Bioconversion of L-phenylalanine to 2-phenylethanol by the novel stress-tolerant yeast Candida glycerinogenes WL2002-5

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ABSTRACT
2-Phenylethanol (2-PE) is a high value aromatic alcohol with a rose-like odor that is utilized in the cosmetics and other industries. Although the chemical routes of 2-PE production have been altered by some microbial transformation processes, the poor tolerance to organic solvents of these microorganisms has limited the 2-PE yield. In this study, the stress-tolerant yeast Candida glycerinogenes WL2002-5 showed a 2-PE tolerance to 4 g/l, which is the highest reported to date. Moreover, the 2-PE titer in a batch fermentation from L-phenylalanine reached 5 g/l, which is the highest level achieved by fermentation without in situ product recovery. These results suggest C. glycerinogenes WL2002-5 is a robust strain for the bioproduction of 2-PE with potential for commercial exploitation.

Introduction
2-Phenylethanol (2-PE) is an important volatile aromatic alcohol with a rose-like odor. It is wildly used in the pharmaceutical industry as a bactericide and also in the food, perfumery, and cosmetics industries to improve character. At present, most commercial 2-PE is synthesized chemically via Friedel-Crafts acylation. However, this produces toxic byproducts that are harmful to health, which is an obvious issue for use in the food and cosmetics industries, and only natural 2-PE extracted from the essential oils of flowers or plants is permitted. However, the scarcity of flowers and plants and the low efficiency of extraction limit the titer of 2-PE produced from natural sources. The price of naturally sourced 2-PE is therefore 1000 US$/kg, compared with only 5 US$/kg for chemical synthesized 2-PE. Much attention has therefore been paid to 2-PE biosynthesis.

Various microorganisms including bacteria, filamentous fungi and yeast possess the ability to synthesize 2-PE. Among the reported 2-PE producers, yeasts such as Saccharomyces cerevisiae, Kluyveromyces marxianus, and Yarrowia lipolytica are the most efficient strains. The 2-PE titer of K. marxianus was improved from 0.39 to 0.5 g/l by culture optimization, while wild-type Y. lipolyticaNCYC3825 produced 1.98 g/l 2-PE in non-optimized batch culture. To further increase 2-PE production, genetic strategies have been applied. Overexpressions of ARO8 and ARO10 in S. cerevisiae SPO810 increased the 2-PE titer from 0.85 to 1.1 g/l. Deletion of ALD3 in the recombinant S. cerevisiae W303-1B improved 2-PE production from 0.11 to 4.8 g/l. Although much attention has been paid to the biosynthesis of 2-PE, the titer remains disappointingly low. Unfortunately, 2-PE is toxic to most microorganisms (log P = 1.36), which limits its production. To reduce the toxicity, in situ product recovery (ISPR) was applied to 2-PE production in K. marxianus CBS 600, and the titer was improved to 26.5 g/l. However, despite the increased yield, extraction of 2-PE from the organic phase proved uneconomical. Bioproduction of 2-PE by a robust and stress-tolerant organism would be a more economically viable and practical approach, and the stress-tolerant yeast S. cerevisiae Ye9-612 has been tested for this purpose.

Candida glycerinogenes WL2002-5 is another stress-tolerant yeast that can tolerate NaCl to 15 %
(w/v) and glucose to 55 % (w/v).\textsuperscript{20,21} As a ‘generally recognized as safe’ industrial strain, \textit{C. glycerinogenes} WL2002-5 has been used commercially for the production of glycerol from glucose for over 20 y in China.\textsuperscript{22} In the present study, the tolerance of \textit{C. glycerinogenes} WL2002-5 to 2-PE was investigated, and the potential for this organism for 2-PE production was assessed.

Materials and methods

Strains and culture conditions

\textit{C. glycerinogenes} WL2002-5 and \textit{S. cerevisiae} W303-1A were stored in our lab and routinely cultivated in yeast extract peptone dextrose (YPD) medium at 30\degree C. For 2-PE synthesis, overnight cultivated \textit{C. glycerinogenes} WL2002-5 was incubated in 30 ml defined medium (50 g/l glucose, 5 g/l L-Phe, 5 g/l KH$_2$PO$_4$, 0.5 g/l MgSO$_4$·7H$_2$O, and 0.17 g/l yeast nitrogen base (YNB) without amino acids) in a 250 ml Erlenmeyer flask at 30\degree C for 50 h.

Analytical methods

The optical density (OD) of cells was determined using a spectrophotometer at 600 nm. For determination of the cell dry weight, fermentation broth was centrifuged at 12,000 \(\times\) g for 5 min in weighed predried tubes. Cell pellets were washed twice and dried at 105\degree C to constant weight. The weight difference is equivalent to the cell dry weight per ml. Identification of 2-PE was performed by gas chromatography-mass spectrometry (GC-MS). The culture supernatant was collected by centrifugation at 10,000 \(\times\) g for 10 min. 2-PE was extracted from the supernatant with pentane:cyclohexane (2:1, v:v) and dried with Na$_2$SO$_4$. The extract was analyzed by GC-MS (Broker SCION SQ, USA) using a pulsed pressure injection of 0.2 \(\mu\)l onto a DB-wax column (30 m \(\times\) 0.25 mm, 0.25 \(\mu\)m). The helium flow rate was 0.8 ml/min, and the inlet port temperature was 250\degree C. The oven program started at 80\degree C for 1 min, followed by an increase of 10\degree C/min to 230\degree C, and retention at 230\degree C for 5 min. Mass detector parameters were as follows: transfer line = 250\degree C, scan mode range = 33-400 m/z, electron impact ionization = 70 eV.

The 2-PE concentration was determined using 1200 series high-performance liquid chromatography (HPLC; Agilent, USA) equipped with a RP-C18 column (250 mm \(\times\) 4.6 mm, 10 \(\mu\)m; Ailite, China). The mobile phase was methanol:water (50:50, v/v), the flow rate was 0.7 ml/min, the temperature was 30\degree C, and the detection wavelength was 260 nm. The glucose concentration was measured enzymatically using a SBA-40E Biosensor.

Batch fermentation

Fresh cultures of \textit{C. glycerinogenes} WL2002-5 were inoculated into in a 5 l bioreactor (Boxing 5BG, China) containing 3 l of medium (90 g/l glucose, 5 g/l KH$_2$PO$_4$, 4 g/l yeast extract, 0.5 g/l MgSO$_4$·7H$_2$O, 7 g/l L-phenylalanine (L-Phe)). The bioreactor was aerated at 1.5 vvm and stirred at 500 rpm, 30\degree C for 50 h.

Results and discussion

2-PE tolerance of \textit{C. glycerinogenes} WL2002-5

To study the tolerance of \textit{C. glycerinogenes} WL2002-5 toward 2-PE, \textit{C. glycerinogenes} WL2002-5 and \textit{S. cerevisiae} W303-1A were cultivated in YPD agar containing various concentrations of 2-PE. As shown in Fig. 1, the cell growth of \textit{C. glycerinogenes} WL2002-5 was not impacted when the 2-PE concentration was lower than 2 g/l, but it gradually decreased at higher 2-PE concentrations. In comparison, the cell growth

![Figure 1](image-url)
of *S. cerevisiae* W303-1A was almost completely repressed at a 2-PE concentration of 2 g/l. Of note, *C. glycerinogenes* WL2002-5 colonies were still observed at 4 g/l 2-PE.

To quantify the difference in tolerance between the organisms, they were cultivated in liquid YPD medium containing 2-PE. As shown in Fig. 2, the cell growth of both strains was reduced slightly at low concentrations of 2-PE. The biomass of *C. glycerinogenes* WL2002-5 was reduced by 40% compared with a reduction of 75% for *S. cerevisiae* W303-1A at a 2-PE concentration of 3 g/l. The growth of *S. cerevisiae* W303-1A was completely repressed at a 2-PE concentration of 3.5 and 4.0 g/l, whereas the biomass of *C. glycerinogenes* WL2002-5 was 34% and 12% that of the control at these concentrations. These results indicated that *C. glycerinogenes* WL2002-5 was more tolerant to 2-PE, and was also higher than that previously reported for *S. cerevisiae* GIV2009, which did not grow at a 2-PE concentration of 4 g/l [23]. As far as we know, *C. glycerinogenes* WL2002-5 is the most tolerant to 2-PE of any reported strain. To our surprise, the concentration of 2-PE in the broth remained stable at all time points (data not shown), even after 80 h, suggesting there might not be a 2-PE degradation pathway in *C. glycerinogenes* WL2002-5.

The organic solvent tolerance of yeast is reported to be due to changes in membrane fatty acids and the cell wall and some other factors, but the exact mechanisms are complex and remain unknown. Some global regulatory mechanisms appear to be responsible for organic solvent tolerance in yeast, such as the high-osmolarity glycerol (HOG) pathway. The presence of glycerol in the medium also contributes to organic solvent tolerance in yeast. We therefore speculate that a high glycerol synthesis capability and efficient HOG pathway in *C. glycerinogenes* WL2002-5 contribute to its excellent 2-PE tolerance.

### Biosynthesis of 2-PE by *C. glycerinogenes* WL2002-5 using different substrates

In order to assess the 2-PE synthesis capability of *C. glycerinogenes* WL2002-5, the strain was cultivated in defined medium (see Material and Methods). As shown in Fig. 3, the 2-PE concentration in broth was 2.5 g/l, as determined by GC-MS, suggesting *C. glycerinogenes* WL2002-5 could synthesize 2-PE. In yeast, there are 2 routes for the biosynthesis of 2-PE from L-Phe: the phenylethylamine (PEA) pathway and the Ehrlich pathway. In the PEA pathway, L-Phe is decarboxylated to PEA by aromatic amino acid decarboxylase and then oxidatively deaminated to phenylacetaldehyde by monoamine oxidase, followed by reduction to 2-PE by alcohol dehydrogenase. In the Ehrlich pathway, L-Phe is transaminated to phenylpyruvate by aminotransferase and then decarboxylated to phenylacetaldehyde by phenylpyruvate decarboxylase, followed by reduction to 2-PE by alcohol dehydrogenase. A previous study revealed a high degree of gene similarity (>99%) between *C. glycerinogenes* WL2002-5 and *Issatchenkia orientalis*, and genomic analysis indicates the Ehrlich pathway is present in *I. orientalis*. We therefore predict that the Ehrlich pathway is responsible for production of 2-PE in *C. glycerinogenes* WL2002-5, and we are currently

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**Figure 2.** Comparison of *C. glycerinogenes* WL2002-5 (A) and *S. cerevisiae* W303-1A (B) for 2-PE tolerance in liquid culture. Cells were incubated in YPD medium at 30°C for 18 h, and diluted to the same concentration. Cells were inoculated into fresh YPD liquid medium containing various concentrations of 2-PE (0 (control), 1, 2, 3, 3.5, 4 g/l), and incubated at 30°C with shaking at 200 rpm. Open square, 0 g/l; solid circle, 1 g/l; open up-pointing triangle, 2 g/l; solid down-pointing triangle, 3 g/l; open diamond, 3.5 g/l; solid left-pointing triangle, 4 g/l.
cloning these genes in our laboratory. Interestingly, the 2-PE concentration was 75 mg/l following cultivation in defined medium without L-Phe (YNB as a sole nitrogen source), indicating the potential presence of a de novo pathway for the synthesis of 2-PE from glucose in C. glycerinogenes WL2002-5.

**Culture optimization**

In order to improve the production titer of 2-PE, the culture process was optimized based on the defined medium. As shown in Fig. 4a, 2-PE accumulation and biomass were higher when the glucose concentration was between 30 and 90 g/l, but lower at a higher glucose concentration (Fig. 4a). Increasing the L-Phe concentration from 3 to 11 g/l also diminished cell growth (Fig. 4b). In contrast, the 2-PE titer peaked at a L-Phe concentration of 7 g/l but was decreased slightly at a higher L-Phe concentration. Yeast extract is cheap and was tested as a nitrogen source instead of YNB. As shown in Fig. 4c, the biomass and 2-PE titer were increased slightly with an increasing concentration of yeast extract, and 2-PE production was improved from 3.2 to 3.6 g/l.

To investigate the effect of temperature on the bio-synthesis of 2-PE, C. glycerinogenes WL2002-5 was incubated in optimized medium (90 g/l glucose, 5 g/l KH2PO4, 4 g/l yeast extract, 7 g/l L-Phe, 0.5 g/l MgSO4·7H2O) at different temperatures. As shown in Fig. 4d, the biomass and 2-PE titer were significantly

![Image](image1.jpg)

**Figure 3.** Gas chromatography of 2-PE produced by C. glycerinogenes WL2002-5 from L-Phe (A). MS spectrum of 2-PE produced by C. glycerinogenes WL2002-5 from L-Phe (B).

![Image](image2.jpg)

**Figure 4.** Effect of glucose (A), L-Phe (B), yeast extract (C) concentration, and temperature (D) on 2-PE production and cell growth. Biomass was measured using a spectrophotometer at 600 nm, and the 2-PE titer was determined by HPLC. Open square, 2-PE; solid triangle, OD600.
reduced with increasing temperature, especially at temperatures above 37°C, and the optimum temperature was 30°C. Even though cell growth was almost completely repressed at 47°C, a 1.5 g/l 2-PE (40% of the maximal titer) was detected in the broth (Fig. 4d). In contrast, 2-PE production in the thermotolerant S. cerevisiae Ye9-596 and some other strains was almost completely repressed (<0.01 g/l) at 40°C. This result suggested that biosynthesis of 2-PE by C. glycerinogenes WL2002-5 is thermotolerant.

**Batch culture**

A batch fermentation was carried out to further enhance production of 2-PE from L-Phe (Fig. 5). Glucose was exhausted and the biomass reached stationary phase at 30 h, and accumulation of 2-PE was coupled with cell growth, as reported previously for other organisms. The maximum titer of 2-PE was 5.0 g/l at 45 h, corresponding to a conversion rate of 0.71 g/g, which is close to the maximum theoretical bioconversion yield of 0.75 g/g. This 2-PE titer and bioconversion rate are higher than that reported for the engineered S. cerevisiae W303-1B (4.8 g/l, 0.48 g/g) for S. cerevisiae Giv 2009 (2.35 g/l, 0.39 g/g), and for other strains. To the best of our knowledge, the 2-PE titer produced by C. glycerinogenes WL2002-5 is the highest reported to date for a batch culture method without the use of ISPR, indicating that C. glycerinogenes WL2002-5 is an excellent strain for the bioproduction of 2-PE. Attempts to further improve 2-PE production by genetic engineering are ongoing in our group.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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