Biosynthesis of Valerenic acid by Engineered Saccharomyces cerevisiae

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Research Article

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Abstract

Objective To produce valerenic acid (VA) in *Saccharomyces cerevisiae* by engineering a heterologous synthetic pathway.

Result Valerena-4,7(11)-diene synthase (*VDS*) derived from *Valeriana officinalis* was expressed in *S. cerevisiae* to generate valerena-4,7(11)-diene as the precursor of VA. By overexpressing the key genes of mevalonate (MVA) pathway *ERG8*, *ERG12* and *ERG19* and integrating 4 copies of *MBP* (maltose binding protein)-*VDS*-*ERG20* gene expression caskets into genome, the production of valerena-4,7(11)-diene was improved to 75 mg/L. On this basis, VA synthases from *Lactuca sativa* was expressed to produce VA and the most effective VA production strain was used for fermentation, and the yield of VA reached mg/L in the flask and 6.8 mg/L in a 5-L bioreactor fed glucose and ethanol.

Conclusions The heterologous synthesis of VA in *S. cerevisiae* increases the production of VA and provides a reference method for the biosynthesis of other sesquiterpenes.

Introduction

Valerenic acid (VA) is the most important short-chain fatty acid in valerian extract. It is a sesquiterpene derivative and mainly exists in *V. officinalis*. VA has a therapeutic effect on diseases such as insomnia and epilepsy (Onyszkiewicz et al. 2020). Recent studies have also shown that VA can lower blood pressure, improve immunity against cancer and reduce neuroinflammation in MPTP-induced Parkinson's disease (Scott et al. 2012; Han et al. 2020; Shi et al. 2021; Rodríguez-Cruz et al. 2020). Although the remarkable physiological effects of VA have attracted research attention, the extraction and purification of VA from *V. officinalis* is difficult because of its naturally low concentration. Recently, the rapid development of synthetic biology and metabolic engineering strategies have provided technical expertise and tools for solving these problems. Among these, *Saccharomyces cerevisiae* has become a preferred host in synthetic biology because of its clear genetic background (Ignea et al. 2011).

For a long time, studies regarding VA biosynthesis were limited. As shown in Fig. 1, sesquiterpene acids can be synthesized via the mevalonate (MVA) pathway of *S. cerevisiae*, in which the isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are generated by condensation reactions and then combined to produce the farnesyl diphosphate (FPP) by FPP synthase. The sesquiterpene synthase gene *VoTPS* screened from valeriana was codon optimized to *VoVDS* and expressed in yeast to transform FPP into valerena-4,7(11)-diene, the precursor of valeric acid (Pyle et al. 2012; Yeo et al. 2013; Ricigliano et al. 2016). By expressing cytochrome P450 monooxygenases (VoCYP71DJ1), also derived from valeriana, in the valerena-4,7(11)-diene-producing yeast chassis, trace amounts of hydroxylated VA were detected. Subsequently, the aldehyde dehydrogenase (AaALDH) and alcohol dehydrogenase (AaADH) derived from *Artemisia annua* was applied in yeast to decrease the hydroxylated acid into VA (Wong et al. 2018). Notably, the finding of LsGAO2 from *Lactuca sativa*, which can oxidize valerena-4,7(11)-diene to VA, provided a direct way to obtain VA (Nguyen et al. 2019).

Figure 1

In this study, we developed a biosynthesis method to produce VA in engineered *S. cerevisiae.* Vallena-4,7(11)-diene synthase (VDS) from *V. officinalis* was expressed in *S. cerevisiae* to produce valerena-4,7(11)-diene. Subsequently, we overexpressed and regulated the expression of key genes related to *ERG8*, *ERG12* and *ERG19* in the MVA pathway. Then, VA synthase (LsGAO2) from *L. sativa* was expressed to produce VA. Finally, the VA production process was optimized and scaled up in a 5-L fermenter. This study demonstrated the heterologous synthesis of VA in *S. cerevisiae*, which may act as a potential alternative method to plant extraction.

Materials And Methods

Reagents, Strains, and Media

VoTPS (GenBank: JQ437840.1), *LsGAO2* (GenBank: KF981867), and *Arabidopsis thaliana CPR1* (*AtCPR1*; GenBank: BT008426.1) were synthesized with codon optimization by GenScript Biotech Corporation Ltd. (Nanjing, China) and were cloned into plasmid pUC57. *S. cerevisiae* 3HP was used as the parent strain for the construction of all engineered strains. Primers were synthesized by GENEWIZ (Beijing, China). Phanta Max Super-Fidelity DNA Polymerase was purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). DNA gel mini purification and mini plasmid extraction kits were purchased from TIANGEN (Beijing, China). The engineered yeast strains were grown in auxotrophic SD plates containing 20 g glucose/L, 20 g agar/L, 6.7 g yeast nitrogen base/L, and a 2 g amino acid mixture/L (without uracil, histidine, leucine, tryptophan, or adenine for auxotrophs as appropriate).

Strain Construction

The expression cassettes consisted of a promoter, structural gene, and terminator and were constructed using fusion PCR. Next, 40 base pair overlaps were designed between the adjacent fragments such that they could be assembled into the *S. cerevisiae* genome. All promoters (p), terminators (t), and *HO, TRP1, DPP1* and *LPP1* genes were amplified from genomic DNA of *S. cerevisiae* W303.P_{TEF1}-*VDS*-T_{ADH2}, P_{PGK1}-*ERG8*-T_{ADH2}-P_{TEF1}-*ERG12*-T_{CYC1}-P_{TDH3}-*ERG19*-T_{ADH1}, P_{TEF1}-*MBP-VDS-ERG20*-T_{ADH2}, P_{TEF1}-*MBP-VDS-ERG20*-T_{PGK1}-P_{PGK1}-*MBP-VDS-ERG20*-T_{CYC1}-P_{TDH3}-*MBP-VDS-ERG20*-$T_{ADH2,}$ and P_{PGK1}-*LsGAO2*-T_{PGK1}-P_{TDH3}-*AtCPR1*-T_{ADH2} expression cassettes were constructed by fusion PCR and integrated into the *S. cerevisiae* genome. DNA fragments were transformed into yeast cells using the lithium acetate method (Burke et al. 2000). All strains used in this study are listed in Table 1.

Table 1
Strains constructed in the study

Strain	description	Source
W303-1a	*MATa; leu2-3,112; trp1-1; can1-100; ura3-1; ade2-1; his3-11,15*	our lab
3HP	W303-1a, *ade2::P_{PGK1}-tHMG1-T_{PGK1}, T_{TDH3}-ERG20-T_{ERG20}; PERG9::P_{HXT1}-ERG9-PEST;*	our lab
Z01	3HP, *HO::P_{TEF1}-VDS-T_{ADH2}*	this study
Z02	3HP, *TRP::P_{PGK1}-ERG8-T_{ADH2}, P_{TEF1}-ERG12-T_{CYC1}, P_{TDH3}-ERG19-T_{ADH1}*	this study
Z03	Z02, *HO::P_{TEF1}-VDS-T_{ADH2}*	this study
Z04	Z02, *HO::P_{TEF1}-MBP-VDS-ERG20-T_{ADH2}*	this study
Z05	Z04, *DPP1::P_{TEF1}-MBP-VDS-ERG20-T_{PGK1}, P_{PGK1}-MBP-VDS-ERG20-T_{CYC1}, P_{TDH3}-MBP-VDS-ERG20-T_{ADH2}*	this study
Z06	Z05, *LPP1::P_{PGK1}-LsGAO2-T_{PGK1}, P_{TDH3}-AtCPR1-T_{ADH2}*	this study

Table 1

Yeast Strain Cultivation And Fermentation

A single colony grown on SD plates was inoculated into 3 mL of yeast peptone dextrose medium (YPD) and incubated with shaking at 220 rpm overnight at 30°C. Then, aliquots were transferred to 250-mL shake flasks containing 30 mL of YPD culture medium at an initial optical density at 600 nm (OD600) of 0.05 and cultivated with shaking at 220 rpm at 30°C for 4 days. Each sample was analysed in triplicate. OD600 was measured with a spectrophotometer (Oppler, 752N, China). CDW (g/L) = OD600 × 0.33.

Strain Z06 was used for batch and fed-batch fermentation in a 5-L bioreactor. A single colony obtained from the plate was inoculated into 30 mL YPD medium overnight, and then the culture was transferred to a 500-mL flask containing 100 mL of YPD medium and cultured for 18 h. Then the culture was used as a seed and inoculated at 10% (v/v) into a 5-L bioreactor containing 2 L YPD culture medium. Fermentation was performed at an initial pH of 5.5 and an air flow rate of 2 L/min at 30°C. Dissolved oxygen was maintained at approximately 35% by stirring. The pH was maintained between 5 and 5.5 by the addition of NaOH or H_2SO_4 (Gietz et al. 1995).

For fed-batch fermentation, a mixed glucose solution was fed into the bioreactor at a speed of 0.4 mL/min to promote the production of cell biomass when the glucose was depleted 12 h after the fermentation began and ended at 48 hours (Zeng et al. 2020). The glucose solution mixture contained concentrated glucose solution (500 g/L, 1000 mL), glutamate solution (10 g/L, 12 mL), microelement stock solution (12 mL/L, 10 mL), and vitamin stock solution (10 mL/L, 12 mL).

Extraction, Gas Chromatography-mass Spectrometry (Gc-ms) Analysis, And Quantification Of Valerena-4,7(11)-diene

Due to the volatility of valeradiene, biphasic fermentation of valeradiene-producing strains was carried out. n-Dodecane was used as the extractant, and the upper organic phase was taken for detection. For extracellular detection of valerena-4,7(11)-diene, 2 mL of the upper organic phase was centrifuged at 12000 rpm for 2 min. The upper layer of n-dodecane was aspirated into a new centrifuge tube and filtered through a 0.22 μm organic film for analysis.

The valerena-4,7(11)-diene structure was further confirmed by GC-MS analysis. A 1 μL aliquot of sample was analysed at a split ratio of 10 on a Shimadzu GCMS-TQ8030 equipped with an Agilent 19091S-433UIHP-5 ms Ultra Inert column using helium as the carrier gas. The injection temperature was set at 250°C, and the oven temperature program was as follows: 40°C for 2 min, flow rate 1 mL/min, starting at 40°C, ramp rate of 10°C/min to 250°C with a hold for 5 min. The ion source temperature was 250°C, and spectra were scanned from m/z 50–600. The production of valerena-4,7(11)-diene was quantified by normalizing the peak area to the standard peak area.

Extraction, Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis and Quantification of VA

For extracellular detection of VA, 5 mL of fermentation broth was centrifuged at 12000 rpm for 10 min. The supernatant was aspirated into a fresh Eppendorf centrifuge tube, 0.5 mL hexane was added, and the tube was vibrated for 5 min. After full contact extraction, the extraction system was centrifuged at 12000 rpm for 10 min, and the upper layer of hexane was aspirated into a new centrifuge tube and filtered through a 0.22 μm organic film for analysis. For intracellular detection of VA, *S. cerevisiae* cells were collected after centrifugation, and an equal volume of quartz sand was added. Hexane was added as the extraction solvent, and the mixture was vortexed thoroughly for 30 min and then centrifuged at 12000 rpm for 10 min. The hexane supernatant was aspirated and filtered through a 0.22 μm organic film for analysis.

The VA structure was further confirmed by LC-MS analysis. For LC-MS detection, the mobile phase was 60% acetonitrile aqueous solution containing 0.1% formic acid; the source type was ESI; ion polarity was positive; nebulizer was set to 2.0 bar; capillary voltage was set to 4500 v; the dry heater was set at 180°C,

and the scan began at 50 m/z. The production of VA was quantified by normalizing the peak area to the standard peak area.

Results And Discussion

Construction of valerena-4,7(11)-diene-producing S. cerevisiae strain

As the accumulation of valerena-4,7(11)-diene is very important for the production of VA, we constructed and optimized the engineering yeast to produce valerena-4,7(11)-diene. Strain 3HP, constructed by our lab from *S. cerevisiae* W303-1a before, was selected as the heterologous expression host (Zhang et al. 2020). The valerena-4,7(11)-diene synthase (VDS) gene from *V. officinalis* was codon optimized and inserted into the *HO* site of 3HP, resulting in strain Z01, which was quantified to produce 1.0 mg/L of valerena-4,7(11)-diene (Fig. 2a). Because the MVA pathway limits the production of exogenous terpenoid compounds, we overexpressed the MVA pathway key genes, *ERG8*, *ERG12* and *ERG19*, under strong promoters to increase the flux to FPP (Fig. 2b), resulted in strain Z03, but it only increased the titer of valerena-4,7(11)-diene to 4 mg/L.

Figure 2

The maltose binding protein (MBP) can increase the yield of valerena-4,7(11)-diene by fusing with VDS and ERG20 (Wong et al. 2018). Here, we construct the *MBP-VDS-ERG20* expression caskets and insert it into the *HO* sites of Z02, resulted the strain Z04, whose yield of valerena-4,7(11)-diene was increased to 35 mg/L. Due to the high efficiency of this fusion protein, we further inserted 3 copies of *MBP-VDS-ERG20* module into the *DPP1* sites of Z04 resulting in the strain Z05 which increased the yield of valerena-4,7(11)-diene to 75 mg/L (Fig. 2c).

Selection of P450 Enzyme and CPR to Synthesize VA

The heterologous synthesis of VA from valerena-4,7(11)-diene requires P450 enzyme and CPR, which provides electrons for P450 activity. The P450 VoCYP71DJ1 screened from valeriana has proved to synthetize hydroxylated VA by expressing in the chassis that producing valerena-4,7(11)-diene, but it require an extra reductase to decrease the hydroxylated acid to obtain VA (Wong et al. 2018). Comparatively, LsGAO2 from *Lactuca sativa* is a kind of P450 with extensive oxidation, which can oxidize valerena-4,7(11)-diene to VA directly (Nguyen et al. 2019). Therefore, LsGAO2 was chosen to synthetize VA with AtCPR1 from *Arabidopsis thaliana*. We integrated *LsGAO2* and *AtCPR1* in the *LPP1* sites of Z05 resulting in the strain Z06 which was detected to produce VA by comparing to the standard using LC-MS, and the titer of VA was 2.8 mg/L (Fig. 3).

figure 3

Batch and Fed-Batch Fermentation of Z06 in a 5-L Bioreactor

The pre-experiment was optimized by shaking flask fermentation conditions, and the final fermentation conditions were as following: the inoculation volume is set to 10%, the glucose concentration is 40 g/L, the pH is 5.5, the temperature is 30 ℃ and the rotation speed is 220–500 rpm.

The VA yielding strain Z06 was then used for batch fermentation in a 5-L bioreactor. As shown in Fig. 4a, glucose was consumed rapidly and ethanol was accumulated in the initial stage of fermentation. The VA was produced at 12 h when glucose was exhausted and ethanol began to be consumed, and then the VA was accumulated gradually and reaching the highest yield of 3.12 mg/L with a CDW of approximately 25.07 g/L.

Figure 4

According to the batch fermentation data, the mixed glucose solution was added automatically at the point of glucose depletion (12 h) for fed-batch fermentation, and stopped feeding when the OD600 increased to 50 at the time of 48h, because the high concentration of ethanol was harmful to the growth of *S. cerevisiae*. As shown in Fig. 4b, the final CDW increased to 29.34 g/L and the fed-batch cultivation further increased the VA titer to 6.8 mg/L, which is the highest titer reported in engineered eukaryotes.

Conclusion

In this study, we use *S. cerevisiae* to synthesize VA heterologously. The endogenous *ERG8, ERG12* and *ERG19, MBP-VDS-ERG20* and P450 enzymes involved in the synthesis pathway were combined, regulated, and expressed, which increased the production of VA. Thus, this work provides a reference method for producing VA and heterogeneously synthesizing other natural products from plants in *S. cerevisiae*.

Although we successfully used a genetically engineered *S. cerevisiae* strain to produce VA, the fermentation yield was low. One reason for this may be that the yield of the precursor valerena-4,7(11)-diene was insufficient, and another reason may be the low enzymatic rate of the P450 enzymes that catalyze the conversion of valerena-4,7(11)-diene. Furthermore, the specific P450 enzyme that directly synthesizes VA from valerian has not been found yet. In the follow-up research, the endogenous oxidase of *V. officinalis* could be explored by genome sequencing and transcriptome sequencing, which will be helpful for VA production.

Declarations

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Declarations

Conflict of interest The authors declare no competing interests.

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

Informed consent Informed consent was obtained from all individual participants included in the study.

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Figures

Figure 1

The heterologous synthesis pathway of valeric acid in *S. cerevisiae. ERG12*, mevalonate kinase gene; *ERG8*, phosphomevalonate kinase gene; *ERG19*, diphosphomevalonate decarboxylase gene; *IDI*, isopentenyl-diphosphate δ-isomerase gene; *ERG20*, farnesyl diphosphate synthetase gene; *VDS*, Vallena-4,7(11)-diene synthase gene; *LsGAO2*, VA synthase gene;

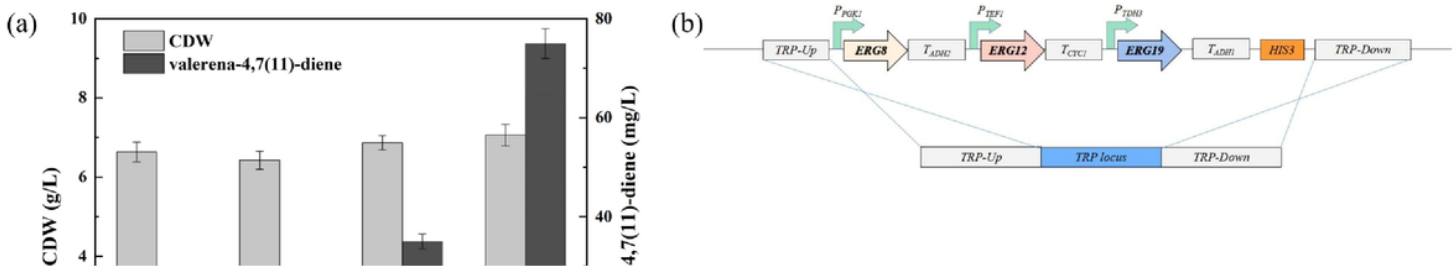

Figure 2

Production of valerena-4,7(11)-diene by strains Z01, Z03-Z05 and construction of regulated strain. All experiments were performed in triplicate, and the error bars represent the mean ± standard deviation

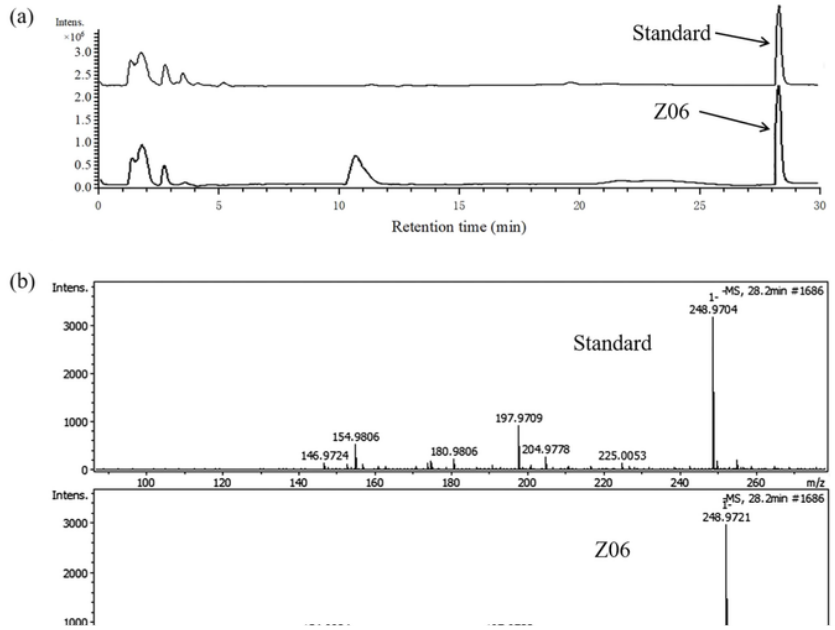

Figure 3

GC-MS analysis of VA produced by engineered *S. cerevisiae* strain. (a) Chromatogram for VA production by strain Z06. (b) GC–MS spectra of standard and the new peak (RT = 28.2 min) produced by Z06.

Figure 4

Production of valerenic acid in batch cultivations (a) and fed-batch (b) cultivations in a 5-L bioreactor. All experiments were performed in triplicate, and the error bars represent the mean ± standard deviation