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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Systematic metabolic engineering for improved synthesis of perillic acid in *Candida tropicalis*

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Abstract

Perillic acid has been studied as an anticancer and antimicrobial drug. Production of perillic acid has attracted considerable attention. Meanwhile, *Candida tropicalis* is an unconventional diploid yeast, most significantly characterized by its ability to metabolize alkanes or fatty acids for growth and proliferation. Therefore, perillic acid's precursor (L-limonene) in *C. tropicalis* was firstly synthesized by expressing a *Mentha spicata* L-limonene synthase gene, *LS_Ms* in this work. Expression of a gene which encoded for a truncated version of *tLS_Ms* increased the production of L-limonene with a 2.78-fold increase in the titer over *C. tropicalis* GJR-LS-01. Compartmentalized expression of the gene *tLS_Ms* inhibited the production of L-limonene in *C. tropicalis* compared to cytoplasmic expression. Cytoplasmic overexpression of seven precursor synthesis genes significantly enhanced the production of L-limonene in *C. tropicalis* compared to their compartmentalized expression (mitochondria or peroxisomes), which increased by 31.7-fold in *C. tropicalis* GJR-tLS-01. The L-limonene titer in *C. tropicalis* GJR-EW-tLS-04 overexpressing the mutant gene *ERG20WW* in the cytoplasm was significantly increased, 11.33-fold higher than the control. The titer of L-limonene for 60 g/L glucose was increased by 1.40-fold compared to the control. Finally, a *Salvia miltiorrhiza* cytochrome P450 enzyme gene *CYP7176* and an *Arabidopsis thaliana* NADPH cytochrome P450 reductase gene *CPR* were heterologously expressed in *C. tropicalis* GJR-EW-tLS-04C for the synthesis of perillic acid, which reached a titer of 106.69 mg/L in a 5-L fermenter. This is the first report of de novo synthesis of perillic acid in engineered microorganisms. The results also showed that other chemicals may be efficiently produced in *C. tropicalis*.

Key points

- *Key genes cytoplasmic expression was conducive to L-limonene production in C. tropicalis.*
- *Perillic acid was first synthesized de novo in engineered microorganisms.*
- *The titer of perillic acid reached 106.69 mg/L in a 5-L fermenter.*

Keywords Metabolic engineering · Synthetic biology · *Candida tropicalis* · L-limonene · Perillic acid

Introduction

Perillic acid, as a potentially pharmacologically active derivative, has been studied as an anticancer and antimicrobial drug (Da Fonseca et al. 2011; Mirata et al. 2009; Yeruva et al. 2007). Microbial bioconversion of limonene has been used to establish cost-effective methods for the production of perillic acid (Ferrara et al. 2013). Many perillyl derivatives are produced selectively by oxidizing the exocyclic methyl group of limonene using various microorganisms (e.g., *Bacillus stearothermophylus*, *Mycobacterium* sp. and *Aspergillus* species) (Duetz et al. 2003; Mirata et al. 2009; Van Beilen et al. 2005). Mars et al. (2001) used *Pseudomonas putida* GS1 to convert limonene to perillic acid using a fed-batch bioreactor and non-limiting amounts

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of limonene, ammonium and glycerol whose concentration in the culture medium reached 64 mM. Three enzymes (two dehydrogenases and cymene monooxygenase) from *P. putida* GS1 cymene degradation pathway were verified to be responsible for the bioconversion of limonene to perillic acid (Mars et al. 2001).

Rapid developments in metabolic engineering and synthetic biology have led to the emergence of microbial cell factories as a sustainable alternative platform for the synthesis of L-limonene (Zhao et al. 2023) and its derivative, perillic acid. Limonene has been reported to be produced by several microorganisms (e.g., *Saccharomyces cerevisiae*, *Escherichia coli*, *Rhodosporidium toruloides*, and *Yarrowia lipolytica*) (Liu et al. 2021; Mirata et al. 2009; Pang et al. 2019; Wu et al. 2019; Zhang et al. 2021). *Candida tropicalis* is an unconventional diploid yeast (Zhang et al. 2016), most significantly characterized by its ability to metabolize alkanes or fatty acids for growth and proliferation (Hara et al. 2001). Wang et al. (2018) used two homologous single exchanges to subsequently replace the promoters of NADPH-cytochrome P450 reductase gene and cytochrome P450 gene by the promoter pGAP, and the titer of long-chain dicarboxylic acids of constructed C. *tropicalis* PJPP1702 reached 32.84 g/L in a 5-L fermenter. Wang et al. (2023) obtained one novel tolerant strain *C. tropicalis* Y31-N against furfural using continuous domestication processes, the xylitol yield of which was increased by 1.848-fold that of the parent strain. Meanwhile, in our previous study, we used combinatorial metabolic engineering strategies to rebuild synthesis pathways of squalene in *C. tropicalis*, and the titer of squalene was increased by 13.5-fold that of the control strain (*C. tropicalis* DC03) (Wei et al. 2024).

In this study, *C. tropicalis* was used for the first time to de novo synthesise perillic acid via systematic metabolic engineering (Fig. 1), which mainly included compartmentalized expression of truncated gene *tLS_Ms*, overexpression of precursor synthesis genes, overexpression of *C. tropicalis* endogenous gene *ERG20*, overexpression of mutant gene *ERG20WW*, and heterologous expression of a cytochrome P450 enzyme gene *CYP7176* from *Salvia miltiorrhiza* and a NADPH cytochrome P450 reductase gene *CPR* from *Arabidopsis thaliana*.

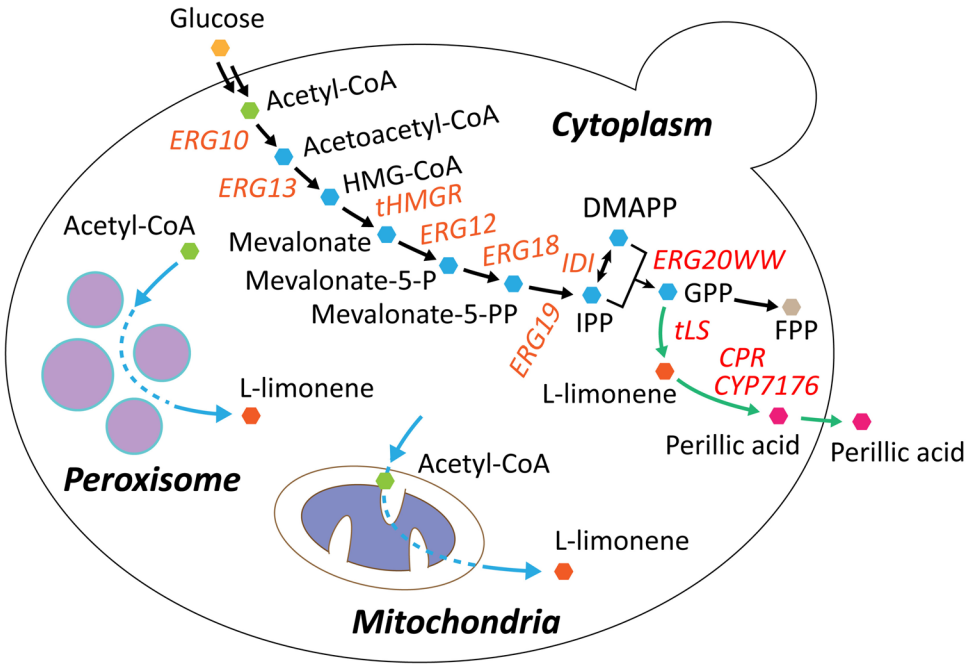

Fig. 1 Synthetic pathway of L-limonene and its perillyl derivative perillic acid in *C. tropicalis*. HMG-CoA, hydroxymethylglutaryl-CoA; Mevalonate-5-P, mevalonate 5-phosphate; Mevalonate-5-PP, mevalonate 5-diphosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl pyrophosphate; FPP, farnesyl diphosphate; *IDI*, isopentenyl diphosphate isomerase gene; *ERG10*, acetoacetyl-CoA synthase gene; *ERG13*, hydroxymethylglutaryl coenzyme A synthetase gene; *tHMGR*, hydroxymethylglutaryl coenzyme A reductase gene; *ERG12*, mevalonate kinase gene; *ERG18*, phosphomevalonate kinase; *ERG19*, mevalonate diphosphate decarboxylase gene; *ERG20WW*, farnesyl pyrophosphate synthase mutant gene; *tLS*, truncated L-limonene synthase gene *tLS_Ms* synthase gene from *M. spicata*; *CYP7176*, the cytochrome P450 enzyme gene from *S. miltiorrhiza*; *CPR*, the NADPH cytochrome P450 reductase gene from *A. thaliana*

Materials and methods

Strains, plasmids, and primers

E. coli JM109 was used for vector construction. Plasmid pMD19-T was used for gene cloning. Plasmids Ts-*gda324-URA3*, Ts-PGAPDH-*carRP-TENO1A*, Ts-POX5-*gda324-URA3*, and Ts-DLD1-24-*gda324-URA3* were constructed in our previous study (Zhang et al. 2022). Gene *gda324* is 324 bp and was obtained from the URA3 gene via PCR using primers Ugda324 and Dgda324. Gene *gda324* was used to eliminate the gene *URA3* from the genome. *C. tropicalis* CU-208 is a *C. tropicalis* ATCC 20336 uracil (URA3) defective strain. The strains constructed in this study are shown in Table S1. The PCR primers used in this study are shown in Table S2.

Media and culture conditions

Lysogeny broth (LB) medium (1 L) included 5-g yeast extract, 10-g tryptone, and 10-g NaCl. Minimal medium (MM) (1 L) included 20 g glucose, 6.7 g yeast nitrogen base (YNB), and 10 g $(NH_4)_2SO_4$. Supplemental medium (SM) included MM and 0.006% (w/v) uracil. SM-5-FOA medium included SM and 0.006% (w/v) 5-fluoroorotic acid (5-FOA). YPD medium (1 L) included 20-g glucose, 10-g yeast extract, and 20-g tryptone. Fermentation medium (1 L) included 20-g glucose, 10-g yeast extract, 20-g tryptone, 2-g $MgSO_4 \bullet 7H_2O$, or 10% (v/v) *n*-dodecane. For *E. coli*, a single colony from solid LB medium was transferred to 25-mL liquid LB medium in a 250-mL flask and cultured at 37℃ and 200 rpm for 8 h. For *C. tropicalis*, a single colony from the solid YPD medium was transferred to 10 mL of liquid YPD medium in a 100-mL flask and cultured at 30℃ and 200 rpm for approximately 24 h until OD_{600} = 8.0. 2% (v/v) seed fermentation liquid was transferred to 30 mL of fermentation medium in a 250-mL flask and cultured at 30℃ and 200 rpm until OD_{600} = 1.0. 10% (v/v) *n*-dodecane was supplemented to the fermentation medium and cultured at 30℃ and 200 rpm for 120 h. For fermentation in a 5-L fermenter, 10% (v/v) seed fermentation liquid was transferred to 2.25 L of fermentation medium. Engineered *C. tropicalis* strains were cultured at 30℃ and 2 vvm in the 5-L fermenter. Stirring speeds were 600 rpm for 0–36 h and 800 rpm for 36–156 h. The pH value (5.5) was controlled by supplying $NH_3 \bullet H_2O$, and the glucose concentration was controlled at 5 g/L.

Constrution of gene knockout cassettes and gene integration cassettes

Acyl coenzyme A oxidase POX5 gene knockout cassette was constructed as example. Gene *POX5* was amplified by PCR using primers POX5-FW and POX5-RS and the genome of *C. tropicalis* ATCC 20336 as a template. Gene *POX5* and plasmid pM19T were linked by solution I. It was verified by restriction endonucleases *Mlu* I and *Pst* I, and the recombinant plasmid pM19T-*POX5* was obtained. Gene fragment *POX5U-Ts-POX5D* was amplified by PCR using primers rPOX5-FW and rPOX5-RS and pM19T-*POX5* as template. The restriction endonucleases *Pst* I and *Mlu* I were used to digest plasmid pM19T-*gda324-URA3* to obtain gene fragment *gda324-URA3*. Gene fragment *POX5U-Ts-POX5D* digested by *Pst* I and *Mlu* I was linked with *gda324-URA3* to obtain the recombinant plasmid pM19T-*POX5-gda324-URA3*. Gene *POX5* knockout cassette *POX5-gda324-URA3-POX5* was obtained using restriction endonuclease *Mlu* I to digest plasmid pM19T-*POX5-gda324-URA3*. Other gene knockout cassettes were constructed using the same method and primers, as shown in Table S2.

Plasmid pM19T-*POX5-gda324-URA3-POX5* was digested by restriction endonuclease *Eco*R I. Plasmid pM19T-P_{GAPDH}-carRP-T_{ENO1A} was digested by restriction endonuclease *Mlu* I. Plasmids pM19T-*POX5-gda324-URA3-POX5* and pM19T-P_{GAPDH}-carRP-T_{ENO1A} were digested and then linked to obtain recombinant plasmid pM19T-*POX5-gda324-URA3*-P_{GAPDH}-carRP-T_{ENO1A}. Gene *POX5-gda324-URA3*-P_{GAPDH}-carRP-T_{ENO1A} was obtained by digesting plasmid pM19T-*POX5-gda324-URA3*-P_{GAPDH}-carRP-T_{ENO1A} using restriction endonucleases *Eco*R I and *Nhe* I. L-limonene synthase gene *LS* was amplified by PCR using LS-FW and LS-RS as primers and plasmid pUC57-*LS* as template. Gene *LS* and *POX5-gda324-URA3*-P_{GAPDH}-carRP-T_{ENO1A} were used to construct plasmid pM19T-*POX5-gda324-URA3*-P_{GAPDH}-*LS*-T_{ENO1A}. Gene *LS* integration cassette *POX5*-P_{GAPDH}-*LS*-T_{ENO1A}-*gda324-URA3-POX5* was obtained by *Mlu* I digestion. Integration cassettes for other genes (*tLS*, *ERG20*, *ERG20WW*) were constructed using the same method, and the primers are shown in Table S2.

LiCl transformation method of gene knockout cassettes and gene integration cassettes in *C. tropicalis*

C. tropicalis was cultured at 30℃ until OD_{600} = 1.0. Cells were collected and washed with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH = 7.4). The collected cells were resuspended in 1 mL of 100 mM LiCl and incubated at 30℃ and 200 rpm for 1 h. 80 µL of resuspended cells were mixed with 10 µL of DNA fragments from the knockout cassette or integration cassette and 2 µL of salmon sperm and incubated at 30℃ for 30 min. 900 µL of 40% (w/v) poly(ethylene glycol) 3350 and 100 mM LiCl were added and incubated at 30℃ and 200 rpm for 1 h. The mixture was heated at 42℃ for 5 min and then cooled to 25℃. Cells were collected and washed using sterile water before being cultured on solid MM medium at 30℃ for 2 days. Colonies were verified by PCR and cultured on solid

SM-5-FOA medium to eliminate gene *URA3*. Positive colonies were obtained by sequencing.

Mutation of gene ERG20

The ERG20 mutant gene *ERG20_F95W/N126W* was amplified by PCR using primers ERG20WW-FW and ERG20WW-RS and pM19T-*EGR20* as templates. The PCR mixture digested by *Dpn* I was transformed into *E. coli* JM109 and cultured on solid LB medium containing ampicillin at 37℃ for 8 days. Positive colonies were verified by colony PCR and sequencing.

Determination of cell density (OD_{600}) and residual glucose

E. coli or *C. tropicalis* culture broth (2 mL) was used to determine the absorbance at 600 nm (OD_{600}). *C. tropicalis* fermentation broth (2 mL) was centrifuged at 12,000 × *g* at 4℃ for 5 min and the supernatant was collected. A residual sugar analyser SGD-III (Shandong Academy of Sciences, Jinan, China) was used to determine the glucose concentration.

Detection of L-limonene and perillic acid

35 mL of the fermentation broth, including cells, was centrifuged at 5000 × *g* at 4℃ for 5 min, and 1 mL of the upper organic phase was collected in a 1.5-mL tube. Anhydrous Na_2SO_4 was used to remove residual water from the organic phase at 12,000 × *g* and 4℃ for 1 min. The organic phase filtered via a 0.22-μm filter membrane was used to determine L-limonene and perillic acid by GC–MS. The TraceGOLD TG-5MS GC column (30 m × 0.25 mm × 0.25 μm) from Thermo Fisher Scientifc Co., Ltd. (Waltham, USA) was used in this study. The column temperature was 50℃ for 1 min and was increased to 100℃ at a rate of 6℃•min^{-1}; it was further increased to 250℃ at a rate of 20℃•min^{-1} with a holding time of 5 min.

Statistical analysis

The means ± standard deviations were reported, and three parallel experiments were independently carried out. Student's *t*-test was used to statistically analyze data. Statistical significance was set at $p < 0.05$.

Results

Construction of engineered *C. tropicalis* producing perillic acid's precursor L-limonene

In this study, the L-limonene synthase gene *LS_Ms* from *Mentha spicata* (Davies et al. 2014) was used to construct a synthesis pathway for perillic acid's precursor L-limonene in *C. tropicalis* and the engineered strain *C. tropicalis* GJR-LS-01c was constructed. Williams et al. (1998) found that when the targeting amino acid residue sequence preceding a tandem arginine pair (R58R59) that is highly conserved in monoterpene synthases was removed, the truncated L-limonene synthase as a tractable pseudomature form had more efficient catalytic efficiency than the native enzyme. Therefore, integration gene expression cassette for the truncated L-limonene synthase gene *tLS_Ms* was integrated into the genome of *C. tropicalis* to construct the engineered strains *C. tropicalis* GJR-tLS-01c. The titer of L-limonene in *C. tropicalis* GJR-tLS-01c reached 0.26 mg/L, which was 2.78-fold higher than that of *C. tropicalis* GJR-LS-01 (Fig. 2A).

Effect of compartmentalized expression of gene tLS_Ms on the synthesis of perillic acid's precursor L-limonene

The C-terminus of gene *tLS_Ms* was fused with a mitochondria-targeting signal peptide gene and a peroxisome-targeting signal peptide gene for compartmentalized expression in mitochondria and peroxisome of *C. tropicalis*, respectively, and two engineered strains *C. tropicalis* GJR-tLS-01 m and *C. tropicalis* GJR-tLS-01p were construced. As shown in Fig. 2B, L-limonene in *C. tropicalis* GJR-tLS-01 m and *C. tropicalis* GJR-tLS-01p was successfully synthesized, but with lower titers than *C. tropicalis* GJR-tLS-01.

Effect of overexpressing precursor synthesis genes on perillic acid's precursor L-limonene synthesis

C. tropicalis ZLH-DC-04, *C. tropicalis* ZLH-DM-04, and *C. tropicalis* ZLH-DP-04 were constructed and derived from *C. tropicalis* CU208 overexpressing precursor synthesis genes *ERG10*, *ERG13*, *tHMGR* (Jiang et al. 2017), *ERG12*, *ERG18*, *ERG19* and *IDI* in cytoplasm, mitochondria, and peroxisome, respectively, in our previous study (Zhang et al. 2022). In this study, seven precursor synthesis genes were overexpressed to enhance the accumulation of precursors IPP and DMAPP in *C. tropicalis*. *C. tropicalis* GJR-tLS-02c, *C. tropicalis* GJR-tLS-02 m, and *C. tropicalis* GJR-tLS-02p were constructed by overexpressing a truncated L-limonene synthase gene *tLS_Ms* in *C. tropicalis* ZLH-DC-04, *C. tropicalis* ZLH-DM-04, and *C. tropicalis* ZLH-DP-04, respectively. The titers of L-limonene in *C. tropicalis* GJR-tLS-02c, *C. tropicalis* GJR-tLS-02 m and *C. tropicalis* GJR-tLS-02p reached 8.17, 2.32, and 3.15 mg/L, respectively (Fig. 3A), which were significantly enhanced conpared with that of *C. tropicalis* GJR-tLS-01.

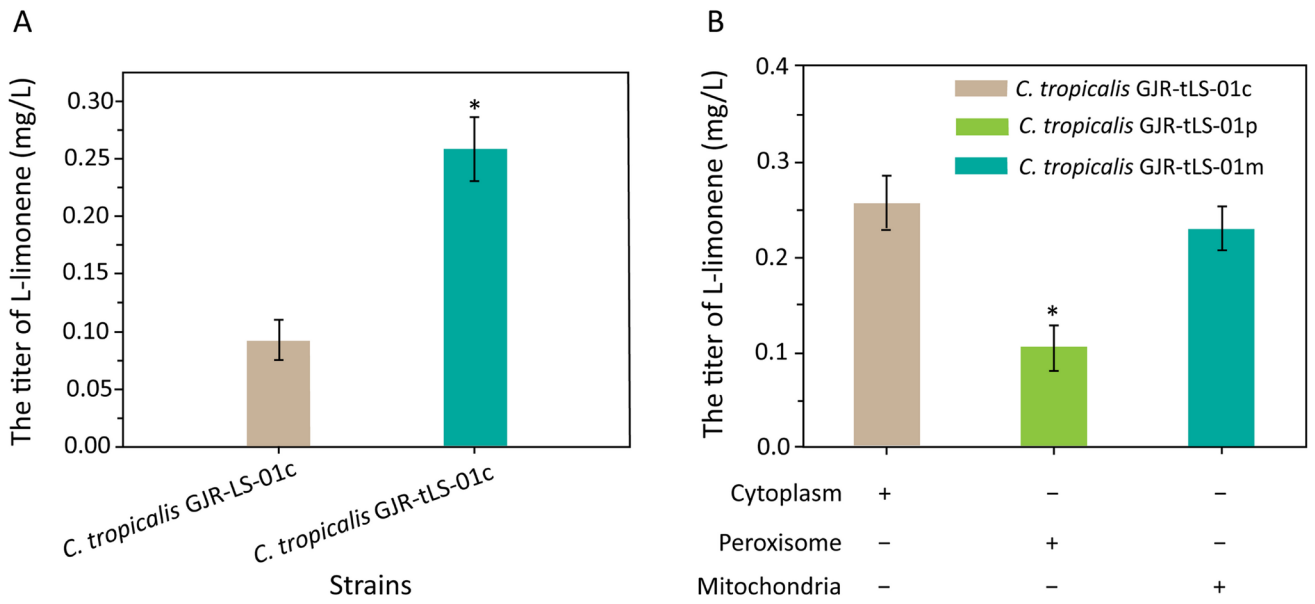

Fig. 2 Construction of engineered *C. tropicalis* producing L-limonene and effect of compartmentalized expression of gene *tLS_Ms* on the synthesis of L-limonene. (**A**) Construction of engineered *C. tropicalis* producing L-limonene. (**B**) Compartmentalized expression of gene *tLS_Ms*. +, expression of gene *tLS_Ms*; −, no expression of gene *tLS_Ms*. *C. tropicalis* GJR-tLS-01c, expessing gene *tLS_Ms* in cytoplasmic of *C. tropicalis*; *C. tropicalis* GJR-tLS-01p, expessing gene *tLS_Ms* in peroxisome of *C. tropicalis*; *C. tropicalis* GJR-tLS-01 m, expessing gene *tLS_Ms* in mitochondria of *C. tropicalis*. Asterisks indicate significant differences compared to the control (*, p value < 0.05). p value < 0.05 was considered statistically significant

Effect of overexpressing *C. tropicalis* endogenous gene ERG20 on perillic acid's precursor L-limonene synthesis

In this study, the endogenous gene *ERG20* from *C. tropicalis* was overexpressed in *C. tropicalis* GJR-tLS-02c, *C. tropicalis* GJR-tLS-02p, and *C. tropicalis* GJR-tLS-02 m to construct *C. tropicalis* GJR-tLS-03c, *C. tropicalis* GJR-tLS-03p, and *C. tropicalis* GJR-tLS-03 m. As shown in Fig. 3B, overexpression of gene *ERG20* significantly reduced the production of L-limonene compared to the control strain, by 90.51% via overexpression in the cytoplasm, 23.52% via overexpression in the peroxisome and 2.76% via overexpression in the mitochondria, respectively.

Effect of overexpressing the mutant gene ERG20WW on perillic acid's precursor L-limonene synthesis

It was found that among all the ERG20P mutants, the mutant ERG20WW with mutation sites F95W and N126W had preferentially GPPS activity over FPP synthase activity (Ignea et al. 2014; Zhao et al. 2016). In this study, the mutant ERG20WW was used to catalyze the synthesis of GPP. The mutant gene *ERG20WW* was overexpressed in the cytoplasm, peroxisome and mitochondria of *C. tropicalis* GJR-tLS-02 to construct the strains *C. tropicalis* GJR-EW-tLS-04c, *C. tropicalis* GJR-EW-tLS-04p, and *C. tropicalis* GJR-EW-tLS-04 m, respectively. As shown in Fig. 4A, overexpression of the mutant gene *ERG20WW* significantly increased the production of L-limonene in *C. tropicalis* GJR-EW-tLS-04 compared to the control strains, with an 11.33-fold increase in its titer in the cytoplasm, and 5.85-fold in the peroxisome.

Enhancing the synthesis of perillic acid's precursor L-limonene in *C. tropicalis* by fermentation optimization

The production of L-limonene in *C. tropicalis* GJR-EW-tLS-04c was enhanced by fermentation optimization. As shown in Fig. S1, the titer of L-limonene gradually increased with prolonged fermentation time, reaching the highest value (100.71 mg/L) in 120 h. However, the cell density OD_{600} was decreased by 96 h. Meanwhile, the glucose concentrations were optimized to enhance the production of L-limonene. As shown in Fig. 4B, the highest titer of L-limonene (141.48 mg/L) was achieved when the glucose concentration in the medium was 60 g/L, which was 1.40-fold higher than the control (20 g/L glucose). When the initial glucose concentration was further increased to 100 g/L, the titer of L-limonene decreased to 72.10 mg/L.

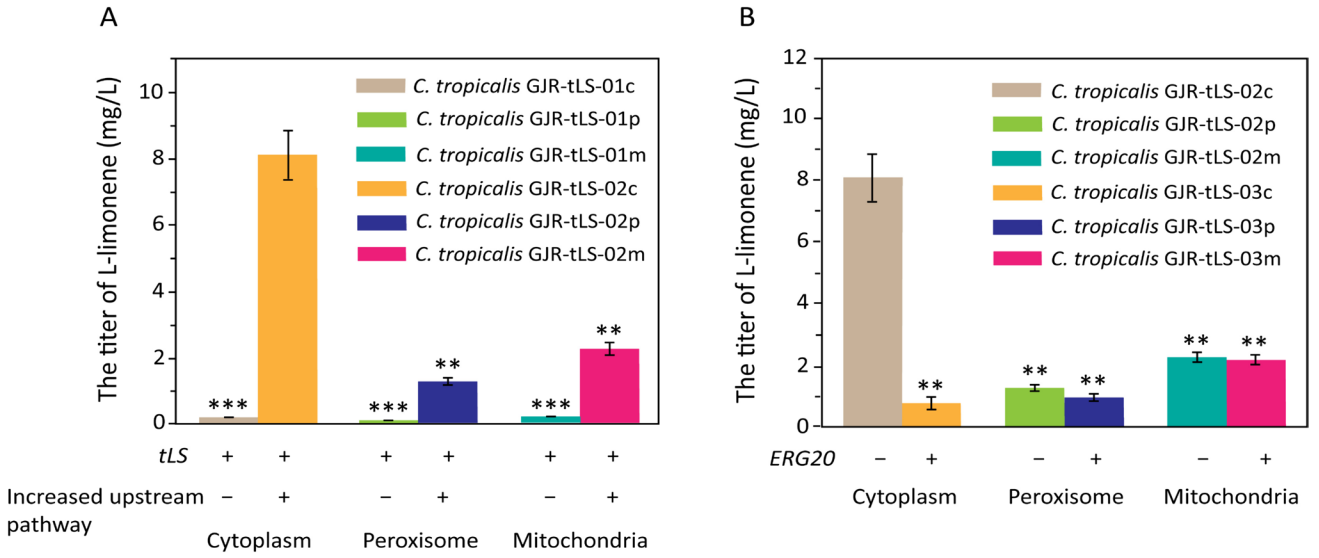

Fig. 3 Effect of overexpressing precursor synthesis genes and *C. tropicalis* endogenous gene *ERG20* on L-limonene synthesis. (**A**) Overexpressing precursor synthesis genes (*ERG10*, *ERG13*, *tHMGR*, *ERG12*, *ERG18*, *ERG19* and *IDI*). (**B**) Overexpressing *C. tropicalis* endogenous gene *ERG20*. +, expression of target genes; −, no expression of target genes. *C. tropicalis* GJR-tLS-01c, expessing gene *tLS_Ms* in cytoplasmic of *C. tropicalis*; *C. tropicalis* GJR-tLS-01p, expessing gene *tLS_Ms* in peroxisome of *C. tropicalis*; *C. tropicalis* GJR-tLS-01 m, expessing gene *tLS_Ms* in mitochondria of *C. tropicalis*; *C. tropicalis* GJR-tLS-02c, expessing seven precursor synthesis genes in cytoplasmic of *C. tropicalis* GJR-tLS-01c; *C. tropicalis* GJR-tLS-02p, expessing seven precursor synthesis genes in peroxisome of *C. tropicalis* GJR-tLS-01p; *C. tropicalis* GJR-tLS-02 m, expessing seven precursor synthesis genes in mitochondria of *C. tropicalis* GJR-tLS-01 m; *C. tropicalis* GJR-tLS-03c, expessing gene *ERG20* in cytoplasmic of *C. tropicalis* GJR-tLS-02c; *C. tropicalis* GJR-tLS-03p, expessing gene *ERG20* in peroxisome of *C. tropicalis* GJR-tLS-02p; *C. tropicalis* GJR-tLS-03 m, expessing gene *ERG20* in mitochondria of *C. tropicalis* GJR-tLS-02 m. Asterisks indicate significant differences compared to the control (**, p value < 0.01; ***, p value < 0.001). p value < 0.05 was considered statistically significant

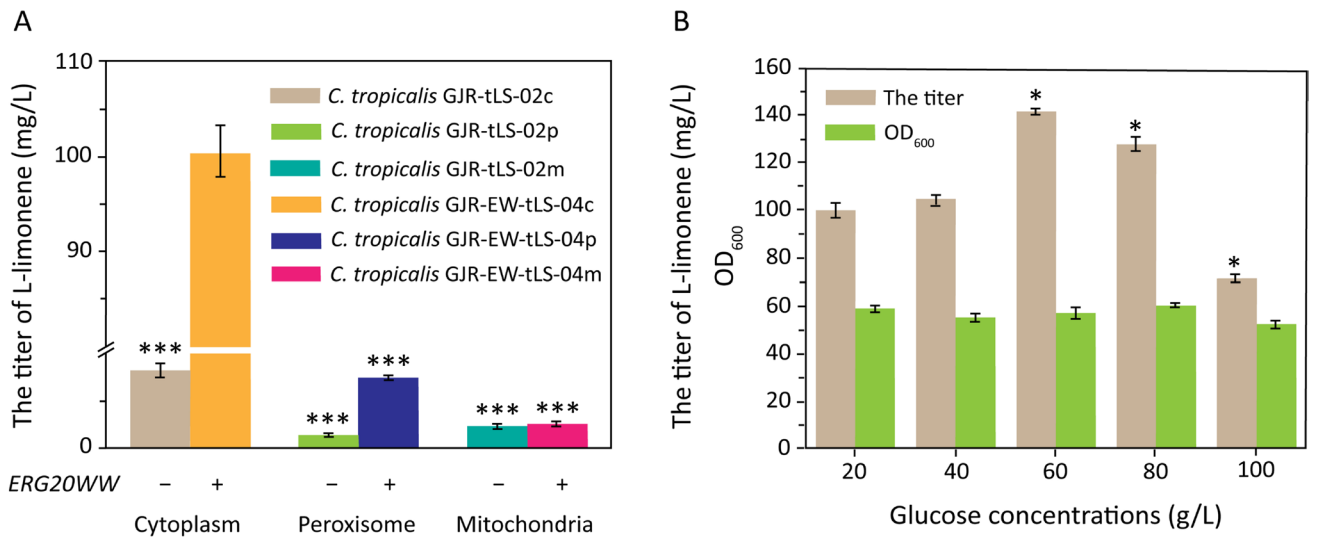

Fig. 4 Effect of overexpressing the mutant gene *ERG20WW* and fermentation optimization on L-limonene synthesis. (**A**) Overexpressing the mutant gene *ERG20WW*. +, expression of gene *ERG20WW*; −, no expression of gene *ERG20WW*. *C. tropicalis* GJR-tLS-02c, expessing seven precursor synthesis genes in cytoplasmic of *C. tropicalis* GJR-tLS-01c; *C. tropicalis* GJR-tLS-02p, expessing seven precursor synthesis genes in peroxisome of *C. tropicalis* GJR-tLS-01p; *C. tropicalis* GJR-tLS-02 m, expessing seven precursor synthesis genes in mitochondria of *C. tropicalis* GJR-tLS-01 m; *C. tropicalis* GJR-EW-tLS-04c, expessing the mutant gene *ERG20WW* in cytoplasmic of *C. tropicalis* GJR-tLS-02c; *C. tropicalis* GJR-EW-tLS-04p, expessing the mutant gene *ERG20WW* in peroxisome of *C. tropicalis* GJR-tLS-02p; *C. tropicalis* GJR-EW-tLS-04 m, expessing the mutant gene *ERG20WW* in mitochondria of *C. tropicalis* GJR-tLS-02 m. (**B**) Fermentation optimization. Asterisks indicate significant differences compared to the control (*, p value < 0.05; ***, p value < 0.001). p value < 0.05 was considered statistically significant

Production of perillic acid in engineered *C. tropicalis* in a 5-L fermenter

L-limonene production by *C. tropicalis* GJR-EW-tLS-04c was used to synthesise perillic acid by heterologously expressing a cytochrome P450 enzyme gene *CYP7176* from *S. miltiorrhiza* and a NADPH cytochrome P450 reductase CPR from *A. thaliana*, respectively, with a recombinant strain *C. tropicalis* GJR-EW-tLS-CYP-05 being constructed. The cytochrome P450 was used as a perillic acid synthase, and the NADPH cytochrome P450 reductase CPR was used as an electron donor. As shown in Fig. 5A/B, the titer of perillic acid in *C. tropicalis* GJR-EW-tLS-CYP-05 in a 250 mL flask was 7.19 mg/L at 120 h. This indicates successful heterologous expression of the cytochrome P450 enzyme gene *CYP7176* from *S. miltiorrhiza* and the NADPH cytochrome P450 reductase gene *CPR* from *A. thaliana*, and catalytic production of L-limonene to perillic acid in *C. tropicalis*. Meanwhile, the titer of perillic acid in *C. tropicalis* GJR-EW-tLS-CYP-05 reached 106.69 mg/L at 144 h in a 5-L fermenter, which was 13.8-fold higher than that in the flask (Fig. 5). This is the first report on the production of perillic acid from glucose in engineered microorganisms.

Discussion

Many monoterpene synthetases include *N*-terminal transporters, which can transfer translation products to the plastids or other organelles in their original host. However, its heterologous expression in *C. tropicalis* is likely to result in confused or misfolded enzyme localization, which affects the catalytic function of heterologous monoterpene synthases (Bohlmann et al. 1998; Turner et al. 1999). Truncating the plastid-targeting sequence at the *N*-terminus of monoterpene synthases is an effective method to improve the substrate conversion efficiency of monoterpene synthase and the yield of L-limonene. Here, the L-limonene synthase gene *LS_Ms* from *Mentha spicata* (Davies et al. 2014) and its truncated L-limonene synthase gene *tLS_Ms* were integrated into the genome of *C. tropicalis*, respectively. The titer of L-limonene in *C. tropicalis*

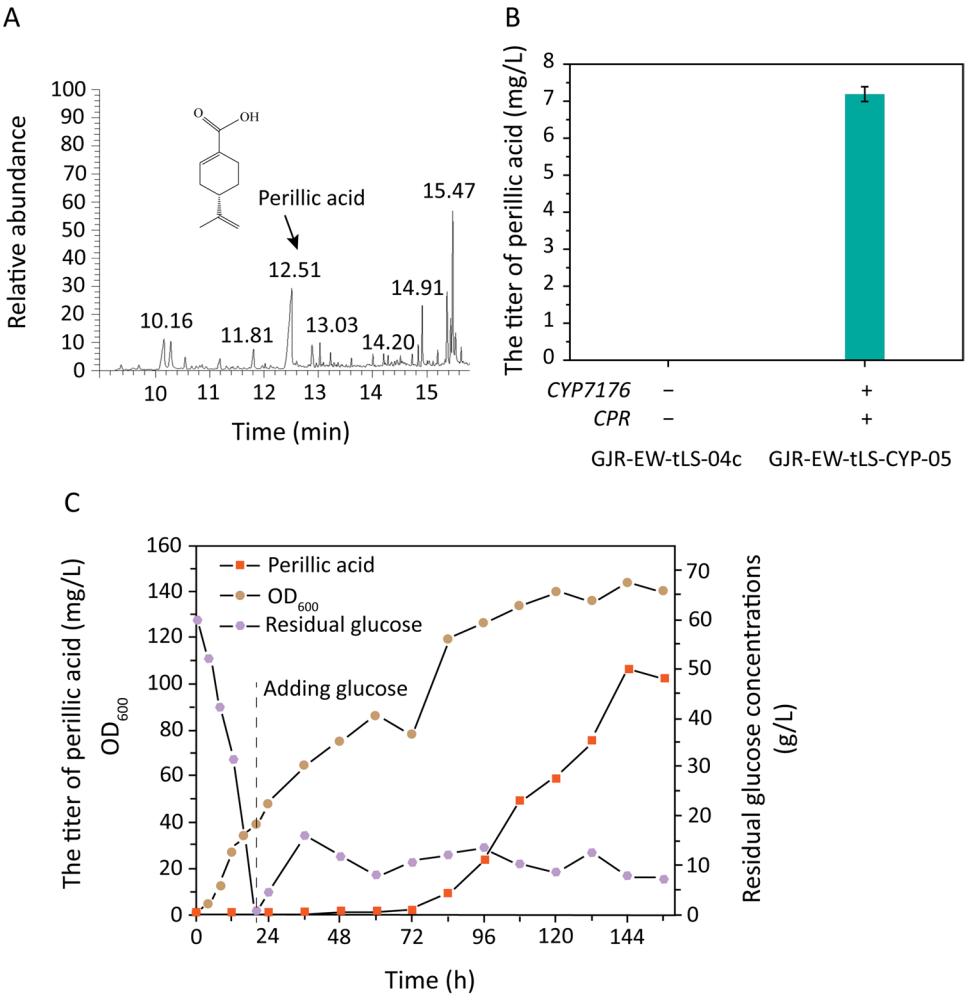

Fig. 5 Production of perillic acid in engineered *C. tropicalis* in a 5-L fermenter. (**A**) Determination of perillic acid by GC–MS. (**B**) Construction of engineered *C. tropicalis* producing perillic acid. +, expression of target genes; −, no expression of target genes. GJR-EW-tLS-04c, expessing the mutant gene *ERG20WW* in cytoplasmic of *C. tropicalis* GJR-tLS-02c; *C. tropicalis* GJR-EW-tLS-CYP-05, expessing the cytochrome P450 enzyme gene *CYP7176* and the NADPH cytochrome P450 reductase gene *CPR* cytoplasmic of *C. tropicalis* GJR-EW-tLS-CYP-04c. (**C**) Production of perillic acid in engineered *C. tropicalis* in a 5-L fermenter

GJR-tLS-01c was significantly enhanced compared with that of *C. tropicalis* GJR-LS-01. This indicates that the truncated L-limonene synthase gene provides a better catalytic element for the biosynthesis of L-limonene.

Cell compartmentalization refers to the restriction of certain metabolic pathways to specific areas of cells, such as mitochondria and peroxisomes. The specific physicochemical properties of some organelles of *C. tropicalis* were used to enhance the production of monoterpenoids in this study. However, the titers of L-limonene for compartmentalized expression in mitochondria and peroxisome in *C. tropicalis* were lower than cytoplasmic expression of gene *tLS_Ms*. Presumably, gene *tLS_Ms* fused with mitochondria-targeting signal peptide gene or peroxisome-targeting signal peptide gene was successfully expressed, and geranyl pyrophosphate (GGP) was available for translocation from cytoplasm into the mitochondria and peroxisomes of *C. tropicalis*. It is similar to FPP or other metabolites reported in previous studies (Bhataya et al. 2009; Deloache et al. 2016; Zhang et al. 2020). In contrast to cytoplasmic expression, their compartmentalized expression inhibits the production of L-limonene in *C. tropicalis*.

Excessive accumulation of precursor GPP is used to improve the synthesis of L-limonene. *C. tropicalis* ZLH-DC-04, *C. tropicalis* ZLH-DM-04, and *C. tropicalis* ZLH-DP-04 were constructed and derived from *C. tropicalis* CU208 overexpressing precursor synthesis genes *ERG10*, *ERG13*, *tHMGR* (Jiang et al. 2017), *ERG12*, *ERG18*, *ERG19* and *IDI* in cytoplasm, mitochondria, and peroxisome, respectively, in our previous study (data not shown). The titers of L-limonene in *C. tropicalis* GJR-tLS-02c, *C. tropicalis* GJR-tLS-02 m and *C. tropicalis* GJR-tLS-02p were 31.7-, 8.91- and 12.1-fold higher than those of *C. tropicalis* GJR-tLS-01 (Fig. 3A). The results indicated that cytoplasmic overexpression of seven precursor synthesis genes significantly enhanced the production of L-limonene in *C. tropicalis* compared with their compartmentalized expression (mitochondria or peroxisomes).

In the yeast endogenous MVA pathway, the synthesis of GPP and farnesyl diphosphate (FPP) is catalyzed by the FPP synthase encoded by gene *ERG20*, the absence of which is lethal to yeast (Grabinska and Palamarczyk 2002; Szkopinska and Plochocka 2005). Here, the production of L-limonene was decreased by overexpression of the endogenous gene *ERG20* from *C. tropicalis*. It is suggested that the overexpression of gene *ERG20* from *C. tropicalis* affects both cytoplasm and organelles, but the cytoplasm is most severely affected. This may be because GPP does not accumulate in *C. tropicalis* cells, but directly generates FPP, which is catalyzed by multiple enzymes to generate squalene, ergosterol, and other substances related to cell membrane synthesis. FPP shunted the flow of GPP, thus leading to an insufficient supply of GPP, the precursor of L-limonene.

Through the above experimental analysis, the double mutant gene *ERG20WW* with mutation sites F95W and N126W was introduced to enhance the synthesis of GPP while attenuating the formation of FPP and maintaining cellular activity. The mutant ERG20WW weakens the FPP synthesis pathway and does not affect the synthesis pathway of GPP from IPP and DMAPP. Here, the mutant ERG20WW was used to catalyze the synthesis of GPP, overexpression of which significantly increased the production of L-limonene in *C. tropicalis* GJR-EW-tLS-04 compared to the control strains. This verifies that overexpression of the mutant gene *ERG20WW* enhances the metabolic flow of the precursor substance GPP and decreases the flow of GPP to FPP, thereby significantly increasing the production of L-limonene.

L-limonene is a metabolite of GPP, and its accumulation requires a certain amount of time. Early termination of the fermentation process will affect the synthesis of L-limonene, and long-term fermentation will cause waste of resources and even lead to the metabolism and decomposition of L-limonene, thus reducing its yield. The production of L-limonene in *C. tropicalis* GJR-EW-tLS-04c was enhanced by fermentation optimization. The cell growth of the engineered strain *C. tropicalis* GJR-EW-tLS-04c was decreased at 96 h, indicating that the cells may have stopped growing and started cleaving. It has been shown that yeasts have low tolerance to monoterpenes and that high concentrations of L-limonene can significantly inhibit the growth of yeasts (Hu et al. 2012; Liu et al. 2023; Parveen et al. 2004). L-limonene with high fat solubility can accumulate in the cell membrane with phospholipid bilayer to destroy the cell membrane structure, decrease the activity of enzymes in the cell membrane and damage cell membrane function, which can disturb the normal metabolic balance of cells and lead to cell death (Hu et al. 2012; Liu et al. 2023; Parveen et al. 2004). Meanwhile, the glucose concentrations were optimized to enhance the production of L-limonene. The highest titer (141.48 mg/L) of L-limonene was achieved for 60 g/L glucose concentration. The results indicated that adjusting the initial glucose concentration of the medium was beneficial to improve the production of L-limonene.

Limonene has been used for the cost-effective production of potentially pharmacologically active derivatives (e.g., perillic acid) and aromatics (e.g., α-terpineol) through microbial bioconversion (Bicas et al. 2009; Mirata et al. 2009). The ease of culture using safe and secure yeasts (e.g., *Arxula adeninivorans* and *Yarrowia lipolytica*) to produce potentially non-toxic anticancer agents has prompted an increasing interest in this specific pathway of biotransformation (Fickers et al. 2005; Gellissen et al. 2005). *Y. lipolytica* ATCC 18942 has been reported to convert limonene

to perillic acid as the sole oxidised terpene product (Ferrara et al. 2013). In this study, a cytochrome P450 enzyme gene *CYP7176* from *S. miltiorrhiza* and a NADPH cytochrome P450 reductase CPR from *A. thaliana* were heterologously expressed in *C. tropicalis* GJR-EW-tLS-04c to synthesise perillic acid, and a recombinant strain *C. tropicalis* GJR-EW-tLS-CYP-05 was constructed. The cytochrome P450 was used as a perillic acid synthase, and the NADPH cytochrome P450 reductase CPR was used as an electron donor. Perillic acid was firstly synthesized in *C. tropicalis* GJR-EW-tLS-CYP-05. This indicates successful heterologous expression of the cytochrome P450 enzyme gene *CYP7176* from *S. miltiorrhiza* and the NADPH cytochrome P450 reductase gene *CPR* from *A. thaliana*, and catalytic production of L-limonene to perillic acid in *C. tropicalis*. The perillic acid titer in *C. tropicalis* GJR-EW-tLS-CYP-05 reached 106.69 mg/L in a 5-L fermenter. This is the first report on the production of perillic acid from glucose in engineered microorganisms. There are several reports on the production of perillic acid, but they are all produced using limonene as a substrate via whole-cell biotransformation. For example, during the production of perillic acid from the muti-step oxyfunctionalization of limonene, Willrodt et al. (2017) evaluated a combination of biologically and technologically inspired strategies to overcome toxicity-related issues, and wild-type *Pseudomonas putida* was used for the bioconversion of limonene, with perillic acid productivity reaching 34 $g/L_{tube}/d$.

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Data availability The datasets generated and analyzed during this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This article is in compliance with ethical standards, and does not contain any studies with animals performed or human participants.

Conflict of interest The authors declare no competing interests.

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