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Rice wine fermentation from Thai low-price rice using *saccharomyces cerevisiae* mutant with enhanced fatty acid ethyl ester production

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ABSTRACT

Thai traditional rice wine, known as Sato, is produced from glutinous rice using a mixed-culture starter called Lookpang. This traditional method often suffers from variability and inconsistent flavor profiles. To address these issues, the study aimed to improve the quality of Thai traditional rice wine by utilizing a defined starter culture composed of high-fatty acid ethyl esters (FAEEs)-producing *Saccharomyces cerevisiae* and *Aspergillus oryzae*. The fermentation process followed a methodology similar to that used in Japanese sake production, aiming to achieve more consistent quality and flavor profiles in the final product. Furthermore, this study involved fermenting rice wine using low-cost Thai rice to enhance its value. The parental *Saccharomyces cerevisiae* strain, 312WT, was isolated from Thai Sugar Industry Co., Ltd., and demonstrated fermentation performance comparable to the commercial wine yeast strain EC-1118. Subsequently, conventional mutagenesis was applied to 312WT, resulting in a mutant strain, 312/6, which overproduces fatty acid ethyl esters (FAEE). Rice wine fermented with the 312/6 strain exhibited the highest concentrations of ethyl hexanoate (28.55 ± 7.91 µg/kg, OAV = 29) and ethyl octanoate (207.22 ± 51.65 µg/kg, OAV = 41). These compounds contribute sweet and sour apple flavors to beverages, respectively. Genomic DNA analysis, compared to the parental strain 312WT, demonstrated that 312/6 harbored several mutations in the fatty acid ethyl ester (FAEE) biosynthesis pathway genes (*FAS1*, *FAS2*, *ACC1*, *EHT1*, and *EEB1*). These genetic changes could account for the overproduction of FAEEs.

Contribution/ Originality: This study contributes to the existing literature by demonstrating the application of a non-GMO FAEE-overproducing *Saccharomyces cerevisiae* mutant in the fermentation of Thai rice wine. It is one of the few studies investigating FAEEs enhancement in low-cost Thai rice using a defined starter system to improve aroma quality.

1. INTRODUCTION

Thailand is a major agricultural country, and rice remains one of its most important crops. The country's advantageous geographical conditions enable substantial rice production. Rice exports constitute a significant source

of revenue for Thailand. In 2023, the nation was the third-largest exporter of rice worldwide, with approximately 8.76 million tons [1, 2]. However, demand in the export market is focused on premium-grade rice, which presents marketing challenges for low-priced rice varieties. Processing rice products is the most effective method for increasing the value of specific low-cost varieties. Rice wine is an alcoholic beverage produced from rice, and its terminology varies across different countries.

Thai rice wine, or sato, is produced from Lookpang, which consists of molds, yeasts, and lactic acid bacteria. Its microbial composition varies widely because it originates from natural sources and is prepared by mixing flour, water, herbs, and a small portion of old starter before sun-drying for several days. The absence of standardization often leads to inconsistent microbial populations, frequent contamination, and the loss of desirable strains during repeated starter production. Consequently, the traditional method frequently yields non-uniform product quality and is unsuitable for industrial-scale production [3].

In contrast, the Japanese sake industry has achieved stable, large-scale production through the use of defined starter systems. Modern sake production commonly employs the Sokujo-style moto, which utilizes pure cultures of *Aspergillus oryzae* and *Saccharomyces cerevisiae*, with added lactic acid instead of lactic acid bacteria. This approach results in faster, more controllable, and cost-efficient fermentation [4, 5]. Premium-grade sake is particularly valued for its fruity and floral notes [6, 7]. The FAEEs, especially ethyl hexanoate and ethyl octanoate, contribute to this fruitiness [8]. FAEEs biosynthesis in *S. cerevisiae* begins with the production of malonyl-CoA by the key enzyme acetyl-CoA carboxylase (encoded by *ACC1*) [9], followed by a condensation reaction catalyzed by fatty acid synthase encoded by *FAS1* and *FAS2*, to produce fatty acyl-CoA. Subsequently, Eht1 and Eeb1 catalyze the esterification of fatty acyl-CoA with ethanol to form FAEEs (see also Figure 1) [10].

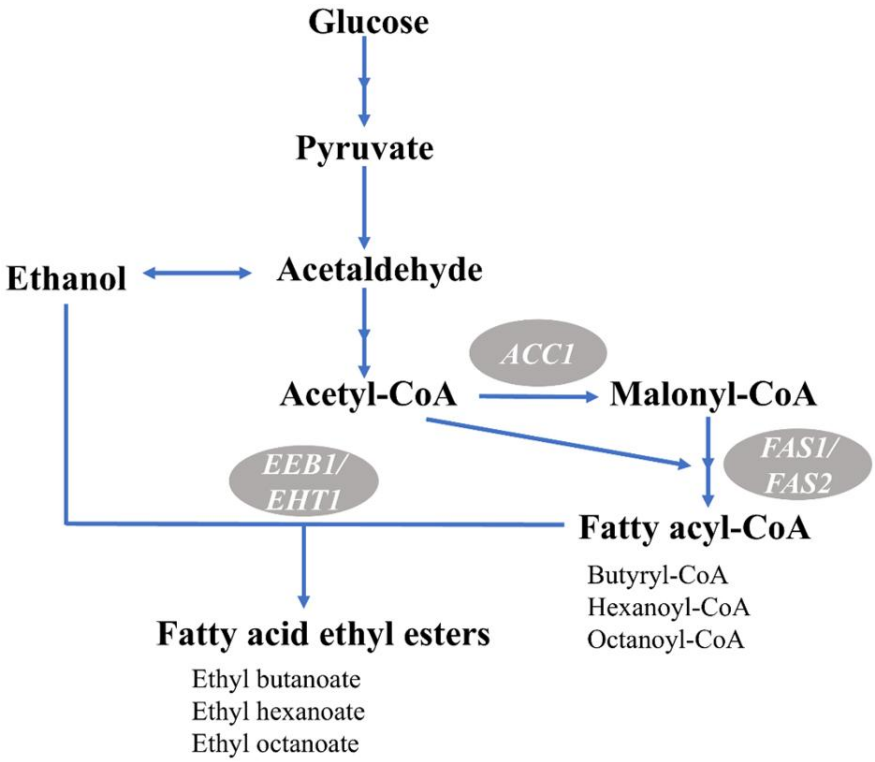

Figure 1. Schematic diagram of the fatty acid ethyl ester (FAEE) biosynthesis pathway in *S. cerevisiae*.

The initial step in FAEE synthesis involves the formation of malonyl-CoA through the carboxylation of acetyl-CoA, a process catalyzed by acetyl-CoA carboxylase, encoded by *ACC1*. Subsequently, acetyl-CoA and malonyl-CoA are directed to the active site of the fatty acid synthase complex, which is encoded by *FAS1* and *FAS2*. This interaction results in the elongation of acyl chains, leading to the production of butyryl-CoA, hexanoyl-CoA, and octanoyl-CoA,

respectively. In the next step, these acyl-CoA molecules react with ethanol to form fatty acid ethyl esters, a reaction catalyzed by ethanol O-acyltransferase enzymes encoded by *EHT1* and *EEB1*.

The levels of these esters, along with higher alcohols and acetate esters, depend strongly on the metabolic characteristics of the yeast strain, and they play a central role in shaping the flavor of rice wines [11]. In general, wild-type *S. cerevisiae* produces a low amount of FAEEs compared to other flavor compounds. [12]. Several yeast strains with enhanced ethanol or flavor production have been reported, including the *S. cerevisiae* isolate 35a14 from Okinawa, which produces vanillin during fermentation [13]. The previous research demonstrated that conventional mutagenesis (non-GMO approaches) could be used to improve aroma formation in fermented beverages. Despite extensive progress in sake yeast development, no published studies have applied a sake-style defined starter system together with an FAEE-enhancing *S. cerevisiae* strain to the fermentation of low-priced Thai rice.

These gaps underscore the need for a systematic evaluation of whether a defined starter culture can improve the consistency and flavor quality of rice wine produced from low-priced Thai rice. Therefore, this study aims to develop yeast strains for rice wine fermentation using low-cost rice from Thailand. This work integrates a defined starter system comprising *A. oryzae* and a non-GMO, FAEE-overproducing mutant of *S. cerevisiae* to assess its impact on both fermentation performance and sensory quality. The parental strain 312WT demonstrated a strong ethanol-producing ability. It also produced higher levels of phenylethyl alcohol and isoamyl alcohol compared with the commercial wine strain *S. cerevisiae* Lalvin EC-1118™ [14].

The mutant strain 312/6 was obtained through UV mutagenesis followed by selection on a cerulenin-containing medium. This approach enriched mutants with altered fatty acid metabolism, which, in turn, increased the accumulation of FAEEs [15]. Because the method does not involve genetic modification, it remains suitable and acceptable for use in food-related industries [16].

2. MATERIALS AND METHODS

2.1. Strain and Culture Media

Aspergillus oryzae MSCU 0046 (Department of Microbiology, Faculty of Science, Chulalongkorn University) was used for the Koji rice preparation step. The mold was reactivated on potato dextrose agar (PDA) (2.4% Potato dextrose broth granulated and 2% glucose) for 7 days before collecting spore suspension [modified from Sivaramakrishnan et al. [17]]. The parental *Saccharomyces cerevisiae* strain used in this study is isolate 312WT, which was obtained from Thai Sugar Industry Co., Ltd. and deposited at the Department of Microbiology, Faculty of Science, Chulalongkorn University (accession no. MSCU 1613). The control strain employed was *S. cerevisiae* Lalvin EC-1118™, a commercial yeast commonly used for wine fermentation. All yeast strains were activated on YPD medium, which contains 1% yeast extract, 2% peptone, and 2% glucose. To support yeast growth in the sample, an enrichment medium was used, comprising 2% glucose, 0.3% polypeptone, 0.3% yeast extract, 3% ethanol, and 0.01% chloramphenicol, with a pH of 5.6. For isolation purposes, an isolation medium containing 10% glucose, 0.3% polypeptone, 0.3% yeast extract, and 0.01% chloramphenicol was employed. Yeast cultures and fermentation tests utilized Yeast Nitrogen Base (YNB) medium, which consists of 0.67% Yeast Nitrogen Base (Difco™) and 2% glucose. To screen for mutants resistant to cerulenin, synthetic dextrose (SD) medium was used, containing 0.17% Yeast Nitrogen Base without amino acids (Difco™), 0.5% ammonium sulfate, and 2% glucose, supplemented with 6 µg/mL cerulenin. When necessary, all culture media were solidified with 2% agar to facilitate handling and incubation.

2.2. Isolation of Parental Yeast Strains from Thai Sugar Industry

Isolation of desired yeast strains for rice wine production from the Thai Sugar Industry Co., Ltd. was performed as follows: 1 g of raw syrup sample was fermented in enrichment media and incubated at 30°C for 7 days under oxygen-limited conditions. The cell suspension was re-streaked to obtain a single colony for further studies.

2.3. Fermentation Test

A single colony of the yeast strain was inoculated into a 15 mL test tube containing 3 mL of YNB medium and incubated at 30°C with shaking at 200 rpm for 24 hours. Subsequently, 600 µL of the pre-culture was inoculated into a 15 mL test tube containing 12 mL of YNB medium supplemented with 25% glucose. Sample weights were measured every 24 hours over a 14-day period to quantify cumulative carbon dioxide loss. Statistical significance was evaluated using one-way ANOVA followed by Bonferroni's post hoc test ($p < 0.05$).

2.4. Yeast Species Identification

The identification of yeast species was conducted by the company. The parental yeast strain was sent to GIBTHAI COMPANY in Thailand and subsequently forwarded to Macrogen Company in Korea. Yeast genomic DNA was extracted using the boiling method with the Instagene matrix. Polymerase Chain Reaction (PCR) was performed to amplify a segment of the ITS region, utilizing the ITS4 primer (5'-TCCGTAGGTGAACCTGCGG-3') and the ITS5 primer (5'-TCCTCCGCTTATTGATATGC-3'). The resulting sequence data were analyzed through a BLASTn Homology Search (http://www.ncbi.nlm.nih.gov/blast), and the sequences were compared with those of related species to determine the yeast species accurately.

2.5. Ethanol Tolerance

To evaluate the ethanol tolerance of yeast strains, a single colony of the yeast was inoculated into a 15 mL test tube containing 3 mL of YPD medium and incubated at 30°C with shaking at 200 rpm for 24 hours. Subsequently, 500 µL of the pre-culture was transferred into a 50 mL test tube containing 10 mL of YPD medium supplemented with 12% and 15% ethanol. Samples were measured by optical density at 600 nm every 24 hours over a period of 3 days to monitor cell growth.

2.6. Isolation of Cerulenin-Resistant Mutant

The experimental model used in this study employed UV-induced random mutagenesis followed by cerulenin-based screening. Cerulenin has been widely applied as a selective agent to enrich mutants with enhanced fatty acid-derived ester production. In contrast to many previous studies that preferentially applied ethyl methanesulfonate (EMS), ultraviolet irradiation was adopted in this work [18].

The conventional mutagenesis method using ultraviolet (UV) irradiation was performed as described in the previous study, with slight modifications [19]. The yeast cells were pre-cultured overnight in YPD media. Afterward, they were harvested, washed twice with sterile distilled water, and centrifuged at 7,000 rpm at 4°C for 3 minutes to collect the cell pellet. The pellets were then diluted to concentrations ranging from 10^{-4} to 10^{-6} using sterile DI water. Subsequently, 1 mL of the cell suspension was transferred onto a sterile plate and exposed to UV radiation at 254 nm for 3 minutes from a distance of 25 cm, resulting in a survival rate of approximately 0.12%. Preliminary tests confirmed that the parental strain 312WT could not grow on SD agar supplemented with 6 µg/mL cerulenin. Therefore, growth on cerulenin-containing medium was used as the initial screening step to isolate mutants with potentially enhanced FAEE production. UV-treated cells were spread onto SD agar containing 6 µg/mL cerulenin and incubated at 30°C for 3 to 5 days.

All colonies on the selective medium were considered cerulenin-tolerant mutants. Their tolerance was confirmed by comparing growth of these mutants and the parental strain 312WT on SD agar with 6 µg/mL cerulenin in a single replicate. Each mutant was then tested for rice wine fermentation and FAEEs production. Among them, one strain exhibited the highest total FAEEs level and was selected for further experiments. This strain was used for rice wine fermentation and compared with the commercial yeast strain EC-1118 and the parental strain 312WT across three independent replicates.

2.7. Rice Wine Fermentation

Commercially available low-cost rice (Kum Ka brand) was purchased from a local store. Rice and water were mixed at a 1:1 ratio, autoclaved at 121°C for 15 minutes, then cooled to room temperature. A 10% spore suspension (1×10^6 spores/ml) was inoculated into the sterilized rice, which was incubated at 30°C for seven days.

The yeast *S. cerevisiae* starter culture for rice wine fermentation was prepared as follows: a single colony of the yeast strain was transferred to 50 mL of Yeast Nitrogen Base (YNB) medium (0.67% Yeast Nitrogen Base Difco™, 2% glucose) and pre-cultured at 30 °C for 24 hours. The resulting cell culture was then transferred into a 1,000 mL Erlenmeyer flask containing 150 mL of YNB medium and incubated at 30 °C with shaking at 200 rpm for 24 hours. The second cell culture was then used for the next stage of rice wine fermentation.

The process of rice wine fermentation was modified from the traditional sake brewing method described by Kuribayashi et al. [8]. In this process, 120 g of mold starter culture (koji) prepared from the previous step was added to 480 g of cooked rice, and 900 mL of water containing 0.03% lactic acid was then added. Subsequently, 10% yeast inoculum was introduced, and the rice wine fermentation was carried out at room temperature for 7 days without stirring, followed by incubation at 10–15 °C for an additional 7 days to develop flavor. After fermentation, the fermented mash was filtered or centrifuged to collect the supernatant as rice wine samples for further analysis [8].

2.8. Analysis of Flavor Compounds

Volatile compounds in rice wine samples were extracted using a solid-phase microextraction (SPME) technique, modified from the method described by Kang et al. [20]. Sodium chloride (NaCl; Qrec, Auckland, New Zealand) was added to each sample until full saturation was achieved. An aliquot of 5 g of the saturated sample was transferred into a headspace vial, followed by the addition of 10 µL of 2-methyl-3-heptanone (100 µg/mL in methanol; Sigma-Aldrich, MO, USA) as an internal standard. The mixture was equilibrated in a temperature-controlled heating block with agitation at 40 °C for 10 minutes to establish phase equilibrium. Subsequently, a DVB/CAR/PDMS SPME fiber (Sigma-Aldrich, MO, USA) was exposed to the vial headspace at 40 °C for 20 minutes to allow adsorption of volatile compounds. Thermal desorption was then performed at the GC injection port at 220 °C for 5 minutes in splitless mode, with the purge flow to the split vent initiated after 1.2 minutes. Gas chromatography–mass spectrometry (GC–MS) analysis was carried out using an Agilent GC 7890B system coupled with an MSD 5977B mass spectrometer (Agilent, CA, USA). Separation of volatile compounds was achieved on a DB-Wax capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent, CA, USA). Helium (ultra-high purity; Air Liquide, Bangkok, Thailand) served as the carrier gas at a constant flow rate of 2.0 mL/min. The oven temperature was programmed from 40 to 220 °C at a ramp rate of 5 °C/min, with both initial and final holding times of 5 minutes. The MS conditions were set as follows: transfer line temperature, 230 °C; electron impact ionization energy, 70 eV; and scan range, 35–350 amu.

Compound identification was conducted by matching the obtained mass spectra with those in the NIST17 library, combined with linear retention index (RI) data. RIs were calculated based on the retention times of a homologous series of C8–C30 n-alkanes analyzed under identical conditions. Odor activity values (OAVs) were determined by dividing each compound's measured concentration by its odor threshold. Compounds reported as not detected (ND) were considered zero for statistical analysis. All experiments were performed in triplicate, and results are expressed as mean ± standard deviation. Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's New Multiple Range Test at a significance level of $p < 0.05$.

2.9. Analysis of Rice Wine Components

The ethanol content in rice wine was analyzed using High-Performance Liquid Chromatography (HPLC, Agilent Technologies, Model 1260 Infinity II). The system was equipped with an Aminex HPX-87H column (300 mm × 78 mm, Bio-Rad Lab), a de-ashing cartridge holder (30 mm × 4.6 mm, Bio-Rad Lab), and a micro-guard cation H^+ refill cartridge holder (30 mm × 4.6 mm, Bio-Rad Lab). The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.6

mL/min, with the oven maintained at 60 °C. A 50-µL sample was injected, and detection was performed using a refractive index detector (Agilent Technologies, Model 1260 RID) at 40 °C. The HPLC method followed previous studies [21, 22]. The pH-meter (Mettler Toledo) was used to assess the acid-base properties of the fermented rice wine.

2.10. Whole Genomic DNA Analysis

To investigate the mechanisms behind the overproduction of FAEEs and key flavor compounds in the mutant strain, whole-genome DNA sequencing was conducted to identify significant mutations compared to the parental strain (312WT). The yeast strains were sent to GIBTHAI COMPANY (Thailand) and then forwarded to Macrogen Company (Korea) for DNA extraction and library preparation. Sequencing was performed using Illumina technology, followed by de novo assembly to generate the genome library of 312WT as a control. The mutant DNA was resequenced and compared to the control genome. All statistical and bioinformatic analyses were carried out by the companies involved. The analysis programs included FastQC, Trimmomatic, Jellyfish, Genomescope, Platanus-allee, Busco, BLAST, MAKER (v3.01.03), and BLAST+ (v2.7.1+).

3. RESULTS AND DISCUSSIONS

3.1. Isolation and Characterization of Parental Yeast Strains from Sugar Industry

Yeasts are vital in industrial processes, especially in producing alcoholic beverages, due to their efficiency in ethanol fermentation and flavor compound production [23]. A yeast strain with strong fermentative ability and the capacity to generate key flavor compounds can significantly enhance product quality. Therefore, strains with fermentation performance similar to commercial starter cultures were initially screened in this study to identify suitable candidates for industrial application.

The isolate designated 312WT, obtained from Thai Sugar Industry Co., Ltd., was evaluated for fermentative performance under laboratory conditions using a high-sugar medium (YNB with 25% glucose) Figure 2. Results showed that strain 312WT caused a carbon dioxide-associated weight loss of 1.46 ± 0.15 g, significantly higher than the control strain EC-1118 (0.91 ± 0.11 g). These findings suggest that 312WT exhibits strong fermentative activity under high-sugar conditions.

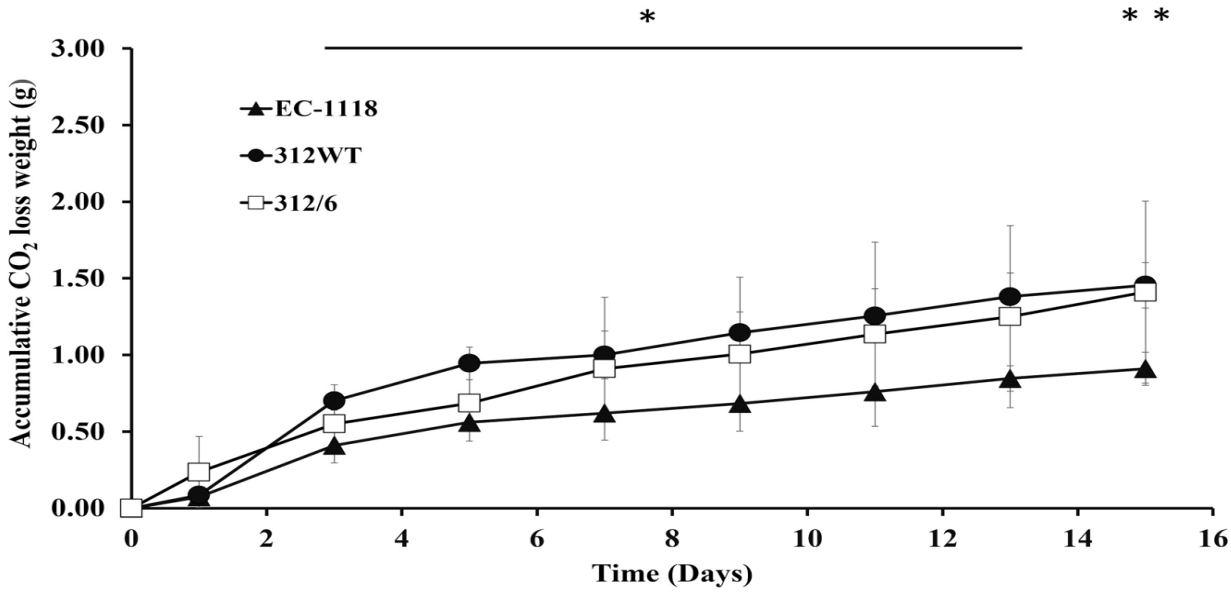

Figure 2. Representation of the accumulation of CO_2 loss weight (g) over time during the fermentation test performed on the 312WT wild-type (Filled-circle), 312/6 mutant (white square), and EC-1118 commercial strain (filled rectangle).

Note: Yeast cells were cultivated in YNB medium with 25% glucose for 15 days. Each point represents the mean with standard deviations from three independent experiments. * Significant difference where $p < 0.05$ for 312WT versus controls (EC-1118), and ** significant difference where $p < 0.05$ for 312WT and 312/6 versus controls (EC-1118) verified by non-repeated measured analysis of variance (ANOVA) followed by the Bonferroni correction.

We examined the ethanol tolerance of the isolated 312WT strain compared to EC-1118. Both strains grew in YPD media containing 12% and 15% (v/v) ethanol (Table 1 and Table S1). Ethanol tolerance is a key characteristic of fermenting yeast, as strains with higher tolerance may demonstrate increased fermentation activity [24].

Table 1. A comparative assessment of ethanol tolerance between the wild-type strain 312WT and the commercial strain EC-1118, based on growth at various ethanol concentrations, was conducted.

Ethanol % (v/v)	Yeast strains	Growth difference (ΔOD_{600}) relative to 0 h		
		24 h	48 h	72 h
12	312WT	++	++	++
	EC-1118	++	++	++
15	312WT	++	+	+
	EC-1118	+	+	+

Note: Symbols (+, ++) represent the magnitude of the increase in optical density (OD_{600}) compared with the initial time point (0 h). (+) indicates a moderate increase ($0.01 < \Delta OD_{600} < 0.1$), whereas (++) denotes a strong increase ($\Delta OD_{600} > 0.1$).

The safety of using the newly isolated 312WT in fermented beverages was confirmed through sequencing analysis of the ITS region and phylogenetic classification based on the NCBI database. BLAST analysis showed that the ITS sequence of 312WT had 95% similarity with *Saccharomyces cerevisiae* HBUA561703 (GenBank accession no. OM348840.1), a yeast strain from Sichuan rice wine. To verify that 312WT is a *S. cerevisiae* strain, a phylogenetic tree was constructed to analyze its relationship with other yeast species. Results demonstrated that 312WT clustered within the same clade as other *S. cerevisiae* strains, confirming its classification as *S. cerevisiae* (Figure 3). Therefore, isolate 312WT appears to be a promising candidate for application in alcoholic beverage fermentation, with potential benefits.

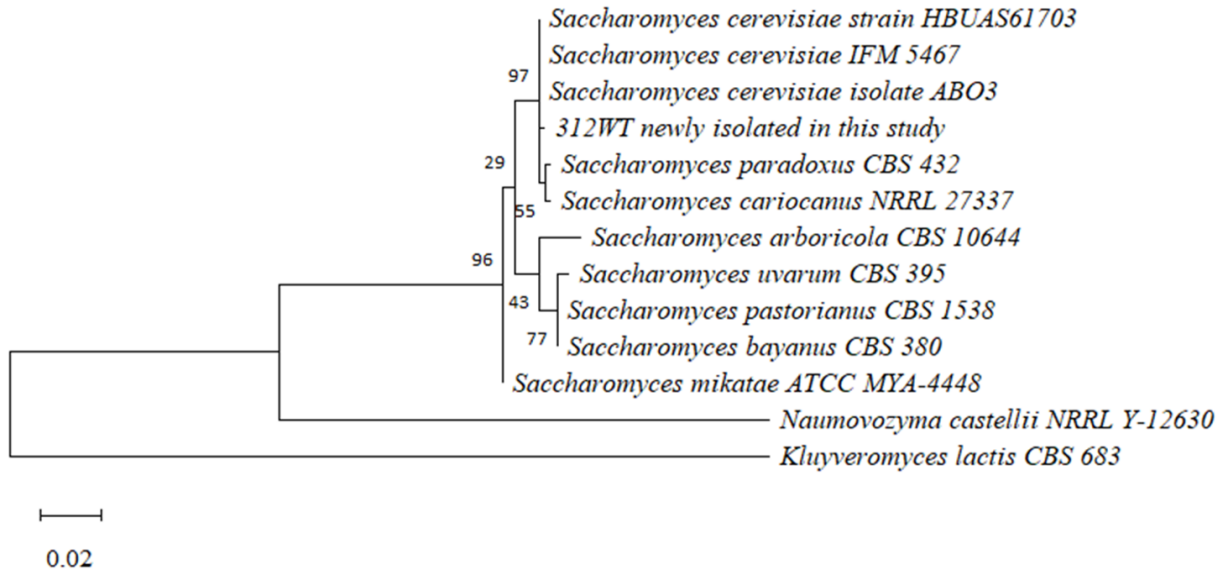

Figure 3. Phylogenetic tree showing the position of yeast isolate 312WT.

The tree was constructed using the maximum likelihood distance-based method with 672 bp of the ITS region. The scale bar indicates 0.02% sequence divergence.

3.2. Screening for Cerulenin-Tolerant Mutants

Fatty acid ethyl esters (FAEEs), including ethyl butanoate, ethyl hexanoate, and ethyl octanoate, substantially enhance the aroma and flavor of alcoholic beverages, particularly by providing fruity flavors [14]. Japanese sake is typically classified based on specific flavor compounds, which determine its quality and type [15]. Consequently, we aimed to develop a mutant with enhanced FAEEs production using isolate 312WT as the parental strain. To generate

cerulenin-resistant mutants, conventional mutagenesis was performed by subjecting the isolated strain 312WT to UV irradiation, followed by selection on culture media containing 6 µg/mL cerulenin. As a result, six mutant colonies were isolated and designated as 312/1, 312/2, 312/3, 312/4, 312/5, and 312/6, respectively. The subsequent mutant colonies demonstrated resistance to cerulenin when cultured on agar media containing cerulenin (Figure 4).

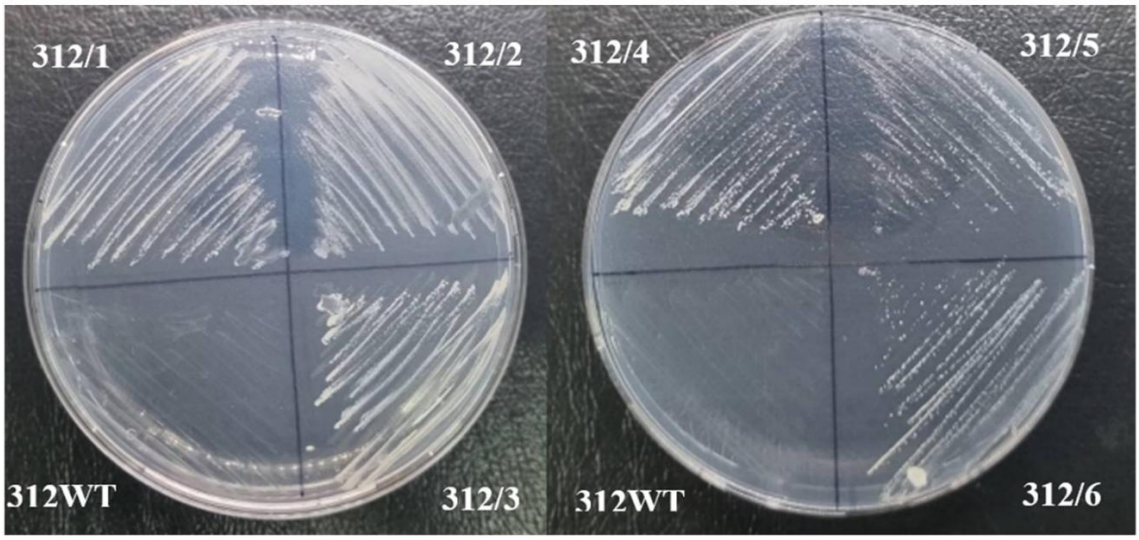

Figure 4. Growth phenotypes of mutants 312/1, 312/2, 312/3, 312/4, 312/5 and 312/6 compared with the control (312WT).

The single colony of yeast strains was streaked on SD agar plates containing 6 µg/mL cerulenin and incubated at 30°C for three days.

The results indicated that all mutants could grow on culture media with 6 µg/mL of cerulenin, whereas the parental strain 312WT failed to grow under the same conditions. Ichikawa et al. [15] evaluated ethyl caproate (ethyl hexanoate) production in sake by EMS mutagenesis of sake yeast *S. cerevisiae* Kyokai No.7, following screening for mutants resistant to 6.28 µg/mL of cerulenin. The selected C-8 mutant exhibited higher ethyl caproate production compared to that of the K-7 strain [15]. Therefore, cerulenin-based screening is an effective strategy for isolating mutants with enhanced FAEEs production. The six mutants (312/1 to 312/6) demonstrated potential to overproduce FAEEs, as shown in the following section.

3.3. Selection of the Desired FAEEs Overproduction Mutant

To select an appropriate mutant for rice wine fermentation, all six cerulenin-resistant mutants were tested simultaneously using low-cost Thai rice and compared with the parental strain 312WT. The fermentation process followed a sake-based protocol, which was adjusted to suit the properties of the Thai rice. Among the tested strains, mutant 312/6 consistently produced the highest levels of fatty acid ethyl esters. The concentrations of ethyl hexanoate, ethyl octanoate, and ethyl nonanoate reached 51.41 µg/kg, 282.39 µg/kg, and 12.62 µg/kg, respectively. In contrast, the parental strain 312WT produced only 28.41 µg/kg of ethyl hexanoate and 94.19 µg/kg of ethyl octanoate, with ethyl nonanoate not detected (Table S2). These results indicate that the 312/6 mutant has a significantly increased ability to form FAEE during rice wine fermentation conditions.

To verify that accumulated mutations did not impair fermentative performance, the fermentation behavior of strain 312/6 was compared with its parental strain, 312WT. By day 15, 312/6's fermentation ability was not significantly different from 312WT and remained higher than that of the commercial strain EC-1118 (Figure 2). A similar outcome has been observed with sake yeasts improved via conventional mutagenesis, where increased production of higher alcohols like 3-methyl-1-butanol and 2-methyl-1-butanol did not impair fermentation capacity.

The developed strains maintained performance comparable to their parental strains [16]. Therefore, the mutant strain 312/6 was selected for further analysis.

3.4. Flavor Compounds Analysis of Rice Wines

Following the identification of 312/6 as a candidate mutant, rice wine fermentation was re-performed triplicated and the resulting wine samples were analyzed for flavor compounds in comparison with those produced by the commercial strain EC-1118 and the parental strain 312WT. The results revealed that 312WT produced key flavor compounds, including isoamyl alcohol (415.65 ± 88.05 µg/kg, OAV = 2), ethyl hexanoate (19.88 ± 2.58 µg/kg, OAV = 20), ethyl octanoate (75.54 ± 52.37 µg/kg, OAV = 15), phenylethyl acetate (47.18 ± 18.23 µg/kg, OAV <1), and 2-methoxy-4-vinyl phenol (86.64 ± 9.95 µg/kg, OAV <1), at levels statistically comparable to those from EC-1118 (442.06 ± 92.27, 23.21 ± 6.23, 49.03 ± 3.57, 66.12 ± 6.31, and 127.17 ± 31.35 µg/kg which calculated as OAV = 2, 23, 10, <1, and <1, respectively for isoamyl alcohol, ethyl hexanoate, ethyl octanoate, phenylethyl acetate, and 2-methoxy-4-vinyl phenol) (Table 2). Notably, 312WT produced significantly higher phenylethyl alcohol (1125.79 ± 189.24 µg/kg, OAV = 1) compared to the value from EC-1118 produced (661.95 ± 372.34 µg/kg, OAV <1).

Table 2. The quantification (µg/kg) of key flavor compounds detected in rice wine fermented with yeast 312/6 mutant, compared to the original strain 312WT and the commercial strain EC-1118.

Compounds	Odor description	Odor threshold (ug/kg)	Concentration (µg/kg)			OAV		
			EC-1118	312WT	312/6	EC-1118	312WT	312/6
Isoamyl alcohol	Alcoholic, banana	250	442.06 ± 92.27[a]	415.65 ± 88.05[a]	356.76 ± 40.91[a]	2	2	1
Ethyl hexanoate	Sweet apple	1	23.21 ± 6.23[a]	19.88 ± 2.58[a]	28.55 ± 7.91[a]	23	20	29
Ethyl octanoate	Sour apple	5	49.03 ± 3.57[b]	75.54 ± 52.37[b]	207.22 ± 51.65[a]	10	15	41
Ethyl nonanoate	Fruity, rose, waxy, rum, wine, natural, tropical flavors.	1300	ND	8.21 ± 0.00[a]	15.99 ± 7.72[a]	ND	<1	<1
Phenylethyl acetate	Roses and honey	250	66.12 ± 6.31[a]	47.18 ± 18.23[a]	38.56 ± 25.05[a]	<1	<1	<1
Phenylethyl alcohol	Sweet, floral, fresh, and bready with a rosy honey aroma.	1000	661.95 ± 372.34[b]	1125.79 ± 189.24[ab]	1438.91 ± 269.47[a]	<1	1	1
2-Methoxy-4-vinylphenol	Sweet, spicy, clove, peppery, smoky, woody, powdery scents.	1100	127.17 ± 31.35[a]	86.64 ± 9.95[a]	94.65 ± 3.73[a]	<1	<1	<1

Note: Each point represents the mean with standard deviations from three independent experiments, with two injections of each replicate.
Data with different superscript letters[a, b, ab] are statistically significantly different, verified by Duncan's New Multiple Range Test (p-value < 0.05).
[ND]Not detectable.

In the analysis, rice wine fermented by the 312/6 mutant exhibited significantly higher levels of FAEEs, including ethyl hexanoate, ethyl octanoate, and ethyl nonanoate, compared to wines fermented by EC-1118 and 312WT. Notably, ethyl octanoate in 312/6 (207.22 ± 51.65 μg/kg, OAV = 41) was approximately five times higher than in EC-1118 (49.03 ± 3.57 μg/kg, OAV = 10) and three times higher than in 312WT (75.54 ± 52.37 μg/kg, OAV = 15). Additionally, phenylethyl alcohol levels from strain 312/6 (1438.91 ± 269.47 μg/kg, OAV = 1) were significantly elevated, surpassing levels in 312WT (1125.79 ± 189.24 μg/kg, OAV = 1) and EC-1118 (661.95 ± 372.34 μg/kg, OAV (Table 2).

Volatile compounds produced during alcoholic fermentation significantly influence consumer perception of traditional Japanese beverages like sake and awamori shochu. Among these, ester compounds largely determine fruity and sweet sensory attributes. FAEEs, especially ethyl hexanoate and ethyl octanoate, are key contributors to fruity aromas and are closely linked to the Ginjo-ka note, which characterizes premium sake quality. Proper understanding of these compounds is essential for quality control and flavor optimization in production [8]. In the present study, the 312/6 mutant exhibited a significant enhancement in FAEE biosynthesis, with ethyl octanoate levels approximately five-fold higher than those observed in the commercial wine strain EC-1118 (Table 1). Previous studies on commercial sake products using similar analytical methods reported ethyl octanoate concentrations ranging from 1 to 412 μg/kg [25]. The rice wine fermented with the 312/6 mutant contained 207.22 ± 51.65 μg/kg of ethyl octanoate, demonstrating this strain's ability to produce a volatile ester profile comparable to commercial-grade products, consistent with the expected range for such fermentations (Table 1).

Beyond mere concentration, the sensory significance of volatile compounds is better understood through the odor activity value (OAV), which indicates their actual impact on human perception. The OAV is determined by dividing a compound's measured concentration by its odor threshold (see Section 2.8). Compounds with OAVs exceeding 1 are considered aroma-active and can influence sensory perception [25, 26]. As shown in Table 1, both ethyl octanoate and ethyl hexanoate produced by strain 312/6 exhibited OAVs above this threshold, indicating that these esters directly contributed to the perceptible apple-like aroma in the fermented rice wine.

Compared to previous studies on Japanese sake, the ethyl octanoate concentration produced by the 312/6 mutant was significantly lower than in earlier research findings [8]. One point worth noting is that the case study used Kyokai no. 901 (K901) as the parental strain. This strain has been long selected for brewing in the Yoshinogawa area and is known among brewers for producing strong aromatic notes. Proper spelling and grammar have been corrected [8]. Apart from genetic factors, raw material composition also influences aroma formation in rice wine [26]. Sake quality is closely tied to rice characteristics; for example, rice with high starch, low protein, and larger grain size generally gives better aroma profiles [25, 27]. Both the K901C8 mutant from the previous report and our 312/6 mutant exhibited approximately a 3.5-fold increase in ethyl octanoate. This consistent trend indicates that targeted mutagenesis is an effective method for enhancing aroma production, even when applied to more affordable local rice varieties. In addition to ester formation, phenylethyl alcohol significantly contributed to the aroma profile of rice wine. Both 312WT and 312/6 produced this compound at OAV values above 1, while the commercial strain EC-1118 remained below the odor threshold (OAV = 0.66). Phenylethyl alcohol is a characteristic volatile component of Japanese sake, responsible for floral and honey-like notes, which are widely regarded as desirable qualities of high-quality products [28]. Taken together, these results confirm that both 312WT and, more notably, the 312/6 mutant are suitable candidates for rice wine fermentation. The 312/6 strain demonstrates a superior ability to enhance key aroma compounds, especially ethyl octanoate and phenylethyl alcohol, compared to the parental strain and the commercial reference strain EC-1118 [29].

3.5. Chemical Analysis of Rice Wines

The chemical characteristics of rice wine change through microbial activity and biochemical transformations during fermentation, which depend on the yeast strain used. Variations in fermentation performance mainly reflect

each strain's ability to metabolize sugars into ethanol. Additionally, ethanol levels and pH are chemical parameters that influence sensory perception, including taste balance and the expression of volatile aroma compounds, affecting the overall quality of the wine [28, 30, 31].

During fermentation, ethanol levels gradually increased in all three strains (312WT, 312/6, and EC-1118) and reached a stable phase by day 14, with final concentrations ranging from 9.74 ± 0.51% to 11.13 ± 0.27% (v/v) (Figure 5). In contrast, glucose levels declined sharply within the first three days and remained relatively constant from day 5 until the end of fermentation in all treatments (Figure 6), following a pattern consistent with earlier reports. Similarly, the non-reducing sugar content also decreased during the early stage and remained stable from day 5 until the completion of fermentation [30].

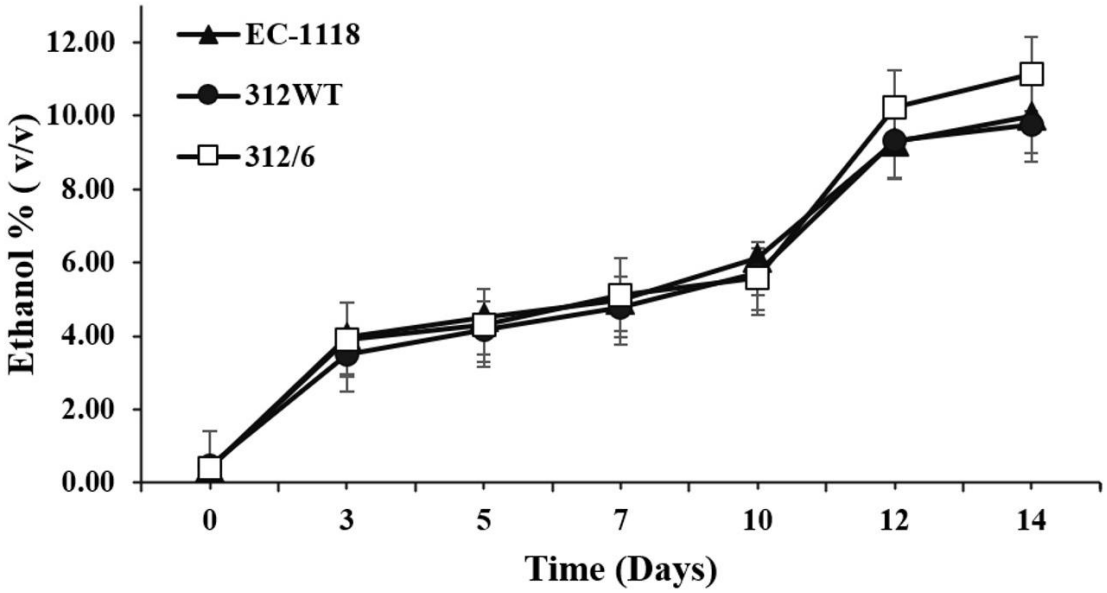

Figure 5. Comparative analysis of ethanol variation during fermentation in rice wine fermented from yeast strains 312WT wild-type (Filled-circle), 312/6 mutant (White-square), and EC-1118 commercial strain (Filled-rectangle).

Each point represents the mean with standard deviations from three independent experiments.

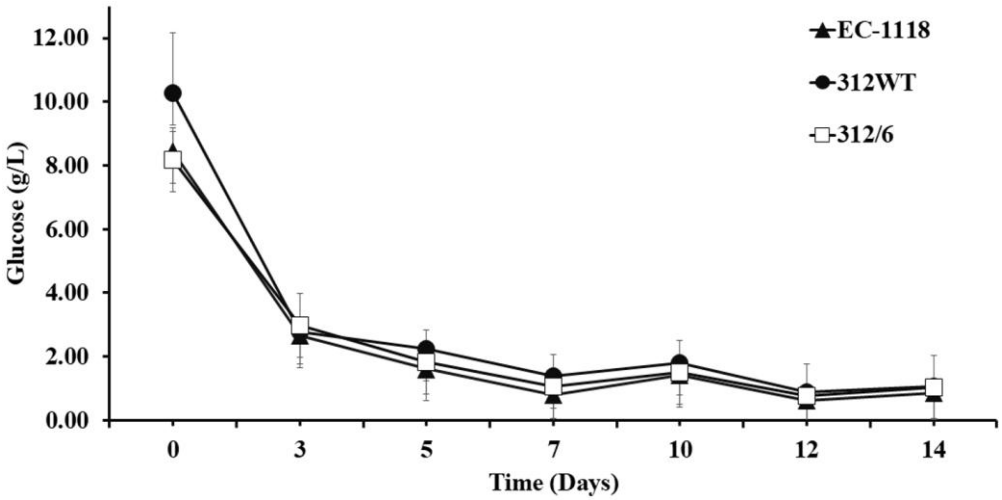

Figure 6. Comparative analysis of glucose variation during fermentation in rice wine fermented from yeast strains 312WT wild-type (Filled-circle), 312/6 mutant (White-square), and EC-1118 commercial strain (Filled-rectangle).

Each point represents the mean with standard deviations from three independent experiments.

The pH ranged from 3.78 ± 0.01 to 5.42 ± 0.35 in EC-1118, 3.62 ± 0.06 to 5.45 ± 0.04 in 312WT, and 3.84 ± 0.03 to 4.95 ± 0.04 in 312/6 during the 14-day fermentation period. The experimental results showed that the pH decreased until day 3 of fermentation and then remained relatively stable until the end of the process (Figure 7). Similarly, consistent with previous studies, the pH of Korean rice wine (makgeolli) decreased rapidly during the first 2–3 days of fermentation [28, 31]. Acetic acid is a by-product of ethanol fermentation, and its accumulation could contribute to the decrease in pH during fermentation [29]. The final pH ranged from 3.63 to 4.00, which falls within the typical pH range of 3.0 to 4.5 for rice-fermented alcoholic beverages [32].

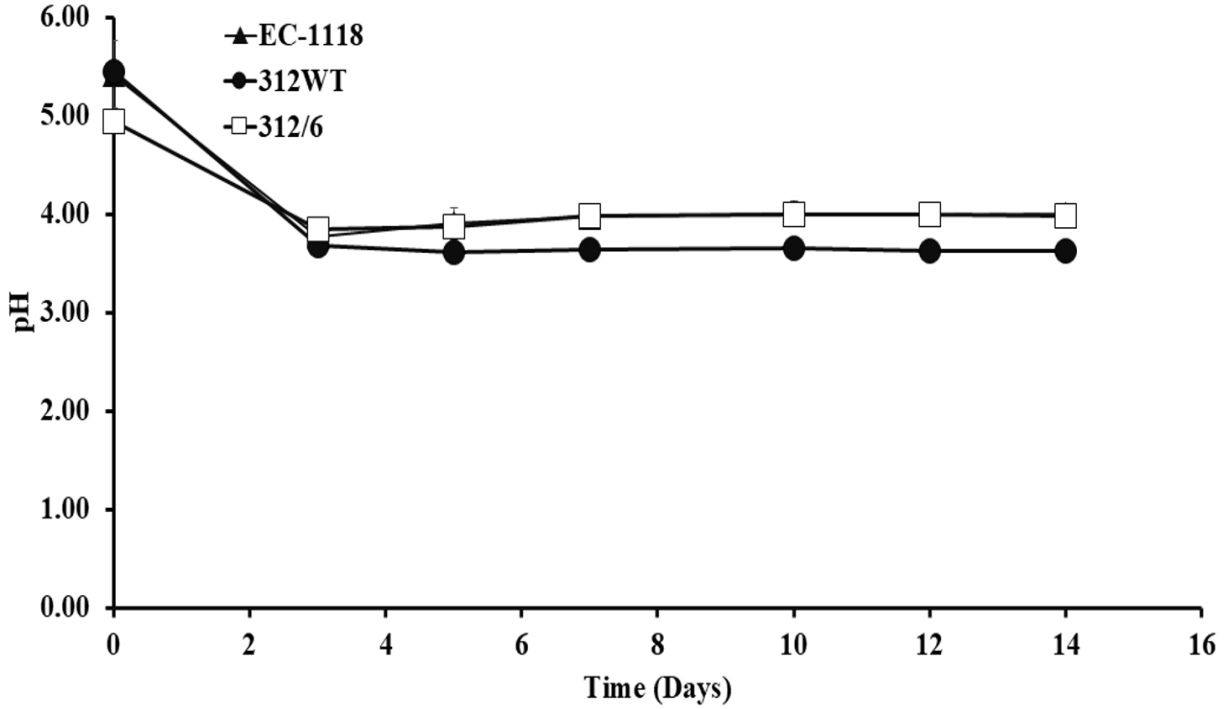

Figure 7. Comparative analysis of pH variation during fermentation in rice wine fermented from yeast strains 312WT wild-type (Filled-circle), 312/6 mutant (White-square), and EC-1118 commercial strain (Filled-rectangle).

Each point represents the mean with standard deviations from three independent experiments.

To assess the fermentation performance of strains 312WT and 312/6 in rice wine production relative to the commercial yeast EC-1118, the final physicochemical parameters of the fermented products were analyzed (Figure 8). The results indicated that rice wine fermented with strain 312WT had a lower ethanol concentration (9.74 ± 0.51% v/v) compared to EC-1118.

This lower ethanol level was associated with the highest residual glucose concentration (1.06 ± 0.10 g/L), significantly greater than in EC-1118 fermentation. These findings suggest that strain 312WT has a reduced capacity for sugar utilization and ethanol production during rice wine fermentation. The final pH of rice wine fermented with 312WT was 3.63 ± 0.11, indicating the highest acidity among the strains and a significantly lower pH than the commercial yeast. Conversely, the 312/6 mutant produced ethanol, residual glucose, and pH values of 11.13 ± 0.27% (v/v), 1.02 ± 0.05 g/L, and 3.99 ± 0.04, respectively, which did not differ significantly from EC-1118. These results demonstrate that the fermentation performance of the 312/6 mutant is comparable to that of the commercial wine yeast strain.

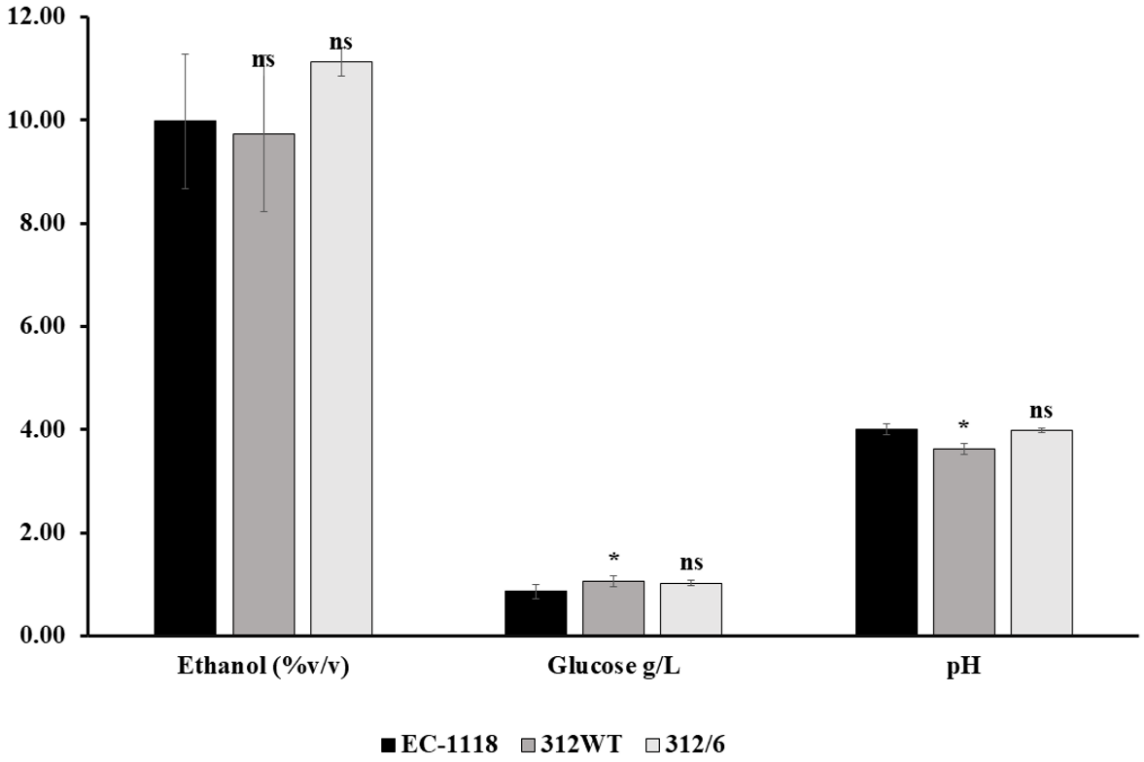

Figure 8. Comparative analysis of the final chemical parameters in rice wine samples fermented with the wild-type strain 312WT (Gray bar), the 312/6 mutant (Grayish-white bar), and the commercial strain EC-1118 (Black bar).

Note: Each value represents the mean ± standard deviation from three independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's correction. Differences were considered non-significant (ns) when $p > 0.05$ and significant (*) when $p < 0.05$ compared with the control strain (EC-1118).

3.6. Identification of Mutations in FAEE Biosynthesis Genes

The entire genomic DNA sequence of strain 312/6 was analyzed and compared to the parental strain 312WT to identify mutations associated with increased production of flavor compounds, especially fatty acid ethyl esters (FAEEs) and phenylethyl alcohol. The analysis revealed 11 missense mutations in the FAEE biosynthesis pathway and 8 in phenylethyl alcohol production, leading to amino acid changes in enzymes of the mutant strain, potentially explaining the enhanced flavor compound synthesis (Table 3). In the FAEEs biosynthesis pathway, 11 mutations were identified: 1 mutation (229 G>A), 3 mutations (335 C>A, 1187 G>A, and 1694 A>G), 5 mutations (82 T>C, 346 A>T, 1028 A>G, 4950 C>G, and 5510 A>C), 1 mutation (92 G>A), and 1 mutation (1151 A>G) in the *FAS1*, *FAS2*, *ACC1*, *EHT1*, and *EEB1* genes, respectively.

The *ACC1* and *FAS* genes encode large, multifunctional proteins with multiple domains. To identify mutation sites across these domains, we compared their gene and protein sequences using databases such as InterPro, PFAM, and UniProt. Our analysis showed that mutations in the *ACC1* gene are located within domains involved in enzymatic reactions. Acetyl-CoA carboxylase, encoded by *ACC1*, is a crucial rate-limiting enzyme in fatty acid biosynthesis, catalyzing the conversion of acetyl-CoA to malonyl-CoA via a two-step carboxylation process [33]. This reaction involved the carboxylation of a biotin group, followed by transferring the carboxyl group to acetyl-CoA. ACCase has two main catalytic domains: biotin carboxylase (BC) and carboxyltransferase (CT).

Mutations in the *ACC1* gene are found in both domains (Table 3). The His343Arg mutation is situated near the active site at position 383 within the biotin carboxylase (BC) domain. In silico structural analysis shows that this substitution occurs in a conserved α-helical segment of the BC domain in ACC1, suggesting that this region is crucial for maintaining enzyme structure and function. DynaMut analysis [34] predicted a positive ΔΔG value of +1.56 kcal/mol and a ΔΔS (ENCoM) of −0.313 kcal/mol/K (Table S3), suggesting that the mutation enhances protein stability while simultaneously reducing molecular flexibility, which could alter active-site dynamics.

Table 3. List of genes containing missense mutations in the FAEEs and phenyl ethyl alcohol biosynthetic enzymes found in 312/6 mutant compared to 312WT.

Gene name	Nucleotide change	Protein function	Amino acid change	Domain region
FAEEs Pathway				
FAS1	229 G>A	Fatty acid synthase subunit beta	Val77Ile	-
FAS2	335 C>A	Fatty acid synthase subunit alpha	Thr112Asn	-
FAS2	1187 G>A	Fatty acid synthase subunit alpha	Arg396Lys	-
FAS2	1694 A>G	Fatty acid synthase subunit alpha	Asn565Ser	-
ACC1	82 T>C	Acetyl-CoA carboxylase	Phe28Leu	-
ACC1	346A>T	Acetyl-CoA carboxylase	Asn116Tyr	Biotin carboxylase domain.
ACC1	1028 A>G	Acetyl-CoA carboxylase	His343Arg	Biotin carboxylase domain.
ACC1	4950 C>G	Acetyl-CoA carboxylase	Asp1650Glu	Acetyl-CoA carboxylase N-terminal domain
ACC1	5510 A>C	Acetyl-CoA carboxylase	Asn1837Thr	Acetyl-CoA carboxylase C-terminal domain
EHT1	1151 A>G	Ethanol O-acyltransferase	Gln384Arg	-
EEB1	92 G>A	Ethanol O-acyltransferase	Arg31Lys	-
Phenyl ethyl alcohol Pathway				
ARO8	20 G>A	Aromatic amino acid transaminase I	Arg7Lys	-
ARO9	77 C>T	Aromatic amino acid transaminase II	Ala26Val	-
ARO9	235 A>G	Aromatic amino acid transaminase II	Asn79Asp	-
ARO9	651 C>A	Aromatic amino acid transaminase II	Asp217Glu	-
ARO9	1360 C>G	Aromatic amino acid transaminase II	Leu454Val	AAT-like domain
ARO10	1182 T>G	Phenylpyruvate decarboxylase	Phe394Leu	-
ARO80	1102 A>G	Zinc finger transcriptional activator	Thr368Ala	-
ARO80	2810 T>C	Zinc finger transcriptional activator	Ile937Thr	-

In parallel, PROVEAN classified this substitution as functionally neutral, indicating that it is unlikely to impair overall protein activity (Table S4) [35]. Collectively, these computational results imply that the His343Arg mutation may modulate catalytic performance, consistent with the elevated FAEEs levels observed experimentally. Nevertheless, experimental validation of the direct kinetic impact of this mutation has not yet been reported. Chou et al. [34] demonstrated that Arg338 in the biotin carboxylase domain of *E. coli ACC* plays a key role in stabilizing the carboxylation transition state, despite being positioned outside the canonical active site [36]. By analogy, the His343Arg substitution in *ACC1*, located near the ATP-grasp motif, may modify active-site conformational behavior, thereby promoting malonyl-CoA accumulation. This key intermediate subsequently feeds into the fatty acid biosynthetic pathway and ultimately enhances flux toward FAEEs formation, providing a plausible explanation for the increased ester production observed in the 312/6 mutant.

Interestingly, while ethyl octanoate levels were markedly increased, the production of other FAEEs, such as ethyl hexanoate, did not show a comparable enhancement (Table 2). These esters form through the condensation of fatty acyl-CoA and ethanol, primarily catalyzed by Eht1 and Eeb1, with Eeb1 showing a higher substrate preference for octanoyl-CoA over hexanoyl-CoA [12]. Sequence analysis showed no amino acid substitutions in the catalytic regions of the EEB1 and EHT1 genes. Variations in enzyme expression, especially increased EEB1 activity, may promote preferential octanoyl-CoA utilization over hexanoyl-CoA, leading to the selective accumulation of ethyl octanoate. Meanwhile, four genes involved in phenylethyl alcohol (*ARO8*, *ARO9*, *ARO10*, and *ARO80*) were found to harbor a total of 8 mutations [1 mutation (20 G>A) in *ARO8*, 4 mutations (77 C>T, 235 A>G, 651 C>A, and 1360 C>G) in *ARO9*, 1 mutation (1182 T>G) in *ARO10*, and 2 mutations (1102 A>G and 2810 T>C) in *ARO80*] in strain 312/6 compared to original strain 312WT. Notably, the 312/6 mutant also harbored a missense substitution, Leu454Val, within the *ARO9* gene, located in the aromatic aminotransferase–like (AAT-like) domain (Table 3). This domain is involved in the transamination of phenylalanine to phenylpyruvate, a key step in phenylethyl alcohol biosynthesis. Since *ARO9* encodes transaminase II [35], alterations in this gene have been associated with changes

in phenylethyl alcohol formation in yeast [36, 37]. Previous work on *S. cerevisiae* HJ, a strain known for high phenylethyl alcohol production, revealed amino acid substitutions at residues 12 (Thr-Ala), 79 (Asn-Asp), 454 (Leu-Val), and 460 (Val-Leu) within the gated channel of the N-terminal domain of Aro9, relative to the laboratory strain S288C. These substitutions were proposed to modify substrate and product tolerance at the active site, thereby enhancing catalytic performance [38].

Similarly, mutations in the 312/6 mutant, especially Asn79Asp and Leu454Val, may enhance tolerance to phenylalanine and phenylethyl alcohol compared to the parental 312WT strain. This increased tolerance could support continuous flux through the Ehrlich pathway, resulting in higher phenylethyl alcohol levels. However, genome sequencing alone cannot determine whether these mutations activate or inhibit enzyme functions. Additional validation, including enzyme activity assays and metabolite profiling, is necessary to confirm their specific biochemical effects.

4. CONCLUSIONS

The yeast strain 312WT, isolated from Thai Sugar Industry Co., Ltd., was identified as *Saccharomyces cerevisiae*, closely related to wine yeast strains. The mutant strain 312/6, derived from 312WT through conventional mutagenesis, was classified as non-GMO and demonstrated superior characteristics in rice wine fermentation, notably producing fruity and floral flavors. Additionally, the rice wine production process adapted from Japanese sake, as proposed in this study, shows potential for Thai traditional rice wine (sato), offering improved quality control over traditional methods. This combined approach, involving yeast strain development and pure-culture fermentation, could be extended to rice wine production using low-cost rice, representing a novel direction for product development and value addition to lower-priced variants. However, this work was conducted solely at the laboratory scale. Moreover, flavor profiles were estimated based only on odor activity values and were not validated by trained sensory panelists. Future research should focus on scaling up the process, optimizing industrial conditions, and conducting sensory evaluations. Additionally, targeted functional assays are necessary to validate the genomic mutations involved.

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REFERENCES

[1] Bangkokbiznews, "Where does Thai rice export stand in the global market in an era where India and Vietnam remain strong?," Retrieved: https://www.bangkokbiznews.com/business/1080192. [Accessed 11 Oct 2024], 2023.

[2] Bangkokbiznews, "In 2023, Thailand exported more than its target of 8.76 million tons of rice, an increase of 13.62%," Retrieved: https://www.bangkokbiznews.com/business/economic/1111240. [Accessed 11 Oct 2024], 2024.

[3] A. Luangkhlaypho, K. Pattaragulwanit, N. Leepipatpiboon, and C. Yompakdee, "Development of a defined starter culture mixture for the fermentation of sato, a Thai rice-based alcoholic beverage," *ScienceAsia*, vol. 40, no. 2, pp. 125-134, 2014. https://doi.org/10.2306/scienceasia1513-1874.2014.40.125

[4] K. Ito, R. Niwa, K. Kobayashi, T. Nakagawa, G. Hoshino, and Y. Tsuchida, "A dark matter in sake brewing: Origin of

microbes producing a Kimoto-style fermentation starter," *Frontiers in Microbiology*, vol. 14, p. 1112638, 2023. https://doi.org/10.3389/fmicb.2023.1112638

[5] M. Suto and H. Kawashima, "Discrimination for sake brewing methods by compound specific isotope analysis and formation mechanism of organic acids in sake," *Food Chemistry*, vol. 381, p. 132295, 2022. https://doi.org/10.1016/j.foodchem.2022.132295

[6] T. Koseki, Y. Ito, S. Furuse, K. Ito, and K. Iwano, "Conversion of ferulic acid into 4-vinylguaiacol, vanillin and vanillic acid in model solutions of shochu," *Journal of Fermentation and Bioengineering*, vol. 82, no. 1, pp. 46-50, 1996. https://doi.org/10.1016/0922-338X(96)89453-0

[7] J. Taira, A. Tsuchiya, and H. Furudate, "Initial volatile aroma profiles of young and aged awamori shochu determined by GC/MS/pulsed FPD," *Food Science and Technology Research*, vol. 18, no. 2, pp. 177-181, 2012. https://doi.org/10.3136/fstr.18.177

[8] T. Kuribayashi *et al.*, "Isolation and brewing properties of a sake yeast mutant with high ethyl caprylate productivity," *Food Science and Technology Research*, vol. 28, no. 3, pp. 217-224, 2022. https://doi.org/10.3136/fstr.FSTR-D-21-00308

[9] S. Shi, Y. Chen, V. Siewers, and J. Nielsen, "Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1," *mBio*, vol. 5, no. 3, p. e01130-14, 2014. https://doi.org/10.1128/mbio.01130-14

[10] P.-T. Liu, B.-Q. Zhang, C.-Q. Duan, and G.-L. Yan, "Pre-fermentative supplementation of unsaturated fatty acids alters the effect of overexpressing *ATF1* and *EEB1* on esters biosynthesis in red wine," *LWT - Food Science and Technology*, vol. 120, p. 108925, 2020. https://doi.org/10.1016/j.lwt.2019.108925

[11] L. Nykänen and I. Nykänen, "Production of esters by different yeast strains in sugar fermentations," *Journal of the Institute of Brewing*, vol. 83, no. 1, pp. 30-31, 1977. https://doi.org/10.1002/j.2050-0416.1975.tb03787.x

[12] S. M. G. Saerens *et al.*, "The saccharomyces cerevisiae EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity," *Journal of Biological Chemistry*, vol. 281, no. 7, pp. 4446-4456, 2006. https://doi.org/10.1074/jbc.M512028200

[13] H. Takagi *et al.*, "Isolation and characterization of awamori yeast mutants with L-leucine accumulation that overproduce isoamyl alcohol," *Journal of Bioscience and Bioengineering*, vol. 119, no. 2, pp. 140-147, 2015. https://doi.org/10.1016/j.jbiosc.2014.06.020

[14] M. Lilly, F. F. Bauer, M. G. Lambrechts, J. H. Swiegers, D. Cozzolino, and I. S. Pretorius, "The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates," *Yeast*, vol. 23, no. 9, pp. 641-659, 2006. https://doi.org/10.1002/yea.1382

[15] E. Ichikawa, N. Hosokawa, Y. Hata, Y. Abe, K. Suginami, and S. Imayasu, "Breeding of a sake yeast with improved ethyl caproate productivity," *Agricultural and Biological Chemistry*, vol. 55, no. 8, pp. 2153-2154, 1991. https://doi.org/10.1271/bbb1961.55.2153

[16] S. Isogai *et al.*, "Improvement of valine and isobutanol production in sake yeast by Ala31Thr substitution in the regulatory subunit of acetohydroxy acid synthase," *FEMS Yeast Research*, vol. 23, p. foad012, 2023. https://doi.org/10.1093/femsyr/foad012

[17] S. Sivaramakrishnan, D. Gangadharan, K. M. Nampoothiri, C. R. Soccol, and A. Pandey, "Alpha amylase production by *Aspergillus oryzae* employing solid-state fermentation," *Journal of Scientific and Industrial Research*, vol. 66, no. 8, pp. 621-626, 2007.

[18] T. Takahashi, Y. Ohara, and K. Sueno, "Breeding of a sake yeast mutant with enhanced ethyl caproate productivity in sake brewing using rice milled at a high polishing ratio," *Journal of Bioscience and Bioengineering*, vol. 123, no. 6, pp. 707-713, 2017. https://doi.org/10.1016/j.jbiosc.2017.01.014

[19] V. Revin, N. Atykyan, E. Lyovina, Y. Dragunova, and V. Ushkina, "Effect of ultraviolet radiation on physiological and biochemical properties of yeast Saccharomyces cerevisiae during fermentation of ultradispersed starch raw material," *Electronic Journal of Biotechnology*, vol. 31, pp. 61-66, 2018. https://doi.org/10.1016/j.ejbt.2017.11.004

[20] J.-E. Kang, K.-H. Kim, E. E. Kwon, and E.-C. Jeon, "Characterization of odor-active volatile compounds in fermented alcoholic beverages using solid-phase microextraction coupled with gas chromatography–mass spectrometry," *Journal of Chromatography A*, vol. 1460, pp. 36–44, 2016.

[21] P. Chinwatpaiboon, I. Doolayagovit, A. Boonsombuti, A. Savarajara, and A. Luengnaruemitchai, "Comparison of acid-, alkaline-, and ionic liquid–treated Napier grass as an immobilization carrier for butanol production by *Clostridium beijerinckii* JCM 8026," *Biomass Conversion and Biorefinery*, vol. 10, pp. 1071-1082, 2020. https://doi.org/10.1007/s13399-019-00491-5

[22] P. Chinwatpaiboon, C. Saejao, A. Boonsombuti, and A. Luengnaruemitchai, "Optimization of ionic liquid 1-ethyl-3-methylimidazolium acetate pretreatment of Napier grass combined with acetic acid and ferric chloride under microwave radiation by response surface methodology," *Biomass Conversion and Biorefinery*, vol. 13, pp. 9877–9888, 2023. https://doi.org/10.1007/s13399-021-01901-3

[23] G. M. Walker and G. G. Stewart, "Saccharomyces cerevisiae in the production of fermented beverages," *Beverages*, vol. 2, no. 4, p. 30, 2016. https://doi.org/10.3390/beverages2040030

[24] T. M. Pais *et al.*, "Comparative polygenic analysis of maximal ethanol accumulation capacity and tolerance to high ethanol levels of cell proliferation in yeast," *PLoS Genet*, vol. 9, no. 6, p. e1003548, 2013. https://doi.org/10.1371/journal.pgen.1003548

[25] K. Takahashi, H. Kohno, and M. Okuda, "Spatial distribution and characteristics of protein content and composition in japonica rice grains: Implications for sake quality," *Rice*, vol. 17, no. 1, p. 26, 2024. https://doi.org/10.1186/s12284-024-00708-w

[26] C. Shen *et al.*, "The dynamic of physicochemical properties, volatile compounds and microbial community during the fermentation of Chinese rice wine with diverse cereals," *Food Research International*, vol. 198, p. 115319, 2024. https://doi.org/10.1016/j.foodres.2024.115319

[27] Y. Zheng, "Structure observation of rice endosperm tissues," *Chinese Journal of Rice Science*, vol. 31, no. 1, pp. 91–95, 2017.

[28] E. H. Park, E. B. Jeon, P. K. Roy, S.-H. Park, and S. Y. Park, "Quality characteristics and antioxidant activity of the Korean traditional rice-oat wine "Makgeolli" supplemented with green seaweed Codium fragile," *Heliyon*, vol. 10, no. 20, p. e39348, 2024. https://doi.org/10.1016/j.heliyon.2024.e39348

[29] P. Romano, M. Ciani, and G. H. Fleet, *Yeasts in the production of wine*. Switzerland: Springer, 2019.

[30] H. Li *et al.*, "Simultaneous saccharification and fermentation of broken rice: An enzymatic extrusion liquefaction pretreatment for Chinese rice wine production," *Bioprocess and Biosystems Engineering*, vol. 36, no. 8, pp. 1141-1148, 2013. https://doi.org/10.1007/s00449-012-0868-0

[31] E. Kim, Y. H. Chang, J. Y. Ko, and Y. Jeong, "Physicochemical and microbial properties of the Korean traditional rice wine, Makgeolli, supplemented with banana during fermentation," *Preventive Nutrition and Food Science*, vol. 18, no. 3, pp. 203-209, 2013.

[32] J. G. L. Dela Rosa and P. M. B. Medina, "Philippine rice wine (Tapuy) made from Ballatinao black rice and traditional starter culture (Bubod) showed high alcohol content, total phenolic content, and antioxidant activity," *Food Science and Technology*, vol. 42, p. e45120, 2022. https://doi.org/10.1590/fst.45120

[33] L. Tong, "Acetyl-coenzyme A carboxylase: Crucial metabolic enzyme and attractive target for drug discovery," *Cellular and Molecular Life Sciences*, vol. 62, pp. 1784-1803, 2005. https://doi.org/10.1007/s00018-005-5121-4

[34] C.-Y. Chou, L. P. C. Yu, and L. Tong, "Crystal structure of biotin carboxylase in complex with substrates and implications for its catalytic mechanism," *Journal of Biological Chemistry*, vol. 284, no. 17, pp. 11690-11697, 2009. https://doi.org/10.1074/jbc.M805783200

[35] I. Iraqui *et al.*, "Aromatic amino acid transamination is catalyzed by the yeast Aro8 and Aro9 aminotransferases," *Molecular and Cellular Biology*, vol. 19, no. 5, pp. 3360–3371, 1999. https://doi.org/10.1128/MCB.19.5.3360

[36] L. A. Hazelwood, J.-M. Daran, A. J. Van Maris, J. T. Pronk, and J. R. Dickinson, "The Ehrlich pathway for fusel alcohol

production: A century of research on Saccharomyces cerevisiae metabolism," *Applied and Environmental Microbiology*, vol. 74, no. 8, pp. 2259-2266, 2008. https://doi.org/10.1128/AEM.02625-07

[37] G. Styger, B. Prior, and F. F. Bauer, "Wine flavor and aroma," *Journal of Industrial Microbiology and Biotechnology*, vol. 38, no. 9, pp. 1145–1159, 2011. https://doi.org/10.1007/s10295-011-1018-4

[38] Q. Yang, S. Liu, Y. Zhao, X. Han, R. Chang, and J. Mao, "Enzymatic properties and inhibition tolerance analysis of key enzymes in β-phenylethanol anabolic pathway of Saccharomyces cerevisiae HJ," *Synthetic and Systems Biotechnology*, vol. 8, no. 4, pp. 772–783, 2023. https://doi.org/10.1016/j.synbio.2023.11.006

APPENDIX

Table S1. OD_{600} values at each time point during the cultivation of EC-1118 and 312WT in YPD media containing 12% and 15% ethanol.

Ethanol %	Yeast strains	Optical Density at 600 nm (OD_{600})			
		0h	24h	48h	72h
12	EC-1118	0.10 ± 0.03	0.34 ± 0.10	0.87 ± 0.22	1.37 ± 0.24
	312WT	0.15 ± 0.00	0.36 ± 0.05	0.54 ± 0.18	0.85 ± 0.15
15	EC-1118	0.08 ± 0.01	0.12 ± 0.02	0.10 ± 0.02	0.12 ± 0.03
	312WT	0.04 ± 0.02	0.19 ± 0.01	0.16 ± 0.02	0.16 ± 0.03

Table S2. Semi-quantification (μg/kg) of key flavor compounds detected in rice wine produced using the FAEEs-overproducing mutant strain developed from 312WT.

Compound	Odor description		Relative concentration (μg/kg)						
		EC-1118	312WT	312/1	312/2	312/3	312/4	312/5	312/6
Ethyl formate	Strong, ethereal-fruity, like rum	29.29	33.23	19.05	ND	7.72	7.24	ND	ND
Ethyl butanoate	Fruity juicy, tutti fruity, pineapple	ND	ND	ND	4.01	4.96	ND	4.69	9.32
Isoamyl alcohol	Alcoholic, banana	355.75	419.19	322.68	352.02	312.25	277.81	292.68	325.16
Ethyl hexanoate	Sweet apple	35.95	28.41	16.70	19.00	38.93	14.43	16.77	51.41
Ethyl octanoate	Sour apple	47.80	94.19	58.31	84.88	79.83	82.62	55.594	282.39
Ethyl phenyl acetate	Roses and honey	ND	38.32	7.20	20.74	ND	9.91	8.31	8.63
Phenylethyl alcohol	Sweet, floral, fresh and bready with a rosy honey	188.84	470.16	307.23	332.67	376.53	420.64	354.64	349.21
Ethyl dodecanoate	Sweet waxy soapy rummy, creamy floral	12.03	33.13	12.04	10.49	9.63	6.49	ND	8.86
2-Methoxy-4-vinylphenol	Sweet spicy clove peppery smoky woody powdery	ND	38.02	30.72	38.86	25.84	66.66	12.97	43.70

Note: Values are the mean of two injections of each replicate.
[ND]not detectable.

Table S3. Predicted effect of amino acid substitutions on protein stability and flexibility.

Original amino acid	Mutated amino acid	Position	Prediction ΔΔG mCSM (kcal/mol)	Prediction ΔΔG SDM (kcal/mol)	Prediction ΔΔG DUET (kcal/mol)	Prediction ΔΔG ENCoM (kcal/mol)	ΔΔS ENCoM ($kacl.mol^{-1}.k^{-1}$)	ΔΔG DynaMut (kcal/mol)
D	E	1650	-0.333	0.01	-0.111	-0.009	0.012	0.258
H	R	343	-0.895	-0.44	-0.716	0.25	-0.313	1.56
N	T	1837	0.103	-0.16	0.331	0.044	-0.055	0.065

The positive and negative values in ΔΔS ENCoM indicate increased and decreased protein flexibility, respectively.

Table S4. Predicted effects of amino acid substitutions on protein function loss.

Name	Mutation	Mutation	Provean
FAS1	Val77Ile	V77I	-0.448
FAS2	Thr112Asn	T112N	-1.779
FAS2	Arg396Lys	R396K	0.299
FAS2	Asn565Ser	N565S	S in query sequence
ACC1	Phe28Leu	F28L	L in query sequence
ACC1	Asn 116 Tyr	N116Y	Y in query sequence
ACC1	His343Arg	H343R	0.127
ACC1	Asp1650Glu	D1650E	0.678
ACC1	Asn1837Thr	N1837T	-0.685
EHT1	Gln384Arg	Q384R	R in query sequence
EEB1	Arg31Lys	R31K	K in query sequence
ARO8	Arg7Lys	R7K	K in query sequence
ARO9	Ala26Val	A26V	V in query sequence
ARO9	Asn79Asp	N79D	0.06
ARO9	Asp217Glu	D217E	0.015
ARO9	Leu454Val	L454V	0.13
ARO10	Phe394Leu	F394L	L in query sequence
ARO80	Thr368Ala	T368A	A in query sequence
ARO80	Ile937Thr	I937T	0.167

Predictions were obtained using PROVEAN with a score below -2.5, indicating a deleterious effect, suggesting potential loss of protein function.

Table S5 presents the raw GC–MS peak table of volatile compounds detected in rice wine fermented by strain 312WT (replicate 1), including retention index, peak areas, calculated concentrations, and odor activity values.

Table S5. GC-MS raw data of flavor compound in rice wine fermentation from 312WT replication-1.

Compound	RI reference	RI calculated	Height (Repeat sample 1)	Height (Repeat sample 2)	Height-average	Peak area ratio	Concentration of internal standard (μg)	Relative concentration (μg/g)	Relative concentration (μg/kg)	Odor threshold	OAV
Isoamyl alcohol	1115-1250	1143.35	1132204	1154201	1143202.50	1.67	1.67	0.33	334.97	250.00	1.34
3-Heptanone, 2-methyl (Internal standard)	1164-1191	1224.00	725645	639486	682565.50	1.00	1.00	0.20	200.00	250.00	0.80
Ethyl hexanoate	1197-1258	1282.06	54027	63560	58793.50	0.09	0.09	0.02	17.23	1.00	17.23
Ethyl octanoate	1486-1569	1481.32	213606	302034	257820.00	0.38	0.38	0.08	75.54	5.00	15.11
Ethyl nonanoate	1509-1601	1583.03	0	0	0.00	0.00	0.00	0.00	0.00	1300.00	0.00
Phenylethyl acetate	1609-1667	1652.07	226370	231560	228965.00	0.34	0.34	0.07	67.09	250.00	0.27
Phenylethyl alcohol	1865-1946	1677.22	3066671	3129530	3098100.50	4.54	4.54	0.91	907.78	1000.00	0.91
2-Methoxy-4-vinylphenol	2145-2212	2010.96	264933	248288	256610.50	0.38	0.38	0.08	75.19	1100.00	0.07

Table S6 presents the raw GC–MS peak table of volatile compounds detected in rice wine fermented by strain 312WT (replicate 2), including retention index, peak areas, calculated concentrations, and odor activity values.

Table S6. GC-MS raw data of flavor compounds in rice wine fermentation from 312WT replication-2.

Compound	RI reference	RI calculated	Height (Repeat sample 1)	Height (Repeat sample 2)	Height-average	Peak area ratio	Concentration of internal standard (μg)	Relative concentration (μg/g)	Relative concentration (μg/kg)	Odor threshold	OAV
Isoamyl alcohol	1115-1250	1143.35	1136320	1119920	1128120.00	2.01	2.01	0.40	402.40	250.00	1.61
3-Heptanone, 2-methyl (Internal standard)	1164-1191	1224.00	651418	469980	560699.00	1.00	1.00	0.20	200.00	250.00	0.80
Ethyl hexanoate	1197-1258	1282.06	62637	62841	62739.00	0.11	0.11	0.02	22.38	1.00	22.38
Ethyl octanoate	1486-1569	1481.32	344081	373048	358564.50	0.64	0.64	0.13	127.90	5.00	25.58
Ethyl nonanoate	1509-1601	1583.03	0	46048	23024.00	0.04	0.04	0.01	8.21	1300.00	0.01
Phenylethyl acetate	1609-1667	1652.07	123784	118116	120950.00	0.22	0.22	0.04	43.14	250.00	0.17
Phenylethyl alcohol	1865-1946	1677.22	3129530	3721767	3425648.50	6.11	6.11	1.22	1221.92	1000.00	1.22
2-Methoxy-4-vinylphenol	2145-2212	2010.96	264933	248288	256610.50	0.46	0.46	0.09	91.53	1100.00	0.08

Table S7 presents the raw GC–MS peak table of volatile compounds detected in rice wine fermented by strain 312WT (replicate 3), including retention index, peak areas, calculated concentrations, and odor activity values.

Table S7. GC-MS raw data of flavor compound in rice wine fermentation from 312WT replication-3.

Compound	RI reference	RI calculated	Height (Repeat sample 1)	Height (Repeat sample 2)	Height-average	Peak area ratio	Concentration of internal standard (μg)	Relative concentration (μg/g)	Relative concentration (μg/kg)	Odor threshold	OAV
Isoamyl alcohol	1115-1250	1143.35	1454666	1398218	1426442.00	2.55	2.55	0.51	509.57	250.00	2.04
3-Heptanone, 2-methyl (Internal standard)	1164-1191	1224.00	561960	557767	559863.50	1.00	1.00	0.20	200.00	250.00	0.80
Ethyl hexanoate	1197-1258	1282.06	56267	55906	56086.50	0.10	0.10	0.02	20.04	1.00	20.04
Ethyl octanoate	1486-1569	1481.32	63582	66115	64848.50	0.12	0.12	0.02	23.17	5.00	4.63
Ethyl nonanoate	1509-1601	1583.03	0	0	0.00	0.00	0.00	0.00	0.00	1300.00	0.00
Phenylethyl acetate	1609-1667	1652.07	86541	88740	87640.50	0.16	0.16	0.03	31.31	250.00	0.13
Phenylethyl alcohol	1865-1946	1677.22	3228815	3756420	3492617.50	6.24	6.24	1.25	1247.67	1000.00	1.25
2-Methoxy-4-vinylphenol	2145-2212	2010.96	252230	269572	260901.00	0.47	0.47	0.09	93.20	1100.00	0.08

Table S8 presents the raw GC–MS peak table of volatile compounds detected in rice wine fermented by strain 312/6 (replicate 1), including retention index, peak areas, calculated concentrations, and odor activity values.

Table S8. GC-MS raw data of flavor compound in rice wine fermentation from 312/6 replication-1.

Compound	RI reference	RI calculated	Height (Repeat sample 1)	Height (Repeat sample 2)	Height-average	Peak area ratio	Concentration of internal standard (μg)	Relative concentration (μg/g)	Relative concentration (μg/kg)	Odor threshold	OAV
Isoamyl alcohol	1115-1250	1143.74	1079712	1057458	1068585	1.85	1.85	0.37	370.46	250.00	1.48
3-Heptanone, 2-methyl (Internal standard)	1164-1191	1224.21	543568	610223	576895.5	1.00	1.00	0.20	200.00	250.00	0.80
Ethyl hexanoate	1197-1258	1281.71	54383	57703	56043	0.10	0.10	0.02	19.43	1.00	19.43
Ethyl octanoate	1486-1569	1480.84	421585	430944	426264.5	0.74	0.74	0.15	147.78	5.00	29.56
Ethyl nonanoate	1509-1601	1582.14	0	40818	20409	0.04	0.04	0.01	7.08	1300.00	0.01
Phenylethyl acetate	1609-1667	1651.96	45490	44794	45142	0.08	0.08	0.02	15.65	250.00	0.06
Phenylethyl alcohol	1865-1946	1677.08	4933672	5040190	4986931	8.64	8.64	1.73	1728.89	1000.00	1.73
2-Methoxy-4-vinylphenol	2145-2212	2010.78	279569	277786	278677.5	0.48	0.48	0.10	96.61	1100.00	0.09

Table S9 presents the raw GC–MS peak table of volatile compounds detected in rice wine fermented by strain 312/6 (replicate 2), including retention index, peak areas, calculated concentrations, and odor activity values.

Table S9. GC-MS raw data of flavor compound in rice wine fermentation from 312/6 replication-2.

Compound	RI reference	RI calculated	Height (Repeat sample 1)	Height (Repeat sample 2)	Height-average	Peak area ratio	Concentration of internal standard (μg)	Relative concentration (μg/g)	Relative concentration (μg/kg)	Odor threshold	OAV
Isoamyl alcohol	1115-1250	1143.74	1085137	1073476	1079306.5	1.95	1.95	0.39	389.07	250.00	1.56
3-Heptanone, 2-methyl (Internal standard)	1164-1191	1224.21	602746	506878	554812	1.00	1.00	0.20	200.00	250.00	0.80
Ethyl hexanoate	1197-1258	1281.71	98038	87807	92922.5	0.17	0.17	0.03	33.50	1.00	33.50
Ethyl octanoate	1486-1569	1480.84	632703	658163	645433	1.16	1.16	0.23	232.67	5.00	46.53
Ethyl nonanoate	1509-1601	1582.14	61453	52025	56739	0.10	0.10	0.02	20.45	1300.00	0.02
Phenylethyl acetate	1609-1667	1651.96	95196	97441	96318.5	0.17	0.17	0.03	34.72	250.00	0.14
Phenylethyl alcohol	1865-1946	1677.08	3900994	3820239	3860616.5	6.96	6.96	1.39	1391.68	1000.00	1.39
2-Methoxy-4-vinylphenol	2145-2212	2010.78	275185	262988	269086.5	0.49	0.49	0.10	97.00	1100.00	0.09

Table S10 presents the raw GC–MS peak table of volatile compounds detected in rice wine fermented by strain 312/6 (replicate 3), including retention index, peak areas, calculated concentrations, and odor activity values.

Table S10. GC-MS raw data of flavor compound in rice wine fermentation from 312/6 replication-3.

Compound	RI reference	RI calculated	Height (Repeat sample 1)	Height (Repeat sample 2)	Height-average	Peak area ratio	Concentration of internal standard (μg)	Relative concentration (μg/g)	Relative concentration (μg/kg)	Odor threshold	OAV
Isoamyl alcohol	1115-1250	1143.74	962068	933938	948003	1.55	1.55	0.31	310.76	250.00	1.24
3-Heptanone, 2-methyl (Internal standard)	1164-1191	1224.21	724869	495357	610113	1.00	1.00	0.20	200.00	250.00	0.80
Ethyl hexanoate	1197-1258	1281.71	99220	100321	99770.5	0.16	0.16	0.03	32.71	1.00	32.71
Ethyl octanoate	1486-1569	1480.84	691143	780474	735808.5	1.21	1.21	0.24	241.20	5.00	48.24
Ethyl nonanoate	1509-1601	1582.14	53719	71049	62384	0.10	0.10	0.02	20.45	1300.00	0.02
Phenylethyl acetate	1609-1667	1651.96	195711	202709	199210	0.33	0.33	0.07	65.30	250.00	0.26
Phenylethyl alcohol	1865-1946	1677.08	3392346	3905733	3649039.5	5.98	5.98	1.20	1196.18	1000.00	1.20
2-Methoxy-4-vinylphenol	2145-2212	2010.78	264485	286740	275612.5	0.45	0.45	0.09	90.35	1100.00	0.08

Table S11 presents the raw GC–MS peak table of volatile compounds detected in rice wine fermented by strain EC-1118 (replicate 1), including retention index, peak areas, calculated concentrations, and odor activity values.

Table S11. GC-MS raw data of flavor compound in rice wine fermentation from EC-1118 replication-1.

Compound	RI reference	RI calculated	Height (Repeat sample 1)	Height (Repeat sample 2)	Height-average	Peak area ratio	Concentration of internal standard (μg)	Relative concentration (μg/g)	Relative concentration (μg/kg)	Odor threshold	OAV
Isoamyl alcohol	1115-1250	1143	1200680	1194500	1197590.00	1.88	1.88	0.38	375.00	250.00	1.50
3-Heptanone, 2-methyl (Internal standard)	1164-1191	1223	610724	666690	638707.00	1.00	1.00	0.20	200.00	250.00	0.80
Ethyl hexanoate	1197-1258	1281	79612	80143	79877.50	0.13	0.13	0.03	25.01	1.00	25.01
Ethyl octanoate	1486-1569	1482	0	0	0.00	0.00	0.00	0.00	0.00	5.00	0.00
Ethyl nonanoate	1509-1601	0	0	0	0.00	0.00	0.00	0.00	0.00	1300.00	0.00
Phenylethyl acetate	1609-1667	1652	184886	194474	189680.00	0.30	0.30	0.06	59.39	250.00	0.24
Phenylethyl alcohol	1865-1946	1678	727709	768303	748006.00	1.17	1.17	0.23	234.23	1000.00	0.23
2-Methoxy-4-vinylphenol	2145-2212	2011	0	0	0.00	0.00	0.00	0.00	0.00	1100.00	0.00

Table S12 presents the raw GC–MS peak table of volatile compounds detected in rice wine fermented by strain EC-1118 (replicate 2), including retention index, peak areas, calculated concentrations, and odor activity values.

Table S12. GC-MS raw data of flavor compound in rice wine fermentation from EC-1118 replication-2.

Compound	RI reference	RI calculated	Height (Repeat sample 1)	Height (Repeat sample 2)	Height-average	Peak area ratio	Concentration of internal standard (μg)	Relative concentration (μg/g)	Relative concentration (μg/kg)	Odor threshold	OAV
Isoamyl alcohol	1115-1250	1143	1258366	647119	952742.50	2.74	2.74	0.55	547.29	250.00	2.19
3-Heptanone, 2-methyl (Internal standard)	1164-1191	1223	49221	647119	348170.00	1.00	1.00	0.20	200.00	250.00	0.80
Ethyl hexanoate	1197-1258	1281	49221	49433	49327.00	0.14	0.14	0.03	28.34	1.00	28.34
Ethyl octanoate	1486-1569	1482	55004	103283	79143.50	0.23	0.23	0.05	45.46	5.00	9.09
Ethyl nonanoate	1509-1601	0	0	0	0.00	0.00	0.00	0.00	0.00	1300.00	0.00
Phenylethyl acetate	1609-1667	1652	125057	125263	125160.00	0.36	0.36	0.07	71.90	250.00	0.29
Phenylethyl alcohol	1865-1946	1678	1527535	1653111	1590323.00	4.57	4.57	0.91	913.53	1000.00	0.91
2-Methoxy-4-vinylphenol	2145-2212	2011	273104	278804	275954.00	0.79	0.79	0.16	158.52	1100.00	0.14

Table S13 presents the raw GC–MS peak table of volatile compounds detected in rice wine fermented by strain EC-1118 (replicate 3), including retention index, peak areas, calculated concentrations, and odor activity values.

Table S13. GC-MS raw data of flavor compound in rice wine fermentation from EC-1118 replication-3.

Compound	RI reference	RI calculated	Height (Repeat sample 1)	Height (Repeat sample 2)	Height-average	Peak area ratio	Concentration of internal standard (μg)	Relative concentration (μg/g)	Relative concentration (μg/kg)	Odor threshold	OAV
Isoamyl alcohol	1115-1250	1143	1299560	1258036	1278798.00	2.02	2.02	0.40	403.90	250.00	1.62
3-Heptanone, 2-methyl (Internal standard)	1164-1191	1223	619337	647119	633228.00	1.00	1.00	0.20	200.00	250.00	0.80
Ethyl hexanoate	1197-1258	1281	53666	49433	51549.50	0.08	0.08	0.02	16.28	1.00	16.28
Ethyl octanoate	1486-1569	1482	229824	103283	166553.50	0.26	0.26	0.05	52.60	5.00	10.52
Ethyl nonanoate	1509-1601	0	0	0	0.00	0.00	0.00	0.00	0.00	1300.00	0.00
Phenylethyl acetate	1609-1667	1652	299447	125263	212355.00	0.34	0.34	0.07	67.07	250.00	0.27
Phenylethyl alcohol	1865-1946	1678	3654022	1653111	2653566.50	4.19	4.19	0.84	838.11	1000.00	0.84
2-Methoxy-4-vinylphenol	2145-2212	2011	327946	278804	303375.00	0.48	0.48	0.10	95.82	1100.00	0.09