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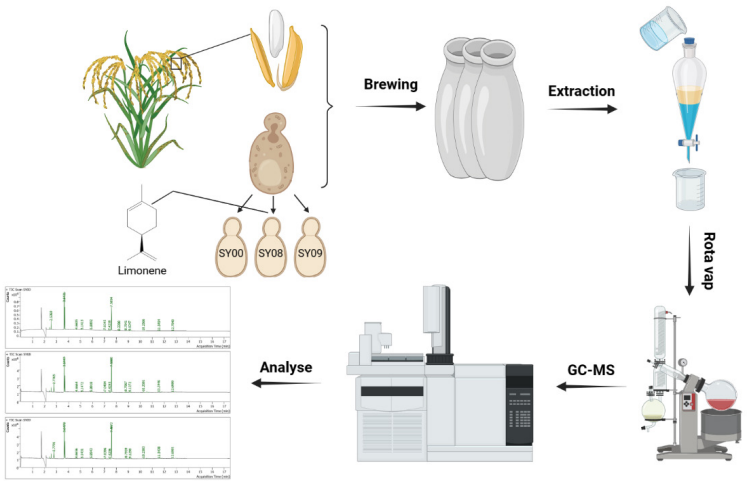
Effects of The Constructed (+)-Limonene Synthesis Pathway in Sake Yeast on The Brewing Flavor of Sake

Hao Xu, Nuo Xu, Yanhui Li, Jie Sun, Tingheng Zhu*, Shijin Wu*

Abstract

Sake is a traditional alcoholic beverage in Japan, and the improvement of sake flavour and quality can be achieved by metabolic engineering modification of strains. Limonene, a terpenoid compound with an aromatic odour, has been detected in plant-based alcoholic products such as wine and brandy, and has flavour-modifying and bioactive effects. As limonene has not been investigated in sake, we used CRISPR/Cas9 technology to construct a limonene synthesis pathway in an industrial sake yeast strain and analysed the modification of sake flavour and composition. Eight strains with limonene yields ranging from 2 μg/L to 2.6 mg/L were obtained using the strategies of increasing the copy of the limonene expression cassette, weakening the competitive pathway, and enhancing the mevalonate (MVA) pathway. Comparative analyses of the sake brewing performance of the starting strain SY00 and the engineered bacteria with different limonene yields, as well as the composition of the sake samples, were carried out using gas chromatography-mass spectrometry (GC-MS) and other analytical methods. The result showed that the total amount of higher alcohols decreased, the phenylethanol content increased, and the flavour was significantly improved in the samples brewed with the limonene synthesising strains. Limonene was detected in the production of sake by two-phase fermentation of the engineered limonene producing strain SY08 (2.6 mg/L), but it was not detected in the final sake samples after fermentation, probably due to volatilisation. In conclusion, this study provides a new strategy for the construction of limonene producing strains by metabolic modification and its application to sake brewing.

Graphical Abstartct



Affiliation:

College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, Zhejiang Province, China

***Corresponding author:**
Tingheng Zhu, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, Zhejiang Province, China.

Shijin Wu, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, Zhejiang Province, China

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Introduction

Sake is a traditional alcoholic beverage in Japan with a history of more than 1,300 years [1, 2] and its production process is based on rice and water as the main raw materials, which are produced by the fermentation of *Aspergillus oryzae* and sake yeast [3, 4]. The brewing process can be divided into five steps, namely brewing, fermentation, filtration, bakelite and maturation, with fermentation being the key step. During this process, sake yeast converts glucose into ethanol under anaerobic conditions, producing a variety of flavour compounds such as higher alcohols, esters, acids and ketones, which give sake its complex flavour [4-7]. The flavour of sake is influenced by a variety of factors, including rice quality, degree of milling, water quality, brewing method and yeast strain used, with the degree of milling of the rice and the yeast strain selected having the most significant effect on the overall flavour of sake [1, 8, 9].

Yeast strains play a crucial role in flavour and other qualities in sake brewing, and improving flavour through strain modification is a common strategy. The improvement of sake yeast strains mainly focuses on increasing the efficiency of alcoholic fermentation, improving aroma and flavour, imparting new functional properties, reducing foam formation and adjusting the colour of sake [10, 11]. In the early days of sake brewing, new yeast strains were mainly obtained by tolerance screening, natural selection, mutagenesis and hybridisation [12-14], but with the development of gene editing technology, genetic engineering has also begun to be applied to sake yeast improvement [15]. Limonene is a plant terpene [16] with a citrus-like aroma and a variety of physiological activities, including anti-inflammatory, antibacterial, anticancer, antioxidant, anti-diabetic, hepatoprotective, and choleolytic [16-19], and is a GRAS food additive [20-22]. Due to its widespread occurrence in plants, limonene is frequently detected in some plant alcoholic products such as brandy and wine [23-26]. Therefore, limonene is a potential flavour modifier and bioactive molecule for alcohol. Studies have also been reported on the production of limone-containing alcoholic products by yeast modification, e.g. Hu et al [27] synthesised limonene by modifying *Saccharomyces cerevisiae* to synthesise limonene and brewed white wine based on health and flavour considerations and found that the construction of the limonene synthesis pathway had no significant effect on the fermentation characteristics of white wine. However, studies on limonene in alcoholic products are still relatively limited and have not been reported in sake.

In plants, limonene is produced from geranyl pyrophosphate (GPP) as a precursor in the mevalonate (MVA) pathway catalysed by limonene synthase [28], and the naturally occurring MVA pathway in *S. cerevisiae* facilitates the biosynthesis of limonene. However, the MVA pathway, as an important isoprenoid synthesis pathway, involves a variety of secondary metabolites, such as carotenoids, vitamin E, and steroids, which may potentially affect the aroma, flavour and colour of wine products. The aim of this study was to investigate the effect of the limonene synthesis pathway on the flavour of sake. We constructed the limonene synthesis pathway in the sake yeast strain SY00 to obtain strains with different limonene yields and analysed the effect of limonene synthesis on the improvement of sake flavour by comparing these constructed strains with the wild-type strains in terms of organoleptic evaluations, fermentation levels and changes in aroma compounds.

Materials and Methods

Strains, plasmids, media and chemicals

The plasmids and strains used in this study are listed in Table 1. Sake yeast (*S. cerevisiae*) SY00 was obtained from the strain conservation centre of Shaoxing Yuanzu Brewing Co. Yeast strains were cultured in YPD medium (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L, pH natural) at 30°C and 180 rpm. Sake fermentation was carried out anaerobically at 30°C in rice semi-solid fermentation medium (rice (g): water (mL) = 1:2, saccharase: 2 g enzyme/1 kg rice). *Escherichia coli* DH5α was used for plasmid construction.

Method for preparing semi-solid fermentation medium for rice

Semi-solid fermentation medium for rice. a) Weigh the appropriate quantity of rice, wash with ultrapure water (UP water), wait until the amalgamated water is no longer cloudy, add the appropriate quantity of UP water and soak for 30 minutes, wrap in gauze to shake dry, then spread out and drain for 15 minutes; b) Place in a steamer for a total steaming time of 1 hour; c) Pour the stewed rice into triangular conical flasks containing 2 times the weight of the rice in sterile UP water. Depending on the quality of the rice before steaming, saccharase is added at a rate of 2 g of enzyme/1 kg of rice, bound with histopathic film and placed in a water bath at 60°C for 10 h for saccharification, shaking well every 2 h.When the saccharification was completed and cooled down to room temperature, it was obtained as a semi-solid fermentation medium of rice.

Plasmids construction

The primers used are listed in supplementary Table S1. The N-terminal signal peptides of the limonene synthase gene *LS*（GenBank ID: AF514287.1) from *Citrus limon* and the nerolidol pyrophosphate synthase gene *CPT1*（GenBank ID: NM001247704.1) from *Solanum lycopersicum* were codon optimised and synthesised (Tianlin Biotechnology,China) on pESC-HIS-(tLs-tCPT1) plasmid. Primers X2Z-F/-R,

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Table 1: Strains and plasmids

Strains and plasmids	Genotype/description	Source
Saccharomyces cerevisiae		
SY00	For sake brewing	Our lab
SY01	SY00, *X4::* (P_{PGK1}-*tLS*- T_{CYC1})-(P_{TEF1}-*tCPT1*- T_{CYC1})	This study
SY02	SY01, *X3::* (P_{PGK1}-*tLS*- T_{CYC1})-(P_{TEF1}-*tCPT1*- T_{CYC1})	This study
SY03	SY02, *XII2::*(P_{PGK1}-*tLS*- T_{CYC1})-(P_{TEF1}-*tCPT1*- T_{CYC1})	This study
SY04	SY03, P_{ERG20}::P_{CYC1}	
SY05	SY03, X3:: P_{PGK1}-*tHMG1*- T_{CYC1}	This study
SY06	SY05, X2:: P_{TEF1}-*ERG20^{WW}*- T_{CYC1}	This study
SY07	SY05 ,X2:: (P_{TEF1}-*ERG20^{WW}*- T_{CYC1})- (P_{TEF1}-*ERG13*- T_{CYC1})	This study
SY08	SY05, X2:: (P_{TEF1}-*ERG20^{WW}*- T_{CYC1})- (P_{TEF1}-*ERG13*- T_{CYC1})-(P_{TEF1}-*ERG12*- T_{CYC1})	This study
SY09	SY00, X2:: (P_{TEF1}-*ERG20^{WW}*- T_{CYC1})- (P_{TEF1}-*ERG13*- T_{CYC1})-(P_{TEF1}-*ERG12*- T_{CYC1})	This study
Escherichia coli		
DH5α	Φ80 lacZΔM15 ΔlacU169 recA1 endA1 hsdR17 supE44 thi-1 gyrA relA1	Our lab
Plasmids		
pESC-HIS-(tLs-tCPT1)	pESC-HIS, (P_{PGK1}-*tLS*- T_{CYC1}),(P_{TEF1}-*tCPT1*- T_{CYC1}) , *AmpR*	This study
gRNA	*HygR*； 2μ ori with P_{TEF1},P_{T3},P_{AmpR}; *AmpR*; P_{SNR52}-sgRNA-T_{SUP4}	Our lab
X4- gRNA	gRNA plasmid carrying *X4* sgRNA	This study
X3-gRNA	gRNA plasmid carrying *X3* sgRNA	This study
XII2- gRNA	gRNA plasmid carrying *XII2* sgRNA	This study
XI3- gRNA	gRNA plasmid carrying *XI3* sgRNA	This study
X2- gRNA	gRNA plasmid carrying *X2* sgRNA	This study
pESC-HIS	pESC,P_{HIS3}-*HIS3*- T_{CYC1}, *AmpR*	Our lab
pESC-HIS-(X2-E20^{WW})	pESC-HIS, (P_{TEF1}- *ERG20^{WW}* - T_{CYC1}), *AmpR*	This study
pESC-HIS-(X2-E20^{WW}-E13)	pESC-HIS-(X2-ERG20^{WW}), (P_{TEF1}- *ERG13* - T_{CYC1}) , *AmpR*	This study
pESC-HIS-(X2-E20^{WW}-E13-E12)	pESC-HIS-(X2-ERG20^{WW}-ERG13), (P_{PGK1}- *ERG12* - T_{CYC1}) , *AmpR*	This study

X2Y-F/R, TEF1-F/R, ERG20WW1-F/R, ERG20WW2-F/R, ERG20WW3-F/R were used to amplify the upstream and downstream homology arms of the *X2* locus, the promoter TEF1 fragment and the farnesyl pyrophosphate synthase mutant (*ERG20$^{F96W\text{-}N127W}$*, *ERG20^{WW}*) fragment from the genome of strain SY00, respectively. The *ERG20^{WW}* expression cassette with sequences upstream and downstream of the X2 locus was obtained by overlapping PCR and constructed as pESC-HIS-(X2-E20WW). The *TEF1* gene promoter and the *ERG13* gene fragment were obtained from the SY00 genome using primers TEF1-F/R and E13-F/R and constructed as plasmid pESC-HIS-(X2-E20WW-E13). The *PGK1* gene promoter fragment was obtained from plasmid pESC-HIS-(tLs-tCPT1) using primer PGK1-F/R; the ERG12 gene fragment was obtained from the SY00 strain genome using primer E12-F/R and constructed as plasmid pESC-HIS-(X2-E20WW-E13-E12). gRNA plasmids were all constructed using reverse PCR, and the corresponding PAM sequences are shown in supplementary Table S2. The plasmids were constructed using a one-step cloning kit (Novozymes, China).

Strains construction

The limonene expression cassette (P_{PGK1}-tLS- T_{CYC1})-(P_{TEF1}-tCPT1- T_{CYC1}) was amplified from the plasmid pESC-HIS-(tLs-tCPT1) with the primers TYLim-F/R. X4Lim, X3Lim and XII2Lim expression cassettes were constructed by overlapping PCR, and cassette homology arm sequences were obtained from the *S cerevisiae* genome using primers X4Z-F/-R, X4Y-F/R, X3Z-F/-R, X3Y-F/R, XII2Z-F/-R, XII2Y-F/R. Primers XI3Z-F/R, XI3Y-F/R, tHMG1-F/R,

and CYC1-F/R were used to amplify the upstream and downstream homologous sequence fragments of the *XI3* locus, the truncated *HMG1* gene fragment, and the *CYC1* terminator fragment, respectively, from the genome of strain SY00, and the overlapping PCR was used to obtain the XI3tHMG1 expression cassette with homologous sequences at the *XI3* locus. The expression cassettes ERG20^{WW}, ERG20^{WW}-ERG13 and ERG20^{WW}-ERG13-ERG12 with homologous sequence at the *XI2* locus was obtained from plasmids pESC-HIS-(X2-ERG20^{WW}), pESC-HIS-(X2-ERG20^{WW}-ERG13) and pESC-HIS-(X2-ERG20^{WW}-ERG13-ERG12) using primers X2Z-F and X2Y-R, respectively. The above gene expression cassettes were integrated into the corresponding loci on the chromosome to obtain a series of strains (Table 1) using the CRISPR/Cas9 system mediated by lithium acetate transformation. Transformants were initially screened using YPD medium containing G418(700 μg/mL) and hygromycin (650 μg/mL) and then confirmed by molecular identification.

Determination of limonene production in recombinant strains

Shake flask fermentation was performed. Single colonies of yeast cells were collected in 5 mL YPD liquid test tubes for activation at 30°C, 180 rpm for 12 h. Yeast cells (1×10^7 cfu) of were transferred to 250 mL triangular conical flasks containing 50 mL of YPD liquid and 10% (v/v) dodecane was added as an organic extractant for a two-phase fermentation, at 30°C, 200 rpm, for 4 d.

Limonene Detection Method

The fermentation broth was centrifuged at 8000 rpm to collect the upper extracted organic phase, and an appropriate amount of anhydrous sodium sulphate was added to remove the water, which was then filtered through a 0.22 μm membrane and analyzed by GC. GC conditions: Shimadzu GC-2010 PlusAF system equipped with an HP-5 column (30 m×0.25 mm×0.25 μm); the carrier gas was N2 at a flow rate of 3 mL/min, and the flow rate of hydrogen was 35 mL/min, the injection volume was 1 μL, the split ratio was 1:20, the gas chamber temperature was 250°C, and the tail-blow flow rate was 30 mL/min. The carrier gas was N2 at a flow rate of 3 mL/min, hydrogen at a flow rate of 35 mL/min, air at a flow rate of 350 mL/min, and a tail blow at a flow rate of 30 mL/min. The injection volume was 1 μL, the split ratio was 1:20, and the temperature of the gasification chamber was 250 °C, and the temperature of the detector was 250 °C. The initial temperature of the column was 100 °C, which was maintained for 1 min, and then it was increased to 110 °C at 10 °C/min, which was maintained for 3 min, then it was increased to 180 °C at 30 °C/min, which was maintained for 2 min. The column temperature was 100°C for 1 min, then increased to 110°C at 10°C/min for 3 min, and then increased to 180°C at 30°C/min for 2 min.

Sake fermentation

Sake fermentation methods include simultaneous saccharification fermentation (SSF) and separate hydrolysis and fermentation (SHF) (29, 30). Simultaneous saccharification fermentation involves the production of amylase by fungi such as *A. oryzae* to hydrolyze starch into fermentable sugars, while yeast utilizes the fermentable sugars for alcoholic fermentation in an anaerobic environment. Separate hydrolysis and fermentation requires thorough saccharification and hydrolysis of the starch in the fermented raw material before the yeast is accessed for alcoholic fermentation to ensure optimal conditions for each step, and the SHF method was used in this study. A single colony of sake yeast was inoculated into a test tube containing 5 mL of YPD liquid for activation at 30°C, 180 rpm for 12 h. After activation, the cells were transferred to a 500 mL triangular conical flask containing 250 mL of YPD liquid at 30°C, 180 rpm for 24 h. The yeast was inoculated into the semi-solid fermentation medium with rice at 1×10^7 cfu/g rice, and a gas check valve was installed to control the gas flow in and out and to record the initial weight. The fermentation was carried out at 30°C and 180 rpm for 24 h to allow the yeast to rapidly consume oxygen and adapt to the fermentation environment. The fermentation was then transferred to a 30°C incubator and weighed every 24h and the weight loss was recorded as CO_2 emission. In other parallel experiments, the incubator placed at 15~20°C.The CO_2 emission was used to monitor the degree of fermentation. When the total amount of CO_2 emission reached 30 g / 100 g rice (DW), the fermentation was considered to be at the end of the fermentation period and the residual glucose was measured. When the residual glucose was less than 25 g/l, the fermentation was stopped and the alcohol content was measured.

Glucose Test Methods

Reducing sugars of the fermentation broth were determined using Ferring's reagent according to GBT13662-2018 (31).

Alcohol Testing

Alcoholic strength of the fermentation broth was determined by the alcoholic gravity method according to GB/T 13662-2018 (31).

Sensory Evaluation of Sake

The sensory evaluation of sake wine is performed in four aspects: color, aroma, taste and style. The temperature of the wine sample is controlled between 15°C and 25°C, and the clarity and colour of the wine are observed in a bright place. The glass is gently shaken to smell the aroma. A small amount of the wine sample is introduced, and the taste is waited until it is clear, then swallowed and the texture and aftertaste are tasted.

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Sake Ingredient Testing

The sample was distilled in a distillation flask, and the distillate was collected to 50 mL, transferred to a separating funnel and extracted with 100 mL, 50 mL and 30 mL of dichloromethane. The organic phase was collected and water was removed by adding an appropriate amount of anhydrous sodium sulfate, and then concentrated by spinning at 22 ℃ and 80 rpm to 0.5 mL, and analyzed by GC-MS (Agilent 8890/7250 GC/Q-TOF system). GC conditions: capillary column DB-5MS (30 m*0.25 mm*0.25 μm); inlet temperature of 280 ℃; programmed temperature increase of 40 ℃ for 2 min, and then increase the temperature to 250 ℃ at 20 ℃/min for 5 min; injection volume of 0.1 μL; shunt ratio of 200:1; carrier gas He, flow rate of 1.0 mL/min; MS conditions: the water was removed from sodium sulfate, and then concentrated to 0.5 mL by spinning at 22 ℃ and 80 rpm, and then analyzed by GC-MS (Agilent 8890/7250 GC/Q-TOF system). /MS conditions: full scan acquisition mode, mass range m/z 25-600 Da; electron bombardment energy 70 eV; source temperature 200 ℃; quadrupole temperature 150 ℃.

Results

Construction of a limonene synthesis pathway in sake yeast

We constructed the limonene synthesis pathway in the sake yeast strain SY00 using CRISPR/Cas9 technology. Strains with different limonene yields were constructed using strategies to increase the copy of the limonene expression cassette, weaken the downstream competing pathway of MVA, and strengthen the upstream pathway of MVA (Fig. 1). The limonene expression cassette X4Lim was integrated into the chromosome X4 locus, and an engineered strain SY01 showed a limonene yield of 2.3 μg/L, indicating that the limonene synthesis pathway was successfully constructed. The strategies and engineered strains with limonene titers (Fig. 2) are described below:

1) Increase the copy of limonene expression cassette

The MVA pathway involves a variety of flavour metabolites, and modification of this pathway affects the aroma and compositional changes of wine, with priority given to the strategy of increasing limonene production by

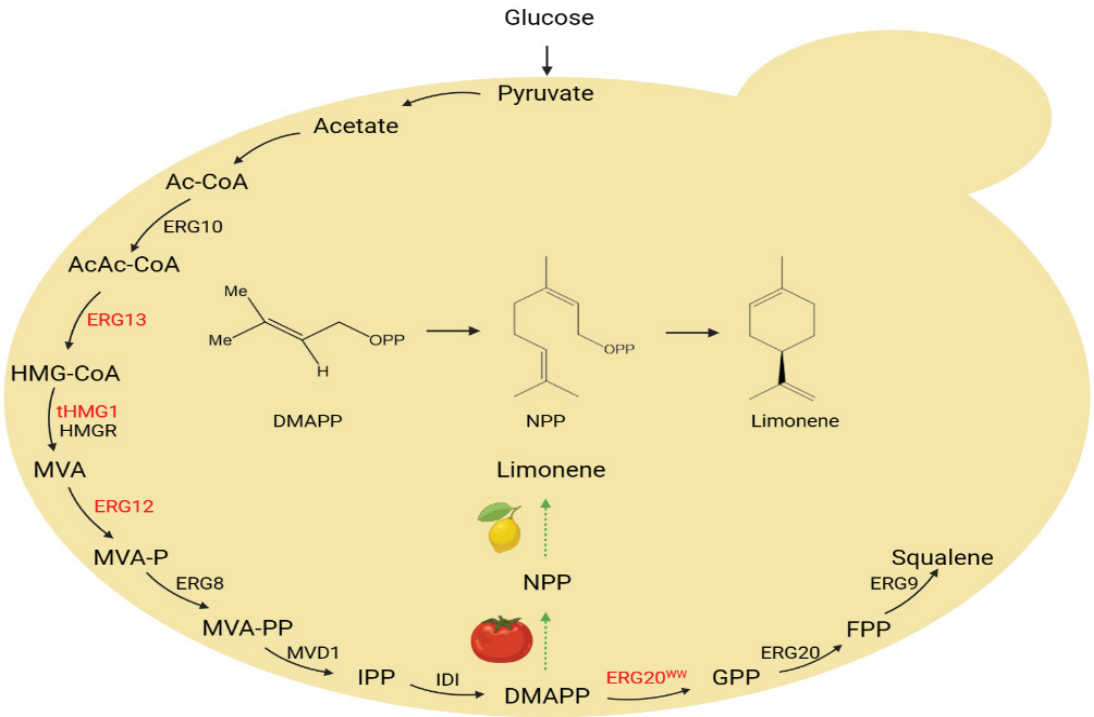

Figure 1: Schematic diagram of genetically modified sake yeast for the production of sake with the bioactive molecule limonene. Black arrows represent native pathways; green dashed lines represent exogenous pathways (or genes); overexpressed genes are highlighted in red. Metabolite abbreviations: Ac-CoA, Acetyl coenzyme A; AcAc-CoA, Acetoacetyl coenzyme A; HMG-CoA, Hydroxymethylglutaric acid monoacyl coenzyme A; MVA, mevalonate; MVA-P, Mevalonate phosphate; MVA-PP, Mevalonate diphosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; NPP, neryl diphosphate; GPP, geranyl diphosphate; FPP, isopentenyl pyrophosphate. Gene abbreviations: ERG10, Acetoacetyl coenzyme A sulfatase; ERG13, hydroxymethylglutaryl coenzyme A; tHMG1, truncated HMG-CoA reductase; HMGR, HMG-CoA reductase; ERG12, Mevalonate kinase; ERG8, Mevalonate kinase; MVD1, Mevalonate-5-bisphosphate decarboxylase; IDI, IPP isomerase; tCPT1, truncated neryl diphosphate synthase from Solanum lycopersicum; tCILS, truncated limonene synthase from Citrus; $ERG20^{WW}$, mutated farnesyl diphosphate synthase; ERG20, FPP synthase; ERG9, squalene synthase.

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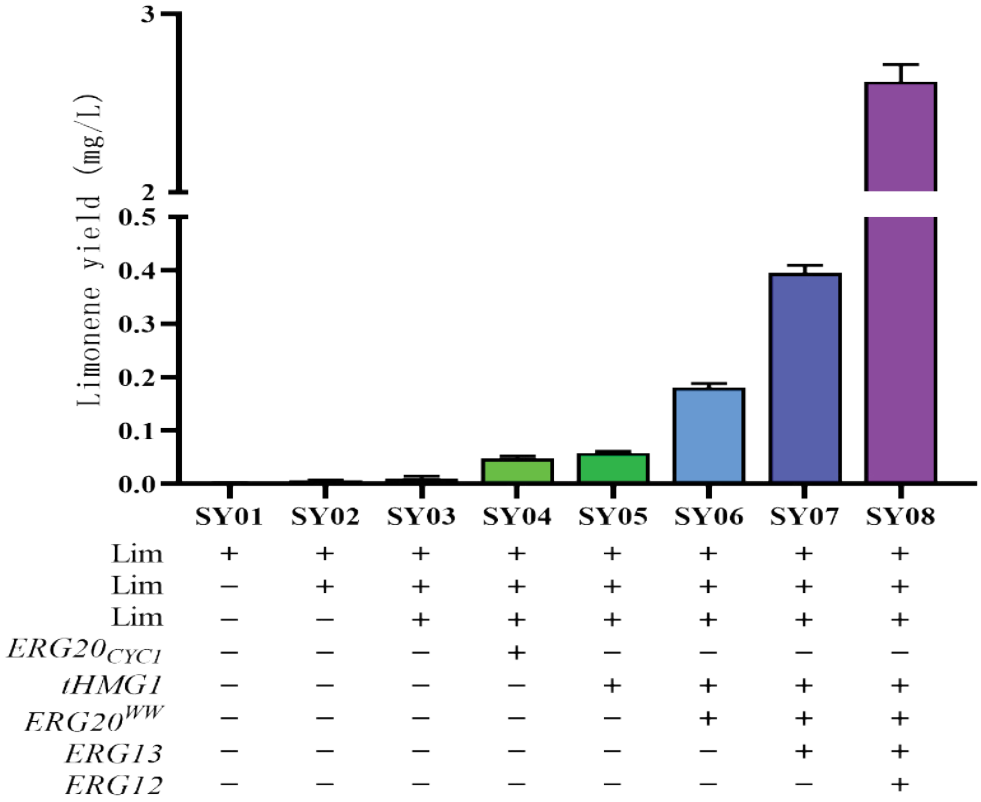

Figure 2: Production of limonene by recombinant strains

increasing the copy of the limonene expression cassette. The recombinant strains SY02 and SY03 were obtained by integrating the limonene expression cassette into chromosome *X3* and *XII2* of strain SY00, respectively, and the limonene titer was 6.9 μg/L and 11 μg/L, respectively, with a limited increase in yield.

2) Weak promoter P_{CYC1} weakens MVA downstream competition pathway

The recombinant strain SY04 was obtained using the weak promoter PCYC1 to replace the endogenous promoter of the *ERG20* gene of strain SY03, with a limonene yield of 49 μg/L, indicating that the attenuation of the downstream pathway of MVA improved limonene production, but a severe growth slowdown of strain SY04 was observed, and it was speculated that the attenuation of the downstream pathway affected the synthesis of squalene, a key substance for cell growth, and thus cell biomass. Due to the low cell biomass and the specificity of the application in this study, the inducible promoters such as the glucose-sensitive promoter P_{HXT1} and the Fe^{2+}-deterrent promoter P_{FET3} could not be used to dynamically regulate the downstream pathway during sake brewing, so the regulation of the competitive downstream pathway was no longer considered.

3) Overexpression of truncated trihydroxy-3-methylglutarate (HMG-CoA) reductase (*tHMG1*)

The truncated *HMG1* gene is the key to terpene modification. Integration of the *tHMG1* expression cassette into the *XI3* locus of strain SY03 resulted in strain SY05 with a limonene yield of 59 μg/L, a slight improvement over strain SY03.

4) Overexpression of mutant farnesyl pyrophosphate synthase ($ERG20^{F96W-N127W}$)

The integration of an $ERG20^{F96W-N127W}$ mutant at the *X2* locus of strain SY05 was used to reduce the use of farnesyl diphosphate (FPP) for sterol biosynthesis, and strain SY06 was obtained with a limonene yield of 0.18 mg/L, which was further improved compared to the limonene yield of strain SY05.

5) Overexpression of hydroxymethylglutaryl coenzyme A synthase (*ERG13*)

ERG13 is a key enzyme in the MVA pathway of *S. cerevisiae* that catalyses the synthesis of hydroxymethylglutaryl coenzyme A (HMG-CoA) from acetoacetyl coenzyme A. By regulating the expression level or enzyme activity of *ERG13*, the production of HMG-CoA in the MVA pathway can be

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regulated, thereby increasing the carbon flux of the MVA pathway. The *ERG13* gene expression cassette was increased at the *X2* locus of strain SY06 to obtain strain SY07, which had a limonene yield of 0.4 mg/L, and the limonene yield was greatly increased compared to strain SY06.

6) Overexpression of mevalonate kinase (*ERG12*)

Mukherjee et al (33) identified *ERG12* as a key enzyme gene affecting the flux of the MVA pathway and its overexpression affects the amount of pentenyl pyrophosphate (IPP) production.IPP is an intermediate of the MVA pathway used for the synthesis of terpenoids such as terpenic alcohols, aldehydes, ketones and acids. These terpenoids play an important role in the metabolism of *S. cerevisiae*, for example, by influencing factors such as the aroma and flavour of wine. Finally, we increased the *ERG12* gene expression cassette at the *X2* locus of strain SY07 to obtain strain SY08 (Supplementary file Fig. S1), which had a limonene yield of 2.6 mg/L, a significant increase in limonene production compared to strain SY07. It was hypothesised that the previous overexpression of *tHMG1*, *ERG20*$^{F96W\text{-}N127W}$, and *ERG13* had caused the strain to accumulate a higher amount of mevalonate, and that the overexpression of *ERG12* relieved this part of the pressure.

Effect of different limonene titres on sake flavor

We selected the original strain SY00 and limonene-producing strains SY06, SY07 and SY08 for sake brewing. Sensory evaluation of the samples showed that the colour, aroma and style of the samples brewed with strain SY00 were within the characteristics of conventional rice wine. Samples brewed with strain SY06 showed a slight improvement in fruity flavour. Strain SY07 showed a further improvement in the fruity flavour, and the rice wine style was in harmony with the fruity components. On the other hand, the samples brewed with strain SY08 further improved the fruity flavour and the coordination between rice wine and fruity flavours, receiving the highest score of 92 points (Table 2). These results suggest that sake flavour was further improved with the increased limonene synthesis capacity. However, these strains differed in the intensity of MVA pathway modification in addition to the limonene synthesis capacity. To further determine whether the limonene synthesis pathway was the main influence on flavour rather than the enhancement of the MVA pathway, we constructed a strain SY09 with the same intensity of MVA pathway modification as strain SY08 and without the limonene synthesis pathway, and then performed sensory evaluations on the wine samples brewed by this strain and found that the wine samples brewed by this strain had some fruity aroma but were slightly astringent and bitter in taste.

Fermentation performance of recombinant strains

To study the effect of genetically modified strains on fermentation performance, three aspects were analysed: CO_2 emission, alcoholic strength determination and residual glucose. In terms of fermentation speed, as the number of overexpressed genes increased, the fermentation speed of sake gradually decreased, with the original strain SY00 completing brewing in 9 days, the highest limonene yielding strain SY08 taking 11 days, and the control genetically modified strain SY09 taking 13 days (Table 3), suggesting that there is a large accumulation of a certain intermediate metabolite in the MVA pathway, enhanced by the overexpressed genes, which has a toxic or inhibitory effect on cells, causing the slow growth of the strain.

Table 2: Sensory evaluation of wine samples brewed with sake yeast strains

Strains	Liquor characteristics	Score
SY00	Slightly yellowish and transparent, with the colour and lustre that rice wine should have; pure rice wine aroma, fruity aroma is not obvious; full body, acidity and astringency are appropriate; rice wine style is obvious, and the body is more harmonious.	80
SY06	Slightly yellowish and transparent, with the colour and lustre that rice wine should have; pure rice wine aroma, with a more pronounced fruity aroma; fuller and richer body; obvious rice wine style, with a harmonious body.	82
SY07	Slightly yellowish and transparent, with the colour and lustre that rice wine should have; its aroma is pure, fruity and harmonious; its body is full, smooth and harmonious; its acidity and astringency are appropriate; its style is clear and its fruity components are harmonious.	88
SY08	Light yellow and transparent, with the colour and lustre that rice wine should have; its aroma is pure, fruity and harmonious; its body is full, smooth and harmonious; its acidity and astringency are appropriate; its style is clear and harmonious with its fruity components.	92
SY09	Slightly yellowish and transparent, with the colour and lustre that rice wine should have; pure rice wine aroma, with a more pronounced fruity aroma; full-bodied, slightly astringent and slightly bitter; obvious rice wine style, with a more harmonious body.	83

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Table 3: Fermentation performance of sake yeast strains with different limonene yields

Strains	CO_2 cumulative weight loss (g)	Alcoholic content (20°C) (%/vol)	Residual glucose (g/L)	Days of fermentation (d)
SY00	28.7	13.75	24.28	9
SY06	28.47	14.03	23.56	10
SY07	28.82	13.34	24.13	10
SY08	28.56	12.8	25.2	11
SY09	27.57	13.65	24.59	13

Sake Sample Composition Identification

To analyse the causes of changes in the flavour of sake brewed with recombinant strains, samples of sake brewed with the original strain SY00, the limonene strain SY08 and the genetically modified control strain SY09 were analysed by GC-MS, and 16 common flavour compounds were selected for comparative analysis (Table 4). Among the four lipids, compared to the original strain SY00, the relative levels of ethyl acetate and phenylethyl acetate were slightly increased in samples brewed by strain SY08, and the relative levels of ethyl caproate and ethyl benzoyl formate were slightly decreased, with ethyl benzoyl formate below the detection threshold. In strain SY09, the relative levels of these four lipids decreased, except for phenylethyl acetate, for which the relative levels increased slightly.

Table 4: Relative contents of major aroma components in samples of sake brewed with different yeast strains

Chemicals	Molecular formula	SY00	SY08	SY09
Ethyl acetate	$C_4H_8O_2$	3.00%	3.28%	2.59%
Isobutanol	$C_4H_{10}O$	6.98%	6.67%	13.74%
3-Hydroxy-2-butanone	$C_4H_8O_2$	0.00%	0.39%	0.58%
3-Methyl-3-buten-1-ol	$C_5H_{10}O$	0.00%	0.40%	1.12%
Isoamyl alcohol	$C_5H_{12}O$	38.22%	24.55%	34.77%
2-Methylbutanol	$C_5H_{12}O$	18.77%	25.87%	24.77%
Isobutyric acid	$C_4H_8O_2$	0.69%	0.16%	0.46%
Isopentenol	C_5H1_0O	0.00%	0.34%	0.81%
3-Pentanol	$C_5H_{12}O$	0.20%	0.32%	0.16%
L-(+)-lactic acid	$C_3H_6O_3$	0.00%	0.29%	0.22%
2-Methylbutyric acid	$C_5H_{10}O_2$	0.00%	0.39%	0.30%
Ethyl benzoylformate	$C_{10}H_{10}O_3$	0.19%	0.00%	0.07%
Ethyl caproate	$C_8H_{16}O_2$	0.67%	0.13%	0.07%
2-Methylhexanoic acid	$C_7H_{14}O_2$	0.47%	2.14%	0.64%
Phenethyl alcohol	$C_8H_{10}O$	30.71%	34.84%	19.53%
Phenethyl acetate	$C_{10}H_{12}O_2$	0.11%	0.23%	0.19%

Comparative analysis of brewing samples of strain SY08 with the original strain SY00 for seven alcohols revealed the following characteristics: the relative contents of isobutanol, 3-methyl-3-buten-1-ol and isopentyl alcohol decreased, with the relative content of isopentyl alcohol decreasing by up to 14%; the relative contents of 2-methylbutanol, isopentenyl alcohol, 3-pentanol and phenylethanol increased, with the relative content of 2-methylbutanol increasing by about 7% and the relative content of phenylethanol increasing by about 4%. The relative content of 2-methylbutanol, isopentenol, 3-pentanol and phenethyl alcohol increased, with the relative content of 2-methylbutanol increasing by about 7% and that of phenethyl alcohol by about 4%. In general, the total amount of higher alcohols decreased, and the decrease in the total amount of higher alcohols helped to reduce the "headiness", and the increase in the relative content of phenylethanol, which is one of the main aromas of sake flavour, helped to improve the quality of sake. In the samples brewed with strain SY09, the total relative content of higher alcohols increased and the relative content of phenylethanol, the main aroma of sake, decreased.

Among the remaining five substances, the relative contents of 3-hydroxy-2-butanone, L-(+)-lactic acid, 2-methylbutyric acid and 2-methylhexanoic acid increased in the brewing sample of strain SY08, while the relative content of isobutyric acid decreased, and the most significant change was in 2-methylhexanoic acid, which, as a substance with a creamy fatty taste, has a positive effect on the improvement of sake quality with an increase in the content of 2-methylhexanoic acid. In the brewing samples of strain SY09, the relative contents of these five substances were slightly increased or decreased, with no major changes. It is noteworthy that limonene was not detected in the final brewing sample of strain SY08, but the yield of limonene in the organic phase was found to be 15.8 mg/L after dodecane extraction during sake brewing, and it was hypothesised that limonene might be volatilised during sake fermentation along with carbon dioxide emission.

Discussion

In the field of sake brewing, improving flavour and quality has always been the goal of brewers. Bioactive molecule research has been a hot research topic in recent years, and the brewing field has begun to focus on how to maintain health and prevent disease through the intake of bioactive molecules from alcoholic beverages. This study is the first to successfully construct the limonene synthesis pathway in sake yeast, which provides a new strategy for metabolically modifying yeast strains and applying them to sake brewing and demonstrates the potential of limonene as a flavour modifier and bioactive molecule. Limonene is a monoterpenoid widely found in plants and has various physiological functions

such as antioxidant, anti-inflammatory, antibacterial and anticancer, etc. Limonene is also an important flavouring agent, imparting a fresh lemony flavour to foods and beverages. Sake is a traditional Japanese rice wine and its flavour is mainly composed of compounds such as alcohols, esters, higher alcohols and phenols. The results of this study showed that the construction of a limonene synthesis pathway in sake yeast was able to reduce the content of higher alcohols in sake, increase the fruity aroma of sake, and improve the flavour and mouthfeel of sake. This may be because limonene synthesis in yeast consumes precursors such as pyrophosphate and acetyl-coenzyme A, thereby reducing the synthesis of higher alcohols.

The strategy used in this study has some generality and can be used to construct different terpene synthesis pathways in other yeasts to produce alcoholic products with specific flavours and functions. Terpenoids are a class of natural products with diverse structures and functions that are widely distributed in plants, animals, microorganisms and other organisms, and the extension of terpene synthesis pathways in yeast for the production of different types of terpenoids can be achieved by introducing terpene synthases from other sources into the yeast MVA pathway.

There are still some limitations to this study, such as there is still room for improvement in the production of limonene, and the effect of the limonene synthesis pathway on other aroma compounds in sake has not been studied in depth. The results in this paper show that limonene production is related to the number of integrated limonene expression cassettes in yeast, the strengthening of the MVA pathway and the weakening of the competitive pathway, and that limonene produced by the modified strains may volatilise during sake brewing. Therefore, the retention of appropriate amounts of limonene in sake is an important direction for future research, and strategies such as the optimisation of limonene metabolic pathways and the development of new sake brewing processes could be adopted. In addition, the physiological activity and safety of limonene in sake need to be further verified. The content and bioavailability of limonene in sake, as well as its interaction with other components in sake, may affect the physiological effects and side effects of limonene. Therefore, more in-depth and detailed studies on the bioactivity and safety of limonene in sake are needed in the future to ensure the efficacy and safety of limonene application in sake. In conclusion, the study in this paper provides a new approach and evidence for improving the flavour of sake by constructing a limonene synthesis pathway in sake yeast, and also opens up a new field and direction for the application of terpenoids in sake.

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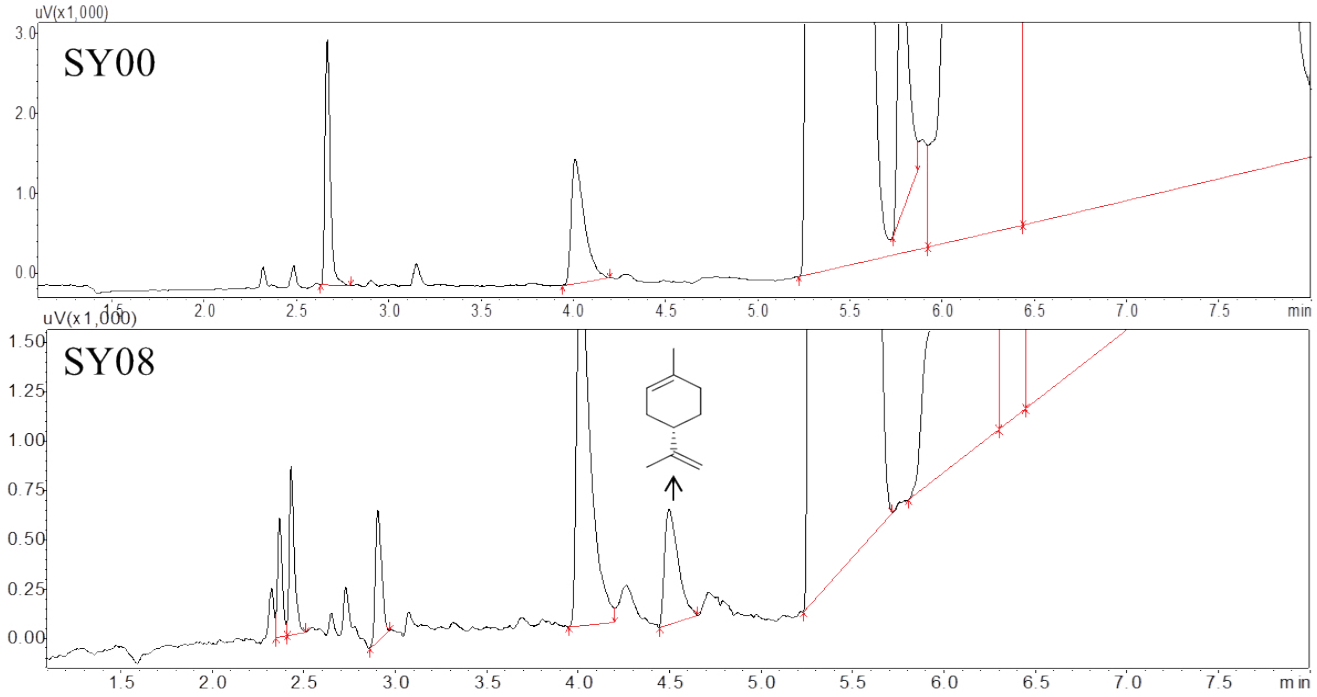

Figure S1: Gas chromatography analysis profiles of the fermentation broths of the wild strain SY00 and the engineered strain SY08. In strain SY08, limonene peaked at 4.5 min.

Table S1: Primers used in this study

Primers	Sequences (5'→3')	Lengths (bp)
X2Z-F	GTGACTGTCCGCTGGAGCAG	814
X2Z-R	GGGTTTTCTTTCACGACACACC	
X2Y-F	GGCTAAGGTCACTTCTTCGTTTCC	778
X2Y-R	CTAGAGAACGAGAGGACCCAAC	
TEF1-F	CAAGCAACAGGCGCGTTGGA	501
TEF1-R	TTTGTAATTAAAACTTAGATTAGATTGC	
ERG20WW1-F	ATGGCTTCAGAAAAAGAAATTAGGAGAGAG	301
ERG20WW1-R	CATCGGCGACCAACCAGTAAGCCTGCAAC	
ERG20WW2-F	GTTGCAGGCTTACTGGTTGGTCGCCGATG	127
ERG20WW2-R	CTCTAACATGAATGCGTCCCAGATGGCAATTTCCCCAAC	
ERG20WW3-F	GTTGGGGAAATTGCCATCTGGGACGCATTCATGTTAGAG	963
ERG20WW3-R	GAGATGAGGTCGTTGCTTTTCC	
E13-F	ATGAAACTCTCAACTAAACTTTGTTGGT	1742
E13-R	CACGTCGGGGTTATGAATGAG	
PGK1-F	GCACAGGCGCTACCATGAGA	941
PGK1-R	TGTTTTATATTTGTTGTAAAAAGTAG	
E12-F	ATGTCATTACCGTTCTTAACTTCTGCAC	1461
E12-R	CTGTCTTCGAAAGCTGAATTGATACTACG	
TYLim-F	GCACAGGCGCTACCATGAGA	4737
TYLim-R	CATTACGACCGAGATTCCCGG	
X4Z-F	CCATCCTCGGGAAGACAGTC	883

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X4Z-R	CGACGGCTGCCATTAGTCAG	
X4Y-F	GAAGTAACAGGCGTGTGCAC	1077
X4Y-R	GCGTTTCCAGTCGCCTATCC	
X3Z-F	GGGTGATGCCTCTTTAGGCG	862
X3Z-R	AGGCCAAGCACATCGTTTAG	
X3Y-F	CGAGAGCCGACATACGAGAC	981
X3Y-R	ACGACGACCGCGATATGACC	
XII2Z-F	ACATAATGTGCCGTTTGAAGC	1020
XII2Z-R	TGCCCGTACATGACATGGTC	
XII2Y-F	GTCTCACTTACTGGGCGACG	981
XII2Y-R	AGTCCAAGCGGGATAAGGTTC	
XI3Z-F	GAATGGGAACAGTCGCGTCT	1009
XI3Z-R	ACGCTTGGCGTCAATATCCT	
XI3Y-F	CCTTACGTGGATTGAGCCAGC	971
XI3Y-R	TGGTAGGCTTTGTTGTGGGC	
tHMG1-F	ATGGACCAATTGGTGAAAACTGAAG	1578
tHMG1-R	TTAGGATTTAATGCAGGTGACGGAC	
CYC1-F	TCATGTAATTAGTTATGTCACGC	248
CYC1-R	GCAAATTAAAGCCTTCGAGCG	

Table S2: Integration sites used in this study

S. cerevisiae loci	Cas9 target sites	PAM
X2	TAGGAAGAGAGTAGTGGGCG	CGG
X4	AAGGTTGTTGAGGGAACAC	TGG
X3	GATCGCCGAATGGCACGCGA	GGG
XII2	GAGTTTCATAACGCGTTACA	CGG
XI3	ATATGTCTCTAATTTTGGAA	GGG