a C-terminal ePTES1 tag. *Bbxfpk*, codon-optimized *Bbxfpk* gene; G6P, glucose-6-phosphate; F6P, Fructose-6-phosphate; PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate-7-phosphate; SHIK, shikimate; CHR, chorismate; PPA, prephenate; PPY, phenylpyruvate; TYR, tyrosine; PAC, phenylacetaldehyde; PYR, pyruvate; TKL2, transketolase; ARO4^{K219L}, feedback-resistant DAHP; ARO1, pentafunctional; ARO2, chorismate synthase; ARO7G139S, feedback-resistant chorismate mutase; TYR1, prephenate dehydrogenase; PHA2, prephenate dehydratase; KDC2, phenylpyruvate decarboxylase; ALD4, phenylacetaldehyde dehydrogenase.

2.4. Microbial fermentation of 2-PE

The engineered strains were inoculated into fresh YPD medium and cultured at 30 °C for 24 h. Subsequently, a 15 % seed culture was inoculated into the 250 mL flask containing 30 mL of fermentation medium and grown under the conditions of 30 °C and 250 rpm for 72 h. For single-phase fermentation, the process was completed after 48 h. For two-phase fermentation, 10 mL of organic solvent was added 8 h into the fermentation to facilitate in-situ extraction of 2-PE.

2.5. Analytical methods

OD₆₀₀ value was assayed by a spectrophotometer. After fermentation, the broth was centrifuged at 12000 rpm. The supernatant was the sample to be tested. To detect the concentrations of glucose, shikimic acid, acetic acid and ethanol, was conducted by using Aminex HPX-87H column (7.8 × 300 mm) at 55 °C with 5 mM sulfuric acid as the mobile phase. The injection volume and flow rate were set as 10 µL and 0.6 mL/min. To detect the concentrations of 2-PE, tyrosol, and L-phenylalanine were conducted by using a ZORBAX Eclipse Plus C18 column (4.6 × 250 mm, 5 µm) at 215 nm under 40 °C column temperature with 50 % methanol as the mobile phase. The injection volume and flow rate were set as 10 µL and 0.6 mL/min. The concentrations of phenylpyruvate was measured by a ZORBAX Eclipse Plus C18 column (4.6 × 250 mm, 5 µm) at 200 nm under 40 °C column temperature. The mobile phase uses two solvents, 20 mM KH₂PO₄ and 1 % acetonitrile, with a flow rate of 0.8 mL/min. Hassing et al. described a gradient elution procedure for phenylpyruvate [9].

2.6. Statistical analysis

Data are presented as mean ± standard error. Student's *t*-tests were

used to determine significant differences, with a *P* value < 0.05 considered statistically significant.

3. Results & discussion

3.1. Reinforcement of the key enzymes to increase 2-PE production

The shikimic acid pathway produces various aromatic compounds, including rosavin [16], resveratrol [17], and *p*-coumaric acid [25], and also supports the de novo synthesis of 2-phenylethanol (2-PE). As an aromatic amino acid derivative, 2-PE shares shikimic acid as a precursor with other aromatic amino acids, which serve as by-products in this process (Fig. 2). To quickly assess potential metabolic bottlenecks in the 2-PE biosynthetic pathway, 2 g/L shikimic acid (SA) were added as a precursor into flask fermentation of the strain GS115, and the change of 2-PE titer was measured. As shown in Fig. 3C, the 2-PE production of strain GS115 did not increase, suggesting catalytic limitations in the downstream synthesis of 2-PE [32,33]. Previous studies have shown that phenylpyruvate decarboxylase is a crucial rate-limiting enzyme in similar microbial pathways. To address this bottleneck, phenylpyruvate decarboxylase genes *KDC1* (XP_002492304.1, from *P. pastoris*), *KDC2* (XP_002493734.1, from *P. pastoris*), *YIARO10* (VBB87765.1, from *Y. lipolytica*), *PPDC1* (AQT03387.1, from *Rose rugosa*), and *PPDC2* (AQT03388.1, from *Rose rugosa*) from various microbial sources were screened by *S. cerevisiae* *ScARO10* (NP_010668.3) amino acid sequence alignment and overexpressed in strain GS115 [34]. All engineered strains overexpressing phenylpyruvate decarboxylase produced 0.34 g/L 2-PE from glucose, a 466 % increase compared to the control strain GS115 (Fig. 3B), confirming that enhanced expression of this enzyme significantly improves 2-PE production. This result was consistent with previous reports [13], highlighting its role as a key step in the pathway.

Notably, the expression of different phenylpyruvate decarboxylase

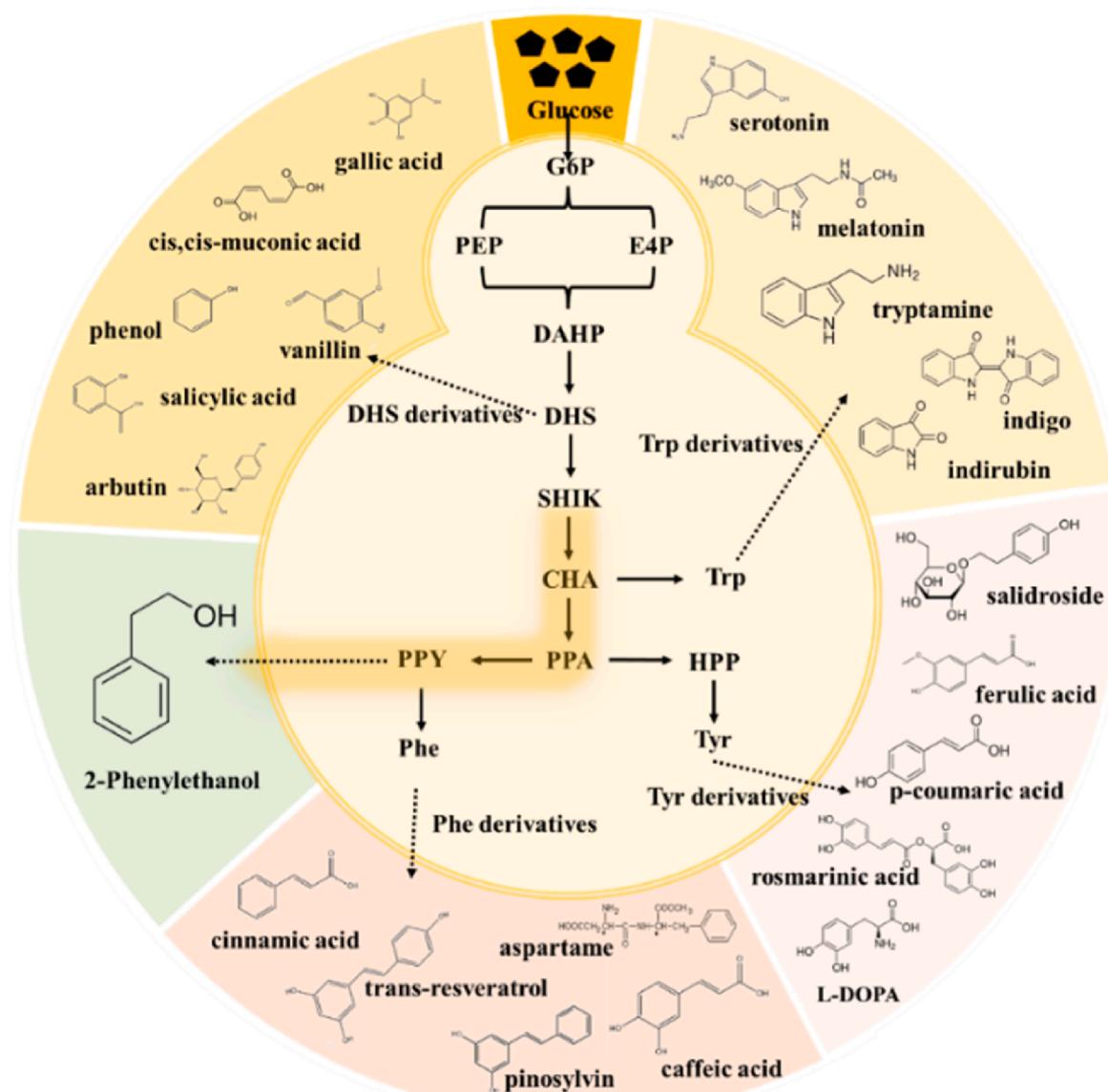


Fig. 2. The biosynthetic routes of aromatic compound.

genes in *P. pastoris* resulted in comparable 2-PE production levels, suggesting the existence of additional metabolic bottlenecks. To assess this problem, 2 g/L shikimic acid were supplemented as a precursor during fermentation, leading to a 52.9 % increase in the 2-PE titer of strain KDC2, from 0.34 g/L to 0.52 g/L (Fig. 3C). These results confirmed that an insufficient supply of shikimic acid still constrained 2-PE biosynthesis.

The 2-PE biosynthesis pathway can be divided into two modules for metabolic reprogramming to drive the increase of carbon flux in the shikimate pathway. Module 1 focuses on alleviating tyrosine feedback inhibition by co-expressing *ARO4^{fbr}* and *ARO7^{fbr}*. Module 2 involves increasing shikimic acid supply by overexpressing key genes, including *ARO1*, *ARO2*, and *PHA2*, to strengthen the pathway.

Previous studies have shown that *ARO4* and *ARO7* in *S. cerevisiae* are feedback-inhibited by tyrosine, restricting the flow of carbon sources into the shikimic acid pathway [23]. Protein engineering of *ARO4* and *ARO7* can result in the creation of feedback-resistant variants, *ARO4^{fbr}* and *ARO7^{fbr}*. However, due to the lack of relevant research alleviating tyrosine feedback inhibition in *P. pastoris*, potential mutation sites for alleviating feedback inhibition were identified by sequence alignment

(Supplementary Fig. 3), using the validated *ARO4^{K229L}* and *ARO7^{G141S}* from *S. cerevisiae* as references. Using the CRISPR genomic editing tool, *ARO4^{fbr}* and *ARO7^{fbr}* mutants were introduced into strain KDC2. However, the individual expression of *ARO4^{fbr}* and *ARO7^{fbr}* variants from *S. cerevisiae* and *P. pastoris* did not enhance the production of 2-PE (Fig. 3D), suggesting that gene regulation strategies may vary in effectiveness across yeast strains with different genetic backgrounds [35]. In *S. cerevisiae*, alleviated feedback inhibition mutants improved 2-PE or aromatic amino acid production [23,36], but similar strategies were less effective in *Kluyveromyces marxianus*; *KmARO7^{G141S}* did not enhance 2-PE synthesis, whereas its combined expression with *KmARO4^{K221L}* increased 2-PE production titers [37]. Next, we co-expressed *ARO4^{fbr}* and *PpARO7^{G139S}* to produce strains Ps1 (overexpressing *ScARO4^{K229L}* and *PpARO7^{G139S}*) and Pp2 (*PpARO4^{K219L}* and *PpARO7^{G139S}*). As shown in Fig. 3D, strains Ps1 and Pp2 produced 0.58 g/L and 0.62 g/L 2-PE, which were 70.6 % and 82.4 % higher than the control strain KDC2, respectively. The combined expression of feedback-insensitive mutants enhanced 2-PE synthesis, with endogenous *P. pastoris* genes showing a greater impact on 2-PE production than non-native homologs from *S. cerevisiae*.

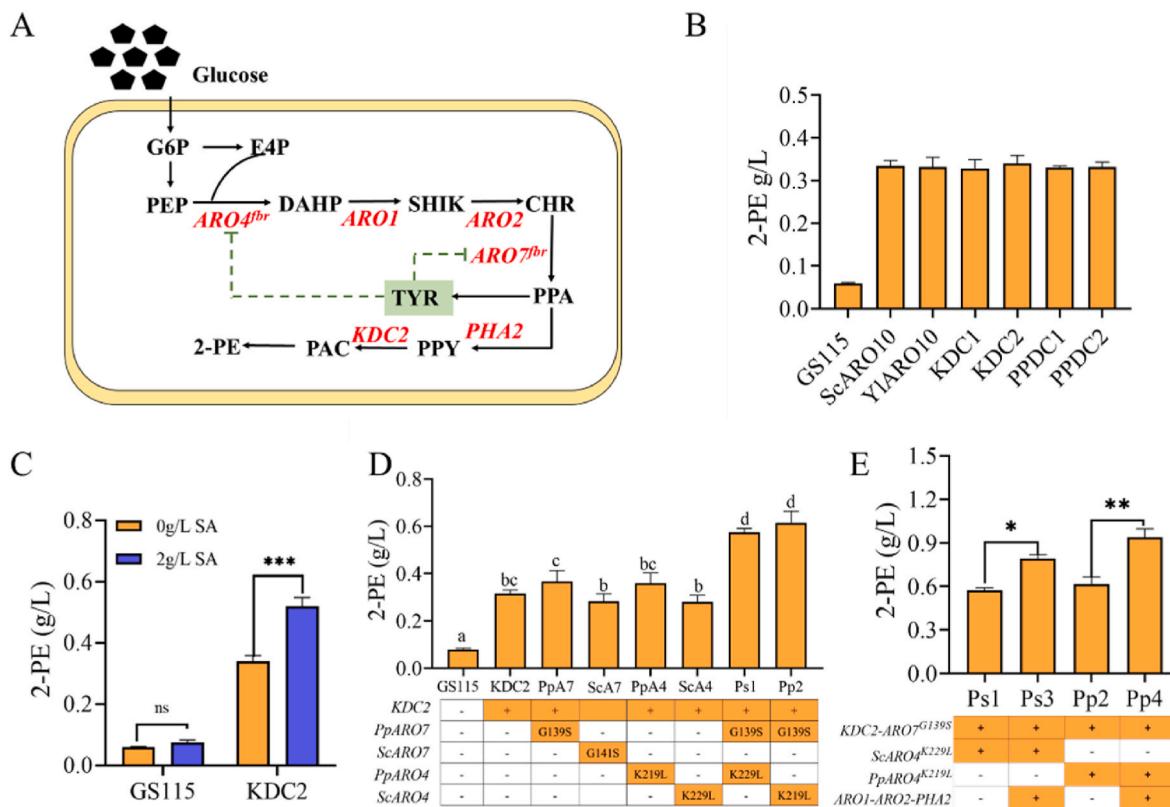


Fig. 3. The production of 2-PE was increased by strengthening shikimic acid pathway. (A) Schematic of the 2-PE synthesis pathway in *P. pastoris*. The genes shown in red are overexpressed. (B) 2-PE production by overexpressing phenylpyruvate decarboxylase genes. (C) 2-PE production by adding precursor shikimic acid. (D–E) Optimizing the shikimic acid pathway increased 2-PE production. Data represent the mean \pm S.D. of three biological replicates. Statistical analysis was performed by using one-way ANOVA (* p < 0.05, ** p < 0.01, *** p < 0.001).

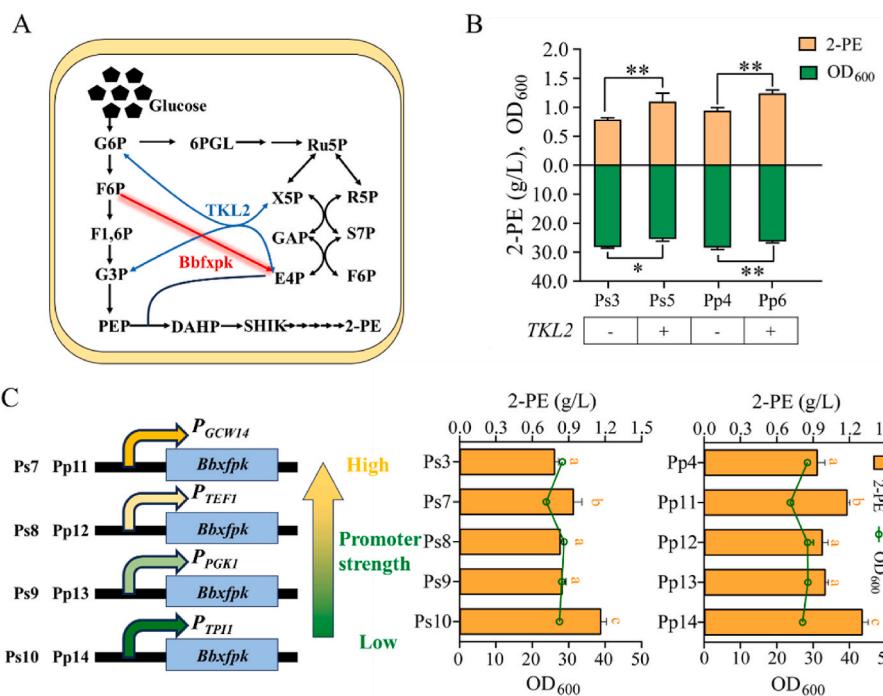


Fig. 4. Regulating the accumulation of E4P and PEP for 2-PE production. (A) The schematic diagram of regulating the supply of E4P and PEP by reforming the pathway. (B) 2-PE production by overexpressing *TKL2* gene. (C) Different strength promoters drive *Bbfpxk* gene to increase E4P concentration to produce 2-PE. Data represent the mean \pm S.D. of three biological replicates. Statistical analysis was performed by using one-way ANOVA (* p < 0.05, ** p < 0.01).

To further increase 2-PE titers, we tested modification strategies originally developed for aromatic derivative synthesis and confirmed their effectiveness for 2-PE biosynthesis [38,39]. By simultaneously overexpressing the penta-functional aromatic protein PpARO1, the bi-functional chorismate synthase PpARO2, and prephenate dehydratase PpPHB2 in strains Ps1 and Pp2, 2-PE production increased to 0.79 g/L in strain Ps3 and 0.94 g/L in strain Pp4 (Fig. 3E). Collectively, our findings demonstrate that enhancing metabolic flux through the shikimic acid pathway significantly increases 2-PE production.

3.2. Enhancing the supply of precursor E4P

Phosphoenolpyruvate (PEP, derived from the glycolytic pathway (EMP)) and erythrose-4-phosphate (E4P, derived from the pentose phosphate pathway (PPP)) are key precursors of the shikimic acid pathway. Since most glucose-6-phosphate is funneled into glycolysis to maintain the metabolic activity of the cell, the metabolic flux through the PPP remains relatively weak. Flux analysis revealed that the available carbon flux toward E4P is at least one order of magnitude lower than toward PEP in yeast [40], a limitation previously shown to restrict aromatic amino acids biosynthesis. This indicates that the enhancement of E4P may be a promising strategy in increasing the flux of the shikimate pathway. To redistribute central carbon metabolism and enhance the shikimic acid pathway (Fig. 4A), *PpTKL2* was overexpressed in strains Ps3 and Pp4, resulting in strains Ps5 and Pp6. This increased E4P flux and elevated 2-PE titers to 1.10 g/L and 1.24 g/L, representing 1.39-fold and 1.32-fold improvements over the control strains (Fig. 4B). This confirmed that addressing E4P limitations effectively enhances the shikimic acid pathway in *P. pastoris*. However, biomass decreased as 2-PE production increased, likely due to the metabolic burden of over-expressing target enzymes and the antimicrobial effects of high 2-PE concentrations [41].

To further augment E4P synthesis and aromatic derivative production, we introduced transketolase to connect the EMP and PPP pathways. *Bbxfpk* from *B. breve* cleaves fructose-6-phosphate into E4P and acetyl phosphate, redirecting EMP flux to PPP while reducing CO₂ release and maximizing carbon flux. Previous studies demonstrated that *Bbxfpk* increases intracellular E4P 5.4-fold in *S. cerevisiae* [42]. We codon-optimized *Bbxfpk* for yeast expression and introduced it into Ps3 and Pp4 strains under the control of four promoters with different transcriptional intensities (PGCW14, PTEF1, PPGK1, and PTPI1), generating strains Ps7 to Ps10 and Pp11 to Pp14 (Fig. 4C).

Strains expressing *Bbxfpk* under the PTPI1 promoter exhibited the highest 2-PE production, with strains Ps10 and Pp14 producing titers up to 1.17 g/L and 1.31 g/L, respectively 1.48-fold and 1.39-fold higher than strains Ps3 and Pp4 (Fig. 4C). In contrast, strains with the strongest promoter (GCW14) showed improved 2-PE production but impaired growth, likely due to excessive metabolic flux from glycolysis to PPP, depleting PEP. Moderate-strength promoters balanced growth and production, emphasizing the importance of precise flux regulation for E4P synthesis. These findings highlight that *Bbxfpk* expression effectively directs carbon flux toward E4P, facilitating 2-PE production. This approach aligns with studies showing that limited E4P flux prevents carbon from entering the shikimate pathway in many microorganisms, and introducing phosphoketolase alleviates this constraint, enhancing downstream product biosynthesis [43,44]. To further increase 2-PE titers, we next focused on its downstream biosynthetic pathway.

3.3. Enhancing KDC activity breaks the downstream flow limit

Overexpression of phenylpyruvate decarboxylase KDC2 has been shown to increase 2-PE production, with no significant differences observed among sources, suggesting similar catalytic activities across variants. However, the catalytic efficiency of *S. cerevisiae* phenylpyruvate decarboxylase (ARO10) has been found insufficient, requiring multiple gene copies to enhance product titers [19]. These findings

highlight the potential of improving enzyme activity as an effective strategy for boosting 2-PE production.

To optimize KDC2, we employed the CAST/ISM (Combinatorial Active-Site Saturation Test/Iterative Saturation Mutagenesis) strategy, which efficiently reduces the size of mutant libraries and minimizes screening effort [45–47]. Using a homology model of KDC2 bound to the substrate phenylpyruvate and product phenylacetaldehyde, 39 residues within 5 Å of the binding pocket were identified (Supplementary Fig. 4). This figure illustrates the spatial positioning of the substrate and product relative to the enzyme active site, providing insights into key residues that influence catalytic activity. Notably, 19 residues were associated with phenylpyruvate binding, while 20 residues were linked to phenylacetaldehyde binding. The large number of potential residues posed a challenge for constructing and screening mutation libraries. To address this, computational evaluation was employed to identify target residues with increased likelihood of improving phenylpyruvate binding and catalytic efficiency.

Optimization efforts were focused on enhancing enzyme activity by promoting catalytically favorable binding of phenylpyruvate while avoiding unfavorable binding of phenylacetaldehyde. This dual approach ensures that the enzyme operates efficiently, thereby maximizing its contribution to 2-PE production. First, Discovery Studio was used to calculate the virtual saturation mutation of the above 39 residues, and the effect of each residue on the theoretical binding energy of phenylpyruvate-KDC2 and phenylacetaldehyde-KDC2 was evaluated by mutating each residue to 20 possible amino acids. The corresponding 780 (39 × 20) mutation binding energies ($\Delta\Delta G_{\text{mut}}$) were calculated, ranging from -1.35 to 4.46 kcal/mol (Fig. 5A and B). Targeting functional residues with high variability instead of strictly conserved residues increases the likelihood of enhancing catalytic properties while maintaining catalytic activity. The average mutation binding energy of each residue replacement was calculated and compared with WT-KDC2 to determine the residues that may increase the affinity of the substrate ligand. The 14 residues were used as CAST engineering sites to improve the binding of phenylpyruvate-KDC2 and increase the ability of the strain to synthesize 2-PE (Fig. 5C).

Seven specific sites (L409, D414, V470, T497, F562, E564, and N569) were identified through the screening of a 14-amino-acid single-site saturation mutagenesis library. Mutants at these sites exhibited catalytic activities ranging from 1.07- to 1.71-fold higher than the WT KDC2 (Fig. 5D). These 12 mutants with enhanced catalytic activity corresponded to the calculated $\Delta\Delta G_{\text{mut}}$ values, confirming the effectiveness of the screening strategy. The mutant V470K, which demonstrated the highest catalytic activity, was used as the template for iterative mutagenesis cycles to further enhance 2-PE synthesis. The remaining six sites (N569F, D414H, L409P, T497M, F562Q, and E563K) were subjected to iterative rounds in this order, with the best-performing mutant from each round serving as the template for the next iteration. The final mutant, M4 (V470K/N569F/D414H/L409P/T497M), achieved a 3.25-fold increase in activity compared to WT, with mutants from the iterative process exhibiting activities between 1.58- and 3.16-fold higher than WT (Fig. 5E). The optimized mutant M4 was integrated into strain Pp14, resulting in a recombinant strain that produced 1.69 g/L of 2-PE, a 1.21-fold improvement over the strain expressing WT KDC2 (Fig. 5F). This demonstrates that enzyme catalytic activity can be significantly improved through rational design and directed evolution guided by structural insights [48]. This approach aligns with findings by Deng et al., who achieved an 11.32-fold increase in N-sulfotransferase activity after eight rounds of iterative mutation using computer-aided design [49]. These results highlight the success of rationally designed protein engineering strategies in enhancing enzyme activity and compound production, offering a more targeted and efficient alternative to traditional methods.

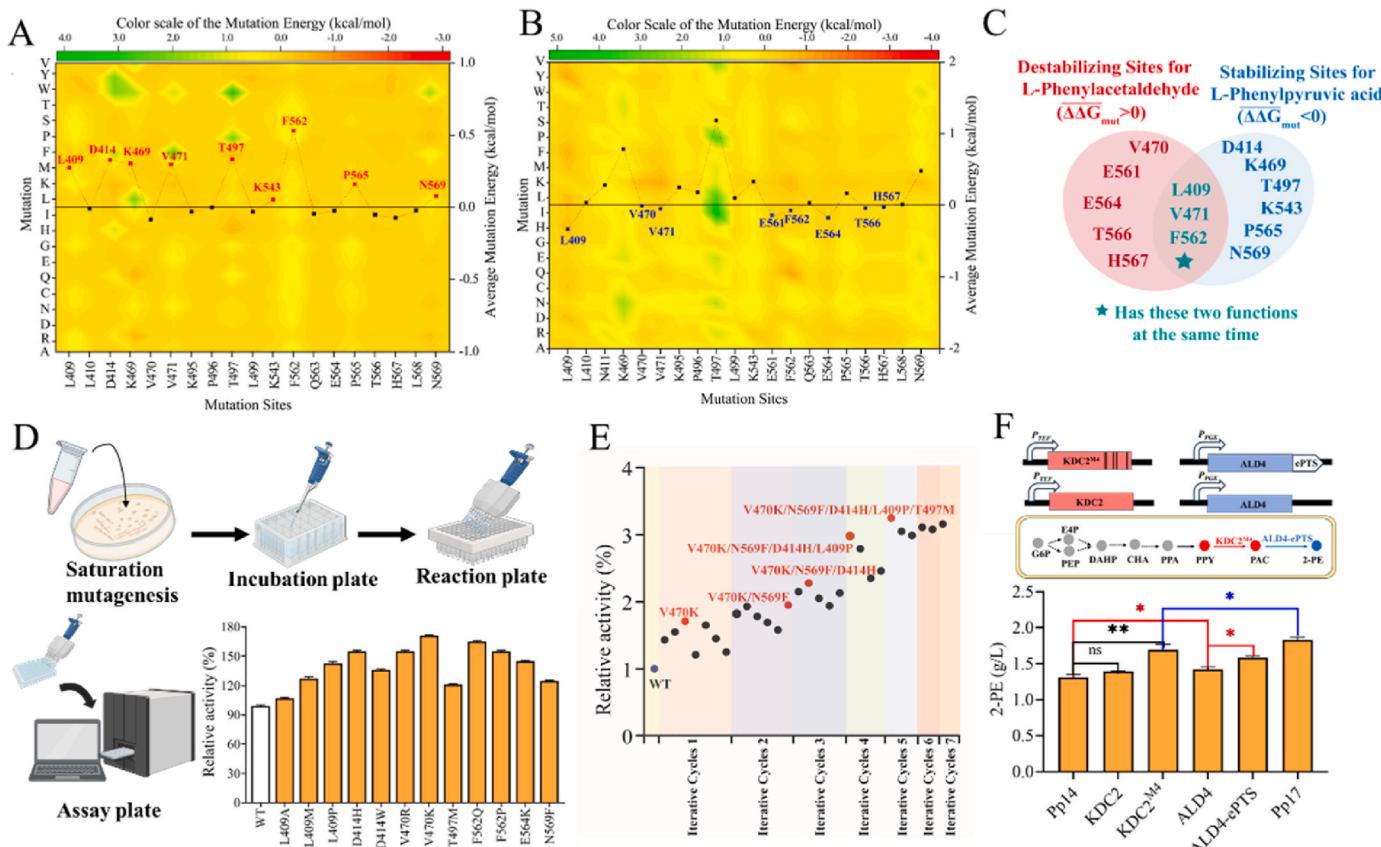


Fig. 5. Directed evolution of phenylpyruvate decarboxylase KDC2. (A–B) Virtual mutant of potential hot spots using Discovery Studio and the average mutation energy of the candidate residues. (C) Variable destabilizing residues are selected. (D) The relative activity of KDC2 variants screened based on random mutagenesis. (E) Evolutionary pathways from WT to the best performing mutant. (F) Reinforcement the 2-PE downstream pathway. Data represent the mean \pm S.D. of three biological replicates. Statistical analysis was performed by using one-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4. Re-location ALD4 into the peroxisome of *P. pastoris* to enhance 2-PE synthesis

Peroxisomes are single-membrane-bound organelles that participate in fatty acid β -oxidation, providing NADPH to other organelles and creating a specialized microenvironment for biochemical reactions. Enzymes of the mevalonate pathway have been successfully localized to peroxisomes, enabling the production of terpene compounds [50]. Studies have also shown that peroxisomes, as subcellular units in *S. cerevisiae*, exhibit detoxification capabilities [51]. Therefore, engineering peroxisomes presents a promising strategy for the efficient production of 2-PE in *P. pastoris*.

Our previous study demonstrated that overexpressing ALD4 in *P. pastoris* increased the 2-PE production titer by 56.9 % [28]. In this study, the introduction of ALD4 successfully increased 2-PE production (Fig. 5F), consistent with our previous finding. This effect was further enhanced by fusing an optimized peroxisome-targeting signal (ePTS1, LGRGRRSKL) [52,53] to the C-terminal of ALD4, leading to a 1.21-fold increase in 2-PE production compared to the control strain. Finally, the production of 2-PE reached 1.83 g/L by the combination of enzyme molecular modification and enzyme molecular repositioning (Fig. 5F).

3.5. Reducing the accumulation of by-products increased the biosynthesis of 2-PE

During the fermentation process, it was observed that, in addition to the accumulation of the target product 2-PE, 0.86 g/L of tyrosol also accumulated (Fig. 6A). Tyrosol is derived from tyrosine, which is essential for yeast growth. Knocking out TYR1 eliminates tyrosol

accumulation but renders the strain tyrosine auxotrophic, an undesirable characteristic. Studies have shown that adding tyrosine to the medium rescues strain growth; however, excessive tyrosine supplementation reduces 2-PE production [9]. Therefore, replacing the natural TYR1 promoter with a weak promoter to fine-tune TYR1 expression could potentially increase 2-PE production while balancing product formation with cell growth. For this purpose, the 2-PE responsive promoters with weak expression intensity were screened. Based on transcriptome data from GS115 under different 2-PE stress conditions, 15 potential dynamic regulation promoters were selected. Their expression intensities were weaker than that of TYR1, and repressed by increase in 2-PE concentration (Table S3). The corresponding promoters were used to replace the natural TYR1 promoter in strain Pp17. Among the constructs, only Pp17-10p showed a 46.5 % reduction in tyrosol formation and a 15.8 % increase in 2-PE production, it produced up to 2.12 g/L 2-PE by utilizing sugar in the medium (Fig. 6A).

In a previous experiment, introducing the *Bbxfpk* gene promoted E4P production and generated acetyl phosphate, increasing acetic acid production and causing undesirable side effects (Fig. 6B). When the medium contained 2 g/L 2-PE, strain growth was significantly inhibited after 24 h of cultivation. When 6 g/L acetic acid and 2 g/L 2-PE coexisted in the medium, the physiological activity of the strain was further impaired. As expected, acetic acid and 2-PE exerted a synergistic toxic effect, with acetic acid further reducing the growth rate of the strain by 82.5 % (Fig. 6C).

To mitigate acetic acid accumulation in the fermentation broth, calcium carbonate was added as a neutralizer to maintain a stable pH. This strategy might enhance cell growth and product synthesis. After shaking flask fermentation, acetic acid production in strain Pp17-10p

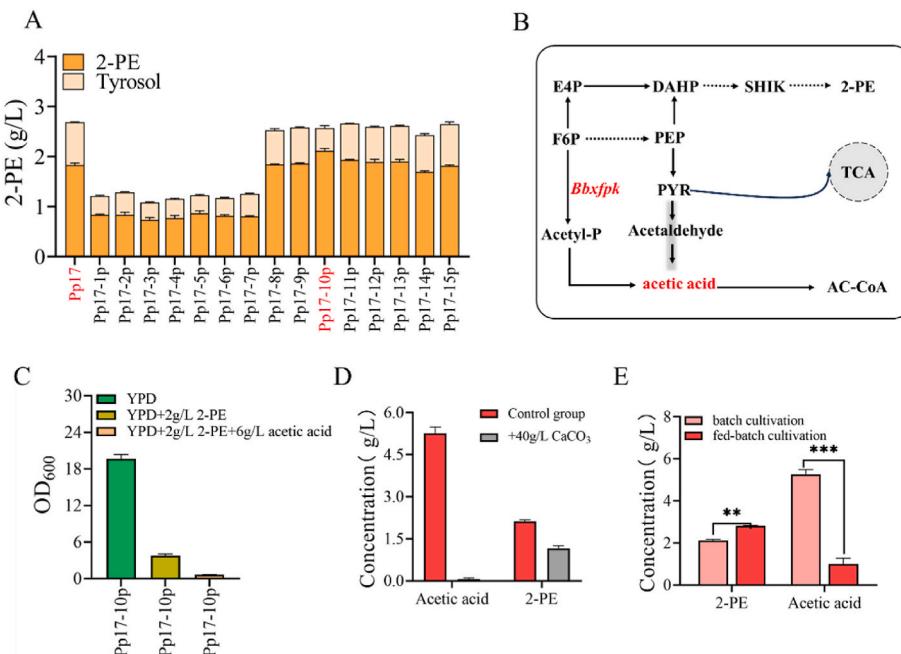


Fig. 6. Modulation of competitive pathway expression. (A) The screening of promoters resulted in a reduction of by-product tyrosol accumulation while enhancing 2-PE production. (B) The schematic of acetic acid metabolic pathway after the introduction of *Bbxfpk*. (C) Effects of 2-PE and acetic acid on the growth of *P. pastoris*. (D) Effect of adding calcium carbonate on fermentation of *P. pastoris*. (E) The synthesis of 2-PE and acetic acid by Pp17-10p strain under two culture strategies. The initial sugar concentrations of batch culture and fed-batch culture were 70 g/L and 20 g/L, respectively. Fed-batch culture was supplemented with 5 g/L glucose every 12 h. Data represent the mean \pm S.D. of three biological replicates. Statistical analysis was performed by using one-way ANOVA ($^{**}p < 0.01$, $^{***}p < 0.001$).

decreased significantly from 5.42 g/L to 29 mg/L. However, 2-PE production also decreased by 45.7 % (Fig. 6D). While acetic acid accumulation was alleviated, the production of 2-PE was greatly reduced. This outcome differs from results obtained with neutralizers used in the biological production of organic acids [54]. This may be because 2-PE fermentation is aerobic process in which the dissolved oxygen level directly affects the product synthesis. Neutralizing the acidity of fermentation broth with calcium carbonate will produce CO₂. Excessive CO₂ accumulation can impair the efficiency of oxygen transfer in the fermentation broth, reducing the overall availability of dissolved oxygen.

Excess carbon sources in the medium can easily lead to acetic acid formation, which inhibits strain growth [55]. To address this, fermentation optimization was carried out to reduce acetic acid accumulation. The initial carbon source concentration in the medium was maintained at 20 g/L, and glucose was restricted during fermentation. This strategy mitigated substrate inhibition from high nutrient concentrations and prevented depletion of limiting nutrients, facilitating a balance between cell growth and product formation. After implementing controlled fermentation, acetic acid accumulation in strain Pp17-10p was effectively reduced, suggesting that regulating sugar concentrations mitigated overflow metabolism. Ultimately, fermentation optimization enabled strain Pp17-10p to achieve a 2-PE production level of 2.81 g/L (Fig. 6E).

3.6. Production of 2-PE in two-phase culture

Our previous study demonstrated that medium containing 2 g/L 2-PE significantly inhibited yeast cell growth [28]. Many studies have reported that the cell morphology of the microbe is changed in response to solvent stress [56,57]. The cell morphology of *S. cerevisiae* under 2-PE stress was irregular, and elongated, with a rough surface [58]. The SEM observations by Zhan et al. revealed that the cell morphology of *B. licheniformis* changed when exposed to 2-PE, exhibiting elongated cells. Furthermore, alterations in cell wall and membrane composition

were also documented, suggesting comparable structural disruptions triggered by these stressors [26]. The engineered strain Pp17-10p cells presented uneven shape and wrinkled surface. Likewise, these morphological changes might reflect that the cells caused cell damage under the toxicity of 2-PE (Supplementary Fig. 5). In-situ extraction technology is considered an effective strategy to alleviate the cytotoxicity of metabolites [59]. In this study, the toxicity of 2-PE was mitigated by employing a two-phase culture system, where 2-PE was extracted in situ from the aqueous phase using organic solvents (organic phase). Several common organic solvents were tested for their ability to extract 2-PE, and polypropylene glycol 1500 (PPG1500) showed the highest efficiency, extracting 91.3 % of 2-PE from the aqueous phase into the organic phase (Fig. 7B). As a result, cells in the aqueous phase experienced minimal toxicity from 2-PE. Although oleic acid has been previously used as an extractant in L-phenylalanine bioconversion, it showed suboptimal performance in the current study, leaving more than 2 g/L of 2-PE in the aqueous phase while achieving 12.6 g/L in the organic phase [60]. Previous studies have consistently demonstrated high extraction efficiency using polypropylene glycol [61,62].

To date, all flask cultures of *P. pastoris* have been grown under single-phase conditions. Compared to single-phase fermentation, two-phase fermentation results in emulsified environments [63], which may interfere with oxygen transfer to the cells. The lack of dissolved oxygen can have a negative impact on the fermentation process of microbial. Studies have shown that the overexpression of *Vitreoscilla* hemoglobin (*vgb*) improves cellular oxygen utilization, enhancing oxygen transport and promoting cell growth and metabolite synthesis [64,65]. Therefore, the recombinant strain Pp18 was obtained by overexpressing *Vitreoscilla* hemoglobin gene *vgb* to improve oxygen utilization. Growth curve results showed that *vgb* overexpression significantly enhanced cell growth (Supplementary Fig. 6), consistent with findings in other reports [66]. PPG1500 was added at 8 h of fermentation for two-phase culture. The content of 2-PE in the organic phase reached 13.4 g/L, indicating successful extraction of 2-PE from the aqueous phase (Fig. 7B). The strain Pp18 produced 7.10 g/L of 2-PE with PPG1500 addition, which was 153

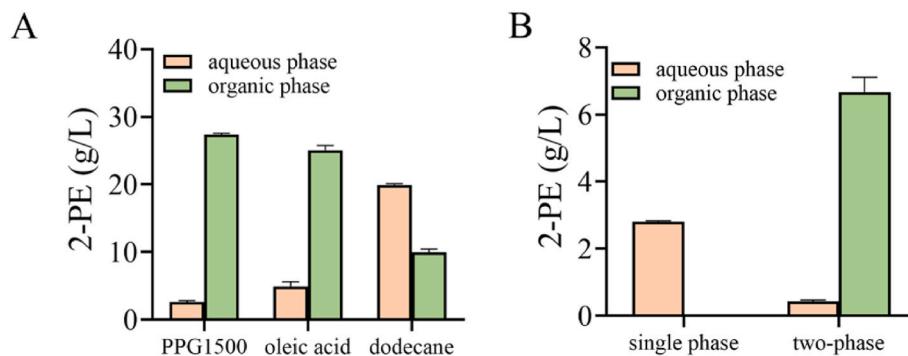


Fig. 7. The diagram of 2-PE fed-batch two-phase fermentation. (A) The ability of different organic solvents to extract 2-PE. (B) The effect of two-phase extraction technology on the synthesis of 2-PE by strain Pp18.

% higher than that without PPG1500 (no extraction). These results demonstrate that two-phase extractive fermentation could effectively alleviate 2-PE toxicity *in situ*, significantly enhancing its production. To date, the *vgb* gene has been successfully used to promote metabolite synthesis in various microorganisms, including *Bacillus thuringiensis* [67], *P. pastoris* [68], and *Bacillus subtilis* [69]. For example, *vgb* expression in *Aeromonas hydrophila* increases polyhydroxyalkanoate synthesis [70], indicating that *Vitreoscilla* hemoglobin improves intracellular oxygen transfer efficiency and promotes growth under oxygen-limited conditions. Finally, the *in-situ* removal of 2-PE using PPG1500 reduced the aromatic compound toxicity to cells, achieving in increased 2-PE concentration and improved fermentation efficiency.

4. Conclusion

In this study, the key steps of the shikimate pathway were systematically analyzed. It was found that the endogenous mutants ARO7 and ARO4, which alleviate feedback inhibition, are more effective for producing 2-PE in *P. pastoris* than the known mutants from *S. cerevisiae*. Additionally, balancing the metabolic flux between the precursors PEP and E4P was shown to drive carbon flux through the shikimate pathway effectively. Phenylpyruvate decarboxylase was identified as playing a crucial role in 2-PE synthesis. The synthetic capacity of the strain was significantly enhanced through rational design and enzyme activity screening. During fermentation, byproducts such as fusel alcohols and acetic acid were detected. To balance product yield with cellular growth, a strategy combining promoter engineering and competitive branch pathway disruption blocking was adopted to reduce byproduct formation during fermentation. Overall, this study improved 2-PE production in yeast strains through systems metabolic engineering approaches, including increasing precursor supply, alleviating feedback inhibition, regulating enzyme expression, and minimizing byproduct formation.

To alleviate the cellular toxicity associated with high 2-PE concentrations, two-phase fermentation was implemented, achieving a 2-PE titer of 7.10 g/L—the highest reported for microbial de novo synthesis of 2-PE. These findings demonstrate that fine-tuning key metabolic nodes through metabolic and enzymatic engineering is effective for enhancing product synthesis. Despite our successes in enhancing 2-PE production, the inherent toxicity of the product to microbial cells continues to pose a significant challenge for industrial-scale applications. Future research should focus on overcoming 2-PE toxicity to enable industrial-scale production by engineering microbial strains with enhanced tolerance through stress-response pathway modifications, membrane engineering, and adaptive laboratory evolution. Dynamic regulation systems, such as feedback-controlled promoters and efflux pumps, could balance production and mitigate intracellular toxicity. Finally, process optimization, such as innovative bioreactor designs and continuous fermentation strategies, can further improve productivity and reduce toxicity effects, paving the way for cost-effective, large-scale

2-PE biosynthesis.

CRediT authorship contribution statement

Lijing Sun: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Conceptualization. **Suyun Liu:** Writing – original draft, Visualization, Validation. **Renjie Sun:** Writing – original draft, Visualization, Validation. **Jinshan Li:** Visualization, Methodology. **Aiqun Yu:** Writing – original draft, Investigation, Conceptualization. **Adison Wong:** Writing – original draft, Visualization, Conceptualization. **Liangcai Lin:** Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition. **Cuiying Zhang:** Writing – original draft, Investigation, Funding acquisition.

Ethics approval and consent to participate

This manuscript does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material

All relevant data generated or analyzed during this study were included in this published article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.synbio.2025.05.004>.

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